# Determination of the relationship between epiphytes and selected filamentous bacteria in activated sludge

**Submitted in fulfillment for the Degree of Masters of Applied Sciences (Biotechnology) in the Department of Biotechnology and Food Technology, Durban University of Technology, Durban, South Africa**


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## REFERENCE DECLARATION

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I, Ms Thobela Precious Conco – 20823074, Prof. Faizal Bux (supervisor) and Prof. Thor Stenström and Dr Sheena Kumari (co-supervisors) hereby declare that in respect of the following dissertation:

Title**: Determination of the relationship between epiphytes and filamentous bacteria in activated sludge**

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

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

## APPROVAL

I hereby approve the final submission of the following dissertation.

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| | |
|---|---|
| AS | Activated Sludge |
| BNR | Biological nutrient removal |
| DAPI | 4', 6-diamidino-2-phenylindole |
| DGGE | Denaturing gradient gel electrophoresis |
| DLVO | Derjaguin‑Landau‑Verwey‑Overbeek |
| DO | Dissolved oxygen |
| EPS | Extracellular polysaccharide |
| F/M | Food to microorganism ratio |
| FISH | Fluorescent *in situ* hybridization |
| LB-EPSs | Loosely bound extracellular polysaccharides |
| MCRT | Mean cell retention time |
| PAOs | Polyphosphate accumulating organisms |
| PFA | Paraformaldehyde |
| PHB | Poly-β-hydyoxybutyrate |
| RAS | Returned activated sludge |
| rRNA | Ribosomal ribonucleic acid |
| S | Sulphur |
| SEM | Scanning electron microscope |
| SRT | Sludge retention time |
| TB-EPSs | Tightly bound extracellular polysaccharides |
| TEM | Transmission electron microscope |
| WWTPs | Wastewater treatment plants |

## OUTPUTS (PUBLICATIONS AND CONFERENCE PRESENTATIONS)

- Evaluation of Epiphytic growth on filamentous bacteria in activated sludge: a morphological approach (Under review in Journal of Basic Microbiology).

- **Thobela Conco,** Sheena Kumari, Thor Stenström, Simona Rossetti, Faizal Bux (2016). Evaluation of Epiphytic growth on filamentous bacteria in activated sludge: a morphological approach. 18th International Conference on Biological, Ecological and Environmental Sciences and Engineering. Holiday Inn Paris Montparnasse Avenue Du Maine, 79-81 Paris, 75014 (Oral Presentation).

- **Thobela Conco,** Sheena Kumari, Thor Stenström, Faizal Bux. Evaluation of epiphytic growth on some selected filamentous bacteria in activated sludge by SEM and TEM. South African Society of Microbiology Biennial Conference, Coastlands Hotel Umhlanga rocks, Durban South Africa, January 2016 (Oral Presentation).

- **Thobela Conco,** Sheena Kumari, Thor Stenström, Faizal Bux. Evaluation of epiphytic growth on some selected filamentous bacteria in activated sludge by SEM and TEM. WISA Biennial Conference, International Convention Center, Durban South Africa, May 2016 (Oral Presentation).

- **Thobela Conco,** Sheena Kumari, Thor Stenström, Simona Rossetti, Faizal Bux. (2016). Evaluation of Epiphytic growth on filamentous bacteria in activated sludge: a morphological approach. IWA Microbial Ecology in Water Engineering & Biofilms joint

specialist conference: Environmental biotechnology: discovering and applying recently discovered microbial physiologies. The National Museum of Denmark, Copenhagen, Denmark, September 2016 (Poster presentation).

# ABSTRACT

Activated sludge (AS) flocs are paramount in biological treatment of wastewater, are comprised of microbial consortia with organic and inorganic material bound together by extra polymeric substances (EPS). The filamentous bacteria play a vital role in the floc formation process by providing the necessary structural support. Presence of epiphytic attachment on selected filamentous bacteria is a commonly occurring phenomenon in activated sludge samples. Different theories have been proposed to describe this phenomenon; however, not much research has been carried out to explore the profundity of the attachment. In this study, an attempt has been made to elucidate the intrinsic nature of the epiphytic attachment between the bacterial rods and filamentous bacteria based on microscopic (morphological and structural) analysis. Characterization of these epiphytes were performed using fluorescence *in situ* hybridization (FISH) at group level using Alpha, Beta and Gamma Proteo-bacterial probes. Morphological characteristics of filament hosts and the bacterial rods at the interface region was assessed using scanning electron microscopy (SEM). The SEM micrographs indicated that the attachment was facilitated by more than the EPS layer. Further ultrastructural examination using transmission electron microscopy (TEM) indicated a possible cell-to-cell interaction between epiphytes and the selected filaments. Fibrillar structures resembling amyloid-like proteins were observed within the filament cell targeted by the epiphytes. An interaction was apparent between the amyloid like proteins and the epiphytes as exhibited by the direction of fibrillar structures pointing towards the

approaching epiphytes. Common bacterial appendages such as pili and fimbria were absent at the interface and further noted was the presence of cell membrane extensions on the epiphytic bacteria protruding towards the targeted filamentous cell. The sheath of host filaments however, remained intact and unpenetrated, during colonization. Amyloid-like fibrils at interface may potentially play the role of attachment sites for the attaching epiphytes, as attachment facilitating appendages were not visualized.

# 1. Chapter One: Introduction

Activated sludge (AS) wastewater treatment plants (WWTPs) are biologically engineered systems in which nutrients and other compounds are removed by microorganisms. The microbial population of AS are described from a functional point of view as aerobic heterotrophs, nitrifiers, denitrifiers, sulphate reducers, iron reducers and phosphate-accumulating organisms (PAOs) (Thomsen *et al.,* 2004). The AS technology is based on the ability of these functional microorganisms to form microbial units known as flocs. These bio-aggregated bacterial units strongly adhere to the filamentous network "backbone" by means of extracellular polymeric substances (EPS) secreted by bacteria. Within these, certain bacterial rods grow attached to other microorganisms (Xia *et al.,* 2008). The occurrence of such attached growth is a common phenomenon in different ecosystems, as in soils or on plant surfaces. In activated sludge, the colonization of certain filament trichomes by epiphytic bacteria has been observed as a common occurrence (Xia *et al,*. 2008).

Presence of epiflora on the trichome has been used as an important criterion for a morphological classification/identification of filamentous morphotypes in AS (Xia *et al.,* 2008). Filamentous morphotypes with attached growth that are identified from AS includes Eikelboom types 0041, 1701, and 1851. These filaments are often regarded as unwanted/nuisance filaments that are involved in bulking and foaming incidence during biological wastewater treatment. In addition, the operational conditions that favor these filamentous bacteria are well documented as low dissolved oxygen (DO) levels, low organic loading rates, low substrate concentration gradients

and nutrient limitation (Guo *et al.,* 2014). However, not many studies have been carried out to date to understand the functional role (if any) of epiflora in AS plants. The role of EPS layer in epiphytic attachment have been proposed by earlier workers (Jenkins *et al.,* 2004). However, not all the filamentous bacteria in the same floc bearing a sheath have these epiphytes attached to their surfaces. Filaments such as *Thiothrix* spp, Type 021N, *Gordonia* spp are among those without such attachment. These observations highlights the need for further research in this area to understand this epiphytic growth beyond the surface attachment, as well as the level of interaction between filamentous bacteria and their epiphytic counterparts. This also includes an understanding in the further conditions inducing such attachment to selected filament morphotypes. The literature currently give limited information on the identity and ecophysiology of microorganisms colonizing filamentous bacteria in AS (Thomsen *et al.,* 2002; Xia *et al.,* 2008).

The aim of this study was therefore to evaluate the mechanisms used by epiphytic bacteria in facilitating attachment on to filamentous hosts in AS. Furthermore the aim was to enhance the understanding of the level of interaction between the filament host and epiphytic bacteria in AS using microscopic observations. This includes evaluating surface and ultra-structural morphology of host filament and that of colonizing epiphytic cell by means of SEM and TEM respectively, and assessing the potential relationship that could exist between the two organisms using a cell viability test (LIVE/DEAD staining technique).

## 1.1 RESEARCH OBJECTIVES

To achieve the overall aim of this study, specific objectives were established and detailed as follows:

I. **To identify and characterize filamentous bacteria with epiphytic attachment from different wastewater treatment plants**.

The objective focused on analyzing activated sludge samples from different wastewater treatment plants to identify and characterize the filamentous bacteria with epiphytic attachment using conventional microscopy and further confirmation using molecular techniques (FISH) in relation to plan operational conditions.

II. **Identification of the structural morphology and possible interaction between filamentous bacteria and epiphytes**.

This objective focused on understanding the structural morphology of filaments and epiphytes using SEM and further observations at the interface between filaments and epiphytes, using TEM to assess the level of interaction. .

III. **Evaluation of the nature of the relationship between filamentous bacteria**.

The objective focused on evaluating the nature of the relationship between filament and its counterpart based on the availability of storage compounds on the targeted cells and the overall viability of colonized filamentous bacteria and epiphytes using LIVE/DEAD staining technique.

## 1.2 OUTLINE OF THESIS

Thesis organization is as follows:

- **Chapter 2:** Background study of current and available literature of wastewater treatment, AS process and AS microbiology. The formation of the microbial floc structure, floc morphology and its direct influence of the overall treatment process. Identification of resident filamentous bacteria and their functional role in AS flocculation. Epiphytic growth and mechanism that facilitates the attachment of epiphytic bacteria to biotic surfaces.

- **Chapter 3:** Identification and characterization of filamentous morphotypes, focusing on morphological characteristics of filamentous bacteria in activated sludge. Evaluation of epiphytic growth on morphotypes prone to attached growth. Characterization of epiphytes by molecular technique (FISH).

- **Chapter 4:** Focuses on evaluating the morphology of filamentous host and epiphytic counterparts by use of SEM and TEM at surface and ultrastructural levels, respectively. Investigation of mechanism involved in attachment of epiphytes at interface. Assessing the selectivity of filament cells colonized by epiphytes all length of filament.

- **Chapter 5:** Evaluation of the interaction between epiphytes and filamentous bacteria under nutritional stress conditions. The determination of the nature of relationship between host and epiphyte. Elemental analysis of surface of epiphytes, interface and colonized filament cells.

- **Chapter 6:** Summary of the general conclusions made in the current study and recommendations for future continuation in further investigation of the present work.

# 2. Chapter Two: Literature Review

## 2.1 Activated Sludge Process.

The emergence of wastewater treatment processes to solve water quality issues led to the development of AS systems, as the main biological process to treat wastewater (Mesquita *et al.*, 2013). The principal of the AS system is based on the action of a highly complex mixture of microbial populations that are crucial for biodegradation and nutrient removal. Bacteria represent around 95% of the bio-volume of AS and play a key role in biological removal of organic carbon, ammonium and phosphate from wastewater. In an activated sludge process, the biodegradation is facilitated by microbial aggregates (flocs) formed by bio-flocculation of suspended microorganisms during biological treatment. This is followed by subsequent settling of the microbial flocs in the secondary clarifiers where the treated effluent is separated from the biomass (Mesquita *et al.*, 2013).

## 2.2 Activated Sludge Flocculation

The AS resident microorganisms aggregate to form microbial units, which are known as activated sludge flocs (Perez *et al.,* 2006). The process in which bacteria aggregate and adhere to one another forming flocs is termed bioflocculation, which is of primary importance for the AS systems (Van Dierdonck *et al.,* 2012). The AS flocs are considered to be a collection of several particles, e.g., primary particles and microcolonies, held together by bridging by EPS and cations- and hydrophobic and Derjaguin‑Landau‑Verwey‑Overbeek (DLVO)-type of interactions (Van Dierdonck *et al.,* 2012). The EPS, a complex high-molecular-weight mixture of polymers, forms an adhesive matrix for the micro-colonies and bacteria to attach each other to and are found both on exterior and interior surfaces of sludge flocs (Sheng *et al,* 2010). The presence of EPS is resourceful as it binds with cells through complex interactions to form a vast net-like structure protecting against toxic substances as well serving as energy sources during nutrient shortage (Sheng *et al.,* 2010). The significant contribution of the EPS towards the formation of strong microbial flocs, is due to its dynamic double layered structure, where tightly bound EPSs (TB-EPSs) forms the inner layer and loosely bound EPSs (LB-EPSs) diffuses into the outer layer (Lin *et al.,* 2014) (Fig 2.1). This contribution is of key importance for an efficient solid-liquid separation of AS treatment systems (Thomsen *et al.,* 2004).

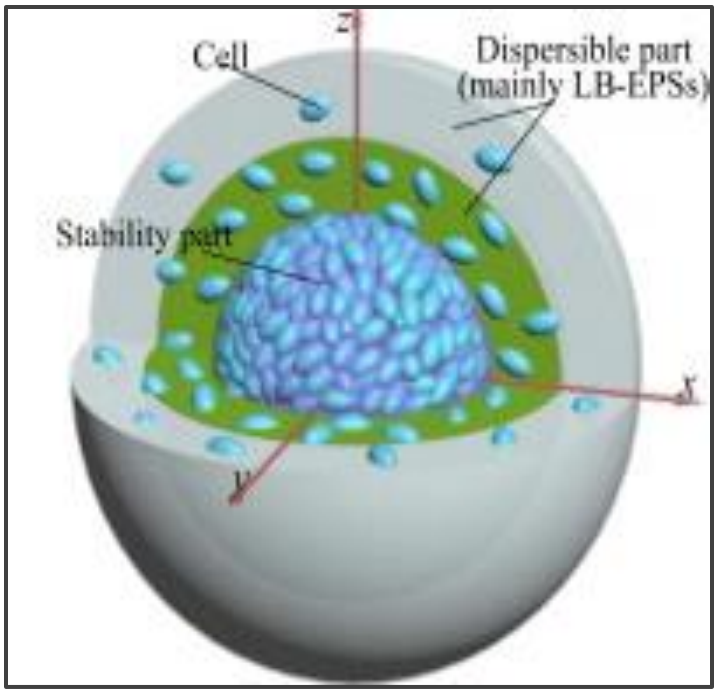


**Figure 2.1** Basic structural composition of sludge floc (Lin *et al.*, 2014).

## 2.3 Floc structure and its microbial composition

The microbial consortium of AS flocs play a crucial role in the structural build-up of the flocs. The floc-formers are both filamentous and non-filamentous bacteria. Filamentous bacteria form a network that serves as the backbone of the floc structure (Fig 2.2). While non-filamentous bacteria make up the microstructure that grows adhered to the filamentous backbone. Additionally resident micro-colonies are firmly held within the floc structure by EPS matrix. Most micro-colony forming bacteria belong to the Betaproteobacteria group; members of this group contribute to the overall floc structure by playing an integral role in floc resistance against shear stress (Thomsen *et al.*, 2004). Further floc strength is derived from the filamentous network, which serves as the backbone of the floc (Perez *et al.*, 2006).

