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# Primary settling changes the microbial community of influent wastewater to wastewater treatment plants

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## ABSTRACT

The continuous immigration of bacteria in influent wastewater strongly impacts the microbial community of activated sludge (AS) in wastewater treatment plants (WWTP), both in terms of species composition and their abundance. Therefore, it is of interest to elucidate the route of immigrating bacteria into the biological tanks, including the effect of primary settlers. These are commonly used pretreatment units that can possibly selectively increase or reduce the relative abundance of certain bacteria. Species-level identification of the microbial composition of influent wastewater before and after primary settling was carried out in four full-scale municipal WWTPs biweekly over one year by 16S rRNA gene amplicon sequencing. Overall, 37–49% of incoming COD was removed in the primary settlers. Most genera and species were present in the wastewater to all four plants and the trend of these were investigated across the primary settlers. Approximately 50% of the genera had the same trend across at least three WWTPs. Few genera significantly increased in relative read abundance (3.7%) after settling, while 22.3% showed a significant reduction in relative abundance. We investigated process-critical species in AS, such as known nitrifiers, polyphosphate-accumulating organisms, and filamentous bacteria. Most taxa were affected similarly in all WWTPs including multiple genera involved in bulking in AS. However, some genera, e.g., important polyphosphate-accumulating bacteria, had inconsistent trends across WWTPs, suggesting that the characteristics of the wastewater are important for the trend of some bacteria through primary settling. In all cases, primary settling changed the microbial community of the influent wastewater, posing an obvious candidate for upstream control to optimize the assembly of the microbial communities in activated sludge.

## 1. Introduction

Most wastewater treatment plants (WWTP) globally utilize biological removal of nutrients and pollutants using activated sludge (AS). The conversion of substrates in the wastewater, and the subsequent separation of sludge flocs and effluent water is highly dependent on the microbial community within the AS (Dueholm et al., 2022; Seviour and Nielsen, 2010; Singleton et al., 2021). Today, WWTP-management is mainly based on optimizing the environmental conditions within the biological tanks to promote bacteria important for plant efficiency (La Cour Jansen, 2019). However, the importance of immigrating bacteria from wastewater streams is often overlooked. Recent studies show that immigration greatly impacts the community structure in AS, indicating that microbial species will only be present if they are regularly added to the WWTP by the influent wastewater or other sources, and that their abundance matters (Dottorini et al., 2023, 2021; Gibson et al., 2023).

These findings highlight the need for a better understanding of the factors that determine the composition and abundance of the bacteria in the influent wastewater and whether their presence or abundance may be manipulated upstream to the biological tank in WWTPs.

Influent wastewater is often treated in a primary settling tank, typically reducing the total suspended solids (TSS) by 60–80% (Tchobanoglous et al., 2014). The settler both reduces the oxygen demand for the biological treatment, and it increases the recovery of organic material for biogas production (Patziger et al., 2016). Currently, primary settling is monitored based on parameters such as hydraulic retention time and the removal efficiency of the chemical oxygen demand (COD) (Griborio et al., 2021; Wilson et al., 2006). The flow is often only regulated if it exceeds the capacity of downstream processes. Chemically Enhanced Primary Treatment (CEPT) is commonly used. In CEPT, chemical coagulants, such as iron chloride, and alum or flocculants, such as polymers, are added to increase the sedimentation velocity and

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removal efficiency (Shewa and Dagnew, 2020).

Along with the COD reduction during primary settling, the bacterial biomass is similarly reduced. However, little is known about the effect of primary settling on the microbial community composition, and the primary settler may act selectively with some taxa experiencing a higher or lower reduction than others. Mechanical processes such as centrifugation have shown to separate bacteria with different motilities (Maggi et al., 2013), therefore primary settling might differently affect bacteria with various sizes, shapes, and physio-chemical properties, leading to either enrichments or reductions in their relative abundances. For example, Shin et al. (2022) found a reduction in both the amount of 16S rRNA gene copies and in the abundances of the different antibiotic resistance genes in wastewater after primary settling, which could be explained by a change in the microbial community after primary settling. However, Lambirth et al. (2018) found no change in the overall microbial community before and after primary settling, but the study was conducted with only four pairs of samplings spread across one year.

This study aims to investigate the impact of primary settling for the microbial community structure of influent wastewater with the hypothesis that the primary settling will promote a reduction or an increase of relative read abundance of certain bacterial species. We focused on process-critical species for AS detected in influent wastewater. We collected samples of influent wastewater before and after the primary settler every two weeks for one year at four Danish municipal WWTPs and applied 16S rRNA gene amplicon sequencing to characterize the microbial community structure. The possible impact of addition of chemicals was also evaluated and compared in the four WWTPs. The results of this study help elucidate potential new parameters for optimizing the primary settling step at WWTP.

## 2. Materials and methods

### 2.1. Sampling and operation of primary settling tanks

Influent wastewater to municipal WWTPs was collected before and after the primary settling units from four full-scale WWTPs using flow-proportional autosamplers for 24 h. Samples were collected between March 2019 and March 2020, approximately every second week (Fig. S1). The WWTPs were in different regions of Denmark, and their characteristics are displayed in Table 1. CEPT was used in Randers and Ejby Mølle and in Randers WWTP iron chloride (13.8% w/w Fe) was dosed with the dosage regulated by the influent wastewater flow (5–6 mL/m<sup>3</sup>, flow < 875 m<sup>3</sup>/h; 8 mL/m<sup>3</sup>, flow > 875 m<sup>3</sup>/h). In Ejby Mølle, iron sulfate and polymers were added independently of flow before the water was pumped to the primary settling unit. In Ejby Mølle 20 kg polymers (NECOLAN CE 663 H, PolyChemie GmbH) and 1300–1500 L iron sulfate (10–12% w/w Fe) were added daily. In dry weather (rain ≤0.5 mm/day), it corresponded to ~35 mL/m<sup>3</sup> iron sulfate or ~0.5 g/m<sup>3</sup> polymer and during wet weather to ~18 mL/m<sup>3</sup> iron sulfate or ~0.3 g/m<sup>3</sup> polymer. All measurements of flow and COD (before and after primary

settler) for the given period were performed at the WWTPs and are available in Supplementary file 1. For Randers, three distinct influent streams were monitored, and some COD measurements were collected before mixing and a weighted mean based on the influent flow was calculated.

The four full-scale municipal WWTPs were characterized by typical wastewater types and settler types across Denmark, but had different percentages of industrial loads, configurations, and operations of the primary settling process. In all catchment areas the sewer lines were characterized by a mixture of gravity and pressure mains, with wastewater consisting of streams from both separate systems (only sanitary wastewater) or combined systems (sanitary wastewater and rainwater). COD removal across the settlers was monitored regularly throughout the sampling period. Reject water may in some cases have been returned to the influent. This was not done systematically, and the amount of reject water was little compared to the influent and has therefore been considered neglectable in this study.

