Bacterial community and filamentous population of industrial wastewater treatment plants in Belgium

Journal name: Applied Microbiology and Biotechnology

Title

Bacterial community and filamentous population of industrial wastewater treatment plants in Belgium

Authors

Karina Seguel Suazo[a], Thomas Dobbeleers[a] and Jan Dries[a*]

Affiliation [a*]University of Antwerp, Department of Applied Engineering, Biochemical Wastewater Valorization and Engineering (BioWAVE), Groenenborgerlaan 171, 2020, Antwerp, Belgium.

Corresponding author Jan Dries[a*] jan.dries2@uantwerpen.be ORCID: 0000-0002-0204-5697

Abstract

The discharge of industrial water requires the removal of its pollutants, where biological wastewater treatment plants (WWTPs) are the most used systems. Biological WWTPs make use of activated sludge (AS), where bacteria are responsible for the removal of pollutants. However, our knowledge of the microbial communities of industrial plants is limited. Understanding the microbial population is essential to provide solutions to industrial problems such as bulking. The aim of this study was to identify at a high taxonomic resolution the bacterial population of 29 industrial WWTPs using 16S rRNA Amplicon Sequencing. Our results revealed that the main functional groups were dominated by *Thauera* and *Zoogloea* within denitrifiers, *Dechloromonas* in phosphate-accumulating and *Defluvicoccus* in glycogen-accumulating organisms. The activated sludge characterization indicated that 59% of the industrial plants suffered from bulking sludge, with DSVI values up to 448 mL/g. From the bulking cases, 72% corresponded to filamentous bulking with *Thiothrix* as most abundant filament, meanwhile the other 28% corresponded to viscous bulking sludge in which *Zoogloea* was the most abundant genus. Furthermore, the bacterial population did not share a core of taxa across all industrial plants. However, 20 genera were present in at least 50% of the plants comprising the general core, including *Thauera*, *Ca.* Competibacter and several undescribed microorganisms. Moreover, statistical analysis revealed that wastewater salinity strongly affected the microbial richness of the industrial plants. The bacterial population across industrial plants differed considerably from each other, resulting in unique microbial communities that are attributed to the specificity of their wastewaters.

Keywords: Industrial activated sludge WWTP, filamentous bacteria, viscous bulking.

Key points

- The general core taxa of industrial plants was mostly made up of undescribed bacterial genera.
- Filamentous bacteria constituted on average 4.1% read abundance of the industrial WWTPs.
- Viscous bulking remains a significant type of bulking within industrial WWTPs.

Introduction

Wastewater treatment plants (WWTPs) ensure that the effluents that are discharged in the local surface waters are properly treated. The activated sludge (AS) process is the most commonly used biological treatment system for industrial and domestic WWTPs. The industry is one of the largest consumers of water worldwide, accounting for 57% of total water consumption in Europe and 19% globally (FAO 2015). The treatment of industrial wastewaters can be challenging due to the large variety of pollutants that it can contain and the seasonal variation in wastewater quality. Furthermore, some wastewaters lack nutrients such as nitrogen and phosphorus, or have high a salinity and contain toxic compounds. These conditions, frequently observed in industrial wastewaters, can inhibit the biological process of wastewater treatment.

Continuous flow activated sludge (CAS) systems are amongst the most commonly used AS systems for industrial wastewater. These systems are susceptible to excessive growth of filamentous bacteria (FB) (Caluwé et al. 2017). This type of treatment system is generally characterized by low substrate concentrations, or diffusion dominated conditions that will result in FB extending from the flocs (Martins et al. 2003). In a recent study of 90 industrial WWTPs in Belgium, it was found that 37% suffered from bulking problems, and 46% of the total number of plants had a CAS system (Cornelissen et al. 2018). Bulking sludge has been defined as sludge that settles slowly and compacts poorly (Martins et al. 2004), it can be classified as filamentous bulking sludge (FBS) and non-filamentous or viscous bulking sludge (VBS). FBS has been shown to occur in different industrial WWTPs worldwide, including different AS systems such as nutrient removal and enhanced biological phosphorus removal (EBPR) systems. The filaments found to cause bulking in industrial plants are Morphotype 021N in the potato processing industry (Levantesi et al. 2004), *Thiothrix* in the brewery and dairy sector, *Sphaerotilus* in pulp and paper making industry (Oppong et al. 2003) and *Kouleothrix* in the tank truck cleaning sector (Tsertou et al. 2022). Additionally, it has also been reported that VBS can cause poor settling in industrial plants including the bottling and brewing processing (Peng et al. 2003), chemical (Lajoie et al. 2000) and winery (McIlroy et al. 2011). VBS sludge has received little attention as in most bulking cases filaments are the major cause, but so far no clear causes of VBS are known.

There is a limited number of studies related to bulking sludge in industrial WWTPs, that have reliably identified filaments and other microorganisms that could be responsible for bulking. In terms of separation problems caused by filaments, there are many contradictions related to the quantity of filaments. Kaetzke et al., 2005 reported that bulking sludge might occur due to a small fraction of some filaments in the biovolume, that is the case of *Ca*. M. Parvicella with relative abundance between 0.54% and 2.47% causing bulking sludge (Zhang et al. 2019). Other studies have shown that sludge only bulks when filaments extend from the surface of the flocs (Vervaeren et al. 2005). Nevertheless, the link between the number of filaments and incidents related to them is still not well understood and continues to be a serious problem for AS plants worldwide (Seviour 2010). Furthermore, compared to the extensive literature that exists for FBS, only limited research has addressed VBS, the focus of such studies has been on EPS content (Shao et al. 2019) or operational parameters leading to VBS (Peng et al. 2003), and little attention has been paid to the microbial population involved in VBS in industrial wastewater treatment plants. Additionally, little is known about the microbial communities of industrial AS plants at higher taxonomic resolution such as species level. There is little information on the groups of functional bacteria found in industrial plants, responsible for the removal of nutrients such as nitrogen and phosphorus.

The objective of this study is to identify the bacterial community of 31 industrial plants from 7 different industrial sectors at high taxonomic resolution using 16S rRNA Amplicon Sequencing. We aimed to identify the core community and the most abundant genera-species within functional groups including nitrifiers, denitrifiers, phosphate-accumulating and glycogen accumulating organisms. Furthermore, we elucidated the microorganisms involved in bulking, including FBS and VBS, and determined the causes of bulking. Additionally, a summary of the composition of industrial wastewaters is presented as well as their impact on the microbial communities. Finally, the impact of the most encountered FB and their relation with settling properties along the different industrial plants are critically presented.

Materials and Methods

Biomass sampling and plant information

Samples including wastewater, activated sludge (AS) and treated effluent were collected from 31 full-scale industrial plants (Table 1, for more details see Table S1 and Table S2). Activated sludge samples (5L) were collected from the homogenized aerobic tanks, and were immediately transported to the laboratory. Samples were taken from each WWTP twice, once during autumn (November-December) and once during summer (June-August). All investigated WWTPs had been in operation for several years under similar conditions. The exact operation time of each plant is however not known.

Sludge characterization

The samples were collected with a maximum of 24 hours before the experiments were carried out, and were kept at 4°C. Biomass concentration was estimated using the mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) (APHA 1998). The morphology of AS samples was characterized using light microscopy, where filaments were identified using the Eikelboom classification based on morphological characteristics observed from phase contrast, Gram and Neisser stain (Eikelboom 2000). Furthermore, the abundance of FB was determined subjectively using a filamentous index (FI, Richard et al., 2003) ranging from 0 (no filaments observed) to 6 (excessive filaments observed). Sludge settleability was determined based on sludge volume index SVI (van Loosdrecht et al. 2016). In order to be able to compare the settleability of the different plants, a diluted SVI (DSVI) was performed, the AS was diluted with effluent until the settled volume was between 150-250 ml L-1 after 30 min as described in (van Loosdrecht et al. 2016).

Most of the information related to the wastewater composition including pH, EC, COD, TN, TP was obtained from the industrial plants. Additionally S^{2-} and VFA were measured using Hach test kit (LCK653 and LCK365), while SO_4^{2-} was measured using Macherey Nagel test kit.

DNA extraction and PCR amplification

DNA was extracted from 2 samples (volume 500 µL) of each WWTPs, using the FastDNA® SPIN kit for soil following the protocol described in (van Loosdrecht et al. 2016). DNA concentration of each extraction was measured using Qubit dsDNA BR Assay kit following the manufacturer's protocol. All samples were then diluted to a final DNA concentration of 7.5 ng/µL. Samples with high DNA concentration (above 100 ng/µL) were diluted twice to minimize dilution error. The 16S rRNA gene amplification was performed targeting the V1-3 region of the 16S rRNA gene, as recommended for AS samples by (Albertsen et al. 2015). Library preparation and sequencing were conducted as described in (Caluwé et al. 2022) using an Illumina MiSeq instrument. The raw sequencing data have been deposited in the NCBI SRA database BioProject ID: PRJNA938135. Amplicon sequencing variants (ASV) were clustered as described in (Dueholm et al. 2020). Taxonomy was assigned using the Microbial Database for Activated Sludge (MiDAS 4) (Dueholm et al. 2022), the obtained dataset was analysed and visualised using R v. 3.2.3 (R Core Team, 2015) with the ampvis2 v. 1.24.0 (Andersen et al. 2018), ggplot2 (Gómez-Rubio 2017) and vegan v.2.5-7 packages. Samples from 2 plants were lost during DNA extraction and therefore no amplification was obtained, resulting in sequencing information of 29 plants.

Data analysis

Data analysis was carried out on 2 samples per plant, one representing winter and one representing summer period. The amplicon data were analyzed using the ampvis2 package (Andersen et al. 2018), samples with less than 10000 reads were discarded, resulting in 58 samples (from 29 WWTPs) and 12301 ASVs with a minimum of 42756 and maximum of 154543 qualified reads per sample. ASVs read counts were normalized to 100 (i.e. percent) per sample using the function amp_subset_samples() from the ampvis2 R package (Andersen et al. 2018). The effective bacterial sequences were taxonomically assigned from phylum to species level. It was possible to classify ASVs as far as species thanks to the high coverage of the specific database for WWTP bacteria. The non-parametric Kruskal-Wallis test was used to assess the statistically significant differences between read abundance of bacterial genera and process type. Alpha diversity indices, including Shannon and Chao1 indices, were calculated using the "amp_alphadiv" function from the ampvis2 R package. Statistical comparison of alpha diversity indices and reactor configuration were performed using a mixed linear model. Spearman's rank correlation coefficient was

calculated to test the correlation between alpha diversity index (Chao1 and Shannon) and operational conditions (pH, EC, COD, TN, TP).

