DETECTION OF FILAMENTOUS BACTERIA IN ACTIVATED SLUDGE

by

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Submitted as partial fulfillment for the Degree

M.Sc. (Microbiology)

in the Faculty of Natural Sciences

(Department of Microbiology and Plant Pathology)

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PRETORIA

AUGUST 1986

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I declare that the thesis herewith submitted
by me for the degree of M.Sc. (Microbiology) at the
University of Pretoria has not been handed in
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A B S T R A C T

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DEGREE FOR WHICH THESIS IS SUBMITTED : M.Sc. (Microbiology)

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A B S T R A C T

Attempts were made to isolate the filamentous bacteria responsible for bulking at Goudkoppies activated sludge plant in Johannesburg, South Africa, in order to allow fluorescent antibody against these microorganisms to be produced and to facilitate and simplify their identification.

Of the filaments only Nocardia could be isolated using standard techiques as the growth requirements of the other filaments present are unknown.

Attempts were made to prepare a fluorescent antibody to the Nocardia isolate and to an isolate of Sphaerotilus natans (Kutzing), but success was only achieved with the latter. The antiserum reacted specifically with S. natans. Subsequently, existing microscopic techniques for the identification of filamentous bacteria occurring in activated sludge were compared with the use of the S. natans fluorescent antiserum. The bacterium was found to be abundant in only one bulking sludge sample. In five other samples, filaments of S. natans occurred but in such low numbers that only the use of the fluorescent antibody allowed their discovery and positive identification.

The use of the fluorescent antiserum proved to be a rapid, simple and accurate test to determine whether S. natans was present in a bulking sludge and whether it played a significant role in the process.

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S A M E V A T T I N G

DIE OPSPORING VAN FILAMENTEUSE BAKTERIEË IN GEAKTIVEERDE SLYK

deur

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SAMEVATTING

Pogings is aangewend om die filamenteuse bakterieë verantwoordelik vir uitdyende slyk by Goudkoppies geaktiveerde slykaanleg in Johannesburg, Suid-Afrika, te isoleer, ten einde fluoresserende teenliggame teen hierdie organismes te produseer met die oog op maklike vereenvoudigde uitkenning.

Van die filamente kon net Nocardia met standaard tegnieke geïsoleer word want die groeivereistes van die ander filamente teenwoordig is nog onbekend.

Pogings om 'n fluoresserende teenliggaam teen die Nocardia-isolaat asook 'n isolaat van Sphaerotilus natans (Kutzing) voor te berei, is ook aangewend, maar sukses is net met laasgenoemde behaal. Dié teenliggaam het spesifiek met S. natans gereageer. Daarna is bestaande mikroskopiese tegnieke vir die identifikasie van filamenteuse bakterieë in geaktiveerde slyk vergelyk met die gebruik van die S. natans-fluoresserende antiserum. Dié bakterie was volop in slegs een uitdyende slykmonster. In vyf ander monsters was filamente van S. natans teenwoordig maar teen sulke lae getalle dat hulle net met die gebruik van die fluoresserende teenliggaam opgespoor en positief geïdentifiseer kon word.

Die gebruik van die fluoresserende teenliggaam is as 'n vinnige, eenvoudige en akkurate toets bewys om te bepaal of S. natans in 'n uitdyende slyk teenwoordig is en of dit 'n betekenisvolle rol in dié proses speel.

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CHAPTER I

INTRODUCTION

Filamentous bulking has long been reported as causing a reduction in the efficiency of activated sludge plants and South Africa is no exception (Blackbeard and Ekama, 1984; Eikelboom and van Buijsen, 1983). However, until recently very little was known about this phenomenon except that it was caused by filamentous microorganisms (Richard, 1984a).

Due to inadequate knowledge those filamentous bacteria observed were usually assigned to named species but sometimes discrepancies with published descriptions occurred (Ruchhoft and Watkins, 1928; Smit 1934). *Sphaerotilus natans* was one of these bacteria. This approach sometimes led to incorrect sewage works operating procedures to try to eliminate the bulking (Beebe and Jenkins, 1981).

More recently researchers have realised that the majority of filamentous bacteria in activated sludges had not previously been described and the majority of these were then characterized according to morphology and staining reactions (Eikelboom, 1977). The occurrence of the specific filaments in bulking sludges were then correlated with specific operating conditions allowing corrective measures to be taken to stop bulking by a less numerous filamentous microorganism present in the activated sludge (Beebe and Jenkins, 1981).

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However, the microscopic identification techniques in present use (Eikelboom, 1977; Richard, 1984a) have severe limitations in the identification of less numerous filaments and require a certain amount of expertise.

Fluorescent antibody techniques have previously been used with success for identification and typing of bacteria (Thomason, 1976; Cloete, 1984). Application of a fluorescent antibody technique to activated sludge might enhance the accurate monitoring of activated sludge plants for filamentous bacteria by relatively unskilled personnel and allow immediate corrective measures to be taken to stop the bulking by one bacterium without these measures causing the bulking by a less predominant microorganism. The application of this technique can only be entirely successful when specific fluorescent antisera have been produced against all the filaments of bulking sludges that cannot be recognised easily by other means.

Isolation of filamentous bacteria in bulking activated sludge in order to provide a range of fluorescent antisera against these microorganisms could facilitate a simpler, quicker and more sensitive identification of the organisms involved in bulking. _S. natans_ is most widely reported as the cause of bulking and has the capacity to exist in both a sheathed and unsheathed form (Buchanan and Gibbons, 1974). It can also easily be confused with other morphologically similar bacteria in activated sludge and be overlooked when present in lower numbers. It could thus be considered to be the most suitable microorganism against which

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fluorescent antibodies should be produced.

The purpose of this study was:

- to isolate and grow the filamentous bacteria occurring in activated sludges in pure cultures;
- to produce fluorescent antibodies against these isolates to facilitate their detection in activated sludge;
- to produce fluorescent antibodies against S. natans using the unsheathed bacterium prior to investigating the possibility of detecting it within a sheath in activated sludges;
- to compare the use of the fluorescent antibody technique to existing ones for the detection of filamentous microorganisms in activated sludge.

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CHAPTER 2

LITERATURE REVIEW

The activated sludge process is a largely aerobic process whereby wastewater organics and inorganics are transformed by an enrichment culture consisting of an assemblage of macro- and microorganisms (Richard 1984a). Process success relies on the enrichment of (mainly) bacteria that grow on wastewater substrates and in a form that can be readily separated and concentrated from the treated wastewater by gravity sedimentation. The efficiency of nutrient, chemical oxygen demand (COD) and suspended solids (SS) removal relies largely on this secondary sedimentation. A failure of the bacteria and associated organisms to settle by gravity would eventually lead to an increase in their concentration in the effluent (Beebe and Jenkins, 1981). This means that the COD and nutrients from the wastewaters would be lost in the effluent after having been removed by these organisms, making the activated sludge process less efficient.

The activated sludge process comprises a large range of reactor sequences including one or more anaerobic zones (where oxygen and nitrate are absent), anoxic zones (where oxygen is absent but nitrate present) and aerobic zones (where oxygenation occurs). After these processes, secondary sedimentation occurs in a final gravity settling tank (secondary clarifier) and the effluent is discharged. Sludge is returned to the beginning of the process to reinoculate the wastewater. Excess sludge is removed for

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drying or anaerobic digestion. Figure 1 gives a diagrammatical representation of the final stages which are common to nearly all activated sludge processes for biological nutrient removal.

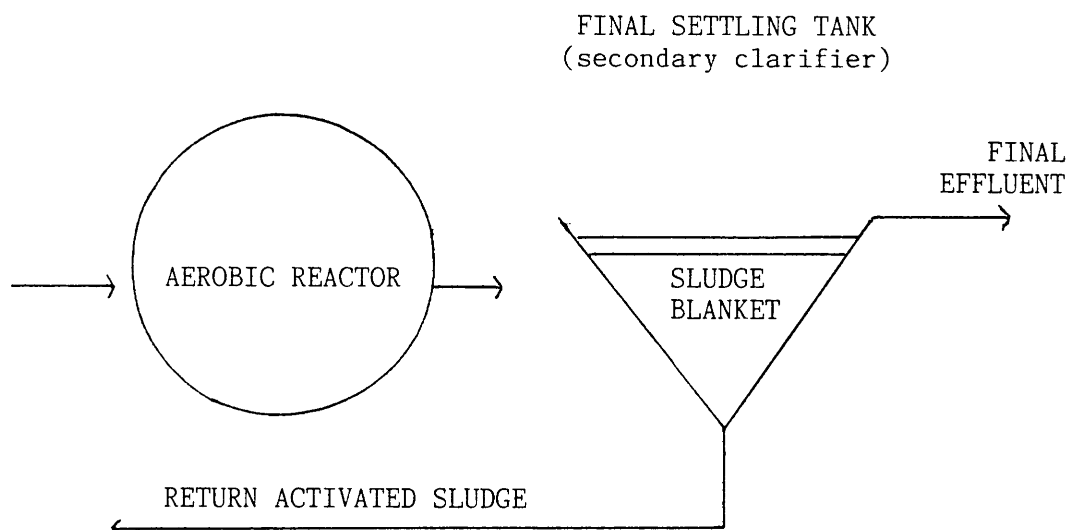

FIGURE 1: A diagrammatical representation of the final stages of the activated sludge process.

(Osborn et al., 1979; Simpkins, 1979)

The ability of the sludge to settle by gravity in the clarifier is primarily dependent on the structure of the activated sludge "flocs". These consist of heterogenous groups of microorganisms adhering to each other, thus aiding sedimentation (Eikelboom, 1977).

Two major groups of microorganisms occur within flocs, namely the spherical or rod shaped floc-formers and the filamentous or

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trichome-forming organisms (Eikelboom and van Buijsen, 1983; Richard, 1984a).

The bulking of activated sludges is where an imbalance of floc-formers and filamentous microorganisms occur resulting in a flocculating, settling and compacting sludge. Two kinds of bulking sludge occur. The first one, i.e. non-filamentous bulking sludge, is characterised by low populations of filamentous organisms (Sezgin, Jenkins and Parker, 1978). Filamentous bulking sludge, by contrast, has excess filamentous organisms resulting in the flocs mechanically repelling each other, hindering settling and compaction. This study is only concerned with filamentous bulking sludges in which the flocs are pushed apart either (i) by bridging or (ii) as a result of open floc structure (Jenkins, Richard and Neethling, 1983). In the former, the filaments link flocs together leaving open spaces between them while in the latter, the flocs, although not connected, cannot compact tightly as protruding filaments keep them physically apart.