### 2.2. Community analysis by amplicon sequencing

DNA extraction, library preparation, and sequencing were performed as described in Dottorini et al. (2021). V1–V3 primers used had the following sequences: 27F 5'-AGAGTTTGATCCTGGCTCAG-3' (Lane, 1991); 534R 5'-ATTACCGGGGCTGCTGG-3' (Muyzer et al., 1993). All raw amplicon reads generated by Illumina MiSeq were processed using the AmpProc5.0 workflow (<https://github.com/eyashiro/AmpProc>) which generated amplicon sequence variants (ASVs). The ASVs were mapped to the full-length ASVs from the database generated by Dueholm et al. (2020), a WWTP specific database using MiDAS 4 taxonomy (Dueholm et al., 2022).

### 2.3. Data analysis

Data analysis was performed in R v4.1.0 (<https://www.R-project.org/>) using RStudio 2022-02.0+443 (<https://www.rstudio.com/>). The R-package 'ampvis2' was used for rarefaction, ordinations, and calculation of alpha diversity measures (Andersen et al., 2018), and the 'ComplexUpset' package was used to make upset plots (Krassowski et al., 2022; Lex et al., 2014). Scripts and data used are available at [https://github.com/MarieRiisgaard/2023\\_PrimarySettling](https://github.com/MarieRiisgaard/2023_PrimarySettling) and bioproject PRJNA946374. Bacteria important in AS were assigned to functional guilds according to Dottorini et al. (2021). The species *Ca. Dechloromonas phosphorivorans* and *Ca. Dechloromonas phosphoritropha* have since been assigned as polyphosphate-accumulating organisms (PAO) (Petriglieri et al., 2021). All samples were rarefied at 60,000 reads. For some samples technical replicates were available, these were merged by taking the mean relative read abundance per ASV. In Randers WWTP three distinct influent streams, each with different influent flow, were sampled separately. To obtain a representative microbial community structure before primary settling, we rarefied the samples to 60,000

**Table 1**

Operational details of the WWTPs. PE is the person equivalents of each WWTP, design is the operational design of the plant (EBPR: Enhanced biological phosphorus removal, BNR: Biological Nutrient Removal), industrial load is measured in % of COD coming from industrial sources, No. of settling tanks in use in the given period, volume of each tank and surface area. The mean surface overflow rate (SOR) was calculated and measured in m<sup>3</sup> per m<sup>2</sup> surface area of the settling tanks per hour. Shape is the shape of the clarifiers, and chemical addition lists chemicals added prior to the clarifier at the treatment plant where '-' denotes that no chemicals were added.

|              | PE      | Design | Industrial load | No. of settling tanks, volume, surface area       | SOR in m <sup>3</sup> /m <sup>2</sup> /h (mean±standard deviation) | Shape       | Chemical addition         |
|--------------|---------|--------|-----------------|---|--|-------------|---------------------------|
| Aalborg West | 330,000 | EBPR   | 25%             | 2 tanks, 1900 m <sup>3</sup> , 404 m <sup>2</sup> | 3.5 ± 1.8  | Rectangular | -                         |
| Ejby Mølle   | 410,000 | EBPR   | 49–55%          | 7 tanks, 1200 m <sup>3</sup> , 464 m <sup>2</sup> | 0.7 ± 0.4  | Rectangular | Iron sulfate and polymers |
| Esbjerg West | 290,000 | BNR    | 60%             | 2 tanks, 1046 m <sup>3</sup> , 440 m <sup>2</sup> | 1.5 ± 0.8  | Circular    | -                         |
| Randers      | 130,000 | EBPR   | 5%              | 2 tanks, 2200 m <sup>3</sup> , 531 m <sup>2</sup> | 0.7 ± 0.3  | Circular    | Iron chloride             |

reads and afterwards took a flow-proportional mean of the sequencing read counts across the three influent samples. Lastly, we normalized the average sequencing read count to percent relative abundance.

A Wilcoxon signed-rank test was performed to test for differences in the relative abundances of bacteria in influent wastewater before and after primary settling. The analysis was conducted plantwise and by pairing samples before and after primary settling by sampling date. The analysis was conducted on both species and genus level and a given taxon was only tested if it had at least 12 observations before or after primary settling. 12 observations corresponded to half of the number of sample pairs in the WWTP with fewest samples. The *p*-values were adjusted by Benjamini and Hochberg (1995) and considered significant if *p*-adjusted < 0.05. In investigations of seasonality, the astronomical seasons for the northern hemisphere were used. In addition, a correlation analysis was made between the daily surface overflow rate (SOR) and the differences in the relative abundances for each of the genera investigated for each WWTP using Spearman's rank correlation coefficient. BH was used for correction of *p*-values and considered significant if *p*-adjusted < 0.05. All results are available in Supplementary files 2 and 3.

### 3. Results and discussion

#### 3.1. Overview of primary settlers

The main design properties and primary settler characteristics are summarized in Table 1. The surface overflow rate (SOR) was calculated for the primary settling tanks during the sample period (Fig. S2). COD removal was used to evaluate the performance and the average COD removal was 37–49%, with lowest removal measured in Esbjerg West WWTP (Fig. 1). Ejby Mølle showed a higher variation in COD removal compared to the other plants, and a strong negative correlation between COD removal and influent flow was found (*p*-value < 0.001, coef.: -0.6) (Fig. S3). As a constant amount of chemicals was added in Ejby Mølle, dilution with rainwater most likely contributed to the reduction in removal efficiency during rain events.

#### 3.2. Effect of primary settling on the overall community of influent wastewater

On average, we observed 1329–1637 unique species or 645–805 unique genera across samples in each WWTP, making up 67–81% or

>94% of the total read abundance of species or genera, respectively. By using the MiDAS 4 reference database, we were able to classify the majority of ASVs to the species level using V1–3 reads, as previously shown for similar systems (Dueholm et al., 2022; Dueholm et al., 2020). The remaining fraction of the total read abundance consisted of >2300 unclassified ASVs on species level or >1100 on genus level (Fig. S4). As typically observed for influent wastewater communities, most species/genera were observed in low relative abundance (<0.1%) while a few constituted most of the reads (Fig. S5). This pattern was strengthened after primary settling in Ejby Mølle and Esbjerg West, where the number of species constituting the biggest cumulative fractions decreased. In all WWTPs, the *Trichococcus* genus constituted >17% of the relative read abundance before primary settling (Fig. S6A). In addition, 22 genera, comprising the 15 most abundant for each WWTPs, represented 67–75% of the mean relative abundance for each WWTP before primary settling. Common for all plants were *Agathobacter*, *Blautia*, *Lactococcus*, *Ruminococcus*, *Streptococcus*, *Subdoligranulum*, and *Trichococcus*, illustrating that the dominant bacteria were very similar across the facilities (except for *Pannonibacter* which was dominant only in Aalborg West). Similar trends were seen after primary settling (Fig. S6B). Process-critical genera in AS systems, such as *Tetrasphaera*, *Ca. Accumulibacter*, and *Dechloromonas*, were all present in influent wastewater samples in low abundance (<0.1%), both before and after primary settling.

