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Seasonal microbial community dynamics complicates the evaluation of filamentous bulking mitigation strategies in full-scale WRRFs

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ABSTRACT

The activated sludge wastewater treatment process has been thoroughly researched in more than 100 years, yet there are still operational challenges that have not been fully resolved. Such a challenge is the control of filamentous bulking caused by the overgrowth of certain filamentous bacteria. In this study, we tested different mitigation strategies to reduce filamentous bulking, caused by two common filamentous genera found in full-scale water resource recovery facilities (WRRF), *Candidatus* Microthrix and *Candidatus* Amarolinea. PAX dosing, ozone addition, hydrocyclone implementation, and the addition of nano-coagulants were tested as mitigation strategies in four parallel treatment lines in a full-scale WRRF over three consecutive years. Unexpectedly, the activated sludge settleability was not affected by any of the mitigation strategies. Some of the strategies appeared to have a strong mitigating effect on the two filamentous species. However, detailed analyses of the microbial communities revealed strong recurrent seasonal variations in all four lines, including the control line which masked the real effect. After removing the effect of the seasonal variation by using a time-series decomposition approach, it was clear that the filamentous bacteria were mostly unaffected by the mitigation strategies. Only PAX dosing had some effect on *Ca.* Microthrix, but only on one species, *Ca.* Microthrix subdominans, and not on the most common *Ca.* Microthrix parvicella. Overall, our study shows the importance of long-term monitoring of microbial communities at species level to understand the normal seasonal pattern to effectively plan and execute full-scale experiments. Moreover, the results highlight the importance of using parallel reference treatment lines when evaluating the effect of mitigation strategies in full-scale treatment plants.

1. Introduction

The activated sludge (AS) process is a well-established biotechnology for wastewater treatment around the world. A well-functioning microbial community is the backbone of the AS process, and the optimal operation of water resource recovery facilities (WRRF) depends on the biomass composition. The increasing number of studies analyzing the microbial community composition (Wu et al., 2019; Nierychlo et al., 2020a; Dueholm et al., 2021), longitudinal studies targeting microbial community dynamics (Jiang et al., 2016; Griffin and Wells, 2017; Jiang et al., 2018; Petrovski et al., 2020; Sun et al., 2021), and ecophysiological studies in full-scale AS systems (e.g., Nierychlo et al., 2019; Speirs et al., 2019; Petriglieri et al., 2021; Singleton et al., 2021), have greatly improved our understanding of the microbial ecology of WRRF. However, there are still several operational problems in WRRF that can be linked to the presence of specific microorganisms, which have not yet been resolved.

Filamentous bacteria are important for the formation of stable flocs in activated sludge plants (Henze et al., 2008). However, overgrowth of filamentous bacteria can cause bulking or foaming (Nielsen et al., 2009) resulting in unstable operation and deteriorated process performance due to inefficient secondary clarification (Henze et al., 2008). Several studies have tried to understand the cause of filamentous bulking by investigating the characteristics and physiology of filamentous bacteria (Rossetti et al., 2005; McIlroy et al., 2013; Miłobędzka et al., 2016; Andersen et al., 2019; Nierychlo et al., 2019, 2020b, 2021), but targeted mitigation strategies are still lacking for many species or they have shown inconsistent results.

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Some of the most common filamentous bacteria causing bulking belong to genus *Candidatus* Microthrix in the phylum Actinobacteria (Nielsen et al., 2009; Wang et al., 2014a; Wágner et al., 2015; Miłobędzka et al., 2016; Wang et al., 2016; Nierychlo et al., 2020b, 2021) and several species in the phylum Chloroflexi (Nierychlo et al., 2019; Speirs et al., 2019; Nierychlo et al., 2020b). *Ca.* Microthrix consumes primarily long chain fatty acids and can store lipids under aerobic and anaerobic conditions (McIlroy et al., 2013). *Ca.* Microthrix is able to store poly-phosphate, and has been found in some cases as the main contributor for phosphorus removal under bulking conditions (Wang et al., 2014b), but does not have a dynamic phosphorus cycling as the typical polyphosphate accumulating organisms (PAOs) (Nierychlo et al., 2021). Low temperature, low dissolved oxygen (DO), long solids retention time (SRT), and low food-to-microorganism (F/M) ratio are reported to be advantageous for their growth (Rossetti et al., 2005; Fan et al., 2020; Nierychlo et al., 2021). There are two common species across the world, *Ca.* Microthrix parvicella and *Ca.* Microthrix subdominans, typically coexisting in WRRFs treating municipal wastewater (Nierychlo et al., 2021). There seem to be some physiological differences between the two species with *Ca.* M. subdominans able to grow better at higher oxygen concentrations, while *Ca.* M. parvicella is likely microaerophilic (Nierychlo et al., 2021).