A bulking sludge could also be defined, according to its settleability, as an activated sludge with a sludge volume index (SVI) of over 150 mℓ/g (Eikelboom and van Buijsen, 1983). This figure however is arbitrary and does not necessarily indicate problem bulking (when the SVI exceeds the limit for optimal sewage plant operation). The SVI is the volume in mℓ occupied by one gram of activated sludge after settling the aerator liquor for 30 minutes (Dick and Vesilind, 1969). This is the most common parameter for

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monitoring waste treatment operation and comparing the settling characteristics of various sludges.

A more accurate method for determining sludge settleability would be to quantify the filaments in terms of total extended filament length (TEFL), but as this is a tedious process, SVI is preferred (Lee, Koopman, Bode and Jenkins, 1983).

Several morphologically distinguishable filamentous microorganisms have been identified in bulking sludges and each may proliferate in response to a specific set of growth conditions. The following major causes for bulking have been described: low aeration basin dissolved oxygen (DO); low organic load i.e. feed to mass ratio (F/M); treatment of septic wastes; insufficient nutrients (especially phosphorus and nitrogen) and low pH (fungi) (Becchari, 1980; Cyrus and Sladka, 1970; Jenkins *et al*., 1983; Richard, 1984a).

As the activated sludge process is a continuous culture system with a mixed population, ecological selection plays a vital role in determining the population of activated sludges. The above selection parameters are related to the conditions under which specific filaments will cause bulking.

The specific population present in sludge digestion does however also alter due to changes in operational procedures, the quality of the wastewater, seasonal influences, etc. (Eikelboom and van Buijsen, 1983).

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Initially, all filamentous bulking was said to be caused by the proliferation of *Sphaerotilus natans* although many early researchers observed differences between *S. natans* and the filaments they studied in bulking sludges (Ruchhoft and Watkins, 1928; Smit, 1934). These researchers were however hampered by the fact that very little was then known about the morphology or physiology of filamentous microorganisms. Subsequent studies have however revealed the presence of many other filaments including algae, actinomycetes, bacteria, fungi and nematodes (Table I).

The majority of these bacteria and actinomycetes are listed in Table 1.

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TABLE 1 : FILAMENTOUS BACTERIA IDENTIFIED IN ACTIVATED SLUDGE

BACTERIUM	REFERENCE
Bacillus	Pipes (1967)
B. macroides	Cyrus and Sladka (1970)
Beggiatoa	Eikelboom (1975), Jenkins et al. (1983), Pipes (1967), Richard (1984a), Sladka and Ottova (1973), van Veen (1973)
Escherichia coli	Pasveer (1969) (sic)
Flavobacterium	Jenkins et al. (1983), van Veen (1973)
Flexibacterium	Eikelboom (1975), Farquhar and Boyle (1971), Sladka and Ottova (1973), van Veen (1973)
Haliscomenobacter hydrossis	Eikelboom (1975), Jenkins et al. (1983), Richard (1984a), van Veen (1973)
Lactic acid bacteria	Farquhar and Boyle (1971)
Micromonospora	Sladka and Ottova (1973)
Micothrix parvicella	Eikelboom (1975), Jenkins et al. (1983), Richard (1984a), van Veen (1973)
Nocardia	Eikelboom (1975), Farquhar and Boyle (1971), Jenkins et al. (1983), Pipes (1967), Richard (1984a), Sladka and Ottova (1973)
Nostocoida limicola	Eikelboom (1975), Jenkins et al. (1983), Richard (1984a), van Veen (1973)
Pelonema subtilissimum	Cyrus and Sladka (1970)

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Table I (continued)

Peloploca	Cyrus and Sladka (1970)
Sphaerotilus species	Farquhar and Boyle (1971)
S. dichotomus	Sladka and Ottova (1973)
S. discophorus	van Veen (1973)
S. natans	Dondero, Philips and Heukelekian (1961), Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a), Sladka and Ottova (1973), van Veen (1973), Waitz and Lackey (1959)
Streptomyces	Sladka and Ottova (1973)
Thiothrix	Eikelboom (1975), Farquhar and Boyle (1971), Jenkins *et al*. (1983), Pipes (1967), Richard (1984a), Sladka and Ottova (1973)
Toxothrix	Farquhar and Boyle (1971)
Vitreoscilla	Farquhar and Boyle (1971)
TYPE 0041	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 0092	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 021N	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)

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Table I (continued)

TYPE 0411	Eikelboom (1975), Jenkins *et al*. (1983)
TYPE 0581	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984)
TYPE 0675	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 0803	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 0914	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 0961	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 1701	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 1851	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)
TYPE 1863	Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins *et al*. (1983), Richard (1984a)

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The identification of many of the bacteria in Table I was based on morphological rather than physiological characteristics. For example the "Types" described by Eikelboom (1975), Eikelboom and van Buijsen (1983), Jenkins _et al_. (1983) and Richard (1984) are differentiated on the basis of the following properties:

a) Presence or absence of a sheath or slime layer
b) Gliding motility
c) Branching (true or false)
d) Nature, length and shape of the filaments
e) Gram stain reaction
f) Diameter, length and shape of the cells
g) Presence or absence and composition of cell inclusions (poly-beta-hydroxybutyrate, polyphosphate or sulphur).

Table 2 and Figure 2 describe the bacteria, using the above parameters, in more detail.

Physiological characterization of these bacteria has proved difficult as many of them have not as yet been isolated.

Richard (1984b) studied 29 activated sludge samples in South Africa and discovered the following filaments to be prevalent (in order of significance):

Type 0092, Type 0041, Type 0675, _Nocardia_ spp, Type 0914, Type 0803, Type 1851, _M. parvicella_, _N. limicola_ II, Type 021N, _Thiothrix_ spp, _H. hydrossis_, Type 1701, Type 0961. This suggests

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that *S. natans* did not appear to play a significant role in bulking sludges in this country. As this bacterium is reported to cause bulking under conditions of low dissolved oxygen (DO) or nutrient deficiencies (Jenkins *et al*., 1983; Richard, 1984a), this might suggest that South African bulking sludges did not have these problems at the time of his investigation. However *S. natans* might have been present, in the activated sludges examined, in low quantities in the sheathed form or as individual rods. The former is prevalent in conditions of low nutrients and the latter when nutrients are abundant (Buchanan and Gibbons, 1974).

Fluorescent antibody techniques have been used successfully for the detection of *Salmonella* in foods by Thomason (1976). In 1984 Cloete extensively reviewed the different fluorescent antibody techniques and successfully applied a fluorescein isothiocyanate (FITC) conjugate in the detection and enumeration of *Acinetobacter* in activated sludges. Labelling of specific antibodies with FITC makes microscopic investigation of reactions between these antibodies and their complementary antigens possible. The reaction products can be observed to fluoresce green when observed through a fluorescence microscope (Cloete, 1984).

CODA:

The presently available techniques for monitoring activated sludge populations have disadvantages as the density of the sludge flocs often prevents detailed microscopic examination of those

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features of filament morphology required for identification. The filaments present also may occur in too low quantities to be positively identified using these techniques.

As the fluorescent antibody technique had previously been successfully applied by other workers (Thomason, 1976; Cloete, 1984) for the detection of specific microorganisms in different environments it could be a more suitable technique for the detection of filamentous bacteria in activated sludges than existing ones.

For this technique to be successful, pure cultures of the filaments must be obtained for the production of antibodies to be conjugated with FITC. For this purpose isolation of the filaments should be carried out or they should be obtained from other sources. The FITC-conjugated antibodies could then be tested for specificity and the fluorescent antibody technique compared to presently available techniques for the identification of specific filaments in activated sludges.

The fluorescent antibody technique might provide a simple, highly specific, visually obvious means of identification which could be used in standard activated sludge population analyses for filamentous bacteria provided that these organisms can be isolated and grown in pure culture.

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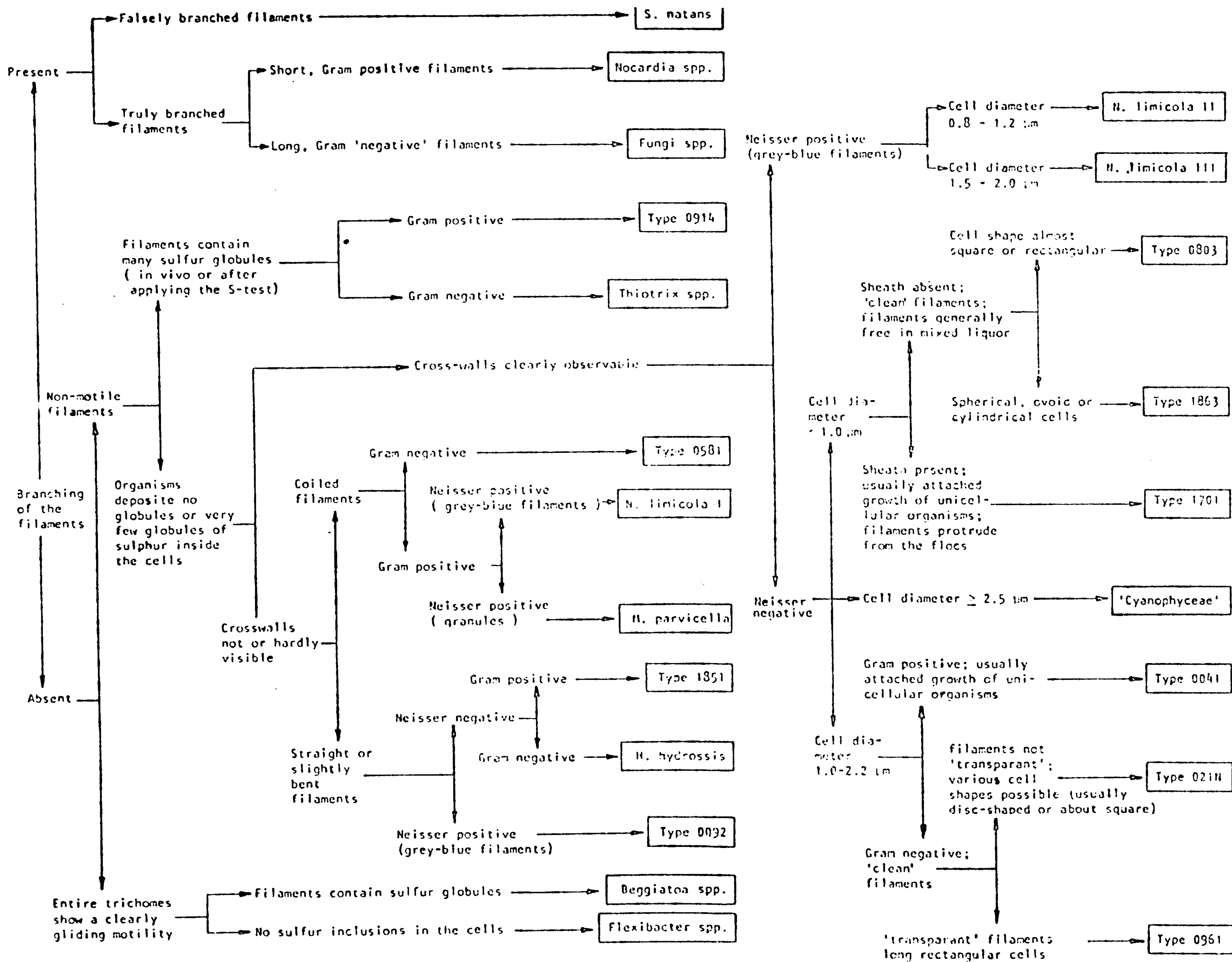