To evaluate the overall community differences before and after primary settling, we visualized the beta diversity of the samples through a Principal Coordinate Analysis (PCoA) (Fig. 2). A PERMANOVA analysis showed that for each plant, the microbial communities differed significantly (*p*-value < 0.001) based on their location before or after primary settling, indicating that the primary settling process altered the abundance distribution of the overall microbial community. In Randers WWTP, primary settling explained 30% of the variation and 13–14% in the remaining WWTPs. Since our recent study has highlighted the importance of seasonality in microbial communities of AS (Peces et al., 2022), this was also assessed using PERMANOVA (Fig. S7). In samples taken before primary settling, seasonality explained 18–32% of the variation across the WWTP. For Aalborg West, Esbjerg West, and Randers WWTP, seasonality could also explain variation for samples taken after primary settling or across all samples independently of primary settling. However, when including all samples, sampling season explained a smaller fraction of the variation indicating that primary settling introduced more variance that could not be explained by

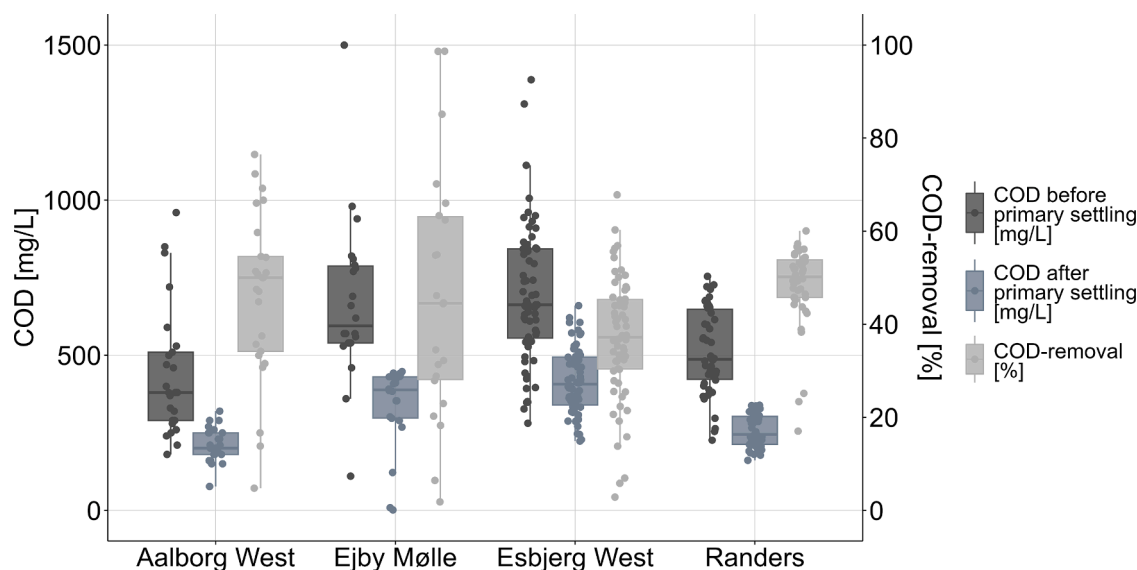
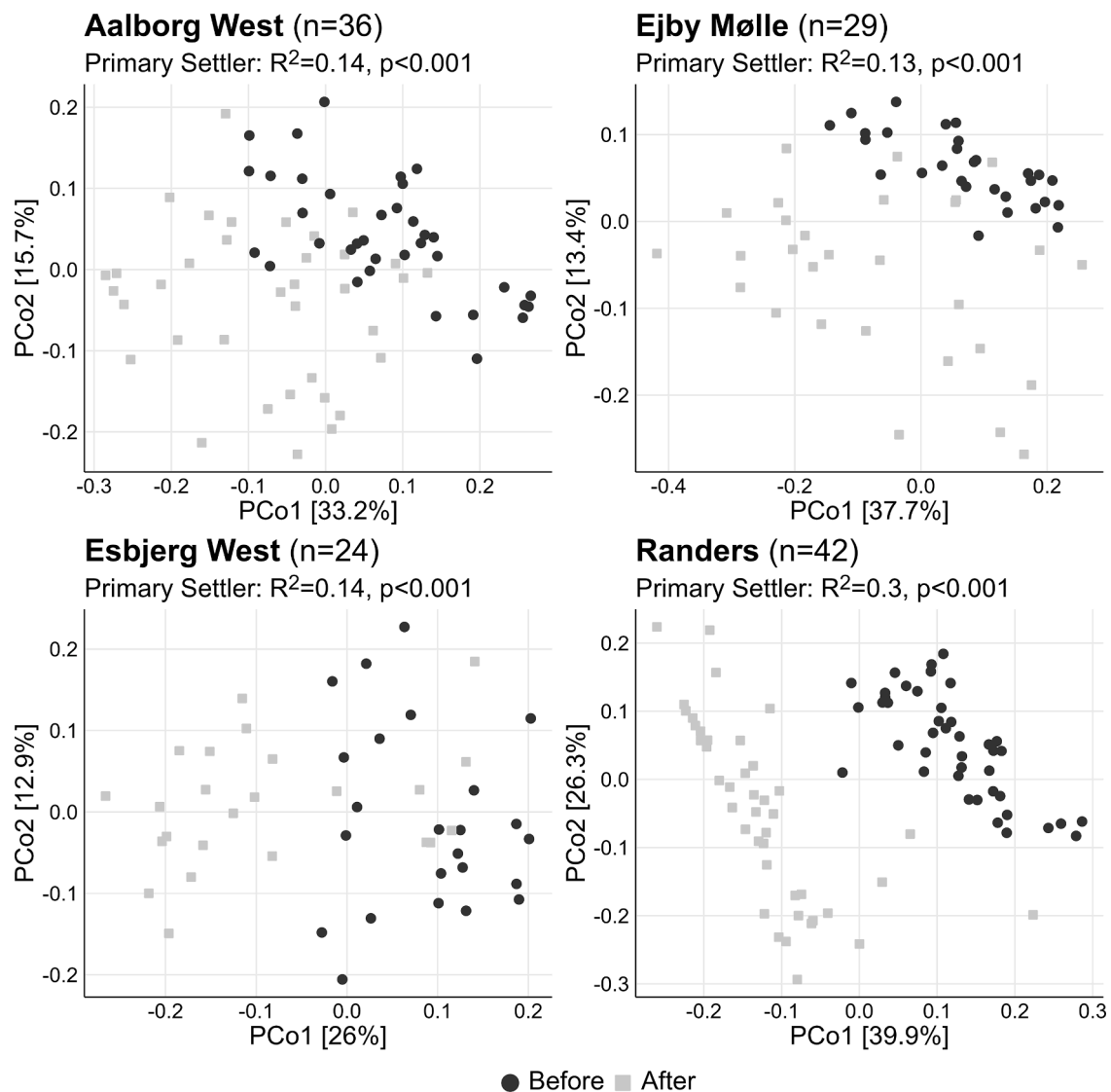


