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Use of molecular techniques for the analysis of foam-causing bacteria in Al Bireh oxidation ditch, Palestine

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Activated sludge foaming, a worldwide problem, usually consists of filamentous bacteria occurring predominantly in the mixed liquor. Because of a lack of pure cultures of most filamentous bacteria and the limited amount of characterisation data, molecular approaches were used to investigate dominant filamentous bacterial strains associated with foaming in Al Bireh Wastewater Treatment Plant in Palestine. Applying denaturing gradient gel electrophoresis (DGGE), 16S rRNA cloning and sequencing showed the dominance of several filamentous bacteria including *Microthrix parvicella*, *Nocardia sp*., *Hyphomicrobium facilis*, *Chloroflexi*, Candidates TM7 and *Nocardioides oleivorans*.

Keywords: Activated sludge; Filamentous bacteria; DGGE; 16S rRNA

1. Introduction

Wastewater treatment is important for healthy human and environmental life. Especially in countries suffering from water scarcity, treated water reuse can be considered as an alternative source of water for many purposes. Sustainable water reuse depends on the water treatment process. It is well known that foaming and filamentous bulking can affect some wastewater treatment plants continuously or seasonally [1]. Filamentous foaming and bulking is caused by the overgrowth of filamentous bacteria, mainly in activated sludge systems [2]. These bacteria are normal components of activated sludge biomass; but they may compete successfully with the floc-forming bacteria under specific conditions [2]. Biological foaming in activated sludge wastewater treatment systems can be described as the formation of a scum layer on the surfaces of aeration basins and secondary clarifiers due to the presence of large quantities of hydrophobic filamentous [3] and possibly non-filamentous microorganisms [4]. It appears that worldwide 20–60% of wastewater treatment plants experience biological foaming from time to time, resulting in sludge wash-out and malfunction of the wastewater treatment process [1].

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Filamentous micro-organisms can be good indicators of conditions prevailing in an activated sludge system on a microbiological level. The indications given by the abundance of filamentous bacteria could be low dissolved oxygen (DO), low food-to-micro-organism ratio (F/M), presence of septic waste, nutrient deficiency, low pH in the system, or high grease and oil load [5,6].

1.1. Common foaming and bulking filamentous bacteria

Foaming and bulking are worldwide phenomena in activated sludge treatment plants [6,7]. They result from the excessive growth of filamentous organisms, like the often reported *Microthrix parvicella*. Foaming problems in activated sludge wastewater treatment systems have been widely reported.

In the United Arab Emirates, Faheem and Khan [8] reported that the dominant filamentous bacteria identified from mixed liquor and foam samples from Dubai included a long branched form of Gram variable nocardioform actinomycetes species, *Thiothrix*, Eikelboom Type 021N, *Sphaerotilus natans*, *Beggiatoa* and *Nostocoida limicola* type I. Occasionally, attached growth forms of Eikelboom type 0041/0675 like filaments were observed in mixed liquor and foam samples especially during warm weather. In Japan, *Nocardia spp.*, *M. parvicella*, *Nostocoida limicola*, type 1851, type 0041, type 1701, type 0675, type 021N, and type 0914 dominated in the foam of a membrane bioreactor [9]. In Bangkok, Thailand, Types 021N, 1701, 0092, 0041 and 0675 were found to be the dominant filamentous microbes in activated sludge treatment [10]. In China, the most frequent filamentous bacterial type observed in foaming activated sludge systems was *M. parvicella* [1].

In South Africa, Blackbeard *et al*. [11] reported that *Microthrix parvicella* and types 1851, 0041, 0675 and 0914 were the dominant filamentous microorganisms in activated sludge systems. In Denmark, Germany, Greece and the Czech Republic, *M. parvicella*, Type 0092 and Type 0041 have been commonly detected [12–15]. In France, Italy and the UK *Microthrix* sp., Type 0041, nocardioform actinomycetes, and *M. parvicella* were the dominant filamentous species [7,16–18].

In North America, *M. parvicella*, Types 0041, 1701, 0092, 021N and 0675 were dominant in most activated sludge systems [19]. In South America, *M. parvicella*, *S. natans*, Types 1701, 0041 and 0675 were dominant [20]. In Australia, according to Seviour *et al*. [21], *M. parvicella*, *Haliscomenobacter hydrossis* and Types 0041, 0675 and 0092 were the most common filamentous species.

