Reliability of Morphological Approach for Bulking Filamentous Bacteria Identification in Activated Sludge Plants

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Abstract

Activated sludge process is the most commonly applied technology for the biological treatment of wastewater. However, operational failures and deterioration of treatment efficiency due to extensive and uncontrolled growth of filamentous bacteria have been reported worldwide as common problems of this method. Over the years, various methods have been developed and applied for the identification of these bacteria in an attempt to find a solution to the bulking problem. In this study, morphological techniques were used to identify dominant filamentous bacteria in activated sludge plants in Ghana. Fluorescence in situ hybridization was subsequently applied to assess the reliability of the morphological results. The findings indicate that the usefulness of morphological approach for routine identification of filamentous bacteria for the purpose of bulking control should not be underestimated since the results of this approach agreed largely with the probe-defined results. Reliability of morphological results, however, can be improved when plant operators would have previously applied molecular techniques to identify these filaments. Where molecular techniques are available, morphological identification can also serve as a preceding rapid screening tool for subsequent probe selection.

Keywords: Activated sludge, filamentous bacteria identification, morphological approach.

1. Introduction

The activated sludge process has grown in popularity since its developed by Ardern and Lockett (1914), and in recent decades has been accredited to be the most widespread secondary biological technology for treating various types of wastewater such as municipal and industrial effluents (Thompson & Forster 2003; Martins et al. 2004). This method is a suspended-growth process that consists essentially of two processes: mineralization and conversion of the biodegradable components of wastewater by a broad variety of microorganisms under aerobic conditions, and separation of activated sludge from the treated wastewater by sedimentation (Bitton 2005). Besides the highly controllable nature of the process which makes it possible to adjust its operation to accommodate a wide range of conditions, it is also able to withstand short-term variations in organic and hydraulic loadings, in addition to its small land requirement, high effluent quality, and flexibility with regard to process configuration (Grady et al. 2011).

However, this method suffers a major operational problem of bulking caused by excessive growth of filamentous bacteria resulting in formation of open flocs and interfloc bridging in the aeration tank (Guo et al., 2012). This phenomenon causes poor settling and compaction in the secondary clarifier which results in deterioration of effluent quality, loss of active biomass and creates a significant burden of the receiving water with organic matter.

Recent surveys indicate that bulking is a worldwide problem, with approximately 60% of all treatment plants experiencing it at one time or the other (Madoni et al., 2000; Seviour et al., 1994; Wagner et al., 1994; Blackbeard et al., 1986). The identification of these filamentous bacteria continues to receive considerable attention in research in an effort to solve this bulking problem.

To this end, various techniques have been developed and applied for the identification of these bacteria. The morphological approach primarily involves the observation of physical features of the bacteria based on the identification keys of Eikelboom (2000) while the fluorescence *in situ* hybridization (FISH) technique entails essentially the application of fluorescently labeled rRNA-targeted oligonucleotide probes (Amann et al. 2001). The objective of this research was to assess the reliability of the morphological technique for the identification of dominant filamentous bacteria in activated sludge plants in Ghana. The findings of this study will be very useful for the application of specific measures for bulking control, especially where the molecular technique is unavailable.

2. Methodology

2.1 Wastewater treatment plants

Two full-scale activated sludge wastewater treatment plants (WWTPs) were investigated in this study. One of the plants is located in Accra and treats municipal wastewater while the other plant is located at the industrial city of Tema and handles wastewater from a food and beverages factory. Both plants operate on the conventional activated sludge system consisting of aeration and sedimentation tanks. Configuration of the plants is based on the completely mixed method and without biological nutrient removal.

2.2 Sample collection and handling

Representative grab samples of the mixed liquor were collected from areas of good mixing during aeration using a long-handled aluminum dipper attached to a wooden handle. Three samples were collected from each plant. Sample containers were three quarters filled so as to maintain aerobic conditions for survival of filamentous organisms during transport. Samples were stored in an ice chest containing ice packs at a temperature below 4^{o}C and transported to the laboratory within 2 hours of sampling, and immediately stored in a refrigerator at 4^{o}C pending analyses.

