Some observations on the morphology and the anatomy of filament type 0041

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Abstract

Some morphological and anatomical characteristics as determined by light, transmission electron and scanning electron microscopy are reported for filament type 0041. The filament is procaryotic, but the dimensions differ slightly from those quoted in literature. The relation between the sheath and the bacterial filament is clearly indicated. The sheath is not tight-fitting as described in literature. As far as could be determined it was the first time that this observation was made. Furthermore the sheath appears to be transparent. The observation on the specimens fixed in Karnovski is important because samples can be preserved for several weeks without the loss of their Gram and Neisser characters.

Introduction

Flocs of bacteria are formed spontaneously when waste water is aerated. The activated sludge process is based on this principle (Eikelboom and Van Buijsen, 1983). Activated sludge flocs consist of bacteria, protozoa, fungi, inorganic and organic material (Eikelboom and Van Buijsen, 1983). The bacteria in the flocs are usually of two types, filamentous and unicellular (Kulpa *et al.*, 1982; Jenkins *et al.*, 1983; Eikelboom and Van Buijsen, 1983; Jenkins *et al.*, 1984). Under certain conditions the filamentous bacteria may give rise to bulking activated sludge (Richard *et al.*, 1981; Jenkins *et al.*, 1983; Eikelboom and Van Buijsen, 1983; Kulpa *et al.*, 1982). Early research on bulking activated sludge incriminated *Sphaerotilus natans* as the causative organism. In his pioneering work Eikelboom (1975) found that most of the filamentous bacteria were not *S. natans*, but other species and types. It was recently confirmed that various filamentous bacteria, as many as 22 different types, can be responsible for this problem (Richard *et al.*, 1983; Eikelboom and Van Buijsen, 1983; Richard, 1984; Jenkins *et al.*, 1984).

It is general practice to examine activated sludge for filamentous bacteria in order to diagnose the cause of sludge bulking. At present this diagnosis is made by microscopic examination under phase contrast of a wet mount of the sludge, augmented with Gram and Neisser stained smears (Eikelboom, 1975; Eikelboom and Van Buijsen, 1983; Richard, 1984; Jenkins *et al.*, 1984).

Unfortunately diagnosis by the light microscope is hampered by technical problems. These problems are aggravated by the phenomenon that filaments show a big variation in morphology, as far as size, attached growth, cross-walls, sheath and staining reaction are concerned (Richard *et al.*, 1981). In order to overcome these problems it was decided to supplement the light microscopic observations with transmission electron microscopic (TEM) and scanning electron microscopic (SEM) studies, as these can provide additional information on the morphology of the filaments.

Blackbeard *et al.* (1986) conducted a survey of the incidence of filament types in 111 South African activated sludge plants. According to their findings type 0041 occurred in 94% of these samples. As this filament is found in the majority of South African activated sludge plants, some morphological and anatomical data on type 0041 are described in this paper.

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It was not an objective of the present study to gain more information on the final classification of this organism. It should be placed in Division 2 *Firmicutes*, Class 2 *Thallobacteria* of the *Procaryotae* (Krieg and Holt, 1984).

It is further not an objective of this paper to comment on bulking sludge as such. As background information it could be mentioned that most of the work was done on the activated sludge plants of Potchefstroom and Lichtenburg. The former is a five-stage Bardenpho plant and the latter is a Pasveer channel. Both treat domestic sewage.

Materials and methods

Light microscopic examination

Samples from the activated sludge plants were brought to the laboratory within two hours after sampling. In the laboratory two smears were prepared for staining by Gram and Neisser procedures and wet mounts were prepared for immediate examination, according to the procedures described by Jenkins *et al.* (1984) and Richard (1984). Wet mounts were studied by means of phase contrast microscopy and photographs were taken under the light microscope (Richard, 1984).

The filaments were identified by means of the key developed by Eikelboom and Van Buijsen (1983).

Washing of the activated sludge flocs

It was soon obvious that most of the fresh samples were unsuitable for light microscopic and electron microscopic examination. Especially in the case of the electron microscope poor results were obtained due to a high debris content. To overcome this problem a simple washing procedure was developed, which included the following steps.

