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Pseudonocardia filamentous bulking sludge in an industrial wastewater treatment plant as revealed by Illumina amplicon sequencing

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Abstract

In this study, 16S rRNA gene amplicon sequencing was performed to identify bulking filamentous bacteria in an industrial wastewater treatment plant, that threats effluents of bioethanol production process from cereal cooking. The presence of Pseudonocardia sp. was confirmed by comparing the 16SrRNA of the most abundantly amplified sequence (OTU; 12.35%) with corresponding nucleotides present in two genomic databases. The Pseudonocardia species identified was closely related to Pseudonocardia spinosa. Over 50 different types of filamentous microorganisms have been found to cause problems with bulking and foaming but *Pseudonocardia* has, until now, not been described to be among them. In addition, the 16S rRNA dataset was analyzed to reveal bacterial community composition during sludge bulking.. *Candidatus* Competibacter was identified as the second most abundant sequence (OUT, 10.04%). Comparative data from samples obtained before and after appearance of *Pseudonocardia* suggests, that a decrease in nutrients could be one of the main factors affecting sludge bulking generated by this species. The outcomes of this study are expected to provide an important insight into the role of *Pseudonocardia* in bulking in industrial wastewater treatment plants.

Keywords: A2/O process, Fluorescence in situ hybridization, Illumina sequencing, Nocardioform bacteria

Introduction

Excessive growth of filamentous bacteria, bulking and foaming, is a a primary concern in aerobic wastewater treatment plants (WWTPs), since they produce poor sludge settleability, resulting ineffluents of low quality and increased costs due to loss of suspended solids (van der Waarde et al. 2002; Martins et al. 2014). More than 30 different filamentous bacteria morphotypes have been found in activated sludge systems of WWTPs receiving domestic wastewater and additional 40 morphotypes have been found in industrial WWTPs (Eikelboom 2006; Guo and Zhang 2012).

Bulking filamentous bacteria are phylogenetically very diverse.. Members of Actinobacteria such as *Candidatus* Microthrix parvicella and *Tetrasphaera* were often found to be the causative organisms of sludge bulking, in treatment plant surveys conducted around the world (Martins et al. 2004; Seviour et al. 2008). In addition to *Actinobacteria*, members of *Proteobacteria* such as *Meganema perideroedes*, *Thiothrix eikelboomii* and type 021N bacteria (Vervaeren et al. 2005; Thomsen et al. 2006; Nielsen et al. 2009) were involved in bulking episodes. Other filamentous members in the phylum *Chloroflexi*, such as *Candidatus* Amarolinea and *Kouleothrix,* have occasionally been associated with bulking incidences (Nittami et al. 2019; Nierychlo et al. 2019). Moreover, the filamentous species *Haliscomenobacter hydrossis* (Bacteroidetes) and *Trichococcus* (*Nostocoida limico*la I) (*Firmicutes*) may also impact biomass settling properties (Kragelund et al. 2008). In fact, different types of filamentous bacteria may exist in WWTPs with or without obvious sludge bulking properties. However, *Pseudonocardia* sp. (non-mycolata branching *Actinobacteria*) has not considered among the different bulking and foaming filamentous bacteria described above. Nocardioform actinomycetes are comprised of different subgroups such as

mycolic acid-containing (mycolata) foaming bacteria, *Pseudonocardia* sp. and related genera.

The identification of bulking filamentous bacteria has relied heavily on classification keys that are based on their morphological characteristics and specific staining reactions (Eikelboom 2000; Jenkins et al. 2004). However, the identification with these methods is limited because a filamentous morphotype can correspond to different taxonomic phyla. For this reason, it has been recommended that fluorescence in situ hybridization (FISH) or other molecular technique, be utlized (Mielczarek et al. 2012). In recent years, next generation sequencing (NGS) has become a popular method because it specifically can provide detailed information on microbial community composition (Ye and Zhang 2013). Few studies using NGS have been performed to investigate the changes in bacterial and filamentous bacterial communities in activated sludge systems treating industrial wastewater (Guo and Zhang 2012; Dunkel et al. 2018). Reliable identification of bulking bacteria represents the first step in developing effective and specific control strategies to help mitigate disturbances in activated sludge systems (Dunkel et al. 2018).

In the present study, NGS and FISH techniques, were used in conjunction to detect and identify *Pseudonocardia* as a potential bulking branched filamentous *Actinobacteria* in an industrial WWTP. The overall goal of this study is to provide valuable information on bulking in full-scale industrial WWTP that treat effluents of bioethanol production process from cereal cooking.

We selected an industrial WWTP located in Galicia (Spain) as case study of *Pseudonocardia* sp. bulking. Two activated sludge samples from an aerated biological reactor, were collected in February 2015 and February 2016.

Materials and methods

Wastewater Treatment PlantP description and sample collection

The investigated WWTP is a full-scale plant located in Galicia (Spain) which consists of a conventional anaerobic/anoxic/aerobic (A2/O) system. This industrial WWTP treats 400 m^3 d^{-1} of effluents from the production of bioethanol from cereal cooking and boiler cleaning. The operational conditions of the WWTP are shown in Table 1. The operating parameters of the WWTP were a very low organic loading rate (F/M ratio) and a high mixed liquor suspended solids concentration (MLSS). These operational conditions were applied to avoid possible toxic effects that can easily appear in bioethanol production residues,

Two activated sludge samples were collected from the aeration tank at different times: February 2015 (S1), and February 2016 (S2). The samples were kept in a portable icebox while being transported to the laboratory. A number of parameters such as total suspended solids (TSS), total nitrogen (TN), total phosphorus (TP), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), sludge volume index (SVI) and V30 were measured according to Standard Methods (APHA 2005).