2.3 Filamentous bacteria identification

2.3.1 Morphological approach

Microscopic examination of detailed morphological structures for filament identification was performed based on the identification keys of Eikelboom (2000) and Jenkins et al. (2004), and visualized with a Nikon Eclipse LV100 (Japan). Wet mounts were observed under phase-contrast at 600x and 1000x magnification. The identification of individual filament types was based on their recognizable microscopic features such as cell shape and size, filament shape and size, presence of cell septa and sheath, true branching, and attached growth (epiflora). Gram staining technique was used to differentiate between Gram-positive and Gram-negative filamentous bacteria. The Gram stain was made according to the procedure described by Adamse (1970). Stained slides were 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. Photomicrographs were captured using Nikon digital camera.

2.3.2 Fluorescence *in situ* hybridization

A range of oligonucleotide probes (Table 1) was applied for molecular identification of filamentous organisms. The synthesis and labeling of the probes was carried out by *Invitrogen*™. Probes were selected based on the results of the morphological identification of the filaments. Detailed information about most of these probes is given in probeBase (Loy et al. 2003). EUB 338, a general probe for most bacteria, was used as positive control, while hybridization without probe was also used as negative control on each slide. FISH was performed based on the procedure described by Amann et al. (1995) and the protocol of the Chair of Biotechnology for Water Treatment, Brandenburg University of Technology, Germany. Detailed procedure is described in Adonadaga, M., & Martienssen, M., (2015).

3. Results and discussion

3.1 *Haliscomenobacter hydrossis*

Straight filaments mostly located at the edges of the flocs and extending outward were observed in the industrial plant. These filaments stained Gram-negative and had a needle-like appearance (Fig. 1A and B) fitting the description of *Haliscomenobacter hydrossis* as "pins in a pin-cushion" by Jenkins et al.(2004). Also, these filaments were sheathed and without any attached growth or branching. Sheath could easily be observed at areas of missing cells when the filament is stained. Application of probe HHY resulted in hybridization to some of these filaments morphologically identified as *H. hydrossis* (Fig 1C). However, other *H. hydrossis*-like morphotypes that were observed under phase contrast were completely missed by the probe.

Haliscomenobacter hydrossis (belonging to the *Cytophaga-Flexibacter*-group of the *Bacteroidetes*) has been identified worldwide in activated sludge samples because of its easily recognizable morphology (Ramothokang et al. 2004; Madoni et al. 2000; Seviour et al. 1994). The appearance of *H. hydrossis* in this study is similar to its morphological descriptions and staining reactions outlined in the identification keys of Eikelboom (2000) and Jenkins et al. (2004). These findings indicate that geographic differences have no significant effect on the morphology of this filament. The positive hybridization obtained from the application of probe HHY confirmed the presence of this filament. The observation of probe HHY completely missing other *H. hydrosssis*-like morphotypes is consistent with results of other studies that applied this probe. For instance, Eikelboom and Geurkink (2002) reported the observation of four filamentous organisms with a *H. hydrossis*-like morphology, but which were HHY-negative. Also, FISH results obtained by Kragelund et al. (2008) showed that large populations of *H. hydrossis* resembling morphotypes observed based on conventional microscopic analysis were frequently completely missed by probe HHY. Taken together, these results obviously indicate that morphotype *Haliscomenobacter hydrossis* includes more species than probe HHY can identify. However, for the purpose of bulking control, the morphological identification is sufficient.

3.2 Thiothrix spp.

Filaments fitting the description of *Thiothrix* spp. were also observed in the industrial plant. Two *Thiothrix*-like morphotypes could be distinguished. The first morphotype was without attached growth, and mostly occurring in the bulk liquid. Rosette formation was very common with this morphotype (Fig 2A and B). It hybridized with only the general probe (EUB 338) and not the specific probe (TNI) (Fig. 2C). The second morphotype was straight or smoothly curved and mostly without any attached growth although small attached growth was sometimes observed at the basal end. This morphotype hybridized with both the general and specific probes (Fig. 2D). It was the dominant filament and responsible for the open floc formation in the industrial plant. Both morphotypes were unbranched and stained Gram-negative. Individual rectangular cells could be seen and, where cells were missing, a sheath could be observed. Also, cell septa could be observed, with individual rectangular cells clearly visible in both morphotypes.