The phylum Chloroflexi includes several filamentous genera where some contribute to poor sludge settling, while others support good floc formation (Nittami et al., 2017; Nierychlo et al., 2019; Speirs et al., 2019). *Candidatus* Villigracilis is mostly found inside the flocs, possibly supporting good floc formation, whereas *Candidatus* Promineofilum and *Candidatus* Sarcinithrix have been connected to bulking events (Nierychlo et al., 2019; Speirs et al., 2019). In particular, *Candidatus* Amarolinea has been reported to cause severe bulking events in Danish WRRFs (Andersen et al., 2019; Nierychlo et al., 2020b), and local foaming events in China (Jiang et al., 2018).

Different mitigation strategies are used to reduce the effect of filamentous bulking. A general treatment method to target most filamentous bacteria is the addition of ozone to the return activated sludge (RAS). Ozone targets the protruding filamentous bacteria with a larger surface area, thus improving the floc structure and consequently, the settleability of the sludge (Nilsson et al., 2014, 2019). However, different filamentous morphotypes are observed to have different responses to ozone treatment, e.g.,*Gordonia* was found to be less affected (Barbarroja et al., 2019). Another approach is to use hydrocyclones to separate the biomass based on density and size. The heavier fraction of the sludge is retained and returned to the process tank while the lighter fraction is removed with the effluent (Kent et al., 2018). Hydrocyclones are commonly used together with the DEMON® process to retain the slow growing anammox bacteria and wash out flocculent biomass (Lackner et al., 2014). Hydrocyclones in full-scale activated sludge systems (Kent et al., 2018) and sequencing batch reactors (SBRs) (Xu et al., 2019) have improved the settling properties of the sludge.

More targeted methods are also used to control specific species. For example, polyaluminum chloride (PAC, referred to as PAX in this study) is dosed to specifically target *Ca.* Microthrix as it affects the exopolymeric layer inhibiting the exoenzyme (lipase) activity and substrate uptake (Nielsen et al., 2005; Hamit-Eminovski et al., 2010; Fan et al., 2020). PAX is applied in many full-scale plants, usually with good results (Roels et al., 2002), but there are also cases reported by the WRRF operators that it can be ineffective. It has been observed that in order to achieve removal of *Ca.* Microthrix, a dosage of 1.5 to 4.5 g Al/kg MLSS and a minimum dosing time of 3 – 3.5 weeks are needed (Roels et al., 2002).

As we will further demonstrate in this study, seasonal patterns of the microbial community are observed in activated sludge systems (Ju et al., 2014; Jiang et al., 2016; Griffin and Wells, 2017; Jiang et al.; 2018; Zhang et al., 2018; Johnston and Behrens, 2020; Petrovski et al., 2020; Peces et al., 2022; Sun et al., 2021). Such variations may challenge traditional full-scale mitigating experiments, as it can be difficult to differentiate the effect of the tested mitigation strategy from the normal seasonal variation, especially if these variations are not well described or if no control line is included. Several studies have shown that specific bacteria belonging to important functional groups, such as nitrifiers, PAOs, and filamentous bacteria, are affected by yearly seasonal variations (e.g., Ju et al., 2014; Jiang et al., 2018; Petrovski et al., 2020; Sun et al., 2021). Moreover, we have recently shown the importance of analyzing seasonality at species level, since species belonging to the same higher taxonomic unit (e.g., genus) or same functional guilds may show different seasonal patterns (Peces et al., 2022). Therefore, there is a need to investigate how yearly seasonal variations impact the evaluation of full-scale testing results at species level.