On arrival in the laboratory the samples were left on the working bench for the sludge to settle down. This usually occurred within 10 min. The upper level of the sample was marked on the sample bottle. The supernatant was discarded and the bottle filled to the mark with tap water. The activated sludge sediment was resuspended by shaking. Sedimentation was allowed to occur – and the washing process was repeated twice. Finally after filling to the mark and mixing, the light microscopic and the electron microscopic examination were carried out. By means of this procedure the flocs retained their characteristic morphology, but the filaments became more obvious as much of the debris and slime was washed away.

Transmission electron microscopy

Washed filaments (*vide supra*) were harvested by centrifugation, at 5 000 rm^{-1} for 5 min in a Sorvall bench centrifuge. The filaments were then fixed for two hours in a 2% glutaraldehyde/formaldehyde mixture prepared in pipes buffer. They were then washed twice in pipes buffer (pH 7,2) and divided into two fractions (Bullock, 1984).

One fraction was examined after negative staining with 0,5% phosphotungstate. The second fraction was prepared for sectioning (Tiedt, 1985). The sections were stained with 2% uranyl acetate and lead citrate (Reynolds, 1963) and examined in a Siemens Elmiskop 101 electron microscope, operating at 100 kV.

Scanning electron microscopy

Five to 10 ml freshly washed sludge (*vide supra*) was sampled and fixed in modified Karnovski's fixative (Hayat, 1972; Bullock, 1984) for at least 24 h. The modified fixative consisted of 1% saturated picric acid added to Karnovski's solution.

Specimens were post-fixed (Boyde and Boyde, 1980), dried, mounted, sputter coated with carbon and gold-palladium. The prepared specimens were studied at 10 kV and 20 kV with a Cambridge Stereoscan 250 SEM.

Results

Light microscopic examination

Fixed specimens (*vide* SEM technique) were stained with Gram and Neisser reagents. These specimens were one to three months old. All the fixed specimens retained their Gram and Neisser characters when compared with fresh specimens. This is the first observation so far to be described. This has the advantage that samples taken far away from laboratories can later be examined at ease without the loss of the Gram and the Neisser characteristics.

A photograph taken under the light microscope of a Gram stained smear of a specimen from the Potchefstroom works is presented in Figure 1. The width of the filament is roughly 1,0 μm, which is less than that measured from a wet mount. Perhaps some shrinkage of the filament occurs during the Gram staining reaction. The width of this filament usually varies from 1,2 to 1,6 μm (wet mount). The length of the cells is more or less 2,3 μm, although it is often less. The attached growth is typical, although it can be much more luxurious.

TEM study

In Figure 2 a TEM micrograph, negatively stained, is displayed. This specimen was fixed. The filament appears to be an empty sheath as no cells can be observed. Further evidence of this is supported by the fact that the implantment of the attached cells can be seen on the other side of the transparent sheath. Many of the attached cells appear to be stalked *Caulobacter*-like bacteria. The diameter of the sheath is 1,9 μm. No sheath could be observed with the light microscope. The diameter is much wider than that measured for Figure 1. This might be ascribed to a sheath fitting rather loosely.

A longitudinal section was prepared (TEM technique, *vide supra*) on a specimen from Potchefstroom (Figure 3) to illustrate the relation between the sheath and the bacterial filament. The relation between the bacterial filament and the sheath is clear in this micrograph. The sheath is not tight-fitting as described in literature (*vide infra*: Morphology and anatomy of type 0041).