Several *Thiothrix* species have been morphologically identified in activated sludge treatment plants. For instance, four *Thiothrix* species were observed by Eikelboom and Geurkink (2002), while Williams and Unz (1985) characterized six different strains of *Thiothrix* from waste water plants in Pennsylvania. Therefore, the observation of different *Thiothrix* morphotypes in the industrial plant by the present study is supported by these previous reports in the literature. The molecular results also indicate the possible occurrence of other *Thiothrix* species that probe TNI can't identify. The second *Thiothrix* morphotype observed in this study, for instance, is similar to the morphotype reported by Williams and Unz (1985) as well as to the isolate CT3(DSM 12730) that was obtained from a WWTP in Italy and considered as a strain of a yet-to-be described *Thiothrix* species (Rossetti 2003). However, additional research on the physiology and genotype of this morphotype is needed before a novel species can be proposed. The attached growth observed at the basal part of the first morphotype should indicate a non-growing region, as observed also by Jenkins et al. (2004), while the negative Gram staining result for both morphotypes is consistent with that reported in the identification keys. As such, the *Thiothrix* morphotypes are identifiable based on their morphology. In addition, similar conditions have been reported as responsible for the growth of all these species. Hence, for the purpose of bulking control, the morphological approach is adequate.

3.3 Eikelboom Type 1851

Filaments fitting the morphological description of Eikelboom Type 1851 were observed in both plants. Gram staining reaction was negative in both plants.

These filaments were very long, straight or smoothly curved, unbranched, and mostly with attached growth perpendicular to the cell surface (Fig. 3A and B). A thick sheath could easily be observed at areas of missing cells with no attached growth. Application of Probe CHL 1851 produced hybridization signals with these filaments in both plants, thereby confirming their identity (Fig. 3C). However, not all the filaments morphologically identified as Type 1851 hybridized with this probe. The morphological features of this filament fitted its descriptions by Eikelboom (2000) and, in particular the observation by Jenkins et al. (2004) of the perpendicular nature of the attached growth. In addition, the Gram-negative reaction of this filament is consistent with findings by Beer et al. (2002) and Kohno et al. (2002). However, some discrepancy exists in the literature with regards the Gram staining reaction of this filament. Irrespective of the staining reaction, this filament was easily identified as the perpendicular epiflora was always a telltale.

3.4 Eikelboom Type 0041

Filaments with morphological features similar to those of Eikelboom Type 0041 were also observed. This filament was about the biggest and longest filament observed. It was characterized by very heavy attached growth all through its length, and was mostly growing beyond the confines of the floc and into the bulk solution. It stained Gram-negative in both plants, and was also observed to sometimes occur in bundles in the municipal plants, unlike in the industrial samples where it mostly occurred as single filaments (Fig 4A and B). It was also associated with the formation of open floc structure. Application of probe G2M produced hybridization signals with this filament (Fig 4C). However, other filaments that under phase-contrast did not have attached growth also hybridized with this probe (Fig. 4D).

Although very few of the sheathed filaments such as Eikelboom Type 0041 have been isolated and characterized, nonetheless, they have been identified based on their morphology. For instance, Lacko et al. (1999) and Madoni et al. (2000) identified this filament in activated sludge plants in South Africa and Italy respectively. Despite the morphological similarity of the filaments identified in this study to those identified in other regions, differences exist in terms of their Gram staining reactions. For instance, whereas Brand et al. (1987) reported this filament as Gram-positive, other studies have described it as Gram variable (Jenkins et al. 2004).The staining results of this filament and that of Type 1851 suggest that Gram staining alone is not reliable for the identification of the sheathed filaments. Notwithstanding, the morphological features of these filaments are adequate for their identification for the purpose of bulking control.