Identification by morphological observation and staining

The samples S1 (without bulking) and S2 (with bulking), which came from mixed liquor from the aeration tank, were used for microscopic observation using a Nikon E200 microscope. Filamentous bacteria were identified by phase contrast microscopy, based on their morphological features and several staining techniques (Gram stain and

Neisser stain) (Eikelboom 2000; Jenkins et al. 2004).

Filamentous bacteria were quantified using the subjective scale of abundance (FI, filament index) proposed by Jenkins et al. (2004). The FI ranges from 0 (no filaments) to 6 (excessive filaments). The filaments (m mL^{-1}) were also counted following the total filament length (TFL) method of Salvadó (2016). *Pseudonocardia* filamentous branching from sample S2 was also examined using a JEOL JSM5410 (JEOL Ltd., Tokyo, Japan) scanning electron microscope operating at 20 kv as previously described (Alonso et al. 2009). Sample S2 was also examined using an Olympus BX50 microscope equipped with Nomarski interference contrast (DIC).

Determination of filamentous bacteria using FISH

The list of oligonucleotide probes applied and respective formamide (FA) concentrations are shown in Supplementary Table S1. For Gram-positive cells (*Microthrix* and filamentous branched filaments), a pre-treatment with mutanolysin was applied. Following dehydration in 50%, 80% and absolute ethanol for 3 min each, 10 µl of mutanolysin (5000 U mL^{-1}) (Sigma, St. Louis, MO, USA) were applied to each well and incubated at 37º C for 20 min in a humid chamber as described in Schuppler et al. (1998). Subsequently, another dehydration series with ethanol was applied. All hybridizations were carried out at 46 ºC in a humid chamber for 2 hours. Following hybridization, excess probes were washed off with standard wash buffer at 48 ºC for 15 min (Manz et al. 1992). Hybridized samples were examined using an epifluorescence microscope (Olympus BX50).

DNA extraction and PCR-based Illumina sequencing

DNA from AS S2 sample was extracted in duplicates, as previously described (Luján-

Facundo et al. 2018), using a commercial kit (FastDNA® SPIN kit for soil, MP Biomedicals, OH, USA). DNA concentration was measured using Qubit® dsDNA BR Assay Kit (Molecular probes, Eugene, OR, USA) and DNA 260/280 ratio was measured using the NanoDrop ND-1000 UV/Vis spectrophotometer (NanoDrop Technologies, DE, USA). The hypervariable V3–V4 regions of bacterial 16S rRNA gene were amplified by Fundación FISABIO sequencing service (Valencia, Spain) using the primers PRO341F and PRO805R (Takahashi et al. 2014). The subsequent amplicon sequencing on the Illumina Miseq platform was also performed by Fundación FISABIO sequencing service (Valencia, Spain) using a 2 × 300 nucleotide paired-end reads protocol.

Bioinformatics analysis of Illumina-generated amplicons

Raw Illumina sequences were analysed using Quantitative Insights Into Microbial Ecology (QIIME™) software package version 1.8.0 (Caporaso et al. 2010). Forward and reverse reads were joined and then checked for chimeras using usearch61 algorithm (Edgar 2010) against SILVA v123 ribosomal database (Quast et al. 2013). The remaining sequences were clustered at 97% similarity into OTUs (Operational Taxonomic Units) using the *denovo* OTU clustering script. The most abundant sequence of each OTU was picked as its representative, and was used for taxonomic assignment against SILVA 123 ribosomal database (Quast et al. 2013) at 97% identity (3% cut-off level) using default parameters.

Sequences of the most abundant OTUs (>0.5%) were also classified at genus level against EzBiocloud 1.5 database (Yoon et al. 2017). Park et al (2012) proposed that NGS results should be analysed by at least two databases.

Phylogenetic analyses

The 16S rDNA OTU1 nucleotide sequence (GBS16) has been deposited in GenBank under accession no. KY213842. The GBS16 sequence was aligned manually against sequences of reference strains of the genus *Pseudonocardia*. A phylogenetic tree was inferred using the neighbour-joining tree-making algorithm from the TREECON software suite programs (Van de Peer and de Watcher 1994) and evolutionary distances matrixes were generated by the neighbour-joining method (Saitou and Nei 1987). The topologies of the phylogenetic tree were evaluated in a bootstrap analysis based on 1.000 replicates (Felsenstein 1993).

Results and discussion

Morphological identification of filamentous bacteria.

The identification of the dominating filamentous species/types was performed with traditional methods based on their morphological characteristics and staining properties. The filamentous bacteria identified in sample S1 were *Haliscomenobacte*r (FI 5), type 0803 (FI 3) and *Microthrix* (FI 2). The dominant filamentous bacteria (FI 6) in S2 formed an extensively branched structure (Fig. 1a) and were Gram positive (Fig. 1b), and Neisser negative. Gram positive filaments aregenerally poorly represented in industrial WWTPs in contrast to their frequent dominance in domestic WWTPs (Wanner et al. 2010). Other filamentous bacteria found in sample S2 were *Haliscomenobacter hydrossis* (FI 5), type 1702 (FI 2) and type 021N (FI 1).

Identification and abundance of filamentous bacteria with FISH

The identity of the filamentous bacteria in sample S2 was further analyzed by FISH using a selection of probes. Using probe HGC1156 targeting *Actinobacteria* (Fig. 1c)

and probe Pse631 mix targeting *Pseudonocardia* (Fig. 1d), a fluorescent signal of branched filamentous bacteria was detected in sample S2 (FI 6). Other types of filamentous bacteria found in sample S2 by FISH were *Haliscomenobacter*-like filaments (probe SAP309) (*Saprospiraceae*) (FI 5) (Fig. S1a and S1b), *Microthrix* (FI 1) (Fig S1c) and type 0803 (*Calidilinea*, phylum *Chloroflexi*) (FI 3) (Fig. S1e and S1f). The identity of the most abundant filamentous *Bacteroidetes* with *Haliscomenobacter* morphology could be detected with probe SAP309. *Haliscomenobacter* abundance data (FI 5) were similar to morphological identification and FISH techniques. *Haliscomenobacter* has been detected worldwide in activated sludge samples because of its easily recognizable morphological appearance as a rigid straight filament and is rarely considered responsible for bulking (Kragelund et al. 2008). Filamentous members of the *Chloroflexi* phylum are frequently observed in activated sludge and contribute to the overall filament index number (Kragelund et al. 2006).

