

Population dynamics of filamentous bacteria identified in Polish full-scale wastewater treatment plants with nutrients removal

A. Miłobędzka and A. Muszyński

ABSTRACT

A comprehensive study of the identity and population dynamics of filamentous bacteria in five Polish full-scale municipal wastewater treatment plants (WWTPs) with nutrients removal had been carried out for 2 years. A quantitative culture-independent, molecular method – fluorescence *in situ* hybridization – was applied to evaluate the structure of different filamentous bacteria populations and their temporal variations. Activated sludge was examined for the abundance of 11 groups of filamentous bacteria. On average, filaments constituted 28% of all bacteria. All samples presented a low diversity of probe-defined filamentous bacteria, usually with significant domination of *Chloroflexi* (with distinction to types 1851, 0803 and others) and/or *Microthrix* (14% and 7% of EUBmix, respectively). *Haliscomenobacter hydrossis*, *Mycolata*, *Skermania piniformis* and TM7 were less abundant, whereas *Curvibacter*, *Thiothrix*/021N and family *Gordonia* have not been detected in any of the samples. The tested WWTPs showed similarity among species found and differences in their abundance. The composition of filamentous populations was rather stable in each plant and similar to those found in other European countries. Little differences between plants were shown by multivariate analysis of variance in terms of *Chloroflexi* and *Microthrix*. No significant general correlations have been found with Pearson product-moment correlation coefficient and Spearman's rank correlation coefficient. Medium correlation strength between the presence of different filaments was recorded only for *Microthrix* and *Skermania piniformis*. Deleterious effect on settling properties of sludge (measured as sludge volume index) was found only for abundance of *Microthrix*; a strong linear correlation was recorded between them. However, no other correlations with wastewater and operational data were revealed.

Key words | activated sludge, bulking, *Chloroflexi*, filamentous bacteria, *Microthrix*, quantitative FISH

A. Miłobędzka (corresponding author)
A. Muszyński
Faculty of Environmental Engineering,
Department of Biology,
Warsaw University of Technology,
Nowowiejska 20, Warsaw 00-653,
Poland
E-mail: aleksandra_milobedzka@is.pw.edu.pl

INTRODUCTION

Activated sludge technology has been used for a hundred years; however, it still brings some serious operational problems. Main issues are bulking and foaming in wastewater treatment plants (WWTPs), which are associated with extensive proliferation of some filamentous bacteria (Jenkins *et al.* 2004; Nielsen *et al.* 2009b; Mielczarek *et al.* 2012). Current studies with molecular methods (e.g. fluorescence *in situ* hybridization (FISH)) have shown that previous identification based on morphology and chemical staining can often be unreliable (Nielsen *et al.* 2009b; Mielczarek *et al.* 2012). Also, laboratory-scale studies of the isolated microorganisms in pure cultures have to be

used carefully, because data from those studies are not easy to extrapolate to full-scale conditions. Ecophysiological studies under *in situ* conditions on probe-defined populations (Eales *et al.* 2005, 2006; Kragelund *et al.* 2005, 2006, 2007a, b; Carr *et al.* 2006) allowed us to link most filamentous bacteria with substrates, electron acceptors and exoenzymes excreted by them and several other aspects.

Studies based on the FISH method revealed the most abundant and frequently occurring groups of filamentous bacteria in Italian, Danish, German and Dutch industrial and municipal WWTPs (van der Waarde *et al.* 2002;

Levantesi *et al.* 2006; Kragelund *et al.* 2007a). *Alphaproteobacteria* have been found in more than half of the tested plants, along with *Chloroflexi*, *Thiothrix* sp. and *Haliscomenobacter hydrossis*. Other *Bacteroidetes*, different from *H. hydrossis*, were not so frequent, as well as *Actinobacteria* and *Microthrix parvicella*, which were detected only in 16% and 21% of WWTPs, respectively. Some bacteria, like *Gordonia amarae*, *Nostocoida limicola*, *Leucothrix* and *Lepthothrix* sp., occurred only in a few plants and were detected in 7%, 6%, 4% and 2% of tested samples, respectively. It seems that the composition of the population of filamentous bacteria may depend on geographic location (Chua and Le, 1994). Research by Mielczarek *et al.* (2012) showed that the percentage of filamentous bacteria in activated sludge was a typical feature of an object and was different among different WWTPs. However, WWTPs in Poland still lack quantitative data about filamentous bacteria. The following survey presents a comprehensive study of the identity and population dynamics of filamentous bacteria in five Polish full-scale municipal WWTPs with nutrients removal.

Aims

The aim of the study was to identify and quantify dominant filamentous bacteria in five full-scale municipal WWTPs in Poland (in the Masovian Province). Plants differed in configuration of reactors and main technological parameters of wastewater treatment process. FISH was used to investigate the population dynamics of filaments. Microbial populations were examined over a 2-year period to describe their temporal variations. All collected data were used to investigate mutual correlations among the abundance of specific filamentous bacteria and wastewater and operational parameters.

MATERIALS AND METHODS

Sampling and WWTP data

The main operational parameters of selected plants are listed in Table 1 (all data provided by the plant operators). The plants ranged in size from 18,000 to 110,000 population equivalents (PE). The fraction of industrial contribution (from food industry mainly) to the organic matter in the influent, depending on the plant, was 0–50%. All the WWTPs had biological N-removal (nitrification and denitrification) and four of them (except

WWTP II) also the well-defined enhanced biological phosphorus removal (EBPR) step. In all plants but one (WWTP V) iron-based coagulants (PIX) were dosed to improve phosphorus elimination. In WWTP V polyaluminium chloride compounds (PAX) were occasionally applied, mostly during autumn and winter, to improve settling properties and to control excessive growth of filamentous bacteria. Operational problems connected with settling of activated sludge were reported in all tested plants, but foaming on the tanks was observed only in WWTP IV.

