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MiniReview

Detection and cultivation of filamentous bacteria from activated sludge

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Abstract

Different applications of the activated sludge treatment process are employed globally for the biological treatment of wastewater. Since the activated sludge process was first developed, different kinds of sedimentation problems, in particular bulking sludge and foaming, have been observed and it is widely accepted that extensive, uncontrolled growth of filamentous bacteria is the main cause of these difficulties. Since the pioneering work of Eikelboom and coworkers who characterized and described seven groups of 26 morphological types of filamentous organisms several attempts have been made to develop new methods for isolation, cultivation and identification of the isolates. Improvement has also been sought in the methods for direct detection, to allow a better understanding of the complex phenomena of sludge bulking and foaming. However, although knowledge of the phylogeny of filamentous organisms has increased recently, only a few attempts have been made to achieve a better understanding of their growth characteristics. The aim of this paper is to give an overview of detection and cultivation methods with special emphasis on the comparison of classical approaches with the modern molecular biological methods, including in situ detection and identification of filamentous bacteria.

Keywords: Filamentous bacteria; Growth requirement; Activated sludge; Isolation; Detection; Cultivation; Taxonomy; Identification

1. Introduction

The settling properties of activated sludge are vital to obtain good separation of the sludge from the treated wastewater. Ever since the activated sludge process was developed, several kinds of sedimentation problems have been observed, in particular bulking sludge and foaming [1,2]. The main reason for the occurrence of bulking sludge and foaming has been thought for some time to be the extensive, uncontrolled growth of different types of filamentous bacteria [2–5]. Several strategies to prevent bulking sludge and foaming have been applied. Chemical treatment, such as precipitation and use of inhibiting chemicals, can solve some of the problems. However, this does not provide long-term solutions, because the main factors stimulating the growth of filamentous bacteria (see Table 1) are not changed. Effective methods against bulking sludge and foaming are still urgently needed. However, this requires a thorough understanding of the biological mechanisms in the activated sludge process [5] and hence a detailed knowledge of the growth characteristics of the different filamentous bacteria. Since the conditions in

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wastewater treatment plants are continuously changing, it seems unlikely that specific parameters will cause the proliferation of certain types of filamentous bacteria. Nevertheless, some causative factors have been suggested (Table 1) [3,6,7]. Even though several theories have been proposed to explain some of the causes of excessive growth of filamentous bacteria, other unexplored causative factors remain undetermined. For these reasons, it is essential to develop new methods for direct detection of filamentous bacteria for process control and monitoring, and to obtain more detailed information on their ecology, taxonomy, physiology, growth requirements and conditions.

2. Detection of filamentous bacteria involved in sludge bulking or foaming

2.1. Microscopy of activated sludge samples

The classical approach for the identification of filamentous bacteria is that of Eikelboom [2], who studied more than 1100 activated sludge samples by phase contrast microscopy. Cell morphological features, like the presence or absence of a sheath or a slime layer, true or false branching, the nature, length and shape of the filaments, and the diameter, length and shape of the cells were noted. In addition, the occurrence of cell inclusions (poly-β-hydroxybutyrate, polyphosphate or sulfur), gliding motility and simple staining procedures were applied to classify the organisms [2,3]. Twenty-six types were distinguished and grouped into seven assemblages (Table 2) using these techniques. About 10–15 of these types are frequently found in activated sludge. Subsequently, simple identification keys [3,4] were constructed, and so it was possible to carry out comparative studies in different treatment plants to detect and identify the most frequently occurring filamentous organisms. Another approach to group filamentous organisms was described by Wanner and Grau [5] (Table 3). All these groupings, however, do not consider the taxonomy of the organisms. These methods are relatively easy and no special laboratory equipment is required, so these keys are still very commonly used for a first characterization. However, they have several limitations. It has frequently been

Table 1
Factors of the activated sludge process favoring the occurrence of filamentous bacteria according to Jenkins [7]

1	Sludge age (mean cell residence time, MCRT; or food to microorganism ratio (F/M): – Low dissolved oxygen (DO) – Low F/M ratio in completely mixed and continuously fed systems
2	Aeration basin configuration (wastewater feeding regime) – Nature of organic substrate (soluble versus particulate; readily biodegradable versus slowly biodegradable)
3	Presence of initial unaerated zone (anoxic or anaerobic) – Septic wastewater, nutrient deficiency, low pH (< 6.5), seeding from surfaces, surface trapping and foam recycle

Table 2
Filamentous organisms in activated slugde grouped and listed according to Eikelboom [2][a]