Fig. 1. COD removal during primary settling for each WWTP. Measured COD values (left axis) and COD removal (right axis) of the participating WWTPs. Mean  $\pm$  standard deviation: Aalborg West: 46%  $\pm$  18, Ejby Mølle 46%  $\pm$  27, Esbjerg West: 37%  $\pm$  13, and Randers: 49%  $\pm$  9.



**Fig. 2.** Effect of primary settling on community structure. PCoA of wastewater samples before and after primary settling for each facility. The number of paired samples by date is shown in the parentheses. Bray–Curtis distance matrix was used and species with a relative abundance  $<0.01\%$  were removed. The fraction of the sample variation explained by primary settling was determined by PERMANOVA (Adonis  $R^2$ -values), and the analysis was made using Bray–Curtis distance matrix of the species composition. Results are printed for each WWTP.

seasonal differences. In Ejby Mølle, primary settling seemed to remove seasonal differences. The analyses were performed using both the Bray–Curtis and Jaccard distance matrixes obtaining very similar results, but with a slightly higher explained variance when using the Bray–Curtis distance matrix.

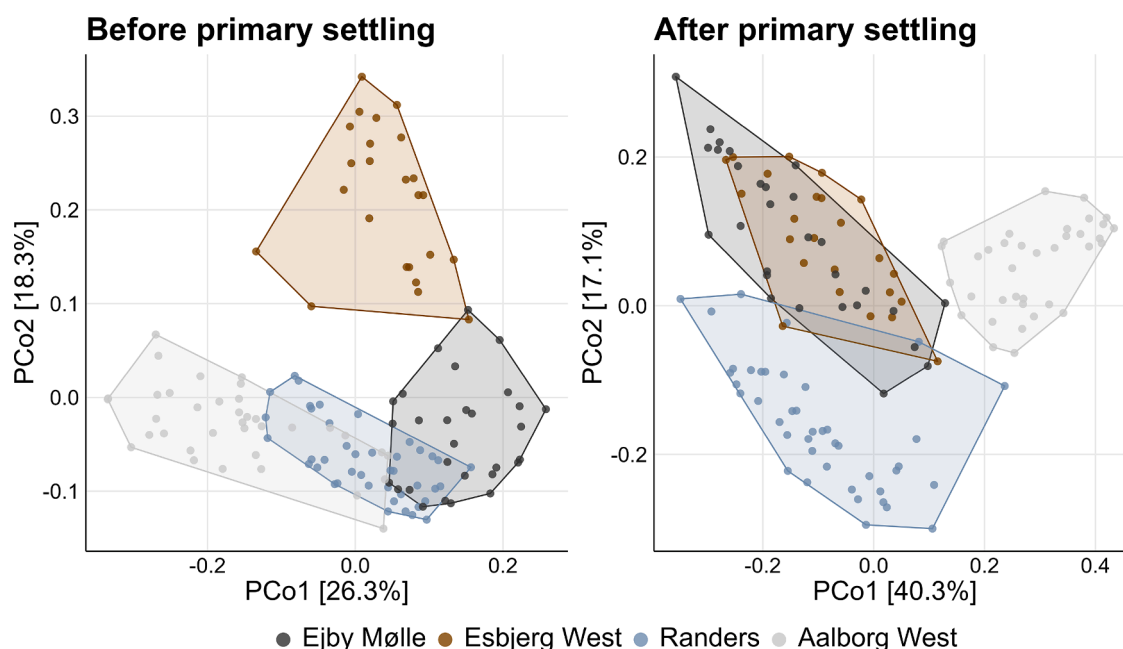
The communities were significantly different from plant to plant when comparing them either before or after primary settling for all WWTPs, in line with the findings for Denmark within the MiDAS project (Nierychlo et al., 2020) (Fig. 3). Microbial communities in Aalborg West WWTP and Randers WWTP were the most similar before primary settling, while Esbjerg West had the most distinct microbial community. However, after primary settling the interrelationship between the communities changed, with Ejby Mølle and Esbjerg West clustering together, while a clear separation of Aalborg West and Randers was seen (Fig. 3).

### 3.3. Primary settling significantly affected many genera and skewed their relative abundances

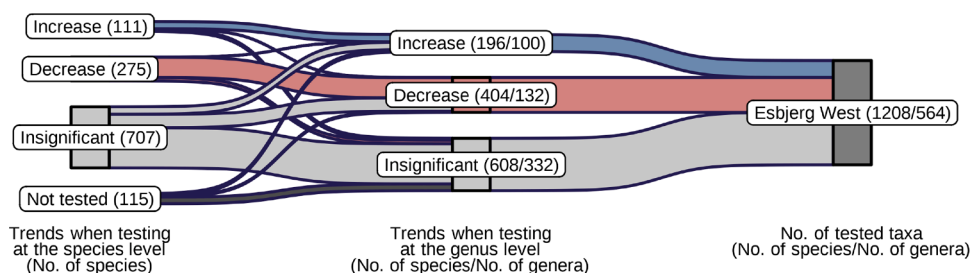
To identify the taxa affected by the primary settler, we compared the

microbial community of each plant considering only bacteria occurring multiple times across all four WWTPs. The overall community change could be ascribed to multiple bacterial taxa that either increased or decreased in relative read abundance after primary settling. Changes were evaluated on both species (1208 species) and genus (564 genera) levels to investigate the consistency of the change at different taxonomic levels. In all WWTPs, 98.6–99.7% of the species followed the same trend as their respective genera. This means that a given species found to increase in relative abundance across the primary settler, belonged to a genus that also increased in relative abundance, and vice versa (Fig. 4, Esbjerg West; Fig. S8, all WWTPs). Consequently, the trends of bacterial reduction or enrichment across the primary settling tank could be described at the genus level. In addition, when the investigation was performed at the genus level, the cumulative abundance of the genera tested accounted for a larger fraction of the total read abundance ( $\geq 93\%$ ). On species level, only 61–78% of the total read abundance was assessed, as many species did not occur enough times to be investigated (Fig. S9).