Activated sludge samples had been collected twice a year in the period from September 2011 to March 2013 (in the beginning of March and in the end of September) from the aerobic process tank, and kept on ice until the fixation for FISH analyses (Nielsen *et al.* 2009b).

FISH identification

FISH analyses were performed according to Nielsen *et al.* (2009a). The 6-Fam labelled EUBmix oligoprobe (equimolar mixture of EUB338, EUB338II and EUB338III) was used to target the entire bacterial community. Filamentous bacteria were identified with a wide selection of 11 oligoprobes: CFXmix (equimolar concentration of GNSB-941 and CFX1223 probes, targeting 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*’), G123 T (*Thiothrix eikelboomii*, *T. nivea*, *T. unzii*, *T. fructosivorans*, *T. defluvii*, Eikelboom type 021N group I, II, III), Myc657 (*Mycobacterium* subdivision *mycolata*), Spin1449 (*Skermania piniformis*), Gor596 (family *Gordonia*), HHY654 (*Haliscomenobacter hydrossis*), Curvi997 (types 1701 and 0041/0675 of *Curvibacter* spp.), TM7905 (type 0041/0675 in *Candidate* division TM7). The specific probes were labelled with Cy3. This selection of probes was based on the recently evaluated probes for filamentous bacteria detected in activated sludge plants (Nielsen *et al.* 2009b; Mielczarek *et al.* 2012). When applicable, a hierarchical approach had been used, i.e. the more general probe was applied first and then the more specific one (e.g. Myc657 followed by Spin1449 and Gor596). Detailed information about the probes used is given in probeBase (Loy *et al.* 2003).

Quantification procedures were performed similarly to Mielczarek *et al.* (2012). Twenty separate images for each probe were captured with a Nikon Eclipse 50i microscope.

Table 1 | Influent and operational parameters of the WWTPs tested in this survey

Parameter		WWTP I	WWTP II	WWTP III	WWTP IV	WWTP V
Size designed (PE)		55,400	83,000	163,500	53,040	22,500
Size actual (PE)		73,400	99,000	110,000	76,000	18,000
Reactor type A2O		A2O	AO	Anaerobic tank + anoxic/aerobic oxidation ditch (ORP controller)	UCT	A2O
Predenitrification		Yes	No	No	No	Yes
Presettling		Yes	Yes	Yes	Yes	No
Fermenter		Yes	No	No	No	No
Aeration		Fine bubble diffusers	Surface (vertical type)	Surface (horizontal rotors)	Fine bubble diffusers	Fine bubble diffusers
P-Precipitation		Occasionally (PIX)	Continuous (PIX)	Occasionally (PIX)	Occasionally (PIX)	No
PAX dosage		No	No	No	No	occasionally (winter)
Wastewater type (% of overall BOD ₅)		Domestic	Domestic industrial (20–25%): 'Slaughterhouse dairy'	Domestic industrial (5–10%) 'fruit & vegetables processing, breweries, landfill leachate'	Domestic industrial (30–50%) 'fruit & vegetables processing sugar refining'	Domestic
SVI (mL/g)	Summer	240 (184–260)	180 (168–198)	220 (185–240)	90 (80–108)	70 (66–81)
	Winter	320 (243–389)	180 (179–182)	190 (148–224)	140 (121–153)	160 (140–188)
COD (mg/L)		1,190 (622–3,650)	940 (303–5,880)	1,530 (457–4,000)	1,440 (508–3,480)	1,420 (228–4,730)
BOD ₅ (mg/L)		540 (220–1,300)	440 (178–2,770)	600 (330–1,320)	890 (360–1,900)	470 (71–920)
N total (mg/L)		82 (37–133)	82 (41–115)	105 (10–196)	81 (36–129)	84 (18–133)
P total (mg/L)		37 (10–124)	13 (7–32)	27 (4–69)	18 (6–64)	21 (5–63)
BOD ₅ /COD (mg/mg)		0.42 (0.24–0.8)	0.45 (0.31–0.60)	0.46 (0.12–0.95)	0.69 (0.36–0.96)	0.37 (0.04–0.99)
BOD ₅ /N _{tot} (mg/mg)		2.8 (1.3–6.3)	4.3 (2.4–10)	5.7 (1.6–46)	11 (2.5–66)	5.4 (0.7–20)
BOD ₅ /P _{tot} (mg/mg)		18 (5.7–36)	29 (12–63)	24 (6.8–82)	76 (8–295)	26 (4–115)
COD/N _{tot} (mg/mg)		6.8 (3.1–14)	10 (5.0–21)	14 (4–115)	15 (5–107)	17 (4–81)
COD/P _{tot} (mg/mg)		44 (16–121)	64 (23–158)	62 (8–375)	97 (13–555)	78 (12–328)
pH		7.8 (7.5–8.0)	7.5 (7.1–8.2)	7.6 (7.4–8.1)	7.9 (7.0–8.6)	7.5 (7.1–7.7)
MLSS (g/L)		3.8 (2.0–5.5)	4.8 (2.5–6.8)	5.9 (3.6–8.7)	6.2 (3.4–9.9)	4.0 (1.6–8.4)
SRT (d)		24 (12–57)	34 (13–94)	37 (10–60)	34 (11–93)	20 (4–82)
Sludge loading						
(gBOD ₅ /gMLSS/d)		0.03 (0.02–0.07)	0.06 (0.03–0.13)	0.03 (0.01–0.08)	0.06 (0.02–0.16)	0.06 (0.01–0.21)
(gCOD/gMLSS/d)		0.08 (0.04–0.20)	0.13 (0.05–0.97)	0.08 (0.02–0.22)	0.09 (0.02–0.32)	0.17 (0.01–0.72)