Group I:	Sheath-forming, Gram-negative bacteria
	1. *Sphaerotilus natans*
	2. Type 1701
	3. Type 1702
	4. *Haliscomenobacter hydrossis*
	5. Type 0321
Group II:	Sheath-forming, Gram-positive bacteria
	6. Type 0041
	7. Type 0675
	8. Type 1851
Group III:	Sheathless curled, multicellular bacteria resembling blue-green algae
	9. Type 021N
	10. *Nostocoida limicola*
	11. Cyanophyceae
Group IV:	Slender, coiled bacteria
	12. *Microthrix parvicella*
	13. Type 0581
	14. Type 0192
Group V:	Straight, multicellular, Gram-negative bacteria
	15. Type 0803
	16. Type 1091
	17. Type 0092
	18. Type 0961
Group VI:	Filamentous bacteria motile by gliding
	19. Type 0914
	20. *Beggiatoa* spp.
	21. Type 1111
	22. Type 1501
Group VII:	Additional types
	23. Type 1863
	24. Type 0411
	25. Fungi
	26. *Nocardia* sp.

[a]For details about morphological characters see [2–4].

Table 3
Assemblages of filamentous organisms according to their morphological/physiological characteristics and the grouped environments by Wanner and Grau [5]

Group		Organisms	Preferred environment
I	Oxic zone growers, S	*Sphaerotilus*-like microorganisms, include types 1701, 0041, and 0675	Prefer high sludge retention times (SRTs), low DO, and high substrate concentrations. Do not often occur in anaerobic/aerobic selector plants
II	Oxic zone growers, C[a]	*Leucothrix*, *Thiothrix*, type 021N	Prefer aerobic conditions, and high SRTs, or if degradable substrate is readily available. Do not often occur in anaerobic/aerobic selector plants but some are able to oxidize sulfur
III	All zone growers	*Microthrix parvicella*-like organisms	Occur in anaerobic and anoxic zones
IV	Foam-forming organisms	*Nocardia*-like organisms	Occur often in the presence of surfactants, grease, or oil. High SRTs and anaerobic/aerobic conditions are exploited

[a]'Cyanophyte-like' organisms.

found that the morphology and staining reactions of microbial cells can vary greatly in differing environmental conditions. Nonfilamentous growth forms are documented for *Haliscomenobacter hydrossis*, *Sphaerotilus natans*, and *Microthrix parvicella* [8,9]. In addition, it has been suggested that the sheath forming capacity is plasmid encoded and so is easily lost [10]. Furthermore, certain filamentous organisms, e.g. *Microthrix parvicella* and Eikelboom type 1863, have variable Gram stain reactions. Another difficulty is that some filamentous organisms, like the sulfur bacteria *Thiothrix* spp., Eikelboom type 021N and *Leucothrix mucor*, are hard to differentiate by cell morphology. *Thiothrix* filaments without sulfur granules cannot be distinguished from *Leucothrix* [11].

2.2. Immunological techniques

One approach for direct detection of certain filamentous organisms is the application of specific antibodies. The main advantage of this technique is the possibility of specific detection of certain filamentous bacterial species or groups. The fluorescent antibody technique was carried out to identify *Sphaerotilus natans* [12], *Gordona amarae* [13], and *Thiothrix* spp. [14] in activated sludge. These approaches are very promising because of their sensitivity and specificity and their speed [14]. However, although it is possible to develop very specific antibodies, the applicability of the immunofluorescence approach also has its limitations. The antibody penetration can be limited by extracellular polymeric substances, which are components of the activated sludge flocs, and also by unspecific binding of antibodies to organic particles or fungal spores. These nonspecific reactions may lead to high levels of background fluorescence. In addition, it must be stated that for the development and testing of new antibodies, reference organisms are necessary with the requirement of their cultivation.

2.3. In situ detection and identification of filamentous organisms using ribosomal RNA targeted oligonucleotide probes

A recent further development is the use of fluorescently labeled ribosomal RNA (rRNA) targeted oligonucleotide probes for in situ identification of microorganisms. A review of the potential of this powerful technique in microbial ecology was given by Amann et al. [15]. The rRNA molecules seem to be ideal targets for nucleic acid probes for several reasons. The sequencing of 16S and 23S rRNA molecules is currently the standard for the classification of new microorganisms. They are functionally conserved molecules present in all organisms. In the primary structures of 16S and 23S rRNA molecules sequence regions of higher and lower evolutionary conservation can be found. Furthermore, 16S rRNA sequences have already been determined for many validly described bacterial species (close to 50% [15]). A main advantage is that their natural amplification with high numbers of copies per cell