Identification of branching filamentous bulking bacteria by NGS

Comparison of the 16S rRNA nucleotide sequence from OTU1, the most abundant retrieved sequence, with corresponding nucleotides sequences of representatives of the genus *Pseudonocardia* confirmed that OTU1 (GBS16, Genbank accession no. KY213842) belongs to the genus *Pseudonocardia* and it is closely related to *Pseudonocardia spinosa* (Fig. 2). The genus currently encompasses 61 recognized species (http://www.bacterio.net/pseudonocardia.html). Single strains representing new *Pseudonocardia* species have been previously isolated from contaminated industrial sludge (Mahendra and Alvarez-Cohen 2005; Cuesta et al. 2013).

Filamentous bacterial abundance could be determined by the total filament length

(TFL) or by subjective scorings of filament levels (FI) and the sludge volume index (SVI) (Nittami et al. 2017). When there is an increase in the number of filamentous organisms, the SVI increases proportionally to TFL (Salvadó 2016). The comparative analytical data before and after the appearance of bulking are shown in Table 1. The sludge settleability was categorized as "no bulking" in sample S1. However, sample S2 suffered from bulking (SVI 176 mL g^{-1}), using as reference value SVI > 150 mL g^{-1} (Jenkins et al. 1993). The *Pseudonocardia* filaments increased from 69.3 (sample S1) to 356 m mL^{-1} (sample S2) interfering with the compaction of the activated sludge and therefore increasing the SVI. Important characteristics of *Pseudonocardia* are its mycelial shape and its cell size. Increasing its concentration, the species can drive the decrease in the settleability of the sludge. In this study, *Pseudonocardia* bulking was found to interfere with the compaction of the activated sludge and affected the activated sludge solids separation in the secondary clarifier.

Factors such as water temperature, dissolved oxygen, sludge retention time, pH, influent quality, sludge loading rate and nutrients ratio are responsible for filamentous sludge bulking (Zhang et al. 2019). The most important differences between samples S1 and S2 were a significant decrease in nutrients (P and N) when *Pseudonocardia* sp. filaments were dominant (sample S2). Thus, nutritional deficiency could be one of the main factors affecting sludge bulking generated by *Pseudonocardia* sp., as suggested by Szeinbaum and Erijman (2007).

Bulking bacteria community composition

A total of 76,594 sequences were generated after amplicon sequencing in Illumina MiSeq for the sample S2 (A2O system) (February 2016), with75,604 sequences

233 remaining after chimeric sequences were removed. The total numbers of OTUs were
234 1,178, which were classified into different bacterial and archaeal taxonomic levels
235 (from phylum to species). In our study 1.17% of OTUs were not assigned to any
236 taxonomic level when clustering against Silva v123 database (Table 2). Bulking
237 bacteria profile described at a similarity cutoff of 97% at phylum level is shown in
238 Table 2. There were 26 phyla assigned against Silva v123 (Table 2). The phyla
239 Proteobacteria, Actinobacteria and *Bacteroidetes* were the most abundant, thus
240 representing 75.01% of the total population. *Proteobacteria* was the predominant
241 phylum in sample S2, accounting for 39.88% of total effective bacterial sequences in
242 the present study. In municipal WWTPs, the phylum *Proteobacteria* has been found to
243 be dominant (Zhang et al. 2012; Hu et al. 2012). The other dominant phyla in sample S2
244 were Actinobacteria (18.72 %) and Bacteroidetes (16.41%). The phyla *Chloroflexi*
245 (7.46%), *Planctomycetes* (5.58%), *Cyanobacteria* (3.95%), *Verrumicrobia* (3.08%) and
246 *Acidobacteria* (1.02%) were also abundant. *Bacteroidetes* were found to be important in
247 degradation of particulate organic matter (Capello et al. 2016). Wang et al. (2016) found
248 that *Actinobacteria* was dominant in a municipal WWTP with a conventional A2/O
249 system where excessive sludge bulking occurred. *Chloroflexi* is a normal member of
250 the activated sludge microbial community (Kragelund et al. 2006). *Cyanobacteria* in
251 biological systems may produce toxic or antibacterial compounds that may affect
252 bacterial communities and thus the efficiency of the treatment (Martins et al., 2011).
253 The remaining phyla showed abundances of <1%.

254 Among *Proteobacteria*, *Gammaproteobacteria* accounted for 18.90% of total
255 effective sequences. *Alphaproteobacteria* was also dominant among *Proteobacteria*
256 (14.76%) (Fig. 3). The class *Betaproteobacteria* had a lower abundance in sample S2
257 (5.05%). In municipal WTP, Betaproteobacteria is the most abundant class, largely

responsible for organic and nutrient removal (Cydzik-Kwiatkowska and Zielinska 2016). The classes *Actinobacteria* (16,91%), *Sphingobacteria* (11,53%) and *Caldilineae* (5,81%) were also abundant. These classes are considered key bacterial groups highly represented in activated sludge samples of domestic and industrial WWTPs (Ju and Zhang 2015).