Beta diversity analysis was used to quantify the similarity between the bacterial communities of the different industrial sectors. Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity was used to visualize the beta diversity among the samples. On the basis of beta diversity, canonical correspondence analysis (CCA) was used to evaluate the impact of environmental factors on microbial community distribution. Where CCA was constrained to a given variable including EC, pH, COD, TN, TP, SO_4^{2-},S^{2-}, VFA, MLSS and MLVSS. Prior to the analysis, ASVs that were not present in more than 0.01% relative abundance were removed. The data was first transformed by applying the Hellinger transformation (Legendre and Gallagher 2001). PCoA and CCA plots were generated using the ampvis2 package.

Results

Industrial WWTPs and their bacterial community

The 31 surveyed plants were designed for different process types, of which 9 were carbon removal plants only (C), 19 had biological nitrogen removal (nitrification-denitrification; N,DN) and 3 reported biological phosphorus removal activity (P). The WWTPs had wastewater flows ranging from 100 to 32000 $m^3.day^{-1}$. The majority of the AS plants were configured as continuous flow systems including Unitank and continuously stirred tank reactors (CSTR), 8 sequencing batch reactors (SBR) and 6 membrane bioreactors (MBR). 35% of the plants reported the use of chemical coagulants to improve the AS settling properties and combat filamentous bulking, of which iron(III)chloride was the most used, only 1 plant from the chemical sector reported ozone addition and 1 plant from the bioindustrial sector dosed polyaluminum chloride (PAC) as control method. Table 2 provides an overview of the wastewater composition range among the industrial sectors.

In this study, the microbial community composition of 29 industrial WWTPs from 7 different industrial sectors was investigated. The clustering classification resulted in 12301 ASVs, that were taxonomically classified from phylum until species level. A total of 45 known bacterial phyla were identified in 58 sludge samples. There were between 40-44 bacterial phyla with relative abundances of over 1%, accounting for 90.63-94.67% of the total bacteria. The dominant bacterial phyla were *Proteobacteria* (33.7-55.5%), *Bacteroidota* (19.4-38.9%), *Chloroflexota* (1.5-8.1%), *Myxococcota* (1.5-7.4%) and *Acidobacteriota* (1.9-5.4%). The relative abundance of the dominant phyla varied across the different industrial plants and sectors (Fig. 1). Taxonomic classification at the family level revealed that the most abundant families were *Rhodocyclaceae* (*Proteobacteria*) and *Saprospiraceae* (*Bacteroidota*), see Figure S1. The high abundance of *Rhodocyclaceae* was mainly due to the significant presence of the genera *Thauera* and *Zoogloea*, and the undescribed *Denitromonas*. The second most observed bacterial family was *Saprospiraceae*, of which the genera midas_g_6, *Ca*. Epiflobacter and midas_g_1328, were the most frequently found.

At a higher taxonomic resolution, 81.9% of the generated ASVs were classified at genus and 61.3% at species level (Figure S2). Based on mean of relative abundance, it can be observed that the dominant genera are *Thauera*, *Denitromonas* and *Zoogloea*. However, results obtained from the median of relative abundance among industrial sectors suggest that the main organisms found were *Ca*. Competibacter, *Thauera* and *Elin6067*. Since the median results are much lower than that of the mean, it suggests that the distribution of genus relative abundance is negatively skewed within the samples and that the presence of micro-organisms does not depend on the industrial sector to which it belongs, but rather other factors. Among the most commonly encountered bacterial genera are also *Thiothrix* and *Ca.* Competibacter, both belonging to the phylum Proteobacteria. *Thiothrix* is a well-studied bulking filamentous bacteria that was encountered mainly in the vegetable, dairy and bioindustrial sectors. *Ca.* Competibacter is a glycogen accumulating organism (GAO) that was abundant in the tank-truck cleaning, meat and brewery sectors with mean of 2.4, 1.6 and 1.4%RA and median of 1.5, 0.7 and 0.8%RA respectively.

Core community

Cumulative analysis was used to determine whether abundant ASVs made up a large proportion of the reads. Fig. 2a presents the cumulative read abundance at genus-level ASVs, across samples from winter and summer (58 samples). In each sample, the 10 most abundant ASVs made up 7.8% of the total read abundance, and the 100 most abundant genus-ASVs made up only 28.7%. The core community was determined as described by (Dueholm

et al. 2022), with a minimum relative abundance threshold of 0.1% of the genus-ASVs to be considered part of the core community. The occurrence cutoff was 80% corresponding to strict core, while 50% as general core and 20% loose core across the 56 samples. A large number of ASVs was observed in one or few samples (Fig. 2b), 105 genus-level ASVs made up the loose core and only 20 genus-level ASVs comprised the general core community while no ASVs were found among strict core community.

The 20 genera that made up the general core community are presented in Fig. 2c. Only few of the general core micro-organisms have been studied in AS systems, for which their in situ physiology is known. These include the genera *Thauera*, *Ca*. Epiflobacter, *Ca*. Competibacter and *Nitrospira*. However, a large number of microorganisms that have not been described in the literature comprise the general core taxa, including *Ferruginibacter, Terrimonas, Nannosystis* and *Haliangium*.

Functional guilds

The functional groups of organisms recognized as involved in nutrient removal were identified across the different process types (Fig. 3). It was observed that the complexity of the treatment process which included removal of carbon (C, 9 plants), carbon and nitrogen (C, N, DN , 17 plants), carbon, nitrogen and phosphorus (C, N, DN, P, 1 plant), and carbon and phosphorus (C, P, 2 plants) played a key role in the abundance of functional guilds. The microorganisms responsible for carbon and nitrogen removal differ in abundance from those found in municipal plants. This was also the case for plants with biological P activity, where the abundance of well described PAO was lower than those reported in the literature.

The main genera within nitrifiers were the ammonia oxidizing bacteria (AOB) *Nitrosomonas* and the nitrite oxidizing bacteria (NOB) *Nitrospira.* Only one undescribed specie (midas_s_11707) accounted for all *Nitrosomonas* abundance, while 16 species corresponded to *Nitrospira* from which *N. nitrosa* and *N. defluvii* were the most abundant (see Figure S3). Furthermore, *N. nitrosa* has been described as a commamox (complete ammonia oxidizer) like organism (Daims et al. 2015). However, based only on 16S sequences, it cannot be stated that the specie *N.nitrosa* represents a true commamox organism. Further research in the role of *N.nitrosa* in industrial plants is needed to determine whether complete ammonia oxidation occurs. Denitrifying bacteria *Zoogloea* were significantly abundant within carbon removal plants (C, 9) however, none of these plants presented biological nitrogen removal, suggesting that their presence was not due to their denitrifying activity. Furthermore, *Thauera* was the dominant denitrifier in the C and N removal systems (C, N, DN, 17 plants). 15 species were found within the genus *Thauera*, of which 2 undescribed species were the most abundant of this genus (midas_s_1356 and midas_s_256).

In the three plants where biological phosphorus removal was reported, the most abundant phosphate-accumulating organisms (PAOs) were *Dechloromonas*, followed by *Tetrasphaera* (recently renamed to *Phosphoribacter*, Singleton et al. 2022) and *Ca*. Accumulibacter. From which, *Ca*. Accumulibacter has been extensively studied and is often considered as the most important PAO genus (Petriglieri et al. 2022). Additionally, we investigated the presence of known putative PAOs (Rubio-Rincón et al. 2017; Stokholm-Bjerregaard et al. 2017) among these plants. Our results revealed that the most encountered putative PAOs in the industrial samples are *Thiothrix* (10.6%), *Gemmatimonas* (0.5%) and *Ca*. Obscuribacter (0.1%) (see Figure S3). Moreover, these plants treat sulfide rich wastewaters (2-16 $mgS.L^{-1}$) which could contribute to the mixotrophic metabolism of *Thiothrix* that combines sulfur oxidation with biological P removal (Rubio-Rincón et al. 2017; de Graaff et al. 2020). The polyphosphate kinase gene (ppk2) has also been encountered in several species within the genus *Thiothrix*, including *T.* Caldifontis (Chernousova et al. 2009; Matsuura et al. 2021). However, it cannot be concluded that the abundance of *Thiothrix* in the industrial plants is due to its mixotrophic metabolism of S and P, for this, further research is needed on the metabolic potential of *Thiothrix*.

Glycogen-accumulating organisms (GAOs) were mainly encountered within C removal (9 plants) from which *Defluvicoccus* and *Ca*. Competibacter were the most abundant genera. The maximum abundance within the industrial plants was 14.3% (sample from meat sector) for *Ca*. Competibacter and 10.4% (sample from tank truck cleaning) for *Defluviicoccus*.

Activated sludge settling properties

The AS characterization revealed that the surveyed industrial plants had DSVI values ranging from 34 to 448 $mL.g^{-1}$. 41% of the studied samples presented good settling properties, with DSVI values below 150 mL.g-1, with mean of 78 mL.g-1 and median of 76 mL.g-1. Few filaments were observed microscopically in the well-settling samples, where some filaments were commonly observed, but were not present in all of the flocs. On average, the filamentous index was 2. However, results from Amplicon sequencing revealed total filaments abundance ranging from 0.003 to 11.3% read abundance, for these well-settling samples. The high abundance of total filaments found in well-settling samples is mainly due to the presence of *Ca*. Villigracilis and *Leptothrix*. *Ca*. Villigracilis was found in 50% of the samples with good settleability with mean of 0.6% and max read abundance of 4.2%. Similarly *Lepthothrix* was present in 67% of the well settling AS, with mean of 0.9% and maximum of 7.2% read abundance.

