



# A comprehensive comparison of microbial communities between aerobic granular sludge and flocculent sludge for nutrient removal in full-scale wastewater treatment plants

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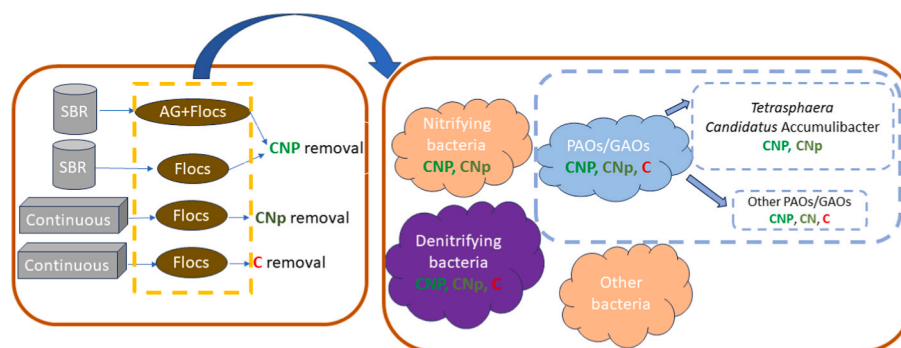
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## HIGHLIGHTS

- Higher richness and diversity of microbial community in granules than flocs.
- Granules enriched more filamentous bacteria, nitrifying bacteria, and GAOs.
- There were equivalent PAOs in granules and flocs.
- Many putative PAOs might not contribute to P removal.
- High abundance of GAOs such as 4.8% did not link with poor P removal.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Understanding the microbial community structure of sludge is crucial for improving the design, operation and optimisation of full-scale wastewater treatment plants (WWTPs). This study aimed to have a comprehensive comparison of microbial communities between aerobic granular sludge and flocculent sludge from two full-scale sequential batch reactors-based WWTPs with nutrient removal for the first time. To better understand key functional bacteria such as polyphosphate accumulating bacteria (PAOs), competitive bacteria such as glycogen accumulating bacteria (GAOs) and nitrifying bacteria for both nitrogen and phosphorus removal, another two full-scale WWTPs with only carbon (C) removal and C and nitrogen (N) removal were compared too. It was found that the richness and diversity of the microbial population in sludge increased with pollutant removal from only C, C and N, to C,N, P removal. For C, N P removal, granule structure led to a more diverse and rich microbial community structure than flocculent structure. Although more abundant nitrifying bacteria were enriched in granular sludge than flocculent sludge, the abundance of total putative PAOs was equivalent. However, the most typical putative PAOs such as *Tetrasphaera* and *Candidatus Accumulibacter* seemed to be more correlated with biological phosphorus removal performance, which might be more proper to be used as an indication for P

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removal potential. The higher abundance of GAOs in flocculent sludge with better phosphorus removal performance might suggest that further investigation is needed to understand the functions of GAOs. In addition, the equivalent abundances of PAOs in the WWTPs with only C removal and with C, N, and P removal, respectively, indicate that many newly reported putative PAOs might not contribute to P removal. This study provides insight into the microbial communities and functional bacteria in aerobic granular sludge and flocculent sludge in full-scale SBRs, which can provide microbes-informed optimisation of reactor operation for better nutrient removal.

## 1. Introduction

Understanding the microbial population of sludge and the functions of key organisms in wastewater treatment plants (WWTPs) is critical for the design, operation, and optimisation of processes to achieve carbon, nitrogen, and phosphorus removal in an environmentally friendly and cost-effective manner. Despite the widespread adoption of enhanced biological phosphorus removal (EBPR) for decades, its lower efficiency and less stable performance compared to chemical phosphorus removal keep chemical precipitation as the dominant method in WWTPs. In some WWTPs that employ EBPR, additional chemical dosing is still necessary to further reduce phosphorus levels to comply with more stringent nutrient regulations.

Aerobic Granular Sludge (AGS), characterized by its granular form, robust structure, and high biomass retention, has gained attention for its efficacy in nutrient removal (Chen et al., 2015; Liu et al., 2010; Liu and Tay, 2012). In the UK, there are a dozen granules-based WWTPs, all operating in sequential batch reactor (SBR) mode. In contrast, flocculent sludge, a more traditional form in wastewater treatment, has been used for nutrient removal in SBR too. The difference between these sludge types may be in their physical characteristics, microbial composition and functionality. Winkler et al. (2013) investigated microbial diversity differences in granular sludge from a pilot-scale plant and activated sludge (AS) from a full-scale plant with the same sewage, using the 16S DNA gene DGGE. They found that *Nitrosomonas* was the dominant AOB in flocculent sludge whereas *Nitrosomonas* and *Nitrosospira* were equally present in granular sludge, suggesting that sludge form (granular or suspended) could affect the abundance of functional bacteria. Studies from lab-scale reactors with activated sludge and granules, respectively, showed that the activity of phosphorus-accumulating bacteria was significantly higher in AGS (Rollemberg et al., 2019). Wang et al. (2015) also found that granular sludge had a higher PAOs content than flocs sludge in two denitrifying phosphorus removal reactors. However, Wei et al. (2021) reported that flocs and small granules had higher polyphosphate-accumulating organisms (PAO) activity while ammonia-oxidizing bacteria (AOB) activities were similar in both flocs and large granules in a lab-scale reactor. These inconsistent results might be attributed to different operational conditions, including different synthetic wastewater mediums. From this aspect, full-scale WWTP studies with actual sewage can reveal more representative microbial information to inform plant operation. While microbial communities have been investigated in full-scale aerobic granular sludge-based WWTPs (Świątczak and Cydzik-Kwiatkowska, 2018), there is very little study on the comparison of microbial communities between granule sludge and flocculent sludge within the same type of reactor and process configuration such as SBRs.

Furthermore, the microbial community of sludge in full-scale WWTPs with EBPR has been extensively studied in the last decade, providing insightful and useful revisions to our understanding of the traditional phosphorus removal model by PAOs and the competition between GAOs and PAOs (Nielsen et al., 2019). More putative PAOs and GAOs are being reported, but most have not been fully validated (Stokholm-Bjerregaard et al., 2017). *Tetrasphaera* and *Candidatus Accumulibacter* are two well-researched PAOs, believed to contribute most to phosphorus removal. However, their presence, alongside other reported putative PAOs and GAOs, has not been well studied in WWTPs without EBPR. While it might seem unnecessary to study PAOs and GAOs in

non-EBPR WWTPs, comparing functional bacteria in both systems could unveil more information about the possible functions of putative PAOs and GAOs and their preferred environment.

This study aimed to investigate the microbial community of sludge from four different full-scale WWTPs, i.e. two with C, N, and P removal in granular and flocculent forms, respectively, in SBRs, one with C, N removal, and one with only C removal by flocculent sludge in continuous flow mode. By conducting a comprehensive analysis of the microbial communities and comparing functional bacteria across these varied WWTPs in the same geographical region with different sludge forms and operational modes, valuable insights were gained into how sludge form and functional bacteria could affect nutrient removal. These findings hold potential for enhancing the design and operational strategies of biological wastewater treatment systems, especially about SBRs, aiming to achieve nutrient removal that complies with regulatory standards without chemical use.

