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In situ Identification of Filamentous Bacteria from Activated Sludge Wastewater Treatment Plants in Ghana

Melvin-Guy Adonadaga[1,2,*], Marion Martienssen[1]

[1]Department of Biotechnology for Water Treatment, Faculty of Environmental Sciences and Process Engineering, Brandenburg University of Technology, 03046 Cottbus, Germany.
[2]Department of Earth and Environmental Science, Faculty of Applied Sciences, University for Development Studies, Navrongo, Ghana
*Corresponding author: madonadaga@gmail.com

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Abstract Filamentous bacteria are important in the activated sludge process as they contribute to proper solids-liquid separation in the secondary clarifier by providing a skeletal matrix for the formation of compact flocs. Morphological and molecular techniques were applied over a one-year period to comprehensively identify filamentous bacteria in municipal and industrial wastewater treatment plants (WWTPs) in Ghana. Several morphologically distinguishable filamentous microorganisms were observed and in most cases, microscopic characteristics of these organisms were in close agreement with those described in published keys. The presence of *Haliscomenobacter hydrossis*, *Thiothrix nivea*, *Sphaerotilus natans*, *Nostocoida limicola* II, Eikelboom Type 1851 and members of the Eikelboom Type 021N group II was subsequently confirmed based on hybridization with their respective oligonucleotide probes. Filaments were not unique to each plant, with higher filament diversity in industrial compared to municipal plants. Additionally, the results revealed that although geographic differences have no significant effect on filament morphology, they likely play a limiting role in the occurrence of specific filaments.

Keywords: *activated sludge process; filamentous bacteria identification; effect of geographic differences; Ghana*

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1. Introduction

The activated sludge process has been accredited as the most widespread technology for the biological treatment of industrial and municipal wastewater since its inception in 1914 [1]. It is an aerobic suspended growth process in which organic matter is oxidized to carbon dioxide, water, ammonia and new cell biomass by a consortium of microorganisms in a biochemical stage, followed by a physical stage in which the microbial flocs are allowed to settle [2]. However, a major process problem with this method is sludge bulking due to proliferation of filamentous bacteria, resulting in deterioration of sludge settleability and loss of active biomass [3].

By using morphological and simple staining techniques, twenty six morphotypes of filamentous bacteria were identified and grouped into seven assemblages [4]. Subsequently, simple identification keys have been constructed based on these techniques, making it possible to identify filamentous organisms in activated sludge samples [5,6]. However, morphology-based identification has some limitations such as non-filamentous growth for some bacteria, loss of sheath-forming capacity and variable staining reactions [7]. This has sometimes led to incorrect identification of filamentous bacteria [8]. Despite these limitations, morphological methods are still very commonly used because they are relatively easy with no special laboratory equipment required [9].

In an effort to overcome these limitations, fluorescence *in situ* hybridization (FISH) technique was developed [10]. This cultivation independent molecular method involves the use of fluorescently labeled ribosomal ribonucleic acids (rRNA) targeted oligonucleotide probes [11,12]. Its applicability in identifying bacteria in activated sludge samples has been confirmed by several studies [7,13,14].

Morphological and molecular surveys on the occurrence of filamentous bacteria in activated sludge WWTPs in Europe have reported the presence of *Microthrix*, *Thiothrix*, *H. hydrossis*, *S. natans* and the unnamed organism of the Eikelboom type 021N among others [7,14,15]. It is known, however, that environmental factors do affect microorganisms by either limiting their occurrence to particular geographic regions or necessitating changes in their morphology [16]. Research by [8] on pure cultures confirmed this phenomenon prompting the authors to ask if these morphological changes were limited to pure cultures or if they occur also in activated sludge. As such, it is important to identify filamentous bacteria in different regions in order to determine the effect of geographic differences on their

occurrence and morphology. This information is necessary also for the application of specific strategies towards filamentous bulking control. Therefore, the aim of this research was to 1) identify filamentous bacteria occurring in full-scale activated sludge plants treating industrial and municipal wastewater in Ghana, and 2) compare their occurrence and morphology to those identified in different geographic regions.