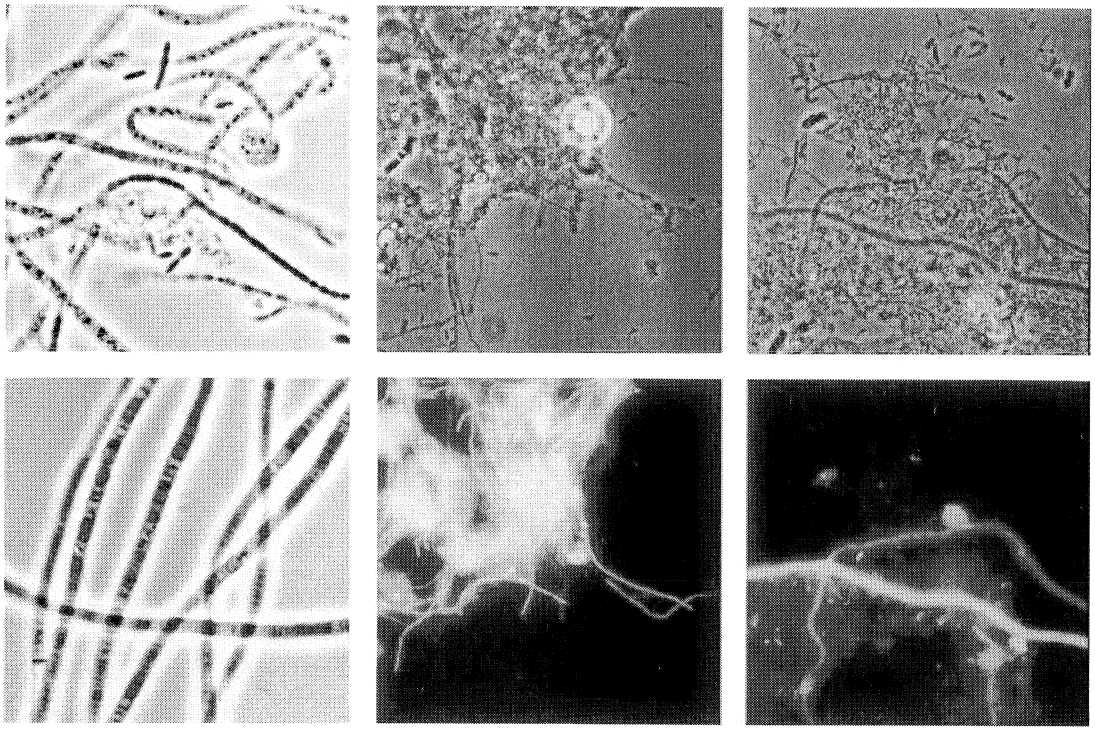

Fig. 1. Top pair of panels: In situ identification of *Leucothrix mucor* and type 021N in an activated sludge sample (from reference [16] with permission). An identical field was studied by phase contrast microscopy (top left) and epifluorescence microscopy (top right). Hybridization was performed with fluorescein-labeled (pale green-yellow) probe LMU and tetramethylrhodamine-labeled (orange) probe 21N (Table 4). 0.5 cm = 10 μm. Middle panels: In situ identification of *Haliscomenobacter hydrossis* in an activated sludge sample (from reference [16] with permission). An identical field was studied by phase contrast microscopy (middle left) and epifluorescence microscopy (middle right). Hybridization was performed with fluorescein-labeled (pale green-yellow) probe HHY (Table 4). 0.5 cm = 10 μm. Bottom panels: Phase contrast microphotograph of *Leucothrix mucor* (DSM 2157) grown in *Leucothrix* medium [11], 0.5 cm = 5 μm (bottom left); phase contast microphotograph of a 021N isolate grown on GMBN medium [41], 0.5 cm = 10 μm (bottom right). (Photographs by M. Ziegler.)

(usually more than 10 000) is the reason for the reliable detection of individual microbial cells (including filaments) by a combination of relatively insensitive fluorescently monolabeled, rRNA targeted oligonucleotides and standard epifluorescence microscopy. By comparison of a newly retrieved rRNA sequence to the continuously growing rRNA data bases the oligonucleotide probes can be designed in a directed way [15]. The use of rRNA targeted probes is a cultivation independent way to monitor the structure and dynamics of the microbial community [15,16]. With respect to the detection of filamentous organisms, oligonucleotide probes were designed for *Haliscomenobacter hydrossis, Sphaerotilus natans, Leptothrix discophora, Leucothrix mucor, Thiothrix nivea* and the unnamed organism of the Eikelboom type 021N [16]. The probe sequences and target sites are given in Table 4. For visualization of the specifically stained cells, standard epifluorescence microscopy and scanning confocal laser microscopy (SCLM) was used [16] which in addition gave more accurate information about the spatial arrangement of the filaments in the sludge floc. As a result it could be shown that in situ hybridization with nine activated sludge samples led to specific detection of filamentous bacteria without the necessity of cultivation. The in situ identification of type 021N, *Leucothrix mucor* and *Haliscomenobacter* is shown in Fig. 1.