## 2. Material and methods

### 2.1. Wastewater treatment process configurations, sludge types and wastewater characteristics in four full-scale WWTPs

Four full-scale WWTPs with different nutrient removal processes and sludge type were investigated. The operational conditions and wastewater quality of the WWTPs were shown in Table S1. The first two WWTPs employed SBR operation for biological C, N and P removal, with one using AGS technology at Birmingham and the other one using conventional AS at Petersfield. The SBR cycle in two plants included anaerobic, anoxic and aerobic phases and cycle duration varied according to influent quality and treatment performance. These two plants had similar excellent N removal efficiency (almost 100% ammonium-N removal) without external carbon addition. For P removal during the sampling period, Petersfield WWTP had stable and excellent biological removal with a phosphate-P removal efficiency of almost 100% while Birmingham WWTP had a combined phosphate-P removal efficiency of 97% through biological P removal ferric precipitation. The third one used conventional activated sludge with a 4-stage Bardenpho (Anoxic/Aerobic/Anoxic/Aerobic) process for biological C and N removal at Millbrook with a soluble N removal efficiency of 93% by adding external carbon source such as methanol in anoxic lanes. Although this Bardenpho process was not designed for biological P removal, there was still around 29% of phosphate-P removal excluding assimilation. The fourth WWTP at Portswood used conventional ASP with two aeration lanes for biological C removal only. Four WWTPs are located in the south of England at a distance each other less than 120 miles away. All plants received municipal wastewater from combined sewer systems with similar wastewater quality.

### 2.2. Sludge collection from four full-scale WWTPs

Mixed liquor in the reaction tanks at the end of the c batch cycles was collected from Birmingham and Peterfield WWTPs with SBR operation. Returned activated sludge from Millbrook and Portswood WWTPs with continuous operation was collected. Since the sludge from Birmingham WWTP was a mixture of large granules and suspended sludge, the sludge was separated into granular sludge (>2 mm in diameter) and flocculent sludge by using a sieve with a mesh of 2 × 2 mm. No obvious granules

were visually observed in the flocculent sludge portion. After sludge collection, approximately 2 mL of sludge samples were washed with buffer solution (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) and centrifuged at 2300 g for 5 min. Approximately 0.5 g wet pellet of washed sludge samples was re-suspended in 600  $\mu$ L extraction buffer solution (0.1 M Tris-HCl, 0.1 M Na<sub>2</sub>-EDTA, 0.1 M Sodium phosphate, 1.5 M NaCl, pH 7.5) and stored at  $-20^{\circ}$  C for subsequent DNA extraction.

### 2.3. Extraction and quantification of genomic DNA

Genomic DNA was extracted from 0.25 g sludge sample (wet weight) in duplicate using DNeasy® PowerSoil® Pro Kit (Qiagen, Manchester, United Kingdom) according to the manufacturer's instructions. Then, the extracted DNA samples were purified by using NucleoSpin™ gDNA Clean-up kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). A Nanodrop™ ND-1000 spectrophotometer (Thermo Fisher Scientific, USA) was used to measure DNA concentration, which ranged from 92 to 410 ng/ $\mu$ L. The DNA samples with absorbance ratios at 260/280 nm ranging from 1.84 to 1.91 were considered pure enough for sequencing.

### 2.4. Library preparation and sequencing of 16S rRNA gene

Extracted DNA samples were sent to the Environmental Sequencing Facility (National Oceanography Centre, University of Southampton, United Kingdom) for library preparation and sequencing of 16S rRNA genes. Firstly, the extracted DNA samples were diluted to 5 ng/ $\mu$ L. Then, the V3–V4 region of the gene encoding the 16S rRNA subunit was amplified by a target-specific PCR, using primers pro341F (5'-CCTACGGNBBGCASCAG-3') and pro805R (5'-GACTACNVGGGTATC-TAATCC-3') as well as Illumina adapters (Forward overhang adapter 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-3', Reverse overhang adapter 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG-3'). To enable sequencing of multiple samples together on the same platform and allow demultiplexing of the sequences in data analysis, a second PCR was followed, in which barcodes or index sequences were tagged into each amplicon sequence by using Nextera XT v2 (Illumina, USA) indexed primers. All PCR amplifications took place in an Applied Biosciences Veriti thermal cycler (Thermo Fisher Scientific, UK). The quality of the prepared libraries was checked and the concentration of PCR products was quantified using an Agilent Bioanalyzer (Agilent, USA) and a Qubit 3.0 fluorometer (Life Technologies, UK). The PCR products were then normalised, pooled, and sequenced on a 2 x 300 bp paired-end sequencing run using the MiSeq System (Illumina, USA).

### 2.5. Bioinformatics analysis

Amplicon sequences were demultiplexed using Local Run Manager Generate FASTQ Analysis Module (Illumina). The demultiplexed sequences were then processed using QIIME2 software package version 2022.2 (Bolyen et al., 2019). The sequences were denoised using DADA2 with default setting and 270 bp (forward read) and 220 bp (reverse read) were truncated. 16S DNA sequences were filtered using the VSEARCH method (Rognes et al., 2016) with 0.80 identity and 0.97 alignment based on reference sequences from the Silva 132 database (Quast et al., 2013). The average read count was 64,082 sequences per sample after quality control and filtering. Taxonomy assignment was performed against the classifier fitted with Naive-Bayes based on the Silva 132 database. Taxa were summarized as a percentage abundance of the total reads. Core metrics phylogenetic was processed with a sampling depth of 33,000 reads to obtain alpha and beta diversity. Alpha diversity was presented as ACE and Chao1 (species richness estimates), Simpson and Shannon (diversity indices), Faith phylogenetic diversity index or Faith pd (diversity index based on phylogenetic relationship, which is defined as a sum of the length of all branches on phylogenetic tree that span the member of a set of species) and Pielou's evenness index (evenness of species distribution). The rarefaction curve was visualized to

approximate species richness in a given sequencing depth. All the raw sequences generated from 16S rRNA sequencing were deposited in the NCBI Sequence Read Archive (SRA) under accession number SAMN41452656 - 41452665.

### 2.6. Statistics analysis

Hierarchical clustering analysis was performed in R program version 2022.12.0 (Posit Software, PBC) using *mia* and *vegan* packages based on Bray Curtis and Weighted Unifrac distances. Venn diagram of OTUs in different sludge samples was constructed using Venny version 2.1 (Oliveros, 2015). Spearman's rho correlation analysis was performed in IBM SPSS Statistics version 28.0.1.1 (IBM Corp., Armonk, NY) to examine the correlation between the abundance of functional genera and wastewater operating variables.

## 3. Results and discussion

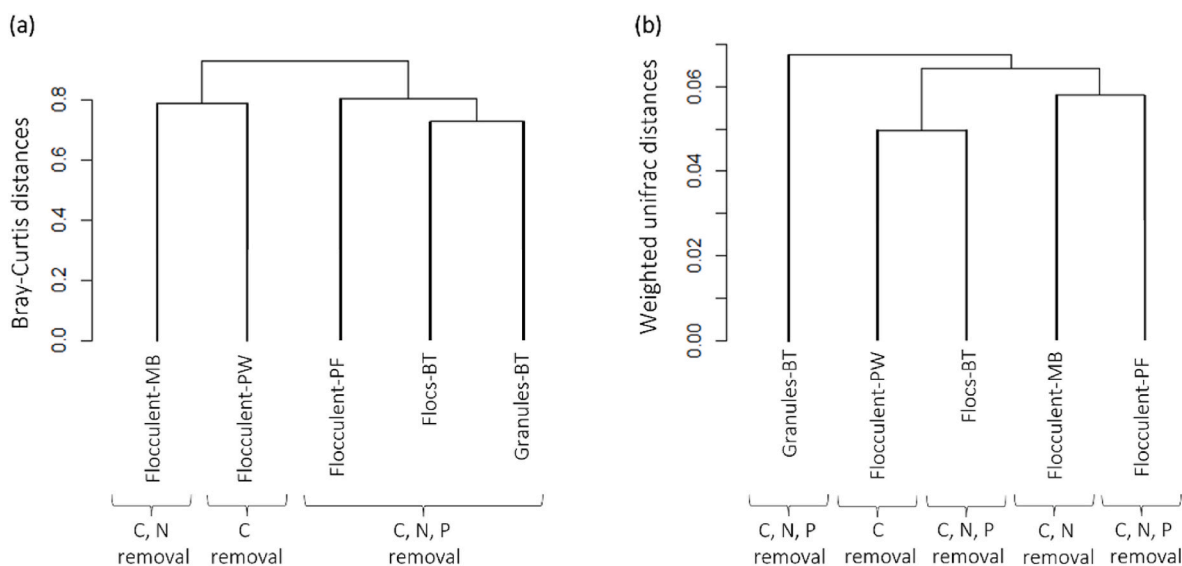
### 3.1. Effects of different nutrient removal processes and sludge type on the diversity of sludge microbial communities