59% of the AS samples presented poor settling properties, with DSVI values ranging from 165 to 448 mL.g-1, considered as bulking sludge (BS). In most of the bulking cases a high FI was determined, however in some cases poor settleability was not related to filaments extending out of the flocs (Fig. 4a). In 72% of the bulking cases a high abundance of filaments was microscopically observed (FI 3-6), therefore this type of bulking is referred to as filamentous bulking sludge (FBS). The mean and median of total filaments among FBS were 6.08% and 1.69% respectively. These results show that the abundance of filaments was not uniformly distributed resulting in few cases (7 cases) with high abundance of filaments (10-27.4%), while the majority (17 cases) of FBS presented abundance lower than 5% of total filaments encountered with amplicon sequencing. The remaining 28% of the bulking cases had FI values below 2 and also low abundance with mean and median of 1.5%, therefore it is considered as VBS. According to microscopy results, FBS and VBS presented a very different floc morphology, in which FBS had filaments extending from the flocs while VBS exhibited open poorly compacted flocs with no filaments. This was also confirmed by Amplicon results, were the filament *Thiothrix* was the dominant genus encountered within FBS. The most abundant genera within VBS (Fig. 4b) samples were *Zoogloea* (mean 4.84%) and *Ferruginibacter* (mean 3.26%). These bacteria are well known floc formers involved in extracellular polymeric substances (EPS) production (Han et al. 2018). VBS was observed in plants designed for C (4) and C,N,DN (3) process types, operating under food to microorganisms in the range of 0.05 to 0.18 g COD/g MLSS.day (mean and median 0.12), and the majority of the affected plants were configured as continuous flow feeding systems. A wide range of bacteria produce different types of EPS, including carbohydrates and proteins, that affects microbial aggregates resulting in VBS. Further research into the type of EPS produced and microbial characterization is needed to address the organisms responsible for VBS in industrial plants. Although FB were responsible for most cases of bulking, VBS remains a significant type of bulking within industrial WWTPs.

Filamentous bacterial population

Filamentous bacteria made up a significant part of the microbial community with read abundance ranging from 0.003% to 27.4%, and an average read abundance of 4.1% among all the studied samples (58 samples from summer and winter, 29 plants) (Fig. 5c). The most abundant filaments, based on mean relative abundance were *Thiothrix*, *Leptothrix*, *Kouleothrix*, *Ca.* Villigracilis and *Ca*. Sarcinithrix (Fig. 5a), we evaluated the impact of process type and period of sampling on the abundance of the top 5 most abundant FB (see Figure S4 and Table S3). *Thiothrix* (*Proteobacteria)* was found to be the most abundant genus within the bioindustry, dairy and vegetable sectors, being encountered in 31 samples with mean read abundance of 2.28%, reaching 27% in the potato processing industry. *Thiothrix* presence was positively correlated with bulking (correlation coefficient 0.48, $p<0.05$) and was microscopically observed in high abundance (FI 4-6) as presented in Fig. 5b.Thiothrix abundance was strongly influenced by the process type (see Figure S4 and Table S3), being more abundant in complex systems such as nutrient removal plants. Several genera from the *Chloroflexota* phylum were encountered among the dominant filamentous bacteria, from which *Kouleothrix* was found abundant in the meat (mean 0.6%) sector in plants with C and N removal and in the tank-truck cleaning sector (mean 3% and max 18%) where only C removal was performed, *Ca*. Villigracilis found throughout all industrial sectors with a max read abundance of 4.2% in the meat sector, *Ca.* Sarcinithrix found only in the dairy (mean 0.1%), brewery (mean 0.2%) and in the meat (mean 0.8%RA and max of 14.4%) sectors. Only *Ca.* Sarcinithrix abundance was positively correlated ($p<0.05$, Figure S4 and Table S3) to process type being abundant in nutrient removal plants. No impact of winter and summer samples was observed in the abundance of the described abundant filaments (see Table S3).

Among the most abundant filaments only two genera do not always present filamentous morphology in-situ, these include species belonging to the genera *Leptothrix* and *Trichoccocus* (Nierychlo et al. 2020). *Leptothrix*,

known to have a variable morphology including straight rods cells and filamentous growth, was encountered in 51 samples with the highest relative abundance (>1%) in the brewery, meat, dairy and chemical sectors respectively. *Leptothrix* abundance was on average 0.99%, median of 0.23% and maximum of 7.2% in the brewery sector. Additionally, for 4 samples (2 plants) from the chemical sector no abundance of total filaments was identified based on the 16S RNA gene, however a large number of FB extending from the flocs were observed microscopically.

External factors affecting the microbial community

Alpha diversity analysis was conducted to estimate the microbial richness and diversity within the samples, where the Chao1 measures the microbial richness and Shannon the microbial diversity. Chao1 richness index ranged from 355 to 2432 and Shannon diversity index ranged from 1.95 to 5.92 with an overall lower richness and diversity found in winter samples compared to summer samples (see Figure S5). A linear mixed model was used as quantitative trait association between microbial diversity or richness and process type, industrial sector. No significant association between industrial sector or process type was encountered with microbial richness and diversity (data not shown) among the industrial plants. Additionally, Spearman rank correlation was used to find association between wastewater composition (pH, EC, COD, TN, TP and VFA) and microbial richness and diversity. The results showed that there is a significant correlation between wastewater salinity EC and microbial richness Chao1 (Spearman correlation coefficient 0.42, $p = 0.002$), indicating that high salinity has a negative effect on microbial richness, but no significant correlation was found between EC and diversity.

Beta diversity analysis was used to quantify the similarity between microbial communities, PCoA revealed a wide taxonomic diversity across industrial sectors (Fig. 6a) where samples from the same industrial sector did not cluster strongly. Winter and summer samples from individual plants cluster together, indicating an overall microbial stability within a period of 6 months, which was the time between the two samples. However, the first two principal coordinates represented only 10% of the total variation, indicating a lower degree of microbiome similarity. The higher sample dispersion was encountered within the meat sector presumably due to the wide range of its wastewater composition (Table 1), while the dairy, chemical, vegetable and bioindustry did cluster together. These results suggest an overall greater microbial complexity within industrial AS plants.

Constrained correspondence analysis CCA was used to determine the influence of external factors on microbial community composition. CCA of the 58 samples, that corresponded to all the studied AS plants, revealed that environmental factors including EC, SO_4^{2-}, and TP had significant effects on the bacterial population belonging to chemical, brewery and bioindustrial sectors (Fig. 6b). Whereas the lower TP encountered in one brewery plant led to a more specific microbial community. Similarly the higher EC and SO_4^{2-} of the chemical wastewater resulted in a more specific microbial community. However, CCA showed that the investigated environmental variables explained only 22.37% of the variability in the industrial WWTPs, suggesting that other factors not considered may explain the microbial variability of the other industrial plants. This can be attributed to the specificity of the incoming industrial wastewaters that will result in unique microbial ensembles. Additionally, CCA analysis did not reveal significant effect of environmental factors on bulking bacterial population, which in turn can be related to the high microbial specificity that exists between the plants, and does not allow for a common pattern to be established.

Discussion

In this study we aimed to gain more insight into the microbial community of industrial full-scale WWTPs. Our findings indicate that several yet undescribed taxa were highly abundant and comprised the general core community of the studied industrial plants. The main functional groups were dominated by *Thauera* and *Zoogloea* within denitrifiers, *Dechloromonas* in the PAO group and *Defluvicoccus* in the GAO group. Furthermore, no significant seasonal variations were observed in the overall microbial community of each plant from winter to summer samples. Additionally, our findings revealed the extent of the problem of bulking sludge for industrial plants with 59% of the samples presenting settleability problems, from which 72% corresponded to FBS and 28% to VBS.

The complexity of the industrial wastewater seems to depend to a large extent on its sector of origin, e.g. wastewater originating from breweries are characterized by high COD concentrations but lack nutrients as

previously reported (Stes et al. 2018). Similarly, wastewaters originating from the vegetable industry were rich in COD, P and N (Dobbeleers et al. 2017). The wastewaters from the dairy sector contained high COD and N concentrations, but lacked P. The chemical sector was characterized by high salinity wastewaters, with an average electrical conductivity of 16 mS.cm-1, and a maximum up to 50 mS.cm-1. Among the various ions that contributed to the high salinity, chlorides (up to 20 $g.L^{-1}$) and sulfates (up to 5 $g.L^{-1}$) stand out. However, there was also a significant difference in the composition of the wastewater coming from the same sector, which may be the result of the different industrial processes that lead to a wide range of pollutants.

Industrial plants revealed unique bacterial communities

Microbial community analysis revealed that the most abundant phylum were *Proteobacteria*, *Bacteroideota* , *Chlororflexota* and *Acidobacteriota,* these findings are in line with previous studies (Ibarbalz et al. 2013; Selvarajan et al. 2018; Wang et al. 2020; Kristensen et al. 2021). However, there are still several abundant phyla that have not yet been described within AS systems, including the *Myxococcota*, *Patescibacteria*, *Gemmatimonadota* and *Latescibacterota*. At the family taxonomic classification, *Rhodocyclaceae* (*Proteobacteria*) and *Saprospiraceae* (*Bacteroidota*) were highly abundant, suggesting an important role in the industrial AS plants. The genus *Thauera* was the most abundant representative of the family *Rhdocyclaceae* reaching up to 51% read abundance in the chemical sector. The high abundance of *Thauera* can be attributed to the versatility of substrates that can be used, including aromatic compounds, amino acids and organic substrates (Thomsen et al. 2007). The abundant representatives found for the family *Saprospiraceae* include midas_g_6 and *Ca.* Epiflobacter, which have been described to possess a broad metabolic potential, including the degradation of proteins, polysaccharides and complex molecules (Kondrotaite et al. 2022), which may give them an advantage in industrial plants, resulting in high abundance.

At a higher rank such as genus and species classification, there were several abundant microorganisms for which no ecophysiological information in AS systems exists (see Figure S2), including *Denitromonas* highly present in the chemical sector, *Ferruginibacter*, *Terrimonas* and Ellin6067 in the tank truck cleaning sector. Ellin6067 is an uncultured genus of the *Nitrosomonadaceae* family, and potential nitrogen-transforming bacteria present in both natural water bodies (Mankiewicz-Boczek and Font-Nájera 2022) and in AS systems (Dueholm et al. 2022). A loose core consisting of 20 microorganisms, most of them undescribed, was also found. These results are in line with those of previous industrial studies (Ibarbalz et al. 2013), suggesting that the bacterial populations across industrial plants differ considerably from each other, resulting in a unique composition of microbial assembles that share only few genera as core community. Additionally, the alpha diversity analysis revealed a much lower richness and diversity (see Figure S5) than those from municipal plants (Wang et al. 2016). This may be because industrial waters contain less variety of contaminants which in turn will select for a more specific microflora as previously reported in the MiDAS project (Dueholm et al. 2022). These results seem to be consistent with the PCoA analysis, that revealed a very specific microbial community even within the same industrial sector, indicating lower degree of microbiome similarity among all industrial plants.