As mentioned above, a basic requirement for designing a specific probe is the knowledge of (preferably complete) 16S rRNA or 23S rRNA sequences, and hence the phylogeny of the organism under study. This information can be obtained from rRNA sequence data bases, which are still incom-

Table 4
Probe sequences and target sites for different filamentous bacteria [16]

Organism	Probe	Sequence (rRNA positions)	Target site[a]
Haliscomenobacter hydrossis	HHY	5′-GCCTACCTCAACCTGATT-3′	16S, 655–672
Leptothrix discophora	LDI	5′-CTCTGCCGCACTCCAGCT-3′	16S, 649–666
Leucothrix mucor	LMU	5′-CCCCTCTCCCAAACTCTA-3′	16S, 652–669
Sphaerotilus natans	SNA	5′-CATCCCCCTCTACCGTAC-3′	16S, 665–673
Thiothrix nivea	TNI	5′-CTCCTCTCCCACATTCTA-3′	16S, 652–669
Type 021N	021N	5′-TCCCTCTCCCAAATTCTA-3′	16S, 652–669

[a]*Escherichia coli* numbering (for details, see reference [16]).

plete and need to be permanently updated. An important factor is specificity testing of probes using, for example, dot blot hybridization against nucleic acids of multiple target and nontarget reference strains [15]. Especially with respect to filamentous bacteria such testing poses a major problem because it implies a requirement for reference strains of filamentous bacteria, and hence their cultivation. The isolation and cultivation of many filamentous bacteria is still a difficult task, if possible at all. In addition, detailed information about their physiology, growth requirements, etc. can only be obtained in pure culture studies. For these reasons, isolation, cultivation and physiological investigations cannot be totally replaced by the molecular methods.

3. Isolation and cultivation of filamentous bacteria involved in sludge bulking or foaming

3.1. Isolation methods

Isolation of several filamentous organisms is a prerequisite for the investigation of nutritional and growth parameters and is still difficult. Exceptions are some representatives of the *Sphaerotilus-Leptothrix* group and some organisms of the nocardioform bacteria (*Gordona* (formerly *Nocardia*) *amarae*, *Skermania piniformis* (formerly *Nocardia pinensis*) and some other nocardioform bacteria which are easiest to isolate and keep in culture) [8,17–20].

In the majority of published studies, single isolates of various filamentous bacteria were isolated and physiologically characterized (for detailed lists of references see [2,19,20]). With respect to the method of isolation, in most cases direct dilution plating on different media is not successful [19–21], because the majority of filamentous organisms are overgrown by more rapid growers, or their full nutritional requirements are not provided by the chosen medium. Van Veen [22] developed a method for isolation of filaments based on specific dilution procedures. Williams and Unz [19] suggested some additional pretreatments prior to plating samples on solid media. In mineral-salt-vitamin solution (MSV) [2], diluted or undiluted mixed liquor samples were spread or streaked onto solid media. In a second step, one of two procedures was recommended by Williams and Unz [19] to increase the density of filamentous organisms. As an alternative, narrow and short filaments or filaments scarce in the sludge flocs were concentrated by centrifugation (at 10°C). Samples containing filaments can be diluted 1:5 in MSV, sonicated at 30 W for 10 s, and washed three times by centrifugation at $1900 \times g$ for 2–5 min. The final pellet was resuspended in fresh MSV and centrifuged at $300 \times g$ for 2–5 min. The supernatant containing free filaments can be used as the inoculum.

Ziegler et al. [20] adapted these methods with minor modifications and introduced some new procedures, as follows. Several washes of the sludge by centrifugation at $2000 \times g$ for 5 min, sonication on ice for 30–60 s (30 W) and subsequent dilution. Gathering filaments with a very thin sterile pipette under a stereoscopic (40×) microscope and subsequent washing steps. Ultra-turrax pretreatment (1 min) on ice or several minutes of vortex mixing in the presence or absence of a few small glass beads in a tube.

Another method for selective isolation is micromanipulation with special microtools under a microscope. This technique was used for the isolation of *Gordona* (*Skermania*) [21] and *M. parvicella* [23]

Table 5
Taxonomy, isolation, cultivation and identification of filamentous bacteria frequently found in bulking or foaming sludge