(continued)

Table 1 | continued

Parameter	WWTP I	WWTP II	WWTP III	WWTP IV	WWTP V
Comments	Severe sludge bulking poor N removal (winter)	Moderate sludge bulking sludge overloadings shock loadings unstable N removal	Severe sludge bulking landfill leachates and septic tanker trucks: shock loadings	Minor sludge bulking severe foaming (winter) industry activity: seasonal sludge overloadings shock loadings	Seasonal (winter) sludge bulking seasonal hydraulic overloading

Description: BOD – biological oxygen demand; COD – chemical oxygen demand; ORP – oxidation-reduction potential; PE – population equivalent; PAX – polyaluminium chloride-based coagulants; PIX – iron based coagulants; SVI – sludge volume index; MLSS – mixed liquor suspended solids; SRT – sludge retention time; UCT – University of Cape Town; process configurations A2O – anaerobic-anoxic-aerobic, AO – anaerobic-aerobic. Mean values and ranges (in parentheses) of influent parameters are shown.

ImageJ software (Collins 2007) was used to determine the biovolume of bacteria, which was relative to the pixel area of cells positive for the specific probe. The microbial abundance (expressed as % of EUBmix probe) was then quantified as a percentage of the pixel area for all bacteria positive for the EUBmix probe and was calculated as a mean of 20 separate measurements. The standard error of the mean was calculated as a standard deviation of the percentage abundance of the specific bacteria divided by a square root of 20 measurements.

Statistical measures and methods

To find the strength of relationship among quantified filamentous bacteria populations, correlation analyses (with Pearson product-moment correlation coefficient and Spearman's rank correlation coefficient) have been performed. Differentiating factors have been searched by analysis of variance (ANOVA) and multivariate analysis of variance (MANOVA) with significance level alpha 0.05. Standard statistical comparisons and graphing were performed in Microsoft Excel, correlation analyses were performed in STATISTICA™ from StatSoft®.

RESULTS

Identity and abundance of filamentous bacteria

Filamentous bacteria were abundant in all tested plants and constituted on average $28 \pm 3\%$ of all bacteria identified by EUBmix probe. The structure of filamentous bacteria community in the tested plants was similar to each other. Most often the filaments belonged to phylum *Chloroflexi* and *Microthrix* morphotype. The average abundance of phylum *Chloroflexi* in the tested WWTPs was 14% (expressed as a percent of EUBmix) and ranged between 3.4 and 3.5% (Figure 1). Phylum *Chloroflexi* was further investigated with more specific probes, T0803 and CHL1851, for abundance of types 0803 and 1851, respectively. Types 0803 and 1851 accounted for 14 and 1.4% of the whole population of *Chloroflexi*, respectively, whereas the rest of this phylum (about 85%) was not further identified (Figure 1).

In all of the tested WWTPs, bacteria classified to morphotype *Microthrix* were found. It was the most abundant genus among the determined filamentous bacteria and constituted on average more than 7% of the total bacterial population (varied from 1.8 to 15% among the WWTPs).

