Application of a novel molecular technique to characterise the effect of settling on microbial community composition of activated sludge

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Abstract

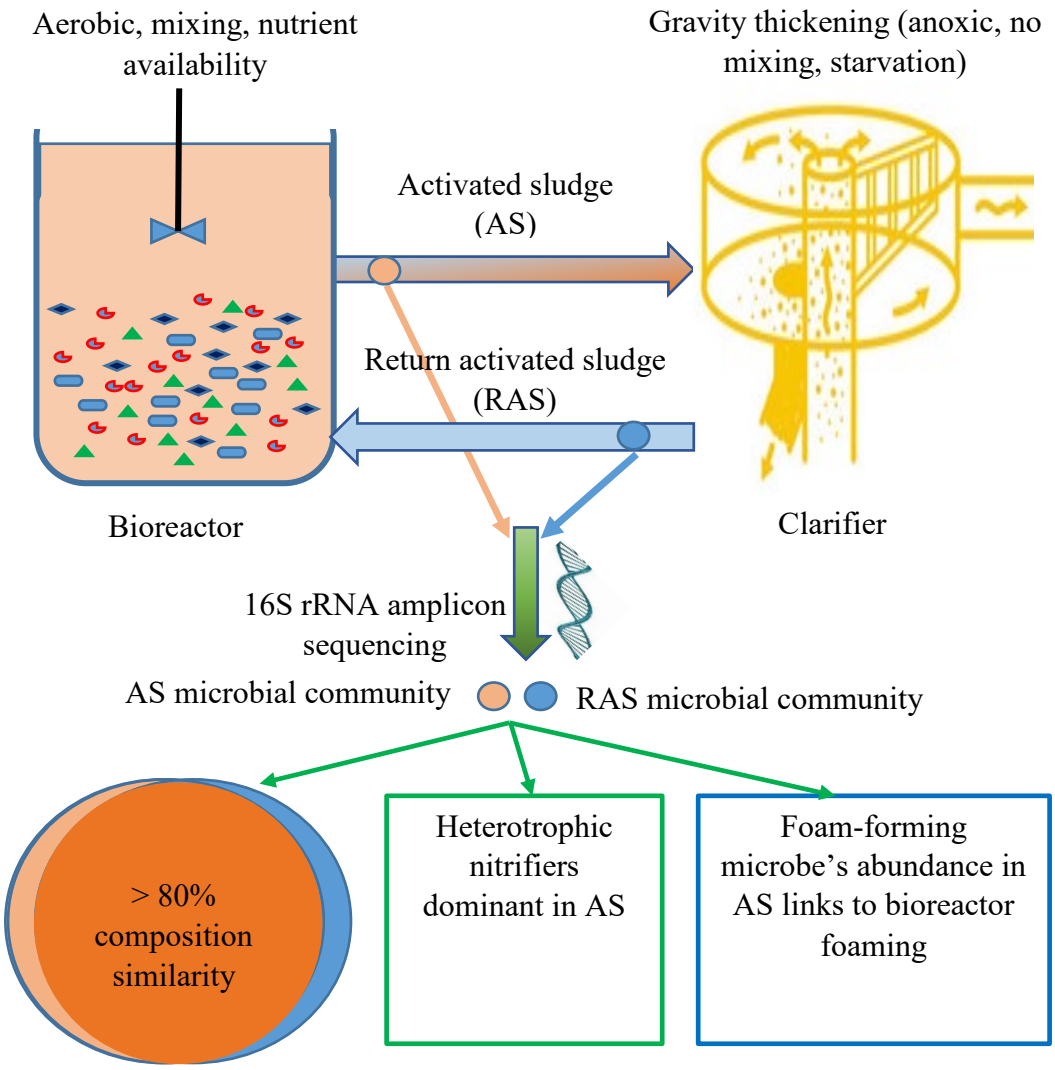
Activated sludge (AS) and return activated sludge (RAS) microbial communities from three full-scale municipal wastewater treatment plants (denoted plant A, B and C) were compared to assess the impact of sludge settling (i.e. gravity thickening in the clarifier) and profile microorganisms responsible for nutrient removal and reactor foaming. The results show that all three plants were dominated with microbes in the phyla of *Proteobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria, Chloroflexi, Firmicutes, Nitrospirae, Spirochaetae, Acidobacteria and Saccharibacteria.* AS and RAS shared above 80% similarity in the microbial community composition, indicating that sludge thickening does not significantly alter the microbial composition. Autotrophic and heterotrophic nitrifiers were present in the AS. However, the abundance of autotrophic nitrifiers was significantly lower than that of the heterotrophic nitrifiers. Thus, ammonium removal at these plants was achieved mostly by heterotrophic nitrification. Microbes that can cause foaming were at 3.2% abundance, and this result is well corroborated with occasional aerobic biological reactor foaming. By contrast, these microbes were not abundant (< 2.1%) at plant A and C, where aerobic biological reactor foaming has not been reported.

Keywords: Activated sludge; 16S rRNA sequencing, Microbial community; Return activated sludge; nitrifiers; foaming.