1861 OTUs in total were assigned in 5 sludge samples from 4 different WWTPs, with a sequence identity of 97% and above. As shown in Table 1, the OTU number of sludge samples from different WWTPs ranged between 787 and 1565. It was found that granular sludge from the Birmingham WWTP had the highest OTU number whereas flocculent sludge from Portswood WWTP had the lowest OTU number. ACE and Chao1 are the indexes indicating species richness in the community while Simpson and Shannon diversity indices describe diversity and complexity of the community, respectively. Moreover, Faith pd represents the biodiversity based on phylogenetic differences of species in the community and Pielou's evenness describes how evenly the species are distributed in the community. It was found that all indices except for Pielou's evenness were the highest in granular sludge followed by the flocculent sludge from the same tank at Birmingham WWTP, the flocculent sludge from Petersfield WWTP with C, N and P removal, flocculent sludge from Millbrook with C and N removal and flocculent sludge from Portswood with C removal only. This consistent trend on indices indicates that granular structure is better for higher richness, higher evenness, and diversity. It was also found that ACE, Chao1 and Shannon values of flocs from the same tank at Birmingham WWTP were lower than those in granular sludge but higher than flocculent sludge from Petersfield. This suggests that the compact and three-dimensional structure is key to retain more diverse species due to a more stable and confined microenvironment in granules. It has been reported that granular sludge could maintain a dynamic balance between granules and flocs due to disintegration and re-formation (Yong Qiang Liu et al., 2010; Liu and Tay, 2012). From this point of view, it is not surprising that the microbial structures of granular sludge and flocculent sludge in the same tank influence each other, resulting in higher richness and diversity of flocculent sludge at Birmingham WWTP than Petersfield although both plants were for COD, N and P removal. In addition, the consistent trend on indices also suggests that process design for more types (e.g., N and P) of pollutant removal promotes the richness, diversity, and phylogenetic difference of microbial population.

Both Bray Curtis and Weighted Unifrac distance were performed as shown in Fig. 1 by clustering analysis to study the beta diversity of the microbial community of sludge. Bray Curtis distance matrix is a quantitative measure of community dissimilarity whereas Weighted Unifrac distance matrix is a quantitative measure of community dissimilarity that incorporates phylogenetic relationships between the features. It can be seen from Fig. 1a that microbial communities were clustered into two groups with a distance of  $\sim$ 0.9 based on C, N, and P removal processes and C and N removal or C removal processes. This indicates that the beta diversity based on Bray Curtis distance was driven more by process design and operation with/without P removal or continuous flow or

**Table 1**  
Alpha diversity of the microbial community in different sludge types.

Biological nutrient removal	Treatment processes	WWTP	Sludge type	Number of OTUs	ACE	Chao1	Simpson	Shannon	Faith pd	Pielou's Evenness
C, N, P	AGS-SBR	Birmingham (BT)	Granular	1565	1650	1647	0.996	9.13	108	0.87
			Flocs	1244	1334	1333	0.994	8.69	84	0.85
C, N, P	AS-SBR	Petersfield (PF)	Flocculent	1146	1188	1187	0.994	8.65	83	0.85
C, N	ASP	Millbrook (MB)	Flocculent	1001	1018	1017	0.990	8.33	81	0.84
C only	ASP	Portsmouth (PW)	Flocculent	787	793	792	0.994	8.32	62	0.87



**Fig. 1.** Hierarchical clustering analysis based on (a) Bray-Curtis distance and (b) weighted unifrac distance of microbial diversity in granular and flocculent sludge from four WWTPs.

SBR. Zhang et al. (2012) also reported that the microbial community in activated sludge from 15 WWTPs could be clustered up on similar/different environmental conditions such as geography and sewage properties. In addition, it was noticed that granular and flocculent sludge from Birmingham WWTP had a Bray Curtis distance of  $\sim 0.7$  although they were from the same tank. This suggests that sludge type still had an important effect on microbial diversity. Similarly, Winkler et al. (2013) revealed that granular sludge from SBR and flocculent sludge from continuous process, respectively, treating the same wastewater with the same relative hydraulic variations had very dissimilar bacterial populations.

On the contrary, beta diversity between sludge samples was driven by sludge types more than operational conditions based on Weighted Unifrac distance where a phylogenetic relationship was involved. As shown in Fig. 1b, microbial communities in sludge based on Weighted Unifrac distance where phylogenetic relationship was involved could be clustered into two main groups of granular sludge and flocculent sludge; even though one flocculent sludge was from the same reactor as granular sludge. This suggests that microbial communities in flocculent sludge from different treatment processes could share similar microbial structure and functions, meanwhile, microbial communities in granular sludge contained more unique taxa that could play unique roles for the formation of granule structure on top of performing wastewater treatment. Among different types of flocculent sludge, however, a distance of 0.04–0.06 was observed, indicating the effects of processes and nutrient removal on Beta diversity. The result differences of the two clustering analysis methods indicate that flocculent sludge from different plants are phylogenetically close to each other although they demonstrate large dissimilarity based on Brey Curtis clustering.

### 3.2. The comparison of microbial population in sludge from different WWTPs

At the phylum level, a clear difference between granular and flocculent sludge was noticed with Chloroflexi of 19% relative abundance in granular sludge (Fig. 2a). The two most abundant classes in phylum Chloroflexi in granular sludge was Anaerolineae and Chloroflexia, which were mainly consisted of facultative/anaerobic filamentous bacteria. The layered structure of granules with anaerobic, anoxic and aerobic zones is conducive to the retainment of facultative/anaerobic bacteria (Tay et al., 2002). No Chloroflexi was detected in the sludge from the process with only C removal. This result is in agreement with the studies that Chloroflexi was consistently abundant in WWTPs with nutrient removal, long SRT and sludge exposed to anaerobic conditions (Speirs et al., 2019). It was reported that Chloroflexi might make a substantial contribution to nutrient transformations during wastewater treatment (Nierychlo et al., 2019). Also, it was speculated that the abundance of filamentous bacteria in granules was observed to have a higher ability for nitrification, denitrification and carbon metabolism (Song et al., 2022), but filamentous bacteria belonging to the phylum Chloroflexi at a certain level was reported to cause sludge bulking with reduced sludge settling ability. Thus, excessive Chloroflexi is definitely not wanted in WWTPs, but a low level of filamentous Chloroflexi might be helpful for the formation of flocs and conducive to forming the backbone for granulation or maintaining the stable structure of granules (Xia et al., 2018). It needs to be pointed out that filamentous bacteria such as *Kouleoithrix* and *Caldilineaceae* (family) had relative abundance of 3.0% and 4.0% in granules, which was 10 and 4 times higher than in flocs from the same tank, respectively. A higher abundance of *Kouleoithrix* (2.2–4.8%) in granules compared to that in flocs (1.3–2.5%) was