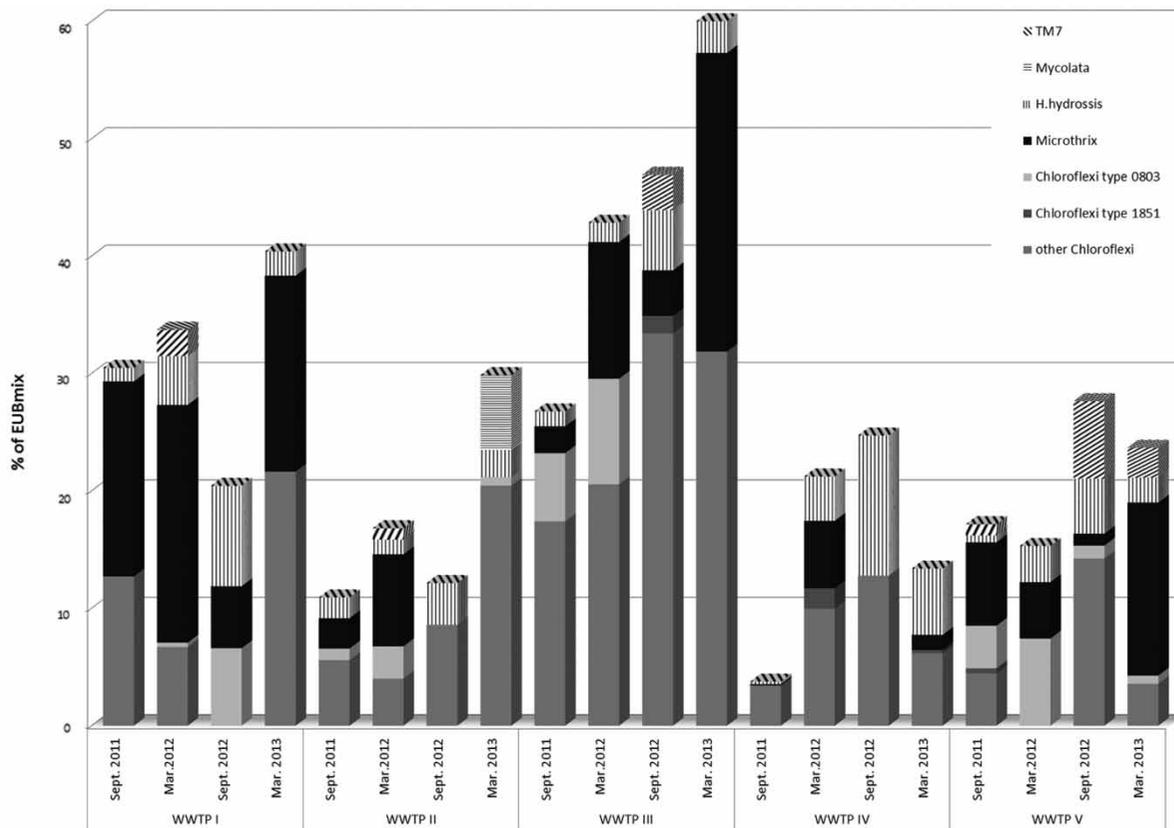


Figure 1 | The filamentous bacteria community in activated sludge of five Polish full-scale WWTPs in the period September 2011–March 2013. Abundance of individual populations was determined by quantitative fluorescence *in situ* hybridization with specific oligoprobes against the EUBmix probe. Bacteria belonging to *Skermania piniformis*, which accounted for a minor fraction, are not shown.

Another species with a significant contribution to overall filamentous community was *H. hydrossis* (average 3.6%, range 2.2–5.4%). When the number of *Microthrix* was dropping, the abundance of *H. hydrossis* increased from 0.6 to even 12%. In most sample types 0041/0675 of TM7 were also observed, with an average abundance of 0.8% (Figure 1). *Mycolata* and *Skermania piniformis* were detected only in WWTP II and III, respectively, where they constituted on average 1.6 and 1% of the bacterial community. *Curvibacter* (types 1701 and 0041/0675), *Thiothrix*/021N and family *Gordonia* have not been found in any of the tested samples.

Seasonal variations

Generally, the percentage of filamentous bacteria was higher after the winter season than after summer. Some small changes in the abundance of probe-defined populations in all five plants were observed during the survey (2011–2013). However, composition of the populations was rather stable in each plant despite the differences in

abundance of specific bacteria (Figure 1). For instance, in WWTPs II and III the relative abundance of *Chloroflexi* in the filamentous bacterial population was consistently high, whereas in WWTPs I and V it was low most of the time (Figure 1). Some seasonal variation in the abundance of *Microthrix* was observed and confirmed by ANOVA. *Microthrix* was more abundant during winter (in samples collected in March). No other statistically significant seasonal pattern had been discovered for the remaining probe-defined populations.

Differences among treatment plants and intercept effect of two differentiating factors

The only statistically significant influence of WWTP on bacterial populations had been found for *Chloroflexi*, which was the most abundant filamentous bacteria phylum. To find out whether or not each plant had a unique filamentous population, MANOVA was carried out on all samples for all seasons. The filamentous communities were considered as a multivariate response, with

treatment plant and season as explanatory variables. It proved that those two variables differentiated populations of *Microthrix* and *Chloroflexi*. The analysis showed also a tendency level for HHY654 ($p = 0.093$), which could be significant in further studies based on larger sample quantity.

Correlation analyses

Spearman's and Pearson's coefficients were tested to reveal mutual correlations between bacterial groups. No statistical dependence has been shown by Spearman's rank correlation coefficient. There were some weak correlations, but they were not statistically significant (Figure 3). One medium correlation ($r > 0.5$ with a significance level $\alpha = 0.011$) between *Microthrix* and *Skermania piniformis* (identified by probes MPAmix and Spin1449, respectively) was obtained by the Pearson's correlation coefficient analysis. Other correlations, despite being strong, did not achieve proper significance level $\alpha (>0.1)$. Strong linear correlation has been found between sludge volume index (SVI) and probe-define population of *Microthrix* ($r = 0.759$ with a significance level $\alpha = 0.011$). No strong relationships between the abundance of filamentous bacteria and biological oxygen demand (BOD), chemical oxygen demand (COD), total N or total P were found. Statistical analyses did not reveal meaningful correlations with other operational parameters (data not shown), similarly to the results obtained by Mielczarek *et al.* (2012) for Danish plants.

DISCUSSION

Bulking problems are reported worldwide and usually they are associated with extensive growth of filamentous microorganisms (Eikelboom 2000; van der Waarde *et al.* 2002; Jenkins *et al.* 2004; Mielczarek *et al.* 2012). This is the first long-term survey of the filamentous bacteria community in Polish municipal full-scale WWTPs, which was carried out with molecular identification by FISH. Recent studies have proved that the vast majority of filamentous bacteria in WWTPs could be identified by existing FISH probes (Kragelund *et al.* 2011; Mielczarek *et al.* 2012). This survey was carried out using a wide selection of 11 oligoprobes, which are widely applied for identification of those bacteria (Nielsen *et al.* 2009a, Kragelund *et al.* 2011; Mielczarek *et al.* 2012). In Polish WWTPs, filamentous communities

constituted on average $28 \pm 3\%$ of the entire biomass as analysed by EUBmix, which is similar to the results obtained by Nielsen *et al.* (2010) and Mielczarek *et al.* (2012) for Danish plants (28% and 24%, respectively).