Organism	Phylogenetic lineage	Method of isolation	Cultivation medium	Identification method	Remarks
I. Gram-positive organisms					
Microthrix parvicella	Subphylum: Actinomycetes new Gram-positive phylum [23,33]	Micromanipulation [23]	Freshly prepared R2A [23,33] NTM medium [33]	Morphological identification see [2,4,5] 16S rRNA sequence[a] [23,33]	Characteristic morphology irregular and winding filaments, polyphosphate inclusions [3,4]
Gordona amarae (formerly *Nocardia* amarae)	Genus: *Gordona* (Nocardiaceae) (Actinomycetales) [34,36]	Micromanipulation [34] Spread plate techniques [17]	TYG medium [26,34] YG medium [26,34] Other media [17,25]	Chemotaxonomic methods [36] in addition to morphological and physiological tests 16S rRNA sequence [36]	Nocardioform morphology, moderately branching, can be cultivated easily [17,34,36], often found in biological foams
Skermania piniformis (formerly *Nocardia pinensis*)	Genus: *Skermania* (Nocardiaceae) (Actinomycetales) [34,35]	Micromanipulation [34] can enhance isolation efficiency	TYG medium [26,34] YG medium [26,34] Other nutrient-rich media	Chemotaxonomic methods [26,34] in addition to morphological and physiological tests [26] 16S rRNA sequence [34,35]	Nocardioform morphology Filaments look like pine needles [26,34], often found in biological foams or aeration tanks
II. Gram-negative organisms					
Sphaerotilus natans type 1701[b]	*Rubrivivax* subdivision of the β-Proteobacteria [37]	Spread plate technique Centrifugation [19] or vortex mix [20] pretreatment For type 1701 centrifugation and micropipette transfer [19]	I, SCY media [2] Stokes [16] SS, CGY [19] R2A [28] GMBN [30,41] Other media [8]	Morphological identification [2–4], sheath formation For additional tests see [8,18,19]	Not able to oxidize Mn^{2+} to Mn^{4+}, nutritionally versatile, can utilize a wide variety of carbohydrates [8,18]
Leptothrix spp.	*Rubrivivax* subdivision of the β-Proteobacteria [37]	Spread plate technique Centrifugation [19] or vortex mix pretreatment [20]	I, SCY media [2] Rouf/Stokes [16,39] SS, CGY [19] R2A [28] GMBN [30,41] Other media [8]	Morphological identification [2,3], sheath formation For additional tests see [18,19,38] 16S rRNA sequences [38]	Able to oxidize Mn^{2+} to Mn^{4+}, encrusted sheaths, poor response to added nutrients [8,18]
Type 0803	*Rubrivivax* subdivision of the β-Proteobacteria [40]	Micromanipulation [40] Centrifugation [19]	SUC medium [19] R2A agar [40]	Morphological identification [2,3] 16S rRNA sequence [40]	Morphology similar to *M. parvicella* [19]

Table 5 (*continued*)

Organism	Phylogenetic lineage	Method of isolation	Cultivation medium	Identification method	Remarks
Leucothrix sp.	γ-Proteobacteria	Spread plate technique on media containing low nutrients [11]	*Leucothrix* medium [11]	Morphological identification [11]	Typical morphology [11], rarely found in bulking sludge [3]
Thiothrix sp. and type 021N[c] *Beggiatoa* sp.	γ-Proteobacteria [16,43]	Spread plate technique Micropipette transfer [19] Vortex mixing [20]	I medium [22] LT, GS, AcS media [19] GMBN [30,41]	Morphological identification [2–4] Physiological differentiation [41,42,46] For *Thiothrix* spp. 16S rRNA sequences are available [43]	Typical morphology [44,45] Type 021N is very often detected in bulking sludge
Type 1863	γ-Proteobacteria [47]	Micromanipulation [47]	R2A [28,47]	Morphological identification [2–4] 16S rRNA sequence [47]	Morphologically similar filaments may belong to different taxa [47]
Haliscomenobacter hydrossis	*Saprospira* subgroup of the *Flexibacter-Cytophaga-Bacteroides* phylum [48]	Vortex mix pretreatment [20]	I, GS, AcS media [20] GMBN [30]	Morphological identification [2–4] Physiological characterization [8,49] 16S rRNA sequence [48]	Characteristic morphology [2]
Type 0092	*Cytophaga* subgroup of the *Flexibacter-Cytophaga-Bacteriodes* phylum [40]	Micromanipulation [40]	R2A [28,40]	Morphological identification [2–4] 16S rRNA sequence [40]	
Type 0411	*Flexibacter* subgroup of the *Flexibacter-Cytophaga-Bacteriodes* phylum [40]	Micromanipulation [40]	R2A [28,40]	Morphological identification [2–4] 16S rRNA sequence [40]	
Herpetosiphon sp.	*Chloroflexus* subdivision of the green nonsulfur lineage [40]	Spread plate technique [50] Micromanipulation [40,50]	R2A [40] Other media [50]	Morphological identification [50] Physiological characteristics [50] 16S rRNA sequence [40]	The filaments show gliding motility [50]

[a]In most cases the accession numbers of 16S rRNA sequences are given.
[b]Type 1701 was not studied for phylogenetic position.
[c]Type 021N was not studied for phylogenetic position.

and a modified method was applied by Hornby and Horan [24] for isolation of other filaments. Details of the methods can be obtained from the original descriptions. The recommended methods for the most frequent filamentous organisms are given in Table 5.

3.2. Cultivation of filamentous bacteria

Some Gram-positive filamentous bacteria, like the nocardioform organisms *G. amarae* and *Skermania piniformis*, and also *Rhodococcus rhodochrous* can be grown on nutrient rich media, like yeast glucose agar

and tryptone yeast extract agar [17,21,25,26]. *Sphaerotilus natans* can also be cultivated on nutrient rich media, like CGYA [27], and media with moderate nutrient content like R2A agar [28].