In many cases, the trend for a given genus was shared among two or more WWTPs (Fig. 5). We observed that the abundance of 62 genera



**Fig. 3.** Difference in community structure across WWTPs before and after primary settling. PCoA of influent wastewater samples either before (left) or after (right) primary settling of all WWTPs. Bray–Curtis distance matrix was used and the relative abundance of the species >0.01%. The points are colored by WWTP and sample points for each WWTP are collected in a colorframe (dark gray: Ejby Mølle, brown: Esbjerg West, blue: Randers, and light gray: Aalborg West). Groupings of samples within each plant were investigated with a permutation test and found significant if  $p < 0.001$ .



**Fig. 4.** Consistency of trends during primary settling of species within the same genus. Sankey diagram for Esbjerg West WWTP of the consistency between trends in the primary settling process when the change in relative abundance was investigated on either species or genus level. The first column displays the result when the analysis was conducted at species level. The second column displays the trends when the analysis was conducted at genus level, and the third column displays the number of species and genera tested.

were consistently reduced in all WWTPs, while only 4 genera were enriched during primary settling. 31 genera showed no change (insignificant) after primary settling in all WWTPs, thus 17.2% behaved consistently across all plants. Common between any combination of three WWTPs (for example Ejby Mølle, Aalborg West, and Esbjerg West), 64 genera additionally showed a decrease, 17 an increase and 127 no change (Fig. 5). Thus, 54% of the genera tested behaved similarly across three or more WWTP, 22.3% showing a decrease, 3.7% an increase, and 28% no change. One of the genera consistently enriched was *Arcobacter* which is a potential pathogen frequently found in wastewater and WWTP effluent (Kristensen et al., 2020). Consistently reduced genera included some with known function (one filamentous genus, two glycogen accumulating organisms (GAO) and two polyphosphate-accumulating organisms (PAO)), but for the vast majority both function and the *in situ* physiology in AS are currently unknown (Fig. S10).

In Randers, most genera were significantly affected by primary settling, and many were only affected in this plant (97 increased and 99 decreased, Fig. 5). This was observed both when all samples from Randers were included ( $n = 42$ , Fig. 5) and when we subsampled to the same number of samples in all plants ( $n = 24$ , Fig. S11). Randers differed from the other plants by a flow-regulated addition of iron chloride prior to the settling tank which potentially could promote reduction of specific bacteria. In Ejby Mølle different chemicals than in Randers were

added (iron sulfate and polymers) before primary settling. Also, in Ejby Mølle, the amount of added chemicals was not adjusted to the influent wastewater flow, which most likely contributed to the high variation in COD removal (Fig. S2). Variation in wastewater flow mildly affected the microbial community, and 25 of the 564 genera investigated correlated with SOR in Ejby Mølle (Supplementary file 2). Most of these genera had a positive correlation, meaning that during days with high flows, a lower reduction in the relative abundance was seen after primary settling. In comparison, 6 genera were affected in Aalborg, 9 in Esbjerg West and none in Randers.

The WWTPs were compared pairwise to find the number of bacteria showing an opposite trend across plants, meaning that a bacterial genus increased in one WWTP and decreased in the other (Fig. S12). In all comparisons with Randers WWTP, we observed the most inconsistent results with 48–85 bacterial genera showing opposite trends. Comparing the other plants without Randers, only 8–34 genera showed opposite trends.

In terms of relative read abundance, the cumulative abundance of the genera found to decrease after primary settling constituted, on average, 14–21% less after primary settling than before primary settling (Fig. 6). The reduction was significant in all WWTPs (Table S1). For example, in Randers, the 293 genera reduced in relative abundance during primary settling constituted 35% of the abundance before primary settling, while after primary settling the same genera comprised

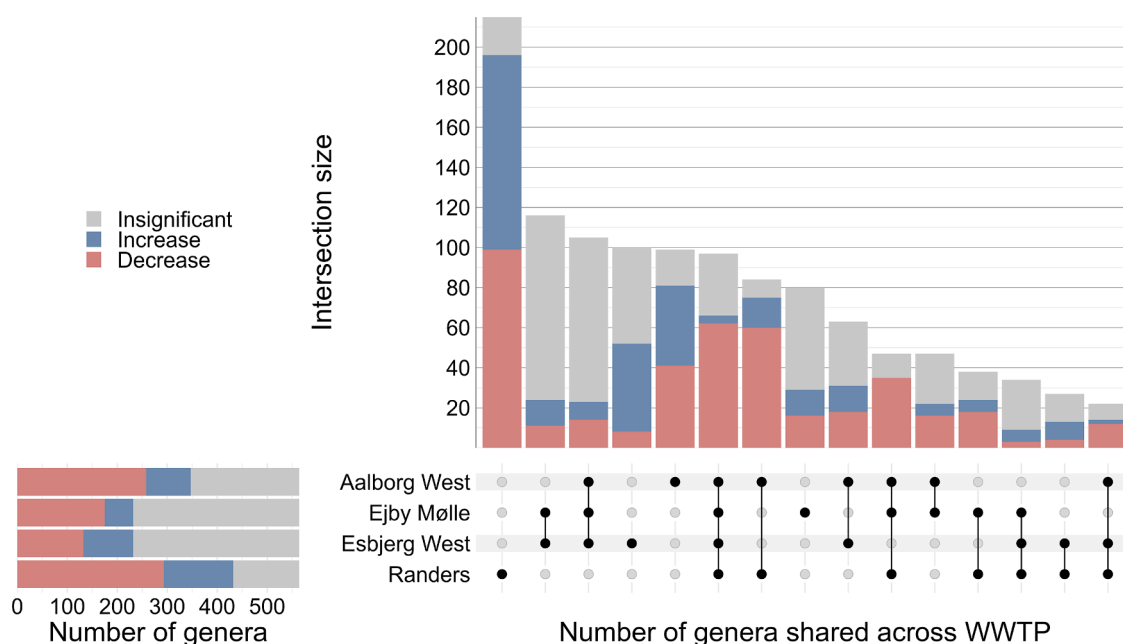


Fig. 5. Genera with shared trends in multiple WWTPs. Upset plot of genera shared between the different WWTPs and with consistent trends across primary settling. Colors indicate the trend after primary settling, either increase in relative abundance (blue), decrease (red) or not significant change (gray). The bars on the left indicate the number of genera with either trend for each WWTP.