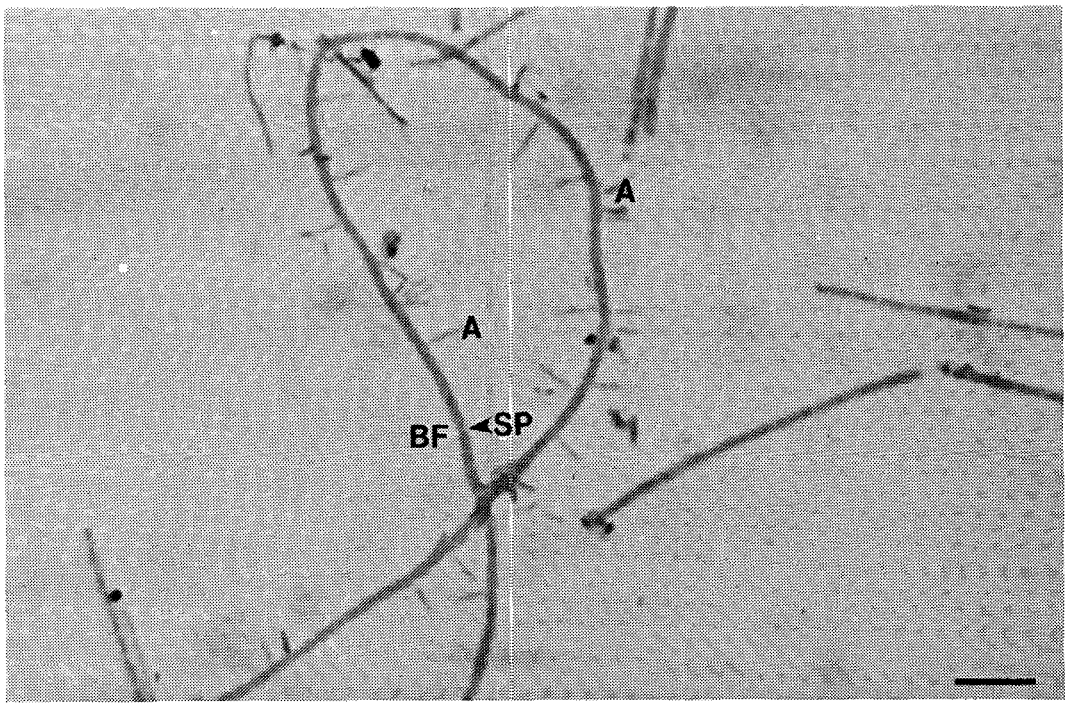

Figure 1
Light microscopic appearance of a Gram stained smear
A = attached growth
BF = bacterial filament
SP = septum
Bar = 4,6 μm

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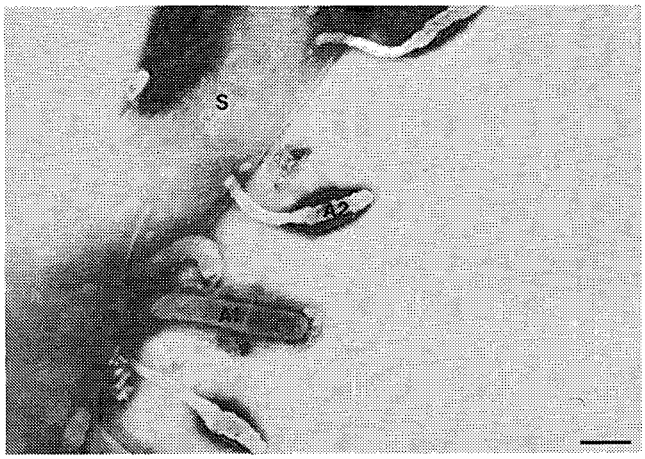

Figure 2
TEM micrograph
A1 = *attached growth*
A2 = *attached growth, stalked* Caulobacter-*like bacteria*
S = *sheath*
Bar = *0,7 μm*

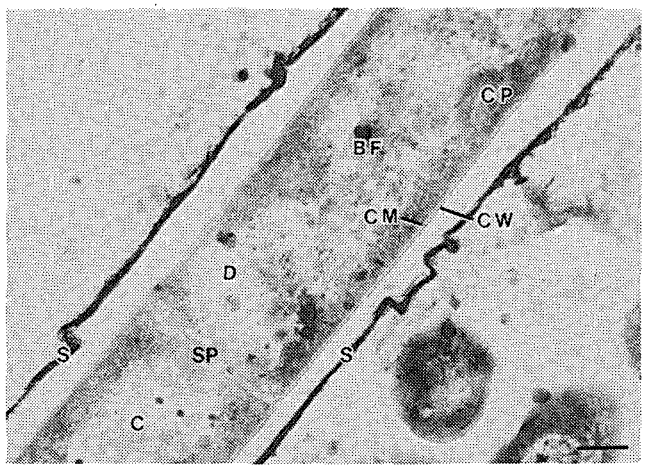

Figure 3
Longitudinal section through type 0041
BF = *bacterial filament*
S = *sheath*
SP = *septum*
C = *cell*
CW = *cell wall*
CM = *cytoplasmic membrane*
D = *DNA*
CP = *cytoplasm*
Bar = *0,2 μm*

This is confirmed by Figure 4 as well as by the SEM micrograph presented in Figure 8.