4. Conclusion

The usefulness of morphological techniques for routine identification of filamentous bacteria in activated sludge plants should not be underestimated since the morphological identification results agreed largely with the probe-defined results. Hence, strategies suggested for the control of settling problems caused by these filaments identified based on the morphological approach should be considered. The added value of FISH in bulking control is in identifying those morphologically indistinguishable filaments whose proliferation is caused by different operational conditions. Although the Gram staining results for the sheathed Eikelboom types 1851 and 0041 have added to the existing inconsistency in the literature, this is of little relevance since these filaments, irrespective of their Gram staining reaction, can be identified based on their morphology. These results, however, seem to bring into question the usefulness of the Gram staining technique for identifying especially the sheathed filaments.

References

Adamse, A. D. (1970). Some characteristics of arthrobacters from a dairy waste activated sludge. *Water Research*, *4*(12), 797-803.

Adonadaga, M., & Martienssen, M. (2015). *In situ* identification of filamentous bacteria from activated sludge wastewater treatment plants in Ghana. doi:10.12691/jaem-3-3-375–381

Amann, R., Fuchs, B. M., & Behrens, S. (2001). The identification of microorganisms by fluorescence *in situ* hybridisation. *Current Opinion in Biotechnology*, *12*(3), 231-236.

Amann, R. I., Ludwig, W., & Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological reviews*, *59*(1), 143-169.

Ardern, E., & Lockett, W. T. (1914). Experiments on the oxidation of sewage without the aid of filters. *Journal of the society of chemical industry*, *33*(10), 523-539.

Beer, M., Seviour, E. M., Kong, Y., Cunningham, M., Blackall, L. L., &Seviour, R. J. (2002).

Phylogeny of the filamentous bacterium Eikelboom Type 1851, and design and application of a 16S rRNA targeted oligonucleotide probe for its fluorescence *in situ* identification in activated sludge. *FEMS microbiology letters*, *207*(2), 179-183.

Bitton, G. (2005). *Wastewater microbiology*. John Wiley & Sons.

Blackbeard, J. R., Ekama, G. A., & Marais, G. V. R. (1986). A survey of filamentous bulking and foaming in activated-sludge plants in South Africa. *Water pollution control*, *85*(1), 90-100.

Brand, P. A. J., L. R. Tiedt, and V.L.H.-A., 1987. Some observations on the morphology and the anatomy of filament type 0041. *Water S. Afr.*, 13, pp.1–6.

Eikelboom, D. H. (2000). *Process control of activated sludge plants by microscopic investigation*. IWA publishing.

Eikelboom, D., & Geurkink, B. (2002). Filamentous micro-organisms observed in industrial activated sludge plants. *Water Science & Technology*, *46*(1-2), 535-542.

Grady Jr, C. L., Daigger, G. T., Love, N. G., & Filipe, C. D. (2011). *Biological wastewater treatment*. CRC Press.

Guo, J., Peng, Y., Wang, Z., Yuan, Z., Yang, X., & Wang, S. (2012). Control filamentous bulking caused by chlorine-resistant Type 021N bacteria through adding a biocide CTAB. *Water research*, *46*(19), 6531-6542.

Jenkins, D., Richard, M. G., & Daigger, G. T. (2004). Manual on the causes and control of activated sludge bulking, foaming, and other solids separation problems3. ed. Lewis.

Kohno, T., Sei, K., & Mori, K. (2002). Characterization of type 1851 organism isolated from activated sludge samples. *Water Science & Technology*, *46*(1-2), 111-114.

Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., ...& Nielsen, P.H. (2008). Identity, abundance and ecophysiology of filamentous bacteria belonging to the Bacteroidetes present in activated sludge plants. *Microbiology*, *154*(3), 886-894.

Lacko, N., Bux, F., & Kasan, H. C. (1999). Survey of filamentous bacteria in activated sludge plants in KwaZulu-Natal. *WATER SA-PRETORIA-*, *25*, 63-68.

Loy, A., Horn, M,. & Wagner, M., (2003). probeBase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Research, 13, 1*.