Highlights:

- AS and RAS communities shared above 80% similarity at all 3 WWTPs
- Settling of the biomass (RAS) had virtually no effect on the microbial composition
- Ammonia removal was achieved mostly by heterotrophic nitrifiers
- Slightly higher (than usual) foaming microbes abundance linked to reactor foaming

1. **Graphical abstract**



2. Introduction

Activated sludge (AS) microbial community is a core component, determining the function, performance and stability of the AS process in wastewater treatment plants (WWTPs) (Briones & Raskin, 2003; Guo et al., 2017). Though microbial respiration, organic matter, ammonium, nitrogen and phosphorus are converted to biomass (i.e. solids) or gases, thus, are removed from wastewater. The AS process has protected natural ecosystems and human health (Joicy et al., 2019; Keerthisinghe et al., 2019; Saunders et al., 2016).

For over a century, the AS process has been arguably the heart of municipal wastewater treatment; nevertheless, key aspects of the AS microbial community, including composition and dynamics, have not been fully understood. This is mainly due to the limitation of traditional research techniques relying on microbe culturing. Most microorganisms responsible for microbial respiration during wastewater are not cultivable. High throughput amplicon sequencing targeting the 16S rRNA genes (present in all prokaryotes) has recently provided a more reliable method for exploring microbial community structure and composition of environmental samples (Fan et al., 2017; Guo & Zhang, 2012; Zhang et al., 2012). Application of high throughput sequencing technologies for studying microbial communities in full-scale wastewater treatment plants (WWTPs) has recently gained an upward trajectory. For example, using 454 pyrosequencing, Ye & Zhang (2013) provided the detailed composition of bacterial communities in AS, digestion sludge, influent and effluent samples of full-scale WWTPs receiving saline wastewater. Bacterial diversity and composition in AS samples from WWTPs have been reported for diverse locations around the world such as the USA (Sanapareddy et al., 2009), Singapore, Canada, and China (Zhang et al., 2011) and Denmark (Albertsen et al., 2011).

In the operation of WWTPs, biomass from the bioreactor settles in the clarifier through gravity thickening. A portion of settle activated sludge is returned to the bioreactor to maintain the biomass concentration sufficient for the desired degree of treatment. Return activated sludge (RAS) is a routine operation at WWTPs, which is either controlled by a constant percentage of the influent flow or fixed flow rate independently from the influent flow. The chemical conditions in the clarifier are different from those in the bioreactor. During thickening that typically has a hydraulic retention time of 5 - 15 hours, depending on plant operation, the sludge becomes anoxic as it reaches up to around 2 wt% increase, and is non-homogenous due to lack of mixing. Furthermore, it can reach a starvation condition (limited organic and nutrient availability). These conditions are hypothesised to alter the community composition in RAS

and may influent the AS community. Compared to recent studies focusing on AS microbial community as listed above, there is no study comparing the community of AS and RAS at full-scale WWTPs.

Nutrient removal and foam-foaming bacteria are important groups for the function of WWTPs. The former bacteria contribute to the removal of nutrients such as ammonia and phosphorus, while the later bacteria can cause foaming in the bioreactor. The abundance of nutrient removal bacteria influences the removal efficiency (Albertsen et al., 2011; Wells et al., 2011). Likely, in the AS process, foam-forming bacteria exist in amounts that may cause foaming incident that is a significant operation problem (Di Bella & Torregrossa, 2013). However, most bacteria responsible for phosphorus and nitrogen removal, as well as foaming formation, are currently not fully characterized (Albertsen et al., 2011; Guo et al., 2017). Recently developed high throughput sequencing technologies are expected to identify these bacteria in the AS process.

In this study, state-of-the-art sequencing technology was used to profile the microbiota within AS from bioreactors at three full-scale WWTPs and their respective RAS to establish if the settling process in the clarifier impacted the microbial community composition. The data generated contribute to a better understanding of the function of RAS in the AS process. The abundance of the key organisms involved in nutrient removals was determined. The abundance of foam-forming bacteria in AS of the plants with and without foaming occurrence was analysed. The data can help to predict foaming events and prevent their debilitating effects on WWTP operation.

3. Materials and methods

3.1. AS and RAS sampling

AS and RAS samples were taken from three full-scale municipal WWTPs (denoted as plant A, B and C) in Sydney, Australia (Fig 1). Detailed characteristics of these plants were presented in Table 1. The AS samples were the mixed liquor suspended solids at the aerobic zones in the bioreactor that was before the clarifier (Fig 1). Mixed liquor suspended solid was subjected to gravity thickening in the clarifier with the HRT of 9.6, 11 and 11.3 hours at plant A, B and C, respectively (Table 1). The conditions in the clarifier were anoxic, non-homogenous due to lack of mixing, and under starvation. At plant A, B and C, 60, 50 and 80% of the settled sludge were returned to the bioreactor, respectively. High RAS return ratio (i.e. 80%) was implemented at plant C due to the small treatment capacity. AS and RAS sampling was

designed to observe any impacts of the clarifier conditions on the AS microbial community. Of particular note, operators at plant B have reported occasional foaming associated with the bioreactor. Bioreactor foaming has not been previously observed at plant A and C. Therefore, the observation of foam-forming bacteria at the three plants allows for an inference of relationship between the abundance of foam-forming bacteria and foaming.

Table 1: Characteristics of the three WWTPs.