Figure 2: Key to the identification of filamentous organisms in activated sludge (Eikelboom and van Buijsen, 1983)

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TABLE 2 : SURVEY OF THE CHARACTERISTICS OF THE FILAMENTOUS MICROORGANISMS INCLUDED IN THE IDENTIFICATION KEY. (Eikelboom and van Buijsen, 1981)

	PHASE CONTRAST MICROSCOPE; 700–1000 ×																BRIGHT FIELD			
			shape filaments						diameter cells					sulfur deposited in the cells			Gram stain		Neisser stain	
	branching	motility	straight or slightly bent	coiled or twisted	length filaments <200 µm	attached growth of unicellular organisms	sheath present	crosswalls clearly visible	< 1.0 µm	1.0-2.2 µm	> 2.5 µm	rectangular or square cells	cells rounded (ovoid, spherical, disc-shaped or rod-shaped)	in vivo	after applying the S-test	PHB[9] granules present	positive	negative	positive	negative
Beggiatoa		+	+	+	±			+3)	+	+	+	+3)		+	+			+		+
"Cyanophyceae"		±	+				?	+			+	+					±	+		+
Flexibacter		+	+	+	+			±	+	+		+	+					+		+
Fungi	+		+					+		+	+	+					4)	4)		+
M. hydrossis			+		+	±	+		+			∞	∞					+		+
M. parvicella				+	+	±			+			∞	∞				+		+6)	
Nocardia	+			+	+				+			∞	∞				+			+
N. limicola I			+	+	±				+				+				+		+7)	
N. limicola II				+	±			+	±	+			+				+		+7)	
N. limicola III				+	±			+		+			+				+		+7)	
S. natans	+1)		+			±	+	+		+			+			±		+		+
Thiothrix	2)		+		±		±	+3)	+	+		+3)	+3)	±	+			+		+
Type 0041			+		±	±	+	+	±	+		+			±8)		+	±		+
Type 0092			+		+				+			∞	∞					+	+7)	
Type 021N	2)		+		±			+	±	+		±	+		±8)			+		+
Type 0581				+	+				+			∞	∞					+		+
Type 0803			+		±			+	+			+					±	+	±6)	+
Type 0914			+		+			+3)	+			+3)		+			+			+
Type 0961			+				?	+		+		+						+		+
Type 1701	±1)		+	±	+	+	+	+	+				+			±		+		+
Type 1851			+		±	+	+	±	+			∞	∞				+5)			+
Type 1863			+	+	+			+	+				+					+		+

± = sometimes; ? = unknown; ∞ = cell shape usually not visible by phase contrast microscopy;

1) falsely branched; 2) sometimes formation of rosettes; 3) only visible after removal of the sulfur inclusions

4) Fungi do not stain by the Gram stain; 5) faintly; 6) granules; 7) filaments grey-blue; 8) small granules;

9) poly-β-hydroxy butyric acid

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CHAPTER 3

MATERIALS AND METHODS

3.1 GROWTH MEDIA AND BUFFERS

3.1.1 *Beggiatoa* enrichment medium (Weissner, 1981)

- Prepare grass hay extract by boiling 10g hay in 250mℓ tap water for two, consecutive, 30 minute periods. (The water must be replaced with clean water each time).
- Retain the final extract and autoclave at 121°C for 15 minutes.
- Add penicillin G and cycloheximide in concentrations of 30 mg/ℓ to prevent the growth of contaminants.

3.1.2 *Beggiatoa* growth medium (Weissner, 1981)

NH_4Cl	50 mg	H_3BO_3	0.1 mg
K_2HPO_4	100 mg	$Co(NO_3)_2$	0.01 mg
$CaSO_4.2H_2O$	2 mg	$NaMoO_4.2H_2O$	0.01 mg
$MgSO_4.7H_2O$	10 mg	$CuSO_4.5H_2O$	0.0005 mg
$ZnSO_4.7H_2O$	0.1 mg	$FeSO_4.7H_2O$	7 mg
EDTA (disodium salt)	9.2 mg	$MnSO_4$	0.02 mg
Beef extract (Difco)	0.5 mg	Peptone (Difco)	0.5 mg
		Agar (Bacto)	12 g

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- Dissolve in 900 mℓ distilled water
- Autoclave at 121°C for 15 minutes
- Add 1 000 Sigma units catalase in 100mℓ distilled water after filter sterilisation
- Pour plates

3.1.3 Flexibacter water agar (Reichenbach and Dworkin, (1981)

$CaCl_2$	1 g
Agar (Bacto)	12 g

- Dissolve in 1 ℓ distilled water
- Adjust the pH to 7.2 with KOH
- Autoclave at 121°C for 15 minutes

3.1.4 Flexibacter CYT agar (Reichenbach and Dworkin, 1981)

Casitone (Difco)	1 g	$CaCl_2.2H_2O$	0.5 g
Yeast extract (Difco)	0.5 g	Agar (Bacto)	15 g
$MgSO_4.7H_2O$	0.5 g		

- Dissolve in 900 mℓ distilled water
- Adjust the pH to 7.2 if necessary
- Autoclave at 121°C for 15 minutes
- Add 25 mg cycloheximide in 100 mℓ distilled water after filter sterilization
- Pour plates

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3.1.5 Haliscomenobacter medium I (Mulder and Deinema, 1981; van Veen, 1973)

Glucose	0.15 g	$MgSO_4.7H_2O$	0.05 g
$(NH_4)_2SO_4$	0.5 g	KCl	0.05 g
$Ca(NO_3)_2$	0.01 g	$CaCO_3$	0.1 g
K_2HPO_4	0.05 g	Agar	10 g

- Dissolve in 900 mℓ distilled water
- Autoclave at 121°C for 15 minutes
- Add 10.5 x 10^{-4}g vitamin B_{12} and 4 x 10^{-4} g thiamine dissolved in 100 mℓ distilled water and filter sterilize
- pour plates

3.1.6 Haliscomenobacter SCY Agar (Mulder and Deinema, 1981; van Veen, 1973)

Sucrose	1.0 g	Casitone (Difco)	0.75 g
Yeast extract (Difco)	0.25 g	Agar	10 g
Tripticase soy broth without dextrose	0.25 g	Vitamin B_{12}	10^{-5} g
Thiamine	4 x 10^{-4} g		

- Dissolve all ingredients except the vitamin B_{12} and thiamine in 900 mℓ distilled water
- Autoclave at 121°C for 15 minutes
- Add the vitamin B_{12} and thiamine in 100 mℓ distilled water after filter sterilization
- Pour plates

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3.1.7 *Haliscomenobacter hydrossis* maintenance medium (American Type Culture Collection (ATCC) Catalogue, 1982)

Solution A :

Trypticase soy broth without dextrose	0.25 g
Casitone (Difco)	0.75 g
Agar	10 g

- Dissolve in 1 ℓ distilled water
- Adjust the pH to 7.0
- Autoclave at 121°C for 15 minutes
- Pour slopes

Solution B :

Sucrose	1 g
Vitamin B_{12}	0.01 mg
Thiamine	0.4 mg
Yeast extract	0.25 mg

- Dissolve in 100 mℓ distilled water
- Adjust the pH to 8.5
- Filter sterilize
- Pipette 1 to 2 mℓ onto slopes of Solution A after the latter have been inoculated.