In sample S2, 190 genera with a relative abundance higher than 0.001% were identified (Table 2). The higher number of genus-level OTUs corresponded to the phylum *Proteobacteria* (95 genera), followed by the phyla *Bacteroidetes* (22 genera) and *Firmicutes* (22 genera). In a recent Illumina MiSeq-based study of 13 municipal WWTP across Denmark, it was shown that the plants contained a core community of 63 abundant genus-level OTUs (Saunders et al. 2016). According to Zhou et al. (2010), influent composition, operational parameters and environmental conditions influenced the microbial community structure in WWTP.

OTUs 1 to 23 represented 79.00% of the obtained sequences. These OTUs were also identified at genus level according to the online EzBioCloud 1.5 database (Table 3). The OTUs (abundance >0.5%) identified at genus level in sample S2 were: OTU1, OTU2, OTU3, OTU5, OTU9, OTU10, OTU11, OTU15, OTU21, OTU22 and OTU23 (Table 3). These OTUs represented 51.74% of the obtained sequences, more detailed information can be found in Table 3. The most abundant phylotype (OTU1) was *Pseudonocardia,* representing 12.35% of the total population (Table 2). *Pseudonocardia* sp. strains which are widely distributed are nocardioform actinomycetes that are abundant in many environments, such as activated sludge, soils, plant tissues and marine sediments, and are known to degrade a wide range of pollutants (Vainberg et al. 2006; Zhang et al. 2014). Significant contribution to the overall

filamentous bacteria community in sludge was made by OTU3 (*Saprospiraceae*, 9.28%). Needle-like thin filaments identified with FISH (probe SAP309,*Saprospiraceae*) were also ranked as abundant (FI 5). Zhang et al. (2019) found that the relative abundance of *Saprospiraceae* was the highest in oxidation ditcheswith long sludge retention time. The family *Saprospiraceae* includes the genera *Phaeodactylibacter, Saprospira, Haliscomenobacter, Lewinella, Portibacter, Aureispira* and *Rubidimonas* (Chen et al., 2014). Members of this family are considered to be important members of the bacterial community involved in ecophysiological activities in a variety of natural environments (Xia et al., 2008). Chloroflexi filamentous bacteria detected at genus level were *Litorilinea* (0.28%) (*Anaerolineaceae*) and *Ornatolinea* (0.003%) (*Caldilineacaeae*). The family *Caldilineaeceae* in sample S2 was represented by 24 OTUs (abundance 5.81%). Type 0803 and genus *Caldilinea* (class *Caldilineae*) are represented by many sequences in different activated sludge clone libraries (Bjornsson et al., 2002). *Chloroflexi* filaments identified with the CFXmix probe were ranked as abundant (FI 4) with FISH. Filamentous members of the *Chloroflexi* phylum are frequently observed in activated sludge and contribute to the overall filament index number (Kragelund et al. 2006). Relative abundances (< 0.5%) of other filamentous bacteria were: *Candidatus* Microthrix (0.18%), *Candidatus* Alysiosphaera (0.12%) (*Nostocoida limicola* II), *Leptothrix* (0.001%), Type 1863 referred to *Chryseobacterium* (0.001%) and *Acinetobacter* (0.005%). Guo and Zhang (2012) found bulking filamentous bacteria ranging from 1.86% to 8.99% in sludge samples from 14 WWTPs. In many industrial sludge samples, large populations of 2-4 filamentous species were simultaneously present (Eikelboom and Geurkink 2002).

Potentially functional groups of bacteria were also identified. Heterotrophic glycogen accumulating organisms (GAO) accounting for 10.94% were identified as

Candidatus Competibacter (OTU2). The microbial community structure of bulking sludge showed low relative abundance of nitrifiers with more abundance of denitrifiers. Denitrifiers were represented by OTU11 (2.74%), OTU3 (1.43%) and OTU15 (1.20%) (*Paracoccus*), and OTU22 (*Hyphomicrobium zarvazinii*) (0.68%). Some denitrifiers were assigned to different genera based on two databases used. OTU3 (5.12%) was assigned to *Nitratireductor* with Silva v123 and *Mesorhizobium* with EzBioCloud 1.5. The average relative abundance of nitrifying bacteria was 0.01% for ammonia-oxidizing bacteria (*Nitrosomonas* and *Nitrosococcus*) and 0.004% for nitrite-oxidizing bacteria (*Nitrolancea*). The lower relative abundance of nitrifiers in the bioethanol WWTP indicates higher sensitivity to toxic compounds from the bioethanol production process than to denitrifiers. Nitrifying bacteria are well known for their slow growth rate and high sensitivity to inhibitory compounds and environmental factors.

Conclusion

This study provides an important insight into the role of *Pseudonocardia* in bulking of industrial WWTPs. Fluorescent in situ hybridization and 16S rRNA amplicon sequencing results have demonstrated that sludge bulking is produced by *Pseudonocardia* filaments. The added value of NGS was in the accurate identification of filaments and abundance detection, thus allowing the assessment of the relationship between filament growth, chemical parameters and process condition at the community scale.

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332

333 **Conflict of interest**

334 The authors declare that they have no conflicts of interest.

335

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505 **Table 1.** Comparative analytical results obtained at the study before

506 and after the appearance of *Pseudonocardia* bulking

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Parameter[a]	Aeration tank	
	Sample S1 Without bulking	Sample S2 With bulking
TN (mg L^{-1})	10.35	4.32
TP (mg L^{-1})	4.59	0.38
MLSS (mg L^{-1})	6757	5243
F/M ratio (kg BOD_5 kg $MLVSS^{-1}$ d^{-1})	0.049	0.047
MLVSS (mg L^{-1})	5879	5033
V30	743	923
SVI (mL g^{-1})	110	176
Filamentous count (m mL^{-1})	69.3	356

508 [a]TN; total nitrogen; TP, total phosphorus; MLSS, mixed liquor suspended solids; F/M ratio, food

509 to microorganism ratio; MLVSS, mixed liquor volatile suspended solids; V30, volume of the

510 settled sludge after30 minutes; SVI, Sludge Volume Index.