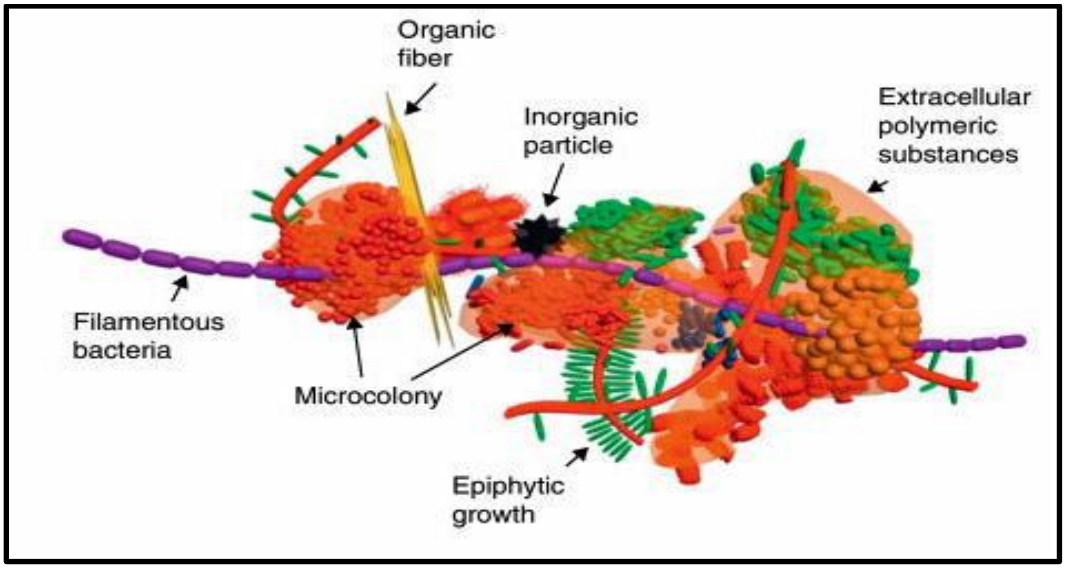


**Figure 2.2** Activated sludge floc representation and depiction of epiphytic growth colonizing selected filamentous bacteria (Nielsen *et al.*,2012).

Changes in operational conditions significantly influence the floc formation, which include formation of pinpoint flocs and filamentous overgrowth leading to bulking sludge (Mesquita *et al.,* 2011). Pinpoint flocs are formed by the flocculation of floc-forming bacteria in the absence of filamentous bacterial backbone; these are small and mechanically fragile (Jenkins *et al.,* 2003) (Fig 2.3b). However their proliferation and overgrowth leads to problem such as bulking (Levantesi *et al.,* 2004; Guo *et al.,* 2012). Bulking flocs are also formed by an overgrowth of filamentous bacteria, in the presence of fewer floc forming bacteria (Fig2.3a). Both pinpoint flocs and filamentous bulking are the main biological problems plaguing AS treatment plants, by negatively impacting floc formation and contributing to poor sludge settling ability (Madoni *et al.,* 2000, Mesquita *et al.,* 2011). Therefore the presence of filamentous bacteria in moderate levels is required for healthy AS flocs (Pal *et al.,* 2014).

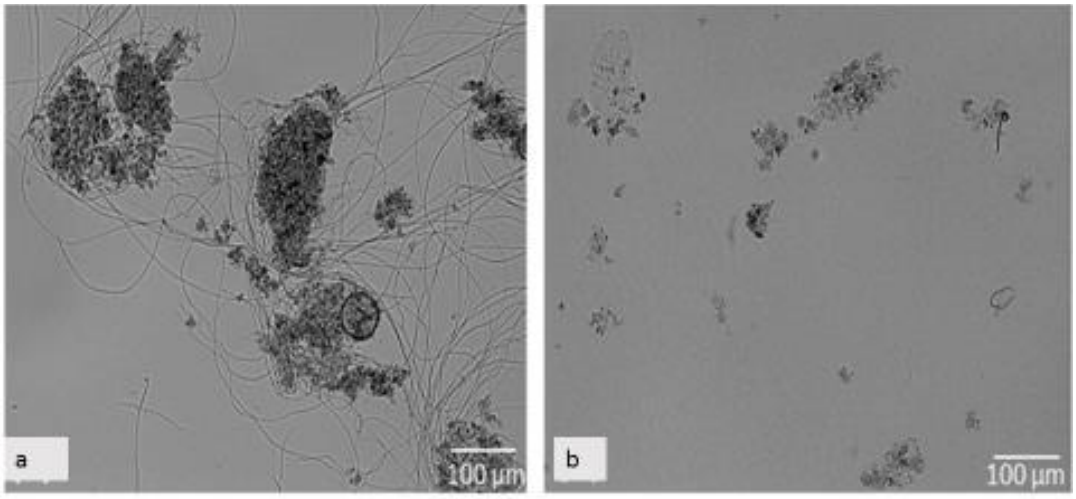


**Figure 2.3** Illustration of filamentous overgrowth (a) and pinpoint flocs. (Mesquita *et al.,* 2011).

## 2.4 Filamentous bacteria

Filamentous bacteria are an important component of the AS process, maintaining the rigid ‘‘backbone’’ structure of AS flocs (Juang 2005; Pal *et al.,* 2014). Their absence or presence in excessive numbers significantly affects the AS floc structure and further leads to process problems that affect the overall treatment process, these include bulking and foaming. A wide range of filaments are identified as causative agents of bulking. Among these *Microthrix parvicella,* Types 0092, 0041 and 0675 are apparently the major morphotype filaments, mainly responsible for the bulking events observed in biological nutrient removal (BNR) activated sludge systems (Martins *et al.,* 2004). The latter two morphotyes 0041 and 0675 are observed with epiphytic bacteria in AS (Nielsen *et al.,* 2009). This unique morphological trait is an important identification criterion for these morphotypes. However, information surrounding the identity of the epiphytic bacteria attaching to these morphotypes is still unclear (Xia *et al*., 2008). Therefore filament-type specific control strategies require a complete understanding of each filamentous bacteria, based on proper identification of the filamentous bacteria involved (Bradford *et al.,* 1996; Martins *et al.,* 2004).

## 2.5 Epiphytic growth and its occurrence in activated sludge

Epiphytic growth is a general and widespread phenomenon occurring in many regimes in the biosphere. The growth on human tissues in relation to diseases and that on plant surfaces, where interaction between the microorganism and plants are well described. Their presence in water bodies where they play an important role in the degradation of organic substances has been documented (Lalke-Porczyk and Donderski, 2003).

In AS, the presence and growth of rod-shaped bacteria colonizing selected filamentous morphotypes has been observed over the years (Xia *et al.*, 2008). The sheaths of large filamentous bacteria (e.g. *Beggiatoa* and *Thioploca*) are often colonized by the epiphytes which are believed to utilize the sheath material for their growth (Xia *et al.*, 2008). Eikelboom morphotypes Type 0041, Type 1701 and Type 1851 implicated in sludge bulking have also been observed to be colonized by epiphytic bacteria (Fig 2.4) (Xia *et al.*, 2008). A common trait among the epiphytic bacteria, is their consistent 90° angle of attachment on host filaments (Xia *et al.*, 2008). The adherence of these epiphytes to other microbial cells is thought to be facilitated by the EPS layer (Nongkhlaw and Joshi, 2014). However, attachment may also rely on differences in surface structure or other, unknown, factors (Xia *et al.*, 2008). With very limited information available on the identity and ecophysiology of these epiphytic bacteria their presence on selected filamentous bacteria has been adopted as an identification criterion (Eikelboom, 2000; Jenkins, 2004).

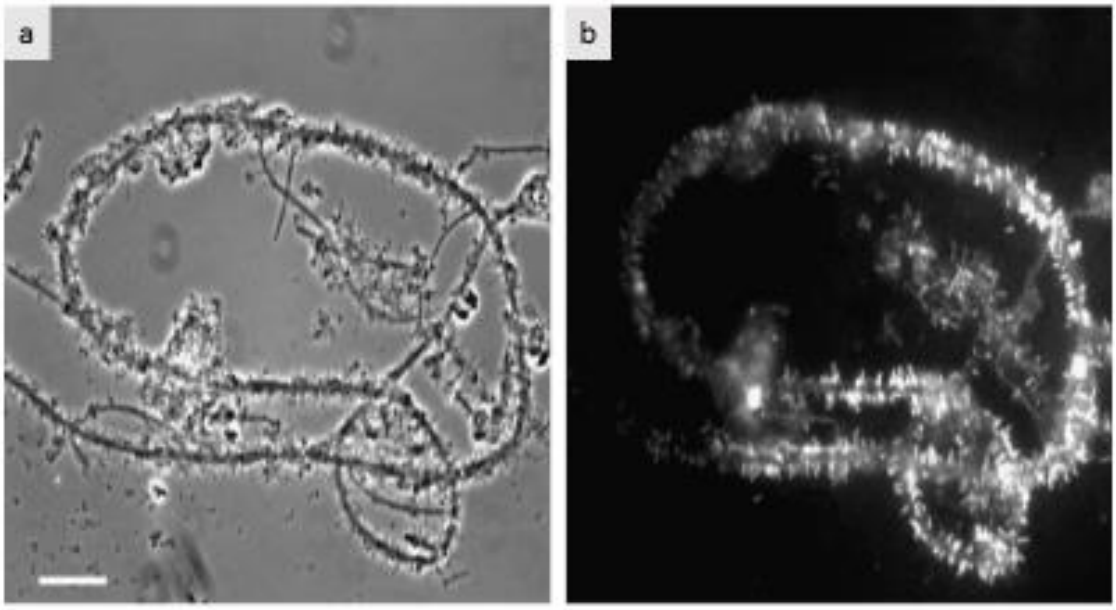


**Figure 2.4** a) Bright field image depicts a densely colonized filamentous morphotype found in a AS sample. b) Depicts the same densely colonized filament fluorescently labelled for FISH analysis. (Xia *et al.*, 2007).

### 2.5.1 Comparison of epiphytic growth with branching of filamentous bacteria in activated sludge

In AS, species from the two genera *Gordonia* and *Skermania* are frequently encountered, often associated with severe foaming episodes. Their morphotypes are commonly described, respectively, as the right-angled branching *Gordonia* amarae-like organisms and the acute-angled pine tree-like organism (Nielsen *et al.* 2009). The presence of branching is an important characteristic used to ascertain the identity of these morphotypes. Due to the right angle appearance of true branching, it often resembles epiphytic growth exhibited by bacterial rods on certain filamentous bacteria (Fig 2.5). Epiphytic attachment is consistently at a 90° angle, where bacterial rods adhere to surfaces along the length of filamentous bacteria (Xia *et al.*, 2008). True branching however occurs at any random point on the filament surface.

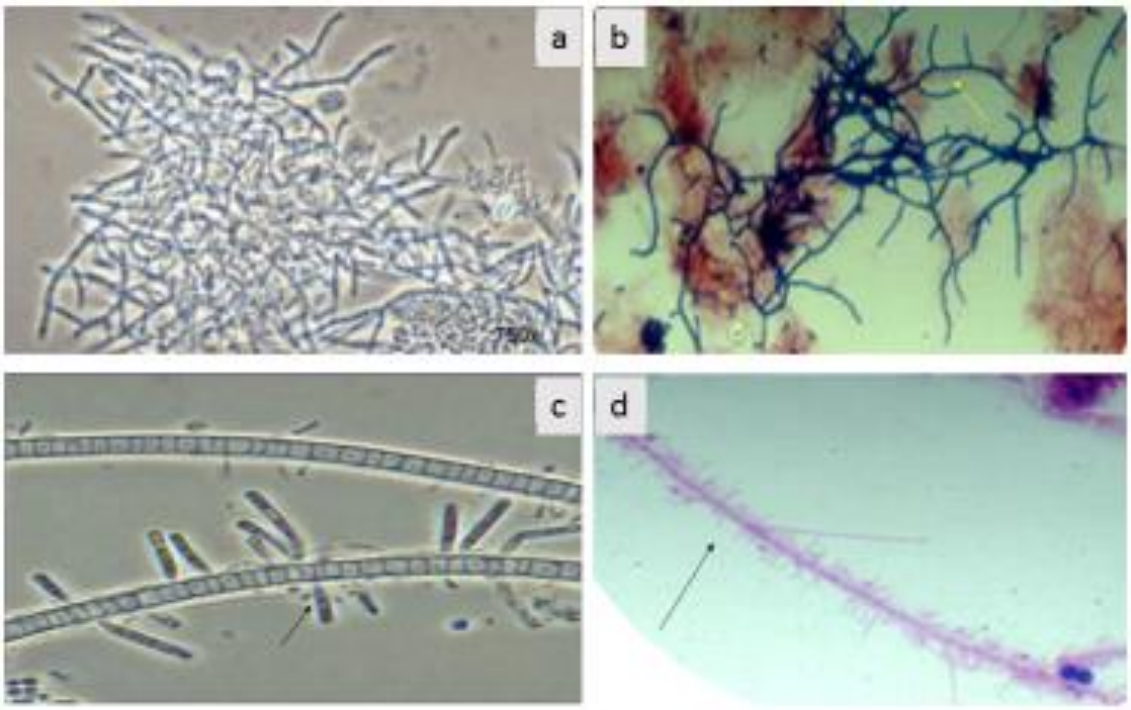


**Figure 2.5.** Illustration of true branching of *Nocardia* spp and epiphytic attachment of morphotype 0041. a and b depicts the random positions of true branches while, c and d shows the uniform 90° angle in the attachment of bacterial rods on filament trichomes (Jenkins, 2004).

## 2.6 Bacterial adhesion and structures that mediate epiphytic attachment

Bacteria generally exist in one of two types of population: planktonic, freely existing in bulk solution, and sessile, as a unit attached to a surface (Garret *et al.,* 2008). The immediate attachment of bacteria to an inert or living surface is the initial step in microbial adhesion and colonization (Hori and Matsumoto, 2010; Habimana *et al.,* 2014). Microbial adhesion is beneficial in bioreactors for wastewater treatment where the common growth pattern of bacteria in AS is on available surfaces rather than in the surrounding aqueous phase (Katsikogianni and Missirli, 2004; Hori and Matsumoto, 2010). Mechanisms by which bacteria are transported to a surface can include Brownian motion, sedimentation due to differences in specific gravity between the bacteria and the bulk liquid, or convective mass transport, by which cells are physically transported towards the surface by the movement of the bulk fluids (Hori and Mastumoto, 2010; Habimana *et al.,* 2014).

Upon approaching a surface, bacteria must overcome an energy barrier to establish direct contact with the surface (Habimana *et al.,* 2014). Physiochemical variables further define the interaction between the bacterial cell surface and the surface of interest (Dunne, 2002). The final outcome of adhesion is, however determined by the attraction and repulsion forces existing between the preferred surface and that of the bacterial cell (Dunne, 2002; Garrett *et al.,* 2008). In instances of repulsion, the repulsion charge between two negatively charged surfaces is minimized, when bacteria approach a surface with the smaller face of one of their poles, initiate attachment, and

either remain attached only at the pole (Young, 2006). Furthermore the net repulsion between two surfaces can be overcome by specific molecular interactions mediated by adhesins located on structures extending from the cell surface, such as pili (Dunne, 2002). These cell surface appendages, viz, bacterial pili are commonly used by bacteria as surface grabbing devices to facilitate attachment (Young, 2006). Additionally the use of amyloid-like adhesions have been utilized by bacteria in the formation of environmental biofilms (Jordal *et al.*, 2009).

### 2.6.1 Bacterial pili

Pili are non-flagellar, proteinaceous, multi-subunit surface appendages, with lengths between hundreds of nanometers to several micrometers that form long fibrous structures (Hori and Matsumoto, 2010; Kline *et al.*, 2010). Pili are used by bacteria both as common surface attachment appendages and for specific attachment. When utilized by bacteria for adhesion purposes, pili are capable of piercing energy barriers as well as altering cell adhesion behavior (Hori and Matsumoto, 2010). In addition, these pili are responsible for maintaining contact during the first stages of bacterial colonization (Bullitt and Makowski, 1998). This is achieved by the successful recognition and binding of these specialized surface structures to their host receptors (Bullitt and Makowski, 1998).