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3.1.8 *Nocardia* modified CZAPEK'S Agar (Goodfellow and Minnikin, 1981)

$NaNO_3$	2 g	$FeSO_4$	0.01 g
K_2HPO_4	1 g	Sucrose	30 g
$MgSO_4.7H_2O$	0.5 g	KCl	0.5 g
Yeast extract (Difco)	2 g	Agar (for plates)	15 g

- Dissolve in 1 ℓ distilled water
- Adjust the pH to 7.2
- Autoclave at 121°C for 15 minutes
- Add filter sterilized cycloheximide to make a final concentration of 30mg/ℓ

3.1.9 *Sphaerotilus natans* STOKE'S MEDIUM (Dondero *et al*., 1961; Mulder and Deinema, 1981)

Glucose	1 g	$CaCl_2$	0.05 g
Peptone (Difco)	1 g	$FeCl_3.6H_2O$	0.01 g
$MgSO_4.7H_2O$	0.2 g	Agar (for plates)	12.5 g

- Dissolve in 1 ℓ distilled water
- Autoclave at 121°C for 15 minutes
- Pour plates
- Dry plates at 35°C overnight
- Half strength medium is obtained by halving all ingredients except the agar and distilled water

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3.1.10 *S. natans* Enrichment medium (Dondero *et al.*, 1961; Mulder and Deinema, 1981)

- Boil 2 to 3 cm lengths of lucerne hay in 250 mℓ tap water as for *Beggiatoa* enrichment (3.1.1)
- Autoclave at 121°C for 15 minutes
- Add 30 mg/ℓ cycloheximide (to some media bottles) after filter sterilization

3.1.11 *S. natans* CGY medium (Dondero *et al.*, 1961)

Pancreatic digest of casein	5.0 g	Glycerol	10.0 g
Yeast extract	1.0 g	Agar (if plates or slopes are to be used)	15.0 g

- Dissolve in 1 ℓ distilled water
- Autoclave at 121°C for 15 minutes
- Pour plates
- Dry plates at 35°C overnight
- Half strength medium is obtained by halving all ingredients except the agar and the water

3.1.12 *S. natans* DSM medium 51 (Deutsche Sammlung von Mikroorganismen (DSM) Catalogue, 1977)

Beef extract 5 g

- Dissolve in 1 ℓ distilled water
- Adjust the pH to 7.0
- Autoclave at 121°C for 15 minutes
- To obtain half strength medium 2,5 g beef extract is used

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3.1.13 LMB Medium (Richard, 1984a)

(For preservation of *Thiothrix* cultures supplied by Richard (1984a)

Glucose	1.0 g	NH_4Cl	0.36 g
$MgSO_4.7H_2O$	0.1 g	$CaCl_2$	0.05 g
$FeCl_3$	0.002 g	EDTA (disodium salt)	0.003 g
K_2HPO_4	0.110 g	KH_2PO_4	0.110 g
*Vitamin mixture	1 mℓ	$NaHCO_3$	0.42 g

- Add 12 g/ℓ agar when slopes are required for storage
- Dissolve solid ingredients in 1 ℓ distilled water
- Autoclave all ingredients except the vitamin mixture

* Vitamin mixture :

Ca pantothenate	10^{-4} g
Niacin	10^{-4} g
Biotin	5×10^{-6} g
Cyanocobalamin (B12)	5×10^{-6} g
Folic acid	5×10^{-6} g
Pyridoxine	10^{-4} g
p-aminobenzoic acid	10^{-4} g
Cocarboxylase	10^{-4} g
Inositol	10^{-4} g
Thiamine	10^{-4} g
Riboflavin	10^{-4} g

- Dissolve in 1ℓ distilled water
- Filter sterilize

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3.1.14 Carbonate buffer 0,5M pH 9.0 (Holborow and Johnson, 1971)

Solution A:

Na_2CO_3 5.3 g

Distilled water to 100 mℓ

Solution B:

$NaHCO_3$ 4,2 g

Distilled water to 100 mℓ

Theoretically, a pH of 9.0 should result from mixing 4.4 mℓ of solution A with 100 mℓ of solution B. In practice, it is sometimes necessary to add as much as 17 mℓ of solution A to 100 mℓ of solution B. The pH should be checked on a meter.

3.1.15 Phosphate buffered saline (PBS) 0,01M, pH 7.2 (Holborow and Johnson, 1971)

Solution A:

Na_2HPO_4 1.4 g

Distilled water to 100 mℓ

Solution B:

$NaH_2PO_4.H_2O$ 1.4 g

Distilled water to 100 mℓ

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- Add 84.1 mℓ of solution A to 15.9 mℓ of solution B.
- Add 8.5 g of NaCl
- Add distilled water to make 1ℓ

3.2 BACTERIAL CULTURES

Pure cultures of bacteria used are listed in Table 3 together with their source.

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TABLE 3 : BACTERIA USED FOR CROSS REACTION WITH S. NATANS ANTISERUM AND THEIR SOURCES

	Microorganism	Source
1	21 Strains of Acinetobacter	University of Pretoria
2	Escherichia coli	University of Pretoria
3*	Haliscomenobacter hydrossis	American type culture collection (ATCC) no 27775
4	H. hydrossis	University of California, Berkeley
5	Microthrix parvicella	University of California, Berkely
6	Nocardia	University of California, Berkely
7*	Sphaerotilus natans Kutzing, 1833 (On rich medium - mostly rods)	Deutsche Sammlung von mikroorganismes (DSM) culture collection
8*	S. natans Kutzing, 1833 (On half strength medium - mostly filaments)	DSM Culture collection
9*	S. natans	University of California, Berkeley
10*	Thiothrix (2 strains)	University of California, Berkeley
11	Type 0041	University of California, Berkeley
12	Type 0961	University of California, Berkeley
13	Type 021N	University of California, Berkeley
14*	Type 1701	University of California, Berkeley

* - These cultures were obtained in a lyophilized form.

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3.3 SLUDGES EXAMINED

Five activated sludge samples were supplied as course material by Richard (1984a). These sludges were obtained throughout South Africa and selected for the wide variety of filaments represented. Three of the samples were taken from full scale activated sludge plants at Bellville (Cape), Cape Receife (Port Elizabeth) and Olifantsvlei (Johannesburg). The other two samples were obtained from activated sludge plants at Cape Town University and Pietermaritzburg. In addition, bulking activated sludge samples were supplied by Johannesburg Municipality from module 3 of their Goudkoppies full-scale activated sludge plant. All samples were extracted from the aerobic zone or the secondary clarifier.

3.4 ISOLATION PROCEDURES

3.4.1 *Beggiatoa* isolation

Beggiatoa enrichment medium (3.1.1) was inoculated with one and ten percent (v/v) bulking activated sludge from Goudkoppies module 3 and incubated at 25°C for one week. Flocs from this enrichment medium were transferred with a sterile Pasteur pipette to plates of *Beggiatoa* enrichment medium (3.1.2).

Colonies formed on the above were examined microscopically

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for the presence of filamentous bacteria and intracellulary stored sulphur.

3.4.2 *Flexibacter* isolation

Bulking sludge from Goudkoppies module 3 was streaked onto *Flexibacter* CYT agar (3.1.4) and *Flexibacter* water agar (3.1.3).

Cytophagas (including *Flexibacter*) could be identified by gliding motility, filament formation and their bright yellow, orange or red colonies. Many *Flexibacter* colonies turn from orange to purple upon the addition of alkali.

3.4.3 *Haliscomenobacter* isolation

A sample of 0.1 to 0.5 mℓ of sludge was pipetted into tubes containing 10 mℓ of autoclave sterilized tap water and stirred for several minutes before being allowed to settle. This procedure was repeated until sufficient filaments were observed microscopically in the upper layers of the supernatant. Small droplets were transferred to previously dried plates of *Haliscomenobacter* medium I (3.1.5) with a Pasteur-pipette and spread with a sterile glass rod. The plates were incubated for three to four weeks at 20°C. Small colonies were transferred to SCY (3.1.6) agar and examined for filamentous growth.

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Haliscomenobacter spp. form pinkish, smooth or slightly filamentous colonies of one to three millimetres in diameter on SCY agar. The cells are less than one micrometer wide in a filament surrounded by an almost invisible sheath.

3.4.4 *Nocardia* isolation

Goudkoppies bulking sludge was streaked onto modified Czapek's agar (3.1.8).

Nocardia strains without aerial hyphae are difficult to distinguish from corynebacteria, mycobacteria and rhodococci without diagnostic sugars and diamino acid analyses of whole organisms or cell walls (Goodfellow and Minnikin, 1981).

Those that produce aerial hyphae can also be confused with *Actinomadura*, *Pseudonocardia* and *Streptomyces*. Isolates obtained by the above techniques that fitted the morphological description of *Nocardia* in Table 2 were thus called *Nocardia*.

3.4.5 *Sphaerotilus natans* isolation

Initial enrichment was carried out by inoculating *S. natans* enrichment medium (3.1.10) with one and ten percent (v/v) Goudkoppies module 3 bulking sludge and incubated for two weeks at 25°C. Flocs from these suspensions were streaked

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onto Stoke's medium (3.1.9.) and GCY medium (3.1.11) plates.

Direct streaking of sludge dilutions onto these plates as well as plates of their half strength medium was also carried out to ensure filament growth. In some cases the sludge was homogenized in an Ultra-turrax prior to inoculation.

The morphological characteristics in Table 2 were used in attempts to identify S. natans microscopically.

3.5 PRODUCTION OF A FLUORESCENT ANTIBODY AGAINST S. NATANS

3.5.1 Bacterial cultures

A culture of S. natans Kutzing (1833) from the DSM was tested for purity using Gram-stain and ability to produce filaments on half-strength DSM-medium 51 (3.1.12). In addition a Nocardia (identification based on morphology) which was isolated from a bulking sludge from Goudkoppies module 3 was used as an antigen.

3.5.2 Production of antigen (Campbell, Garvey, Cremer and Sussdorf, 1970)

The S. natans bacterial isolate was inoculated into 400mℓ liquid medium containing 5 g/ℓ beef extract in distilled

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water (DSM medium 51) (3.1.12) and incubated for seven days at 25°C. To test for filament production, half strength DSM medium 51 (3.1.12) was used and the culture examined under phase contrast.

The culture was then washed twice by centrifugation at 8000 rpm in a Sorvall centrifuge with phosphate buffered saline (3.1.15) for ten minutes.

The culture was subsequently boiled for one hour at 100°C, then washed three times with PBS. The density of the washed culture was then adjusted to McFarland number 4 with sterile PBS (McFarland, 1970). The same procedure was followed with the _Nocardia_ culture after it had been grown on modified Czapek's Agar (3.1.8.).

3.5.3 Injection of rabbits (Campbell _et al_., 1970)

This was carried out with a 28-gauge needle and 2mℓ syringe. Before intravenous injection of the antigen, the rabbit's ear was disinfected with 70% ethanol and the syringe flushed with one part per thousand adrenalin to prevent anaphylactic shock.

The following inoculation programme was carried out with four rabbits (two per antigen):

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Day 1 : 0,5 mℓ antigen

Day 4 : 1,0 mℓ antigen

Day 7 : 1,5 mℓ antigen

Days 10, 13, 16, 19, 22 : 2,0 mℓ antigen

On day 29 a titre test was carried out.