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516 **Table 2** Phyla and genera assigned against Silva 123 database

Phylum	Abundance relative %	Number of Genera	Most abundant Genus	Abundance relative %
Proteobacteria	39.875	95	*Candidatus Competibacter*	10.984
Actinobacteria	18.724	15	*Pseudonocardia*	12.411
Bacteroidetes	16.413	22	*Phaeodactylibacter*	9.455
Chloroflexi	7.455	5	*Litorilinea*	0.280
Planctomycetes	5.580	4	*Planctomyces*	0.192
Cyanobacteria	3.947	NA[a]		
Verrucomicrobia	3.082	4	*Opitutus*	0.784
Acidobacteria	1.020	5	*Candidatus* Solibacter	0.431
Gemmatimonadetes	0.770	NA		
Firmicutes	0.667	22	*Acetobacterium*	0.161
*Latescibacteria*_WS3	0.299	NA		
Spirochaetae	0.245	5	*Spirochaeta*	0.201
*Hydrogenedentes*_NKB19	0.243	NA		
Chlorobi	0.152	1	*Ignavibacterium*	0.001
Deinococcus-Thermus	0.118	1	*Truepera*	0.118
Gracilibacteria	0.071	NA		
*Saccharibacteria*_TM7	0.054	NA		
*Parcubacteria*_OD1	0.026	NA		
TM6	0.017	NA		
Synergistetes	0.015	1	*Thermovirga*	0.001
Chlamydiae	0.011	2	*Candidatus* Protochlamydia	0.005
Tenericutes	0.009	1	*Acholeplasma*	0.007
*Microgenomates*_OP11	0.001	NA		
Fibrobacteres	0.001	NA		
*Omnitrophica*_OP3	0.001	NA		
Euryarchaeota	0.036	7	*Methanobrevibacter*	0.012

Unassigned; Other	1.168

[a]NA: Not assigned to genus level

Table 3 Closest similarity to the genera level (bold letter) against EzBioCloud 1.5 and Silva 123 databases. OTUs 1 to 23 (abundance >0.5%) represented 76.79% of obtained sequences

OTU number[a]	Size (bp)	Silva[b] Relative abundance %	EzBioCloud 1.5 closest similarity[c]	Silva123 database closest similarity
1	446	**12.35**	***Pseudonocardia***[d]	***Pseudonocardia***
2	465	**10.94**	*Plasticicumulans*	***Candidatus* Competibacter**
3	460	**9.28**	*Saprospiraceae*	***Phaeodactylibacter***
4	465	7.16	*Xhantomonadaceae*	*Xhantomonadaceae*
5	440	**5.12**	***Mesorhizobium***	***Nitratireductor***
6	463	4.60	*Phycisphaerae*	*Phycisphaerae*
7	443	3.93	*Cyanobacteria*	*Obscuribacterales*
8	456	3.72	*Caldilineaceae*	*Caldilineaceae*
9	449	**3.48**	***Rudaeicoccus***	*Dermacoccaceae*
10	465	**3.28**	***Aquabacterium***	*Comamonadaceae*
11	440	**2.74**	***Paracoccus***	*Rhodobacteraceae*
12	460	1.90	*Flavobacteriales*	*Flavobacteriales*
13	440	**1.43**	*Rhodobacteraceae*	***Paracoccus***
14	441	1.40	*Caldilineaceae*	*Caldilineaceae*
15	465	**1.20**	***Hydromonas***	*Neisseiraceae*
16	464	1.08	*Chthoniobacterales*	*Chthoniobacteraceae*
17	460	1.00	*Flavobacteriales*	*Bacteroidetes*
18	440	0.79	*Rhodobacteraceae*	*Rhodobacteraceae*
19	464	0.79	*Pedosphaera*	*Verrumicrobia*
20	460	0.79	*Sphingobacteria*	*Sphingobacteriales*
21	464	**0.71**	***Opitutus***	***Opitutus***
22	440	**0.68**	***Hyphomicrobium zavarnii***	***Hyphomicrobium***
23	441	**0.53**	***Ilumatobacter***	*Acidimicrobiaceae*

[a]OTUs number in Supplementary Table S1

[b]OTUs relative abundance in Supplementary Table S1

[c]sequence similarity to reference sequences: species ($x \geq 97\%$), genus ($97 > x \geq 94.5\%$), family ($94.5 > x \geq 86.5\%$), order ($86.5 > x \geq 82\%$), class ($82 > x \geq 78.5\%$), and phylum ($78.5 > x \geq 75\%$).

[d]OTUs identified at genus level are indicated in bold letter and represented 51.74% of relative abundance