We showed that the structure of filamentous community in Polish WWTPs is similar to those found in other European countries, as could be anticipated due to similar climate (Wanner *et al.* 2009). Most filamentous bacteria detected in Polish WWTPs belonged to phyla *Chloroflexi*, *Actinobacteria* (*Microthrix* and *Mycolata*), TM7 and *Bacteroidetes* (*H. hydrossis*). However, unlike the countries mentioned in the Introduction, *Microthrix parvicella* was more frequent (present in all tested plants), whereas *Thiothrix* and filamentous *Curvibacter* (both of phylum *Proteobacteria*) were not found. The most abundant, *Chloroflexi* and *Microthrix*, on average, accounted for 14% and 7% of all bacteria, respectively. Similar results were obtained by Nielsen *et al.* (2010) and Mielczarek *et al.* (2012) for Danish municipal WWTPs with nutrients removal; *Chloroflexi* and *Microthrix* constituted in Denmark about 10% and 5%–6% of biomass targeted by EUBmix probe, respectively. Those two bacteria are the most frequently reported filaments causing bulking in activated sludge systems treating primarily municipal wastewater (Wanner *et al.* 2009). In previous studies, filamentous *Chloroflexi* was often the most abundant phylum of the filamentous organisms and constituted 1–25% (Beer *et al.* 2006), 8% (Kong *et al.* 2007) or even up to 30% of the entire bacteria population in nutrients removal plants (Morgan-Sagastume *et al.* 2008; Nielsen *et al.* 2010; Kragelund *et al.* 2011). *Chloroflexi* and *Microthrix* constituted the great majority (64–81%) of filamentous bacteria investigated in this survey (Figure 2). These two groups of filaments utilize different substrates – the latter takes up lipids whereas the former utilizes proteins, polysaccharides and dead cell debris. They use different substrates – there is no competition for source of energy between them, because they occupy different niches and therefore may co-dominate in sludge (Nielsen *et al.* 2009a).

Bacteria belonging to phylum *Chloroflexi* dominated in almost all of the plants investigated in this study (except WWTP I). The impact of *Chloroflexi* filaments on sludge floc structure is species dependent. Some bacteria from this phylum serve as backbones to which other microorganisms can adhere and form strong and dense flocs. Other morphotypes are located at the edge of the sludge flocs and protrude out into the bulk liquid, which has a negative impact on sludge settling properties (Miura *et al.* 2007; Kragelund *et al.* 2007a; Speirs *et al.* 2009; Nielsen *et al.* 2009a).

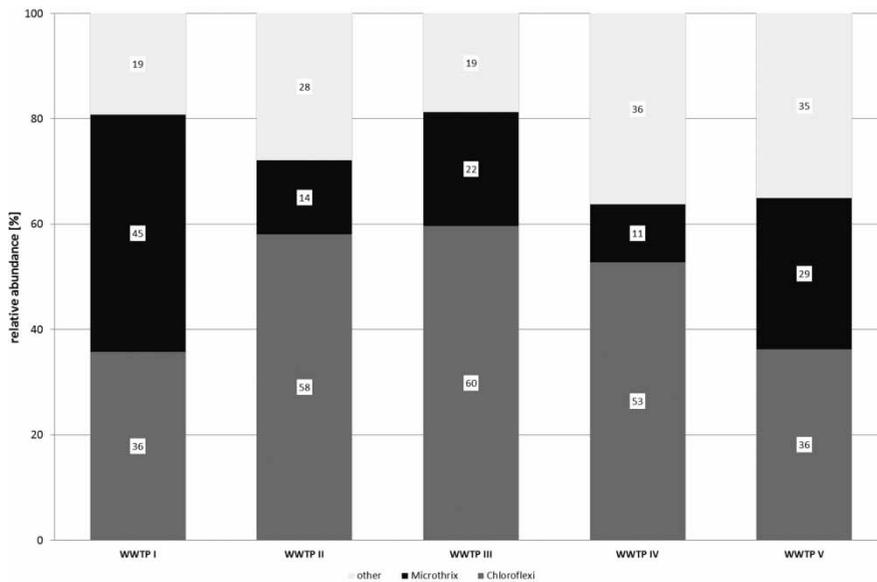


Figure 2 | Relative composition of filamentous bacterial population in the tested plants (average for 2011–2013).

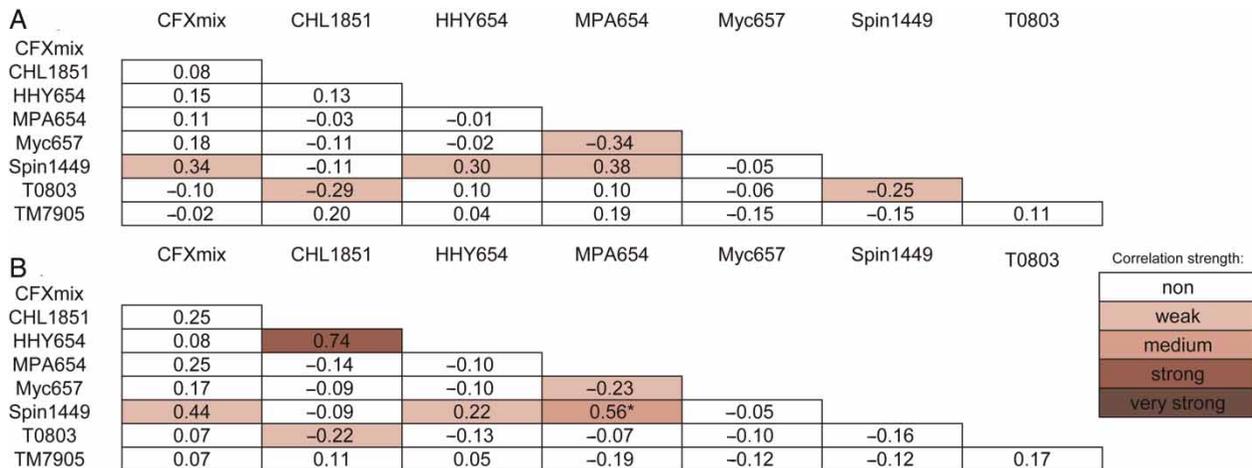


Figure 3 | Spearman's (A) and Pearson's (B) correlation analyses showing strength of mutual relations between probe-defined groups of filaments (2011–2013).