3.5.4 Titre test

3.5.4.1 Preparation of the serum

The rabbit's ear was swabbed with xylene (to dilate the vein), disinfected with alcohol and smeared with vaseline to prevent the rabbit's ear from being coated in blood.

The vein was then cut across with a sterile sharp blade and 5 mℓ blood was drained into a sterile test tube. (This process was repeated with each rabbit).

To prepare the serum, the blood was left standing for about 30 minutes until clotted. The blood was then centrifuged at 1000 rpm for 10 minutes and the serum was then collected.

3.5.4.2 Agglutination test to determine the titre

a) A dilution series of rabbit serum was made in sterile test tubes as explained in Table 4 using sterile pipettes.

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TABLE 4 : COMPOSITION OF TITRE TEST DILUTION SERIES

TEST TUBE NO	A	B	C	D	E	F	G
PBS (mℓ)	3.9	2	2	2	2	2	2
SERUM (mℓ)	0.1	2 mℓ of A	2 mℓ of B	2 mℓ of C	2 mℓ of D	2 mℓ of E	-
DILUTION	1:40	1:80	1:160	1:320	1:640	1:1280	CONTROL

One mℓ of the contents of each of the test tubes was discarded to attain a final volume of 1 mℓ.

One drop of antigen was then added to each test tube and the test tubes incubated for 18 hours in a waterbath at 55°C. A white precipitate was considered to be positive.

The titre is the reciprocal of the last dilution with precipitate and if less than 320, a booster injection should be given to the rabbit and the titre test repeated after seven days. If the titre is 320 or higher, the serum can be used. However, if the titre is lower, too low a quantity of reactive antiserum has been isolated from the experimental animal to achieve meaningful results (Cloete, 1984).

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3.5.5 Preparation and purification of FITC-antiserum conjugate (Holborow and Johnson, 1971)

3.5.5.1 Harvesting of serum

Fifty mℓ blood was collected from the heart of each rabbit using a sterile 50 mℓ syringe and an 18 gauge needle. (Two of the three rabbits had a titre of over 320).

The blood was left to stand for two to three hours to allow it to clot and centrifuged twice in a Sorvall centrifuge at 1000 rpm for 20 minutes.

3.5.5.2 Precipitation of immunoglobulin fraction

The volume of serum was determined and 12% (v/v) polyethyleneglycol 6000 was added. This mixture was then centrifuged at 2000 rpm for 20 minutes before discarding the supernatant. The precipitate was then dissolved in the minimum volume of 0.01 M phosphate buffer (pH 7.2) for complete solution before adding another 12% (v/v) polyethyleneglycol and repeating the centrifugation. The final precipitate contained the immunoglobulin (Ig) fraction and was redissolved in a volume of PBS equal to one third of the initial volume of serum. The above process was carried out at 4°C.

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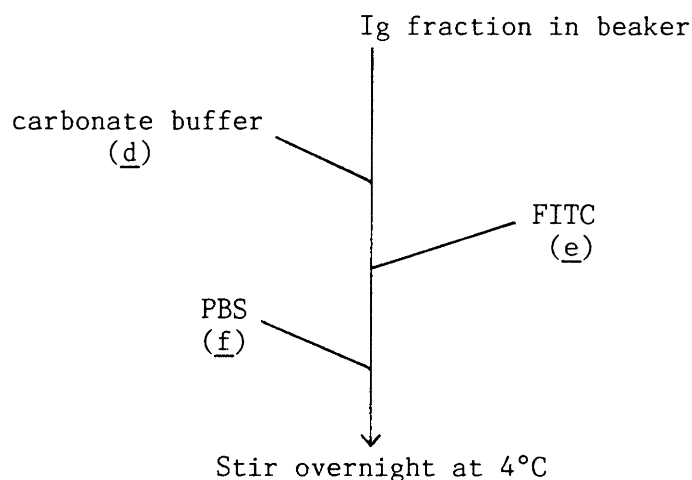
3.5.5.3 Preparation of the conjugate (Cloete, 1984).

A Beckman DU 8 spectrophotometer was used to determine absorbance at 260 and 280 nm of the Ig fraction and the conjugate constituents were determined using the following formulae:

Protein concentration (mg $m\ell^{-1}$)	=	1.45 x (Abs_{280}) - 0.74 (Abs x 10 (dilution)	----- a
Total protein	=	a x Initial volume serum	-- b
Volume required to obtain a protein concentration of 10 mg/ml	=	$\frac{b}{10}$	--------- c
Volume carbonate buffer (3.1.13) added	=	$\frac{c}{10}$	--------- d
Volume powdered fluorescein isothiocyanate added (FITC)	=	b x 0.5	--------- e
Volume PBS added	=	c - (Initial serum volume + d)	--------- f

Mixing was carried out in the following order:



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3.5.5.4 Purification of the labelled immunoglobulin

Conjugated FITC was separated from the unbound FITC by dialysing the combined FITC and Ig fraction in PBS using Union Carbide 18 FO seamless cellulose dialysing tubing.

Fifteen mℓ of conjugate were dialysed for 18 hours at 4°C with six changes of PBS (pH 7). At this stage, the dialysing fluid showed no fluorescence. The purified fraction was then preserved by adding 0.03% NaN_3 and was subsequently ready for staining.

3.5.6 Staining of antigen

For each bacterial suspension examined for the presence of an antigen-body reaction, a drop of the suspension was heat-fixed on a clean microscope slide and an area selected for viewing. This area was marked with a diamond pencil and a loopful of antibody-FITC conjugate placed on top of it. The slide was then incubated in a petri-dish with wet filter paper (to prevent drying out) for 20 minutes at room temperature. The stained smears were then washed with PBS and mounted under a coverslip in 90% glycerol buffered with 10% PBS. Care was taken not to let the smear dry out. The stained smear was then examined using a Zeiss phase contrast microscope equipped with epifluorescence UV-illumination. The presence of a positive fluorescent reaction was tested by staining the non-filamentous and filamentous *S. natans*

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pure cultures in the above manner.

3.5.7 Testing the purity and specificity of the conjugate

The purity of the conjugate and its specificity was tested by checking for cross reactions between it and all of the pure cultures in Table 3.

3.5.8 Fluorescent staining of activated sludge samples

The *S. natans* fluorescent antiserum was used to stain samples of the activated sludges in 3.3 using the techniques described in 3.5.5.

The above technique was also applied to Goudkoppies module 3 activated sludge using a fluorescent antiserum produced against *Acinetobacter* by Cloete (1984) to produce a nonspecific reaction. Using a fluorescent antibody produced against *Escherichia coli* on Goudkoppies module 3 sludge and the bacteria in Table 3 the fluorescent staining procedures were repeated.

3.5.9 Staining procedures

The Gram stain (Hucker modification) and Neisser stain (modified) were applied to thin smears of air dried activated

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sludges as indicated by Eikelboom and van Buijsen (1983) before examining under oil immersion with direct illumination (not phase contrast).

3.6.1 Sulphur storage test (Eikelboom and van Buijsen, 1983)

Equal volumes of activated sludge and Na_2S solution (200 mg $Na_2S.7H_2O$ per 100 mℓ) were mixed and allowed to stand for 10 to 15 minutes with occasional shaking to keep the sludge in suspension. Phase contrast microscopy (oil immersion) was then used to see if sulphur granules were present in the cells. These could be observed as large, highly refractile, granules within the filaments. This test was applied only if prior phase contrast examination for sulphur granules had proven negative.

3.7 PHASE CONTRAST MICROSCOPY

This was carried out using a Zeiss phase contrast microscope. Most of the characteristics could only be observed with a magnification of 700 to 1000 times (Richard, 1984a). The filaments in the activated sludge flocs were examined for motility, branching, attached growth of unicellular microorganisms, presence of a sheath, crosswalls between the cells and the dimensions of the filaments. The cell sizes of each filament examined were noted as well as their shape and the microscopic observation of intracellular polyhydro-

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xybutyrate (PHB) or sulphur granules (Eikelboom and van Buijsen, 1983). All these observations were carried out using phase contrast microscopy. In addition Neisser and Gram stains were carried out and observed under bright field illumination as well as fluorescent antibody staining for *S. natans*. All the information obtained was noted down and the identification keys of Eikelboom and van Buijsen (1983) were used to identify the bacteria (see pages 15 & 16).

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CHAPTER 4

RESULTS AND DISCUSSION

4.1 ISOLATIONS

Eight attempts to isolate Sphaerotilus natans, Flexibacter, Beggiatoa and Haliscomenobacter using standard and half strength media failed. This was possibly due to the fact that the enrichment procedures used for these microorganisms also enriched for a multitude of other bacteria that grew more rapidly under the same circumstances. For example, numerous spore-forming aerobic rods were isolated on GCY-agar in attempts to isolate S. natans. In this situation the filamentous bacteria formed too small an inoculum.

Another difficulty is that S. natans has the tendency to form filaments only under conditions of nutrient deficiency (Buchanan and Gibbons, 1974). Normally, it grows as individual rods without sheaths and may thus be easily overlooked.

Another explanation, for the lack of success with Goudkoppies modue 3 sludge, was that these filaments were not in fact present. This conclusion was supported by microscopic examination of the sludge, using Eikelboom and van Buijsen's (1983) techniques, which showed that bulking at Goudkoppies was due to the proliferation of Type 0675 (a small version of Microthrix parvicella,) Type 0041, Type 0092 and Nocar-

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dia. Use of the fluorescent antiserum specific to *S. natans* did however indicate the presence of this bacterium in filamentous form in minor quantities in Goudkoppies module 3 sludge. Numerous actinomycetes with the morphological characteristics of *Nocardia* were successfully isolated from Goudkoppies module 3 and the fastest growing isolate was chosen as an antigen to induce antibody production in rabbits. Of the filamentous bacteria observed in Goudkoppies module 3 activated sludge only Type 0041 and *Nocardia* had been previously isolated by Richard (1984a).

Previous attempts to isolate the filamentous bacteria from activated sludge met with limited success as early published information suggests that the filaments in bulking sludge were all bacteria previously documented and described in journals and textbooks. In fact only a few of the filaments observed in activated sludges had been described (Table 1). The application of published isolation techniques thus failed repeatedly. Difficulties also occur because of the variation in population composition with differences in environmental conditions.