Parameter	Plant A	Plant B	Plant C
Peak treatment capacity (ML/d)	171	95	24
Average dry weather flow (ML/d)	50	35	17
Bioreactor arrangement	Anoxic/aerobic	Anoxic/ aerobic	Aerobic/anoxic/ aerobic
Dissolved oxygen (mg/L)	0.5 – 1.2	1.6 – 2.0	1.3 – 2.0
Sludge retention time (day)	6.1	5.3	6.4
HRT in aerobic zones (h)	7	4	6
Average HRT in the clarifier (h)	9.6	11	11.3
RAS return ratio (% of influent flow)	60	50	80

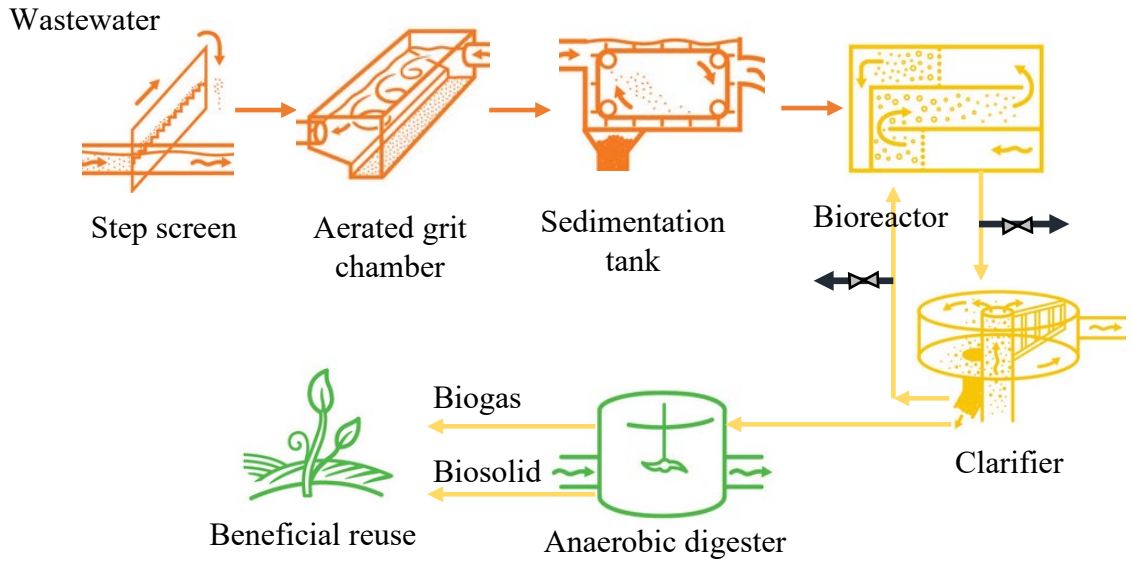


Fig 1. Generic diagram of the WWTPs in this study. The primary and secondary treatments are displayed in orange and yellow, respectively, while the biosolids handling is illustrated in green.

3.2. Microbial community analysis

Microbial communities of samples from the bioreactor (aerobic zones) and the RAS were analyzed in this study (Fig. 1). AS and RAS were collected into 50 mL sample bottle and mixed with 100% ethanol (1:1 v/v) to preserve the cells (Nguyen et al., 2019). Samples were stored in an ice bag during transport and immediately transferred to - 20 °C freezer upon arrival to the laboratory. Genomic DNA was extracted using DNeasy PowerSoil Pro Kit (QIAGEN Pty Ltd, Australia) following the manufacturer's instruction. The integrity, purity and concentration of the extracted DNA were determined by spectrophotometry (Nanodrop ND2300). The amount of DNA in the samples was higher than 10 μg, and the concentration was normalized to 50 ng/μL using DNA/RNA free water. Samples were stored at - 20 °C until DNA sequencing.

The variable regions (V3-V4) on the 16S rRNA genes of extracted DNA were amplified using the universal primers Pro341F (*5'-CCTAYGGGRBGCASCAG-3'*) and 806R (*5'-GGACTACNNGGGTATCTAAT-3'*) (Takahashi et al., 2014). The amplified fragments were sequenced on the Illumina MiSeq sequencing platform at the Australian Genome Research Facility, Australia. Raw paired-end (2×300 bp) 16S rRNA gene sequence data were analyzed according to the Quantitative Insights into Microbial Ecology (QIIME2) pipeline (Caporaso et al., 2010). In brief, raw sequences were denoised using DADA2 with the following parameters: trim left-f = 17, trim left-r = 20, trunc-len-f = 280, trucn-len-r = 220, and all other parameters at their default setting. The sequences were clustered into representative OTUs based on a 97% nucleotide identity cut-off. The 16S rRNA gene sequencing generated 120,000 to 450,000 sequences per sample after pre-processing. The taxonomical assignment was performed against MiDAS database version 2.1 (McIlroy et al., 2017). The 16S rRNA gene sequences were deposited in GenBank with the accession numbers PRJNA507317. Principal coordinate analysis and compositional similarity index were performed in PASS software with the Bray-Curtis index. Statistical analysis was performed in Microsoft Excel using Student's unpaired *t*-Test, with a two-tailed distribution.

4. Results and discussion

4.1. Global activated sludge microbial profile

Amplicon sequencing from total genomic DNA samples resulted in the identification of over 97% of all microorganisms in the biological reactor of all three WWTPs in this study. These microorganisms can be classified into ten major phyla, including *Proteobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria, Chloroflexi, Firmicutes, Nitrospirae,*

Spirochaetae, Acidobacteria and Saccharibacteria (Table 1). Composition of these major phyla was similar amongst the three plants investigated in this study (Table 2). The results corroborated from the literature also show that vicinity WWTPs in the same region usually have a similar microbial composition in the activated sludge reactor.