2. Materials and Methods

2.1. Wastewater Treatment Plants

Three full-scale activated sludge WWTPs located in Greater Accra Region of Ghana were investigated in this study. Two of the plants treat wastewater from hotels (municipal) while the third plant handles wastewater from a food and beverages factory (industrial). All the plants operate on the conventional activated sludge design, consisting of aeration and sedimentation tanks, without biological nutrient removal. Air is supplied to all the aeration tanks by means of diffuse aerators. DO concentrations in the industrial plant range between 1.3-1.7 mg/l while that in the municipal plants is between 0.4-0.9 mg/l. Average temperature of the mixed liquor in each of the plants was 30 ^{o}C. Whereas the effluent from the municipal plants is used for watering of plants and general landscaping on site, that of the industrial is discharged directly into the central sewerage system.

2.2. Sample Collection and Handling

Field samples were collected from all three WWTPs over a one-year period. Sample collection was done monthly from May to July 2014 and from November 2014 to January 2015, coinciding with the wet and dry seasons respectively in Ghana. Representative grab samples of mixed liquor were taken during aeration and stored half-full in pre-sterile plastic bottles so as to maintain aerobic conditions for survival of filamentous organisms during transit. Samples were stored in an ice chest at a temperature below 4°C in order to reduce any change to their characteristics.

2.3. Morphological Identification

Microscopic examination of detailed morphological structures for filament identification was performed based on the identification keys of [5] and [6], and visualized with a Nikon Eclipse LV100 (Japan). Wet mounts were studied under phase-contrast at 600x and 1000x magnification. Filament abundance was subjectively rated using the scoring technique outlined by [6]. Filament abundance ratings were determined for each filamentous organism type. Each filament was scored on a scale between 0 and 6 (integer scores had the following meanings: 0 = none; 1 = few; 2 = some; 3 = common; 4 = very common; 5 = abundant; and 6 = excessive).

2.4. Gram Staining

Gram staining technique was used to differentiate between Gram-positive and Gram-negative filamentous bacteria. The test was performed according to the procedure described by [17], but with some modifications. 60 µl of appropriately diluted sample was placed on a ready-to-use slide (Menzel Superfrost, 76×26 mm), smeared across the slide and allowed to air dry. The sample was then completely flooded with working solution 1C (mix of crystal-violet solution (1A) and sodium hydrogen carbonate solution (1B) 1:1) for 60 s. The slide was carefully rinsed with distilled water for 5 s after which it was completely covered with stabilizing solution 2 (iodine solution) for 60 s. The stabilizing solution acts as a mordant to form the crystal violet-iodine complex. This step is referred to as fixing the dye. The slide was again carefully rinsed with distilled water for 5 s, and the sample subsequently decolorized by washing off the dye for 5-10 s using decolorizing solution (a mixed solvent of ethanol and acetone). The decolorization step was properly timed to ensure that all dye which is not trapped is washed off, but also that it is not too long to remove the dye from both the Gram-positive and negative cells. The slide was again rinsed carefully with distilled water for 5 s and then counterstained by covering it completely with fuchsin solution for 15-30 s. The slide was finally rinsed with distilled water, shaken to remove excess water and allowed to air-dry. The sample was then visualized under bright-field using Nikon Eclipse LV 100 microscope at 600× magnification. Gram-positive filamentous bacteria appear blue or purple, while Gram-negative are red or pink.

2.5. Fixation of Cells

Mixed liquor samples for molecular analysis were fixed immediately upon arrival in the laboratory according to the procedure described by [18], and the protocol of the Chair of Biotechnology for Water Treatment, BTU. Each sample was centrifuged at 5000rpm at room temperature for 5 min and the resulting pellet washed three times in 1 x phosphate buffered saline (PBS) (130 mM NaCl, 10 mM $NaPO_4$, pH 7.2). The pellet was re-suspended in 1ml of freshly prepared 4% PFA in 3×PBS (390 mM NaCl, 30 mM $NaPO_4$, pH 7.2-7.4). Fixation was done overnight at 4°C. Fixed samples were then washed three times in 1×PBS, each centrifugation step carried out at 10000rpm for 3 min. The final pellet was then gently suspended in 500 µL 1×PBS and 500 µL pure ethanol and stored at -21°C.

2.6. Identification by FISH

A range of oligonucleotide probes (Table 1) was applied to identify filamentous organisms present in all samples. Probes were selected based on the morphological identification results. Synthesis and labeling of probes was carried out by Invitrogen™, Life Technologies. Detailed information about most of these probes is given in probeBase [19]. EUB 338, a probe specific for most bacteria, was used as positive control, while hybridization without probe was also used as negative control on each slide.