The majority of filamentous bacteria, however, prefer media with low (<0.5 g l^{-1} of specific and/or complex carbon sources) or moderate nutrient concentrations (0.5–5 g l^{-1} of specific and/or complex carbon sources) [19,20]. Media often used for cultivation are: I medium, and SCY medium [22], GC, AcS, and SS medium [29], LT, CGY, and GS medium [19], GMBN medium [30], medium of Slijkhuis [31], and R2A medium [32]. In most cases an aerobic incubation at 20°C or 25°C (sometimes up to 30°C) is chosen (for up to 6 weeks). The growth of filamentous colonies can be better observed under a stereoscopic (40×) or high-power microscope (160×).

4. Taxonomy of filamentous bacteria causing foaming and bulking problems in the activated sludge process

On the basis of 16S rRNA sequencing studies, knowledge about taxonomy, including phylogeny, has increased during the last few years. Conversely, several filamentous organisms are still very poorly characterized, mainly due to the problems of cultivation and maintenance of cultures.

4.1. *Microthrix parvicella*

Microthrix parvicella is a Gram-positive, straight filamentous bacterium mostly found in Europe in connection with sludge bulking and especially foaming [3,33]. Morphologically it can be characterized by its thin filaments (0.3–0.7 μm diameter), which can be hundreds of micrometers long with a characteristic winding appearance [2–4,23,31,33]. *M. parvicella* is very difficult to cultivate and to maintain. For isolation, micromanipulation is recommended [23]. Based on 16S rDNA sequencing it was found to be a novel deep branching member of the actinomycetes within the Gram-positive phylum of the domain Bacteria [23,33]. It grows best, with great difficulty, on fresh R2A or NTM medium [33]. Because of the lack of information of phenotypic features the name 'Candidatus *Microthrix parvicella*' was proposed [33]. At present no reference strains are available from culture collections. Presumptive identification at present is mainly based on morphological criteria [2–4] and a few phenotypic characteristics [31,33].

4.2. *Nocardioform organisms (Gordona amarae, Skermania piniformis) and other organisms*

Nocardioform actinomycetes play an important role in the formation and stabilization of biological foams in the activated sludge process [17,21,26,34]. The most frequently found organisms are *Gordona amarae* and *Skermania piniformis* (recently proposed for *Nocardia pinensis* [35]) in addition to *Rhodococcus rhodochrous* and a few other nocardioforms [17,21,26,34]. They are relatively easy to isolate with spread plate techniques (Table 5), although micromanipulation can facilitate isolation [21]. They grow at 25–30°C even on relatively nutrient rich media (>5 g l^{-1} of specific and/or complex carbon sources), like yeast glucose agar and tryptone yeast glucose agar [26,34,35], and can be maintained by lyophilization. Several phylogenetic studies on the basis of 16S rRNA sequences have been published [34–36]. For genus or species identification, especially chemotaxonomic investigations (analyses of isoprenoid quinones, polar lipids, cell wall diamino acids, and fatty acids) are essential [35,36], in addition to morphological and physiological characterization. Reference strains are available in culture collections and should be included for control purposes in identification procedures of unknown isolates.

4.3. *Sphaerotilus-Leptothrix, type 1701, type 0803*

Historically, organisms belonging to *Sphaerotilus natans* (at present the only species of the genus) have been regarded as the principal agents of filamentous bulking [19], but it is now clear that a wide variety of filamentous bacteria contribute to this problem. Organisms assigned to the genera *Sphaerotilus* and *Leptothrix* are characterized by their ability to form sheaths. The formation of the sheaths is affected by the composition of the culture medium [8,18]. *S. natans* can be grown on a wide variety of media (Table 5), with a preference for media with moderate nutrient concentrations [8,18].