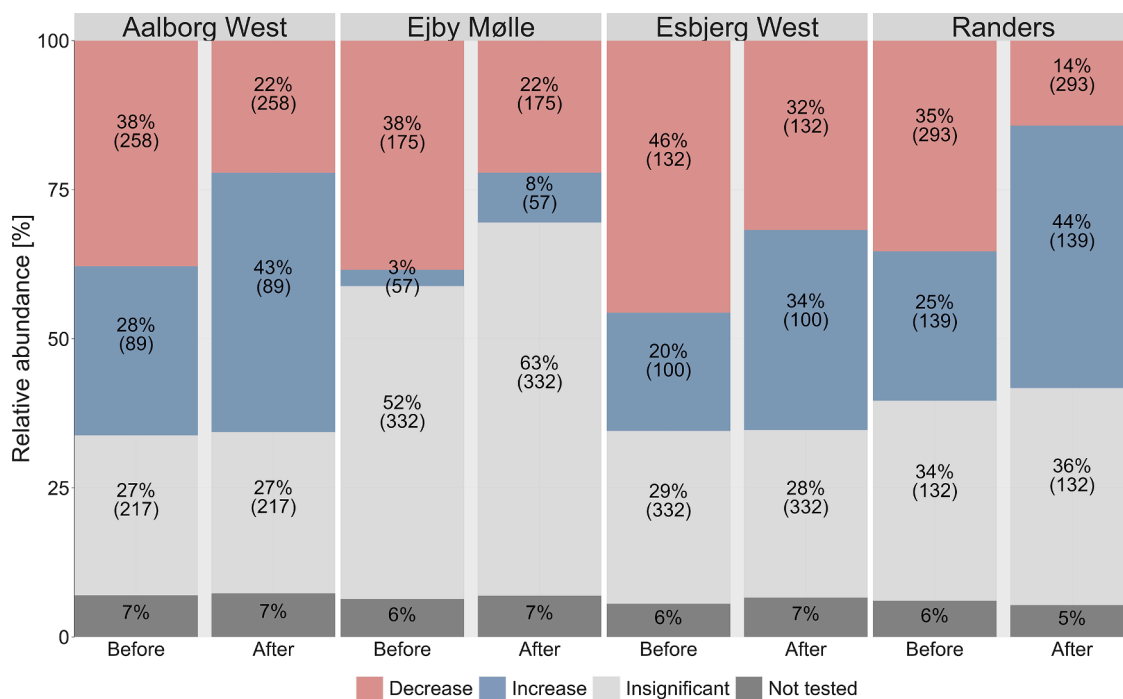


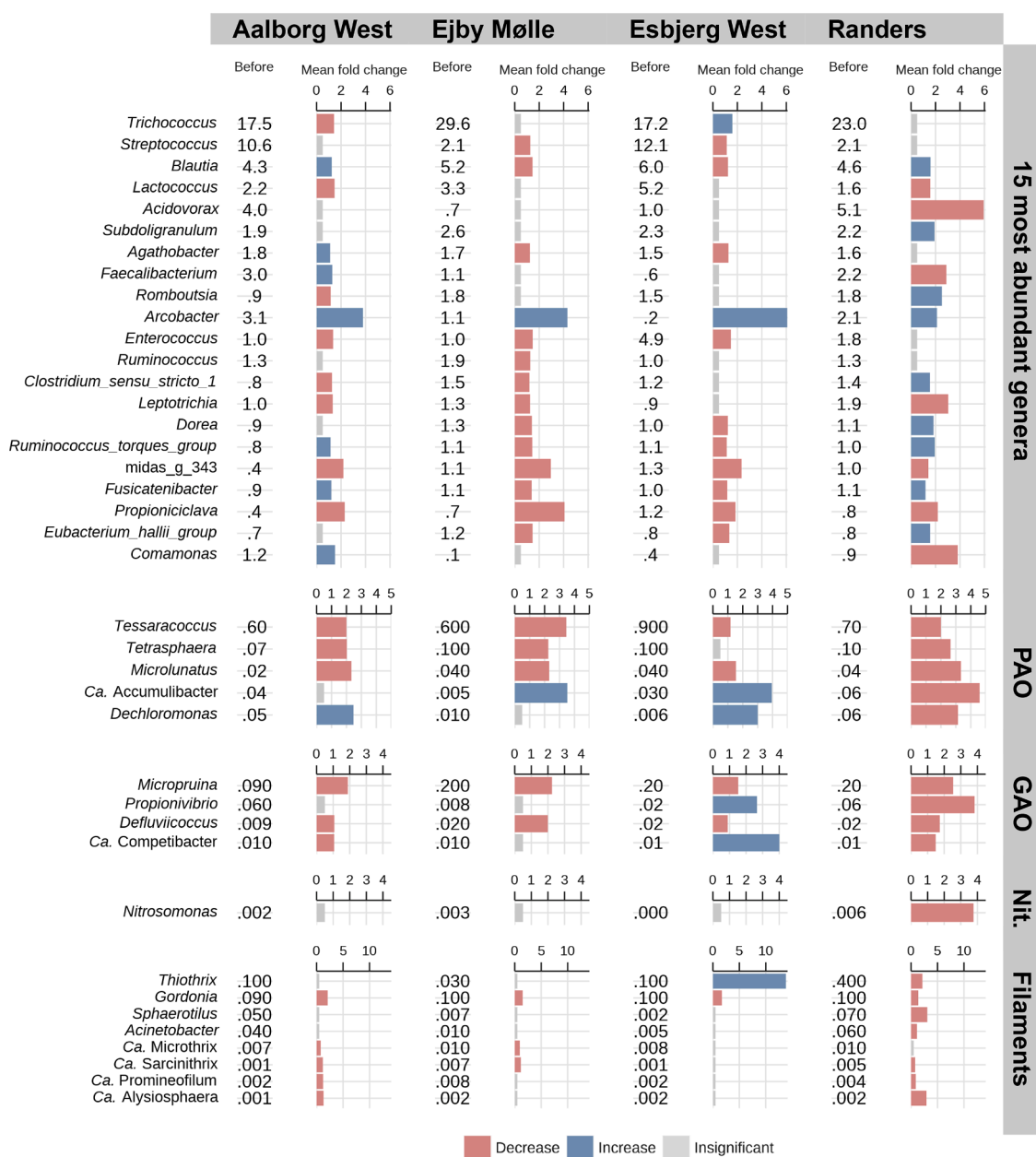
Fig. 6. Cumulative abundance of bacterial genera reduced/enriched by primary settling. The cumulative abundance of the genera before and after primary settling is shown for each WWTP and colored by trend; decrease (red), increase (blue), insignificant (light gray), and not tested (dark gray). The abundances were calculated as the mean abundance across samples within each group. The number of genera tested within each group is shown in a parenthesis.

only 14% of the abundance. Conversely, the cumulative abundance of the genera found to be enriched, was significantly higher after primary settling in all WWTPs with an average increase of 5–19% across the WWTPs. No difference in the cumulative abundance was found for the genera showing no change during primary settling for Aalborg West, Esbjerg West, and Randers. However, in Ejby Mølle a significant increase was observed for genera with no trend across the primary settler, and before primary settling these constituted a higher fraction (52%) of the

abundance compared to the genera with no trend in the other WWTPs (27–34%).