The ecosystem-specific reference database for activated sludge and anaerobic digesters, MiDAS 3, provides reproducible species-level identification of the bacteria in WRRFs (Nierychlo et al., 2020a). Using this tool, the aim of the study was to assess the effect of different mitigation strategies to reduce filamentous bulking in a full-scale WRRF with four independent parallel treatment lines during a 3-year survey period. We investigated the effect of PAX dosing, ozone dosing, hydrocyclones, and nano-coagulants on (i) the activated sludge settleability, (ii) the changes in overall microbial community composition, and (iii) the changes in the most abundant filamentous bacteria, i.e., *Ca.* Microthrix and *Ca.* Amarolinea. Moreover, we investigated the effect of seasonal microbial variation on the evaluation and interpretation of the experimental results.

2. Materials and methods

2.1. Description of Damhusåen WRRF

Damhusåen WRRF (Copenhagen, Denmark) has a design capacity of 350,000 population equivalent (PE) and treats municipal wastewater with approximately 15–20% industrial contribution (expressed as chemical oxygen demand (COD) fraction, estimated based on the amount of industrial water in the catchment area and the types of industries). The WRRF has four parallel treatment lines (A, B, C, and D) with dedicated secondary clarifiers and return sludge on each line, so it is possible to test different treatment methods in full-scale by comparing independent parallel lines. The WRRF is designed as an enhanced biological phosphorus removal (EBPR) system with alternating Bio-denitro™ operation known as Biodenipho™ (Bundgaard et al., 1989). Each treatment line consists of one anaerobic tank, two aeration/denitrifying tanks, and six secondary clarifiers. In lines B, C, and D, the return sludge is collected from their respective secondary clarifiers and it is directed back to the beginning of the treatment line where it is mixed with the wastewater (EBPR process operation). Line A is operated as side-stream hydrolysis (SSH), where part of the return sludge is collected and directed into an anaerobic tank, operated with a long residence time (approximately 24 h). More about how the SSH is operated and what are the advantages of this operation can be found in Vollertsen et al. (2006). In line A, the influent wastewater is added only after the anaerobic tank. More details on the design and operation of Damhusåen WRRF can be found in **Table S1**, Supporting Information (**SI**). Average influent wastewater characteristics are also shown in the SI (**Table S2, SI**). Suspended solids in the process tanks were relatively constant over the whole experimental period, with a median value of 3.3 g SS/L (supporting **Fig. S1, SI**).

2.2. Experimental design

Four full-scale experiments were conducted to test the control of filamentous bulking. PAX dosing and nano-coagulant dosing were tested in line A, ozonation in line D, and hydrocyclones in line C (**Fig. S2, SI**). Line B was left in all cases as a reference line without any treatment.

Polyaluminum chloride (PAX15, Kemira, Finland) was dosed in the return sludge just before entering the treatment line A (**Fig. S3, SI**). First, the recommended dose by the supplier was used, however, it was halved when a white tint started appearing in the sludge. The recommended daily dose of 1.5 g Al/kg SS was added in the period 06-03-2018 to 12-04-2018 (37 days). The dosing was lowered to half the amount in the consecutive period 13-04-2018 to 18-06-2018 (66 days).

Ozone treatment was conducted using a Primozone GM18 ozone generator (Primozone Production AB, Sweden, **Fig. S4, SI**). Approximately 8–15% of the return sludge in line D was mixed with the ozone in a contact tank with a daily dose of 4.5–5 g ozone/kg SS. The return sludge flow to the contact tank was between 20 and 36 m^3/h and the residence time in the contact tank was 5-10 minutes. Two dosing periods were conducted between 10-08-2018 and 21-09-2018 (42 days) and between 26-03-2019 and 10-04-2019 (15 days).

Six hydrocyclones (**Fig. S5, SI**) were installed in line C to separate the heavy sludge (underflow) from the loose, light fraction (overflow). Part of the return sludge was led through the hydrocyclones with an inflow of 21 m^3/h. The overflow sludge was directed away with a flow of 14 m^3/h while the underflow sludge was led back to the treatment line with a flow of 7 m^3/h. The normal waste activated sludge removal was stopped in this line as itwas done via the overflow of the hydrocyclones. Two test periods were conducted between 15-10-2019 to 27-10-2019 (12 days) and between 18-01-2020 to 10-03-2020 (52 days).