Ready-to-use glass slides (Thermo scientific, Menzel-glasses Superfrost ® Plus, Germany) were used for immobilization of cells in this study. 10 µL of fixed sample was spotted on a prepared slide, air-dried and dehydrated stepwise in 50, 80 and 98% ethanol, each dehydration step lasting 3min. The slide was again allowed to air dry. Hybridization was then performed according to the procedure described by [20], and the

protocol of the Chair of Biotechnology for Water Treatment, BTU. Briefly, 10 µL of hybridization buffer (20 mM Tris, 0.9 M NaCl, 0.01% SDS, pH 7.2-7.4), probe and formamide (Calbiochem® - Novabiochem, Germany) was added to the dehydrated sample. The hybridization buffer and formamide concentration were optimized in each case depending on the stringency of the probe. The slide was then placed in a 50 ml polypropylene screw top tube (Eppendorf, Germany), and incubated at 46°C for 180 min in a thermostat (Eppendorf ThermoStat Plus). The slide was removed from the chamber and hybridization immediately stopped by rinsing the probes off with washing solution (5M NaCl, 1M Trias/HCl, 10% SDS, pH 7.2-7.4) pre-warmed to the washing temperature.

Table 1. FISH probes, target microorganisms, target sites and formamide concentration

Probe	Target microorganism	Probe sequence (5'-3')	Target site (16S rRNA positions)	% formamide
TNI	*Thiothrix nivea*	CTCCTCTCCCACATTCTA	652-669	45
SNA	*Sphaerotilus natans*	CATCCC CCTCTACCGTAC	656-673	45
HHY	*Haliscomenobacter hydrossis*	GCCTACCTCAACCTGATT	655-672	20
21N	Eikelboom type 021N strain II-26	TCCCTCTCCCAAATTCTA	652-669	35
MPA	*Microthrix parvicella*	CCGGACTCTAGTCAGAGC	645-661	20
EUB 338	General bacteria	GCTGCCTCCCGTAGGAGT	338-355	20
CHL 1851	Eikelboom type 1851	AATTCCACGAACCTCTGCCA	592-611	35
G2M	Group II isolates of Eikelboom type 021N	GCACCACCGACCCCTTAG	842-859	35
N.LIM II 175	*Nostocoida limicola* II strains except strain Ben 70	GGCTCCGTCTCGTATCCG	175-192	40

The stringency of the washing solution was optimized according to that of the probe. The slide was then put in polypropylene screw top tube filled with 50 ml of the pre-warmed (48°C) washing solution for at least 15min to remove all unhybridized probes. Slides were then allowed to air dry, and a drop of antifade reagent (SlowFade® Gold, Life technologies, USA) applied on each spot to reduce photobleaching. A glass cover slip (VWR, 24×24 mm) was then placed over the spots and pressed gently to remove any excess oil and blotted out with tissue paper. Hybridization was then visualized with Nikon Eclipse epifluorescence microscope (LV100), using a Nikon Intensilight C-HGFI as a light source.

3. Results

3.1. Morphological Identification of Filamentous Bacteria

A total of 18 activated sludge samples were analyzed for presence of filamentous microorganisms. Based on morphology and staining properties, several filament types were identified, with higher diversity in industrial compared to municipal plants.