### 3.4. Effect of primary settling on the most abundant genera of influent wastewater and of process-critical genera of activated sludge

Some genera were present in high relative abundance (>1%) in all plants (Fig. 7). These included *Trichococcus*, *Streptococcus*, *Blautia*,



**Fig. 7.** Summary of enrichments and reductions of selected microbial groups at genus level. The genera are divided into the 15 most abundant genera in terms of median relative abundance, and into their function in AS consisting of: polyphosphate-accumulating organisms (PAO), glycogen accumulating organisms (GAO), nitrifiers (Nit.), and filamentous bacteria (Filaments). Across all WWTP, the 15 most abundant genera comprised 22 genera. The 'Before' column indicates the median percent relative abundance before primary settling. The 'Mean fold change' column displays the mean of the fold change between samples before and after primary settling paired by date. The bars are colored by trend, where the colors blue and red indicate a significant increase or decrease after primary settling, respectively. The gray bars indicate an insignificant result, and all are set to 0.5. *Streptococcus*, *Lactococcus*, and *Trichococcus* were amongst the top 15 genera but are also characterized as filamentous bacteria. In samples with an abundance of zero a pseudo-count of 0.001% relative abundance has been introduced to calculate the mean fold change.

*Lactococcus*, *Subdoligranulum*, *Agathobacter*, and *Ruminococcus*. Only *Arcobacter* and *Propioniciclava* had a consistent trend across all plants, while the remaining abundant genera showed inconsistent trends. *Acidovorax* had a very distinct behavior in Randers WWTP, where it had a 6-fold reduction, while being unaffected in the other WWTPs (Fig. 7).

Since process-critical bacteria for AS (such as PAO, GAO, nitrifiers, and filamentous bacteria) are continuously immigrating from the influent wastewater, we evaluated if and how much the primary settler affected their abundance. These bacteria were generally present in low abundance (<0.1%) in the influent wastewater. Nevertheless, many were affected by primary settling. PAOs and GAOs generally decreased in relative abundance, but some plant-specific differences were found

(Fig. 7). For example, the abundance of the PAO *Tetrasphaera* decreased in all but Esbjerg West, where no change was seen. *Dechloromonas* and *Ca. Accumulibacter* showed inconsistent trends; while both bacteria were reduced in Randers, they were either enriched or showed no change in Esbjerg West, Aalborg West, and Ejby Mølle. Among the putative PAOs, *Tessaracoccus* and *Microlunatus* were significantly reduced across all plants. The GAOs, *Micropruina* and *Defluviicoccus* were reduced in all plants. The trend of *Propionivibrio* and *Ca. Competibacter* differed in Esbjerg West and Randers. Both genera were enriched in Esbjerg, while being reduced in Randers.

The nitrifiers were present in very low abundance in the influent (<0.006%), and rarefaction of read counts at 60,000 reads in all samples



did not give sufficient resolution to investigate them. An exception was *Nitrosomonas* which decreased in relative abundance in Randers (Fig. 7). In that plant, the available sequencing resolution was higher than in the other plants, and we could rarefy the dataset to 100,000 reads, which allowed to determine that *Nitrotoga* as well as *Nitrospira* decreased in relative abundance during primary settling in Randers (Fig. S13).

The filamentous bacteria were generally reduced or unchanged in relative abundance. The only exceptions were *Trichococcus* and *Thiothrix*, both enriched in Esbjerg West (Fig. 7). *Ca. Microthrix*, the most common filamentous bacteria in Danish WWTPs, was reduced in Aalborg West and Ejby Mølle. Multiple other bacteria related to bulking or foam formation in AS were also consistently reduced, including *Gordonia*, *Tsukamurella*, and *Millisia* (Goodfellow and Kumar, 2015; Soddell et al., 2006).

#### 4. Discussion

Primary settling is a well-known mechanical treatment commonly present at many WWTPs to reduce the oxygen demand for the biological treatment and increase the recovery of organic material for biogas production. In the four WWTPs investigated, primary settling reduced the COD with 37–49% in the incoming wastewater. The values fall within the expected, which for CEPT is around 60% dependent on chemical and dosage, and non-CEPT approximately 40% (Shewa and Dagneu, 2020; Tchobanoglous et al., 2014; Wan et al., 2016). When designing primary settling tanks, the SOR is generally expected within 1–2 m<sup>3</sup>/m<sup>2</sup>/h for both circular and rectangular tanks (Wilson et al., 2006). Only in Aalborg West, the SOR surpassed this range. However, the plant performed equally well in terms of COD removal, and only 6 of 564 genera were correlated with SOR. Apart from design and operation, wastewater characteristics have a great effect on the COD removal (Wilson et al., 2006), and variation of >20% in COD removal, despite similar operating conditions, has previously been observed (Patziger et al., 2016).

While removing the organic fraction, the primary settler also removes microorganisms contained in it. The reduction of COD therefore sets a new total amount of biomass which the relative read abundance refers to after primary settling. We found that primary settling partially explained the difference in microbial community structure in the influent wastewater before and after settling. Thus, when studying the assembly of AS communities, primary settling should be considered as an important factor as it alters the composition and amount of microbes added to the process tanks. Considering a bacterial taxon reduced in primary settling, the percentage decrease is even more pronounced when including the concurrent reduction in COD as the smaller fraction of read abundance is taken from a smaller amount of biomass. To give a rough estimate of the change in the mass flow of a given genus, we must assume that the fraction of bacterial biomass to organic matter (COD) was similar before and after primary settling. Thus, if no change in the relative abundance was observed across the primary settler, the mass flow of the bacteria would be reduced proportionally to the COD. *Arcobacter* in Aalborg West was enriched with 9% in relative abundance across the primary settler. Considering the increase in abundance and the average COD reduction in the plant of 46%, the mass flow of *Arcobacter* was reduced with only 33%. Reversely, if it had been a 9% reduction in abundance, the reduction in mass flow would have been 59%.

