

Differences in populations of filamentous bacteria involved in foaming and bulking of activated sludge

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Bulking and foaming of activated sludge are related to excessive proliferation of a specific group of activated sludge biocenosis – filamentous bacteria. The research was carried out to compare filamentous bacteria populations in foam and activated sludge in a full-scale municipal wastewater treatment plant located near Warsaw (Poland). Fluorescent in situ hybridization (FISH) – a quantitative, culture-independent, molecular method was applied to evaluate the structure of filamentous populations. Activated sludge and foam were examined for the abundance of eleven groups of these microorganisms, which occur in wastewater treatment plants in Europe. Filamentous bacteria constituted 18% and 24% of all bacteria detected in sludge and foam, respectively. The structures of filamentous bacteria populations in sludge and foam were different, although the same bacteria were found in both types of samples. The most abundant filaments belonged to phylum *Chloroflexi* (targeted by the CFXmix probe) and genus *Microthrix* (targeted by the MPAmix probe) in sludge and foam, respectively. The third significantly abundant bacteria was *Haliscomenobacter hydrossis* (targeted by HHY654).

Key words: FISH, filamentous bacteria, sludge bulking, sludge foaming

Introduction

Activated sludge is a microbiological structure consisting of a mixed consortium of micro- and macro-organisms that remove inorganic and organic contaminants from wastewater. Typical biomass contains about 95% of bacteria and 5% of higher organisms, such as protozoa, micrometazoa and lower invertebrates. The activated sludge process relies on the growth of floc-forming bacteria which biodegrade the organic material contained in wastewater. These bacteria, together with the protozoa, are able to flocculate and settle down, leaving a clarified supernatant and a thickened sludge for recycling.

Filamentous bacteria constitute a significant part of the biomass in activated sludge, often more than 25% of all bacteria [1]. They serve as a sort of backbone for the flocs which become stronger and more resistant to tearing. Moreover, filaments adsorb pollutants contained in wastewater, oxidize them and decompose complex compounds to simpler substances, which can already be used as substrates for other bacteria. On the other hand, an excessive growth of certain groups of filamentous bacteria changes the structure of flocs in activated sludge and results in bulking and foaming [2,3,4].

Filamentous bulking and foaming of activated sludge is a common problem in wastewater treatment plants (WWTPs). Bulking impairs flocculating and settling properties of flocs in the secondary clarifier, making solid particles get into treated wastewater [5,6,7]. Foaming is usually caused by *Candidatus* 'Microthrix parvicella' [8] and *My-*

colata [9] and occurs mainly in municipal and industrial wastewater treatment plants without nutrient removal [10]. In Poland, activated sludge bulking coincided with high abundance of bacteria from genus *Microthrix* [11].

In this study these two phenomena were observed simultaneously in one WWTP and filamentous bacteria responsible for them were investigated and compared to each other. The study presents a new approach to research on activated sludge by using molecular methods, it also provides information about the abundance of specific bacteria in sludge and foam, which is not feasible to be obtained by conventional methods.

Materials and methods

Sampling

Activated sludge and foam were sampled from an aerobic tank and a secondary clarifier, respectively, then kept below 4°C until fixed properly for FISH analyses [12].

WWTPs data

The WWTP had an anoxic and an aerobic (AO) reactor type, biological N-removal (nitrification and denitrification). The plant's designed size was 83 000 population equivalents (PE), while the actual size is 99 000 PE. The fraction of industrial contribution (mainly slaughterhouse and dairy wastewater) to the organic matter in the influent was 20-25% (expressed as COD). To improve phosphorus elimination, iron based coagulants (PIX) were dosed when necessary. Operational problems connected with settling of

the activated sludge were reported, they included both poor settling and foaming on secondary clarifiers.