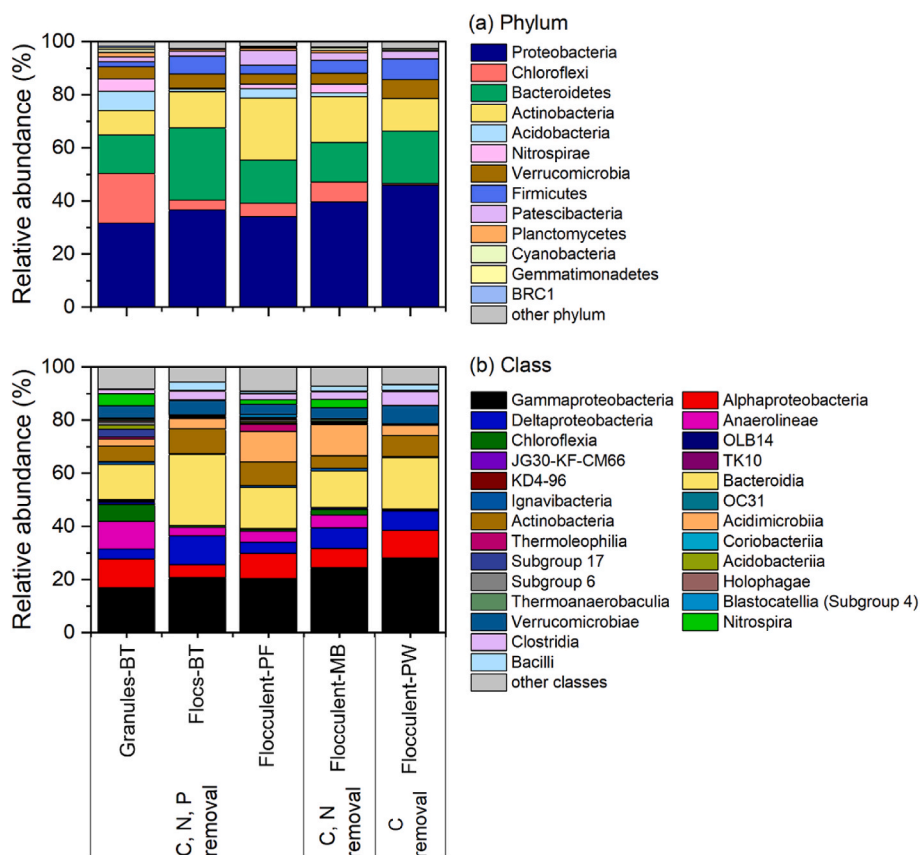


Fig. 2. Relative abundance of microbial phyla (a) and classes (b) in granular and flocculent sludge from four WWTPs.

also reported in the study by Layer et al. (2019). Qiu et al. (2022) also found a high abundance of *Kouleothrix* (8%) in mature granular sludge, which might facilitate granulation by providing a skeleton for granule interior structure and allowing a stable environment for diverse microorganisms to attach to the entangled filaments and maintain structure of granules.

Additionally, it was interesting to note from Fig. 2 that the microbial community structure of flocs in the granule dominant tank in Birmingham WWTP was more similar to those from flocculent sludge-based processes in other plants although those flocs were from the same tank of granular sludge. The Venn diagram of OTUs presented in granules and flocs from Birmingham WWTP as shown in Fig. 3 suggests only 36.8%

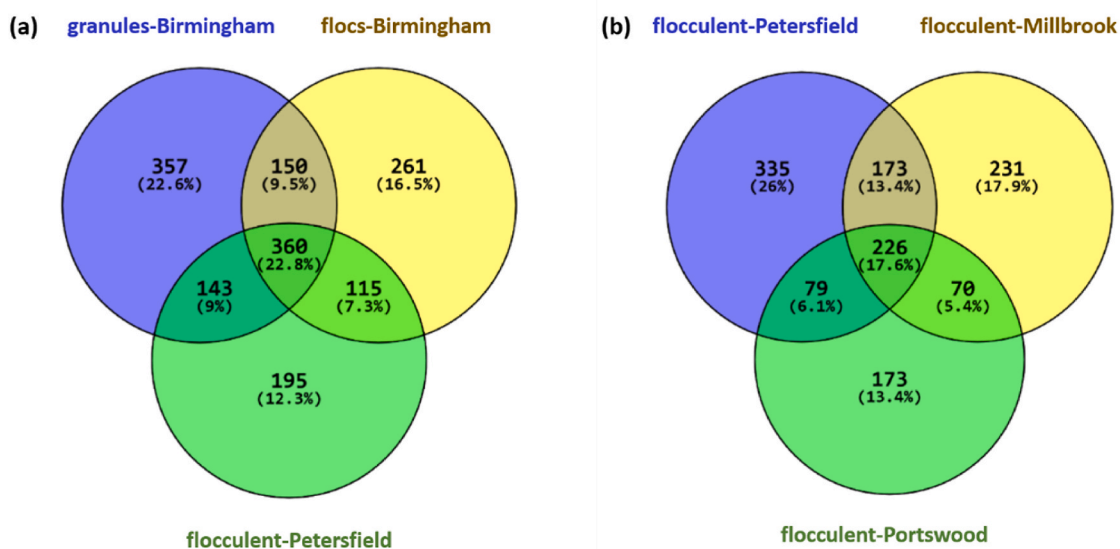


Fig. 3. Venn diagrams of OTUs in different types of sludge from four different WWTPs: (a) granular and flocculent sludge from WWTPs for C,N and P removal at Birmingham (Granules + flocs) and Petersfield (flocculent sludge), and (b) flocculent sludge from WWTPs for C,N and P removal at Peterfield, C and N removal at Millbrook, and for C removal at Portswood.

shared OTUs among total OTUs found in granules and flocs. All of these indicate that the sludge structure can significantly affect microbial community structure. The presence of mixed granular sludge and flocs in WWTP in Birmingham WWTP with a more diverse microbiome could benefit pollutant removal more than pure flocs. Among the shared OTUs between granules and flocs in the same tank, the top 3 shared species with the highest abundance in granules were *Nitrospira defluvii*, *Tetrasphaera* sp., and *Kouleothrix* sp. *Nitrospira defluvii* belongs to Nitrospira sublineage I, which was originally believed as a nitrite-oxidizing bacteria (NOB) (Daims et al., 2001) for sewage treatment while it was discovered in the last decade (Mehrani et al., 2020) for being capable of performing complete ammonium oxidation (comammox). *Tetrasphaera* sp. has recently been determined to have at least equal functional importance as *Candidatus Accumulibacter* for being polyphosphate-accumulating organisms (PAOs) (Singleton et al., 2022). *Kouleothrix* sp. is a filamentous organism under phylum Chloroflexi, which helps with floc formation and settling at low levels but cause sludge bulking at high levels (Nittami et al., 2019). These three species were all important functional species for nitrogen and phosphorus removal as well as helping with the formation of flocs or granules. Their presence in both granules and sludge implies that both flocs and granules contributed to nutrient removal. However, the dominance of granules (i.e., 88% of the total sludge) determined its major role in nutrient removal.