1.2. Foaming and bulking control strategies

Suggested solutions to control filamentous foaming and bulking in activated wastewater treatment systems include both non-specific and specific strategies [6]. Non-specific strategies provide temporary solutions that are potentially detrimental for all the biomass in the treatment system. These include chlorination, ozonation, and the addition of hydrogen peroxide, metal salts and synthetic polymers [7,22–25]. Specific strategies focus on understanding and adjusting the relationships between the dominant filamentous bacteria and the operational parameters of the treatment plant [7,22,24]. These parameters include: dissolved oxygen concentration, F/M ratio, grease and oil amounts, pH and nutrients.

1.3. Laboratory identification of filamentous bacteria

Micro-organisms in foaming wastewater treatment plants include filamentous species that have been traditionally identified by their morphology and simple staining reactions [26]. Micro-organism morphology is a poor descriptive attribute that can vary widely depending upon nutritional conditions [27]. The majority of filamentous bacteria in sludge, however, are still unidentified beyond these simple characteristics [28].

The development of culture-independent molecular techniques, like fluorescence *in situ* hybridisation (FISH), analysis of 16S rRNA genes by polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) has improved the analysis of microbial communities in wastewater treatment systems and environmental samples [29] including filamentous bacteria. In bioreactors, where stability and performance depend on complex microbial interactions, the development of these molecular techniques provides the opportunity to establish the connection between the microbial structure and the functional characteristics of the system [30].

The 16S rRNA genes, which are found in all bacteria, have slow mutation rates. Highly polymorphic regions of the 16S rRNA provide a unique signature to any bacterium and useful information about the relationship between them [31]. On the other hand, since 16S rRNA have certain conserved regions found in all known bacteria, PCR primers may be then designed to recognise these conserved bacterial 16S rRNA gene sequences and used to amplify intervening, variable, or diagnostic regions [32,33]. This procedure avoids the need to grow the bacterium and requires no pre-existing phylogenetic information [34].

1.4. Foaming in Al-Bireh wastewater treatment plant (BWWTP)

In Palestine, Al-Bireh wastewater treatment plant (BWWTP) handles municipal wastewater to meet increasingly stringent environmental requirements and to reuse both reclaimed effluent and stabilised biosolids in the future. BWWTP, an oxidation ditch system, has a modified single activated sludge system for simultaneous nitrification and denitrification with aerobic sludge stabilisation. The liquid line consists of a preliminary treatment stage (coarse screening, two aerated grit chambers), one biological unit and two secondary settling tanks. During this study, treatment capacity of BWWTP was calculated at 35,000 population equivalent (PE), exceeding the actual design capacity (25,000 PE). More technical data on process description of BWWTP and other operational parameters can be found in Al-Sa‘ed and Hithnawi [35].

BWWTP manifests the biological foaming phenomenon associated with excessive growth of unknown filamentous bacteria, which lead to treatment process instability and effluent quality deterioration [36]. There is a lack of studies that identify micro-organisms responsible for this phenomenon. Identifying the filamentous microorganisms behind this biological phenomenon can help the operational staff at BWWTP to control and optimise the treatment process, so that a stable effluent quality is achieved. This study aimed at applying modern molecular techniques (DGGE, 16S rRNA-gene cloning and sequencing) to identify foam-causing filamentous micro-organisms in biosolid samples from BWWTP. In addition, morphological analyses using the microscope were applied.

2. Materials and methods

2.1. Sampling

Activated sludge foam samples were collected from the secondary clarifier of BWWTP. In addition, samples for oil and grease content analysis were collected from the aeration tank and secondary clarifier.

2.2. DNA extraction, 16S rRNA gene amplification and DGGE

Genomic DNA was extracted according to the method of Oude Elferink *et al*. [37], which is based on mechanical disruption by bead beating and phenol/chloroform/iso-amyl-alcohol extraction. The 16S rRNA gene (approximately 1500 bp) was amplified from genomic DNA by PCR as described by Roest *et al*. [38] using primers 0007-f and 1492-r (table 1). The following thermocycling program was applied: pre-denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 sec, annealing at 48°C for 20 sec, elongation at 72°C for 40 sec and post-elongation for 7 min. Primers 968-f-GC and 1401-r (table 1) were used for partial (V6–V8 region) 16S rRNA-gene amplification for bacterial DGGE analysis [38].