An inorganic coagulant based on nanotechnology, Norfloc NF1® (Alumichem, Denmark), was dosed during one year between 14-04-2020 and 13-04-2021 in line A (364 days). The dosage of the coagulant was controlled based on the actual flow, and approximately 12 mg/L of coagulant was dosed in the first few months which was then slowly reduced to 4 mg/L in the colder winter months. The nano-coagulant was dosed directly in the aeration tank in line A instead of the iron chloride that is usually added to the treatment lines.

2.3. *Sample collection and analysis*

Metadata were collected from Damhusåen WRRF, including online sensor data and laboratory measurements. Chemical analyses were done in the laboratory at the WRRF. Total COD, total nitrogen, total phosphorus, NH_4-N, NO_3-N, and PO_4-P in the influent and effluent water were analyzed with Hach® test kits (Germany) and samples were measured in Hach® AP3900 multi laboratory robot. The online sensors used for the analysis of PO_4-P, NH_4-N, and NO_3-N were Phosphax, Amtax and Nitratax, respectively, supplied by Hach®. Suspended solids (SS) were measured online with Solitax from Hach®. Diluted sludge volume index (DSVI) and total suspended solids (TSS) were measured according to the standard methods (APHA et al., 2012). The DSVI was measured weekly or twice a week (from 26-07-2017 to 26-05-2021).

Activated sludge samples at the WRRF were collected from the process tank bi-weekly, weekly or twice a week (from 06-09-2017 to 24-08-2020) in 2 ml tubes and were kept frozen at -20 °C until analysis. In total 600 samples were collected in the four lines (150 per line).

The detailed protocol for the DNA extraction from activated sludge (version: aau_wwtp_dna_v8.0) can be found on https://www.midasfieldguide.org/guide/protocols. In short, DNA extraction from the biomass was done as follows. Samples were homogenized prior to DNA extraction using Heidolph RZR 2020 (Heidolph Instruments, Germany). 160 μl of activated sludge sample (target of 0.6 mg total solids) was used to extract DNA with the FastDNA® spin kit for soil (MP Biomedicals, USA). FastPrep 96 bead beater (MP Biomedicals, USA) was used for bead beating of the samples for 3 times 2 min at 1800 RPM. The DNA was stored at -20°C until library preparation.

Amplicon sequencing was carried out using the V1-V3 primer (Lane, 1991; Muyzer et al., 1993) to analyze the community structure, using the protocol (version: aau_b16S_v1.2) available on https://www.midasfieldguide.org/guide/protocols. The extracted DNA quality was evaluated using Nanodrop1000 (Thermo Fisher Scientific, USA), and Tapestation 2200 (Agilent, USA) gel electrophoresis. The DNA concentration was measured with Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, USA) and on an Infinite® M1000 PRO (Tecan, Switzerland) microplate reader. Amplicon library PCR was run on duplicates for each sample with approximately 10 ng of extracted DNA. The PCR program was run at 95 °C for 2 min, then 30 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 60 s, followed by at 72 °C for 5 min. The amplicon libraries were cleaned up and the samples were pooled. The library was sequenced on MiSeq (Illumina, USA).

The amplicon data were processed with the AmpProc v.5.1.0 workflow (https://github.com/eyashiro/AmpProc). Taxonomy was assigned to Amplicon Sequencing Variants (ASVs) using the MiDAS 3 reference database (Nierychlo et al., 2020a), generated by the AutoTax method (Dueholm et al., 2020). The database is built on high-quality full-length 16S rRNA gene sequences from activated sludge and anaerobic digester systems, and it proposes unique species-level names for all microorganisms.

2.4. *Data treatment*

Data analysis and visualization were performed in R-Studio® with R version 3.5.1 (R Core Team, 2018). The *ampvis2* v.2.5.7 (Andersen et al., 2018) R-package was used to load the ASV count table into R, with its corresponding taxonomy. Samples with less than 10,000 reads were removed during the data pre-treatment. Taxa are shown as relative read abundance (%). Time-series data visualisation was performed with the *ggplot2* v.3.3.3 package (Wickham et al., 2019) and the *ampvis2* package. Principal coordinates analysis (PCoA) was performed with the *ampvis2* package using Bray–Curtis dissimilarity distance measure without data transformation. The *ggseas* v.0.5.4 package was used for time-series decomposition (Ellis and Sax, 2018), and *Harmonic.Regression* v.1.0 (Lück et al., 2014) was used for time-series harmonic regression.