Functional bacteria in industrial plants

The presence of functional guilds among the different treatment processes revealed that *Zoogloea* and *Thauera* were highly abundant denitrifiers in C and C,N removal plants. *Zoogloea* was abundant within C removal plants, where no biological nitrogen removal occurred, suggesting that their presence was not related to their denitrifying activity. *Thauera* was the dominant denitrifier within C,N removal plants. The distribution of denitrifying organisms in our study appears to be distinct from the global MiDAS study that includes mostly municipal plants, where *Rodophherax* are the main denitrifiers (Dueholm et al. 2022). Within the PAO phylotype, *Dechloromonas* was the most abundant representative of this group. Interestingly, the presence of recognized PAOs does not exceed 2% read abundance in our surveyed Bio-P plants. The low presence of recognized PAOs genera can be explained as a result of the low Bio-P activity in the mentioned industrial plants. The biological P removal activity differs from that of EBPR plants. The mentioned plants (3) reported lower bio-P release (15-20 $mgP.L^{-1}$) and much higher COD-P ratio (40-60) than conventional EBPR systems. The putative PAO *Thiothrix* (Rubio-Rincón et al. 2017; Mardanov et al. 2020) was abundant in the bio-P plants, suggesting that the mixotrophic metabolism of *Thiothrix* including sulfur oxidation and biological P removal may play an important role within industrial plants, possibly contributing to the biological P removal. In addition, only a very small number of industrial plants had

biological P removal, since chemical removal was used for the most part. Furthermore, the configuration of the industrial plants, mostly CAS type, was not suitable for the growth of PAOs resulting in a low overall abundance of this group of bacteria among all the industrial plants.

GAO representatives were found abundant in one C-removal plant from the tank-truck cleaning sector, and in nutrient removal plants. This can be attributed to the unaerated feeding strategy implemented in the tank-truck cleaning plant (Caluwé et al. 2022), or by the presence of anaerobic/anoxic zones in the latter. *Defluvicoccus* (*Alphaproteobacteria*) and *Ca*. Competibacter (*Gammaproteobacteria*) were the most abundant genera. *Defluviicoccus* possess a broad substrate affinity including acetate, propionate, pyruvate and glucose, and have been described as potential carbon competitors of PAOs and other GAOs (Burow et al. 2007). However, our results show that these bacteria cohabit the same system, suggesting that there is no such competition for substrate. The well-known GAO, *Ca.* Competibacter (McIlroy et al. 2013) was found among the top 10 most abundant microorganisms (Fig. 1) and in more than 50% of the samples with read abundance higher than 0.1% and also comprising the general core community in the studied industrial plants.

The efficiency and performance of a WWTP depends primarily on the composition and activity of its microbial community. The functional community described in this study aligns with established bacterial guilds, including PAOs, GAOs, and denitrifiers responsible for nutrients removal. The bulking community commonly consists of filaments, which not only have a structural role, but also aid in nutrient removal in the AS system. Our findings indicate that the abundance of *Thiothrix* and *Ca.* Sarcinithrix is influenced by process design, with higher abundance found in complex systems designed for N and/or P removal. This aligns with existing literature regarding the *Chloroflexota* phylum filaments (Petriglieri et al. 2023). The presence of certain filaments in nutrient removal plants can be attributed to their substrate storage capacity, giving them similar advantage as for well-known nutrient removal bacteria. Previous studies have reported the storage capacity of filaments including lipids and polyphosphate storage by *Ca*. M. Parvicella (Jon McIlroy et al. 2013), sulfur and polyphosphate by *Thiothrix* (Rubio-Rincón et al. 2017) and glycogen by *Chloroflexota* filaments (Petriglieri et al. 2023). However, the precise contribution of these filaments to nutrient removal in industrial WWTPs is not yet established, and therefore further studies are necessary to determine their functional role.

Settling properties and microbial community

Bulking sludge was observed in 55% of the plants in winter and 62% in the summer. These findings differ from previous studies where bulking is commonly observed in the winter season (V.Tandoi et al. 2006). These differences can be explained as a result of industrial or municipal wastewaters, whereas municipal plants suffer from overgrowth of the filament *Ca*. M. Parvicella, which was not found in the industrial plants studied. Additionally, our study only evaluated samples from winter (November-December) and summer (June-August) in Belgium with a different temperature range than previous studies, more samples should be considered to evaluate in detail the seasonal effect on bulking sludge.

The majority of bulking cases corresponded to FBS (72%), our findings suggest that despite the low abundance (<5%) of total filaments, FBS can still occur. The low abundance of total filaments can be attributed to different biases occurring in the amplicon sequencing such as gene copy number, targeting region of the 16S rRNA gene or DNA extraction biases. Furthermore, the samples considered as FBS presented a high abundance of filaments (FI>3) observed microscopically, suggesting that the special distribution of filaments in the flocs rather than abundance affects the sludge settling properties, as previously described (Wágner et al. 2015). The phylum *Chloroflexota* was the third most encountered phylum, and it has recently been reported that almost all of its species have a filamentous morphology (Petriglieri et al. 2023). *Kouleothrix, Ca.* Villigracilis and *Ca*. Sarcinithrix were the most abundant filamentous genera from *Chloroflexota*. *Kouleothrix* and *Ca*. Sarcinithrix were observed extended from the flocs, causing bulking and contributing to an increased FI (Fig. 5b). The negative effect of *Kouleothrix* on sludge settleability in municipal nutrient removal plants have been associated to low oxygen concentration in the aeration tank (<1.1 mg O_2.L-1) and the low temperatures being able to grow even at 7°C (Nittami et al. 2020). However, seasonality between winter and summer samples was not a statistically significant factor influencing the abundance of *Kouleothrix,* based on Kruskal-Wallis analysis (see Table S3). *Ca*. Sarcinithrix abundance was significantly correlated with plant configuration, being highly abundant in more complex plant configurations such as nutrient removal, which may be due to their previously reported facultative anaerobic

metabolism (Nierychlo et al. 2019). *Ca*. Sarcinthrix is a recurrent filament in municipal Danish plants (Nierychlo et al. 2019) and also found in domestic plant operating under long sludge age (McIlroy et al. 2011).

Amplicon sequencing also revealed the presence of described FB in well settling samples, with total filaments accounting for up to 11.3% read abundance, with *Leptothrix*, *Ca*. Villigracilis and *Trichococcus* genera being abundant. The genus *Trichococcus* has been reported to present variable cell morphology in AS plants, growing as single cell or filament (Nierychlo et al. 2020), while *Leptothrix* have also presented single cell morphology in AS systems (Wagner et al. 1994). Our findings suggest that *Leptothrix* did not presented filamentous morphology extended from the flocs and did not contribute to bulking, we suggest that it did not present filamentous morphology at all or it was filamentous but only inside the flocs, not being able to classify using conventional microscopy. The morphology of *Ca*. Villigracilis has been confirmed in situ, found predominantly located within the flocs, suggesting their structural role in well-settling sludge (Nierychlo et al. 2019). In the global MiDAS survey (Dueholm et al. 2022), *Leptothrix and Ca*. Villigracilis were found to be the second and fourth most abundant FB worldwide and are also part of the general core (with >0.1% RA in 50% of all plants) of AS systems. However, these bacteria were not part of the general core taxa of the industrial plants studied in this survey. From our results *Ca*. Villigracillis and *Leptothrix* were not microscopically observed but were found to have high read abundance based on amplicon sequencing results. These filaments appear to play a structural role in industrial AS systems, helping to maintain a well-settling sludge. In addition, these results suggest that when filaments are not observed microscopically, it does not mean that their abundance is low.

According to our results, unknown filaments were found which were observed microscopically but amplicon sequencing results did not reveal the presence of known filaments. On the one hand these findings can be explained by the non-amplification of the filamentous bacteria by 16S RNA amplicon sequencing. The latter may be due to the targeted region of the 16S rRNA gene (V1-3) which may not be suitable for this organism or due to bias in the DNA extraction. On the other hand, it is possible that filamentous bacteria were amplified and found to be abundant but not classified as filamentous because their morphology has not been reported in the literature. It is therefore important to combine different identification techniques, such as molecular and morphological approaches. Additionally, our results revealed a different abundance of FB than that found in municipal plants, a good example of this is the absence of *Ca*. Microthrix in the studied industrial plants. The excessive growth *Ca*. Microthrix is commonly associated with bulking and foaming problems in municipal treatment systems (Nierychlo et al., 2020, Dueholm et al., 2022). However our results do not reveal a high abundance of *Ca*. Microthrix in the surveyed industrial plants, which was encountered in low abundance (0.002-0.4% read abundance) within the meat and dairy sector. Additionally, *Ca*. Microthrix is one of the easiest filament to identify using conventional light microscopy, and it was seldomly observed within the mentioned samples.

VBS accounted for 28% of the bulking cases, observed in C (4) and C,N,DN (3) process type plants operating under food to microorganisms ratios ranging from 0.05 to 0.18 g COD/g MLSS.day (mean and median 0.12), the majority of the affected plants were configurated as continuous flow feeding systems. This resulted in VBS plants enriched in genera belonging to the phyla *Proteobacteria* and *Bacteroidota*. The *Rhodocyclaceae* (*Proteobacteria*) family, including *Zoogloea* and *Thauera* were abundant in VBS samples (Fig. 4b), *Zoogloea* spp. such as *Z. ramigera* have been widely associated with VBS due to their excessive production of EPS (Rosselló-Mora et al. 1995), similarly to *Thauera* (Allen et al. 2004). Comparative genome analysis revealed that clusters of EPS biosynthesis genes were found in the genomes of members of the *Rhodocyclaceae* family (An et al. 2016), highlighting the importance of members of this family in VBS. Similarly, representatives of the *Bacteroidota* phylum, including *Ferruginibacter* and *Terrimonas* were abundant in VBS samples, however the *in situ* physiology of these and other VBS genera has not been described. The identification of the abundant microorganisms found in VBS samples from industrial AS plants will serve a basis for further study of their role in EPS production and bulking.