PCR products were separated in a 1% agarose gel, stained with SYBR Green and visualised with a gel documentation system (Kodak gel logic 100E). Amplicons of expected size (approximately 500 bp) and reasonable yield were used for DGGE analysis as described by Roest *et al*. [38].

2.3. Clone library construction

The amplified 16S rRNA gene products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany), and cloned in One Shot® OmniMax 2–T1® chemically competent *Escherichia coli* (Invitrogen, Barcelona, Spain) by using the pCRII® – TOPO® plasmid (Invitrogen, Barcelona, Spain). Thereafter, 50 µl and 100 µl of the cloning mixtures were spread on LB agar plates with ampicillin and blue/white selection. Plasmids of about 37 different clones were isolated using a QIAprep spin kit (Qiagen GmbH, Hilden, Germany).

2.4. Sequencing and BLAST search

The 16S rRNA genes were partially sequenced in Hy Laboratories Ltd – Israel, using pCRII®–TOPO targeted Sp6 primer. Similarity searches of 16S rRNA gene sequences derived from clones against sequences deposited in publicly accessible databases were performed using the NCBI Blast search tool at http://www.ncbi.nlm.nih.gov [41].

Table 1. Primers used in this study

Primer	Sequence (5'→3')	Reference
0007F	AGA GTT TGA T**YM** TGG CTC AG	[39]
1492R	CGG CTA CCT TGT TAC GAC	[39]
0968F-GC	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G AAC GCG AAG AAC CTT AC	[40]
1401R	CGG TGT GTA CAA GAC CC	[40]

M = A, C ; Y = C, T

2.5. Hexane extractable material (HEM; oil and grease)

The oil and grease content of wastewater from BWWTP was determined in aeration and secondary settling tank according to EPA 1664 Standard Method. A 1L sample was acidified with hydrochloric acid to pH <2 and extracted with n-Hexane in a separation funnel. The extract was dried over sodium sulphate. The solvent was distilled and the HEM was dried and weighed.

2.6. Gram staining

Different foam samples were gram stained during summer and winter periods to monitor microscopically the dominant filamentous bacteria in the secondary clarifier.

3. Results

The filamentous micro-organism most frequently observed by gram staining during cold seasons and with high abundance in the secondary clarifier foam sample was *Microthrix parvicella* (figure 1). Other filaments like *Nocardia Spp*. were abundant during the hot seasons (figure 2).

The oil and grease content of wastewater was 100 mg/L and 30 mg/L in the aeration tank and secondary sedimentation tank, respectively.

The dominant clones of the bacterial domain were determined by comparing the clones to the total DGGE profile of the foam sample. A total of 20 dominant clones were chosen for 16S rRNA sequencing using the forward SP6 primer and ABI prism sequencer. The sequences obtained were trimmed, aligned and compared to available databases by the use of the Basic Local Alignment Search Tool (BLAST) [42] and Ribosomal Database Project-II (RDP). Clones with the greatest similarity to the clone sequences (from the BLAST search) which represent filamentous bacteria were selected (table 2, figure 3).

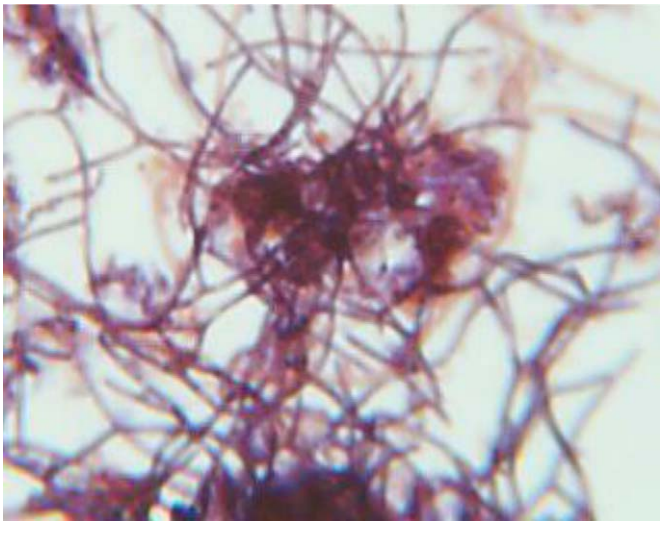

Figure 1. Gram stain of scum filamentous bacteria in BWWTP (winter), showing the dominance of *M. parvicella*.