Madoni, P., Davoli, D., & Gibin, G. (2000). Survey of filamentous microorganisms from bulking and foaming activated-sludge plants in Italy. *Water Research*, *34*(6), 1767-1772.

Martins, A. M., Pagilla, K., Heijnen, J. J., & van Loosdrecht, M. C. (2004). Filamentous bulking sludge—a critical review. *Water research*, *38*(4), 793-817.

Ramothokang, T. R., Drysdale, G. D., & Bux, F. (2004). Isolation and cultivation of filamentous bacteria implicated in activated sludge bulking. *Water Sa*, *29*(4), 405-410.

Rossetti, S., Blackall, L. L., Levantesi, C., Uccelletti, D., & Tandoi, V. (2003). Phylogenetic and physiological characterization of a heterotrophic, chemolithoautotrophic *Thiothrix* strain isolated from activated sludge. *International journal of systematic and evolutionary microbiology*, *53*(5), 1271-1276.

Seviour, E. M., Williams, C., DeGrey, B., Soddell, J. A., Seviour, R. J., & Lindrea, K. C. (1994). Studies on filamentous bacteria from Australian activated sludge plants. *Water Research*, *28*(11), 2335-2342.

Thompson, G., & Forster, C. (2003). Bulking in activated sludge plants treating paper mill wastewaters. *Water research*, *37*(11), 2636-2644.

Wagner, M., Amann, R., Kämpfer, P., Assmus, B., Hartmann, A., Hutzler, P., ...& Schleifer, K.

H. (1994). Identification and in situ detection of gram-negative filamentous bacteria in activated sludge. *Systematic and Applied Microbiology*, *17*(3), 405-417.

Williams, T. M., & Unz, R. F. (1985). Isolation and characterization of filamentous bacteria present in bulking activated sludge. *Applied microbiology and biotechnology*, *22*(4), 273-282.

Table 1: FISH probes, target microorganisms, probe sequences and target sites.

Probe	Target microorganism	Probe sequence (5'-3')	Target site (16S rRNA positions)
TNI	*Thiothrixnivea*	CTCCTCTCCCACATTCTA	652-669
HHY	*Haliscomenobacterhydrossis*	GCCTACCTCAACCTGATT	655-672
EUB 338	General bacteria	GCTGCCTCCCGTAGGAGT	338-355
CHL 1851	Eikelboom type 1851	AATTCCACGAACCTCTGCCA	592-611
G2M	Group II isolates of Eikelboom type 021N	GCACCACCGACCCCTTAG	842-859