The width of the total trichome is 1,1 μm, while that of the bacterial filament inside the sheath is 0,8 μm. The length of the cell is in excess of 2,5 μm. Note the typical procaryotic cell structure. The width of the filament is less than that quoted in literature.

This micrograph (Figure 4), has been included to compare the result with that of the SEM (Figure 8). This specimen came from Potchefstroom, while that in Figure 8 was taken at Lichtenburg. The same results were obtained. The total trichome has a diameter of 2,4 μm, whilst the bacterial filament measured a width of 1,4 μm. The length of the cells varies from 2,0 to 2,4 μm. These dimensions agree more favourably with those described in literature than the other TEM micrographs. Note the transparent and loose-fitting sheath.

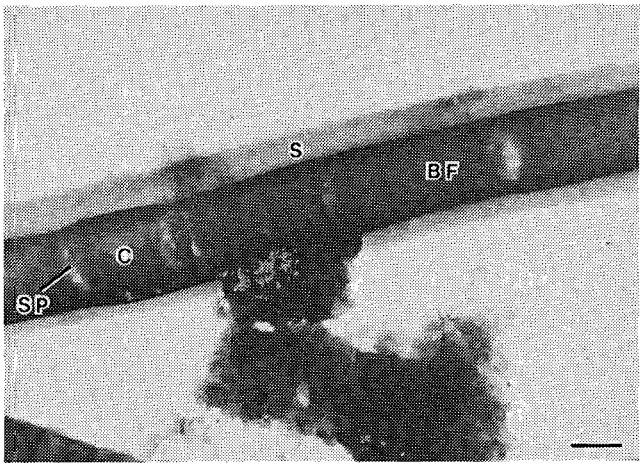

Figure 4
Type 0041 with a sheath
BF = *bacterial filament*
S = *sheath*
C = *cell*
SP = *septum*
Bar = *0,8 μm*

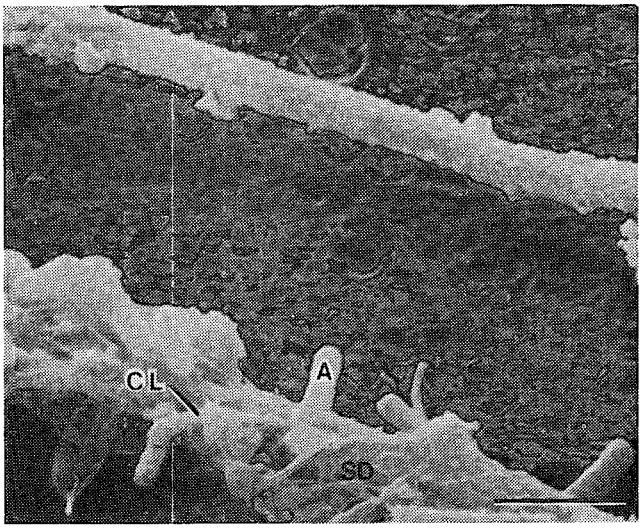

Figure 5
Type 0041 with attached growth
A = *attached growth*
CL = Caulobacter-*like attached cells*
SD = *Slime and debris*
Bar = *2 μm*

SEM study

Several SEM micrographs are presented, starting with an unwashed specimen of type 0041 from Johannesburg Northern Sewage Works (Figure 5). This works is a five-stage Bardenpho activated sludge plant. Note the luxurious attached growth and the amount of slime and debris surrounding the filament. No cells and sheath are discernible. The diameter of the filament could not be determined. The phenomenon of slime has been described by Ross (1984).

Some filaments are devoid of attached growth (Figure 6, Lichtenburg). The rectangular cells are clearly seen in this figure. The diameter of the cells is 1,4 μm, while the length is 2,3 μm. The septa are obvious, but no sheath is noticeable. The filament in the background is type 1863.

As soon as the filament carries attached growth the morphology of the trichome becomes obscure (*vide* Figure 7). The trichome has a diameter of 1,6 μm. The type 0041 filament to the right only has a diameter of 1,1 μm. In the left corner a *Nostocoida limicola III* can be seen.

The relation of the bacterial filament to the sheath is indicated in Figure 8. This rare micrograph illustrates how the results of SEM and TEM supplement each other (compare Figure 4). The diameter of the total trichome is 1,7 μm and that of the bacterial cells 0,9 μm. The septa can be clearly seen. The length of the cells varies from 1,1 to 2,4 μm.