The preferred incubation temperatures range from 20 to 28°C. Reference strains are available from culture collections. Isolation can be successful using spread plate techniques [8], centrifugation pretreatment [19] or vortex mix pretreatment of activated sludge [20]. Several studies on the phylogenetic position of the Gram-negative genera *Sphaerotilus* and *Leptothrix* have been published recently [27,37,38]. Both genera are placed within the *Rubrivivax* subdivision of the β-subgroup of the Proteobacteria. The genus *Leptothrix* includes six species, from which *L. discophora, L. cholodnii* and the recently described *L. mobilis* [38] are available from culture collections. Strains of the remaining species *L. ochracea, L. pseudo-ochracea*, and *L. lopholea* have never been grown in axenic culture [27]. Representatives of the culturable species can be grown on nutrient poor media, like PTYP medium [27] and the medium described by Rouf and Stokes [39], incubated at 20–30°C (temperature optimum) [8]. *Leptothrix* and *Sphaerotilus* share a number of features (i.e. motile cells, formation of poly-β-hydroxybutyrate (PHB), requirement for vitamin B_1), but can be clearly differentiated by the ability of *Leptothrix* to oxidize Mn^{2+} to Mn^{4+}, in addition to several other features listed by Mulder and Deinema [8]. Identification of species is based largely on morphological and cytological criteria [8]. However, for culturable representatives, several phenotypic characters have also been described [8,18,37,38]. Further information is given in Table 5. Bacteria of the type 1701 described by Eikelboom [2] share nearly all features with the *Sphaerotilus-Leptothrix* group (Table 5), especially with *S. natans* [2,19,20]. Eikelboom [2] stated that especially on I medium, colonies of type 1701 closely resemble *S. natans*. Cells of type 1701 are smaller [3]. Detailed taxonomic studies are required to clarify the relationship of type 1701 to *S. natans*. Type 0803 was recently also shown to belong to the *Rubrivivax* subdivision of the β-subgroup of the Proteobacteria [40]. Morphologically this filament may be confused with *M. parvicella* [19]. Nutritional requirements are largely unknown.

4.4. *Leucothrix-Thiothrix-Beggiatoa, type 021N, type 1863*

The Gram-negative organisms of the genera *Leucothrix*, *Thiothrix*, *Beggiatoa*, and type 021N belong to the filamentous sulfur bacteria. *Thiothrix* and type 021N are especially frequently found in bulking sludge and their occurrence has been described in connection with specific plant operational factors [3,4,41]. They can be differentiated by several morphological and cytological characteristics [42]. Phylogenetic studies have shown that they belong to the γ-subdivision of the Proteobacteria [43]. Organisms of the genus *Leucothrix* are rarely found in wastewater treatment plants [3]; however, they can be differentiated from *Thiothrix* and type 021N by their salinity tolerance and response to nutrient conditions [11]. At present only one species has been described, *Leucothrix mucor* (Fig. 1). A detailed morphological and physiological description is given by Brock [11]. Isolation can be performed by spread plate techniques on media with low nutrient concentrations [11]. The organism also grows on media containing moderate carbon and nitrogen concentrations. Bacteria of the genera *Thiothrix* and *Beggiatoa* are also characterized by their distinct morphological features [44,45]. *Thiothrix* organisms form ensheathed filaments which accumulate sulfur in the presence of sulfide. At present two species are described, *T. nivea* and *T. ramosa*, and their phylogenetic position has been clarified recently [43]. Media with low levels of single carbon sources and thiosulfate or sulfide can enhance recovery (for details see Table 5). A micropipette transfer [19] or a vortex mix pretreatment can facilitate recovery (Table 5). Only one *Beggiatoa* species has been described, *B. alba*. Williams and Unz [19] isolated strains of *Beggiatoa* without pretreatment on LT, GS and AcS media. Organisms of the type 021N are very similar to *Thiothrix*. They also have a distinct morphology (Fig. 1). Eikelboom [2] isolated these organisms on I medium and SCY medium. In a further study [19] GS, AcS and LT media were used. The detailed nutrition of *Thiothrix*, type 021N, *Beggiatoa* and *Leucothrix* was given by Williams and Unz [42,46]. Some isolates of type 1863 were also shown to belong phylogenetically to the γ-subdivision of the Proteobacteria; however, it has also been reported that other filaments of this type belong to the *Flexibacter-Cytophaga-Bacteroides* phylum [47]. Type 1863 can be isolated by micromanipulation and can be cultivated on R2A agar [47].

4.5. *Haliscomenobacter hydrossis, type 0092, type 0411*

Filaments of the species *Haliscomenobacter hydrossis* (at present the only species of the genus) are characterized by their straight, thin needle shaped cells [8]. It is Gram-negative and can be identified by its unique cell morphology. Recently it was shown that the organism phylogenetically belongs to the *Saprospira* subgroup of the *Flexibacter-Cytophaga-Bacteroides* phylum [48]. Isolation can be facilitated by using a centrifugation or vortex mix pretreatment [20]. The organisms grow on I medium [22] and GMBN medium [30,49] at 25°C. A detailed nutritional study has shown that few carbon sources are utilized [30,49]. Types 0092 and 0411 also belong to the *Flexibacter-Cytophaga-Bacteroides* phylum [40]. Type 0092 belongs to the *Cytophaga* subgroup and type 0411 to the *Flexibacter* subgroup. Filaments of both types could be isolated by micromanipulation and were grown on R2A agar [40]. Type 0411 has also been grown on I medium and on SCY medium [2] (Table 5). Detailed growth requirements are not known.