Proteobacteria is the dominant phylum (by at least 50%) of the AS community in this study as well as the literature (Table 2). The *Proteobacteria* phylum made up of at least eight classes including β-*proteobacteria,* α-*proteobacteria*, λ-*proteobacteria* and δ-*proteobacteria*. Most genera that significantly correlate with the functions and performance of WWTPs belong to the *Proteobacteria* phylum (i.e. *Nitrosomonas, Nitrobacter, Comamonas* and *Thauera*). The *Proteobacteria* phylum also contains bacterial groups that are responsible for nutrient removal including ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and phosphorus accumulating organisms. In this study, ammonia-oxidizing bacteria are represented by the *Nitrosomonas* genus, while nitrite-oxidizing bacteria are represented by the *Nitrobacter* and the *Nitrospira* (*Nitrospirae* phylum) genera. Phosphorus accumulating organisms (*β-proteobacteria* class) are capable of immobilising phosphorus from the mixed liquor using nitrate and oxygen as an electron acceptor in the anoxic and aeration zones of the bioreactor, respectively. By using nitrate as a final electron acceptor, the phosphorus accumulating organisms also contribute to denitrification by producing nitrogen gas. Nitrification occurs in the aerobic zone, where *Nitrosomonas* and *Nitrobacter* convert ammonium into nitrate and nitrite (Sanapareddy et al., 2009).

Apart from the *Proteobacteria* phylum, there is a striking similarity between abundant phyla in AS and those in human gut microbiota. Members of the gram-positive *Firmicutes* and the gram-negative *Bacteroidetes* phyla are the most common organisms in human gut microbiota. Several others phyla, including the *Verrucomicrobia* and *Actinobacteria*, commonly occur in human gut microbiota (Eckburg et al., 2005) also present in the microbial community of AS in this study (Table 2).

Chloroflexi is another phylum that is frequently detected in the AS community (Table 2) as well as in the community of marine and freshwater sediments (Hug et al., 2013). The phenotype of *Chloroflexi* member includes carbon cycling, organohalide respiration, fermentation, CO_2 fixation and acetogenesis (i.e. production of volatile fatty acids and acetate) with ATP formation by substrate-level phosphorylation (Hug et al., 2013). Member of *Chloroflexi* phylum has the ability to degrade a wide range of complex organic matters (Graber & Breznak,

2005). The abundance of *Chloroflexi* and their phenotype suggests their role in organic carbon removal in the AS process.

The phylum *Saccharibacteria* was present at 0.5 to 2% of the total bacteria in the AS community. Members of *Saccharibacteria* can degrade various organic compounds in aerobic, anoxic and anaerobic conditions (Ohashi et al., 2016). In the AS community, *Saccharibacteria* members could contribute to organic carbon removal and nitrate reduction in the AS process.

The phylum *Acidobacteria* was present at less than 2% of the total bacteria in the AS community (Table 2). This phylum adapts to oligotrophic environments and contributes to carbon and nitrogen cycles (Eichorst et al., 2018). Bacteria of *Acidobacteria* phylum carry carbon metabolism-associated genes involved in the degradation of polysaccharides and aromatic compounds (Hester et al., 2018; Janssen et al., 2002). The phylum *Acidobacteria* is characterised as slow-growing microbes due to low energy generation in their metabolisms (Fierer et al., 2007; Jones et al., 2009). Their low growth rate could make it hard for them to compete with other phyla in the AS community, explaining their low abundance. The RAS could probably help to maintain this phylum in the AS microbial community.

Table 2: Comparison of microbial community of activated sludge revealed by the next-generation sequencing.

Sample	**Sequencing platform**	**Country**	**Taxonomic classification databases**	**Dominant phylum (relative abundant %)**	**Ref**
Activated sludge from three WWTPs	Illumina HiSeq	Australia	MiDAS database version 2.1	*Proteobacteria* (49 -54), *Bacteroidetes* (20-29), *Verrucomicrobia* (3-6), *Actinobacteria* (6-8), *Chloroflexi* (1-4), *Firmicutes* (1-4), *Nitrospirae* (0.5- 2), *Spirochaetae* (0.5 -2), *Saccharibacteria* (0.5 -2) *Acidobacteria* (0- 2)	This study
Activated sludge sample from nitrogen and phosphorus removal WWTPs	Illumina HiSeq 2000	China	NCBI NT	*Proteobacteria* (70), *Nitrospirae* (15), *Bacteroidetes* (8.6), *Actinobacteria* (2)	(Guo et al., 2017)