The broad phylum probe (CFXmix) covers several different morphotypes, which could be investigated with more specific probes e.g. type 0803 (genus *Caldilinea*, Kragelund *et al.* (2011)) and type 1851 (close relative of *Roseiflexus castenholzii*, in class *Chloroflexi*, Beer *et al.* (2002)), targeted by T0803 and CHL1851, respectively. Out of these two morphotypes, the more abundant one in Polish WWTPs is type 0803, which accounted for up to 9% of all bacteria (an average abundance of 2% in the tested WWTPs). Type 0803 is common in municipal WWTPs; its straight filaments are mainly inside the flocs, but sometimes it can occur in rosettes and cause bulking (Eikelboom 2000; Jenkins *et al.* 2004). Type 0803 may worsen the settling properties

especially during winter, when it proliferates and is responsible for creating open-floc structures (Kragelund *et al.* 2011). An average abundance of type 0803 in Danish full-scale municipal WWTPs is about 2.6–2.9% (Kragelund *et al.* 2011; Mielczarek *et al.* 2012), which corresponds well with the results obtained for Polish WWTPs (average 2% of EUBmix). Type 1851 is less abundant in both Polish (0–1.7%) and Danish WWTPs (average 0.5% – Kragelund *et al.* (2011)). Definitely, the more common population of phylum *Chloroflexi* is type 0092, which can reach up to 10% of all bacteria in sludge (average 3.6%) (Kragelund *et al.* 2011; Mielczarek *et al.* 2012). Interestingly, type 0092 does not respond to the EUBmix probes (Speirs *et al.*

2009; Nielsen *et al.* 2009b). Speirs *et al.* (2009) used probes CFX197 and CFX223, and showed that this morphotype is very common in both EBPR and non-EBPR full-scale plants in Australia. It should be stressed that for the time being about one-third of *Chloroflexi* cannot be identified beyond phylum level (Mielczarek *et al.* 2012). Unidentified population of *Chloroflexi* in our research was even higher (about 85%), but we did not use probes targeting type 0092, which can constitute above one-third of bacteria targeted by broad probe CFXmix (Mielczarek *et al.* 2012).

Microthrix can assimilate long-chain fatty acids under both aerobic and anaerobic conditions (Nielsen *et al.* 2009a); therefore it occurs exclusively in activated sludge plants with EBPR process (anaerobic-aerobic (AO) biomass cycling), where it is associated with both bulking and foaming incidents (Eikelboom 2000; Jenkins *et al.* 2004). *Microthrix* was the most abundant genus (above 7% of the total bacterial population) among the determined filamentous bacteria in this survey. However, in some EBPR WWTPs, which suffered from severe sludge bulking, abundance of these bacteria occasionally exceeded 20% of the entire biomass as analysed by EUBmix probe. It was correlated with SVI, which is easy to explain by the morphology and occurrence of *Candidatus* 'M. parvicella' and C. 'M. calida'; these characteristic coiled and twisted filaments are long, thin and usually present around flocs. Mielczarek *et al.* (2012) showed that *Microthrix* was present in all samples from 28 Danish WWTPs and its abundance varied substantially among the plants (0.3–16% of EUBmix), but it was the most abundant during winter and spring. Similar results, confirmed by ANOVA, were obtained in this study and in earlier observations (Levantesi *et al.* 2006; Nielsen *et al.* 2009a; Kragelund *et al.* 2011). *Microthrix* constituted 11–45% of all filaments in this survey, which was in some cases substantially more than in Danish WWTPs (11–22%, Mielczarek *et al.* 2012). The temporal changes in composition of probe-defined populations in the individual plants were minor and no statistically significant seasonal variations were noticed for other filaments, similarly to research carried out by Mielczarek *et al.* (2012). Another common probe-defined population in this study was *H. hydrossis*. These filaments accounted for 3.6% of the bacterial biovolume, which is higher than their abundance in Danish WWTPs (average 1.9%) revealed by Nielsen *et al.* (2010) and Mielczarek *et al.* (2012). We showed that when the number of *Microthrix* was dropping the abundance of *H. hydrossis* increased from 0.6% to even 12%.

Other filamentous bacteria investigated in this survey, which altogether accounted for 19–36% of filamentous community, seem to play a minor role in activated sludge. The

ranges of their abundance corresponded with the respective results obtained by Nielsen *et al.* (2010) and Mielczarek *et al.* (2012), with exceptions for TM7 and *Curvibacter*. TM7 were less abundant (<1%) than in Danish WWTPs (5%), whereas filamentous *Curvibacter*, constituting on average 0.7% of the bacterial biovolume as described by Mielczarek *et al.* (2012), were not detected in this study.