Fluorescent *in situ* hybridization identification

FISH analyses were carried out according to Nielsen *et al.* [12]. The selection of probes was based on the recently designed probes for filamentous bacteria detected in activated sludge plants [13,14]. The 6-Fam labelled general EUBmix probe (equimolar mixture of EUB338, EUB338II, and EUB338III) was used as universal probe to target the great majority of bacterial community. Filamentous bacteria were identified with CFXmix (equimolar concentration of GNSB-941 and CFX1223 probes, targeting phylum *Chloroflexi*), CFX197 (Type 0092 in phylum *Chloroflexi*), T0803 and CHL1851 (Types 0803 and 1851, respectively, in phylum *Chloroflexi*), MPAmix (equimolar concentration of MPA645, MPA223 and MPA60, targeting *Candidatus* 'Microthrix parvicella' and *Candidatus* 'M. calida'), HHY654 (*Haliscomenobacter hydrossis*), Myc657 (*Mycobacterium* subdivision *mycolata*), Spin1449 (*Skermania piniformis*), Gor596 (family *Gordonia*), Curvi997 (Types 1701 and 0041/0675 of *Curvibacter* spp.), TM7905 (Type 0041/0675 in *Candidate* division TM7), G123T (*Thiothrix eikelboomii*, *T. nivea*, *T. unzii*, *T. fructosivorans*, *T. defluvi*, Eikelboom Type 021N group I, II, III). The specific probes were labelled with Cy3. Detailed information about the probes used is given in probeBase [15].

Quantification of filamentous bacteria

Twenty separate images for each probe were captured with a Nikon Eclipse 50i microscope. Quantification procedures were performed similar to Muszyński *et al.* [16] and Mielczarek *et al.* [14]. The biovolume of bacteria, relative to the pixel area of the cells positive for the specific probe, was evaluated by image analysis using ImageJ software [17]. The percentage abundance of a specific bacteria (in % of EUBmix probe) was calculated as a mean of 20 independent measurements. The standard error of the mean (SE) was calculated as a standard deviation of the percentage abundance of the specific bacteria divided by a square root of 20 (number of measurements).

Results and Discussion

Over 30 different morphotypes have been found in activated sludge in various wastewater treatment plants [2,3, 18,19]. In plants with enhanced biological phosphorus removal investigated by Nielsen *et al.* [1] the most abundant filamentous bacteria belonged to phylum *Chloroflexi* and TM7, genera *Curvibacter*, *Haliscomenobacter*, *Microthrix* and *Mycolata*.

Filamentous bacteria were abundant in all samples and constituted 18% and 24 % of all bacteria identified by the EUBmix probe in sludge and foam, respectively. Filaments were more abundant in foam, whereas average abundance in sludge was lower than mean percentages ($28 \pm 3\%$) ob-

tained for Polish WWTPs with nutrients removal [20] or for Danish plants (24-28%), analyzed by Nielsen *et al.* [1] and Mielczarek *et al.* [14].

The same three main groups of filamentous bacteria (*Chloroflexi*, *Microthrix* and *Haliscomenobacter hydrossis*) were detected both in foam and sludge samples. Although filamentous populations were the same, their relative abundance was different (Figure 1). Previous research, carried out by Miłobędzka and Muszyński [20], Nielsen *et al.* [1] and Mielczarek *et al.* [14], showed that the most abundant filamentous bacteria in sludge in Polish and Danish WWTPs also belonged to phylum *Chloroflexi* and genus *Microthrix*. Those two groups of bacteria occurred most frequently during bulking of activated sludge in systems treating primarily municipal wastewater [21].

Chloroflexi was the most abundant filament in sludge ($10 \pm 3\%$ of all bacteria; 57% of all filamentous bacteria (Figures 1 and 2)). Bacteria from this phylum are important protein-hydrolyzing organisms, they excrete exoenzymes like esterase, phosphatase, glucuronidase, galactosidase, chitinase, and protease which help them utilize polysaccharides, proteins, and other complex macromolecules. Usually, these filaments do not cause serious operational problems because they are hidden inside flocs. However, sometimes they can form open structures in flocs, which results in deterioration of sludge settling properties. Morphotypes 0041, 0092, 0803, 1701, 1851, and *Nastocoida limicola* are embraced by *Chloroflexi*. Some of them - morphotypes 0041, 1851 and 1701 are covered by epiphytic *Epiflobacter* [1].