### 2.6.2 Amyloid like structures

Amyloid can be defined as orderly repeats of protein molecules arranged in a cross-β structure, in which the β strands are perpendicular to the fiber axis (Fig 2.6) (Otzen and Nielsen, 2007; Freire *et al.,* 2014). The outcome of the presence and formation of amyloid proteins has been associated with diseases such as Alzheimers, Diabetes and Parkinsons in humans, where each disease is as a result of specific protein or peptide aggregation (Fowler *et al.,* 2007; Rambaran and Serpell, 2008). From bacteria to humans, the characteristic cross-β-strand structure of amyloids, has been observed to be common (Romero *et al.,* 2010; Villar-Piqué and Ventura, 2012). The expression of amyloid in their various kinds are believed to be important for aggregation and for making strong attachment to the surfaces (Otzen and Nielsen, 2007). Curli which are amyloid fibers encoded by *Escherischia coli* and other Enterobacteriaceae such as *Salmonella* spp. have been proposed as a virulence factor in human disease (Hung *et al.,* 2014).

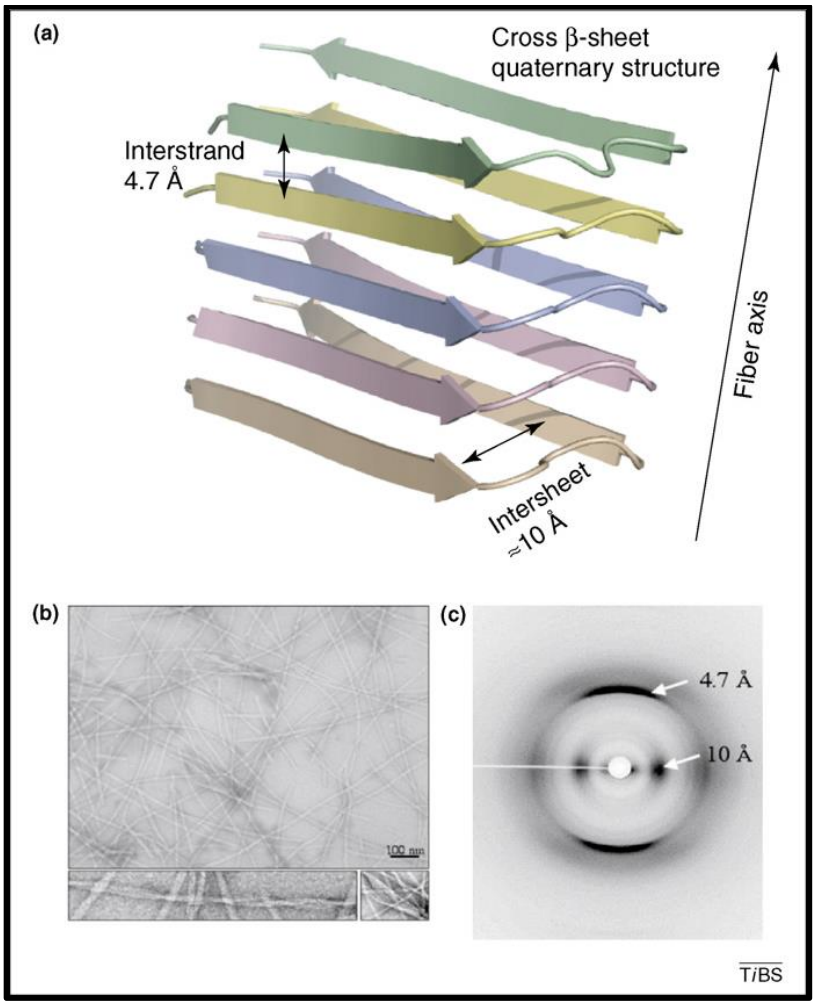


**Figure 2.6** Typical structure of amyloids fiber. a) Depicts the characteristic cross β-sheet amyloid structure. b) A transmission electron micrograph of negatively stained amyloid fibers. c) An X-ray fiber-diffraction pattern from partially aligned amyloid fibers. (Fowler *et al.*, 2007).

Amyloid has been observed in bacteria from wastewater, where Otzen (2010) identified their occurrence in 5-40% of assessed total bacterial species. In general functional amyloids often may aid in adhesion to surfaces and tissues as well as changing surface properties (Blanco *et al.,* 2012). During bioremediation, amyloids play a pivotal role in forming aggregative clusters in the matrix of AS-derived flocs (Blanco *et al.,* 2012). Filamentous bacteria from the phylum *Chloroflexi,* have been observed to express amyloids as part of the sheath or close to the septum between the individual cells in the filaments (Otzen, 2010). A unique attribute of these *Chloroflexi* filaments is the presence of epiphytic bacteria e.g. Candidatus *Epiflobacter* spp. (*Saprospiraceae*, *Bacteroidetes*) that specialize in protein degradation, excreting high levels of proteases (Otzen 2010). Otzen (2010), speculated that the excreted proteases feasted on the accessible amyloid. However an interesting observation made by Jordal *et al.* (2009) revealed that amyloids may be deeply embedded in the cell envelope and not easily accessible (Jordal *et al.,* 2009).

## 2.7 Filamentous identification

The conventional identification of AS filamentous bacteria has relied on the characterization of morphological traits and response to different stains (Nielsen *et al.,* 2009). These characteristics have been incorporated into identification keys by Jenkins *et al.* (1993, 2004) and Ekeilboom (2000). Most are still referred to as morphological types (e.g. Type 021N, Type 1863), using a naming system based exclusively on their microscopic features (Beer *et al.,* 2002).

### 2.7.1 Conventional Identification

Conventional staining techniques such as Gram and Neisser stains has been central in identifying filamentous bacteria in AS (Jenkins, 1993) combined with microscopic direct visualization and crude characterization (Moter and Göbel, 2000) where filament dimensions, the presence of a sheath and attached particles (epiphytic bacteria) on the sheath are central features as outlined by Sevior (2010) in Table 2.1.

Additionally, staining with special stains for the detection of intracellularly stored products aids in further characterization. These include sulphur globules Sulphur oxidation test (S test) where the globules appear bright yellow upon addition of sodium sulphide ($Na_2S.9H_20$) solution. The presence of sulphur granules is a characteristic of *Thiothrix* spp. (Wagner *et al.,* 1994). Further poly-*β*-hydroxyl butyrate (PHB) staining is used, where polyhydroxybutyrate, appears as bluish

globules within filament trichomes. This feature is a characteristic of *Sphaerotilus natans* (Jenkins, 1993; Pandolfi *et al.,* 2007).

**Table 2.1** Commonly observed filamentous bacteria identified by morphological and staining characteristics

| Filament type | Filament shape | Cell septa | Sheath | Attached growth | Gram stain | Neisser stain | | PHB | Sulfur granules |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Filament | Granule | | |
| S. *natans* | St | + | + | - | - | - | - | + | - |
| Type 1701 | St/B | + | + | ++ | - | - | - | + | - |
| **H.hydrosis* | St/B | - | + | -/+ | - | - | - | - | - |
| Type 0914 | St | + | + | -/+ | -,+ | - | -/+ | + | - |
| Type 1851 | St/Sc | +/- | + | -/+ | +weak | - | - | - | - |
| Type 0041 | St/Sc | + | + | ++/- | +/V | - | -/+ | - | - |
| Type 0675 | St/Sc | + | + | ++/- | +/V | - | -/+ | - | - |
| Type 0092 | St/B | -/+ | - | - | - | - | - | + | - |
| Type 021N | St/Sc | + | - | - | - | - | -/+ | + | + |
| *M. parvicella* | C | - | - | - | + | - | + | + | - |
| Nocardioforms | I | + | - | - | + | - | + | + | - |
| *N. limicola* I | C | + | - | - | + | + | - | + | - |
| *Thiothrix* I | St/Sc | + | + | - | -/+ | - | -/+ | + | + |

**Haliscomembactor hydrosis*

***** St- Straight, SC- Smoothly curved,

B- Bent, C- Curved, I- Irregular

(+)- Positive response, (-)- Negative response, V- Variable

Traditional methods to identify filamentous bacteria in sludge samples based on direct (or after staining) microscopic observation are useful, cost effective and allow ease of use for routine research analysis, however suffer from low sensitivity (Guo and Zhang, 2012). This is seen in its

failure to differentiate between morphologically similar organisms with phylogenetic variance. Further, these methods are limited in differentiating the same species whose morphology has been altered by changes in environmental conditions (Martins *et al.,* 2004). This morphological approach for identification by use of staining alone, has therefore proved to be unreliable in the identification of filamentous bacteria (Mielczarek *et al.,* 2012).

### 2.7.2 Molecular Characterization of Filamentous Bacteria in Wastewater

The use of molecular techniques has complemented conventional identification techniques by a non-destructive *in situ* analysis (Merkel *et al.,* 1999). Further, this has provided the possibility of identifying specific populations of microorganisms in their native habitat without the need to isolate them (Sanz and Köchling, 2007). Molecular techniques such as FISH and Denaturing gradient gel electrophoresis (DGGE) have been extensively applied to the study of microbial consortia of flocs in activated sludge (Sanz and Köchling, 2007) (Martins *et al.,* 2004). FISH is a cytogenetic technique developed in the early 1980s, for the targeting of specific nucleic sequences (Hu *et al.,* 2014). FISH relies on the use of fluorescently labelled oligonucleotide probes, that target and hybridize to highly conserved regions of microbial rRNA within intact cells (Moter and Göbel, 2000; Schmidt *et al.,* 2012). The 16 rRNAs sequence domains are the main target molecules for the FISH procedure beacuase of their stability and high copy numbers (Amann *et al.,* 2001). Hybridization which is by far the most crucial step in the procedure (Fig 2.7), facilitates the binding under a stringent set of conditions in order to ensure proper annealing of fluorescence labelled, target-specific probe to the target sequence (Moter and Göbel, 2000; Chen and Chen, 2013).

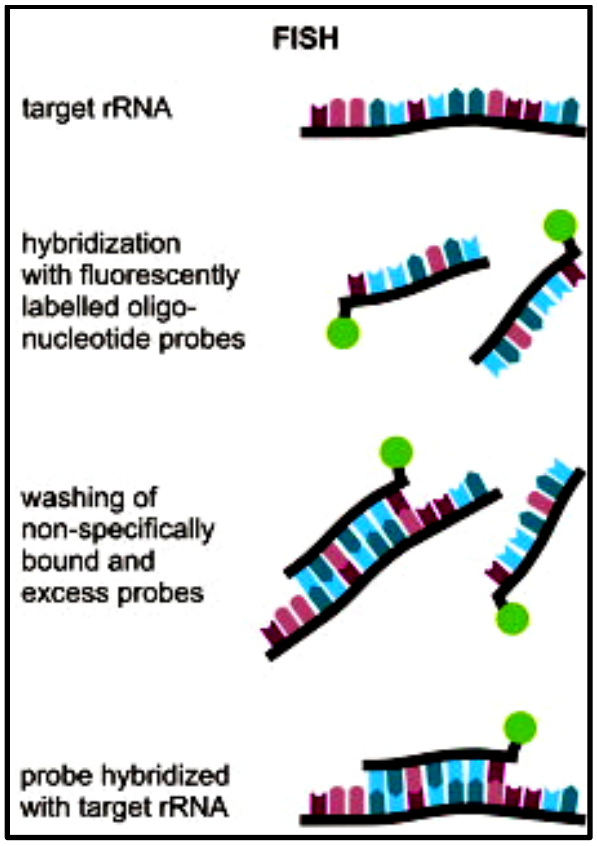


**Figure 2.7** Typical hybridization step of FISH procedure (Eickhorst and Tippkötter, 2008).

The specificity of both probe and target sequence has made FISH a method of choice in the reliable identification and enumeration of individual bacterial cells, together with microscopic characterization in environmental matrices (Wagner *et al.,* 2003; Wilén *et al.,* 2003; Gougoulias and Shaw, 2012). Currently available oligonucleotide probes used for the detection of filamentous bacteria in activated sludge as outlined in Table .2.2.

**Table 2.2 List of some available FISH probes for the identification of filamentous bacteria (Martin *et al.*, 2004)**

| Filamentous bacteria | Probe name | Reference |
|---|---|---|
| **Type 1851** | CHL 1851 | Beer *et al.*, 2002 |
| **Type 021N** | 21N | Wagner *et al.*, 1994 |
| **Type 0041/0675** | TM7905 | Hugenholtz *et al.*, 2001 |
| **Type 1701** | AQS977 | Thomsen *et al.*, 2004 |
| **Type 0092** | CFX223 | Hugenholtz *et al.*, 2001 |
| ***S. natans*** | SNA23a | Wagner *et al.*, 1994 |
| ***Haliscomenobacter spp.*** | HHY | Kim *et al.*, 2002 |
| ***Gordonia* spp.** | Gor-0596 | De los Reyes *et al.*, 1998 |
| ***N.limicola*** | N.limII175 | Liu *et al.*, 2001 |
| ***M.parvicella*** | MPA60;MPA223;MPA645 | Erhart et *al.*, 1997 |
| ***Acinetobacter* spp.** | ACA | Wagner *et al.*, 1994 |
| ***Leptothrix discophora*** | LDI | Wagner *et al.*, 1994 |
| ***Leucothrix mucor*** | LMU | Wagner *et al.*, 1994 |

# 3. Chapter Three: Identification and characterization of filaments and epiphytic bacteria in activated sludge

## 3.1 Introduction

The external colonization of Eikelboom morphotypes 0041, 0675 and 1851 commonly termed as attached growth has for a long time been used in identification keys, for the characterization of these morphotypes (Eikelboom, 2000; Jenkins, 2004). This has been helpfully in identifying filamentous morphotypes in AS, which are believed to be causative agents of bulking (Kragelund *et al.,* 2007). Additionally, the existence of these morphotypes has been identified in conditions of low dissolved oxygen, low food to microorganism and long mean cell retention time using their unique appearance (Guo *et al.,* 2014). However, very limited information is available in literature on the identity and ecophysiology of microorganisms colonizing filamentous bacteria in activated sludge (Xia *et al.,* 2008).

Conventional identification based on staining (Gram and Neisser) and microscopy is limited in ascertaining the species level identification of epiphytes on filamentous bacteria. This is further complicated by the lack of pure culture representatives, as knowledge surrounding the exact environments for their growth is currently unavailable (Stewart, 2012).

The use of molecular methods in ascertaining the identity of epiphytic bacteria, is crucial in bridging gaps availed by traditional microscopic and culture dependent techniques (Martins *et al.,*

2004). In a detailed study by Xia *et al.* (2008) investigating the presence of epiphytic bacteria colonizing filamentous bacteria it was found that most epi-flora bacteria hybridized with an oligonucleotide probe designed to target most of the members of the family *Saprospiraceae* in the phylum *Bacteriodetes* (Xia *et al.*, 2008). The aim of this aspect of the study was to implement culture independent FISH techniques for the group level characterization of filamentous morphotypes and epiphytic bacteria attaching to their surfaces in activated sludge samples from in and around Durban, South Africa.

## 3.2 Materials and Methods

### 3.2.1 Sample collection

AS samples were collected from the aeration tanks and returned activated sludge (RAS) streams of different WWTP treating both domestic and industrial wastewaters in Kwa-Zulu Natal, South Africa (Table 3.2). Grab samples of 750 mL were collected and stored at 4°C during transportation for microscopic analysis within 24 h period of collection.

### 3.2.2 Preliminary characterization and identification of filamentous bacteria with epiphytic attachment using conventional staining techniques

Preliminary identification and characterization of filamentous morphotypes prone to epiphytic growth was achieved by the use of microscopy and staining techniques. Grams staining (Appendix 1) and Neisser staining (Appendix 2) was performed on air dried samples, to assess the presence and abundance of epiphytic growth on filamentous bacteria in and around the floc. Morphological assessment of the morphotypes taken into consideration in the identification exercise were presence of sheath and filament shape, as outlined by Eikelboom (2000) and Jenkins *et al.* (2004) (Table 2.1). Filamentous bacteria prone to epiphytic growth were characterized and identified based on the morphological features and staining reactions to both stains in accordance with the guidelines of Jenkins *et al.* (2004).