The Venn diagram in Fig. 3a demonstrates that among a total of 1581 OTUs, 618 of them were shared between granule dominant sludge at Birmingham (granules + Flocs) and flocculent sludge at Petersfield for C, N and P removal, which accounted for 39% of total OTUs. Among these shared OTUs, *Nitrospira defluvii*, *Tetrasphaera* sp., and *Propionivibrio* sp. were the top 3 abundant species. *Nitrospira defluvii* and *Tetrasphaera* sp. were believed to contribute to biological N and P removal. *Propionivibrio* sp. was referred to GAO (Albertsen et al., 2016; Roy et al., 2021), but it was also reported to be capable of fermenting organic molecules to VFAs (Albertsen et al., 2016) to facilitate biological N and P removal or to be putative PAO (Coats et al., 2017; C. Li et al., 2019). The result that the most abundant overlapped OTUs in two different WWTPs are putative functional organisms for N and P removal is interesting. Both plants adopted SBR technology treating similar sewage although one was based on granular sludge while the other was based on conventional suspended flocculent sludge with different operating conditions. The results imply that the similar wastewater quality, geographical region, weather condition and operating mode (SBR) were important for the enrichment of same types of functional organisms for N and P removal. In addition, 768 OTUs or 49% of total OTUs were found uniquely in granule-dominant sludge (both granules and floc) at Birmingham WWTP while 195 OTUs or 12% were found uniquely in flocculent sludge at Petersfield WWTP, suggesting more diverse microbial community in granule-dominant sludge to contribute to granule structure or to be attributed to the layered structure with 3-dimensional anaerobic, anoxic and aerobic microenvironment. In contrast, as shown in Fig. 3b and 31% of OTUs were shared between flocculent sludge from Petersfield WWTP for C, N, P removal and flocculent sludge from Millbrook for C, N removal, which was lower than that with granule dominant sludge at Birmingham, but higher than that with flocculent sludge from Portswood WWTP for C removal only. The dominant OTUs shared between Bsludges at Petersfield and Millbrook WWTPs were *Nitrospira defluvii* (1.6–3.2%), and *Dokdonella* sp. (0.3–0.8%), which were associated with nitrification and denitrification. The third dominant and shared genus was *Kouleothrix* sp. (0.3–0.6%), which was conducive to the formation of flocs at the low level. Again, from different plants, the dominant and shared OTUs were functional organisms for biological N removal and flocs formation as the sludge at Millbrook WWTP was not designed for biological P removal. In addition, it was noticed that only 17.6% of the total OTUs were shared in flocculent sludge from Petersfield WWTP for C, N, P removal, Millbrook WWTP for C, N removal and Portswood WWTP for C removal only although sludge

was all flocculent based, treating similar sewage at similar weather conditions. This implies from another perspective that microorganisms for COD removal could be more diverse and their enrichment and presence were probably more random or related to the interaction with functional organisms for nutrient removal and flocs formation.

### 3.3. Functional bacteria for N and P removal in sludge from four WWTPs

#### 3.3.1. Comparison of dominant functional genera in granules and flocs from the aerobic granules-based plant at Birmingham WWTP

The relative abundances of key or functional microorganisms in granules and floc from aerobic granules based Birmingham plant were presented in genus level in Fig. 4. It was observed that bacteria that facilitate floc formation such as *Zoogloea* and *Ferruginibacter* were more abundant in flocs than in granules. For nitrification, it was observed that the relative abundance of nitrifying bacteria such as *Nitrospira* (comammox bacteria), *Nitrosomonas* (ammonium oxidizing bacteria (AOB)), and *Candidatus Nitrotoga* (Nitrite oxidizing bacteria (NOB)) was higher in granules than in floc by 14, 4 and 8 times, respectively. Other literature also reported that *Nitrospira* was enriched in granules more than in flocs (Ali et al., 2019; Layer et al., 2019). Higher abundances of *Nitrosomonas* and *Candidatus Nitrotoga* were also reported in granules with abundances of 0.8% and 1.6% compared to 0.2% and 1.3% in flocs, respectively, in a low-SRT system treating anaerobic digester dewatering centrate (Figdore et al., 2018a). This suggests that autotrophic slow-growing nitrifying bacteria were more easily enriched in granules than flocs due to the longer actual SRT of granules and their tendency to form relatively dense and strong microcolonies (Larsen et al., 2008). Thus, granular sludge is expected to play a positive role for the retention of nitrifying bacteria, benefiting nitrification. PAOs were also enriched in both granules and flocs. One of the dominant PAOs in both granules and flocs was *Tetrasphaera* with relative abundances of 4.0% in granules and 4.4% in flocs. *Dechloromonas* (PAO) and *Candidatus Accumulibacter* (PAO) were also highly abundant, particularly in granules (1.6% and 1.3%).

The total abundance of PAOs in flocs was almost the double of granules, which was mainly because of the putative denitrifying phosphate-accumulating organisms (DPAOs) *Flavobacterium*. *Flavobacterium* was reported as putative denitrifying PAO and is also important for granular formation due to extracellular polymeric substances (EPS) production (Suhonen et al., 2023), but its much higher abundance (6.5% in flocs) than granules (0.5%) suggests that the physiology of *Flavobacterium* in N and P removal needs further investigation. Similar trends were reported by Figdore et al. (2018b). However, Layer et al. (2019) found that the abundance of *Tetrasphaera* was higher in granules than in flocs whereas the abundance of *Candidatus Accumulibacter* was higher in flocs than in granules. Other putative PAOs were also found in either granules or flocs. For example, *Gemmatimonas* was found mainly in granules with abundance of 0.1%, whereas *Acinetobacter*, *Pseudomonas*, *Halomonas*, and *Tessaracoccus* were found only in floc with an abundance of 1.5%, 0.4%, 0.1% and 0.01%, respectively. These indicate that unlike nitrifying bacteria, granule structure is not key for the abundance of specific type of PAOs and DPAOs. Since it is well-known that the SRT of flocs is much shorter than granules, it is generally believed that flocs are unfavorable for retaining slow-growing PAOs (Kreuk and Loosdrecht, 2018). But the equivalent abundance of PAOs excluding *Flavobacterium* or much higher abundance including *Flavobacterium* in flocs than granules may indicate that the enrichment of PAOs as heterotrophic bacteria was not affected by the difference of SRT of flocs and granules. In general, the shorter SRT of flocs made flocs harder to be retained in the reactors compared with granules. By considering the volume ratio of granules and flocs in the granule-dominant sludge at Birmingham WWTP, the total abundance of PAOs was 6.5% in granules and 1.7% in flocs (Fig. S1), suggesting granules still contributed more to P removal due to their volume dominance. Except PAOs, GAOs such as *Candidatus Competibacter*,