Only a limited number of sludge filaments described have to date been isolated and too little is known about the growth and physiological requirements of the remainder to allow isolation without the application of trial and error techniques.

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4.2 FLUORESCENT ANTIBODY ACTIVITY

4.2.1 Nocardia

The titre was 160 and the initial volume of serum was 4 mℓ. In spite of the low titre, a protein determination was carried out to determine if there were enough anti-bodies to produce fluorescent antiserum. The absorbance of the immunoglobulin fraction at 260 nm was 1.277 and that at 280 nm was 1.264.

Calculations (See page 35)

$$\text{Protein concentration} = [(1{,}45 \times 1{,}264) - (0.74 \times 1{,}2779)] \times 10$$
$$= [1.8328 - 94498] \times 10$$
$$= 8.878 \text{ mg m}\ell^{-1}$$

This protein concentration was considered too low to produce good antibody-antigen cross reactions and further experimentation with this serum was terminated here due to unforseen circumstances.

4.2.2 S. natans

The result of the titre test here was 640 and the initial serum was 4 mℓ. The absorbance of the immunoglobulin fraction at 260 nm was 3.312 and that at 280 nm was 3.284.

Calculation (See page 35)

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Protein concentration = [(1.45 x 3.284) - (0.74 x 3.312)]x10

= [4.7618 - 2.4509] x 10

= 23.109 mg ml^{-1}

Total protein = 23.109 x 4

= 92.436

Volume to obtain correct protein concentration = $\frac{92.436}{10}$

= 9.244 mℓ

Volume carbonate buffer added = $\frac{9.244}{10}$

= .924 mℓ

Volume PBS added = 9.244 - (4 + .924)

= 4.32 mℓ

Volume FITC added = 92,436 x 0.05

= 4.62 mg

4.2.3 Cross reaction results

These were carried out to test the specificity of the *S. natans* fluorescent antibody (See Table 3). A 4 + fluorescence according to Thomason (1976) was obtained when it was reacted with the *S. natans* culture from the DSM.

The cells stained brightly whether they occurred singly (full strength DSM medium), or within sheaths (half strength DSM medium) although in the latter, the cell cytoplasm could not be distinguished from the cell walls. Empty sections of sheath stained faintly and gaps between the cells were clearly visible (Plates 2 and 3). The *S. natans* culture

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obtained from Richard (1984a) gave similar results except that the fluorescence was only 3+ but the antibody was already 12 months old when it was reacted with this culture.

All other bacteria used to test the antibody specificity in Table 3 yielded negative results. It was thus concluded that the fluorescent antibody produced was specific to *S. natans* and that the *S. natans* from America was serologically related to the one from Germany as both reacted positively with the specific fluorescent antibody produced against the DSM culture of *S. natans*.

4.2.4 Microscopic sludge investigation results

The following results in Tables 5, 6, 7, 8, 9 and 10 were obtained using the microscopic techniques of Eikelboom and van Buijsen (1983) and Richard (1984a).

Certain characteristics here were difficult to ascertain e.g. filament length and the presence of a sheath. Others were variable e.g. attached growth of microorganisms on the filaments often depended on sludge age (unicells may occur at one end of a filament but not the other). Certain characteristics in Tables 5 to 10 were not determined (ND) for one of the above reasons or because it was not necessary for positive identification of the filament being studied. When a filament type was considered to be more significant

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or more dominant than another in an activated sludge (Tables 5 to 11) it was visually observed as being more numerous.

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TABLE 5 FILAMENTS IDENTIFIED IN SLUDGE SAMPLES FROM BELLVILLE SEWAGE WORKS (CAPE)

WET MOUNT 1000X PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE) 1	2	3	4	5	6
1 BRANCHING	-	-	-	True branching	-	-
2 MOTILITY	-	-	-	-	-	-
3 FILAMENT SHAPE	coiled	straight	straight or slight-ly bent	ND	straight	curved
4 LOCATION	coiled around flocs	ND	ND	ND	ND	ND
5 ATTACHED UNICELLS	few	ND	-	ND	+ (few)	+
6 SHEATH	-	ND	-	ND	+	+
7 CROSSWALLS	faint	+	-	ND	+	-
8 FILAMENT (μm) DIAMETER	0.8	1.4	0.8	ND	2.1	1.0
9 FILAMENT (μm) LENGTH	<200	ND	ND	ND	<200	ND
10 CELL SHAPE	ND	ND	ND	ND	Rectan-gular	ND
11 CELL SIZE (μm)	ND	ND	ND	ND	ND	ND
12 SULPHUR DEPOSITS	-	-	-	ND	-	-
13 OTHER GRANULES	-	-	-	ND	-	-
<u>STAINS 1000 X</u>						
14 GRAM	+ (strong)	+	-	+	+	-
15 NEISSER	+	+	+	-	-	+
IDENTIFICATION	<u>Microthrix parvicella</u>	Type 0675	Type 0092	<u>Nocardia</u>	Type 0041	Type 1851

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 6 : FILAMENTS IDENTIFIED IN SLUDGE FROM CAPE RECEIFE (PORT ELIZABETH)

WET MOUNT 1000x PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE)		
	1	2	3
1 BRANCHING	True branching	-	-
2 MOTILITY	-	-	-
3 FILAMENT SHAPE	ND	straight to slightly curved	straight to slightly curved
4 LOCATION	ND	ND	ND
5 ATTACHED UNICELLS	ND	+	+
6 SHEATH	ND	-	+
7 CROSSWALLS	ND	-	+
8 FILAMENT (μm) DIAMETER	ND	0.7	2.1
9 FILAMENT (μm) LENGTH	ND	<200	ND
10 CELL SHAPE	ND	ND	rectangular
11 CELL SIZE (μm)	ND	ND	4 - 7
12 SULPHUR DEPOSITS	ND	-	-
13 OTHER GRANULES	ND	-	-
STAINS 1000x			
14 GRAM	+	-	+
15 NEISSER	-	+	+
IDENTIFICATION	*Nocardia*	Type 0092	Type 0041

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 7 FILAMENTS IDENTIFIED IN SLUDGE FROM GOUDKOPPIES MODULE 3 (JOHANNESBURG)

WET MOUNT 1000x PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE) 1	2	3	4
1 BRANCHING	-	-	-	True branching
2 MOTILITY	-	-	-	-
3 FILAMENT SHAPE	straight	straight and slightly curved	straight	ND
4 LOCATION	ND	ND	extending	ND
5 ATTACHED UNICELLS	sometimes	+ few	-	-
6 SHEATH	ND	+	-	-
7 CROSSWALLS	+	+	-	-
8 FILAMENT DIAMETER (μm)	1.4	2.1	0.7	0.8
9 FILAMENT LENGTH (μm)	ND	ND	<200	ND
10 CELL SHAPE	rectangular	rectangular		
11 CELL SIZE (μm)	ND	ND	ND	ND
12 SULPHUR DEPOSITS	-	-	-	-
13 OTHER GRANULES	-	-	-	-
STAINS 1000X				
14 GRAM	+	+	-	+
15 NEISSER	+	-	+	-
IDENTIFICATION	TYPE 0675	TYPE 0041	TYPE 0092	*Nocardia*

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 8 FILAMENTS IDENTIFIED IN SLUDGE FROM LABORATORY SCALE PLANT (CAPE TOWN)

WET MOUNT 1000X PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE)					
	1	2	3	4	5	6
1 BRANCHING	False branching	-	-	-	-	-
2 MOTILITY	-	-	-	-	-	-
3 FILAMENT	straight to coiled	coiled	straight	curved	straight to curved	straight
4 LOCATION	ND	ND	ND	ND	ND	ND
5 ATTACHED UNICELLS	+	-	+	-	-	-
6 SHEATH	+	-	+	+	+	-
7 CROSSWALLS	+	+	vague +	+	+	-
8 FILAMENT (µm) DIAMETER	1.2	2.1	0.7	1.0	1.4	0.7
9 FILAMENT (µm) LENGTH	ND	ND	200	ND	ND	ND
10 CELL SHAPE	sausage	rectangular and discoid at tips of filaments	ND	Rectangular	square	ND
11 CELL SIZE (µm)	ND	ND	ND	ND	ND	4 - 7
12 SULPHUR DEPOSITS	-	-	-	-	-	-
13 OTHER GRANULES	-	-	-	-	-	-
<u>STAINS 1000 X</u>						
14 GRAM	-	-	-	-	+ or -	-
15 NEISSER	-	-	-	-	-	-
IDENTIFICATION	<u>Sphaerotilus natans</u>	Type 021N	<u>Haliscomenobacter hydrossis</u>	Type 1701	Type 0041	<u>Thiothrix</u>

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 9 FILAMENTS IDENTIFIED IN SLUDGE FROM OLIFANTSVLEI (JOHANNESBURG)

WET MOUNT 1000x PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE) 1	2	3
1 BRANCHING	-	-	-
2 MOTILITY	-	-	-
3 FILAMENT SHAPE	straight or sligthly curved	straight	straight
4 LOCATION	extending from floc	extending from floc	extending from floc
5 ATTACHED UNICELLS	present or absent	-	present or absent
6 SHEATH	+	-	+
7 CROSSWALLS	+	-	-
8 FILAMENT (µm) DIAMETER	2.0	0.7	0.9
9 FILAMENT (µm) LENGTH	ND	ND	ND
10 CELL SHAPE	rectangular	ND	ND
11 CELL SIZE (µm)	4 - 7	ND	ND
12 SULPHUR DEPOSITS	-	-	-
13 OTHER GRANULES	-	-	-
STAINS 1000x			
14 GRAM	+ or -	-	+
15 NEISSER	-	+	-
IDENTIFICATION	TYPE 0041	TYPE 0092	TYPE 1851

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 10 FILAMENTS IDENTIFIED IN SLUDGE FROM LABORATORY SCALE UNIT 2 (PIETERMARITZBURG)