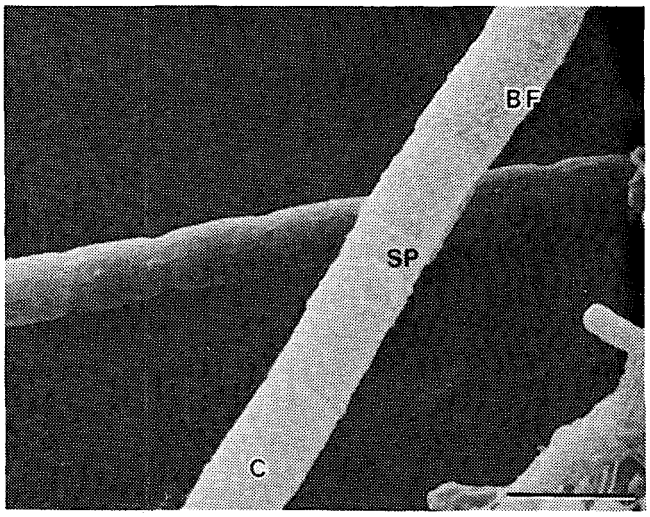

Figure 6
Type 0041 without attached growth
BF = bacterial filament
C = cell
SP = septum
Bar = 2 μm

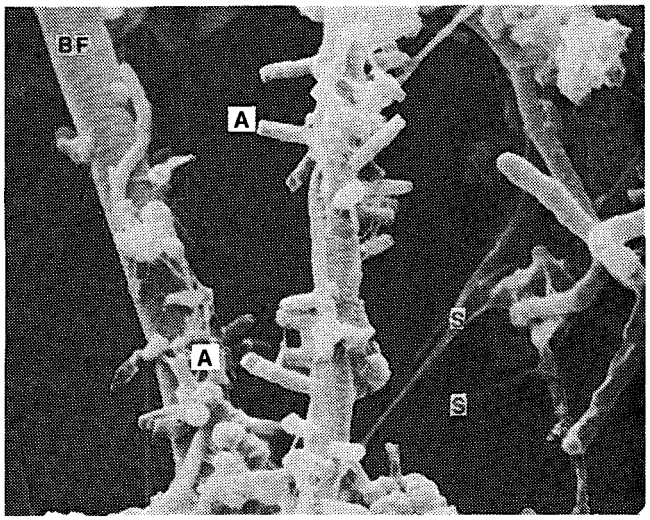

Figure 7
Type 0041 with attached growth
A = attached growth
BF = bacterial filament
Bar = 4 μm
S = slime

Discussion

Morphology and anatomy of type 0041

Eikelboom (1975), Eikelboom and Van Buijsen (1983), Richard (1984), Richard *et al.* (1981) and Jenkins *et al.* (1984) describe the morphology and the anatomy of this filament (trichome). The straight or slightly bent filament is septated and multicellular. The length of the trichome may vary from 100 to 500 μm. The filaments are found in the liquid between the flocs as well as in the flocs. Branching is incidental. The square to rectangular cells are 1,5 to 2,5 μm long and 1,2 to 1,6 μm wide. The cells are surrounded by a clear, tight-fitting sheath, which may be strongly electron dense and difficult to observe. The end cells of the filament are not enclosed by the sheath.

Frequently heavy attached growth is observed, but sometimes this may be absent. Occasionally the septa are masked by the attached growth. The filaments give a negative Neisser reaction and a positive Gram staining reaction. Atypically a positive Neisser staining covering may be observed. Parts of the filament with abundant attached growth often stain Gram variable. It tends to be Gram positive inside the floc and Gram negative when extending into the liquid.

There might be a resemblance between filaments without attached growth and type 021 N. The latter, however, always shows a Gram negative character.

Type 0041 grown in culture has cell diameters of 1,0 to 1,5 μm and lengths of 2,0 to 4,0 μm, while the diameter of the filament may vary from 1,0 to 1,5 μm and 2,0 to 3,0 μm. The sheath is heavy-walled (Richard *et al.*, 1981). Cells are almost square in broth media and more rectangular in agar media.