Microbial populations were analysed by a few statistical approaches to show possible rules governing them. Mutual correlations have been searched for, but no significant general correlations have been found between the presence of different filaments. Medium correlation strength, showed by Spearman's rank correlation coefficient, was recorded only between *Microthrix* and *Skermania piniformis*. Similar analysis, carried out by Mielczarek *et al.* (2012), showed no strong or even medium correlation between bacteria in Danish WWTPs. In our study, we found differences in filamentous populations among treatment plants shown by ANOVA, but only for *Chloroflexi*. We were not able to confirm that each treatment plant had unique population composition throughout the period of FISH analyses, as was shown by Mielczarek *et al.* (2012). The relative abundance of filamentous species in individual plants was rather similar. Small differences between plants were noticed, mostly in the case of *Chloroflexi* and *Microthrix* (shown by MANOVA). The conclusion that filamentous community is relatively constant and similar in different plants during long-term observation contradicts the assumption of Bellucci & Curtis (2011) about chaotic microbial composition of heterotrophic bacteria despite a constant function. However, studies of Bellucci & Curtis (2011) were carried out in laboratory-scale reactors and it may be misleading to extrapolate results from laboratory-scale reactors to full-scale plants. As was shown by Muszyński *et al.* (2012), some of the populations identified in laboratory-scale reactors are detected mainly in those conditions and seem to play a questionable role in full-scale EBPR systems. That is why application of knowledge about the structure and functions of activated sludge community, obtained from laboratory-scale studies, can be unreliable when applied to work out the bulking control methods in full-scale WWTPs.

This is the first molecular, long-term study on the presence of filamentous bacteria in full-scale WWTPs in Poland. Prior to this study filaments were identified only to morphotypes, using conventional light microscopy. In this paper, an attempt was made to find connection between abundance of filamentous bacteria with operational data by statistical method for not only linear, but also non-linear

correlations. The results of this study must be considered as reliable only on the assumption that FISH probes are sufficiently specific and target single (or closely related) species or ecotypes. Furthermore, FISH analysis does not provide enough resolution to observe fine-scale variability from plant to plant. Only methods with deep sequencing of full genomes or metagenomes can show microdiversity of bacterial groups over time or between the plants (Albertsen *et al.* 2011). It would also be interesting to compare the results obtained by FISH with information about microbial community structure collected with stable-isotope probing, which has been successfully used for *in situ* identification of denitrifying bacteria (Ginige *et al.* 2004), fatty acid-degrading anaerobic bacteria (Hatamoto *et al.* 2007) or fermenting bacteria (Nielsen *et al.* 2012).

CONCLUSIONS

1. Filamentous bacteria are abundant in activated sludge of Polish full-scale WWTPs and constitute on average 28% of all bacteria. The structure of filamentous community is similar to those found in other European countries.
2. The most abundant filamentous bacteria belong to phylum *Chloroflexi* and genus *Microthrix* (targeted by probes CFXmix and MPAmix, respectively).
3. The relative abundance of filamentous species in individual plants is rather similar. Small differences between plants were noticed, mostly in terms of *Chloroflexi* and *Microthrix* (shown by MANOVA).
4. No significant general correlations have been found between the presence of different filaments. Medium correlation strength was recorded only between *Microthrix* and *Skermania piniformis* (identified by probes MPAmix and Spin1449, respectively).
5. The strong linear correlation between *Microthrix* and SVI confirms deleterious effect of these bacteria on settling properties of sludge. It is associated with the characteristic morphology of these twisted filaments producing a diffuse open-floc structure and protruding from flocs.

ACKNOWLEDGEMENTS

The study was financially supported by the Polish National Science Centre (grant No. N N523 736540). We would like to thank Ewa Łukomska for technical help with preparation of activated sludge samples.

REFERENCES

- Albertsen, M., Hansen, L. B., Saunders, A. M., Nielsen, P. H. & Nielsen, K. L. 2011 A metagenome of a full-scale microbial community carrying out enhanced biological phosphorus removal. *ISME J.* **6**, 1094–1106.
- 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 Microbiol. Lett.* **207**, 179–183.
- Beer, M., Stratton, H. M., Griffiths, P. C. & Seviour, R. J. 2006 Which are the polyphosphate accumulating organisms in full-scale activated sludge enhanced biological phosphate removal systems in Australia? *J. Appl. Microbiol.* **100**, 233–243.
- Bellucci, M. & Curtis, T. P. 2011 Research on nitrification and related processes. *Meth. Enzymol.* **496** (B), 269–286.
- Carr, E. L., Eales, K. L. & Seviour, R. J. 2006 Substrate uptake by *Gordonia amarae* in activated sludge foams by FISH-MAR. *Water Sci. Technol.* **54** (1), 39–45.
- Chua, H. & Le, K. Y. 1994 A survey of filamentous foaming in activated sludge plants in Hong Kong. *Water Sci. Technol.* **30** (11), 251–254.
- Collins, T. J. 2007 ImageJ for microscopy. *Biotechniques* **43**, 25–30.
- Eales, K., Nielsen, J. L., Kragelund, C., Seviour, R. & Nielsen, P. H. 2005 The *in situ* physiology of pine tree like organisms (PTLO) in activated sludge foams. *Acta Hydrochim. Et Hydrobiol.* **33** (3), 203–209.
- Eales, K. L., Nielsen, J. L., Seviour, E. M., Nielsen, P. H. & Seviour, R. J. 2006 The *in situ* physiology of *Skermania piniformis* in foams in Australian activated sludge plants. *Environ. Microbiol.* **8** (10), 1712–1720.
- Eikelboom, D. H. 2000 *Process Control of Activated Sludge Plants by Microscopic Investigation*. IWA Publishing, London.
- Ginige, M. P., Hugenholtz, P., Daims, H., Wagner, M., Keller, J. & Blackall, L. L. 2004 Use of stable-isotope probing, full-cycle rRNA analysis, and fluorescence *in situ* hybridization-microautoradiography to study a methanol-fed denitrifying microbial community. *Appl. Environ. Microbiol.* **70**, 588–596.
- Hatamoto, M., Imachi, H., Yashiro, Y., Ohashi, A. & Harada, H. 2007 Diversity of anaerobic microorganisms involved in long-chain fatty acid degradation in methanogenic sludges as revealed by RNA-based stable isotope probing. *Appl. Environ. Microbiol.* **73**, 4119–4127.
- Jenkins, D., Richard, M. & Daigger, G. 2004 *Manual on the Causes and Control of Activated Sludge Bulking, Foaming and Other Solids Separation Problems*. IWA Publishing, CRC Press, London.
- Kong, Y., Xia, Y., Nielsen, J. L. & Nielsen, P. H. 2007 Structure and function of the microbial community in a full-scale enhanced biological phosphorus removal plant. *Microbiology* **153**, 4061–4073.
- Kragelund, C., Nielsen, J. L., Thomsen, T. R. & Nielsen, P. H. 2005 Ecophysiology of the filamentous *Alphaproteobacterium Meganema perideroedes* in activated sludge. *FEMS Microbiol. Ecol.* **54** (1), 111–122.