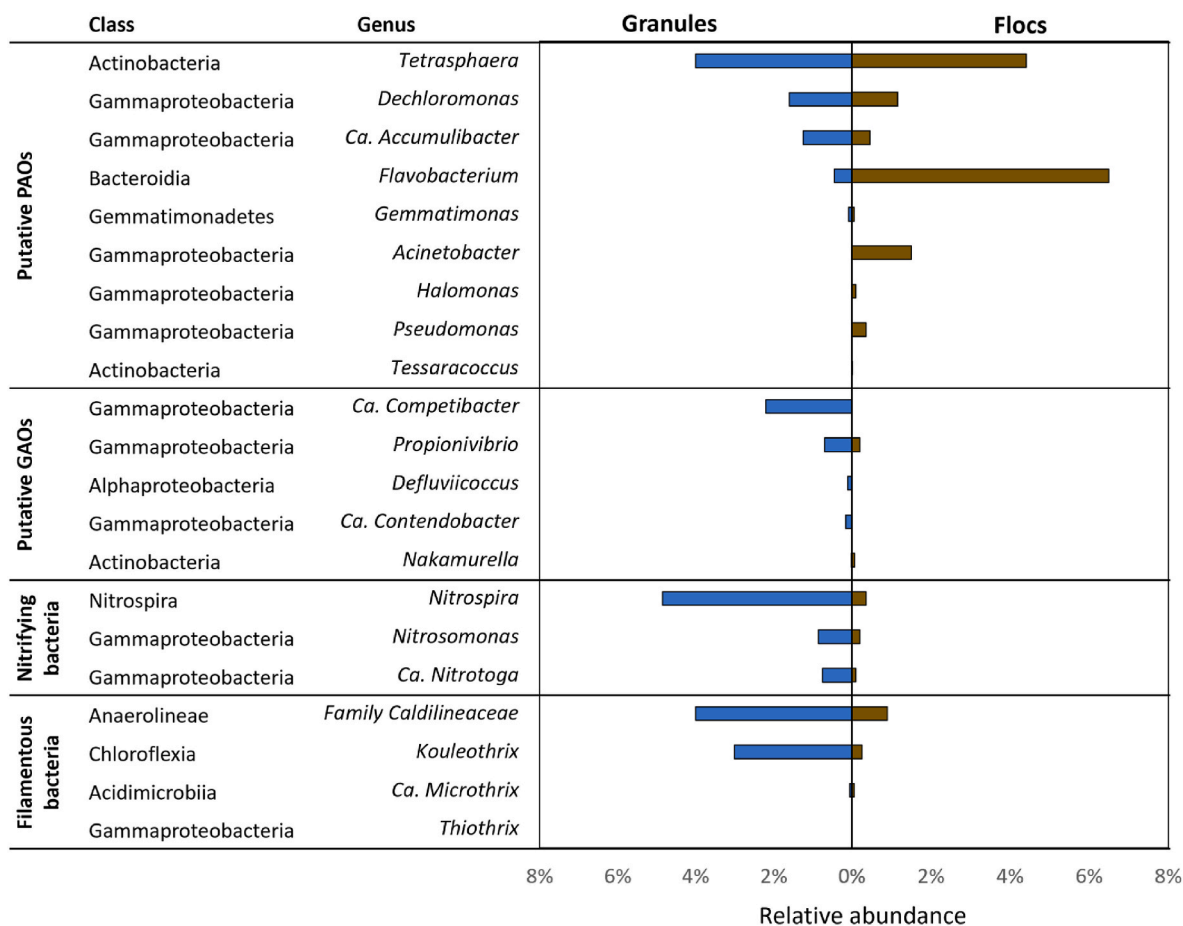


Fig. 4. Relative abundance of important genera in granules and flocs, respectively, from Birmingham WWTP.

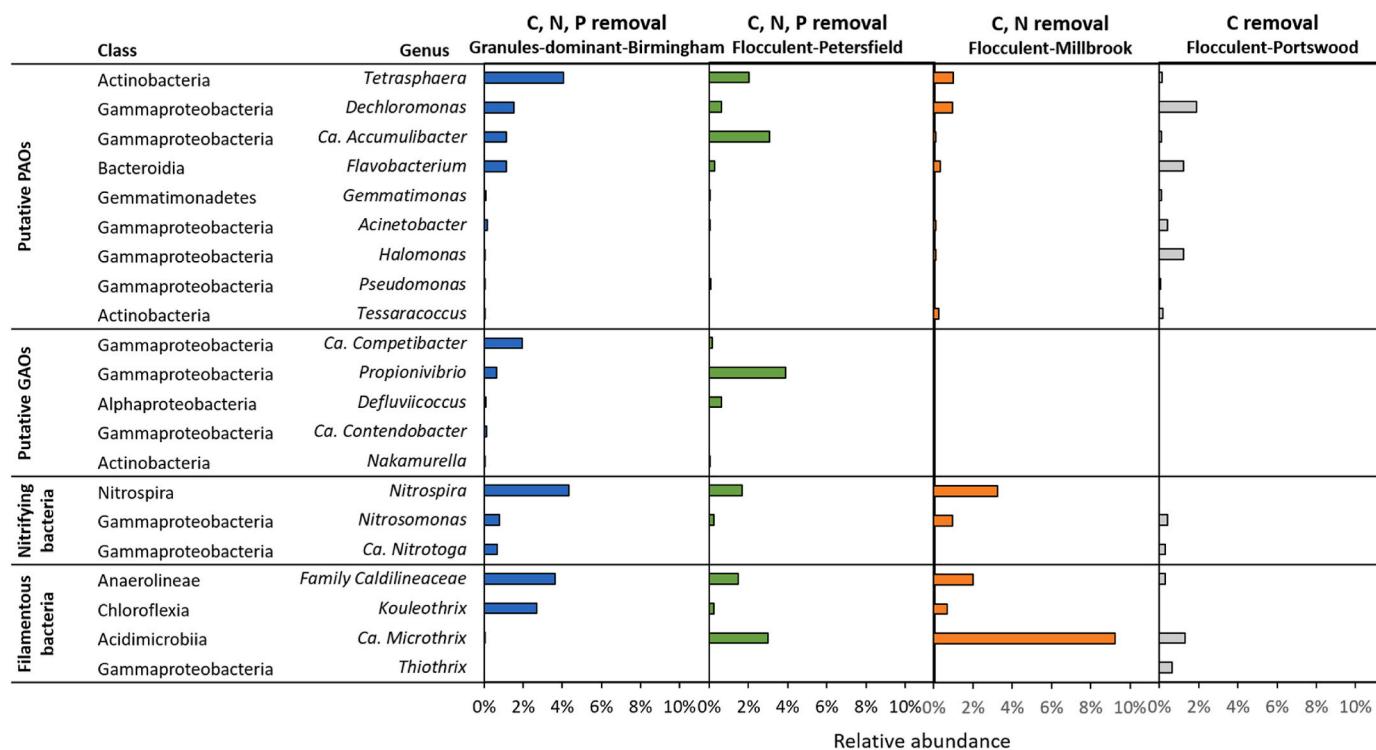
*Propionivibrio*, *Defluviicoccus*, *Candidatus Contendobacter*, and *Nakamurella* were also observed with abundances of 1.9%, 0.6%, 0.1%, 0.1%, and 0.02%, respectively, in granule-dominant sludge from Birmingham WWTP and the GAOs were mainly found in granules rather than in flocs. This is in agreement with previous studies that GAOs, i.e., *Candidatus Competibacter*, were more abundant in granules (0.8–2.6%) (Figdore et al., 2018b; Layer et al., 2019). It appears that GAOs tend to grow in granular sludge more than in flocs when both of them co-exist in a system. The total abundance of GAOs in flocs was 3.2%, which was 2.3 times lower than the total abundance of PAOs in granules. More recent research has begun to challenge the typical understanding regarding the competitiveness of GAOs with PAOs and their negative effects on phosphorus removal (Nielsen et al., 2019). From a sludge structure perspective, granules and flocs in this study did not show much difference in terms of PAOs but more GAOs in granules.

### 3.3.2. Comparison of dominant functional genera in sludge from different WWTPs

In Fig. 5, it can be seen that *Nitrospira* was enriched in flocculent sludge for C, N, and P removal at Petersfield and C, N removal at Millbrook WWTP but not for C removal only at Portswood WWTP due to a much shorter SRT (3–8 days). A previous study reported that the minimum HRT for the enrichment of *Nitrospira* should be around 14–16 h (Winkler et al., 2017). *Nitrospira* was reported as the most dominant comammox bacteria and functionally dominated ammonia oxidation in 25 other full-scale WWTPs (Zheng et al., 2023). Abundances of *Nitrospira* were reported in a range of 0.8–6.1% in sludge for C, N removal (Xu et al., 2017) and 0.2–5.7% in sludge for C, N, P removal (Kong et al., 2007; Lemaire, 2007; Nielsen et al., 2010), respectively. AOB such as *Nitrosomonas* was found in sludge for N removal, but *Nitrosomonas* was

three times more abundant in granules dominant sludge at Birmingham than flocculent sludge at other WWTPs. Also, NOB such as *Candidatus Nitrotoga* was found only in granules dominant sludge. *Candidatus Nitrotoga* was also reported in another full-scale aerobic granules-based WWTP in Sweden (Ekholm et al., 2022) and it was reported to be a cold-adapted NOB (Spieck et al., 2021). However, in this study, samples were taken in summer and *Candidatus Nitrotoga* was also detected in flocculent sludge without much nitrification capability for only C removal at Portswood WWTP. The conditions for the enrichment of *Candidatus Nitrotoga* needs further investigation because it seems that temperature is not the only factor to influence its enrichment. Winkler et al. (2013) reported that only *Nitrosomonas* was the dominant AOB in flocculent sludge, whereas both *Nitrosomonas* and *Nitrospira* were found in granular sludge with comparable abundances. These suggest that aerobic granular sludge could enrich nitrifying bacteria better than flocculent sludge (Liu et al., 2015) in terms of abundance and diversity in C, N and P removal and C, N removal processes when they are in the same geographic region with similar sewage quality. It could be due to a more stable environment and the high retention capacity of slow-growing bacteria such as nitrifying bacteria in compact granule structures (Xia et al., 2018). No nitrifying bacteria was detected from sludge for C removal only at Portswood WWTP, indicating that the presence of nitrifying bacteria could be used as an indicator for nitrification. The abundance of nitrifying bacteria from three WWTPs with N removal varied from 2% (Petersfield) to 5.8% (Birmingham) although three WWTPs including Millbrook performed excellent N removal. From this aspect, the abundance of nitrifying bacteria could not be directly used to indicate nitrogen removal efficiency.