Multiple studies have investigated primary settling and its effect on the wastewater quality (Ma et al., 2013; Oakley, 2019; Shewa and Dagneu, 2020) as well as the efficiency of downstream processes (Kosar et al., 2022). However, these studies only rely on classic wastewater measures such as COD, TS or bacterial cell counts and are unable to distinguish which and how much different bacterial taxa are affected. To our knowledge, only Lambirth et al. (2018) has assessed the effect of primary settling on bacterial abundances, but in contrast to our study, no significant changes were found. The discrepancy could result from

the sampling size, limited to only four pairs of samplings spread across one year, which reduced the power of the study. In accordance with other studies, we found a clear seasonality of influent wastewater (LaMartina et al., 2021; Peces et al., 2022). It was not possible to see any effect of the seasonality on the community change during primary settling.

Primary settling affected many of the immigrating bacteria by consistent reduction or enrichment in relative abundance, and the trends on genus levels were representative of the trends at higher taxonomic resolution, e.g. species level. The change in composition at the genus level could be explained by the close to zero aberrant trends to the change in composition when the analysis was performed on the species level, underlining that a close taxonomic relation likely leads to similar sizes and properties of the bacterial cells. Moreover, closely related bacteria are more likely to exhibit the same growth form (planktonic/biofilm) under the same conditions (Lennon et al., 2012; Martiny et al., 2013). We hypothesize that the consistent pattern of reduction or enrichment of species within a genus are most likely due to specific morphological or physiological properties of the bacteria, which result in similar settling velocities and thus similar trend in a sedimentation process.

The consistent enrichment of bacterial genera across the investigated WWTPs could refer to motile bacteria living in a planktonic state and may indicate the *in situ* growth in the influent wastewater. Some of the genera with the highest fold changes across all plants were *Pseudoarcobacter* (2–12 fold increase) and *Arcobacter* (2–6 fold increase). Bacteria in both genera can be motile and have previously been shown to be present in raw sewage (Fisher et al., 2014). *Arcobacter* is a known contaminant of effluent from WWTPs, while *Pseudoarcobacter* species have been isolated from marine waters (Kristensen et al., 2020; Levican et al., 2015) and their presence in the water phase most likely explains the increase in abundance.

The consistent reduction of some bacterial genera could be typical of bacteria growing in dense flocs or foaming bacteria. The reduction of foaming bacteria may be caused by the mechanical skimming of floating matter as a part of the primary settling process and is exemplified by the reduction of *Gordonia* and *Ca. Microthrix*. Many other genera were also consistently reduced in relative abundance, but very few investigations have been made in wastewater systems, and the *in situ* morphology or surface properties in influent wastewater are similarly unknown (Nierychlo et al., 2020). The GAO, *Micropruina*, and the putative PAOs, *Microlunatus*, and *Tessaracoccus*, had some of the highest relative reductions across all WWTP (1.5–3.5 fold decrease). All these genera are non-motile bacteria and can occur as single cells or clusters (Nierychlo et al., 2020). Thus, our findings suggest that non-motile bacteria, and bacteria growing in clusters (or pieces of biofilms) are more likely to sediment and thus be reduced during primary settling. However, we did not investigate these morphological characteristics directly in the wastewater and further investigations are needed.

A certain fraction of influent bacteria/genera were differentially enriched or reduced across the different primary settlers, highlighting the presence of plant-specific (or bacteria-specific) patterns. We hypothesize that a lack of consistency in reduction or enrichment patterns could be due to different characteristics of the settlers at the WWTPs. No obvious patterns were observed in terms of design, and variation in SOR only had a minor effect on the trends, particularly in Ejby Mølle. Differences in wastewater composition may contribute to plant-specific patterns by affecting the sedimentation or suspension of bacteria. This includes chemicals added in CEPT, remains from industrial contributions, or chemicals used in the sewer system. CEPT has been shown to increase the removal rate of typical pathogen and fecal indicators (Oakley, 2019), and could help to explain the many inconsistent trends seen at Randers WWTP. This is supported by the finding that addition of iron chloride gave a distinct composition of antibiotic resistance genes compared to when no chemicals were added (Shin et al., 2022). However, the wastewater composition was not assessed in detail, and our

study fails to explain plant-specific patterns, e.g., why Ejby Mølle, also using an iron-based chemical, did not show the same patterns as Randers. As multiple process-critical genera, such as the PAOs *Tetrasphaera*, *Dechloromonas*, and *Ca. Accumulibacter* showed plant-specific patterns, it is of great interest to further explore which factors influence their faith in the primary settling process to prevent reduction of these genera.

Given the impact of immigrating bacteria from the influent wastewater for the structure of the microbial community in AS (Dottorini et al., 2023, 2021; Gibson et al., 2023), the study of bacteria in influent wastewater is highly relevant. While the efficiency of the primary settler in removing organic matter is well established, its impact on the immigrating bacteria in the influent wastewater has not been investigated. It is important to understand how primary settling affects the flow of bacteria, and the findings of the consistent reduction of foaming bacteria and the plant-specific reduction of putative PAOs is an incentive to address the effect of primary settling further to optimize and ensure stable operation of WWTPs.

## 5. Conclusions

Our study targets influent wastewater to four municipal WWTPs in Denmark where primary settling reduced the incoming COD with 37–49% across the WWTPs. During this biomass reduction, the relative composition of the microbial community of the influent water was significantly changed, and primary settling explained 13–30% of the community variation in influent wastewater for each WWTP. The behavior of bacteria could be described at genus level, as species within the same genus behaved similarly. More than 93% of the biomass (in terms of read abundance) was constituted by genera present in the wastewater to all WWTPs. Of these, 54% of the 564 genera investigated behaved consistently across at least three WWTPs with 3.7% genera increasing in relative abundance after primary settling, while 22.3% decreased in relative read abundance. Special focus was on the process-critical microbes in activated sludge, such as known nitrifiers, polyphosphate accumulating organisms, glycogen accumulating organisms, and filamentous bacteria. Most process-critical taxa showed the same trends in all four WWTPs including genera associated with bulking or foaming in activated sludge (*Gordonia*, *Tsukamurella*, and *Millisia*) which decreased in relative abundance. However, some genera behaved plant-specifically, suggesting that either plant-specific operational parameters or the characteristics of influent wastewater of a specific plant (e.g., composition of organic compounds) was important for the trend of bacterial genera during primary settling. The effect of primary settlers should be included in the understanding and potential in regulation in the assembly of microbial communities in activated sludge.

## Declaration of Competing Interest

The authors declare no financial interests/personal relationships which may be considered as potential competing interests.

## Data availability

All data are available and accesses is given in the paper.

Links to all data and code used are provided in the materials and methods.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120495.

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