Several approaches exist to control AS settling properties, with nonspecific control methods such as the addition of chemicals and disinfectants, while specific methods include adjustment of wastewater composition, selectors and feeding strategy. Wastewater composition significantly influences the microbial community of AS systems, however many industrial effluents can be deficient in macro and micronutrients, leading to FBS and VBS, therefore a BOD_5:N:P ratio of 100:5:1 is recommended (Richard et al., 2003). Additionally, in certain scenarios,

modifying the composition of wastewater to avoid FBS achievable by employing pretreatment techniques, including Dissolved Air Flotation (DAF) or a fat and grease trap system. These methods aid in eliminating lipids and preventing bulking caused by Actinobacteria filaments. In other cases, however, where the effluent consists of diverse substrates that are difficult to modify, addressing bulking issues proves challenging and requires consideration of additional factors. When it comes to dealing with filaments in wastewater from the petrochemical, tank truck cleaning and brewing industries, switching to an anaerobic feeding has proven to be successful strategy (Caluwé et al. 2017; Stes et al. 2018; Poelmans et al. 2023). Therefore, various operational factors must be taken into account when addressing bulking in industrial WWTPs.

This study provides a deeper insight into the microbial community composition of full-scale industrial WWTPs. With focus on functional groups of bacteria responsible for nutrient removal, and microorganisms involved in bulking cases. No common core taxa were found across all the plants, but a general core of mostly undescribed bacteria was found, suggesting that industrial plants have unique microbial communities derived from their specific wastewaters. These results highlight the need for future research to investigate the physiology of important undescribed taxa in full-scale plants. Finally, this study contributes to the overall goal of understanding the ecology of full-scale AS systems.

Supplementary data

Supplementary data associated with this article can be found in the online version of this paper.

Author contribution TD and JD conceived and designed the research. KS conducted the experiments, data analysis and wrote manuscript. TD and JD revised and edited the manuscript. All authors read and approved the manuscript.

Data availability The datasets generated during this current study are available from the corresponding author on reasonable request.

Acknowledgments This work was funded by the University of Antwerp through the University Research Fund (BOF-STIMPRO), supporting fundamental research. This study was carried out at the research group BioWAVE, part of the faculty of Applied Engineering.

Compliance with Ethical Standards

Funding: This study was funded by the University of Antwerp (BOF) grant number 39709.

Conflict of Interest: Karina Seguel Suazo declares that she has no conflict of interest. Thomas Dobbeleers declares that he has no conflict of interest. Jan Dries declares that he has no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

References

Albertsen M, Karst SM, Ziegler AS, Kirkegaard RH, Nielsen PH (2015) Back to basics - The influence of DNA extraction and primer choice on phylogenetic analysis of activated sludge communities. PLoS One 10(7). https://doi.org/10.1371/journal.pone.0132783

Allen MS, Welch KT, Prebyl BS, Baker DC, Meyers AJ, Sayler GS (2004) Analysis and glycosyl composition of the exopolysaccharide isolated from the floc-forming wastewater bacterium Thauera sp. MZ1T. Environ Microbiol 6(8):780–790. https://doi.org/10.1111/J.1462-2920.2004.00615.X

An W, Guo F, Song Y, Gao N, Bai S, Dai J, Wei H, Zhang L, Yu D, Xia M, Yu Y, Qi M, Tian C, Chen H, Wu Z, Zhang T, Qiu D (2016) Comparative genomics analyses on EPS biosynthesis genes required for floc formation of Zoogloea resiniphila and other activated sludge bacteria. Water Res 102:494–504. https://doi.org/10.1016/J.WATRES.2016.06.058

Andersen K, Kirkegaard R, Karst S, Albertsen M (2018) ampvis2: an R package to analyse and visualise 16S rRNA amplicon data. https://doi.org/10.1101/299537

APHA (1998) Standard Methods for the Examination of Water and Wastewater American Public Health Association, 20th editi. Washington, DC, USA

Burow LC, Kong Y, Nielsen JL, Blackall LL, Nielsen PH (2007) Abundance and ecophysiology of Defluviicoccus spp., glycogen-accumulating organisms in full-scale wastewater treatment processes. Microbiology 153(1):178–185. https://doi.org/10.1099/mic.0.2006/001032-0

Caluwé M, Dobbeleers T, Daens D, Blust R, Geuens L, Dries J (2017) The effect of the feeding pattern of complex industrial wastewater on activated sludge characteristics and the chemical and ecotoxicological effluent quality. Environ Sci Pollut Res 24(11):10796–10807. https://doi.org/10.1007/s11356-017-8712-3

Caluwé M, Goossens K, Suazo KS, Tsertou E, Dries J (2022) Granulation strategies applied to industrial wastewater treatment: from lab to full-scale. Water Sci Technol 85(9):2761–2771. https://doi.org/10.2166/wst.2022.129

Chernousova E, Gridneva E, Grabovich M, Dubinina G, Akimov V, Rossetti S, Kuever J (2009) Thiothrix caldifontis sp. nov. and Thiothrix lacustris sp. nov., gammaproteobacteria isolated from sulfide springs. Int J Syst Evol Microbiol 59(12):3128–3135. https://doi.org/10.1099/ijs.0.009456-0

Cornelissen R, Van Dyck T, Dries J, Ockier P, Smets I, Van Den Broeck R, Van Hulle S, Feyaerts M (2018) Application of online instrumentation in industrial wastewater treatment plants - A survey in Flanders, Belgium. Water Sci Technol 78(4):957–967. https://doi.org/10.2166/wst.2018.375

Daims H, Lebedeva E V., Pjevac P, Han P, Herbold C, Albertsen M, Jehmlich N, Palatinszky M, Vierheilig J, Bulaev A, Kirkegaard RH, Von Bergen M, Rattei T, Bendinger B, Nielsen PH, Wagner M (2015) Complete nitrification by Nitrospira bacteria. Nature 528(7583):504–509. https://doi.org/10.1038/nature16461

de Graaff DR, van Loosdrecht MCM, Pronk M (2020) Stable granulation of seawater-adapted aerobic granular sludge with filamentous Thiothrix bacteria. Water Res 175:115683. https://doi.org/10.1016/j.watres.2020.115683

Dobbeleers T, Daens D, Miele S, D'aes J, Caluwé M, Geuens L, Dries J (2017) Performance of aerobic nitrite granules treating an anaerobic pre-treated wastewater originating from the potato industry. Bioresour Technol 226:211–219. https://doi.org/10.1016/j.biortech.2016.11.117

Dueholm MKD, Nierychlo M, Andersen KS, Rudkjøbing V, Knutsson S, Nielsen PH (2022) MiDAS 4: A global catalogue of full-length 16S rRNA gene sequences and taxonomy for studies of bacterial communities in wastewater treatment plants. Nat Commun 13(1). https://doi.org/10.1038/s41467-022-29438-7

Dueholm MS, Andersen KS, McIlroy SJ, Kristensen JM, Yashiro E, Karst SM, Albertsen M, Nielsen PH (2020) Generation of comprehensive ecosystem-specific reference databases with species-level resolution by high-throughput full-length 16s rrna gene sequencing and automated taxonomy assignment (Autotax). MBio 11(5):1–14. https://doi.org/10.1128/mBio.01557-20

Eikelboom D (2000) Process Control of Activated Sludge Plants by Microscopic Investigation

FAO (2015) Food and Agriculture Organization of the United Nations. https://www.fao.org/aquastat/en/overview/methodology/water-use

Gómez-Rubio V (2017) ggplot2 - Elegant Graphics for Data Analysis (2nd Edition). J Stat Softw 77(Book Review 2). https://doi.org/10.18637/jss.v077.b02

Han X, Zhou Z, Mei X, Ma Y, Xie Z (2018) Influence of fermentation liquid from waste activated sludge on anoxic/oxic- membrane bioreactor performance: Nitrogen removal, membrane fouling and microbial community. Bioresour Technol 250(December 2017):699–707. https://doi.org/10.1016/j.biortech.2017.11.090

Ibarbalz FM, Figuerola ELM, Erijman L (2013) Industrial activated sludge exhibit unique bacterial community composition at high taxonomic ranks. Water Res 47(11):3854–3864. https://doi.org/10.1016/j.watres.2013.04.010

Jon McIlroy S, Kristiansen R, Albertsen M, Michael Karst S, Rossetti S, Lund Nielsen J, Tandoi V, James Seviour R, Nielsen PH (2013) Metabolic model for the filamentous 'Candidatus Microthrix parvicella' based on genomic and metagenomic analyses. ISME J 7(6):1161–1172. https://doi.org/10.1038/ismej.2013.6

Kaetzke A, Jentzsch D, Eschrich K (2005) Quantification of Microthrix parvicella in activated sludge bacterial communities by real-time PCR. Lett Appl Microbiol 40(3):207–211. https://doi.org/10.1111/j.1472-765X.2005.01656.x

Kondrotaite Z, Valk LC, Petriglieri F, Singleton C, Nierychlo M, Dueholm MKD, Nielsen PH (2022) Diversity and Ecophysiology of the Genus OLB8 and Other Abundant Uncultured Saprospiraceae Genera in Global Wastewater Treatment Systems. Front Microbiol 13(July):1–15. https://doi.org/10.3389/fmicb.2022.917553

Kristensen JM, Singleton C, Clegg LA, Petriglieri F, Nielsen PH (2021) High Diversity and Functional Potential of Undescribed "Acidobacteriota" in Danish Wastewater Treatment Plants. Front Microbiol 12. https://doi.org/10.3389/fmicb.2021.643950

Lajoie CA, Layton AC, Gregory IR, Sayler GS, Taylor DE, Meyers AJ (2000) Zoogleal Clusters and Sludge Dewatering Potential in an Industrial Activated-Sludge Wastewater Treatment Plant. Water Environ Res 72(1):56–64. https://doi.org/10.2175/106143000x137112

Legendre P, Gallagher ED (2001) Ecologically meaningful transformations for ordination of species data. 2001(September 2000):271–280. https://doi.org/10.1007/s004420100716

Levantesi C, Beimfohr C, Geurkink B, Rossetti S, Thelen K, Krooneman J, Snaidr J, Van Der Waarde J, Tandoi V (2004) Filamentous Alphaproteobacteria associated with bulking in industrial wastewater treatment plants. Syst Appl Microbiol. https://doi.org/10.1078/0723202042369974

Mankiewicz-Boczek J, Font-Nájera A (2022) Temporal and functional interrelationships between bacterioplankton communities and the development of a toxigenic Microcystis bloom in a lowland European reservoir. Sci Rep 12(1):19332. https://doi.org/10.1038/s41598-022-23671-2