Activated sludge from 14 WWTPs	454 Pyro sequencing	China	Ribosomal Database Project (RDP) Classifier	*Proteobacteria* (36 - 65), *Bacteroidetes* (2.7 – 15.6), *Firmicutes* (1.4 – 14.6), *Actinobacteria* (1.3 - 14), *Verrucomicrobia* (4.2), *Chloroflexi* (3.4)	(Zhang et al., 2012)
Activated sludge from enhanced biological phosphorus removal WWTPs	Illumina GAII	Denmark	Blastp with MEGAN v. 4.30	*Proteobacteria* (28), *Actinobacteria* (30), *Bacteroidetes* (8), *Chloroflexi* (16), *Firmicutes* (4), *Nitrospirae* (2),	(Albertsen et al., 2011)
Activated sludge from 14 WWTPs	454 Pyro sequencing	China	RDP	*Proteobacteria* (21 – 53), *Bacteroidetes* (11 – 64), *Actinobacteria* (1 – 27), *Chloroflexi* (1 – 17), *Verrucomicrobia* (1 – 4), *Planctomycetes* (1 – 3),	(Wang et al., 2012)
Activated sludge from 14 sewage treatment plants	454 Pyro sequencing	China, Hong Kong, Singapore, Canada	RDP	*Proteobacteria* (36 – 65), *Bacteroidetes* (3 – 16), *Actinobacteria* (2 – 14), *Chloroflexi* (3 – 5), *Verrucomicrobia* (4), *Planctomycetes* (2),	(Zhang et al., 2011)

4.2. Impact of settling on AS and RAS microbial community

Gravity settling of AS in the clarifier has no significant impact on the microbial community. The AS and RAS microbial communities at all three WWTPs shared a high composition similarity (above 84% shared genera) (Fig 2). The similarity index (Bray-Curtis similarity index) between the AS and RAS communities was 88 ± 1, 84 ± 5 and 86 ± 2% at plant A, B and C, respectively. Although RAS is operated by either a constant percentage of the influent flow or fixed flow rate independent from the influent flow, this study reports for the first time i) the microbial composition in RAS and ii) high similarity of microbial composition between AS and RAS at WWTPs. The high similarity of AS and RAS community compositions suggests that RAS likely contributes to the maintenance of the bioreactor functionality.

The microorganisms can influence the AS microbial composition in the influent. However, previous research results suggest that influent's microbial community (i.e. the microorganisms in wastewater) only impact the temporal variation of the AS microbial community. Lee et al. (2015) reported only 4.3 to 9.3% similarity between the microbial communities in the influent

and the AS samples. Although the microbial composition of influent was not analyzed in this study, the high similarity of AS and RAS community could indicate a minimal interference of influent microbial communities on the AS.

The AS microbial communities at all three plants also shared a high level of similarity (>70%) (Fig. 2). This observation is in consistence with results reported by Saunders et al. (2016) who used the same technique with this study and found that 13 Danish WWTPs shared 68% of microbial community similarity. In line with the discussion in Section 3.1, there are commonly shared bacterial genera in all AS samples across the world.

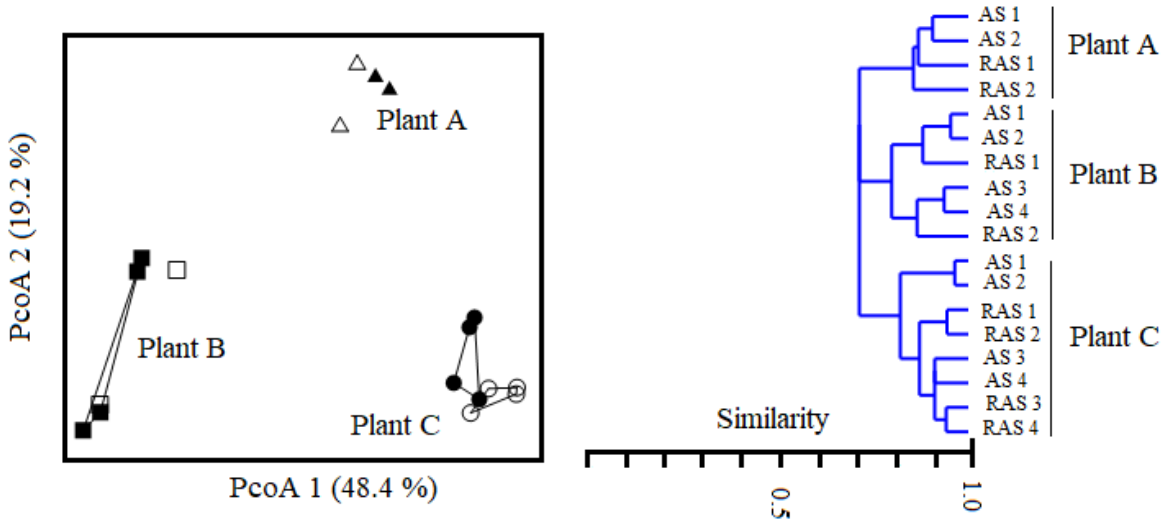


Fig. 2: Principal coordinates analysis (PcoA) and unweighted pair group method with Bray-Curtis similarity index of microbial communities at three plants.

The taxonomical analysis revealed 16 major orders (i.e. abundance > 1% of the total) that have similar abundance in the AS and RAS microbial community (Fig. 3). Consistently, these orders have been regularly reported as the major order in AS samples from WWTPs at different geographic locations. For example, the order of *Acidimicrobiales, Flavobacteriales,* and *Rhodobacterales* were among the most abundant orders found in the AS of eight WWTPs in Canada (Isazadeh et al., 2016). Order of *Caldilineales* and *Acidimicrobiales* were dominant in sludge samples of six WWTP's in China (Zhang et al., 2017). *Burkholderiales, Flavobacteriales, Pseudomonadales, Rhizobiales, Rhodobacterales, Rhodocyclales, Sphingobacteriale and Xanthomonadales* were the most abundant orders accounting for 64-68% of total bacteria in four municipal WWTPs China (Zhang et al., 2017) and in 19 municipal

WWTPs in Brazil (Nascimento et al., 2018). Bacteria in the order of *Sphingobacteriale, Anaerolineales, Rhodocyclales, Burkholderiales, Rhizobiales, Xanthomonadales, Verrucomicrobiales, Clostridiales, Planctomycetales and Myxococcales* presented high abundance (95% of the total bacteria) in the AS from 14 WWTPs (Wang et al., 2012).