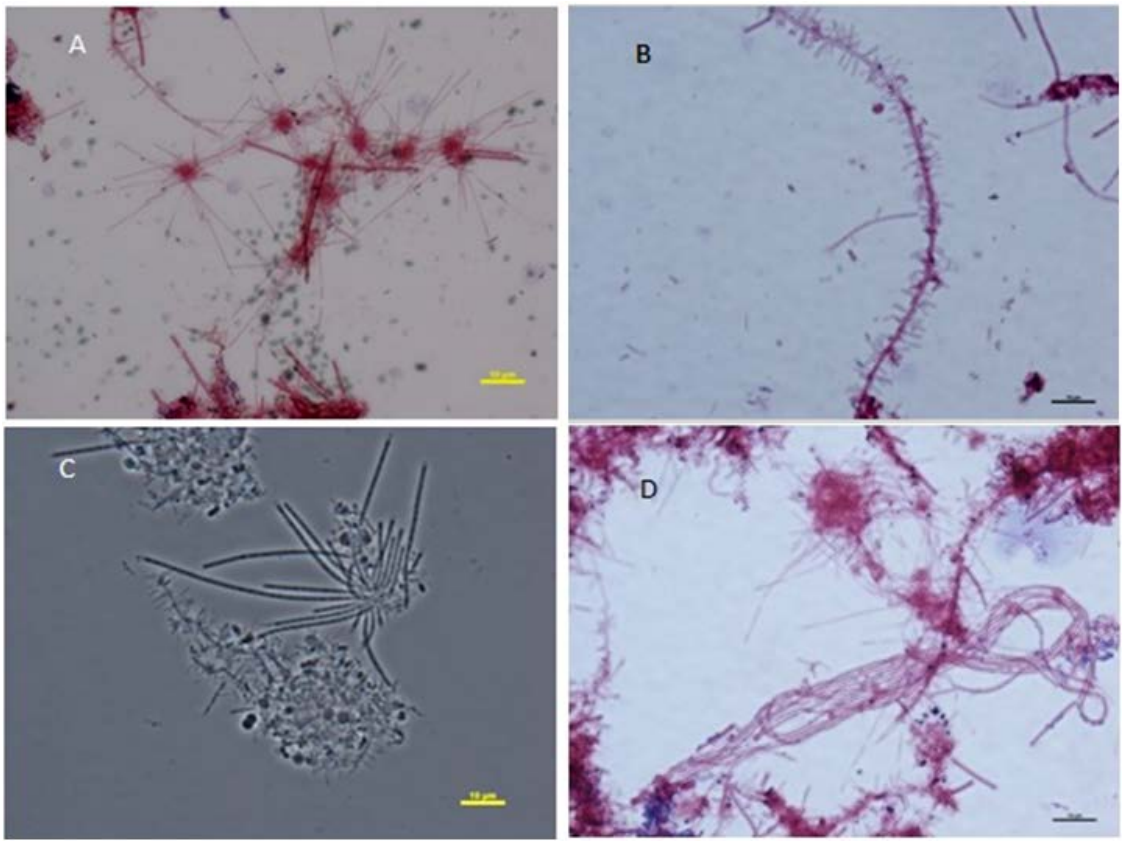

Figure 1. Bright-field and phase-contrast micrographs of filamentous bacteria observed in mixed liquor samples of activated sludge plants. (A) Gram stained *H. hydrossis*, (B) Gram stained Eikelboom Type 1851 with perpendicular attached growth, (C) *Thiothrix* morphotype with rosettes, and (D) Gram stained *N. limicola*. (Bar for all micrographs = 10 µm)

Straight or bent filaments mostly located around the edges of the flocs (Figure 1A) were observed in the industrial but not in the municipal plant, and was one of the co-dominating filaments (FI = 4). This filament stained Gram-negative and had needle-like appearance, fitting the "pins in a pin-cushion" description of *Haliscomenobacter hydrossis [6]*. Sheath could easily be observed at areas of missing cells. The abundance of this morphotype, without Gram staining, can be underestimated even at magnifications of 1000× due to its rather very thin nature.

Eikelboom Types 1851 and 0041/0675 morphotypes were observed in both industrial and municipal plants, although more abundant in the latter. These filaments were co-dominating in the municipal plant, but were few in the industrial plant. They were straight or smoothly curved, unbranched and mostly occurring in bundles. Sometimes, they occurred without attached growth, but when attached growth was present, it was heavier at the base than the growing tip. In the specific case of Type 1851, attached growth was less heavy and mostly perpendicular to the cell surface (Figure 1B). A thick sheath could also easily be observed at areas of missing cells. Gram staining reaction of this filament was negative in all the plants.

Thiothrix-like morphotypes were also observed only in the industrial plant and were one of the co-dominating filaments (FI = 4). Three morphotypes of this filament were distinguished. The first was thin and either straight or smoothly curved. It was without attached growth and mostly occurred freely in the bulk solution. The second morphotype was thicker than the first and had attached growth especially at the basal region, and was always growing outwards from the floc. The third *thiothrix*-like morphotype was similar in size to the second morphotype, but never with attached growth, and was mostly occurring in the bulk liquid. Rosette formation was very common with this morphotype (Figure 1C). All of these morphotypes were unbranched and stained Gram-negative. Also, clearly defined cell septa could be observed, with individual rectangular cells clearly visible, and a sheath also clearly visible at areas of missing cells.

Filaments with sausage-shaped cells having clear indentations at the cell septa, and fitting the description of *Sphaerotilus natans* were observed only in the industrial plant, though their abundance was low. This filament did not have any attached growth and always occurred in the bulk solution, occasionally exhibiting false branching.