Mardanov A V., Gruzdev E V., Smolyakov DD, Rudenko TS, Beletsky A V., Gureeva M V., Markov ND, Berestovskaya YY, Pimenov N V., Ravin N V., Grabovich MY (2020) Genomic and Metabolic Insights into Two Novel Thiothrix Species from Enhanced Biological Phosphorus Removal Systems. Microorganisms 8(12):1–13. https://doi.org/10.3390/MICROORGANISMS8122030

Martins AMP, Heijnen JJ, Van Loosdrecht MCM (2003) Effect of feeding pattern and storage on the sludge settleability under aerobic conditions. Water Res 37(11):2555–2570. https://doi.org/10.1016/S0043-1354(03)00070-8

Martins AMP, Pagilla K, Heijnen JJ, Van Loosdrecht MCM (2004) Filamentous bulking sludge - A critical review. Water Res 38(4):793–817. https://doi.org/10.1016/j.watres.2003.11.005

Matsuura N, Masakke Y, Karthikeyan S, Kanazawa S, Honda R, Yamamoto-Ikemoto R, Konstantinidis KT (2021) Metagenomic insights into the effect of sulfate on enhanced biological phosphorus removal. Appl Microbiol Biotechnol 105(5):2181–2193. https://doi.org/10.1007/s00253-021-11113-4

McIlroy SJ, Albertsen M, Andresen EK, Saunders AM, Kristiansen R, Stokholm-Bjerregaard M, Nielsen KL, Nielsen PH (2013) 'Candidatus Competibacter'-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. ISME J 2014 83 8(3):613–624. https://doi.org/10.1038/ismej.2013.162

McIlroy SJ, Speirs LBM, Tucci J, Seviour RJ (2011) In situ profiling of microbial communities in full-scale aerobic sequencing batch reactors treating winery waste in Australia. Environ Sci Technol 45(20):8794–8803. https://doi.org/10.1021/es2018576

Nierychlo M, McIlroy SJ, Kucheryavskiy S, Jiang C, Ziegler AS, Kondrotaite Z, Stokholm-Bjerregaard M, Nielsen PH (2020) Candidatus Amarolinea and Candidatus Microthrix Are Mainly Responsible for Filamentous Bulking in Danish Municipal Wastewater Treatment Plants. Front Microbiol 11(June):1–17. https://doi.org/10.3389/fmicb.2020.01214

Nierychlo M, Miłobędzka A, Petriglieri F, McIlroy B, Nielsen PH, McIlroy SJ (2019) The morphology and metabolic potential of the Chloroflexi in full-scale activated sludge wastewater treatment plants. FEMS Microbiol Ecol 95(2):1–11. https://doi.org/10.1093/femsec/fiy228

Nittami T, Kasakura R, Kobayashi T, Suzuki K, Koshiba Y, Fukuda J, Takeda M, Tobino T, Kurisu F, Rice D, Petrovski S, Seviour RJ (2020) Exploring the operating factors controlling Kouleothrix (type 1851), the dominant filamentous bacterial population, in a full-scale A2O plant. Sci Rep 10(1):1–10. https://doi.org/10.1038/s41598-020-63534-2

Oppong D, King VM, Bowen JA (2003) Isolation and characterization of filamentous bacteria from paper mill slimes. Int Biodeterior Biodegrad 52(2):53–62. https://doi.org/10.1016/S0964-8305(02)00174-9

Peng Y, Gao C, Wang S, Ozaki M, Takigawa A (2003) Non-filamentous sludge bulking caused by a deficiency of nitrogen in industrial wastewater treatment. Water Sci Technol 47(11):289–295. https://doi.org/10.2166/wst.2003.0617

Petriglieri F, Kondrotaite Z, Singleton C, Nierychlo M, Dueholm MKD, Nielsen PH (2023) A comprehensive overview of the Chloroflexota community in wastewater treatment plants worldwide. bioRxiv :2023.06.26.546502. https://doi.org/10.1101/2023.06.26.546502

Petriglieri F, Singleton CM, Kondrotaite Z, Dueholm MKD, McDaniel EA, McMahon KD, Nielsen PH (2022) Reevaluation of the Phylogenetic Diversity and Global Distribution of the Genus " Candidatus Accumulibacter" . mSystems 7(3). https://doi.org/10.1128/MSYSTEMS.00016-22/SUPPL_FILE/MSYSTEMS.00016-22-S0010.DOCX

Poelmans S, Dockx L, Seguel Suazo K, Goettert D, Dries J (2023) Implementation of an anaerobic selector step for the densification of activated sludge treating high-salinity petrochemical wastewater. Water Sci Technol 87(4):823–833. https://doi.org/10.2166/WST.2023.033

Richard M, Richard G, Jenkins D (2003) Manual on the Causes and Control of Activated Sludge Bulking, Foaming, and Other Solids Seperation Problems, 3rd Edition. CRC Press

Rosselló-Mora RA, Wagner M, Amann R, Schleifer K-H (1995) The abundance of Zoogloea ramigera in sewage treatment plants. Appl Environ Microbiol 61(2):702–707

Rubio-Rincón FJ, Welles L, Lopez-Vazquez CM, Nierychlo M, Abbas B, Geleijnse M, Nielsen PH, van Loosdrecht MCM, Brdjanovic D (2017) Long-term effects of sulphide on the enhanced biological removal of phosphorus: The symbiotic role of Thiothrix caldifontis. Water Res 116:53–64. https://doi.org/10.1016/j.watres.2017.03.017

Selvarajan R, Sibanda T, Venkatachalam S, Kamika I, Nel WAJ (2018) Industrial wastewaters harbor a unique diversity of bacterial communities revealed by high-throughput amplicon analysis. Ann Microbiol

68(7):445–458. https://doi.org/10.1007/s13213-018-1349-8
Seviour R (2010) Microbial Ecology of Activated Sludge
Shao Y, Zhang H, Buchanan I, Mohammed A, Liu Y (2019) Comparison of extracellular polymeric substance (EPS) in nitrification and nitritation bioreactors. Int Biodeterior Biodegradation 143:104713. https://doi.org/10.1016/J.IBIOD.2019.06.001
Singleton CM, Petriglieri F, Wasmund K, Nierychlo M, Kondrotaite Z, Petersen JF, Peces M, Dueholm MS, Wagner M, Nielsen PH (2022) The novel genus, 'Candidatus Phosphoribacter', previously identified as Tetrasphaera, is the dominant polyphosphate accumulating lineage in EBPR wastewater treatment plants worldwide. ISME J 16(6):1605–1616. https://doi.org/10.1038/s41396-022-01212-z
Stes H, Aerts S, Caluwé M, Dobbeleers T, Wuyts S, Kiekens F, D'Aes J, De Langhe P, Dries J (2018) Formation of aerobic granular sludge and the influence of the pH on sludge characteristics in a SBR fed with brewery/bottling plant wastewater. Water Sci Technol 77(9):2253–2264. https://doi.org/10.2166/wst.2018.132
Stokholm-Bjerregaard M, McIlroy SJ, Nierychlo M, Karst SM, Albertsen M, Nielsen PH (2017) A critical assessment of the microorganisms proposed to be important to enhanced biological phosphorus removal in full-scale wastewater treatment systems. Front Microbiol 8(APR):1–18. https://doi.org/10.3389/fmicb.2017.00718
Thomsen TR, Kong Y, Nielsen PH (2007) Ecophysiology of abundant denitrifying bacteria in activated sludge. FEMS Microbiol Ecol 60(3):370–382. https://doi.org/10.1111/j.1574-6941.2007.00309.x
Tsertou E, Caluwé M, Goossens K, Dobbeleers T, Dockx L, Poelmans S, Suazo KS, Dries J (2022) Is building up substrate during anaerobic feeding necessary for granulation? Water Sci Technol 86(4):763–776. https://doi.org/10.2166/wst.2022.236
V.Tandoi, David J, Wanner J (2006) Activated sludge separation problems. IWA Publishing, London
van Loosdrecht MCM, Nielsen PH, Lopez-Vazquez CM, Brdjanovic D (2016) Experimental Methods in Wastewater Treatment. In: Water Intelligence Online. IWA Publishing, pp 9781780404752–9781780404752
Vervaeren H, De Wilde K, Matthys J, Boon N, Raskin L, Verstraete W (2005) Quantification of an Eikelboom type 021N bulking event with fluorescence in situ hybridization and real-time PCR. Appl Microbiol Biotechnol 68(5):695–704. https://doi.org/10.1007/s00253-005-1963-9
Wágner DS, Ramin E, Szabo P, Dechesne A, Plósz BG (2015) Microthrix parvicella abundance associates with activated sludge settling velocity and rheology - Quantifying and modelling filamentous bulking. Water Res 78:121–132. https://doi.org/10.1016/j.watres.2015.04.003
Wagner M, Erhart R, Manz W, Amann R, Lemmer H, Wedi D, Schleifer K, Munich D-, Abfallwirtschaft W- (1994) Development of an rRNA-Targeted Oligonucleotide Probe Specific for the Genus Acinetobacter and Its Application for In Situ Monitoring in Activated Sludge. (35):792–800
Wang P, Yu Z, Qi R, Zhang H (2016) Detailed comparison of bacterial communities during seasonal sludge bulking in a municipal wastewater treatment plant. Water Res 105:157–166. https://doi.org/10.1016/j.watres.2016.08.050
Wang Q, Liang J, Zhang S, Yoza BA, Li QX, Zhan Y, Ye H, Zhao P, Chen C (2020) Characteristics of bacterial populations in an industrial scale petrochemical wastewater treatment plant: Composition, function and their association with environmental factors. Environ Res 189. https://doi.org/10.1016/j.envres.2020.109939
Zhang M, Yao J, Wang X, Hong Y, Chen Y (2019) The microbial community in filamentous bulking sludge with the ultra-low sludge loading and long sludge retention time in oxidation ditch. Sci Rep 9(1):1–10. https://doi.org/10.1038/s41598-019-50086-3

716 **Tables and figures**

717

718 **Table 1** Distribution of the industrial plants across the different sectors.

Sector	**Food**				**Chemical**	**Textile**	**Tank truck cleaning**	**BioIndustry***
	Meat	Vegetable	Brewery	Dairy				
Number of plants	9	2	3	3	5	2	4	3

719 * Bioindustry: Corresponds to 3 industrial plants processing soya beans, malt and rapeseed.