The bacterial genera, which are attributable to the nutrient removal in WWTPs, are ammonia-oxidizing bacteria, nitrite-oxidizing bacteria and phosphorus-accumulating organisms. The microorganisms were detected in the orders *Nitrosomonadales*, *Nitrospirales* and *Rhodocyclales*. The relative abundance of *Nitrosomonadales*, *Nitrospirales* and *Rhodocyclales* was ca. 0.5 – 1.5, 0.5 – 2 and 5 – 10 % in AS and RAS communities (Fig. 3).

Taxonomical and principal coordinates analyses overall showed a consistently high similarity between the AS and RAS microbial community composition at all three plants. Only a notable difference was that *Burkholderiales* and *Opitutales* orders were present at higher abundance in RAS than in AS community. Members of the *Burkholderiales* have been found to survive in limited nutrient environments (Li et al., 2012). The abundance of *Burkholderiales* showed a significant increase in the deeper sediment horizons that has low nutrient and anoxic conditions (Atashgahi et al., 2015). The *Opitutales* order belongs to the *Verrucomicrobia* phylum, previously described as being common in the human gut microbiota. Hester et al. (2018) showed that the abundance of *Opitutales* in the rhizosphere soil increased with low nutrient availability. *Opitutales* have been described as anaerobic polysaccharide-utilizing bacteria capable of denitrification (Chin et al., 2001). In the anoxic environment of the clarifier, *Opitutales* may take advantage of the remaining organic carbon in the wastewater and play a role in the denitrification process occurring. The capacity of *Burkholderiales* and *Opitutales* to proliferate in anoxic conditions probably explain their enrichment in the clarifier (i.e. RAS samples). The observation of *Burkholderiales* and *Opitutales* at high abundance in RAS samples is consistent with the denitrification occurring in the clarifiers at WWTPs.