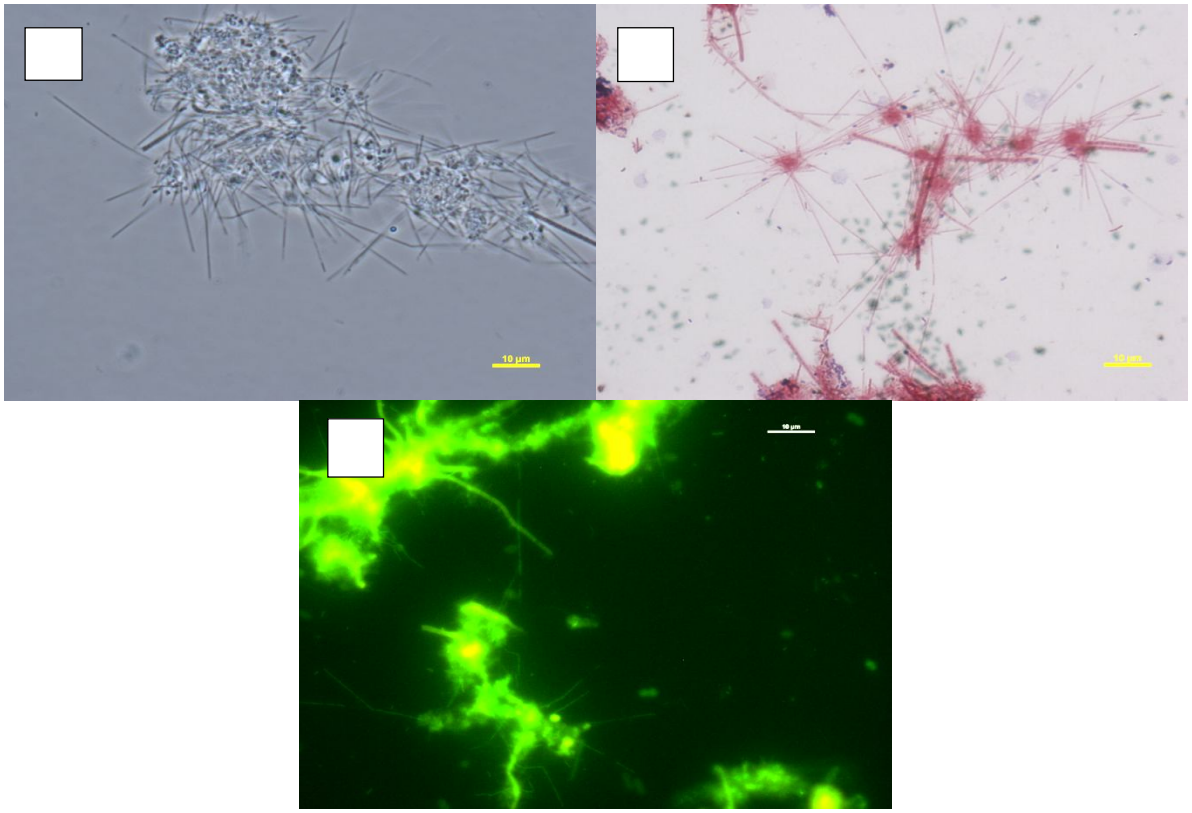

Fig. 1: (A) Phase-contrast, (B) Gram stain micrographs of *H. hydrossis* and (C) FISH micrographs showing hybridization with probe HHY.