720

721

722

723

724

725

726 **Table 2** Range of wastewater composition in different industrial sectors.

Industrial Sector	**COD ($mg_{O2}.L-1$)**	**VFA ($mg.L^{-1}$)**	**P total ($mg.L^{-1}$)**	**N total ($mg\ N.L^{-1}$)**	**S total ($mgS.L^{-1}$)**	**EC ($mS.cm^{-1}$)**	**pH**	**COD/N**	**COD/P**
Meat	768-5200	578-1162	2-46	15-1090	1-2042	1.1-32	5.2-11.5	5-73	56-636
Vegetable	1992-4682	440-880	6.4-59	38-72	11-22	0.7-3.4	4.3-5.2	43-62	59-418
Brewery	2974-5756	853-1528	5-14	13-47	2-12	2.6-4	6.6-7.5	86-96	227-538
Dairy	894-2252	504-804	6.5-21	75-148	23-38	2.4-3.4	5.7-7.9	7-28	104-249
Bio-industry	702-11304	65-788	8-120	25-126	6-232	2.8-16	5.1-8.7	28-90	25-140
Chemical	561-3504	61-624	4.1-13	6-191	0-1113	1.9-50	6.7-9.5	8-250	176-421
Textile	250-1260	50-290	1-13	11-51	62-142	3.8-5	6,5-8	18-130	114-250
Tankcleaning	864-5120	974-1500	2-10	9-18	18-297	2-3.5	4.2-8.2	75-311	304-557

727

728

729

730

731

732

733

734

735

736

Figure captions:

Fig. 1. a) Bar chart of 10 most abundant phyla across the 29 plants of 7 industrial sectors b) Heatmap of 10 most abundant genera for each industrial plant, values were calculated as averages of read abundance (%)

Fig. 2. a) Rank abundance plot presenting the cumulative read abundance across 56 activated sludge samples, including mean and SD of genus-level ASVs b) Core community plot highlighting strict, general and loose core community based on observation frequency c) Heatmap of the general core community at genus-level ASVs across industrial sectors.

Fig. 3. Heatmap presenting the functional groups (at genus classification) along the different process type and their respective number of plants. Results are the mean of read abundance.

Fig. 4. a) High-low plot describing settling properties (DSVI) and abundance of filaments (FI) in 58 samples (Range high-low and average are presented); b) Abundant genera in filamentous bulking sludge (FBS) and viscous bulking sludge (VBS) samples

Fig. 5. a) Heatmap visualising the abundance of the top 10 filaments genera and phyla across the industrial sectors, b) heatmap presenting most abundant FB distributed along FI and c) Boxplot showing the total filaments abundance across industrial sectors

Fig. 6. a) CCA plot, the arrow length represents the strength of the correlation between the environmental variables and the microbiome. The relative contribution (eigenvalue) of each axis to the total inertia in the data as well as to the constrained, respectively, are indicated in percent at the axis titles. b) PCoA of bacterial communities, the first two principal coordinates are plotted representing 10% of the total variation.

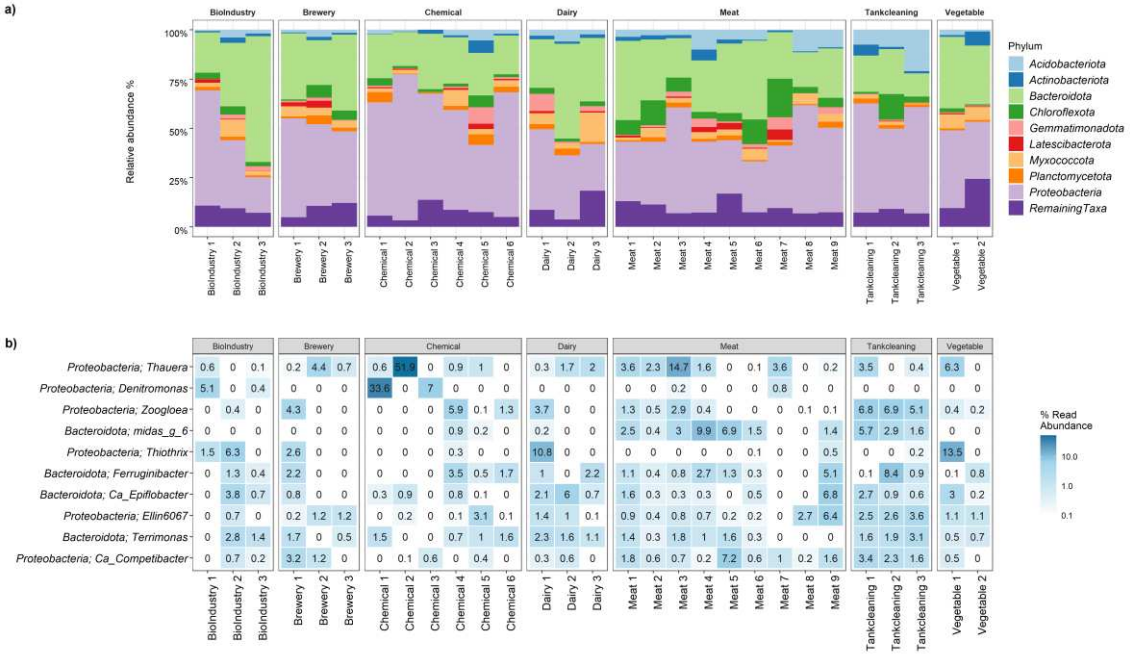


Fig. 1. a) Bar chart of 10 most abundant phyla across the 29 plants of 7 industrial sectors b) Heatmap of 10 most abundant genera for each industrial plant, values were calculated as averages of read abundance (%)

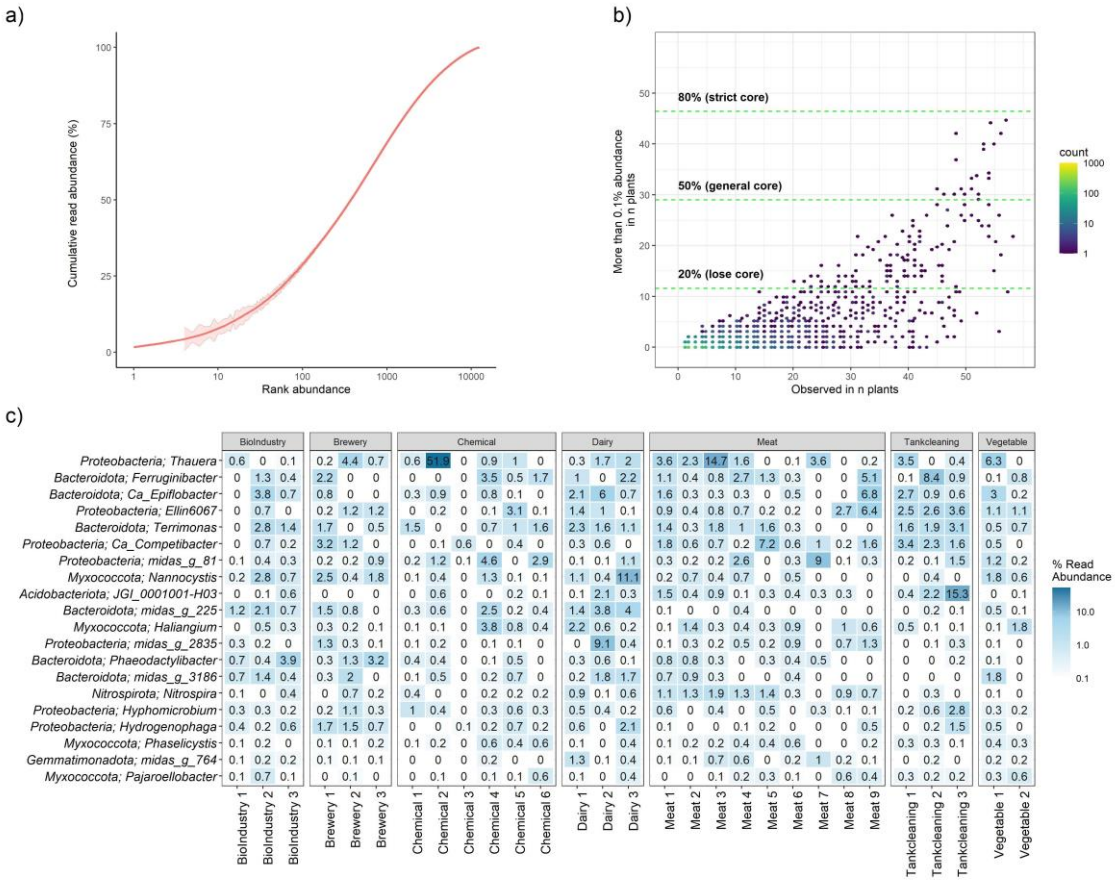
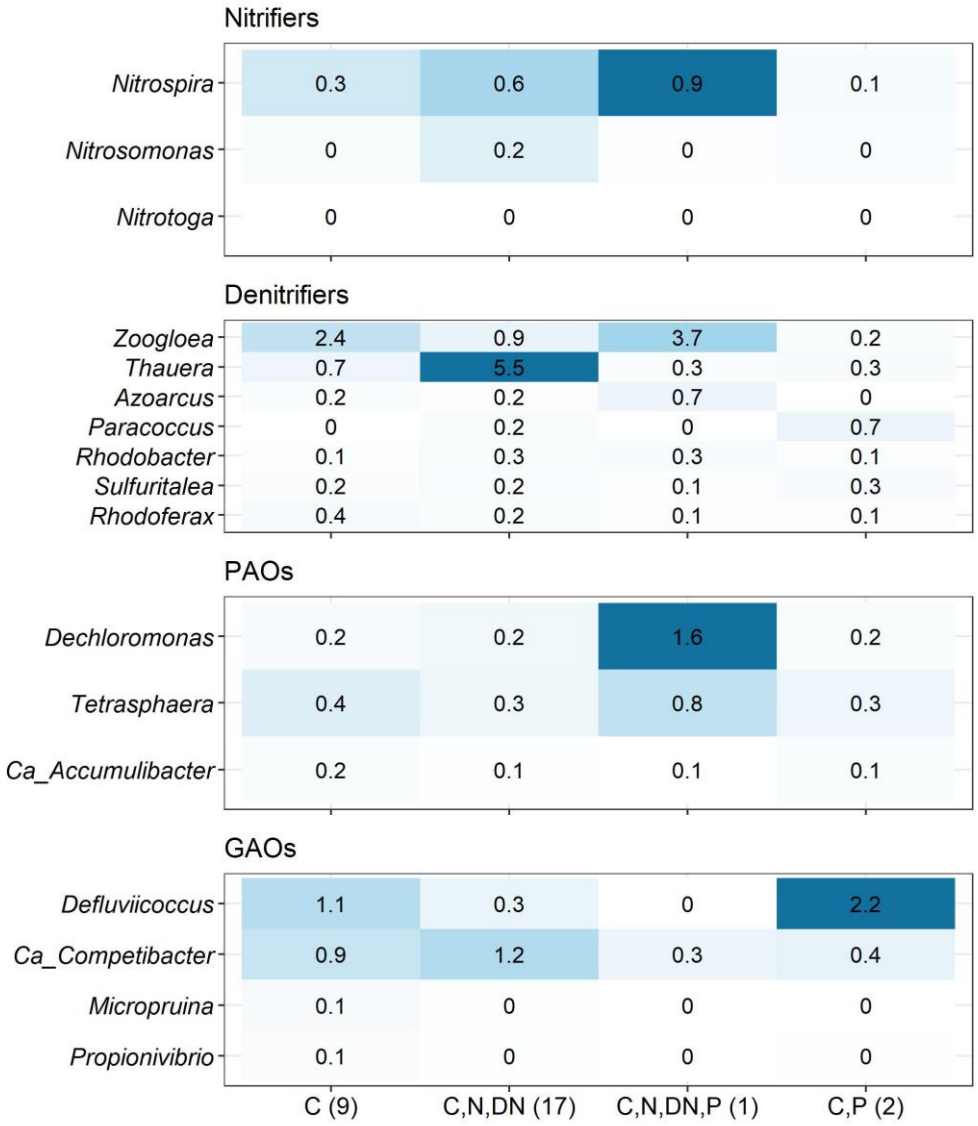