- Kragelund, C., Kong, Y., van der Waarde, J., Thelen, K., Eikelboom, D., Tandoi, V., Thomsen, T. R. & Nielsen, P. H. 2006 Ecophysiology of different filamentous Alphaproteobacteria in industrial wastewater treatment plants. *Microbiology* **152** (10), 3003–3012.
- Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., Kong, Y., van der Waarde, J., Krooneman, J., Rossetti, S., Thomsen, T. R. & Nielsen, P. H. 2007a Identity, abundance and ecophysiology of filamentous Chloroflexi species present in activated sludge treatment plants. *FEMS Microbiol. Ecol.* **59** (3), 671–682.
- Kragelund, C., Remesova, Z., Nielsen, J. L., Thomsen, T. R., Eales, K., Seviour, R., Wanner, J. & Nielsen, P. H. 2007b Ecophysiology of mycolic acid-containing Actinobacteria (*Mycolata*) in activated sludge foams. *FEMS Microbiol. Ecol.* **61** (1), 174–184.
- Kragelund, C., Thomsen, T. R., Mielczarek, A. T. & Nielsen, P. H. 2011 Eikelboom's morphotype 0803 in activated sludge belongs to the genus *Caldilinea* in the phylum *Chloroflexi*. *FEMS Microbiol. Ecol.* **76** (3), 451–462.
- Levantesi, C., Rossetti, S., Beimfohr, C., Thelen, K., Krooneman, J., van der Waarde, J. & Tandoi, V. 2006 Description of filamentous bacteria present in industrial activated sludge WWTPs by conventional and molecular methods. *Water Sci. Technol.* **54**, 129–137.
- Loy, A., Horn, M. & Wagner, M. 2003 Probebase: an online resource for rRNA-targeted oligonucleotide probes. *Nucleic Acids Res.* **31**, 514–516.
- Mielczarek, A. T., Kragelund, C., Eriksen, P. S. & Nielsen, P. H. 2012 Population dynamics of filamentous bacteria in Danish wastewater treatment plants with nutrient removal. *Water Res.* **46** (12), 3781–3795.
- Miura, Y., Watanabe, Y. & Okabe, S. 2007 Significance of *Chloroflexi* in performance of submerged membrane bioreactors (MBR) treating municipal wastewater. *Environ. Sci. Technol.* **41**, 7787–7794.
- Morgan-Sagastume, F., Larsen, P., Nielsen, J. L. & Nielsen, P. H. 2008 Characterization of the loosely attached fraction of activated sludge bacteria. *Water Res.* **42**, 843–854.
- Muszyński, A., Lebkowska, M., Tabernacka, A. & Miłobędzka, A. 2013 From macro to lab-scale: changes in bacterial community led to deterioration of EBPR in lab reactor. *Cent. European J. Biol.* **8** (2), 130–142.
- Nielsen, P. H., Kragelund, C., Seviour, R. J. & Nielsen, J. L. 2009a Identity and ecophysiology of filamentous bacteria in activated sludge. *FEMS Microbiol. Rev.* **33**, 969–998.
- Nielsen, J. L., Nguyen, H., Meyer, R. L. & Nielsen, P. H. 2012 Identification of glucose-fermenting bacteria in a full-scale enhanced biological phosphorus removal plant by stable isotope probing. *Microbiology (Reading, England)* **158** (Pt 7), 1818–1825.
- Nielsen, P. H., Daims, H. & Lemmer, H. (Eds.) 2009b *FISH Handbook for Biological Wastewater Treatment*. IWA Publishing, London.
- Nielsen, P. H., Mielczarek, A. T., Kragelund, C., Nielsen, J. L., Saunders, A. M., Kong, Y., Hansen, A. A. & Vollertsen, J. 2010 A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. *Water Res.* **44** (17), 5070–5088.
- Speirs, L., Nittami, T., McIlroy, S., Schroeder, S. & Seviour, R. J. 2009 Filamentous bacterium Eikelboom type 0092 in activated sludge plants in Australia is a member of the phylum *Chloroflexi*. *Appl. Environ. Microbiol.* **75**, 2446–2452.
- van der Waarde, J., Krooneman, J., Geurkink, B., van der Werf, A., Eikelboom, D., Beimfohr, C., Snaidr, J., Levantesi, C. & Tandoi, V. 2002 Molecular monitoring of bulking sludge in industrial wastewater treatment plants. *Water Sci. Technol.* **46** (1–2), 551–558.
- Wanner, J., Kragelund, C. & Nielsen, P. H. 2009 Microbiology of bulking. In: *Microbial Ecology of Activated Sludge* (R. J. Seviour & P. H. Nielsen eds). IWA Publishing, London, pp. 191–214.

First received 21 May 2014; accepted in revised form 3 December 2014. Available online 23 December 2014