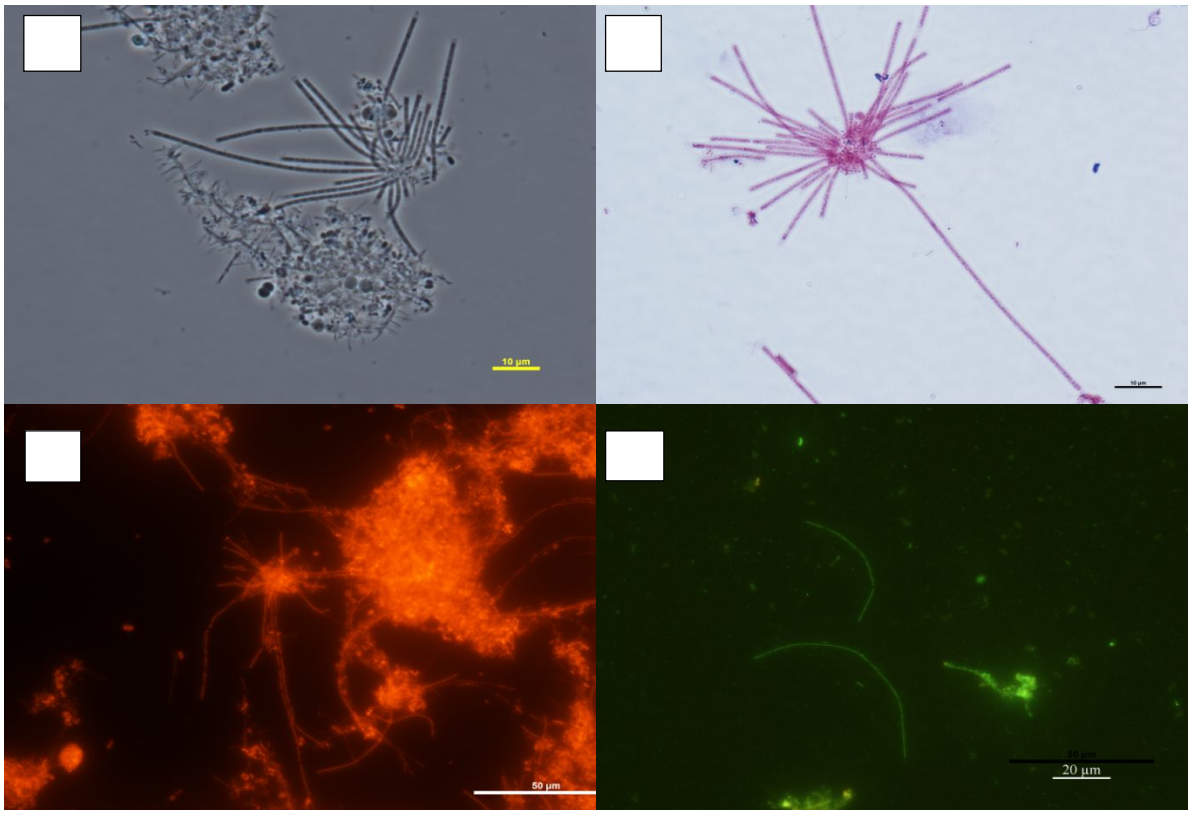

Fig. 2: (A) Phase-contrast and (B) Gram stain micrographs of *Thiothrix*morphotype, (C) hybridization of first morphotype with probe EUB 338, and (D) hybridization of second morphotype with probe TNI.