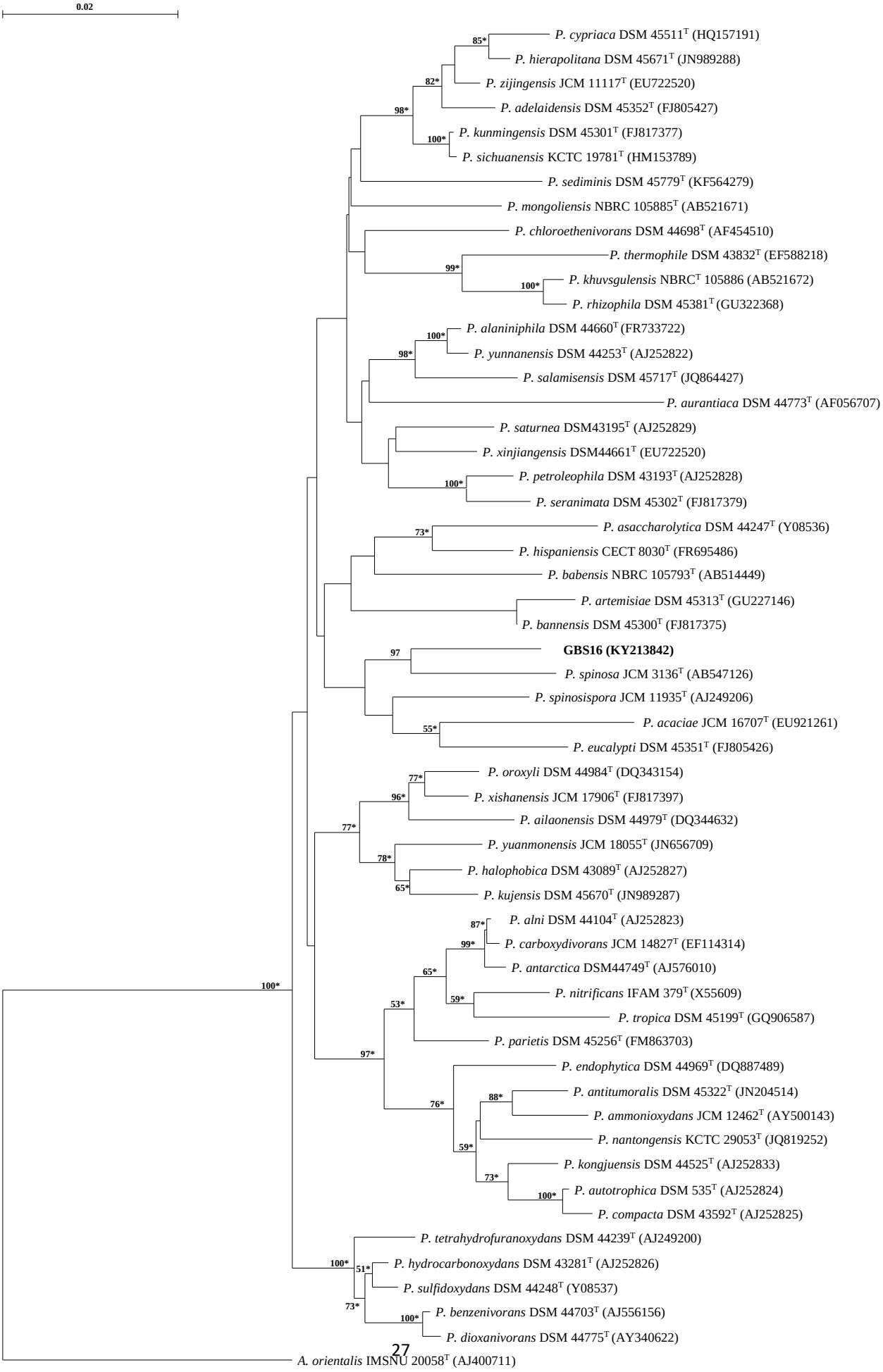
0.02
P. cypriaca DSM 45511T (HQ157191)
P. hierapolitana DSM 45671T (JN989288)
P. zijingensis JCM 11117T (EU722520)
P. adelaidensis DSM 45352T (FJ805427)
P. kunmingensis DSM 45301T (FJ817377)
P. sichuanensis KCTC 19781T (HM153789)
P. sediminis DSM 45779T (KF564279)
P. mongoliensis NBRC 105885T (AB521671)
P. chloroethenivorans DSM 44698T (AF454510)
P. thermophile DSM 43832T (EF588218)
P. khuvsgulensis NBRCT 105886 (AB521672)
P. rhizophila DSM 45381T (GU322368)
P. alaniniphila DSM 44660T (FR733722)
P. yunnanensis DSM 44253T (AJ252822)
P. salamisensis DSM 45717T (JQ864427)
P. aurantiaca DSM 44773T (AF056707)
P. saturnea DSM43195T (AJ252829)
P. xinjiangensis DSM44661T (EU722520)
P. petroleophila DSM 43193T (AJ252828)
P. seranimata DSM 45302T (FJ817379)
P. asaccharolytica DSM 44247T (Y08536)
P. hispaniensis CECT 8030T (FR695486)
P. babensis NBRC 105793T (AB514449)
P. artemisiae DSM 45313T (GU227146)
P. bannensis DSM 45300T (FJ817375)
GBS16 (KY213842)
P. spinosa JCM 3136T (AB547126)
P. spinosispora JCM 11935T (AJ249206)
P. acaciae JCM 16707T (EU921261)
P. eucalypti DSM 45351T (FJ805426)
P. oroxyli DSM 44984T (DQ343154)
P. xishanensis JCM 17906T (FJ817397)
P. ailaonensis DSM 44979T (DQ344632)
P. yuanmonensis JCM 18055T (JN656709)
P. halophobica DSM 43089T (AJ252827)
P. kujensis DSM 45670T (JN989287)
P. alni DSM 44104T (AJ252823)
P. carboxydivorans JCM 14827T (EF114314)
P. antarctica DSM44749T (AJ576010)
P. nitrificans IFAM 379T (X55609)
P. tropica DSM 45199T (GQ906587)
P. parietis DSM 45256T (FM863703)
P. endophytica DSM 44969T (DQ887489)
P. antitumoralis DSM 45322T (JN204514)
P. ammonioxydans JCM 12462T (AY500143)
P. nantongensis KCTC 29053T (JQ819252)
P. kongjuensis DSM 44525T (AJ252833)
P. autotrophica DSM 535T (AJ252824)
P. compacta DSM 43592T (AJ252825)
P. tetrahydrofuranoxydans DSM 44239T (AJ249200)
P. hydrocarbonoxydans DSM 43281T (AJ252826)
P. sulfidoxydans DSM 44248T (Y08537)
P. benzenivorans DSM 44703T (AJ556156)
P. dioxanivorans DSM 44775T (AY340622)
A. orientalis IMSNU 20058T (AJ400711)

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Fig. 2 Neighbor-joining tree based on nearly complete 16S rRNA gene sequences showing relationships between OTU1 (Genbank accession number KY213842) and the related *Pseudonocardia* type strains. Asterisks indicate branches of the tree that were also recovered using the maximum-parsimony tree-making algorithms. Numbers at the nodes indicate the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled datasets; only values above 50% are given. The scale bar indicates 0.02 substitutions per nucleotide position.