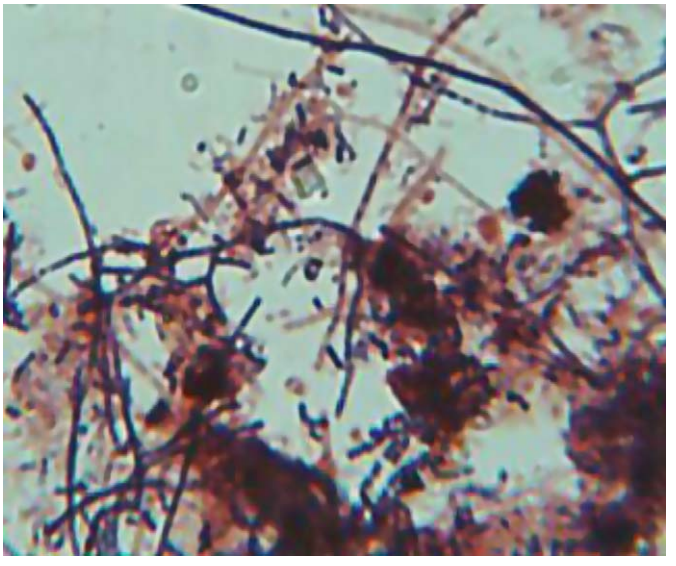

Figure 2. Gram stain of scum filamentous bacteria in BWWTP (summer), showing the dominance of *Nocardia spp*.

4. Discussion

Filamentous micro-organisms can be good indicators of conditions prevailing in an activated sludge system on a microbiological level. The indications given by the filamentous bacteria could be of low dissolved oxygen (DO) (e.g. *Sphaerotilus natans*), low food-to-microorganism (F/M) ratio (e.g. *Microthrix parvicella*, Type 0092), presence of septic waste (e.g. *Thiothrix spp*.), nutrient deficiency (e.g. *Haliscomenobacter hydrossis*), low pH in the system (e.g. fungi) [5], and high grease and oil content (e.g. *Microthrix parvicella* and *Nocardioides oleivorans*) [5,6].

From the sequences of the clones, a wide variety of organisms in the foam sample was identified without prior cultivation. Although only a small number of clones were

Table 2. Blast result of dominant filamentous bacteria in the secondary clarifier

Clone	Microorganism	% Similarity	Accession
PL 2-3B	*Hyphomicrobium facilis*	97	Y14312
PL 3-3B	*Nocardioides oleivorans*	99	AJ698724.1
PL 4-3B	Uncultured bacterium FukuS110	96	AJ289986
PL14-3B	Uncultured candidate division TM7 bacterium clone SM1G12	97	AF445701
PL 17-3B	*Microthrix parvicella*	94	X89560
PL 20-3B	Activated sludge foam clone 47	97	AF513095.1
PL 24-3B	Bacteria: phylum *Chloroflexi*:– Clone SHA-147.	95	AJ306749
Pl 26-3B	*Nocardioides sp*. str. ND6	95	AJ511294.1