WET MOUNT 1000X PHASE CONTRAST CHARACTERISTICS	MICROORGANISMS (IN ORDER OF SIGNIFICANCE) 1	2	3	4	5
1 BRANCHING	-	-	-	-	-
2 MOTILITY	-	-	-	-	-
3 FILAMENT	coiled & twisted	coiled	straight to curved	straight to curved	straight
4 LOCATION	ND	ND	ND	ND	ND
5 ATTACHED UNICELLS	-	-	-	+	+ or -
6 SHEATH	-	-	-	+	+
7 CROSSWALLS	1	where no sulphur is present	-	-	+
8 FILAMENT (μm) DIAMETER	1.4	2.1	0.7	1.0	1.0
9 FILAMENT (μm) LENGTH	ND	ND	ND	ND	<200
10 CELL SHAPE	discoid	discoid	ND	ND	square
11 CELL SIZE (μm)	ND	ND	ND	ND	ND
12 SULPHUR DEPOSITS	ND	+	-	-	-
13 OTHER GRANULES	ND	-	+	-	-
STAINS 1000 X					
14 GRAM	+	-	-	+	-
15 NEISSER	+	-	+	-	-
IDENTIFICATION	Nostocoida limicola	Thiothrix	Type 0092	Type 1851	Haliscomenobacter hydrossis

\+ = present (or positive when referring to a stain reaction)
\- = absent (or negative when referring to a stain reaction)
ND = not determined for reasons stated on page 44

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TABLE 11 PREDOMINANT FILAMENTS IN SIX ACTIVATED SLUDGE SAMPLES

ACTIVATED SLUDGE SAMPLE

FILAMENTS (IN ORDER OF DOMINANCE)	BELLVILLE	CAPE RECEIFE	GOUDKOPPIES	LABORATORY	OLIFANTSVLEI	UNIT 2
1	*Microthrix parvicella*	*Nocardia*	TYPE 0675	*Sphaerotilus natans*	TYPE 0041	*Nostocoida limicola*
2	TYPE 0675	TYPE 0092	TYPE 0041	TYPE 021N	TYPE 0092	*Thiothrix*
3	TYPE 0092	TYPE 0041	TYPE 0092	*Haliscomeno-bacter hydrossis*	TYPE 1851	TYPE 0092
4	*Nocardia*		*Nocardia*	TYPE 1701		TYPE 1851
5	TYPE 0041			TYPE 0041		*Haliscomenobacter hydrossis*
6	TYPE 1851			*Thiothrix*		

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4.2.5 Fluorescent antibody studies of activated sludges

All six bulking sludges were stained four times with fluorescent *S. natans* antibody before examination under oil immersion (1000x magnification) using Ultra Violet illumination. In all six samples filaments exhibiting fluorescence could be observed in spite of their not being observed in five of these samples using Eikelboom's (1975) and Richard's (1984a) microscopic techniques (see Plate 1). The exception was the sludge sample from the University of Cape Town pilot plant (laboratory sample). Here the majority of the filaments were identified as *S. natans* (Table 8) and were 4+ under Ultra Violet illumination (see Plates 2 and 3). The staining was typical of that of *S. natans* filaments as described previously, with the cells exhibiting clear 4+ fluorescence (Thomason, 1976) and the sheaths staining faintly. As before, the cytoplasm and cell walls could not be distinguished and the gaps between cells within the sheath were clear. The number and size of gaps increased with the age of the sludge, probably due to death of cells (see Plate 3).

Plate 4 demonstrates the fluorescent staining of the laboratory sample after three months storage at 7°C. The filaments were faintly stained but no sign of strongly positive staining cells were present. This could indicate a total loss of viability of *S. natans* culture. Stained cells all had the typical shape of *S. natans* cells within the sheaths

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and the filament sizes and shapes corresponded. Much fluorescent antibody was trapped by the flocs as seen in Plate 2 and a few individual cells were stained outside of the sheaths. These cells may be S. natans "conidia", described by Waitz and Lackey (1959) as being vegetative cells having broken loose from filaments to initiate other filaments elsewhere. A large proportion of the filaments however remained unstained by the fluorescent antibodies.

A few N. limicola cells from unit 2 (Pietermaritzburg) stained with 2+ intensity (Thomason, 1976) fluorescence within their sheaths but the majority were not stained. The cell morphology here was however characteristic and the majority of filaments remained non-fluorescent.

No false branching was observed in the filaments that stained positively but this was not surprising as this identifying characteristic of S. natans was rarely seen in the Cape Town Laboratory sample using phase contrast microscopy and not at all in the other bulking sludges.

The fluorescent antibody stained cells could be observed through the smaller flocs, simplifying identification where these flocs obscured the characteristics required for identification using contemporary microscopic techniques (Plate 3).

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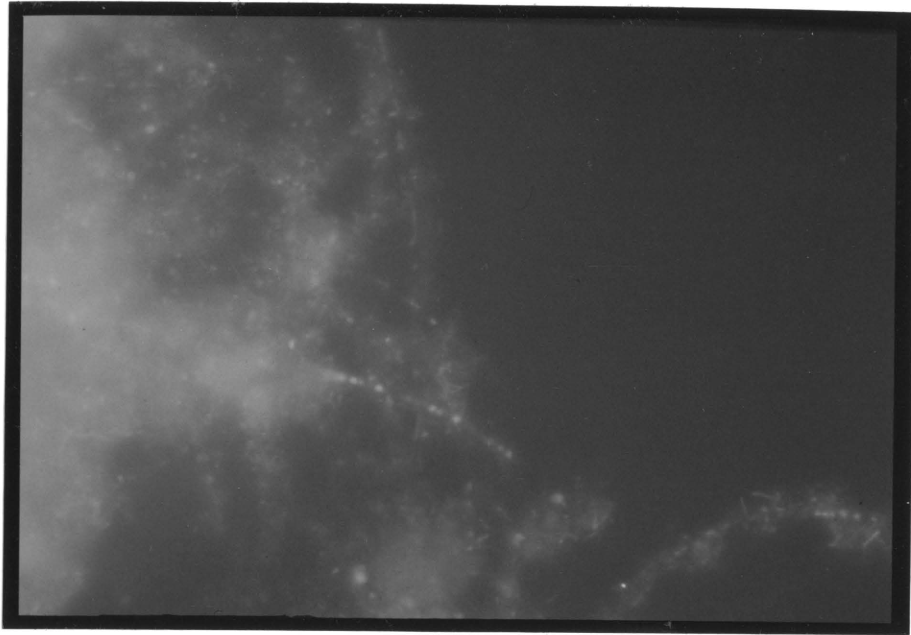

PLATE 1: Protruding filaments of S. natans exhibiting 4+ fluorescence in Belville activated sludge (1000 x magnification).

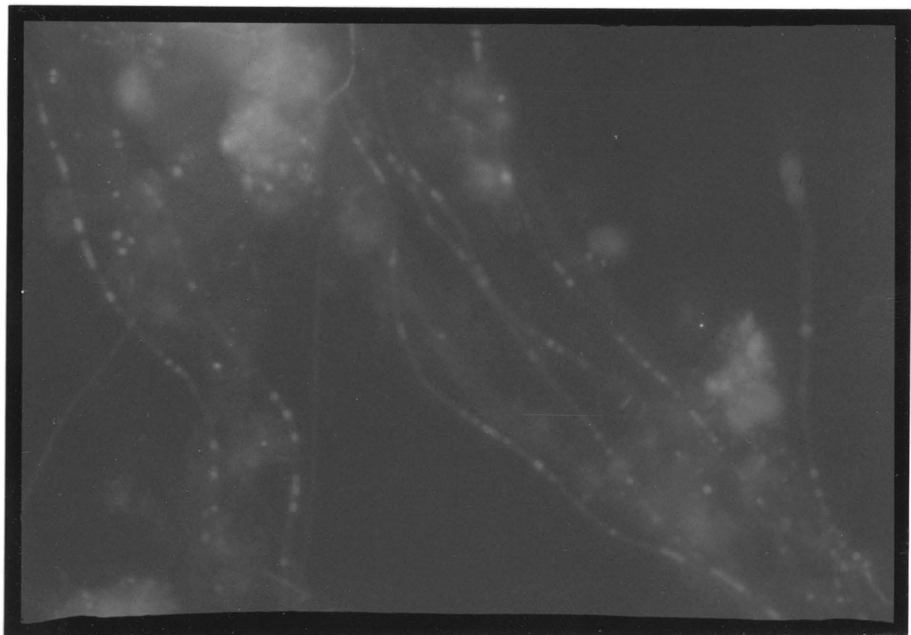

PLATE 2: Filaments of S. natans exhibiting 4+ fluorescence in the Cape Town laboratory scale activated sludge plant where this filament was the major cause of bulking (1000 x magnification).

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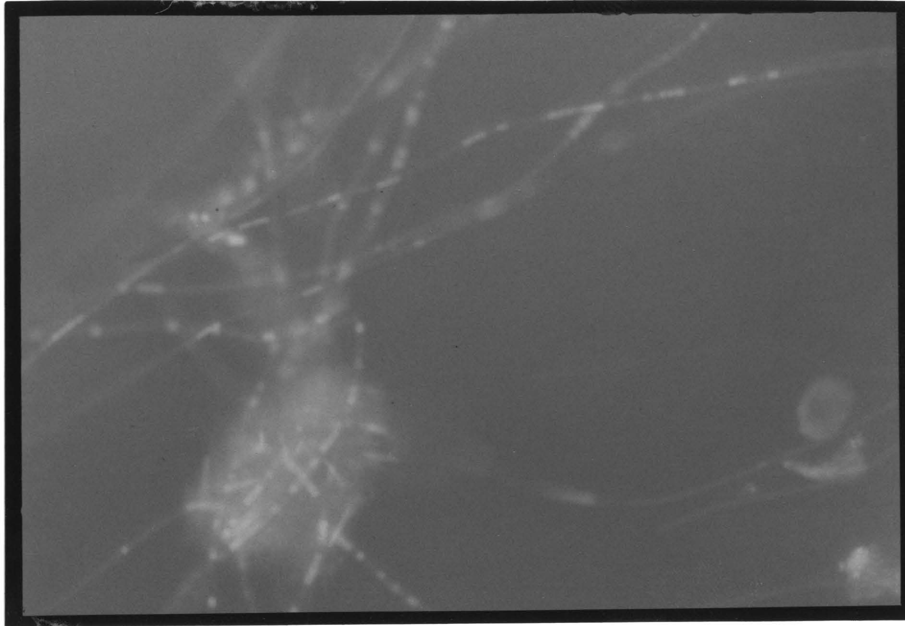