Nostocoida limicola morphotypes were observed in both municipal and industrial plants (Figure 1D). All of those observed in the municipal plants stained Gram-positive, fitting their purple beaded necklace description by [6]. Also, they were either attached to other filaments or occurred within the floc. However, most of those observed in the industrial plant were Gram-negative, and were longer and more coiled than those in the municipal plants. Apart from differences in their staining reactions, these filaments could hardly be distinguished based on their morphological characteristics, especially when embedded in the floc.

Other species such as *Streptococcus* were often observed in municipal plants but were rarely observed in the industrial plant.

3.2. Identification of Filamentous Bacteria by FISH

Most of the morphologically identified filamentous organisms were subsequently confirmed using their respective fluorescently labeled 16S rRNA oligonucleotide probes.

The presence and identity of *Sphaerotilus natans* was confirmed by the specific probe SNA (Figure 2A). All the filaments morphologically identified as *S. natans* hybridized with the probe, producing very bright signals. Whole cell hybridization with probe CHL 1851 also resulted in the confirmation of Eikelboom Type 1851 in both the industrial and municipal plants (Figure 2D). However, not all filaments that were identified morphologically as Type 1851 hybridized with this probe or even the general probe (EUB 338).

Probe N.LIM II 175, specific for *Nostocoida limicola* II strains except strain Ben 70 [21], produced hybridization signals with *N. limicola* morphotypes in only the industrial plant, thereby confirming its identity and presence (Figure 2B). The FISH results clearly showed that the abundance and length of this filament cannot be correctly determined by using phase-contrast microscopy as the filament was sometimes embedded in the floc.

Application of probe HHY, a species-specific probe targeting *H. hydrossis* [7], resulted in the specific visualization of *H. hydrossis* filaments in only the industrial plant. Other filaments whose morphology differed from the typical descriptions of this filament also hybridized with this probe. The shape of these filaments was curved or curled. However, other *H. hydrossis*-like filaments that were observed based on phase-contrast were completely missed by probe HHY.

Hybridization of fixed mixed liquor samples with probe TNI resulted in the staining of the first and second *Thiothrix* morphotypes observed in the industrial plant (Figure 2C). However, the third morphotype (with rosettes formation) did not hybridize with probe TNI but rather with the general probe (EUB 338).

Although some filaments were morphologically identified as Eikelboom Type 021N, application of probe 21N did not yield any hybridization signals. Probe G2M [13] hybridized with filaments in both the municipal and industrial plants confirming the presence of Type 021N group II members. However, other morphologically similar filaments were completely missed by the probe. A list of all filamentous organisms identified is presented in Table 2.

Table 2. Filamentous bacteria observed in industrial and municipal plants.

Filamentous organism	Industrial plant	Municipal plant
H. hydrossis	+	-
Thiothrix II	+	-
Thiothrix I	+	-
Eikelboom type 0041/0675	+	+
Eikelboom type 1851	+	+
N. limicola I	+	+
Eikelboom type 021N group II	+	+
N. limicola II	+	-
S. natans	+	-
Streptococcus	+	+