Fig. 2. a) Rank abundance plot presenting the cumulative read abundance across 56 activated sludge samples, including mean and SD of genus-level ASVs b) Core community plot highlighting strict, general and loose core community based on observation frequency c) Heatmap of the general core community at genus-level ASVs across industrial sectors.

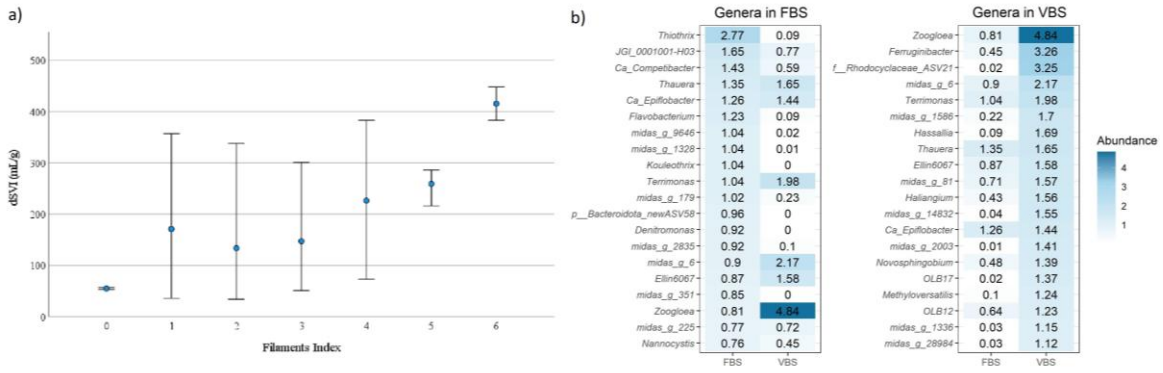


770

771 **Fig. 3.** Heatmap presenting the functional groups (at genus classification) along the different process type and

772 their respective number of plants. Results are the mean of read abundance.

773



774

775 **Fig. 4.** a) High-low plot describing settling properties (DSVI) and abundance of filaments (FI) in 58 samples

776 (Range high-low and average are presented); b) Abundant genera in filamentous bulking sludge (FBS) and

777 viscous bulking sludge (VBS) samples

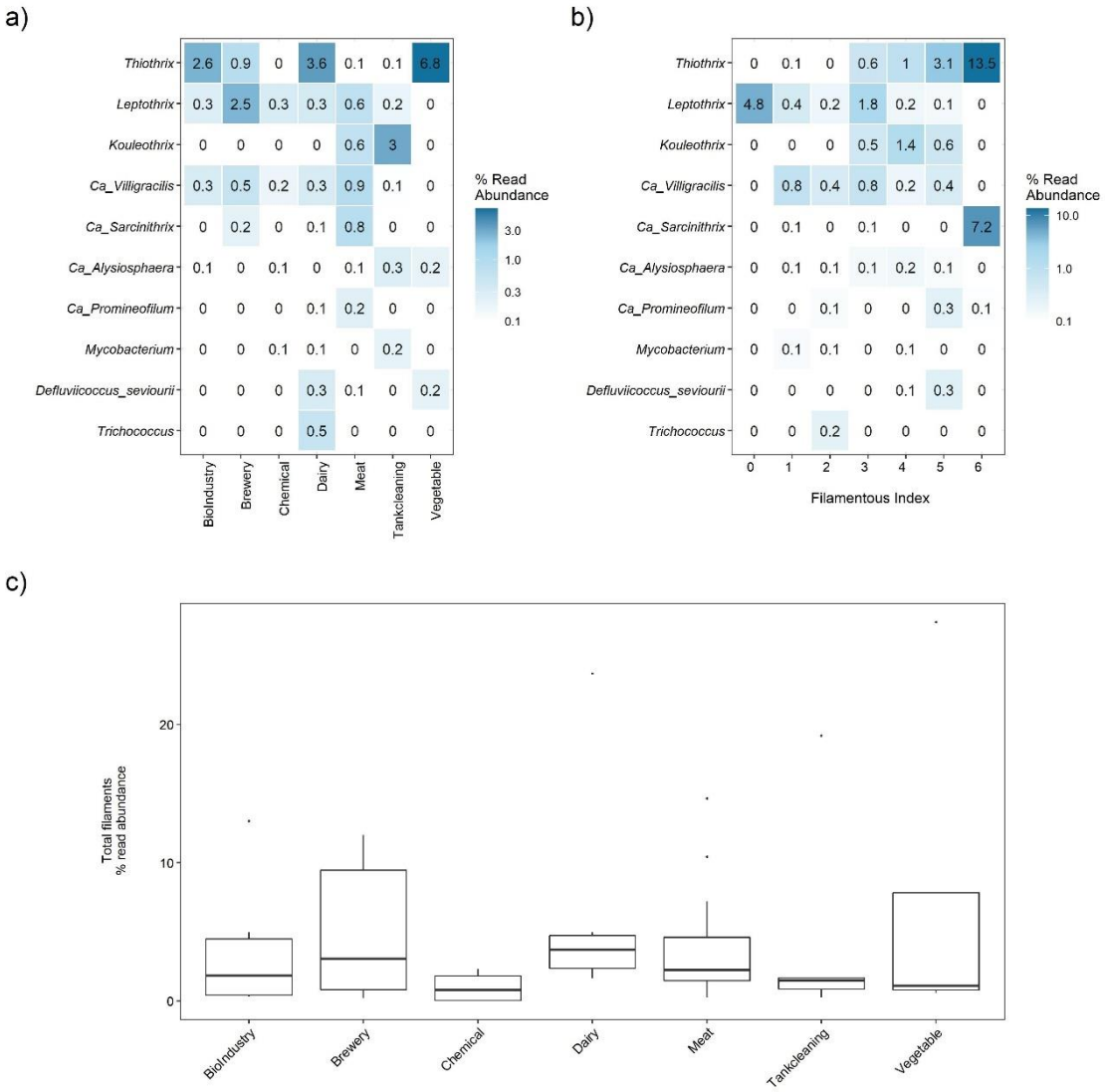
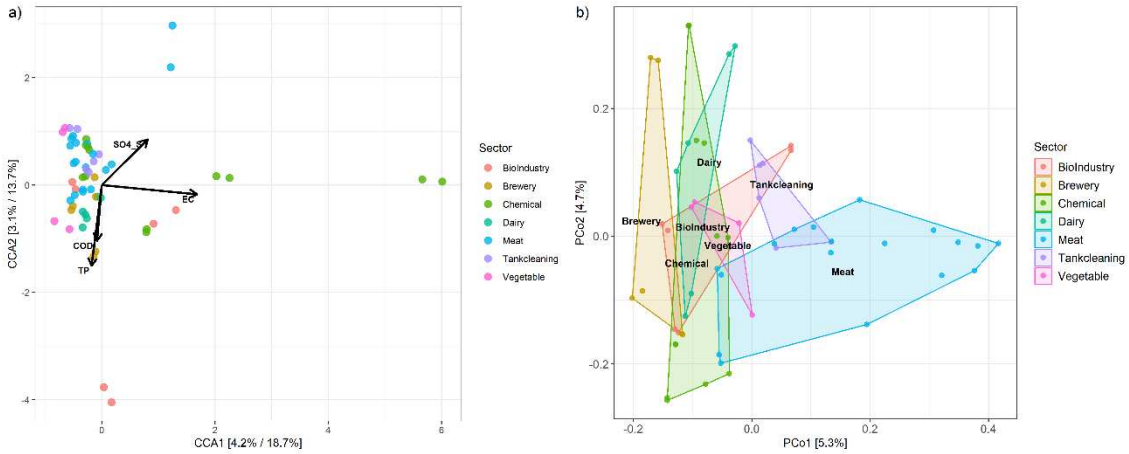


Fig. 5. a) Heatmap visualising the abundance of the top 10 filaments genera and phyla across the industrial sectors, b) heatmap presenting most abundant FB distributed along FI and c) Boxplot showing the total filaments abundance across industrial sectors



786

787 **Fig. 6**. a) CCA plot, the arrow length represents the strength of the correlation between the environmental
788 variables and the microbiome. The relative contribution (eigenvalue) of each axis to the total inertia in the data
789 as well as to the constrained, respectively, are indicated in percent at the axis titles. b) PCoA of bacterial
790 communities, the first two principal coordinates are plotted representing 10% of the total variation.

791

792

793