### 3.3 Molecular characterization of filamentous bacteria with epiphytic attachment

#### 3.3.1 Oligonucleotide Probe Selection

The identity of the epiphytic bacteria was investigated using FISH with universal and group level oligonucleotide probes (Table 3.1) to target all host filamentous bacteria and epiphytes. Probes were labelled with sulfoindocyanine dye (Cy3) (CNR-Italy).

**Table 3.1** 16S rRNA targeted oligonucleotide probes used in this study

| Probe | Sequence(5'-3') | Target organisms | FA (%) | References |
|---|---|---|---|---|
| **EUBmix** | GCTGCCTCCCGTAGGAGT | All bacteria | 35 | (Daims *et al.*, 1999) |
| **ALF968** | GGTAAGGTTCTGCGCGTT | Alphaproteobacteria | 20 | (Manz *et al.*, 1992) |
| **BET42a** | GCCTTCCCACTTCGTTT | Betaproteobacteria | 35 | (Manz *et al.*, 1992) |
| **GAM42a** | GCCTTCCCACATCGTTT | Gammaproteobacteria | 35 | (Manz *et al.*, 1992) |

### 3.3.2 Fluorescence In-Situ Hybridization

#### 3.3.2.1 Sample fixation and dehydration

Samples were fixed in 4% Paraformaldehyde (PFA) and stored at -20ºC for further analysis as per the protocol by Amann (1995a). Previously fixed samples (10 µL) were spotted on wells of teflon slides pre-coated with Poly-L- Lysine (Sigma Diagnostics, USA). Slides were thereafter air dried and followed by dehydration in an ethanol series (50%, 80% and 100%) for 3 min incubation period each, for the removal of excess water.

#### 3.3.2.2 Whole cell hybridization

Hybridization was performed as previously described by Amann (1995). A range of formamide concentrations was used for hybridization buffer preparation (Table 3.1) in 2 mL microcentrifuge tubes. Using the freshly prepared buffers, all probes were diluted to obtain a working solution with a concentration of 50 ng/µL. The buffer and probe mixture was then added to dehydrated samples in wells. Remaining buffer was used to moisten tissue paper placed in 50 mL hybridization chambers. Slides were introduced into hybridization chambers and incubation was carried out at 46°C overnight. Following hybridization, slides were rinsed with warm wash buffers and inserted into wash buffer tubes which were then placed in water bath and incubated at 48°C for 10 min. Afterwards the washed slides were rinsed with cold milliQ water. Following the wash step, slides were air dried in a vertical position. Hybridized samples were counter-stained with 3µL of DAPI

(4’,6-diamidino-2-phenylindole) and allowed to stand at room temperature in the dark for 10 min and thereafter rinsed with warm milliQ water. Dry Vector shield mounting agent drops were added to wells and coverslips placed on; to these pressure was applied to ensure even distribution of mounting agent. The slides were viewed using an Epifluorescence microscope (Olympus BX51).

## 3.4 Results

### 3.4.1 Primary identification and characterization of filamentous bacteria with epiphytic attachment

Conventional staining techniques were employed to identify the specific filamentous bacteria prone to epiphytic attachment as shown in Table 3.2. The common filamentous bacteria with epiphytes viz. Type 0041, 0675, and 1851 were detected from all the samples analyzed and were found to be more abundant in domestic wastewater as compared to the industrial samples. The Eikelboom Type 0041 was found to be the dominant filamentous bacteria with epiphytes both in domestic and industrial samples which was followed by Type 1851. Observations revealed a variation in the abundance of epiphytes colonizing specific filaments in domestic and industrial systems. It was interesting to note that the filamentous morphotype 0041 exhibited dense epiphytic attachment in domestic wastewater samples compared to the industrial sample, while Type 1851 showed sparse attachment in both domestic and industrial samples (Fig 3.1a). The common plant operational conditions that this phenomenon was observed to be prevalent under Low F/M and DO (Table 3.2). Further, the presence of Type 1851 as a single bent filament was accompanied by dense epiphytic attachment observed on the filament when in bundle formation (Fig 3.1 d).

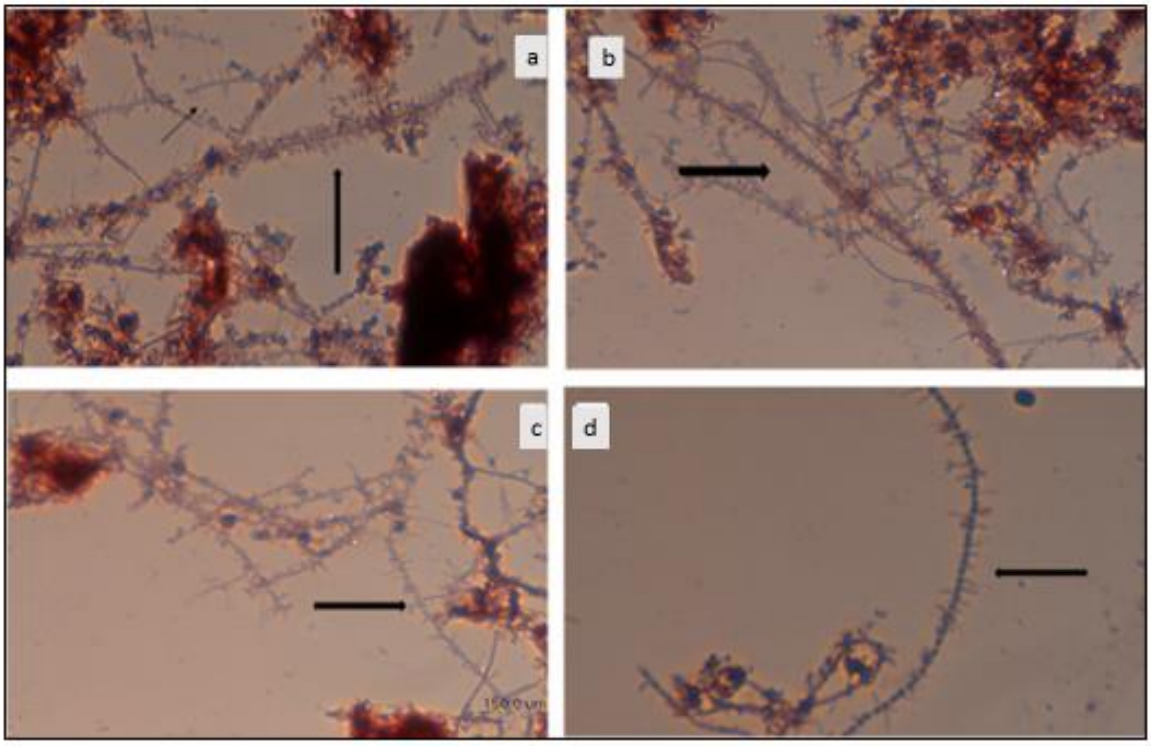


**Fig 3.1.** Conventional staining of filamentous morphotypes prone to attached growth in activated sludge a) Morphotype 0675, exposed region densely colonized by epiphytes, filament cells appear dotted within a sheath holding cells in place, b) Type 0041, densely colonized, by epiphytes, c) Sparsely colonized morphotype 1701, d) Type 1851, present outside floc as a single slightly bent filament with sparse attached growth.

**Table 3.2** Preliminary identification of filamentous bacteria with epiphytes and plant operational conditions

| WWTP'S | Influent type | Composition | Operational conditions | Dominant filament/s |
|---|---|---|---|---|
| **Amanzimtoti** | Municipal 50% Domestic 50% Industrial | Household Brewery Textiles Chemical | Low F/M | *Gordonia* Type 1851 Type 0041 |
| **Craigie Burn** | Domestic | | Low F/M High Oil | *Gordonia* Type 021N Type 0041 |
| **Darvil** | Municipal 90% Domestic 10% Industrial | Household Fats and Oils | Low F/M Nutrient deficiency | Type 021N *Gordonia* Type 0041/0675 |
| **Hammersdale** | Industrial | Poultry Textiles | Low F/M High Oils | *M.parvicella* *Gordonia* Type 0041 |
| **Kingsburg** | Domestic | | Low F/M Low D.O | Type 0092 Type 0041/0675 Type 1851 |
| **Northern** | Industrial | Dyes Textiles | Low D.O Nutrient deficiency Low F/M | *M.parvicella* Type 0092 Type 1851 |
| **Southern** | Domestic | | Low F/M Low D.O | Type 0041 Type 0092 Type 1851 |

*F/M ratio was found to be <0.5, in samples in which colonized filamentous morphotypes present, with a D.O range between 0.10-1.18mg/L

### 3.4.2 Characterization of epiphytic bacteria using FISH

EUB mix and group level probes were used to assess the identity of the epiphytes using FISH (probes tabulated in Table 3.1). The epiphytes that colonized the filamentous bacteria were

dominated by the class Alpha-proteobacteria (ALF 968) both in domestic and industrial samples followed by BET42a and GAM42a.

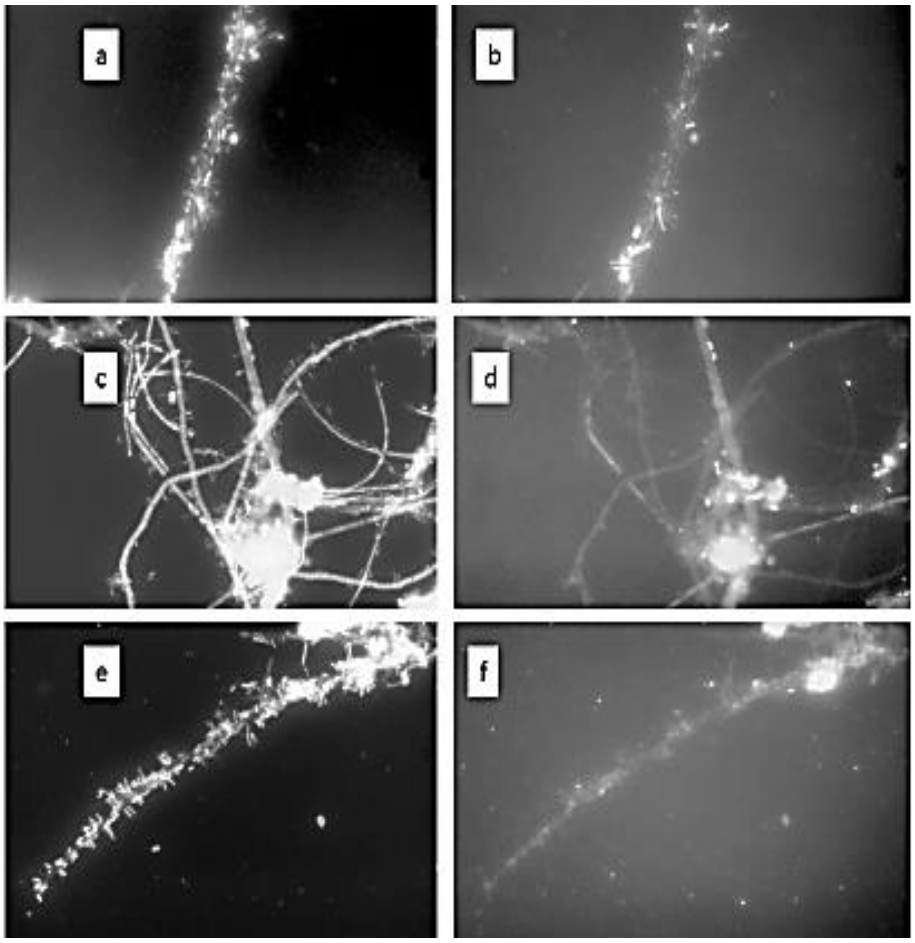


**Fig3.2** a-f Labelling of flocs with EUBmix probe and group level probes (Alpa, Beta and Gammaproteobacteria).

a. All bacteria labelled with EUBmix show positive binding, including heavily colonized filament together with epiphytic growth.

b. Alphaprotoebacteria positive select few epiphytic bacteria, while filament is Alpha negative.

c. EUBmix positive microcolonies, including epiphyte colonized filament.

d. Beta negative filament and epiphytes.

e. Colonized filament is EUB-mix positive.

f. Filament and epiphytes are Gamma negative, with a fraction of the floc showing positive binding with Gammaproteobacteria probe.

### 3.5 Discussion

The common filamentous bacteria prone to epiphytic attachment were present in all samples analyzed from domestic, municipal and industrial plants. The most dominant filaments in all the plants were morphotypes Type 0041, Type 0675 and Type 1851 (Table 3.2). A consistency in the dominant morphotypes was apparent for all samples. This observation was in line with observations of survey conducted by Lacko *et al.* (1999) of AS plants in Kwa-Zulu Natal, additionally this observation was also in line with that of Eikelboom *et al.* (1998) and Madoni *et al* (2000). Observations of epiphytic growth on morphotype 1851 showed a unique pattern, that varied when the filament was seen as a single bent filament, and when it had formed bundles. The epiphytic growth was sparse when the filament was in bundle and dense on single filaments (Fig. 3.1d). This can be attributed to the limited surface exposure for epiphytic attachment when the filament was in bundles.

The epiphytes when visualized under light microscopy, often resembled branching which is a characteristic feature of *Nocardia* spp. (Fig 2.5b). However the consistency of the 90° angle of attachment exhibited by the epiphytes aided in ruling out branching which occur at random points with no uniform pattern. This observation was found to be in line with that of Xia *et al.* (2008).

The identity and ecophysiology of microorganisms colonizing certain filamentous bacteria in AS still remained unclear, based on conventional microscopic observations. This is due to the limited

information available on the epiphytes (Xia *et al.,* 2008a). In a detailed study conducted eight Danish WWTPs, one American WWTP and one Swedish WWTP by Xia *et al.* (2008), it was observed that most epiflora colonizing filamentous bacteria hybridized with a probe designed for members of *Saprospiraceae* belonging to phylum *Bacteriodetes* (Xia *et al.,* 2008). However, certain groups of epiphytes did not hybridize with this probe and group level probes were therefore used in this study as the primary step of molecular characterization of the epiphytic bacteria. In this study epiphytes that hybridized with the Alpha-proteobacteria probe showed a higher binding affinity as compared to the beta and gamma groups. The FISH results also revealed that Alpha positive cells were the heaviest colonizers of specific filamentous bacteria while Beta and Gammaproteobacteria positive cells were the least colonizers prone to this epiphytic attachment. A consistency in the binding affinity of the Alphaproteobacteria probe was apparent between the samples.

Furthermore, FISH observation revealed binding of the universal probe EUBmix to morphotypes with dense epiphytic growth (Fig 3.2e) and those prone to sparse epiphytic attachment varied (Fig 3.2c). Type 0041 which were densely colonized by epiphytes showed a negative binding with EUB probes, while epiphytes colonizing this morphotype 0041 were positively bound with EUBmix probe. The opposites was observed with Type 1851 which is sparsely colonized, both filament and epiphytic counterparts were both bound by the EUBmix probe. In the case of Type 0041 the lack of target sites for all EUBmix probes on *Chloroflexi* filaments can be taken into consideration

(Nielsen *et al,*. 2009). Additionally the possibility of inefficiency of probe to penetrate into the filament cells due to the heavy attachment should also be considered.

### 3.6 Conclusion

This chapter focused on characterization and identification of epiphytic bacteria colonizing Eikelboom morphotypes 0041/0675 and Type 1851 in domestic and industrial AS samples. Morphotype 0041 was found to be heavily colonized by epiphytic bacteria in domestic samples and less dense attached growth in industrial samples. Type 1851 was found to have more epiphytic growth when present as a single bent filament in samples, however sparse growth was observed on this morphotype when present as bundles. FISH aided in the identification of the epiphytes based on their response to group level probes: Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria. A consistency in the binding of Alphaproteobacteria probe was observed, as majority of epiphytes show a high binding affinity with this group level probe. Additionally the Alphaproteobacteria positive cells were seen to colonize all morphotypes prone to epiphytic attachment, however more Alpha positive cells were visualized on morphotype 0041. The binding of EUB mix probes was apparent of the epiphytes colonizing Type 0041, while the host filament was however unbound by this universal probe. The response of this morphotype maybe attributed to the lack of probe penetration due to the heavy epiphytic attachment on the surface of this filament.