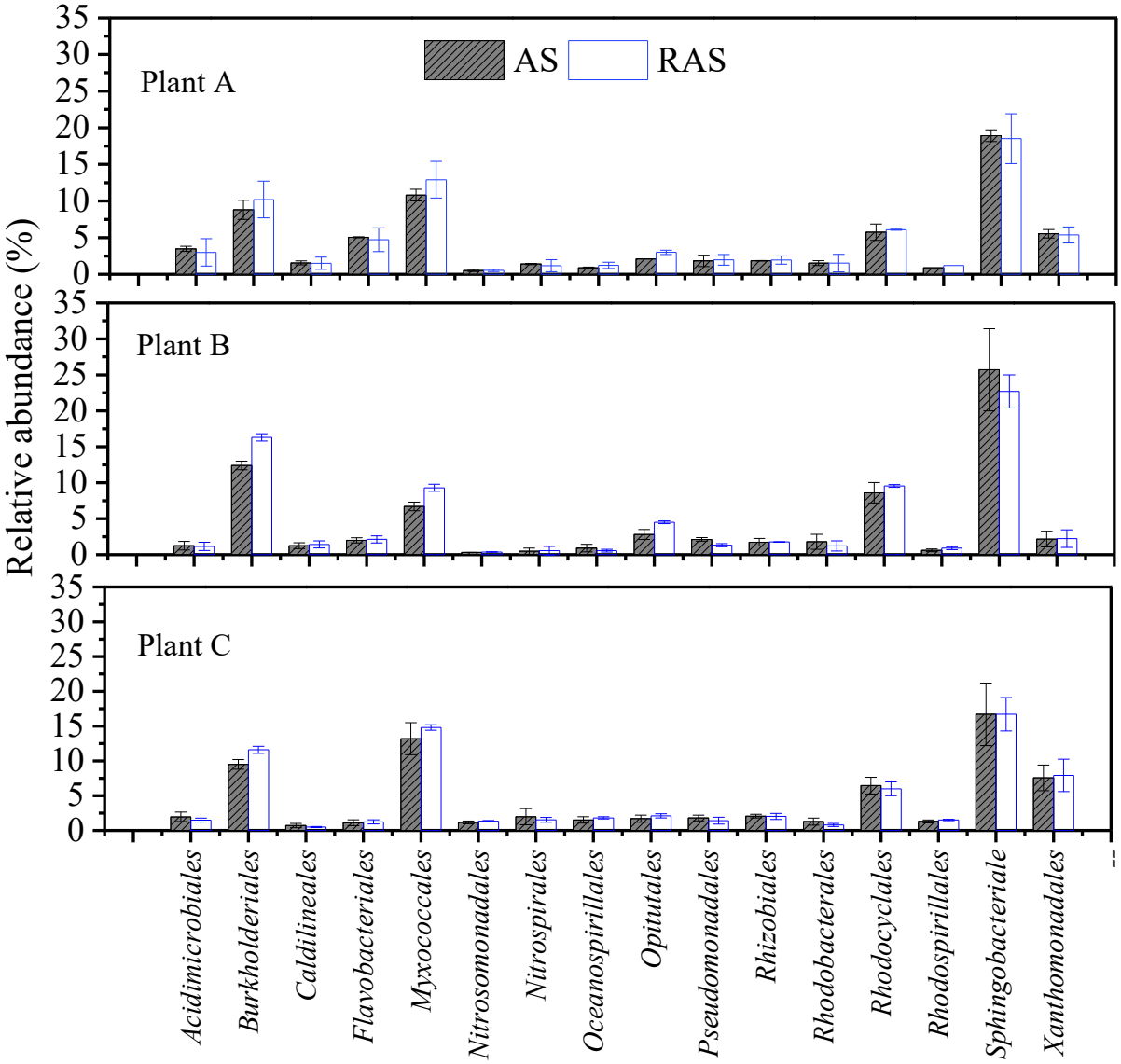


Fig. 3: Relative abundance of major bacterial orders that show similar abundance in AS and RAS microbial communities at three municipal WWTPs.

4.3. Nutrient removal microbes in AS

Results revealed the presence of ammonia-oxidizing bacteria (oxidation of ammonium to nitrite), nitrite-oxidizing bacteria (oxidation of nitrite to nitrate), and complete ammonium oxidizer (Comammox, complete oxidation of ammonium to nitrate) as well as denitrifiers (reduction of nitrate via nitrite and intermediate gaseous nitrogen oxide products to dinitrogen) in the AS microbial community (Table 3). *Nitrosomonas* and *Nitrosomonadaceae* (unclassified) are the main functional groups of ammonia-oxidizing bacteria. Their relative abundance ranged from 0.3 to 1.3% of total bacteria in the AS samples (Table 3). The study of

Hoshino et al. (2006) suggests that the abundance of ammonia-oxidizing bacteria increased from 1 to 6% of total bacteria when operating AS at partial nitrification and complete nitrification, respectively. Therefore, the low population of ammonia-oxidizing bacteria observed in this study may be attributed to the partial nitrification operation at the plants.

Nitrite-oxidizing bacteria (*Nitrospira sp.*) were present at higher abundance than ammonia-oxidizing bacteria (*Nitrosomonas sp.*) in the AS microbial community. This is probably because of the sensitivity of *Nitrosomonas sp.* to low temperature (winter) and dissolved oxygen in the WWTP during this study. Temperature below 15 °C is detrimental to the proliferation of ammonia-oxidizing bacteria (Siripong & Rittmann, 2007). Another factor that may contribute to the abundance of *Nitrospira* sp. in AS is their prevalence in the influent. In total, 3 to 9% of the AS microbial community is shared with the influent of WWTPs in which *Nitrospira* sp. are dominant (Lee et al., 2015). *Nitrospira* represents the most diverse known group of nitrite-oxidizing bacteria. Species of *Nitrospira* globally inhabit terrestrial and limnic environments, marine waters, deep-sea sediments, drinking water distribution systems, corroded iron pipes and WWTPs (Daims et al., 2001). The main ecological function of *Nitrospira* is nitrite oxidation. However, they also have versatile metabolism, including the utilisation of various organic compounds. Recently, research results reported that *Nitrospira* species possess all the enzymes to catalyse complete nitrification (Daims et al., 2015). These species are referred to as 'comammox'. Phylogenetic analyses suggested that comammox *Nitrospira* are present in diverse environments (Daims et al., 2001; Fan et al., 2017). The detection of *Nitrospira* in AS and RAS in this study warrantee future research into the detection of comammox *Nitrospira* that could revolutionize nitrogen removal in WWTPs.

Although autotrophic nitrifiers were present at lower abundance than heterotrophic nitrifiers (Table 3), successful nitrification was achieved at the three plants to satisfy the discharge standard. Results from this study and performance data from the plants suggested that ammonium removal was probably due to the heterotrophic process. Heterotrophic nitrifiers including species in the genus of *Comamonas, Thauera, Accumulibacter* and *Dechloromonas* were present at 5 to 14% of total bacteria in the AS microbial community (Table 3). These genera can produce hydroxylamine oxidase and periplasmic nitrate reductase, facilitating the oxidation of ammonium to nitrate (Chen & Ni, 2011). These species were previously found dominant in AS receiving ammonium-rich influent (Fan et al., 2017; Ma et al., 2015). Ma et al. (2015) observed more than 10% of heterotrophic nitrifiers (i.e. *Comamonas sp.* (6.6%), *Thauera sp.* (4.0%) and *Azoarcus sp*. (7.8%) in six WWTPs receiving high ammonium-bearing

wastewater (i.e. 300 mg/L). Both ammonia-oxidizing and nitrite-oxidizing bacteria were present at low abundance (Ma et al., 2015).

Species of *Accumulibacter sp*. and *Dechloromonas sp.* could also perform phosphorous removal in the AS process. However, their abundance was relatively low compared to the level in the enhanced biological phosphorous removal plant. Thus, phosphorus removal in the studied plants is mainly due to chemical precipitation.

Table 3: Specific nutrient removal bacterial group in AS. Data are mean and standard deviation (*n = 4*).