It is interesting to note that putative PAOs were present in all sludge including the sludge with only C removal at Portswood WWTP, which



**Fig. 5.** Relative abundance of functional microorganisms groups in granule-dominant sludge (including both granule and floc fractions) from Birmingham WWTP and flocculent sludge from Petersfield, Millbrook and Portswood WWTPs. The bars show the mean values of duplicates. The relative abundance of microorganisms in granule-dominant sludge from Birmingham WWTP was calculated from the sum of relative abundance in granules and flocs in the sludge based on the ratios of granules to flocs of 7.6.

included 5.4% PAOs such as 1.3% *Flavobacterium*, 1.9% *Dechloromonas*, 1.3% *Halomonas* and other PAOs, but typical PAOs such as *Tetrasphaera* and *Candidatus Accumulibacter* were only 0.2% and 0.1%, respectively. *Dechloromonas* was reported as the second largest putative PAOs followed *Tetrasphaera* in 18 Danish WWTPs with EBPR (Stokholm-Bjerregaard et al., 2017), but there are few papers to report the comparison between EBPR and C removal-only processes. According to the design, operation, and performance of WWTP at Portswood WWTP, there were not supposed to have enriched PAOs for biological phosphorus removal. Although *Flavobacterium*, *Dechloromonas*, *Halomonas* and others have been reported as putative PAOs, they might be able to demonstrate PAO's function only under alternating anaerobic and aerobic conditions with external carbon sources present. In addition, given shorter SRT and HRT used in the WWTP at Portswood with only C removal, the presence of multiple putative PAOs especially those with abundance higher than 1% indicates that PAOs grow much faster compared with nitrifying bacteria although PAOs were usually claimed as slow-growing bacteria. From the result of this study, they seem not significantly be restricted by SRT used for the operation. But it is still unclear that how much contribution that *Flavobacterium*, *Dechloromonas*, and *Halomonas* could contribute to P removal under appropriate conditions. *Tetrasphaera* and *Candidatus Accumulibacter* have been investigated much more than other genera and have partially demonstrated their phosphorus removal ability. The percentages of *Tetrasphaera* and *Candidatus Accumulibacter* in two WWTPs at Birmingham and Petersfield for C, N, and P removal were 5.2%, followed by the WWTP for C,N removal with a percentage of 1.1% and the WWTP for C removal only with a percentage of 0.3%. The high percentages of total PAOs and tiny percentages of *Tetrasphaera* and *Candidatus Accumulibacter* in sludge for C removal only at Portswood WWTP might indicate that their abundance could be used as an indicator for biological phosphorus removal potential.

Flocculent sludge had a much higher abundance of *Candidatus Microthrix* (3.0% in sludge for C,N and P removal and 9.3% in sludge for

C, N removal), a filamentous organism which is probably the most problematic bulking and foaming organism in nutrient removal plants (Nierychlo et al., 2021) than in granular sludge. However, although a high percentage of *Candidatus Microthrix*, there was no sludge bulking problem in the WWTPs investigated. About 6–17% of PAO abundance was reported in granules from a full-scale aerobic granular plant and the abundance increased with granule size (Ali et al., 2019), while PAO abundances ranging from 5 to 22% were reported in flocculent sludge from 18 full-scale EBPR plants (Stokholm-Bjerregaard et al., 2017). 6.3–8.2% of total PAOs in granular and flocculent sludge fitted in the ranges reported very well. Accordingly, it suggests that the architecture of sludge did not affect overall abundance of PAOs but it did affect compositions of PAOs communities, in which *Tetrasphaera* was the most dominant PAO in granular sludge whereas *Candidatus Accumulibacter* was the main PAO in flocculent sludge in this study. One of the main reasons for this could be that a fermentation tank was before the main reactor for flocculent sludge at Petersfield WWTP while raw sewage was directly fed into the main reactor at Birmingham, causing the higher abundance of VFA utilising *Candidatus Accumulibacter* in Petersfield WWTP.

A higher abundance of putative GAOs (4.8%) including *Candidatus Competibacter*, *Propionivibrio Defluviicoccus*, *Candidatus Contendobacter*, and *Nakamurella* was found in flocculent sludge at Petersfield WWTP than in granular sludge at Birmingham (2.8%). Significant GAO enrichment was reported in aerobic granular sludge reactors with the addition of acetate and propionate (D. C. Li et al., 2020). Thus, the easy access to VFA might be the main reason for the higher abundance of GAOs at Petersfield WWTP. However, Petersfield WWTP had excellent and stable P removal while Birmingham WWTP had to have periodical ferric dosing to meet the phosphorus permit. This phenomenon is consistent with the report that GAOs might not have negative effects on biological phosphorus removal (Nielsen et al., 2019). On the contrary, Nielsen et al. (2019) claimed that a moderate amount of GAOs may be a



good sign for efficient EBPR because they indicated a surplus of organics, which was supported by the result in this study as Petersfield WWTP had better P removal efficiency. However, it has been pointed out that the percentage of *Candidatus* Competibacter in sludge at Petersfield WWTP was only 0.2% while it was 1.9% in sludge at Birmingham WWTP. Similar to PAOs, many putative GAOs need further investigation about their functions and metabolic competition with PAOs.