# 4. Chapter Four: Evaluation of the morphology between epiphytes and filamentous bacteria

## 4.1 Introduction

The degree of association between filament hosts and epiphytic counterparts in AS treatment has for a long time been unclear. Recent studies have shown that some of these epiphytes belong to a protein-hydrolyzing group of bacteria suggesting a potential relationship between the filamentous morphotypes and epiphytes (Xia *et al.*, 2008; Otzen, 2010). The usefulness in assessing the interior microstructure of flocs and that of individual cells with conventional light microscopy is limited (Wilén *et al.*, 2003). Therefore advanced microscopic techniques such as electron microscopy ought to be incorporated in the detailed study of individual cell and floc morphology (Martins *et al.*, 2004).

SEM is a powerful surface visualization tool used in biological imaging. A high resolution 3D image is created as a tightly focused beam of electrons scans over the specimen, and secondary electrons are detected (Denk and Hortsmann, 2004). The use of SEM is pivotal in understanding the surface morphology. However, for insight on the ultrastructural composition of the microbial consortia the use of TEM is essential. TEM makes use of a broad beam of electrons which are directed at a two-dimensional cross section of the sample, that is thin enough to allow a substantial fraction of the electrons to pass through and thereby provide an accurate image of ultrastructure (Denk and Hortsmann, 2004). In this study the morphology (relationship) of filamentous bacteria

with epiphytic bacteria was investigated at surface and intracellular levels using SEM and TEM respectively.

## 4.2 Experimental Procedure

### 4.2.1 Sample preparation for scanning electron microscopy: Concentration of filaments with epiphytes.

The AS samples (50 mL) were subjected to a series of washes with different concentrations of Tween 80 (50 and 80 %), at exposure times between 1-5 mins. The determined optimal concentration of Tween 80 was 50 % at exposure time of 5 min. Following this, the Tween 80 treated samples were transferred to 10 mL tubes and further sonicated at 2 watts for 30 sec to break flocs and further release filamentous bacteria. The sludge samples were thereafter filtered using sieves of different pore sizes (300 µm, 150 µm, 100 µm and 75 µm) rendering a less compact floc. Following this, wet mounts were prepared and viewed using Nikon Eclipse 80i phase contrast microscope with oil immersion.

### 4.2.2 Scanning electron microscopy

The filtered samples were spotted on glass coverslips, air dried and subsequently coated with gold according to Li *et al.* (2005) (Appendix 6). The gold coated slides were viewed at low vacuum under the Zeiss Ultra Plus field emission gun scanning electron microscope (FEGSEM).

### 4.2.3 Sample preparation for transmission electron microscope: Fixation and Infiltration

Two milliliter filtered sample was transferred to an Eppendorf tube and centrifuged at 7500 rpm for 5 mins. The supernatant was discarded and the pellet was fixed in buffered 2.5 % glutaraldehyde 0.1 M phosphate for 24 h. Furthermore, post fixation, infiltration and polymerization was carried out in accordance with Williams and Unz. (1985) and Jang *et al.*(2014) (Appendix 7).

#### 4.2.3.1 Ultrastructure sectioning and image analysis

Ultra-thin sections were obtained using a Leica UC7 Ultra-microtome; the micro-sections were cut to a thickness of 100 nm. These were stained with Toluidine blue and observed under light microscopy and later mounted on grits and coated with gold to make them conductive and observed under JEOL 1010 TEM at 60 keV.

## 4.3 Results

### 4.3.1 Surface morphology of filaments and epiphytes using scanning electron microscope:

SEM analysis was carried out on filtered sludge samples, to assess the morphology of the epiphytic attachment to specific filamentous bacteria. At both lower and higher magnifications, it was noted that the main fractions of organisms were embedded in the dense matrix formed by EPS. Epiphytic attachment was observed on the exposed regions of filaments protruding out of the floc. Under light microscopy attached growth resembled branching which is common in *Nocardia* spp. and other Gram positive bacilli found in activated sludge (Fig 2.5 a and b).

#### 4.3.1.1 Assessment of branching

Branching has been observed among some filamentous species such as *Nocardia* spp. (Figure 4.1a). Branching occurred at 2 major parts, along the length as well as at this tip of filaments of the filamentous bacteria. A distinct difference between the true branching occuring when regions of filament trichome protrude and extend in multiple directions, exhibited by e.g. *Nocardia* spp. in AS samples) (Fig 4.1a) and attached growth (attachment of bacterial rods on surface of filamentous bacteria) (Fig 4.1 b,c and d) were apparent under SEM.

#### 4.3.1.2 Assessment of Attachment

It was noted that the epiphytic rods were attaching perpendicular to the filament host, where the angle of attachment was approximately 90° (Fig 4.1 b,c and d). The frequency of attachment varied between regions of the exposed filament where some became heavily colonized while other regions had very few (1 to 3) epiphytes attached to them. SEM analysis however did not confirm the mode and depth of these attachments, as there were no visible surface adhering appendages detected.

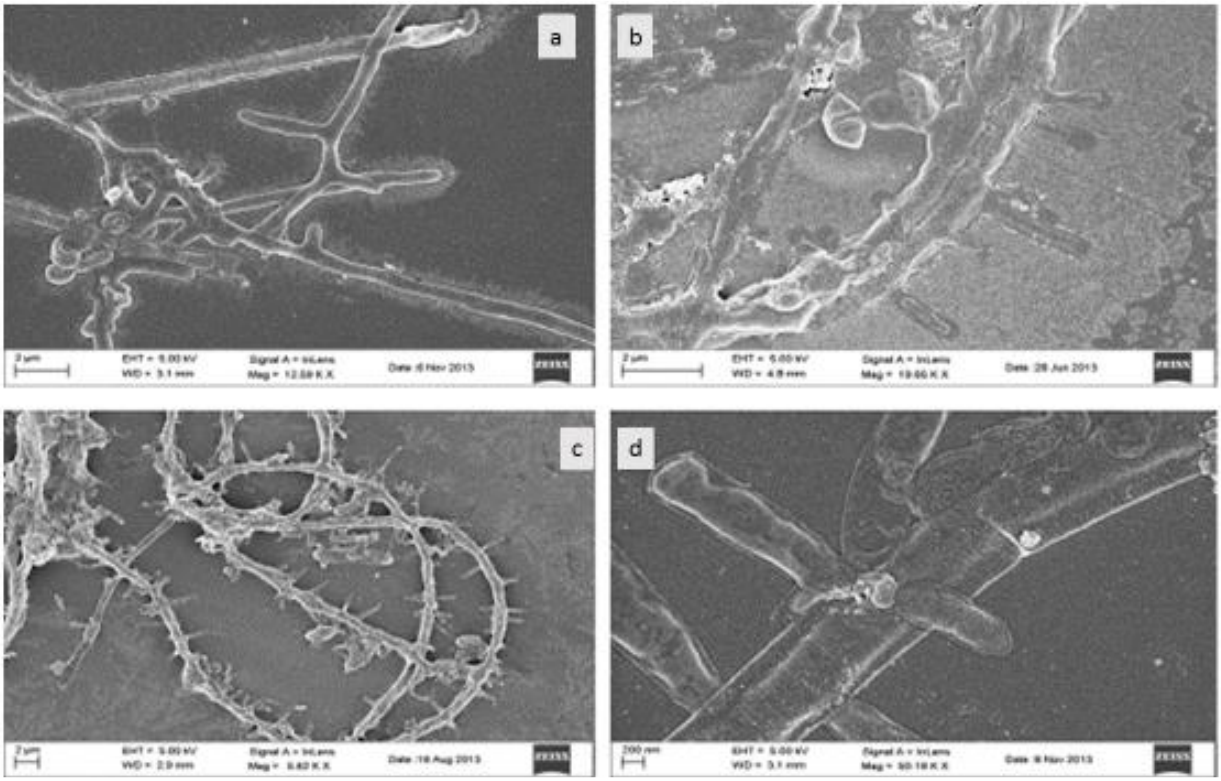


**Figure 4.1** a) Shows the irregular positioning of true branches of *Gordonia* spp. where cells of the filament protrude at random points along the length of the filament. b) Shows bacterial rods encased within individual sheaths while epiphytically attaching to filament bacteria. c and d) Depict the typical epiphytic attachment which conforms to the 90° angle style of attachment

### 4.3.2 Ultra structure analysis using TEM

#### 4.3.2.1 Assessment of attachment at interface

Ultrastructural investigations were further carried out to further understand the mechanism of attachment at the intracellular level. From the TEM images it was obvious that the mode of attachment is independent of the surface protrusions such as fimbria and pili. Furthermore, no penetration of the sheath was observed at the interface between filament host and epiphyte (Fig 4.2 c and f). Though finger-like projections were visualized at the pole end of certain epiphytic bacterial cells as they approached the filamentous bacterial host, the role of these projections in attachment was however not evident.

#### 4.3.2.2 Investigation of adhesion mechanism

Presence of amyloid-like structures (known to be proteinaceous beta sheet folded structures) were observed at the interface of filament cell and epiphyte (Fig 4.2 a-f ). This observation of the presence of intracellular amyloid fibrils is reported for the first time in this study. It was also noted that the epiphytes were attached to those cells with amyloid like structures and the cells with no amyloid like structures showed no signs of epiphytic attachment on their surface. Further, it was noted in the cells colonized by the epiphytes, that the position of fibrillar (amyloid like) structures was either in the direction of the approaching epiphyte or often directly below the point of contact between filaments and epiphytes (Fig 4.2 b, c and d). These amyloid fibrils possibly serve as

potential preferred attachment sites for the epiphytes colonizing selected filaments (Fig 4.2 b, c and d ).

#### 4.3.2.3 Intracellular inclusions

The presence of intracellular storage compounds was noted in most of the filamentous bacterial host cells colonized by epiphytic bacteria (Fig 4.2 b-f). In was interesting to note that the cells observed with amyloid like structures also showed a high amount of intracellular storage compounds. Rarely was intracellular storage observed with un-colonized filamentous bacterial cells.

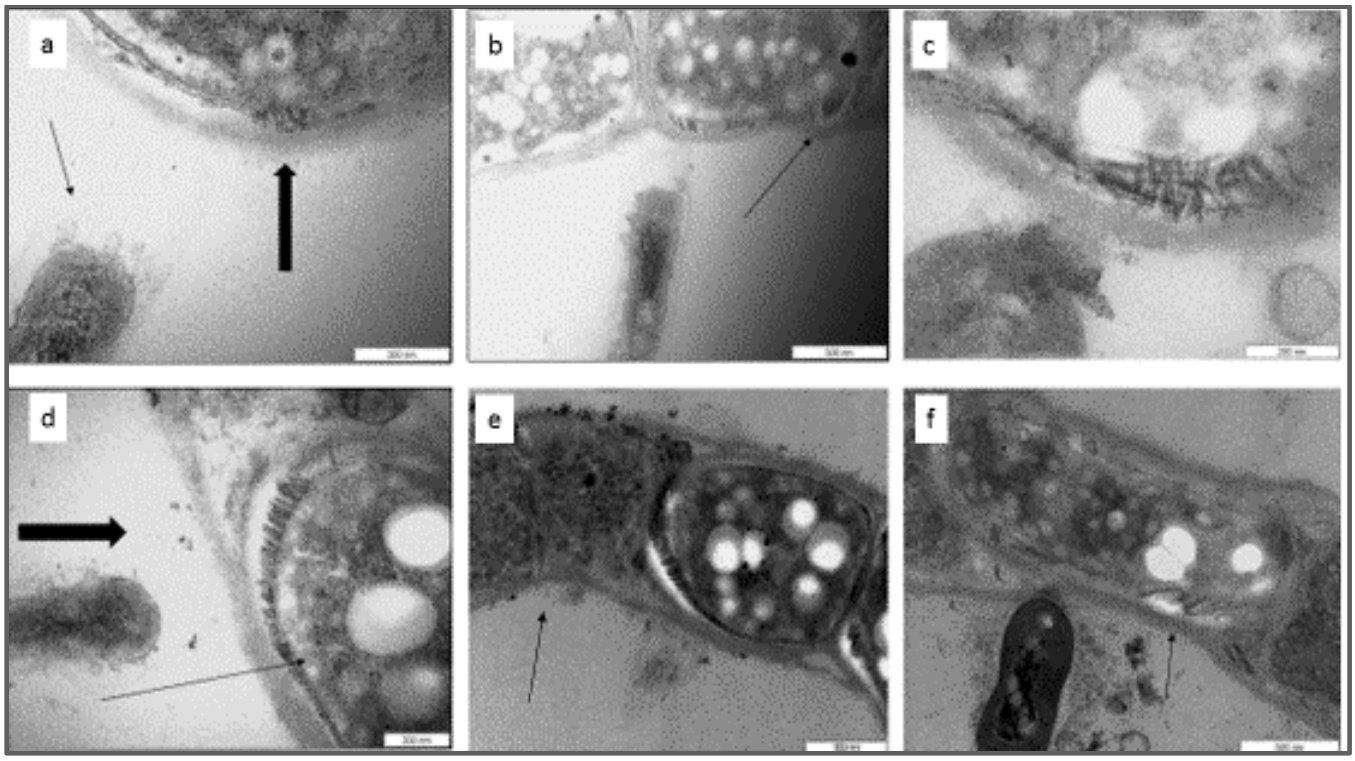


**Fig 4.2** a-f TEM micrographs depicting the presence of fibrillar structures emanating from within filamentous bacterial cells being targeted by bacterial rods, the direction of fibrils is towards the approaching/attached bacterial rod.

a, b. Bacterial rods approaching filament compartment, have finger-like projections extending from cell membrane of epiphytic cell as it approaches the targeted filamentous bacterial cell; protrusion is in the direction of filament compartment.

c and f. TEM micrograph showing the attachment site between the filament and the epiphytic rod bacteria. Intact filamentous sheath was observed.

b,c,d,e and f, depicts the presence of storage compounds within filament compartment being approached and also attached to by epiphytic bacteria.

e. Upper adjacent compartment of filament is devoid of stored compounds.

## 4.4 Discussion

Apart from the basic morphological features, the growth of bacteria as branched or unbranched filaments, living in sheathed or unsheathed chains, or aggregate in primitive or highly organized multicellular composites has been displayed by organisms in AS (Young, 2006). Attached growth is also a common mode of existence for certain bacteria in AS. This has been noted by the growth of bacterial rods on the surface of filamentous bacteria (Xia *et al.,* 2008).

Under light microscopy, the epiphytic attachment resembled branching. Owing to the high magnification and resolution of SEM the distinction between branching which is a characteristic feature of *Nocardia* and many other actinobacterial species (Fig 4.1a) and epiphytic attachment were observed and diffrentiated.

Epiphytic attachment occurred as external entities (bacterial rods) colonizing and attaching at a 90° (Fig 4.1b,c and d ) angle to filamentous bacteria. This perpendicular attachment of epiphytic bacteria to filaments in activated sludge were in line with findings by Xia *et al.* (2008). It was interesting to note that the epiphytic bacteria colonizing filaments were individually enclosed in sheaths (Fig 4.1b and d ).