Occurrence of type 0041 in the present study

During 1984 and 1985 activated sludge samples were examined on several occasions from Potchefstroom (Bardenpho), Lichtenburg (Pasveer) and Schweizer-Reneke (Pasveer). Sludge samples from the Johannesburg Northern Works (5 stage Bardenpho) were analysed once. In all cases type 0041 was present, but it was never the dominant filament type.

Conclusion

The filamentous type 0041 is generally present in South African activated sludge plants (compare Blackbeard *et al.*, 1986; and Hart, 1985). It is usually fairly easy to identify this organism under the phase contrast microscope. Nevertheless, much still re-

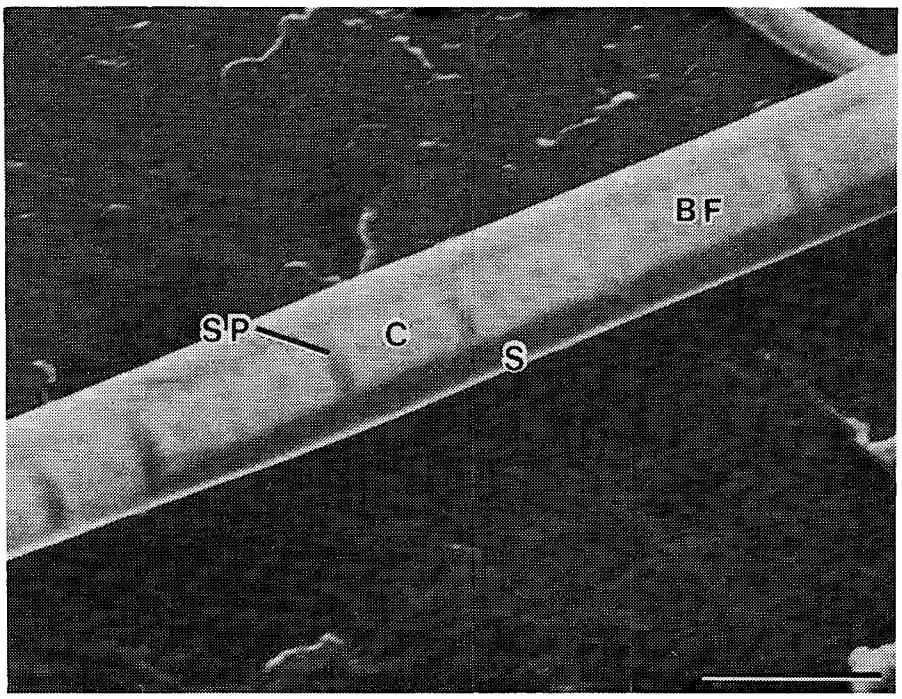

Figure 8
Type 0041 with a sheath
BF = bacterial filament
C = cell
SP = septum
S = sheath
Bar = 2 μm

mains unknown about its anatomy and morphology. The objectives of this study were to clarify some of the uncertainties.

Under the light microscope a sheath is very seldom seen. Even with most of the electron microscopic examinations it is difficult to observe. During the present study it was best observed with the TEM (*vide* Figure 3) only after sections had been made. Examining roughly 20 samples with the TEM and the SEM and scanning several hundreds of microscopic fields it could only be seen twice with the TEM and once with the SEM (*vide* Figures 3, 4 and 8). Jenkins *et al.* (1984) state that the cells of type 0041 are contained in a clear, tight-fitting sheath. Consequently Figures 3, 4 and 8 disagree with this statement. Furthermore it seems as if the sheath is extremely transparent, thus it is difficult to observe.

The cells are clearly seen in the absence of attached growth (*vide* Figure 6). On these occasions the filaments always stain Gram positive, the cells are more rectangular and they appear to be slightly bigger. As soon as luxurious attached growth occurs (*vide* Figures 5 and 7), the Gram character changes to negative or variable and the cells are not easily seen. In general the filaments show attached growth.

As far as the shape and the dimensions of the cells are concerned the present results correlate well with available literature. However, it appears that the dimensions of the cells are smaller when measured with the electron microscope than when measured with the light microscope, especially with the TEM.

It was found that the Gram and the Neisser stain characteristics did not change when samples were fixed as described for the SEM. In this respect it could be advantageous to preserve samples before sending them to a laboratory for examination.

The occurrence of excessive slime surrounding the filaments was observed on various occasions. Here it is illustrated in Figures 5 and 7. This compares well with the results discussed by Ross (1984).

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