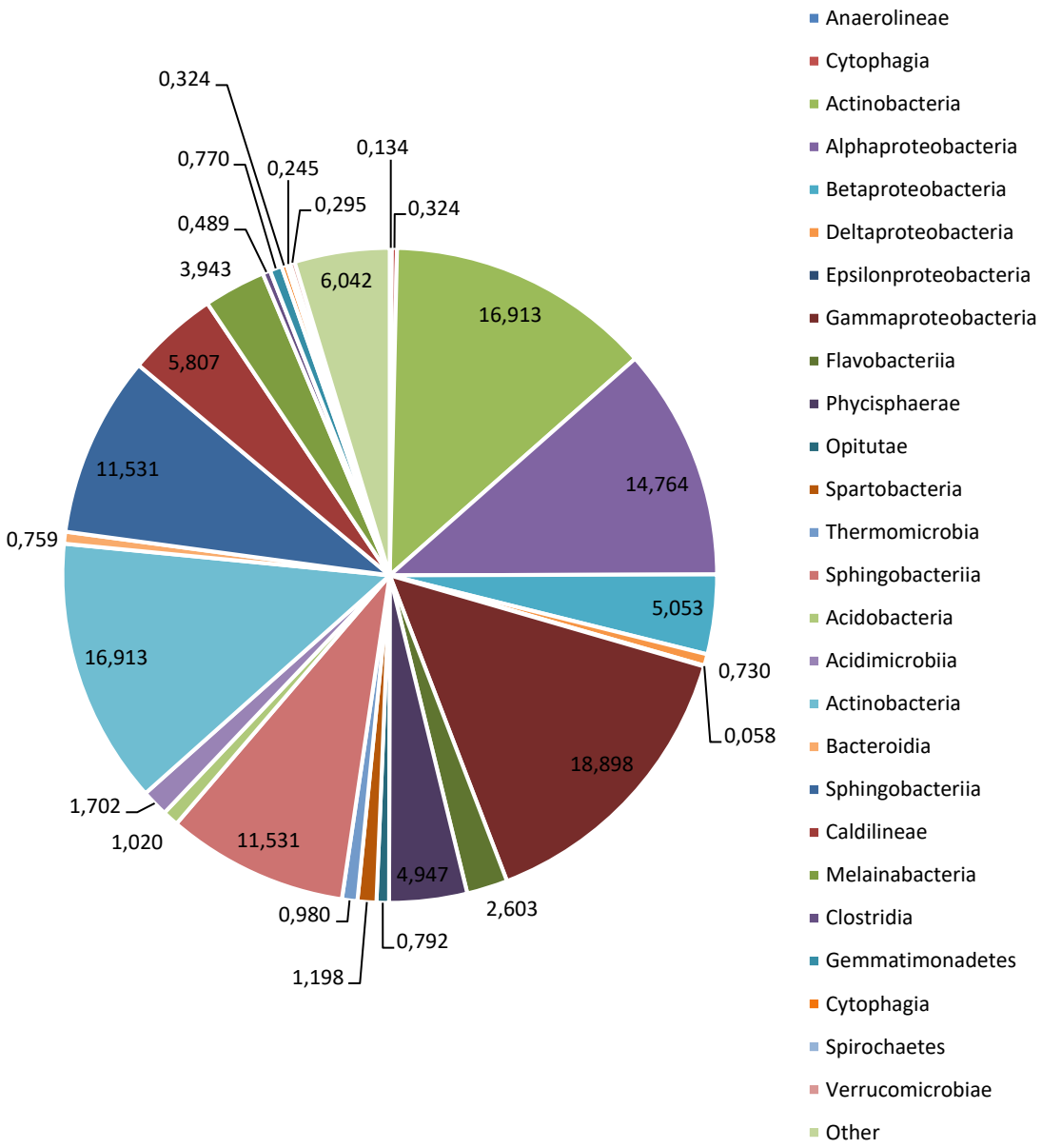

Fig. 3 Microbial community at the class level in activated sludge sample S2

559 **Table S1** List of rRNA-targeted oligonucleotide probes with corresponding specificity
560 and recommended formamide (FA) concentration

Probe name	Sequence (5´-3´)	Target	FA (%)	Reference
EUB 338I	GCTGCCTCCCGTAGGAGT	*Bacteria*	0-50	Amann et al. 1990
EUB338II	GCAGCCACCCGTAGGTGT	*Planctomycetes*		Daims et al. 1999
EUB338III	GCTGCCACCCGTAGGTGT	*Verrumicrobiales*		Daims et al. 1999
EUB338IV	GCAGCCTCCCGTAGGAGT			Schmid et al. 2005
HGC1156	CGAGTTGACCCCGGCAGT	*Actinobacteria*[b]	20	Erhart et al. 1997
GNSB941	AAACCACACGCTCCGCT	*Chloroflexi*	35	Gich et al. 2001
CFX1223	CCATTGTAGCGTGTGTGTMG	*Chloroflexi*		Bjornsson et al. 2002
Pse631-C	AGTCATGCCCGTATCGACCGCA	*Pseudonocardia*	25	Li et al. 2018
Pse631-G	AGTGATGCCCGTATCGACCGCA	*Pseudonocardia*	25	Li et al. 2018
SAP309	TCTCAGTACCCGTGTGGG	*Saprospiraceae*	25	Schauer and Hahn 2005
T0803-0654	ACACC CTCTCACYRCCT	Type 0803	30	Kragelund et al. 2011
T0803ind-0642 [b]	CTGCCTCAAGCTACTCAG	Type 0803	30	Kragelund et al. 2011
CFX67a[b, c]	TTCCGAAGATCAGGTTCG	Type 0914	35	Speirs et al. (2011)
CFX67b[b]	TTCCGAAGATTAGGTTCG	Type 0914	35	Speirs et al. (2011)
CFX197 [c]	TCCCGGAGCGCCTGAACT	Type 0092	40	Speirs et al. (2009)
CFX223 [b]	GGTGCTGGCTCCTCCCAG	Type 0092	35	Speirs et al. (2009)