Furthermore SEM observation at cell-to-cell level of exposed filament regions showed that the attachment mechanism exhibited by the bacterial rods may have been facilitated by additional factors than the presence of EPS, compared to that of the rest of the floc as these regions were not

completely embedded in the EPS matrix, suggesting a morphological mechanism of attachment. This observation was in-line with the hypothesis made by Romero *et al.* (2010) of adhesion being facilitated by a selective mechanism beyond the presence of the EPS layer (Romero *et al.,* 2010).

TEM was used for ultra-structural level investigations of the mechanism of this attachment at the interface between filament cells and epiphytes. Under TEM certain epiphytic cells exhibited cellular membrane extensions and these protrusions were visualized on portions approaching the filament host (Fig 4.2 a and b) and those resting on the surface of the filament cell being colonized (Fig 4.2c). However, observations did not reveal common bacterial surface attachment appendages such as fimbria or pili at the interface between filament host and epiphyte.

TEM observation further postulated the presence of amyloid like structures in the host filament cells being targeted by the epiphyte. The presence of amyloid protein has been detected in AS in relation to biofilm formation and floc aggregation (Larsen *et al.,* 2007).

In addition amyloid fibrils play a functional role in facilitating adhesion, as well as invasion of host cells and host-pathogen interactions (Zhou *et al.,* 2012). Furthermore these proteins are known to also function as attachment sites for the epiphytic bacteria (Nielsen *et al.,* 2009b). The TEM observation in this study suggested the possibility of a selective amyloid-like nature of interaction between the host filamentous bacteria and epiphytes.

It was also interesting to note that the position of the intracellular amyloid-like structure were inclined in the direction of the approaching epiphyte and observed often directly below the interface point between filament and the epiphyte (Figure 4.2 a-f). The amyloid-like structures were furthermore mostly visualized in cells containing an abundance of storage compounds (Figure 4.2 b-f).

Epiphytes showed to selectively attach to those host cells with intracellular storage compound (Figure 4.2 b-f ). These findings suggested a potential nutritional interaction between selected filaments and epiphytes. Additionally the presence of small polyhydroxyalkanoates (PHA) granules have been noted with the *Curvibacter* and *Chloroflexi* filaments, however TM7 filaments have never been examined for PHA storage (Nielsen *et al.*, 2009a). Xia *et al.* (2008) speculates about a possible symbiotic relationship between the filamentous and epiflora bacteria which however, was not verified. It has been noted by Otzen (2010) that the amyloid-protein producing filamentous bacterium *Chloroflexi* is often covered by multiple copies of epiphytic *Candidatus Epiflobacter* species (*Saprospiracea Bacteriodetes*) an epiphyte that specializes in protein degradation (Otzen, 2010). According to a study conducted in Denmark, it was observed that members of the *Saprospiracea* family were in abundance in AS, where they are involved in the breakdown of complex organic compounds (McIlroy and Nielsen, 2014).

## 4.5 Conclusion

The focus of this chapter was on evaluation of the morphology between the filamentous bacteria and their epiphytic counterparts and potentially understanding the mechanism used by the epiphytic bacteria in facilitating attachment to filamentous hosts. The use of electron microscopy (SEM and TEM) to elucidate ultrastructural details between the epiphytes and filamentous bacteria at interface level was fundamental and it proved an important tool in understanding the interaction between the two parties. The observations showed a level of selectivity between epiphytes and filamentous morphotypes colonized, further a more intricate selectivity existed with the type of filament cells that were colonized. Absence of bacterial attachment appendages was noted. However amyloid-like structures were observed in such cells and were believed to be potential attachment sites for the epiphytes. The preferred cells of filaments colonized were rich in storage compound. The findings reported in this chapter suggest that a selective relationship exists between filamentous morphotypes and epiphytes. Therefore, we could postulate that is a certain degree specificity exists between the filament host and the colonizing epiphyte.

# 5. Chapter Five: Evaluation of the interaction between epiphytes and filamentous bacteria under nutritional stress conditions

## 5.1 Introduction

The cellular morphology of filamentous bacteria serves a nutritional function in accessing nutrients that would otherwise be completely out of reach (Young 2006). The added advantage of proliferation, gives filamentous bacteria an edge over non-filamentous bacteria, that is not protruding outside the main floc structure.

Production of storage compounds such as Polyhydroxybutyrate (PHB's) by bacteria under feast-famine conditions is a common phenomenon in AS (Fang *et al.,* 2009). These are carbon and energy reserve material stored intracellularly by a wide range of microorganisms under nutrient-limiting and other environmental stress conditions (Liu *et al.,* 2011).

The presence of EPS layer provide entrapment of organic material (Wilen *et al.,* 2008) but diffusion within EPS are lower than in water, which means that EPS strongly influences the availability of nutrients and extrusion of metabolic products (Sheng *et al.,* 2010). This significantly negatively impacts microbial life within the floc. It is under such conditions of nutrient limitation that filamentous bacteria proliferate in activated sludge (Guo *et al.,* 2014).

Proliferation is a selective advantage for filamentous bacteria over non filamentous ones in the sludge floc, which serves as an enhanced ability for substrate uptake under stress condition. In

activated sludge, the presence of bacterial rods colonizing filamentous bacteria with storage globules has been observed. Sheaths of large filamentous bacteria (eg.*Beggiatoa* and *Thioploca*) are often colonized by other bacteria belonging to the phylum *Bacteroidetes*, which are believed to use sheath material for growth (Xia *et al.,* 2008). Furthermore the presence of epiphytic bacterial rods on filamentous morphotypes such as Type 0041, 0675, 1851 has been reported (Xia *et al.,* 2008; Nielsen *et al.,* 2009).

Combination of operational conditions such as low F/M and extended sludge Retention time (SRT) accelerates the rate and presence of inactive biomass. Live/dead staining methods have proved to be a useful technique in assessing the viability of organisms by detecting active cells based on the cell wall integrity (Sheng *et al.,* 2010). Live/dead staining makes use of two individual fluorescent dyes Propidium Iodide (PI) and Calcein AM, staining damaged and intact cells respectively. PI is a widely used stain for detection of dead cells since the dye penetrate into cells with disrupted membranes and intercalate with DNA, resulting in red fluorescence in the nucleus (Rieger *et al.,* 2010). Cells with intact membranes are stained with Calcein AM, a highly lipophilic vital dye that rapidly enters viable cells, is converted by intracellular esterases to calcein that produces an intense green signal (Bratosin *et al.,* 2005). Since nutrient limiting conditions adversely affects all resident microbes, with filamentous bacteria showing a resilience under stress conditions, the need to evaluate the nutritional interaction between filament hosts and colonizing epiphytes was apparent.

The focus of this chapter is to evaluate the response of resident microbes to nutrient stress and to determine the presence and nature of energy rich storage compounds.

## 5.2 Material and Methods

### 5.2.1 Sample collection and concentration of filaments with epiphytic growth

Grab samples of 750 mL were collected from returned activated sludge (RAS) streams and aeration basins of different WWTP's treating domestic, industrial and municipal wastewaters in Kwa-Zulu Natal. RAS samples were diluted with equal parts of distilled water (1:1). The diluted samples were thereafter sonicated at 2 watts for 30 seconds using a sonicator (Misonix Ultra Liquid Processor, XL-2000 series, Trilab Support, South Africa). The sonicated samples were filtered in descending order through wire mesh sieves (Labotec suppliers, South Africa) of different pore sizes (300 µm; 150 µm; 100 µm and 75 µm) to remove compact flocs. Following this, wet mounts were prepared and viewed using Nikon Eclipse 80i phase contrast microscope under oil immersion.

### 5.2.2 Polyhydroxybutyrate (PHB) staining

PHB stain (Appendix 3) was used in the detection of polyhydroxybutyrate granules stored intracellularly by filamentous bacteria in accordance with Jenkins *et al.* (1993).

### 5.2.3 Live/Dead viability staining of filaments with epiphytic growth

Filtered samples were subjected to viability staining (Appendix 4) using Live/Dead staining kit (Sigma Aldrich). Working solutions of Calcein AM (1µM) and PI (1µM) in 100 µL Phosphate saline buffer (PBS) were prepared. Filtered samples (98 µL) were mixed with 2µL Live/Dead stain mixture (2µL Calcein AM + 2µL PI) and incubated at room temperature in the dark for 15 min. Following the incubation, a drop of stained sample was spotted on glass slides and covered with coverslips for viewing under Axiolab Zeiss fluorescent microscope. Viable cells were fluorescent green and non-viable cells were fluorescent red.

### 5.2.4 Elemental Analysis

Elemental analysis was carried out with two microscopic methods as stated below.

**a) <u>SEM elemental analysis</u>**

AS samples were spotted on circular glass cover slips (Appendix 6) and gold sputter coated in accordance with Li *et al.* (2005). Scanning electron microscopy was conducted on gold coated samples using a Zeiss Ultra Plus field emission gun scanning electron microscope (FEGSEM) fitted with an Oxford X-max 80mm SD detector for elemental analysis. Analysis was carried out at 20 kV under high vacuum on surface of host filamentous bacterial cell and that of epiphytic at the interface point.

**b) TEM elemental analysis**

TEM samples were prefixed in 2.5% glutaraldehyde in 0.1 M phosphate at pH 7.4 and prepared as outline in Williams and Unz (1985) (Appendix 7). Ultra-thin sections of 100 nm thickness were achieved using a microtome (Leica UC7 Ultra-microtome). Further sections were mounted on grits for staining with lead acetate and thereafter viewed under JEOL 1400 fitted with an Oxford X-max SD detector for elemental analysis.

## 5.3 Results

### 5.3.1 Concentration of filamentous bacteria with epiphytic attachment

Successful isolation of filamentous morphotypes with epiphytes attached was achieved by the use of filtration sieve of 75 µm pores size. The broken and less compact flocs were also retained in the filtrate. Filamentous morphotypes colonized by epiphytic bacteria were confirmed to be Type 0041, 0675, and type 1851 in all domestic, industrial and municipal samples using conventional identification keys (Jenkins *et al.*, 2004).

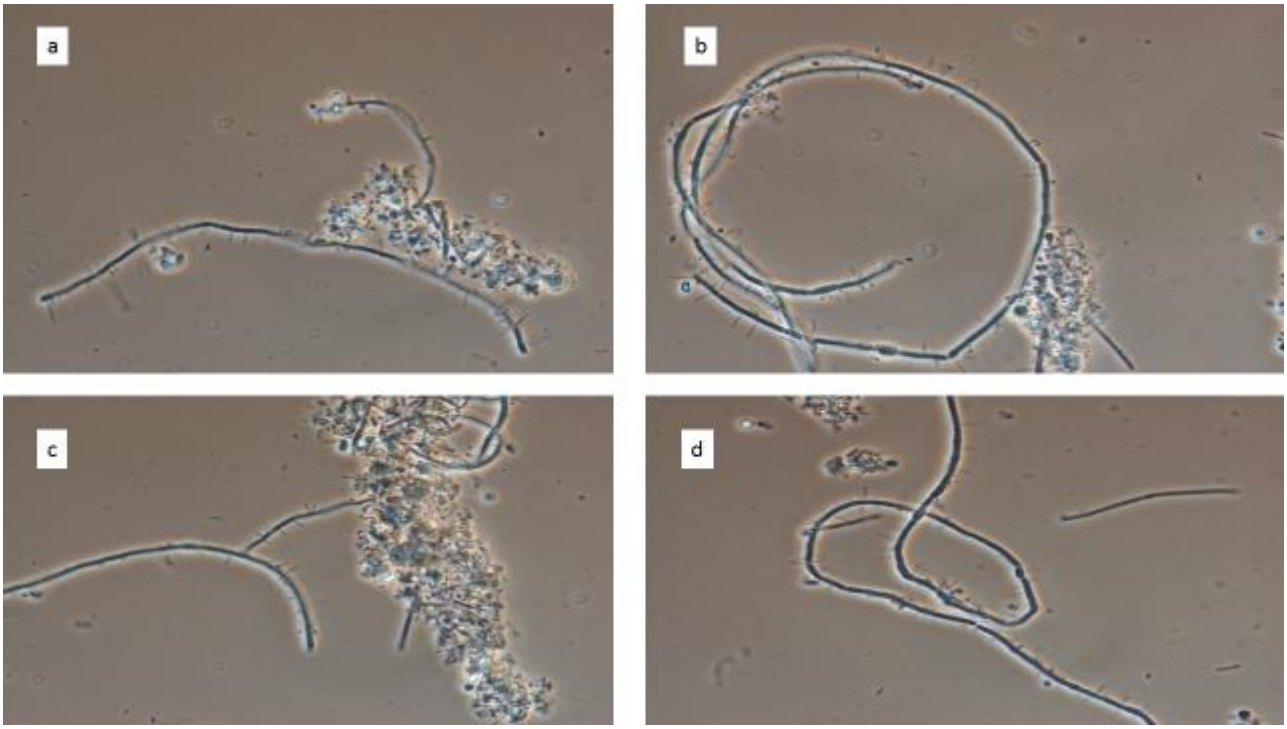


**Figure 5.1.** Filamentous bacteria observed under phase contrast at 1000x after final filtration with 75µm pore sized sieve. a, b and c exhibit less compact and diminished floc .d) depicts complete exposed heavily colonized filament.

### 5.3.2 Overall assessment of Floc viability

Live/Dead staining of RAS and aeration filtrates revealed a consistent pattern of bright red floc units. Majority of the floc forming bacteria in the interior of the floc structure were non-viable lending to the red fluorescence, while very few floc formers on the periphery of the floc structure were viable due to the green fluorescence. Additionally some filamentous bacteria protruding out of the floc structures fluoresced bright green (alive) Fig 5.2 d.

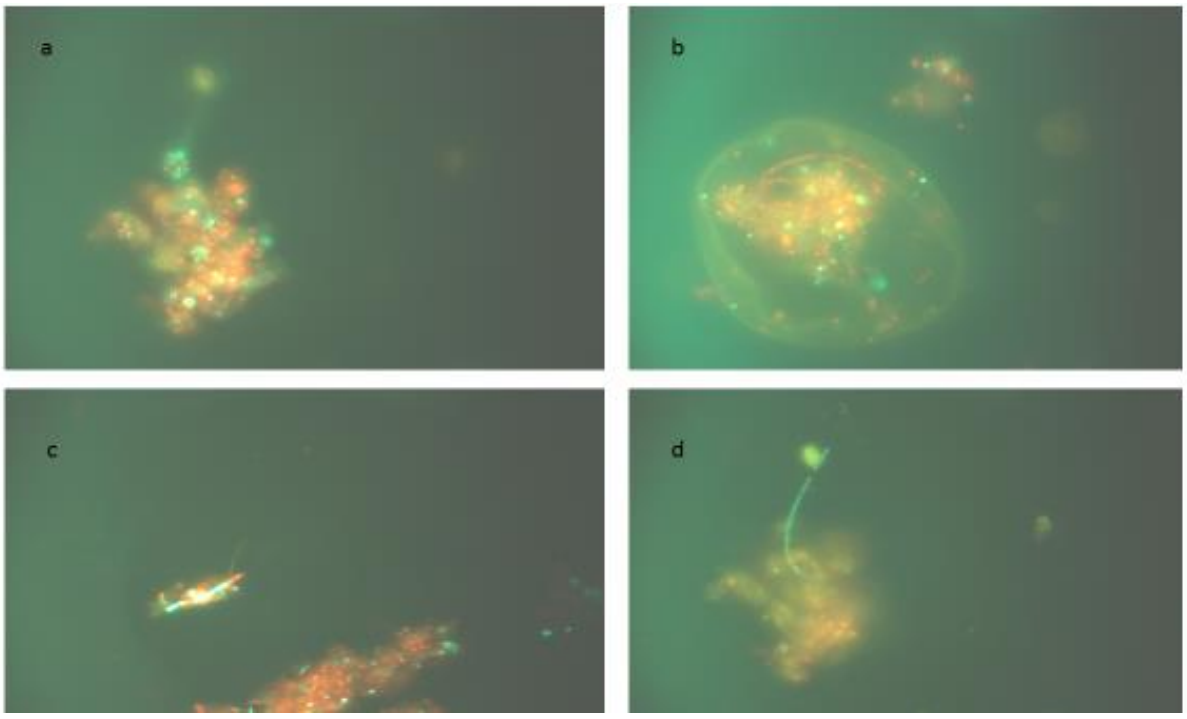


**Figure 5.2.** Depicted in micrographs a, b and c are the non-viable microflora within compact flocs, organisms towards center of flocs fluoresce red, while certain organisms towards periphery fluoresced green signaling viability. d) Depicts viability of a filamentous bacteria as it protrudes out of the floc structure.