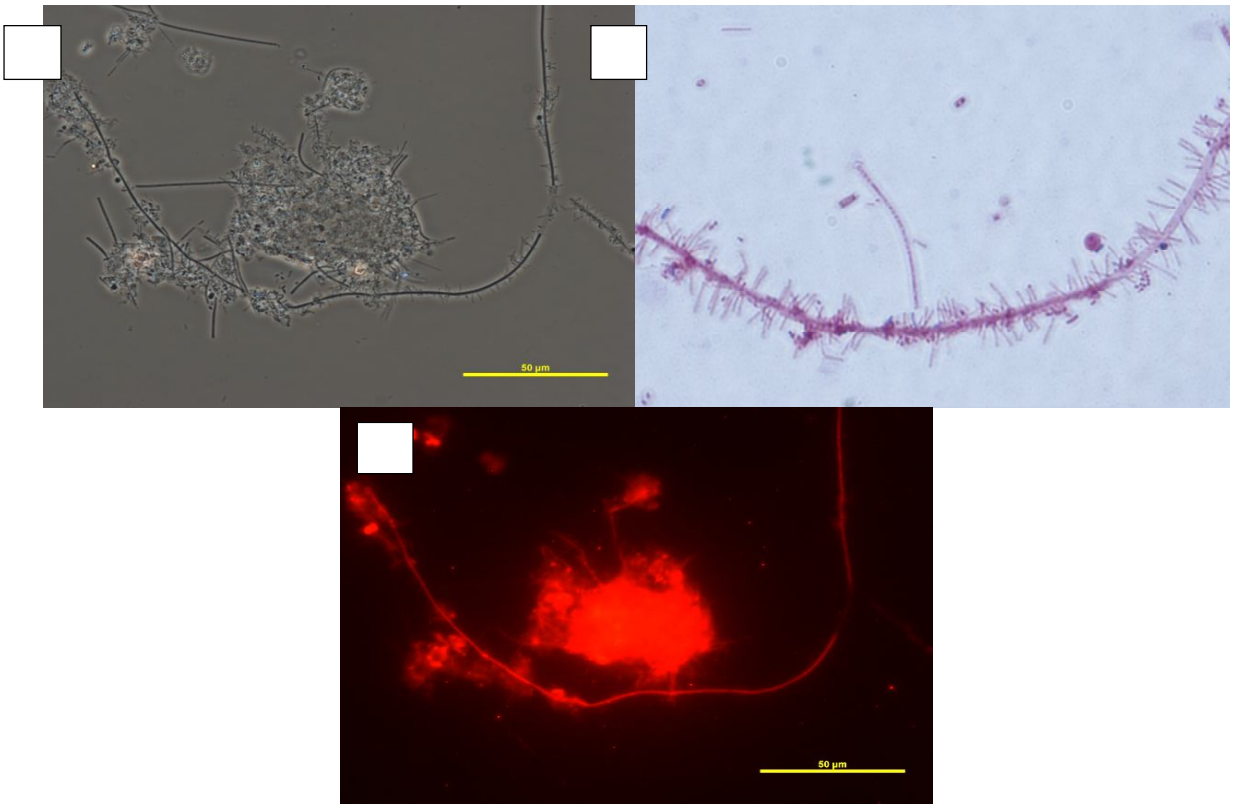

Fig. 3: (A) Phase-contrast and (B) Gram stain micrographs of Type 1851, and (C) FISH micrographs showing hybridization with probe CHL1851.

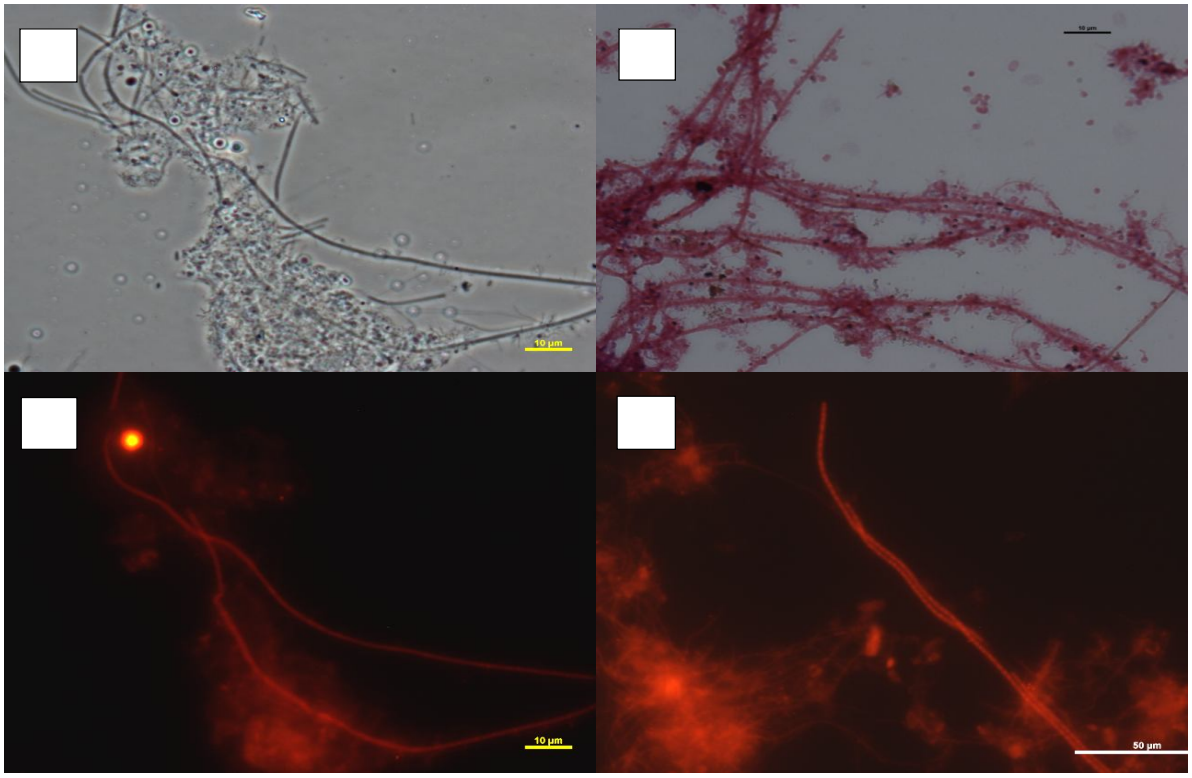

Fig. 4: (A) Phase-contrast and (B) Gram-stain micrographs of Type 0041, (C) FISH image showing Type 0041 hybridizing with probe G2M and (D) hybridization by filaments without attached growth.