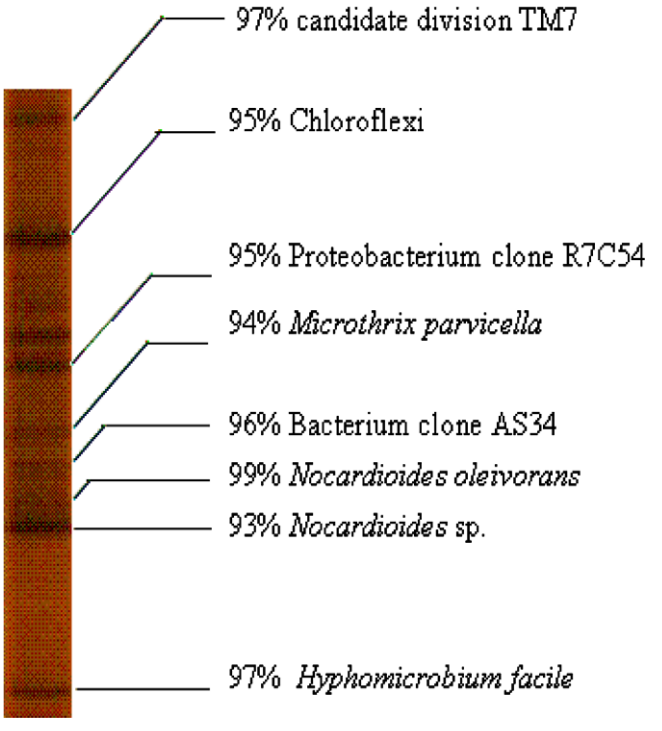

Figure 3. Identification of bands in the bacterial DGGE pattern of activated sludge foam. Percentage values indicate sequence similarity with closest relative present in the sequence database.

sequenced, the results indicated that most of the clones isolated belong to filamentous organisms associated with activated sludge foaming.

As shown in table 2 and figures 1 and 2, the dominant filamentous bacteria found in the secondary clarifier are *Hyphomicrobium facilis* clone (PL 2-3B), *Nocardioides oleivorans* clone (PL 3-3B), TM7 bacterium clone (PL14-3B), *Microthrix parvicella* clone (PL 17-3B) and *Chloroflex*i clone (PL 24-3B).

Nocardia spp. was dominant in summer sampling as shown by gram staining but it was not indicated by 16S rRNA analysis. This may be due to the DNA extraction protocol which might not be efficient enough to break up all gram-positive cells [27]; or perhaps clone scoring difficulties meant that not all dominant clones were covered and sequenced.

Clone (PL 17- 3B) has 94% similarity to *M. parvicella* which is a common cause of foaming in wastewater treatment plants where low food-to-micro-organism ratio (F/M ratio) is recorded [43]. Andreasen and Nielsen [44] found *M. parvicella* to thrive on the long chain fatty acids oleic and palmitic acid and on trioleic acid with oleic acid being used in aerobic, anoxic and anaerobic conditions. For *M. parvicella* fatty acids are toxic at high concentrations. Thus, in order to use high amounts of fatty acids, *M. parvicella* must already have constituted a high biomass concentration. The ground of some *M. parvicella* problems might therefore well be prepared by other scum bacteria and then stabilised by *M. parvicella* [45].

M. parvicella seems to be restricted to strongly substrate-limited conditions below F/M ratio of 0.15 kg BOD/(kg TSS.d). These conditions cause the increase of *M. parvicella* biomass and thus reactors develop thick stable scum layers [46].

The conditions discussed are similar to those of BWWTP where the F/M ratio is below 0.1 kg BOD/(kg TSS.d) and the influent and effluent contains high grease and oil content, 100mg/L and 30 mg/L, respectively. As *M. parvicella* favours temperature below 15°C, it was more concentrated in cold seasons than in hot seasons, as also shown in figures 1 and 2.

Clone (PL 2- 3B) has 97% similarity to *Hyphomicrobium facili*s which is a gram-negative bacterium of particular interest routinely monitored in wastewater treatment systems. Hypertrophic growth (i.e. hyphal elongation) of *Hyphomicrobium* can lead to poor sludge settling and compaction. It is one of the dominant filamentous bacteria found in the scum layer formed in the secondary settling tank. The low F/M ratio in BWWTP as discussed earlier is a good condition for *Hyphomicrobium facilis* to thrive, since the most important factor that supports the presence of this filamentous bacterium is a low F/M ratio.

Clones (PL14- 3B) and (PL 24- 3B) have 95% and 97% similarity to *Chloroflexi* and candidate phylum TM7 respectively. Both have also been shown by molecular methods to have filamentous representatives in sludge and to cause serious operational disorders of bulking and foaming in activated sludge wastewater treatment plants [47–49].

Beer *et al.* [21] showed that the 16S rRNA sequence of Eikelboom Type 1851 from a bulking activated sludge plant is very close to '*Roseiflexus castenholzii*', a member of the phylum 'Chloroflexi', class 'Chloroflexi', previously called the green non-sulphur bacteria. Type 1851, which belongs to the *Chloroflexi* group, is associated with high mean cells residence time MCRTs (>10 days) and low F/M ratios (<0.2 Kg BOD_5 / kilogram of mixed-liquor VSS) a conditions similar to that of BWWTP.