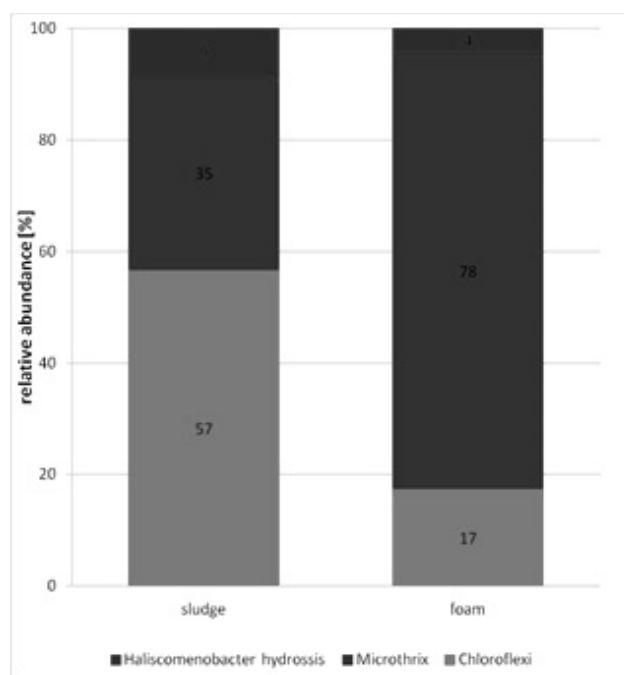


Figure 1. Differences in relative abundance of the filamentous bacteria identified in activated sludge and foam in the tested WWTP

Phylum *Chloroflexi* was further investigated with hierarchical approach, probes T0803, CHL1851 and CFX197 were used to determine abundance of morphotypes 0803, 1851 and 0092, respectively. Type 1851 was not abundant in the tested WWTP, morphotype 0092 accounted for 1% of the whole population of *Chloroflexi* both in sludge and foam, while Type 0803 constituted 5% and 1% in sludge and foam, respectively.

Type 0803, common in municipal WWTPs, may create the open-floc structures or rosettes. It may result in deterioration of settling properties of the sludge, especially when these bacteria extensively proliferate during winter. However, these straight filaments usually occur inside flocs [2,3,6].

Type 0092 of *Chloroflexi* constitutes on average 3-4% of all bacteria in activated sludge [6,14]. However, it was less abundant in sludge and foam of the tested WWTP and did not exceed 1%.

Despite the accuracy of the FISH method allowing to find bacteria not only from type or family, but also particular species, there are still no specific probes for all bacterial species existing in activated sludge (about one third of *Chloroflexi* cannot be identified beyond phylum level) [14]. In our study we were not able to differentiate 40% and 50% of *Chloroflexi* in sludge and foam, respectively.

Microthrix was the dominating filamentous bacteria in foam ($18 \pm 3\%$ of all bacteria; 78% of all filamentous bacteria) (Figures 1 and 3), it also co-dominated in sludge ($6 \pm 2\%$ of all bacteria; 35% of all filamentous bacteria). *Mi-*

crothrix can express high levels of lipases and esterase and grow only on lipids and long-chain fatty acids. What is more, under anaerobic conditions they can store these substrates as lipidic inclusion, which can be utilized for growth under aerobic or anoxic conditions. All these uncommon metabolic characteristics predestinate them to compete with other bacteria. They have a hydrophobic surface and therefore they very often form stable foam on surface of activated sludge tanks [1].

Significant contribution to overall filamentous community in sludge and foam was also made by *H. hydrossis* – 9% and 4% (Figure 1), which corresponds to 2% (Figure 2) and 1% (Figure 3) of all bacteria, respectively. Similar results were obtained in Danish WWTPs, on average 1,9% of all bacteria. These needle-like, thin filaments from phylum *Bacteroidetes* are commonly present in low numbers (occasionally abundant), usually in biological WWTPs with nutrients removal.

TM7, *Mycolata*, family *Gordonia* and *Skermania piniiformis*, *Curvibacter* (Types 1701 and 0041/0675), *Thiothrix/021N* have not been found in the tested WWTP.