561 [a]EUB338I, EUB338II, EUB338III, and EUB338IV were used as a mixture probe
562 (EUBmix). GNSB941and CFX1223 were used as a mixture probe (CFXmix).

563 GNSB941and CFX1223 were used as a mixture probe (CFXmix)

564 [b]Helper probes are required for the application

[c]Competitor probes are required for the application

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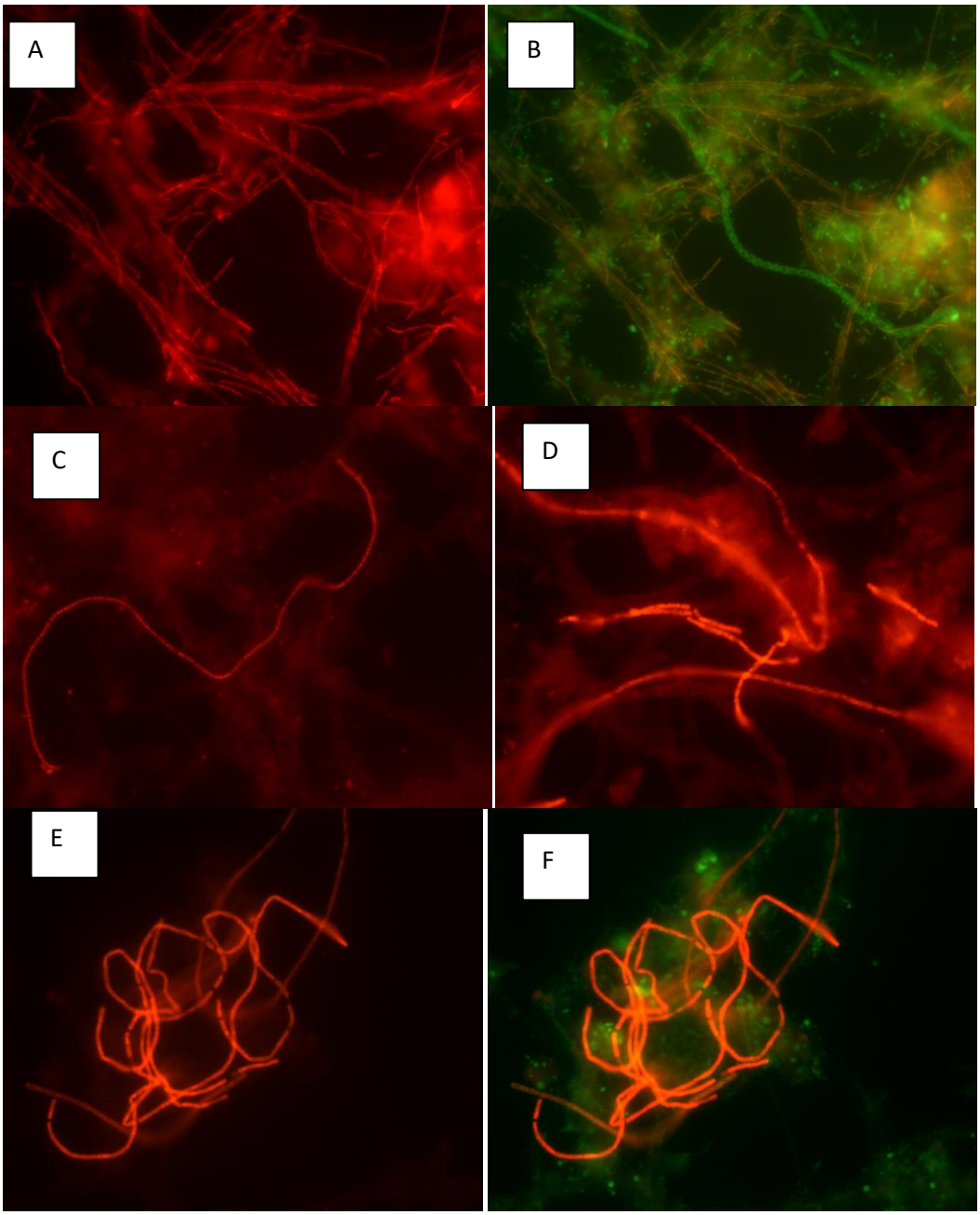

Fig. S1 Identification of filamentous bacteria using FISH 1000X, (a) *Haliscomenobacter* red filaments, probe SAP 309, (b) Same field, *Haliscomenobacter* orange filaments, probes EUB mix + SAP309, (c) *Microthrix* red filament probe, probe mPAll1410, (d) *Chloroflexi* red filaments, probe GNSB941/CFX1223 mix, (e) Type 0803 (*Caldilineaceae*) red filaments, probe 0803-0654 (f) Same field, Type 0803 (*Caldilineaceae*) orange filaments, probes EUB mix + 0803-0654.