### 5.3.4 Viability assessment of filamentous bacteria

Some filamentous bacteria isolated from the floc structure were observed to be viable while others were seen to have damaged cells within their trichomes (Fig 5.2 a and b). Filamentous bacteria colonized by epiphytic bacteria were observed to be viable together with their epiphytic counterparts. Live/dead staining revealed that those viable cells within trichomes of filaments showed the presence of epiphytic growth (Fig. 5.2b), while non-viable cells had no epiphytic attachment.

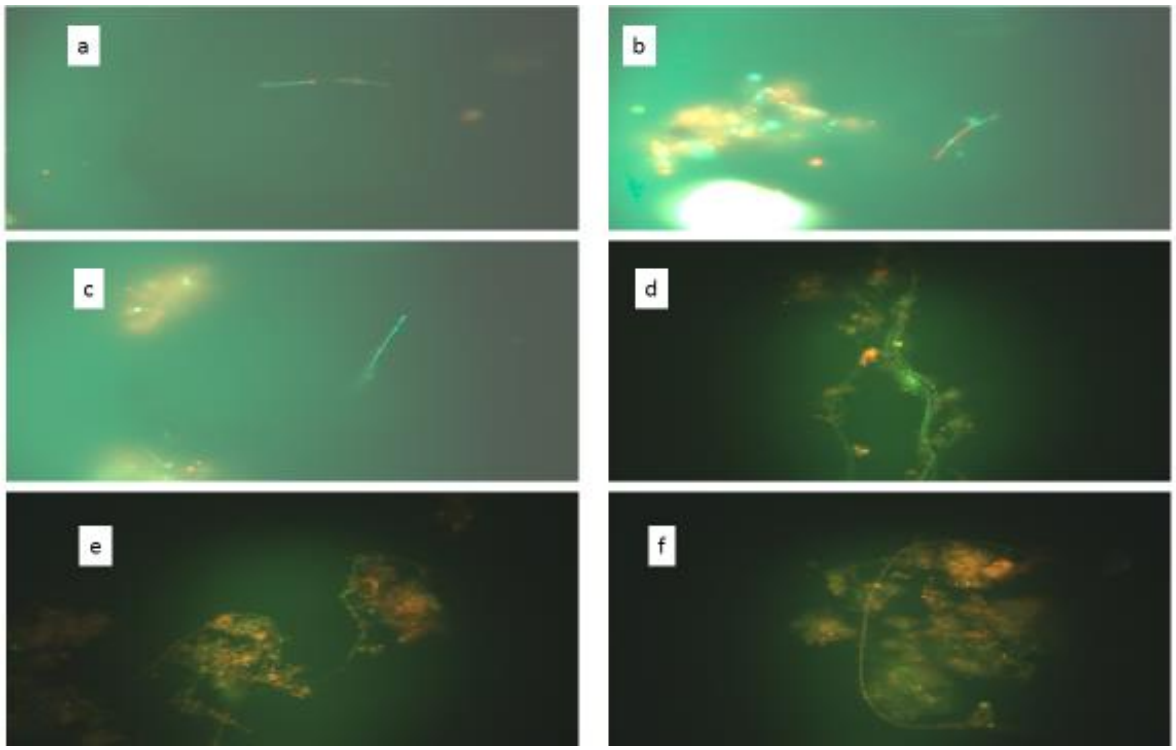


**Figure 5.3** Micrograph illustrates the existence of filamentous bacteria beyond floc structure. a) depicts the presence of non-viable cells among viable cells in a filament trichome. b) shows epiphytic bacteria attaching to only the viable cells of the trichome. c, d, e and f) viable epiphytic growth attaching to the only viable filaments around non-viable flocs.

### 5.3.5 Intracellular inclusions staining

Polyhydroxybutyrate (PHB) staining was carried out on smeared and air dried samples for the detection of intracellular polyhydroxybutyrate granules. Certain filament cells colonized by epiphytic bacteria had a positive reaction as a result of the presence of PHB granules. These appeared as dark blue/black globules within a pale pink background of the cell. Furthermore the epiphytic cells did not have intracellular granules.

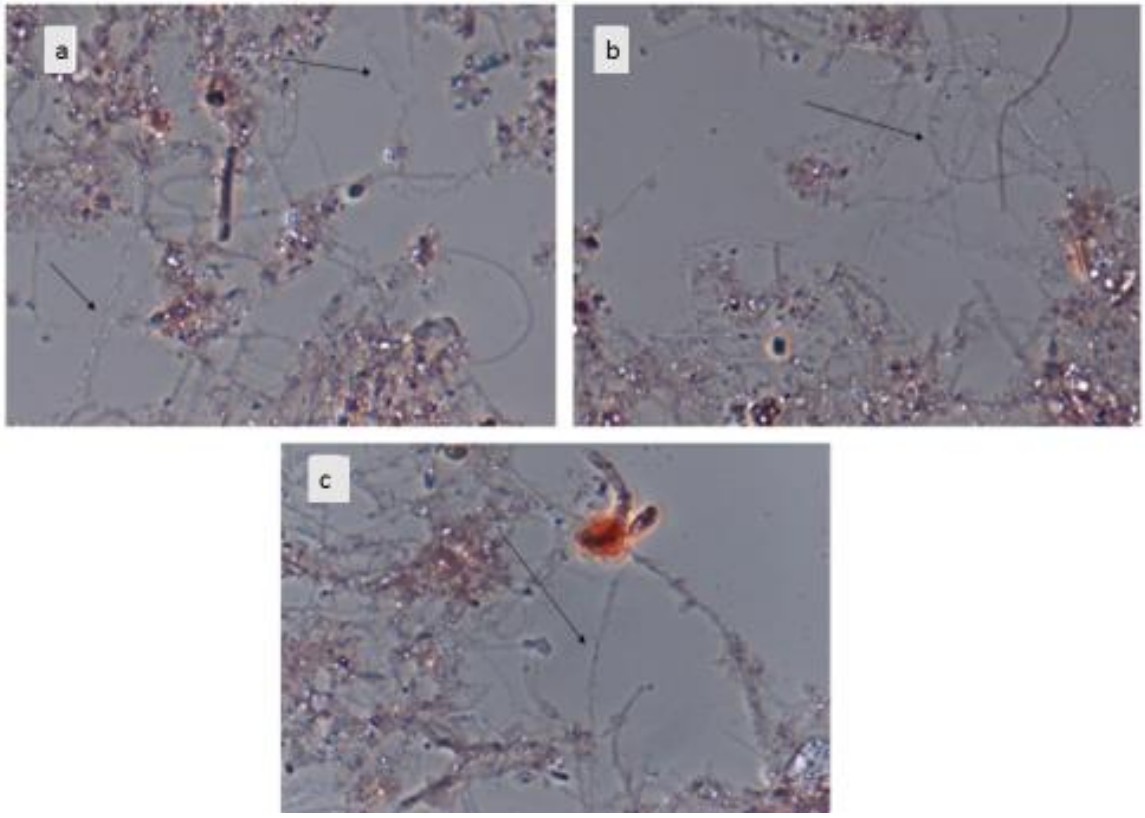


**Figure 5.4** Micrograph a, b and c depicts the presence of PHB granules within cells of heavily colonized filamentous bacteria. In micrograph a. only a select few cells are positive for the intracellular storage of PHB.

### 5.3.6 Elemental Analysis of Intracellular inclusions

Microscopic investigations of the surface of the epiphytic bacteria as well as the interface revealed the presence of various elements, of both organic (Carbon, Oxygen) and inorganic (metals) origin were detected (Fig 5.5 a and b). Further elemental analysis at ultra-structural level revealed the presence of Carbon, Oxygen, Nitrogen and traces of Chlorine (Fig 5.6 a and b). Carbon was detected with the highest weight percentage intracellularly.

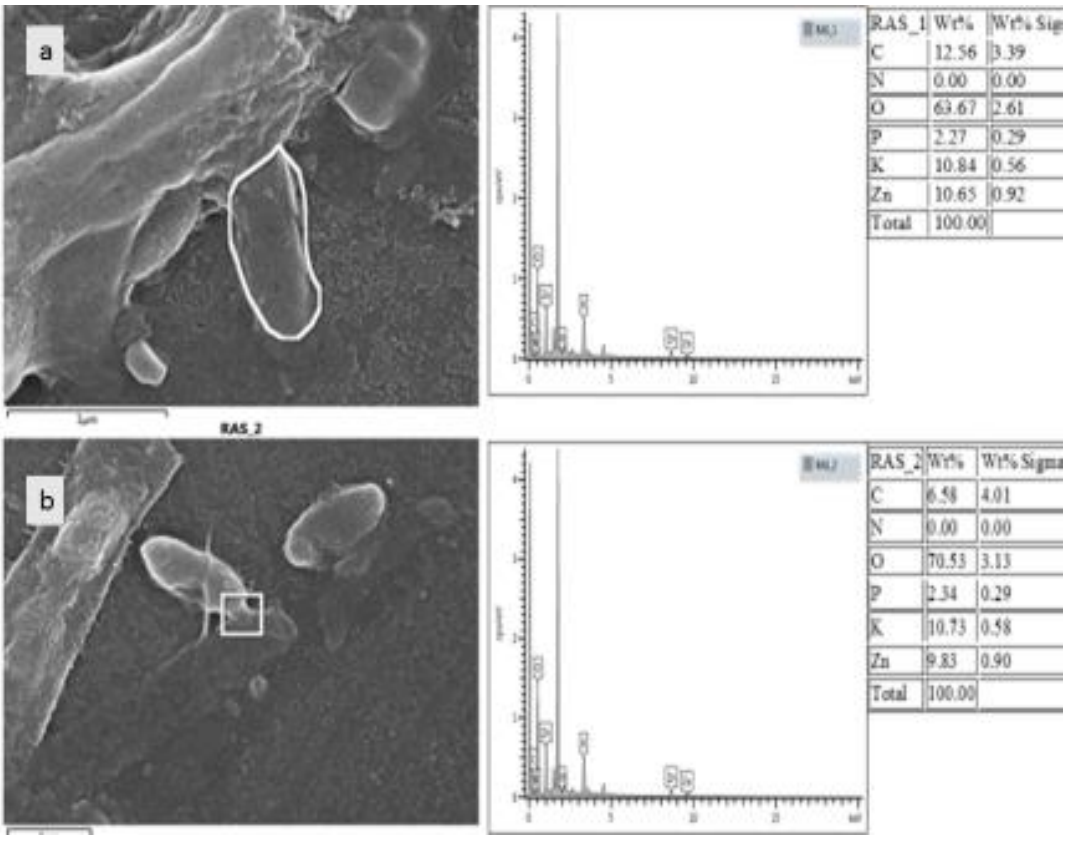


**Figure 5.5.** SEM micrograph depicting the elements present at surface level. Micrograph A) shows the elements present on the surface of the epiphytic bacteria, while B) shows the shows the elements present at the interface between filament host and epiphytic cell.

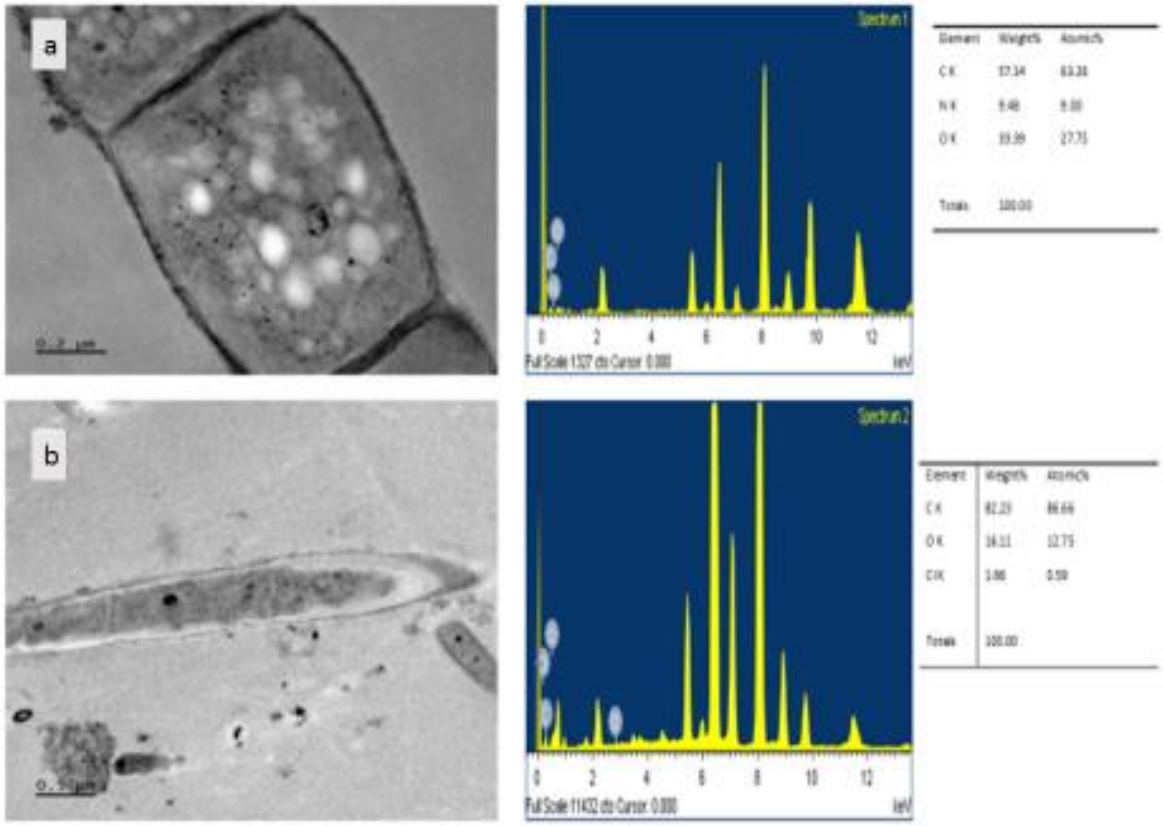


**Figure 5.6** TEM micrograph of elements detected at ultrastructural level. Micrograph a) depicts globular cellular inclusions seen as white patches, micrograph b) shows the elements present within cell targeted by epiphytic bacteria.

## 5.4 Discussion

The EPS layer which is found within and on the surface of the floc (Sheng *et al.,* 2010) is beneficial for the compaction of the bacterial consortia. However, this can also serves as a hinderance in the diffusion of nutrients. Filamentous organisms have an adavantage of protrusion into the bulk solution, thereby having access to nutrients readily available in the bulk solution.

Among the protrduing filaments, morphotypes Type 0041, Type 0675 and Type 1851 were observed to be proned to epiphytic attachment. The abundance of epiphytes colonizing these filaments varied significantly between the samples from domestic and industrial wasterwater analyzed. Live/dead cell stain test was used to assess the viability of the consortia within the floc, where the organisms concentrated within the core of the floc fluoresced red indicating non-viability (Fig 5.1 a, b and c). This observation is in line with an observation made by Sheng *et al.* (2010), stating that strongly bound fraction of cells at the core of activated sludge flocs suffer the fate of lowered diffusion co-efficients as a result of EPS, which may hinder the rapid import of nutrients and expulsion of metabolic products (Sheng *et al.,* 2010).