The filamentous bacteria in plants with enhanced biological phosphorus removal are adapted to decompose and consume macromolecules. Some of them, like *Microthrix*, are extremely specialized in substrate consumption, but others do not present such specialism.

How to control growth of detected filamentous bacteria? Most obvious, but also usually not possible solution to limit proliferation *Microthrix*, is the elimination of lipids

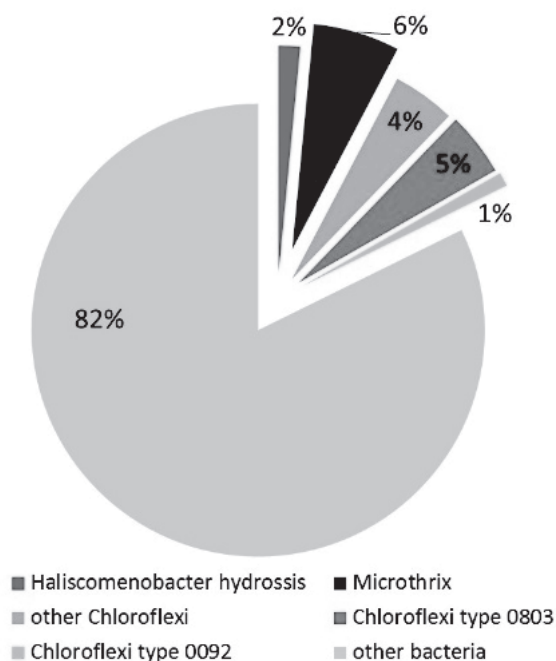


Figure 2. The composition of filamentous bacteria population in activated sludge of the tested WWTP determined by quantitative FISH

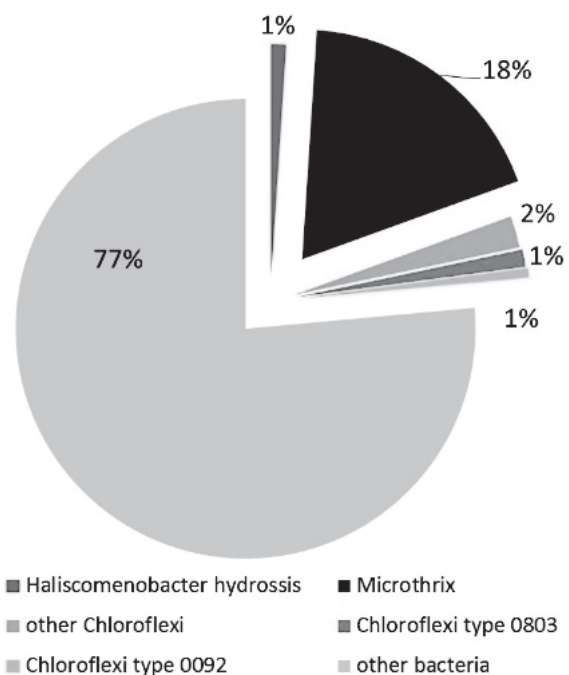


Figure 3. The composition of filamentous bacteria population in foam of the tested WWTP determined by quantitative FISH

from influent. A more frequently used method is treatment with aluminium-based specific chemicals targeting the physiology of *Microthrix* [22]. Another technique to exclude filaments using macromolecules can be to switch to more soluble substrates. Elimination of polysaccharides from the influent is a way to control *Haliscomenobacter*. However, many species from *Chloroflexi* degrade complex macromolecules (essential in activated sludge biostructure) and are very difficult to be selectively removed [1].

Conclusions

Populations of filamentous bacteria causing bulking and foaming in the same WWTP were investigated with quantitative FISH:

1. Filaments constituted significant fractions of bacteria in sludge and foam and accounted for, on average, 18% and 24% of all bacteria, respectively.
2. The structure of filamentous bacteria populations in sludge and foam was different.
3. The most abundant filamentous bacteria in sludge and foam belonged to phylum *Chloroflexi* (CFXmix) and genus *Microthrix* (MPAmix), respectively.

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