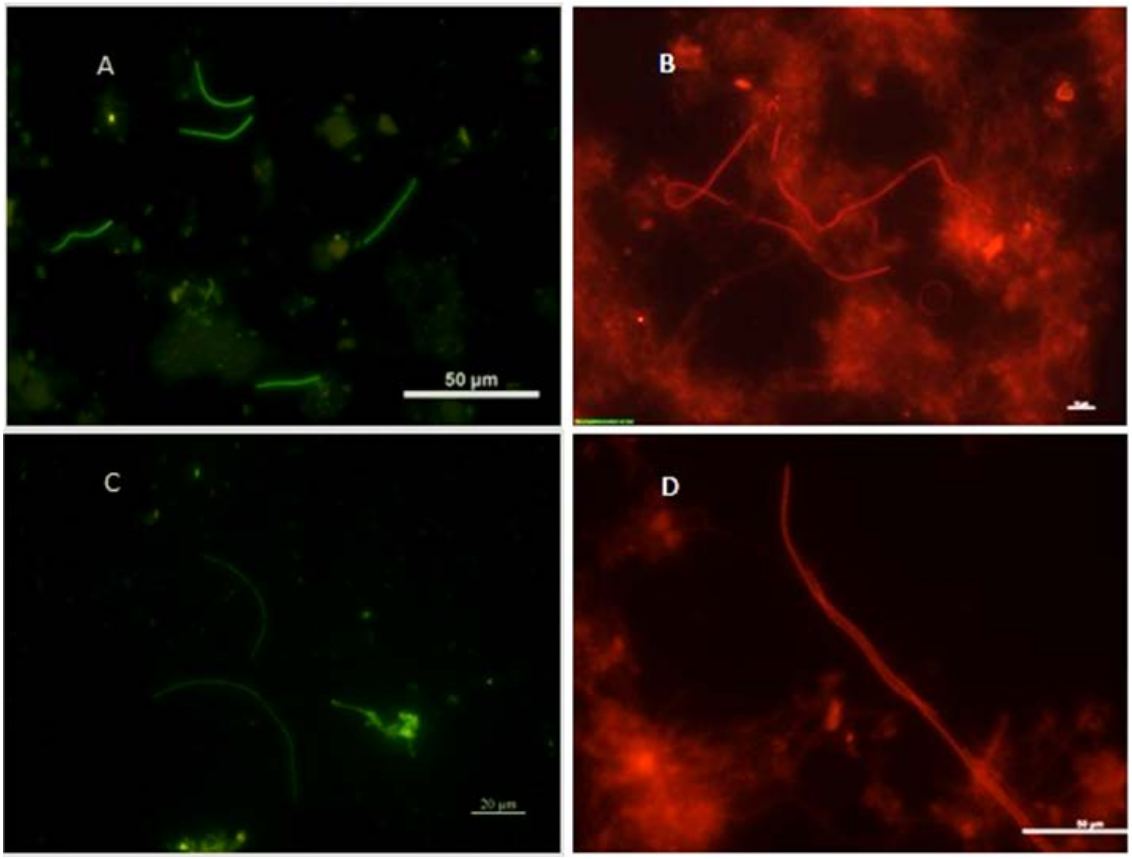

Figure 2. Epifluorescence micrographs of filamentous bacteria observed in mixed liquor samples of activated sludge plants. (A) *S. natans*, bar = 50 µm (B) *N. limicola* II occurring within the floc, bar = 10 µm (C) *Thiothrix nivea* and, bar = 20 µm (D) Eikelboom Type 1851, bar = 50 µm

4. Discussion

4.1. Information by Conventional Microscopy

This is the first investigation in Ghana on filamentous bacteria, involving the application of morphological and molecular techniques. The morphology of most of the filaments identified in this study was similar to their descriptions in published identification keys. Additionally, other studies from several different countries have described the morphology of these filaments in activated sludge samples [22,23,24]. Taken together, these findings indicate that geographic differences have no significant effect on the morphology of these filaments.

Despite this general similarity in morphology, Eikelboom Types 1851 and 0041/0675 stained Gram-negative, contrary to their descriptions by [5]. However, other studies on pure cultures of Eikelboom type 1851 (BEN 52) have reported this filament as staining Gram-negative [25]. Similarly, [26] isolated five morphotypes of Type 1851 from bulking activated sludge plants treating industrial and domestic wastewater and described them as Gram-negative. Other *in situ* studies have observed that unlike in domestic WWTPs where mainly one morphotype occurs meeting the Gram-positive description, a number of Eikelboom Type 0041/0675 resembling morphotypes with different staining reactions do occur in industrial WWTPs [27]. These findings clearly point to the unreliability of Gram staining for identifying sheathed filamentous bacteria.

The observation of *N. limicola* in this study is in line with previous studies, all summarized by [6]. In addition, [28] and [24] have observed this filament in mixed liquor samples from the Czech Republic and South Africa respectively. These findings indicate that *N. limicola* occurs frequently in both municipal and industrial plants.

4.2. Information by FISH

The presence of most of the filamentous microorganisms identified based on their morphology was subsequently confirmed by hybridizations with their respective FISH probes. The fact that most of these filaments were successfully identified using 16S rRNA oligonucleotide probes developed based on isolates from different regions indicates that these filaments are phylogenetically similar. However, the FISH results revealed that the morphological identification technique resulted in a few filaments being incorrectly identified while the abundance of others was underestimated.