Identification and enumeration of filaments using FISH with group-specific 16S rRNA-targeted probes revealed that 14–16% of filaments of the Type 0041 morphotype hybridised with TM7-specific probes in two wastewater treatment plants [50]. Thomsen *et al* [24] also found no significant physiological differences between TM7-positive and TM7-negative. Type 0041 filaments and TM7 filamentous bacteria can take up carbon substrates under aerobic and anaerobic conditions.

From the previous results, it can be noticed that the detected filamentous bacteria found in the scum samples all need comparable conditions to thrive in activated sludge systems. These filamentous bacteria mostly result from low F/M ratio, high sludge residence time (SRT) and high grease and oil content. The analysis of sludge bulking by molecular techniques allows the prompt detection of threatening problems or altered operating conditions. Simultaneous nitrification and denitrification processes are achieved at BWWTP. Denitrification and anaerobic digestion takes place in the secondary settling tank, resulting in release of nitrogen and methane gas as a product. This gas is only slightly soluble in water and small nitrogen and methane gas bubbles form in the settling sludge and cause sludge blanket flotation in the final clarifier. Denitrification and anaerobic digestion problems are more prevalent during the warmer times of the year and can be more severe if filamentous microorganisms are present, because they will trap more extensively the nitrogen and methane gas bubbles. Recently, Al-Sa'ed and Tomaleh [36] suggested possibilities for suitable corrective measures like increasing sludge wastage rate, resulting in a shorter sludge age. An improved control of sludge bulking was achieved with more efficient biological processes.

The oil and grease concentration in BWWTP is high compared to domestic wastewater from other countries. Perhaps the Palestinian community lack awareness of the need to separate oil from other wastes; or perhaps there is more oil and grease because of leakage of runoff water through the sewer system and the industrial wastewater discharged into BWWTP [35]. As an extended aeration system, BWWTP lacks primary settling tanks. This exhibits the problems of sludge bulking and scum formation since most of the lipids

are usually removed in this primary tank. The foam-forming microbial population is specialised in consuming lipids, substrates classified as slowly degradable. When the temperature increases, the rate of lipid hydrolysis becomes sufficiently high for this population to become abundant, accumulate on the surfaces of the aeration basins, and cause biological foaming [51]. Communities with enforced grease and oil ordinances appear to suffer less from wastewater treatment plant foaming problems. The treatment of septage, which contains substantial grease and oil, has also been associated with foaming problems [3].

The scum layer of BWWTP exhibits similar behaviour since the problem of the scum increases in summer as the temperature and the strength of the wastewater increases. The presence of the crude oil degrading bacteria *Nocardioides oleivorans* is supported by the relative high lipid load. This results in scum formation in the secondary settling tank.

The proliferation of filamentous bacteria can be reduced by means of biological selectors. These enhance the growth of floc-forming bacteria over that of the filamentous bacteria and, thus, help control sludge bulking [52,53]. A selector is a biological reactor or compartment in which certain operational parameters (e.g. F/M ratio, electron acceptor) can be manipulated to reduce the growth of filamentous bacteria [54]. Worldwide, there are three common types of biological selectors that have been successfully applied: aerobic, anoxic, and anaerobic [55]. Such a selector – either aerobic or anaerobic – is urgently needed at BWWTP to reduce the annual proliferation of filamentous bacteria in BWWTP, but this depends on securing the finance and having the full support of the decision-makers of the municipality.

5. Conclusions

Culture-independent molecular techniques, like DGGE, 16S rRNA cloning and sequencing, allow better understanding of microbial populations involved in wastewater treatment. These techniques are of great importance especially when uncultured bacteria are involved. Foam samples from BWWTP were found to contain at least eight dominant micro-organisms that are usually associated with foaming problems. The most important of these are the filamentous *M. parvicella* (dominant in winter) and *Nocardia spp.* (dominant in summer). The abundance of these filamentous micro-organisms is supported by factors including the high grease and oil content, low F/M ratio and high SRT.

Finally, solving the foaming problem in BWWTP requires the introduction of a mechanical stirrer (mechanical energy) to reduce the filamentous bacterial successfully, the installing of biological selectors, the rapid increase of F/M ratio, and the sudden decrease in the sludge age.

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