Groups	Ecology function	Genera	Plant A	Plant B	Plant C
Autotrophic nitrifiers	Ammonia oxidizing	*Nitrosomonas*	0.3 ± 0.0	0.0 ± 0.0	0.3 ± 0.0
		Nitrosomonadaceae	0.53 ± 0.11	0.32 ± 0.0	1.26 ± 0.1
	Nitrite oxidizing	*Nitrospira*	1.3 ± 0.5	0.5 ± 0.4	1.8 ± 0.8
Total abundance (%)			2.1	0.8	3.4
Heterotrophic nitrifiers	Ammonia and nitrite oxidizing	*Comamonas*	5.2 ± 0.9	10.2 ± 1.3	3.5 ± 0.4
		Thauera	1.5 ± 0.5	0.1 ± 0.0	1.0 ± 0.3
		Accumulibacter	0.1 ± 0.0	1.3 ± 0.2	0.1 ± 0.0
		Dechloromonas	0.9 ± 0.5	2.1 ± 1.1	0.4 ± 0.0
Total abundance (%)			7.7	13.7	5.0

4.4. Foam-forming microbes in AS

Several bacterial genus, known to cause bioreactor foaming were detected in AS samples from all the three plants (Table 4). The foam-forming bacteria include *Gordonia sp*., *Mycobacterium sp., Nocardia sp.* and *Flavobacterium sp*. The major phenotype of these bacteria is the presence of mycolic acids in their cell walls. The mycolic acids increase cell surface hydrophobicity. Due to this property, the foam-forming bacteria present themselves as a string of bacteria or filaments in the activated sludge that promotes foaming formation (Guo et al., 2015). Guo et al. (2015) reported that foam-forming bacteria were 3.0 to 4.5 times more abundant in the foam than in the AS sample at a Hong Kong WWTP. A four-year study lead by Ju et al. (2014) released the outburst of *Gordonia sp*. during a foaming episode at the sludge treatment plant. The enrichment of these genera in the foam suggests their major roles in foam formation in the AS process.

Foaming formation in the AS process probably depends on the foam-forming bacteria abundances. In the three plants, foam-forming bacteria presented at 0.5 to 3.2% of the total bacteria in AS samples (Table 4). Notably, foaming has not been previously observed at the bioreactor of plant A and C. Consistently, the total foaming bacteria abundances were 0.5 and

2.11% in AS samples. On the other hand, a higher foaming bacteria abundance was observed at plant B, where foaming occasionally occurred in the bioreactor. This observation could indicate that a threshold level of foaming bacteria abundance exists beyond which foaming occurs in the AS process. The observation of 3.2% total foaming bacteria at plant B is likely within the threshold levels. Previous studies have suggested that the likelihood of foam formation depends on a threshold in foam-forming bacteria cell number (Petrovski et al., 2011). A concentration ranging from 10^7 to 10^9 cells per mL sample (i.e. estimated at 0.1% of cell abundance) can initiate foaming formation (Petrovski et al., 2011). However, this inference to foam formation may be a rough estimate due to the limitation imposed by the low detection limits of traditional molecular methods. Recently, improvement in metagenomic sequencing allows more insight into the AS microbial community as well as foam-forming bacteria (Guo & Zhang, 2012). The abundance of foam-forming bacteria can be quantified precisely and correlated with the sludge volume index (SVI) as an indicator of foaming. Above 300 mL/g SVI occurred at AS with 2% of foaming bacteria (Guo & Zhang, 2012; Ju et al., 2014). In this study, the SVI value at plant B averaged at 525 ± 111 mL/g, while the values at plant A and C were 105 ± 30 mL/g and 145 ± 40 mL/g, respectively. The SVI value was 5 times higher at plant B than at plants A and C, partly explaining the foaming observed and high foam-forming bacteria abundance at plant B. It is acknowledged that the emergence of foam-forming bacteria is an aftereffect. Change in weather, influent quality, as well as operating conditions, are likely the primary factors. The modern molecular techniques employed in this study can be used in a future study with a specific condition to identify the cause of foaming clearly.

Table 4: Relative abundance (%) of foam-forming bacteria in the AS microbial community. Data are mean and standard deviation (*n = 4*).

Family	Genus	Plant A	Plant B	Plant C
Nocardiaceae	*Gordonia*	0.0 ± 0.0	0.85 ± 0.39	0.48 ± 0.34
Nocardiaceae	*Nocardia*	0.0 ± 0.0	0.01 ± 0.00	0.00 ± 0.00
Mycobateriaceae	*Mycobacterium*	0.27 ± 0.12	0.6 ± 0.18	1.1 ± 0.5
Intrasporangiaceae	*Tetrasphaera*	0.17 ± 0.09	1.8 ± 0.6	0.5 ± 0.6
Total abundance (%)		0.45	3.20	2.11

5. Conclusion

AS microbial community at three full-scale WWTPs was dominated by *Proteobacteria, Bacteroidetes, Verrucomicrobia, Actinobacteria, Chloroflexi, Firmicutes, Nitrospirae, Spirochaetae, Acidobacteria* and *Saccharibacteria* phylum. AS microbial profile is consistent with the diverse distribution of the phyla in other plants. AS and RAS microbial communities were similar, suggesting the role of RAS in maintaining the stability of AS microbial community. Autotrophic nitrifiers occurred at low abundance, while heterotrophic nitrifiers presented at a high level in AS communities, indicating that ammonium removal mainly occurs through the heterotrophic process. Foam-forming bacteria accounted for 3.2% of the total population at plant B that has occasional foaming.

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