Time-series decomposition based on LOESS local smoothing regression (Cleveland et al., 1990) was used to find the underlying trend, seasonal pattern, and residual pattern of each species in each line. Time-series decomposition was carried out using an s.window = “periodic” and frequency = 52. Prior to time-series decomposition, data were transformed using robust-centered log-ratio (rclr) transformation to account for the compositional and sparse nature of the data (Martino et al., 2021). Only species with a relative abundance > 0.05% in at least one sample were used for time-series decomposition. After time-series decomposition, the extracted seasonal pattern was used to assign species into seasonal cohorts based on the temporal location of the maximum seasonal peak. Seasonal cohorts were defined based on yearly process tank temperature variation (**Fig. S6, SI**), using as a reference the definition of astronomical seasons for the northern hemisphere (Peces et al., 2022). Additionally, the seasonal component was used to deseasonalize species times-series for further analyses by subtracting the seasonal signal to the rclr transformed time-series. Further details about the time-series decomposition and data transformation can be found in Peces et al. (2022).

2.5. *Additional WRRFs and PAX dosing data*

Randers WRRF (Randers, Denmark) has a design capacity of 130,000 PE and treats municipal wastewater with approximately 5% industrial contribution. It has biological nitrogen removal system with internal recirculation between the aerobic and anoxic tanks, and it includes SSH tanks.

Aalborg West WRRF (Aalborg, Denmark) has a design capacity of 330,000 PE and treats municipal wastewater with approximately 25% industrial contribution. It has an alternating Bio-denitro™ system and it includes SSH tanks.

Polyaluminum chloride (PAX-XL 100, Kemira, Finland) was dosed at Randers WRRF with a daily dose of 1.15 g Al/kg SS, and this was added in the period 28-03-2017 to 18-05-2017.

Polyaluminum chloride (NORDPAC, Nordisk Aluminat A/S, Denmark) was dosed at Aalborg West WRRF in three periods between

2015-2017. The specific periods and the applied dose are shown in **Table S3, SI**.

3. Results and discussion

3.1. Effect of mitigation strategies on sludge settleability

The settling properties (assessed by DSVI values) were most of the time above or around the threshold in all four lines considered to indicate sludge with poor settling characteristics (120 mL/g (Mielczarek et al., 2012)). Dosing of PAX, nano-coagulant, and use of hydrocyclone did not affect the settleability of the sludge in the respective line compared to the other lines (Fig. 1). Ozone dosage in line D seemed to cause a decrease of the DSVI, but by comparing with the other three lines, it was clear that all lines had low and similar DSVI values during the same period. Besides this very small effect of ozone, the increase of DSVI after ozone dosing was slightly delayed compared to the reference lines (Fig. 1).

These results highlight the importance of having parallel reference lines to evaluate the effect of mitigation strategies. We observed a clear decrease in DSVI in all lines after the mitigation strategies were implemented but not only in the line with treatment. If we had used only one line to evaluate the treatment strategies, for example line A in case of the PAX and nano-coagulant dosing, and line C in case of the hydrocyclone implementation, the conclusions would have been that they were very efficient. Thus, it is important to draw conclusions very carefully in full-scale with only one treatment line and no reference line. The use of reference lines to compare mitigation measures of bulking in full-scale systems are very rare (e.g., Levén et al., 2016), but necessary to draw correct conclusions.

3.2. Microbial community composition and settling properties

The microbial communities in the four treatment lines were very similar during the 3-year period investigated as seen by PCoA ordination (Fig. 2**a**). Lines B, C, and D clustered tightly together suggesting that they were very similar in microbial structure throughout the years, while line A was slightly different. This is also visualized and supported by the top 25 most abundant genera with some dissimilarity in line A in the abundances but not identity (Figs. 2**b, S7, SI**). Line A is operated as SSH as opposed to the traditional EBPR scheme for the other lines, hence this could result in the differences observed.

The most abundant genera were typical for Danish EBPR plants with the PAO *Tetrasphaera* and the filamentous *Ca*. Microthrix among them (Nierychlo et al., 2020a). Several genera showed periodic increase and decrease in relative read abundance. Some of the most abundant genera *Ca*. Microthrix, *Rhodobacter*, midas_g_70 (class Gammaproteobacteria), *Trichococcus, Rhodoferax,* and OLB8, among others, showed clear periodicity related to seasonal variations (**Fig. S8, SI**). In line A, which is operated as SSH, we observed several genera among the most abundant that were in lower abundance compared to the other three lines (*Tetrasphaera*, midas_g_70, *Ca*. Competibacter, midas_g_399 and *Fodinicola*), however, the trends for these genera are still identical among the lines (**Fig. S8, SI**).