Other studies based on application of probe HHY have identified *H. hydrossis* in several wastewater samples. For example, a comprehensive FISH survey in The Netherlands, Denmark, Germany and Italy by [29] confirmed that *H. hydrossis* was present in most of the samples. The lack of hybridization of this probe with other *H. hydrossis*-like morphotypes has also been reported by other studies. For instance, [27] observed four filamentous organisms with *H. hydrossis*-like morphology but were HHY negative. Also, the observation of probe HHY rather targeting filaments whose morphology differed from the typical needle-like appearance of this filament and instead

missing *H. hydrossis* resembling filaments has been reported by [30].

Probe 21N was developed by [7] for specific *in situ* detection of Eikelboom type 021N bacteria in environmental samples. The fact that this probe did not hybridize with any filament can only confirm the absence of group II isolate T1-4, the only Eikelboom type 021N isolate whose 16S rRNA was found fully complementary to this probe, based on its reevaluation by [13]. Probe TNI was developed complementary to a region of the 16S rRNA of *T. nivea*. Although the filament that was missed by this probe is morphologically similar to the Italian isolate (strain CT3) that was considered as a strain of a yet-to-be-described *Thiothrix* species by [31], further studies on it are needed before any conclusions can be made. Also, [13] reported that probe TNI equally targets members of Eikelboom type 021N group II. However, this probe did not hybridize with any of the filaments that were identified with probe G2M in this study. This could suggest that Eikelboom Type 021N group II contains many more members than probe TNI can identify.

4.3. Filaments Occurrence in Different Geographic Regions

The filamentous bacteria identified in this study were compared with those of other surveys conducted in different geographic regions to determine whether or not geographic differences have a role in the occurrence of specific filaments. In a one-year survey of a German municipal WWTP, Eikelboom Types 0041 and 0411, and *N. limicola* III were the commonly occurring filaments, while *Thiothrix* and Eikelboom type 021N were observed only occasionally (Martienssen et al. unpublished data) . A similar research by [27] observed *S. natans*, *H. hydrossis*, *Thiothrix*, *N. limicola* and Eikelboom Types 0041/0675 and 1851 as occurring in industrial plants. The fact that all these filaments were also observed in the Ghanaian plants indicates that geographic differences have no effect on their occurrence. The differences in their abundance in these regions should therefore be due to differences in operating conditions of the various plants. Even though *N. limicola* morphotypes were identified based on their morphological features and staining reactions in the Ghanaian plants, it is inconclusive if *N. limicola* III was present since a specific probe for this filament was not applied.

Microthrix parvicella is reported as the most frequently observed filament in European WWTPs [14,23]. This filament has been associated with low temperature and low loading municipal plants with biological nutrient removal. For instance, in a survey of 38 Danish plants (all with biological nutrient removal), *M. parvicella* was the most dominant filament and also the most abundant in the winter [32]. This tendency to thrive at low temperature was confirmed in an activated sludge study in Italy [33]. This filament was also completely eliminated at a temperature of 29°C in both bench and pilot scale nutrient removal plants [34]. Although the municipal plants in this study lacked nutrient removal, the absence of *Microthrix parvicella* should be due to the effect of temperature since these plants operate at low loading rates (<0.2 KgBOD/KgMLSS.day) and low DO concentration (0.4-0.9 mg/l) which are factors that should favor the growth of this filament. Considering the fact that growth of *M. parvicella* is favored by low temperatures, and whereas the average mixed liquor temperature throughout this study was 30°C, it can be argued that this high temperature possibly selected against *M. parvicella* in this region.

5. Conclusions

A survey of filamentous bacteria in full-scale municipal and industrial activated sludge WWTPs in Ghana was conducted using morphological and molecular techniques and the following conclusions made:

Filamentous bacteria were abundant in both municipal and industrial plants, with overall abundance and also diversity higher in industrial plants. Filament composition was stable for each plant but not unique to each plant.

Filament morphology is not significantly affected by geographic differences. Hence the usefulness of morphological techniques for routine identification of filamentous bacteria in wastewater should not be underestimated. Most of the filaments could also be identified using existing probes, indicating that they are phylogenetically similar to those occurring in other regions. The fact that *Microthrix parvicella* was not observed in this study suggests that geographic differences play a role in the occurrence of filamentous bacteria.

Further research should be carried out to investigate correlations between the presence of specific filamentous bacteria, process parameters and wastewater composition for possible bulking control since some filaments recorded high filament index.

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Statement of Competing Interest

The authors declare that they have no competing interest.

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