PLATE 3: S. natans filaments in the laboratory sample with the cells stained 4+ by fluorescent antibody. The gaps between the cells within the sheath show clearly as does the filaments capacity to be imbedded within a floc to cause bridging (1000 x magnification)

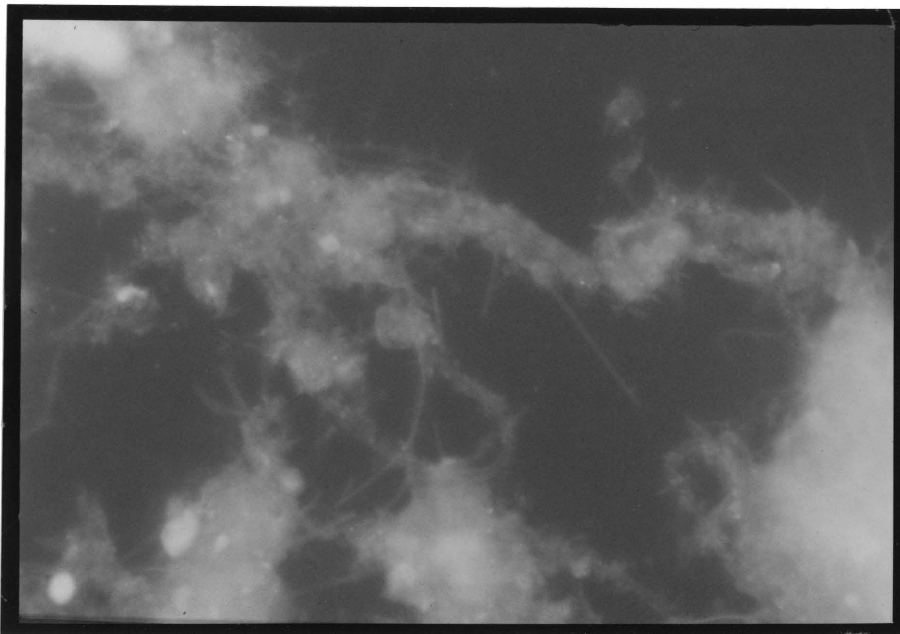

PLATE 4: Empty S. natans sheaths stained faintly in the laboratory sample after storage at 7°C for three months (1000 x magnification)

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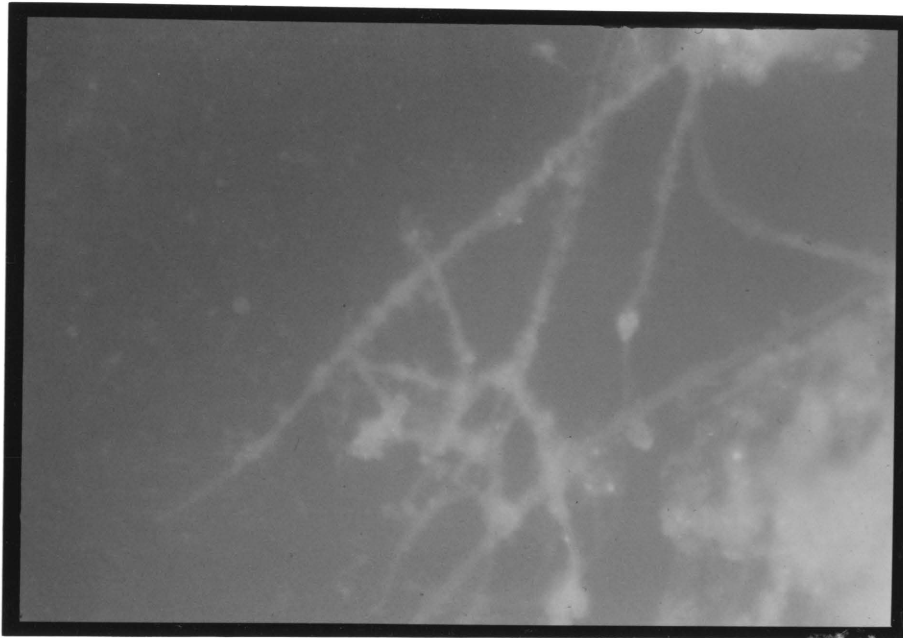

PLATE 5: Non-specific fluorescent staining of Goudkoppies module 3 activated sludge by a fluorescent antibody produced against Acinetobacter (1000 x magnification).

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Plate 5 was taken of Goudkoppies activated sludge stained with the fluorescent antiserum produced against *Acinetobacter* by Cloete (1984). This showed that a dull non-specific staining of the filaments and flocs was also possible.

The microscopic techniques of Eikelboom and van Buijsen (1983) and Richard (1984a) used in this study were sufficient for the identification of the predominant filaments in activated sludges. The less numerous species were possibly overlooked, especially where the filaments were morphologically similar. For example, *S. natans* and Type 1701 are similar in many respects (Richard 1984a, Table 2).

Wet preparations have the disadvantage that filaments may be partially or totally embedded within the floc obscuring identifying characteristics such as false branching which is more common in *S. natans* than Type 1701 (Eikelboom and van Buijsen, 1983). Filament length determinations were also difficult.

Another problem that arose from these microscopic identification techniques was the fact that the specific filament observed under phase contrast microscopy could not be examined for Neisser or Gram stains.

The resulting difficulty in identifying these less numerous filaments means that sewage works operation may alter conditions to control the more dominant filaments and, in so

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doing, cause the proliferation of a previously infrequent species. Prior identification of the latter may thus prevent their causing bulking. Application of the above microscopic techniques also requires a certain amount of expertise and experience.

The fluorescent antiserum produced against the S. natans from Germany was shown to be highly specific to S. natans irrespective of whether it was isolated in Germany or America, or observed in South Africa. In addition, it was shown to react with filaments resembling S. natans in each bulking sludge examined.

However, isolation of S. natans from Goudkoppies module 3 proved unsuccessful and use of phase contrast microscopy, Neisser and Gram stains revealed no S. natans in the sludges with the exception of the laboratory sample. The possible explanations for this have been discussed. The labelled antibody was also shown to be capable of penetrating bacterial sheaths to stain the cells individually, the effect of which could be observed through flocs or other suspended matter which hinder identification using other microscopic techniques.

The results of the microscopic techniques of Eikelboom and van Buijsen (1983) and the fluorescent antibody studies overlapped significantly. This was particularly apparent in

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the Cape Town Laboratory sample, where both techniques showed S. natans to be the predominant filament causing bulking. Using both of these techniques it was also shown that many of the filaments present were not S. natans. In addition, the use of these techniques indicated that other filaments predominated in the remaining bulking sludges. Use of the fluorescent antibody technique indicated the presence of S. natans in minor quantities in these sludges while use of conventional microscopic techniques failed to detect it. All filaments stained by fluorescent antiserum resembled S. natans (as described by Eikelboom and van Buijsen, 1983) when examined under phase contrast microscopy.

The use of a fluorescent antiserum to identify filaments of S. natans in activated sludges proved to be a rapid and simple technique the accuracy of which relied entirely on the specificity of the antibody produced. This method could be used to monitor sewage works accurately once fluorescent antibodies have been produced against all the filaments occurring in activated sludges.

The faint staining of S. natans sheaths by fluorescent antibody produced against cells of this bacterium and the ability of the majority of the activated sludge filaments to be stained non-specifically by fluorescent antisera produced against Escherichia coli and Acinetobacter indicated non-specific adsorption of antiserum to, or possible antigenici-

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ty of, these sheaths. The production of specific fluorescent antibodies against _S_. _natans_ cultivated within a sheath might thus have been complicated or made impossible due to the production of antibodies against the sheaths as well as the cells. This should be taken into consideration if the fluorescent antibody technique is to be applied to other sheathed bacteria in activated sludges.

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CHAPTER 5

SUMMARY

1. Standard techniques for the isolation of bacterial filaments were applied to Goudkoppies activated sludge but only *Nocardia* could be successfully isolated. This was also the only filamentous microorganism observed in this activated sludge (using standard microscopic techniques) that has been previously isolated. Fluorescent staining revealed small quantities of *Sphaerotilus natans* filaments in this sludge but the inoculum was too low for isolation of this organism and the competition for nutrients on the isolation media from other microorganisms too great.

2. Fluorescent antiserum was prepared successfully against *S. natans* antigen in rabbits but unsuccessfully against *Nocardia* antigen. The titre and protein content in the latter was too low due to a lack of reaction by the rabbit's immune system.

3. The *S. natans* fluorescent antiserum was shown to be highly specific, showing no reaction with either pure cultures of similar filamentous bacteria or entirely unrelated microorganisms. The antiserum did however show a lack of strain specificity as it reacted with *S. natans* isolates from Germany and America and filamentous bacteria in South African activated sludges which appeared morphologically identi-

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cal to this organism. *S. natans* thus appears, within the scope of this study, to have little serological variation within the species.

4. Fluorescent antiserum is capable of penetrating the sheath of *S. natans* to stain the cells individually. This can easily be observed through the sheath as 4+ fluorescence although the cell wall cannot be so easily distinguished from the cytoplasm. The sheath can also be faintly stained by the antiserum. Individual cells of *S. natans* were also stained 4+ but here cell cytoplasm could be distinguished from the cell walls.

5. The use of fluorescent antiserum in the identification of *S. natans* filaments obscured by activated sludge flocs and other suspended matter was simple as the cells stained brightly and could be observed through the less dense matter while the use of other microscopic techniques would be hampered by these obstructions.

6. *S. natans* occurred in six activated sludges in South Africa but in such minor quantities that it could not be identified by any technique other than the specific fluorescent antiserum technique. The sludges have to be examined when fresh.

7. The previously described microscopic identification techniques for filaments in bulking sludges were shown to be

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excellent for identifying the predominant filaments but were laborious and required practiced expertise. Fluorescent antiserum staining, however, proved to be rapid, accurate and easy to apply. If *S. natans* was present, even in low numbers or partially obscured within sludge flocs, it could easily be observed by a relatively unskilled worker.

8. With the aid of *S. natans* fluorescent antiserum, the *in situ* study of this organism's ecology in activated sludges and other aqueous environments could be greatly enhanced. This technique could therefore give us tremendous insight into the autecology of the organism.

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