Similar to many other Danish WRRFs, the most abundant filamentous bacteria in Damhusåen WRRF were *Ca.* Microthrix and *Ca.* Amarolinea in relative read abundances up to 30% followed by other typical filamentous bacteria found in Denmark (Nierychlo et al., 2020b) and globally (Dueholm et al., 2021) (Figs. 2c, **S9, SI**). Line B, C, and D were very similar with minor abundance differences among some filamentous bacteria in line A (**Fig. S10, SI**).

The most abundant filamentous bacteria had a profound effect on the settling properties. The DSVI values correlated well with the abundance of *Ca*. Microthrix and *Ca*. Amarolinea (Figs. 3, **S11, SI**), where high abundance caused poor settling activated sludge. The sum of the abundance of *Ca.* Microthrix and *Ca.* Amarolinea showed moderate correlation with DSVI in all lines ($R^2 \geq 0.5$). There was a low or no positive correlation between the DSVI and other filamentous bacteria ($R^2 \leq 0.4$). We found similar results in all lines (Figs. 3, **S11, SI**). This suggests that the sludge settleability was strongly shaped by *Ca.*

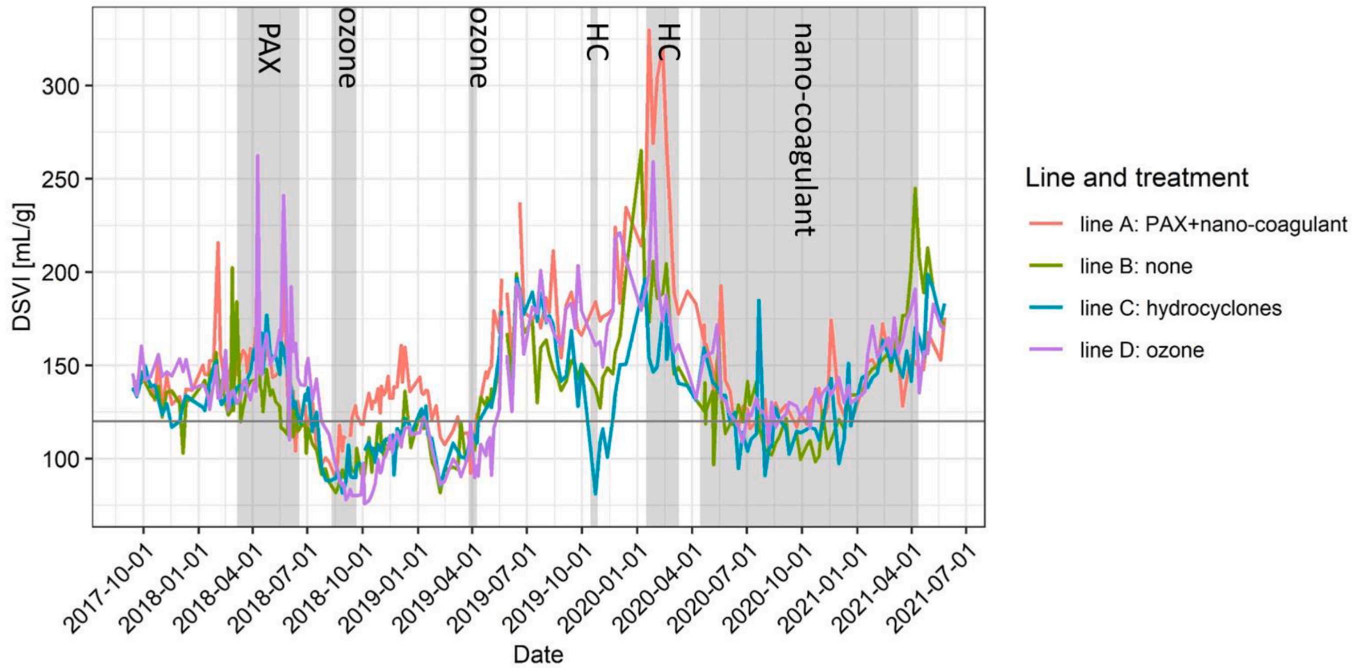
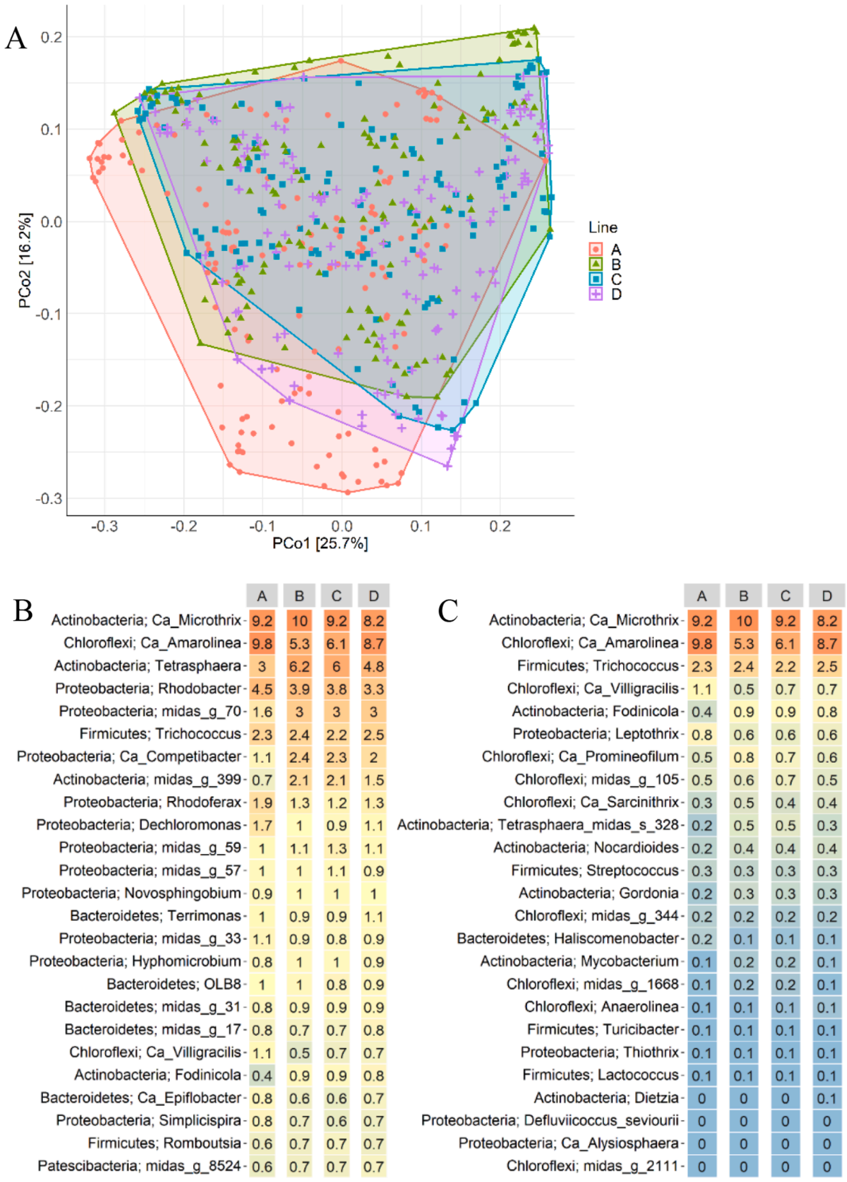


Fig. 1.. Activated sludge settleability in the four lines of Damhusåen WRRF. The dilute sludge volume index (DSVI) measurements were taken weekly or twice a week. The periods with different treatments are marked with grey shading. Only line A received the PAX dosing and the nano-coagulant dosing and the line is marked with red. Line B did not receive any treatments and is marked with green. Only line C got the hydrocyclone (HC) implementation and it is marked with blue. Only line D got the ozone treatment and it is marked with purple. The horizontal grey line shows DSVI value of 120 mL/g, the threshold that above there can be problems with sludge settleability.