4.6. *Herpetosiphon*

The occurrence of bacteria belonging to the genus *Herpetosiphon* in bulking sludge was reported by Trick and Lingens [50] for the first time. In their study they characterized their strains morphologically and physiologically. The isolation can be performed by spread plate techniques [50], but also by micromanipulation [40]. *Herpetosiphon* belongs phylogenetically to the *Chloroflexus* subdivision of the green nonsulfur lineage [40]. Additional information is given in Table 5.

4.7. *Other filamentous organisms*

For several other filamentous bacteria only a little information can be obtained from literature. Type 0041 was isolated and characterized by Williams and Unz [19]. The isolation could be improved by using centrifugation or micropipette transfer [19]. The isolates grew on I, SS, and SUC media [19]. Cultivation of type 0581 on I and SCY media was described by Eikelboom [2]. These organisms have not been studied for their phylogenetic position until now. For types 1702, 0321, 0675, 1851, 0192, 1091, 0961, 0914, 1111, 1501 and *Nostocoida limicola* [2] no pure cultures are available.

5. Specific growth requirements of some filamentous bacteria

In only a few studies [19,30,42,46] have the detailed growth requirements of filamentous organisms been investigated. The majority of culturable filamentous organisms can grow on carbohydrates like glucose or fructose. Often, growth is poor and only moderate concentrations of the carbon sources (0.2–2 g l^{-1}) lead to visible biomass production [19]. This has been observed for *Sphaerotilus* [8,20], *Haliscomenobacter* [8,20,49], and type 021N [42,46]. Growth responses to organic acids like acetate may be different among filamentous bacteria. No growth with acetate was observed for the majority of *Haliscomenobacter* strains, whereas all other groups utilized acetate, in congruence with literature data [49].

A strict dependence on calcium, magnesium, and phosphate in the medium has been documented by Kämpfer et al. [30]. Ammonia has been found by Williams and Unz [46] to be the best nitrogen source for the filamentous bacteria in their study. High ammonia concentrations (>2 g l^{-1}) inhibited the growth of type 1701, the pink pigmented organisms, and *Haliscomenobacter* sp. [30], whereas the other groups did not show significant growth differences at different ammonia concentrations. Phosphorus concentrations of >0.01 g l^{-1} satisfied the phosphorus requirements of several filamentous bacteria [30], but *Haliscomenobacter* preferred a distinct range of phosphorus concentration (0.05–0.2 g l^{-1}) and type 021N showed better growth at concentrations ranging from 0.01 to 0.4 g l^{-1} [30,46]. Interestingly a dependence on calcium and magnesium was observed, which was more pronounced for calcium. The presence of nitrate, sulfide, and thiosulfate was not necessary for growth of the filamentous organisms, however, for nitrate, better growth was observed at a low concentration (<0.1 g l^{-1}) [30]. High concentrations of sulfide and thiosulfate inhibited growth of several isolates. Very different growth responses were obtained when peptone or yeast ex-

tract was added to the media in different concentrations. The nocardioform organisms and *Sphaerotilus* showed enhanced growth responses with increasing nutrient concentration [30], whereas *Haliscomenobacter* did not grow at concentrations of 0.5 g l^{-1}, type 1701 developed better at moderate concentration (0.05–0.2 g l^{-1}) and type 021N showed no significant growth differences at concentrations <1 g l^{-1}, but did not grow at all at concentrations >2 g l^{-1} [30].

6. Conclusions and outlook

Complex microbial communities are directly responsible for the effectiveness and success of the wastewater treatment process (organic matter reduction, nitrogen and phosphorus removal). Community structure and function also have a direct effect on the quality of the final effluent, which is essentially based on the ability of the activated sludge to settle and to be properly separated in the secondary clarifier. In the last few years several 16S rRNA sequences have been published [23,27,33–36,38,40,43,47,48]. These data are very important and form the basis of a phylogenetically based classification. The development of 16S RNA oligonucleotide probing [15,16] and fluorescent antibody techniques [12–14] has provided new tools for in situ identification of filamentous bacteria without the necessity for cultivation. Results of these investigations have outlined a high degree of genetic diversity hidden by identical morphology. In situ monitoring of bulking or foaming with these new molecular tools may considerably improve our understanding of these complex processes and may even help in finding causative factors. The use of new optical methods like SCLM may even produce detailed information about the spatial arrangement of the filaments in sludge flocs. This information is essential to monitor the presence, the various roles and the development of different filaments in the flocs. In addition, more detailed phenotypic characterization is urgently needed, both for a correct taxonomic description of the various Eikelboom types and for the improvement of our knowledge of their physiology. Such studies require new cultivation techniques and, most importantly, techniques to maintain and conserve these cultures. A combination of new molecular methods, new microscopic techniques and improved cultivation, maintenance and physiological information about filamentous isolates may lead to detailed insights into the activated sludge process. The ultimate goal is the identification of causative factors to prevent the excessive filamentous growth that causes bulking and foaming.

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