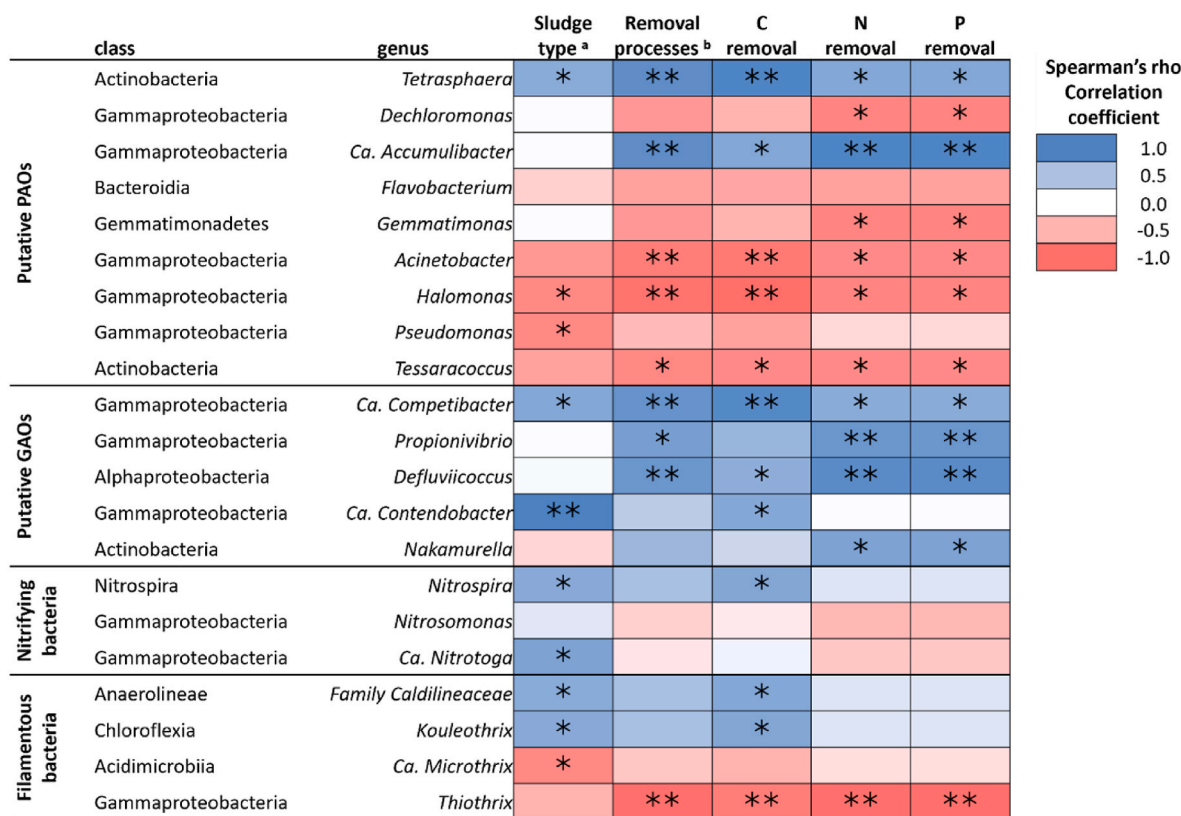
Fig. S2 shows the relative abundance of denitrifying bacteria from four WWTPs. It can be seen that denitrifying bacteria were enriched in the Portswood WWTP with a relative abundance of 15% although Portswood WWTP did not possess nitrification and denitrification capability. This indicates that many denitrifying bacteria such as *Rhodoferrax*, *Rhodobacter*, *Zoogloea*, *Iamia*, *Arcobacter*, and *Acidovorax* had versatile metabolisms, capable of utilising different electron acceptors and growing under aerobic conditions (McIlroy et al., 2016; Midori et al., 2009; E. Rosenberg et al., 2014; Willems and Gillis, 2015; Zhu et al., 2020). This is the main reason that denitrification rarely could be a problem as long as denitrification conditions are provided such as sufficient carbon source and low dissolved oxygen. Therefore, the relative abundance of denitrifying bacteria is not as meaningful as nitrifying bacteria, PAOs and GAOs for indicating nutrient removal performance.

The Family Saprospiraceae is crucial for treating real sewage due to its ability to hydrolyse and utilize complex carbon sources. It was found that the Family Saprospiraceae (uncultured) was one of the most dominant families in all WWTPs studied. It was ranked first at the Petersfield WWTP for CNP removal, fourth at the Birmingham WWTP with CNP removal, third at the Millbrook WWTP with CN removal, and second at the Portswood WWTP with C removal only. The most

dominant genus at the four WWTPs was as follows: *Nitrospira* at Birmingham (granule dominant), Family Saprospiraceae at Petersfield (with a fermentation tank), *Candidatus* Microthrix at Millbrook (continuous operation for CN removal), and *Hydrogenophaga* at Portswood (continuous operation for C removal only). *Zoogloea*, known for its role in floc formation through extracellular polysaccharide excretion, was the second most dominant (6.2%) at Millbrook and fifth (2.8%) at Portswood in continuously operated tanks. In contrast, its dominance was only 0.4% at Birmingham and 0.2% at Petersfield WWTPs with CNP removal in SBRs, which have excellent sludge-settling ability. This suggests that a single floc forming genus could not imply the sludge settleability.

3.4. Relationship between operational variables and microbial community in sludge

To further investigate the relationship between the abundance of important genera and processes for nutrient removal, sludge type and removal efficiencies, correlation analysis was conducted based on Spearman's rho coefficient (Fig. 6). It was found that only *Tetrasphaera* and *Ca. Accumulibacter* were positively correlated with C, N, P removal process and P removal efficiency with which all other putative PAOs were negatively correlated. This result highlights the importance of *Tetrasphaera* and *Ca. Accumulibacter* as PAOs for P removal efficiency and C,N, P process investigated in this study. Meanwhile, it questions the validity or reliability to use the total abundance of all putative PAOs reported in the literature to interpret EBPR performance and the competition between PAOs and GAOs. Thus, we would like to highlight the importance to compare microbial community of EBPR with that in



<sup>a</sup> sludge types were coded with 1 – flocculent sludge and 2 – granular-dominant sludge.  
<sup>b</sup> removal processes were coded with 1 – C removal, 2 – C, N removal, and 3 – C, N, P removal.  
 \*\* Correlation is significant at p-value < 0.01.  
 \* Correlation is significant at p-value < 0.05.

Fig. 6. Correlation between the abundance of important genera and wastewater treatment processes (C,N,P removal or without P removal), sludge types (granules or flocculent), and removal efficiencies of C, N and P, based on Spearman's rho coefficient.

the processes without EBPR for better understanding the possible functions and versatility of putative PAOs. In addition, the positive correlation of GAOs with the process for C, N, P removal, P and COD removal efficiency suggests that GAOs were more likely to co-exist with PAOs as the literature reported (Santos et al., 2020; D. Wang et al., 2019) and contributed to COD removal by taking up COD. From this aspect, it seems that the presence of a certain level of GAOs might be beneficial for wastewater treatment as well as P removal efficiency, which supports the report by Nielsen et al. (2019) that there was no well-documented case of GAO-induced full-scale EBPR problems in the literature. Our understanding of GAOs' function needs further investigation for the better control of processes for stable and excellent EBPR. For sludge type, granular sludge was generally more positively correlated with GAOs and nitrifying bacteria, suggesting granular sludge is a better medium to retain nitrifying bacteria and GAOs for N and C removal. But it has to be pointed out that other environmental factors also affect nutrient removal even when functional bacteria were retained sufficiently (Yong Qiang Liu and Tay, 2012) just as the sludge in Birmingham WWTP. This implies the higher abundance of key functional general does not necessarily lead to better performance, thus, operational conditions should be optimised to create the best environment for nutrient removal.

#### 4. Conclusions

- The richness and diversity of the microbial population in sludge increased with pollutant removal from only C, C and N, to C, N, P removal processes. For C, N and P removal, granule structure led to a more diverse and rich microbial community structure than flocculent structure.
- The microbial community structure of granules and flocs had a distinct difference with more filamentous bacteria, nitrifying bacteria and GAOs enriched in granules than flocs from the same tank while with an equivalent abundance of PAOs if excluding putative PAO *Flavobacterium*. This indicates sludge structure, i.e., granules or flocs, and relevant SRT affected the enrichment of key bacteria such as filamentous bacteria, nitrifying bacteria and GAOs, but not PAOs.
- The abundance of PAOs from sludge for only C removal at Portswood WWTP was just slightly lower than those from other WWTPs for C, N and P removal, suggesting many putative PAOs might not contribute to phosphorus removal, and they can grow faster and be retained in tanks under shorter SRT. The most typical putative PAOs such as *Tetrasphaera* and *Candidatus Accumulibacter* seemed to be more correlated with biological phosphorus removal, which could be used as an indication for P removal potential.
- The putative GAOs are the most abundant at Petersfield WWTP with the best biological P removal performance. This supports the claim reported in the literature that a certain level of GAOs might be a good sign of excess organic carbon in EBPR for good P removal efficiency. But *Candidatus Competibacter*'s abundance at Petersfield WWTP was the least, so the function of GAOs especially *Candidatus Competibacter* and its competition with PAOs need further investigation.

#### CRediT authorship contribution statement

**Tararag Pincam:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Yong-Qiang Liu:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization. **Alexander Booth:** Writing – review & editing. **Yi Wang:** Writing – review & editing. **Guihong Lan:** Writing – review & editing. **Ping Zeng:** Writing – review & editing.

#### Declaration of competing interest

There is no conflict of interest.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.chemosphere.2024.142644>.

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