Furthermore, the bacteria in direct contact with the bulk solution, fluoresced green which made apparent their status of viability ( Fig 5.1d). This observation was also in line with that of Liang *et al.* (2010), stating loosely bound cells present on the surface of the floc have an advantage of ease of access to nutrients present in bulk solution (Liang *et al.,* 2010). The proliferation of filamentous bacteria may be influenced by nutrient limitation within the floc structure, which is in line with the observation of Young (2006).

It was further noted that individual filamentous bacteria in the bulk solution not forming part of the floc often showed a positive reaction in the viability test (Fig 5.2 a,c and d ). Non-viable

filaments were also present in the bulk solution and yet others having just a few cells with their trichome fluorescing red among a string of green cells (Fig 5.2 a and b). The protruding colonized regions of filamentous morphotypes were found to be viable, together with their epiphytic counterpart as they extented into the bulk solution (Fig 5.2 d, e and f). The attachment of epiphytes to viable cells on filamentous trichomes was apparent and rarely was this attached growth visualized on damages and non viable filament cells (Fig 5.2 d,e and f ).

PHB staining was conducted on aeration and RAS samples with a SRT of 25 days, to assess the intracellular storage capacity of filaments prone to piphytic growth. Filament cells colonized by attached cells exhibited the presence of PHB granules internalized within cell compartments (Fig 5.3a,b and c) . Trichomes of filaments together with their epiphytic counter parts stained pink in line with findings by Liu *et al.* (2011).

SEM elemental analysis at both the interface and surface of epiphytic bacterial cell revealed the presence of carbon, however at a lower weight percentage. Further, elemental analysis of filament compartments using TEM made apparent a significantly higher carbon content within the filament cell (Fig 5.6 a and b). This observation suggested more carbon is internalized within filament compartments than that present at the interface. According to Sujatha *et al.* (2004) carbon is an important building block in the synthesis of PHB's (Sujatha *et al*., 2004). Therefore the presence of higher volumes of carbon within filament cells may be an indication of the presence of energy rich PHB reserves.

It was further interesting to note that phosphorus in low concentrations, while nitrogen was completely absent. This observation was in line with that from a study conducted in China by Liu *et al.* (2011), stating that in the absence of the nitrogen and or phosphorous sources PHB

production increases (Liu *et al.*, 2011). These observation collectively point towards the possibility a relationship founded on nutritional benefit for epiphytes under nutrient limiting condition in AS.

## 5.5 Conclusions

This chapter evaluated the interaction between the filamentous morphotypes and their epiphytic bacterial counterparts under nutrient-limiting conditions. Techniques implored in evaluating this interaction included sonication and filtration for the breaking up of compact sludge flocs and concentration of filaments prone to epiphytic attachment. Filtration proved beneficial in this study as it allowed less compact flocs to pass through series of sieve with descending pore size and allowed for greater exposure of morphotypes with epiphytic growth. Further Live/dead viability test revealed the status of resident floc formers at different levels of floc structure, with results showing floc members densely concentrated at floc core fluorescing red (non-viable) and floc memebers located on the surface of the flocs as well as filamentous bacteria protruding beyond the floc surface fluorescing green (viable). Elemental analysis using SEM and TEM revealed the presence of carbon at interface and intracellularly within filament compartments. This element was however present in abundance within filament cells targeted by epiphytic bacteria. The presence of globular structures within filament compartments analyzed for elements further confirmed the findings of PHB staining which was positive for the presence of storage compounds under light microscopy. The findings suggests the interaction may potential border on nutrional stress and survival adaptation on the part of the epiphytes.

# 6. Chapter Six: General summary and conclusions

Occurrence of epiphytic growth on filamentous bacteria is a common phenomenon in AS plants worldwide. Eikelboom morphotypes 0041, 0675, 1701 and 1851 are identified as the common filament with their trichomes colonized by epiphytic bacteria. Traditionally the epiphytic growth has been used as a morphological attribute for their characterization. These morphotypes are present in all types of wastewater treatment plants including domestic, municipal and industrial wastewater treatment plants where epiphytic colonization varies from dense to sparse respectively. Conditions that favor the growth of these morphotypes are well documented in literature, including their presence in bulking sludge which has earned these Eikelboom morphotypes the term of "problem filaments", however, relatively little is known thus far about this interaction. This study was undertaken to investigate a potential existence of a relationship between colonized filamentous morphotypes and epiphytes, further evaluate the level of interaction between filament host and epiphytic counterpart.

Most filamentous bacteria in activated sludge are encapsulated by translucent hallow sheath where some are prone to epiphytic attachment and others not. Those prone to and colonized by epiphytic growth were consistently observed to Eikelboom Types 0041, 0675, 1851 filament morphotypes and occasionally on *S. natans* and Type 1701. The exposed regions of these filament morphotypes extending into the bulk region was colonized, while colonization of the portions embedded in the floc structure could not be visualised. Dense colonization was particularly observed with Type 0041 in domestic sludge, while sparse growth was observed on this same morphotype in industrial sludge. Additionally Type 1851 was observed with sparse growth in domestic sludge, however, in

industrial sludge epiphytic growth on this particular morphotype was fewer when present and sometimes completely absent. The FISH observations revealed that Alpha positive epiphytes were the majority epiphytic bacteria colonizing filamentous morphotypes prone to this attachment. Type 1851 showed a positive binding with EUB mix probe, while Type 0041 showed an opposite response to the same probe. This may be due to lack of binding sites or resultant effects of colonization. Therefore in depth investigation is crucial in understanding the outcome of both morphotypes when hybridized with same probe.

SEM analysis proved to be successful in differentiating between branching (which was previously visualized by use of light microscopy) and epiphytic attachment. The attachment consistently was to a 90° angle. Further TEM revealed the presence of fibrillar structures in-vivo in the filaments next to attachment sites of epiphytes, resembling amyloid fibrils. These fibrillar structures were visible within filament cells with storage compounds at the interface between filament and epiphytes, additionally in the direction of the approaching epiphyte. Additionally TEM made apparent the absence of bacterial pili and other surface attachment commonly used by bacterial to facilitate attachment.

In conclusion there seem to exist a level of selectivity in the filamentous morphotypes being colonized by epiphytes in activated sludge, where the presence of amyloid fibrils was observed particularly in the colonized cells at the interface. Absence of structural appendages that facilitate attachment was evident, which suggested attachment being potentially facilitated by fibrillar structures observed at interface. Further the association of the epiphyte to the specific filament could be based on a symbiotic relationship.

It should be noted that the investigations in determining the relationship between filamentous morphotypes and epiphytes in activated sludge was not detailed due to the non-availability of facilities and lengthy time frame beyond that allocated for this qualification required to conduct a more detailed investigation. However proposed future work in this area will be in-depth.

## 6.1 Significant Findings

Very limited information has been documented in current literature surrounding the interaction between filamentous bacteria and epiphytes. The following findings can be considered as novel:

- A clear relationship between epiphytic growth and viable filament cells. It was observed that the presence of epiphytes was on viable filament cells and absent on non-viable filament cell.
- Consistent presence of amyloid-like structures at interface between certain filament cells and epiphytes was apparent.
- Only certain cells within filament trichome were specifically colonized, particularly those containing storage compounds and exhibiting amyloid-like structures.

These findings can be considered as significant and making a novel contribution to the field of study. Further these can serve as the basis for future research.

## 6.2 Future Recommendations

The presence of epiphytic growth on selected filamentous morphotypes in activated sludge is a common occurrence, however, the exact identity of epiphytic bacteria and conditions surrounding and promoting their growth is still unclear. The use of methods such as RT-PCR and Pyro-sequencing would be beneficial for the characterization of epiphytic bacteria colonizing filamentous bacteria which can only be done through micromanipulation. Further, their identity would shed light on the preferred substrates utilized by the respective epiphytic groups. Understanding the particular substrate uptake would further contribute to understanding the conditions that promote dense epiphytic growth on some morphotypes and sparse epiphytic growth on other morphotypes.

Additionally, the effects of such colonization on filamentous bacterial health are still in need of in-depth investigation. This need may be addressed by use of bacterial Quorum Sensing. This approach may be capable of bringing to our understanding the extent of interaction between the filament hosts and epiphytic counterparts, furthermore revealing the nature of relationship between these two groups of bacteria.

The presence of the amyloid-like structures were observed at interface, however confirmation of these structures is in need and pivotal in understanding their role in attachment of epiphytic bacteria. Detection and confirmatory studies using conformational antibody staining would be advantageous for future studies of these fibrillary structures used as docking sites for epiphytic bacteria.

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## Appendix 1: Gram Staining

(Jenkins *et al.*, 1993)

Reagents

Solution (1)

a) Crystal violet 2g

Distilled water 80mL

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

- Smear mixed liquor sample onto microscope slides and allow to air dry.
- Flood sample smears with 1 ml solution (1) and allow to stand for 60 seconds; rinse with $H_2O$.

- Add 1 ml solution (2) and allow to stand for 60 seconds: rinse well with $H_2O$.
- Decolorize with 95% ethanol onto the smeared sample for 30 seconds, by holding slide at an angle and adding the ethanol in a drop-wise procedure. Blot dry.
- Add solution (3) for 60 seconds, rinse well and blot dry.

## Appendix 2: 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 3: 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:

- Prepare thin sample smears on microscope slides and allow to air dry.
- Then satin slides for 10 minutes with solution 1 and the rinse well with water for 1 second. Stain this preparation with solution 2 for 10 seconds, rinse well with water, blot dry and view under 1000x magnification.

## Appendix 4: Live/Dead staining

### 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 $1x10^5$ to $1x10^6$ 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.

## Appendix 5: FISH

Pretreatment of slides (8 welled Teflon coated slides (Merck, Germany) were used for FISH technique)

1. Clean the slide surface by soaking in warm detergent solution for 1 hr.
2. Rinse with water and air dry.
3. Place clean slides in a 1: 10 diluted solution of Poly-P-Lysine (Sigma Diagnostics, USA).
4. Allow the slides to soak for 5 minutes at room temperature.
5. Remove and allow the slides to air dry.

**Cell Fixation**

(Amann, 1995)

Solutions

1 x Phosphate Buffered saline (PBS) pH 7.2

10 mL 10X PBS

90 mL sterile deionized water

3 x Phosphate Buffered Saline (PBS) pH7.2

30 mL 10X PBS

70 mL sterile deionized water

4% Paraformaldehyde

98% Ethanol

99,99% Ethanol

**Preparation of paraformaldehyde Fixative**

Preparation of 4% paraformaldehyde:

- heat 33 mL of deionized water to 60-65$^{o}$C
- add 2g paraformaldehyde while stirring
- add 2M NaOH drop-wise until paraformaldehyde is dissolved completely
- add 16.5mL 3 x PBS buffer adjust the pH to 7.2 - 7.4 at 20$^{o}$C filter through 0.45µm filter use within 24hrs, store in ice until use.

**Fixation** (Gram negative bacteria):

- harvest cells by centrifugation(3500rpm, 4min) and discard the supernatant.
- wash the pellet with 1 x PBS, centrifuge again and re-suspend pellet in an appropriate volume of fresh 1 x PBS
- add 3 volumes of fresh paraformaldehyde solution
- incubate for 3hrs or overnight at 4$^{o}$C
- spin down the cells (5mins)
- discard supernatant
- wash the pellet with 1 x PBS, centrifuge again
- resuspend the pellet in fresh 1 x PBS
- add 1 volume of ice-cold $EtOH_{abs}$ store at -20$^{o}$C

**Ethanol Fixative** (Gram positive bacteria):

- Harvest cells by centrifugation.
- Wash the pellet in 1 X PBS, and centrifuge.
- Resuspend the pellet in an appropriate volume of fresh 1 X PBS.
- Add one volume of ice-cold $EtOH_{abs}$.
- Store at $-20^0$C.

**Immobilization of cells**

(Amann 1995)

- Prepare specimen on a Teflon coated slide: spot fixed cells (2-15 µL, 10 µl more ideal).
- Spread the sample evenly in the well and dry for about 10 mins at $46^0$C.
- Dehydrate the cells by successive passages through increasing alcohol series 50, 80 and 100% ethanol washes for 3 minutes each.
- Allow to air dry. The slides can be stored at room temperature.

**Whole Cell Hybridization**

(Amann, 1995)

Materials

- 50 ml Polypropylene screw top tube
- Whatman 3MM paper
- Hybridisation buffer pH 7.2

- Formamide and ultrapure water depending on applied stringency

**Preparation of hybridisation buffer** (for in situ hybridization at 46° C).

Pipette into a 2mL Eppendorf reaction tube:

5 M NaCl 360µL

1 M Tris HCl pH 8.0 40µL

add formamide and MQ (ultrapure water), as per 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 4µL (Add last; added onto lid of the tube, less mixing to prevent formation of foam by the surfactant (SDS).

- Thaw the oligonucleotide probes
- Drop 9µL of hybridization buffer onto the wells (cover the well without touching the surface of the wells; change tips each time)
- Add 1µL of each probe (working solution, concentration 50ng/µL for FLOUS labeled probes) without scratching the Teflon-coated surface (Mix the 1 µL probe with 9 µL of the hybridization buffer. For EUB, add 1 µL of each probe to 7 µL of the hybridization buffer.
- Prepare a hybridization chamber (50mL sterile falcon tube) by folding a piece of tissue or Whatmann 3MM 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$^{o}$C) for 3 hours.
- Prepare the washing buffer and preheat this buffer at 48$^{o}$C in a water bath.

**Preparation of washing buffer** (for in situ hybridisation at 48$^{o}$ C)

Mix in a 50mL Falcon tube:

1mL of 1M Tris/HCl pH 8.0

5M NaCl and 0.5M EDTA pH 8.0 accordingly

| %Formamide in hybridization buffer | [NaCl] in 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 | No NaCl, only 350µl EDTA |

*For formamide concentrations of 20% and higher in the hybridization buffer add 500µL (not critical though) of 0.5M EDTA (for stabilization of probe)

50µL of 10% (w/v) SDS  preheat the washing buffer at 48$^{o}$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$^{o}$C preheated water bath
- Remove the washing buffer with distilled water without detaching the cells and dry the slide quickly with compressed air.
- Subsequently stain with DAPI.

**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 6: SEM

**Concentration of filaments**:

- Aeration and RAS samples were filtered using sieves of descending pore sizes (300µm; 150µm; 100µmand 75µm).
- Spot filtrates on glass coverslips.
- air dried at room temperature.

**Sputter coating**

(Li *et al.*, 2005)

- Mount glass coverslips with sample onto aluminum stubs
- Sputter coat with gold.

## Appendix 7: TEM

Harvesting of cells:

- Two milliliter filtered sample was transferred to an Eppendorf tube.
- centrifuged at 7500 rpm for 5 mins
- The supernatant was discarded and the pellet was fixed in buffered 2.5 % Glutaraldehyde for 24 h.

**Post fixation**

(William and Unz- 1985)

- Osmium tetroxide fixation incubated at room temperature for 1 hour.
- Phosphate buffer washes for 5 min repeated 3 times.
- Acetone series dehydration :

  (2x 5 min. 30% Acetone)

  (2x 5 min. 50% Acetone)

  (2x 5 min. 75% Acetone)

  (2x 10 min. 100% Acetone).

**Infiltration and Polymerization**

(Jang *et al.*,2014)

- Equal parts Resin and Acetone infiltration for 4 hours.
- Whole resin incubation for 18-24 hours.
- Orientation of specimen in whole mould resin
- Place in oven for polymerization at 70°C for 8 hours.