B

	A	B	C	D
Actinobacteria; Ca_Microthrix	9.2	10	9.2	8.2
Chloroflexi; Ca_Amarolinea	9.8	5.3	6.1	8.7
Actinobacteria; Tetrasphaera	3	6.2	6	4.8
Proteobacteria; Rhodobacter	4.5	3.9	3.8	3.3
Proteobacteria; midas_g_70	1.6	3	3	3
Firmicutes; Trichococcus	2.3	2.4	2.2	2.5
Proteobacteria; Ca_Competibacter	1.1	2.4	2.3	2
Actinobacteria; midas_g_399	0.7	2.1	2.1	1.5
Proteobacteria; Rhodoferax	1.9	1.3	1.2	1.3
Proteobacteria; Dechloromonas	1.7	1	0.9	1.1
Proteobacteria; midas_g_59	1	1.1	1.3	1.1
Proteobacteria; midas_g_57	1	1	1.1	0.9
Proteobacteria; Novosphingobium	0.9	1	1	1
Bacteroidetes; Terrimonas	1	0.9	0.9	1.1
Proteobacteria; midas_g_33	1.1	0.9	0.8	0.9
Proteobacteria; Hyphomicrobium	0.8	1	1	0.9
Bacteroidetes; OLB8	1	1	0.8	0.9
Bacteroidetes; midas_g_31	0.8	0.9	0.9	0.9
Bacteroidetes; midas_g_17	0.8	0.7	0.7	0.8
Chloroflexi; Ca_Villigracilis	1.1	0.5	0.7	0.7
Actinobacteria; Fodinicola	0.4	0.9	0.9	0.8
Bacteroidetes; Ca_Epiflobacter	0.8	0.6	0.6	0.7
Proteobacteria; Simplicispira	0.8	0.7	0.6	0.7
Firmicutes; Romboutsia	0.6	0.7	0.7	0.7
Patescibacteria; midas_g_8524	0.6	0.7	0.7	0.7

C

	A	B	C	D
Actinobacteria; Ca_Microthrix	9.2	10	9.2	8.2
Chloroflexi; Ca_Amarolinea	9.8	5.3	6.1	8.7
Firmicutes; Trichococcus	2.3	2.4	2.2	2.5
Chloroflexi; Ca_Villigracilis	1.1	0.5	0.7	0.7
Actinobacteria; Fodinicola	0.4	0.9	0.9	0.8
Proteobacteria; Leptothrix	0.8	0.6	0.6	0.6
Chloroflexi; Ca_Promineofilum	0.5	0.8	0.7	0.6
Chloroflexi; midas_g_105	0.5	0.6	0.7	0.5
Chloroflexi; Ca_Sarcinithrix	0.3	0.5	0.4	0.4
Actinobacteria; Tetrasphaera_midas_s_328	0.2	0.5	0.5	0.3
Actinobacteria; Nocardioides	0.2	0.4	0.4	0.4
Firmicutes; Streptococcus	0.3	0.3	0.3	0.3
Actinobacteria; Gordonia	0.2	0.3	0.3	0.3
Chloroflexi; midas_g_344	0.2	0.2	0.2	0.2
Bacteroidetes; Haliscomenobacter	0.2	0.1	0.1	0.1
Actinobacteria; Mycobacterium	0.1	0.2	0.2	0.1
Chloroflexi; midas_g_1668	0.1	0.2	0.2	0.1
Chloroflexi; Anaerolinea	0.1	0.1	0.1	0.1
Firmicutes; Turicibacter	0.1	0.1	0.1	0.1
Proteobacteria; Thiothrix	0.1	0.1	0.1	0.1
Firmicutes; Lactococcus	0.1	0.1	0.1	0.1
Actinobacteria; Dietzia	0	0	0	0.1
Proteobacteria; Defluviicoccus_seviourii	0	0	0	0
Proteobacteria; Ca_Alysiosphaera	0	0	0	0
Chloroflexi; midas_g_2111	0	0	0	0

Fig. 2.. Community diversity and composition in the activated sludge of the four treatment lines of Damhusåen WRRF. (A) Principal Coordinates Analysis (PCoA) of the activated sludge collected in the four lines based on Bray–Curtis dissimilarity distance measure without data transformation. The axes show the percentage of the total variation described by each component.(B) Heatmap of the 25 most abundant genera in the four lines. Values were calculated as average relative read abundance over three years of sampling in each line. (C) Heatmap of the 25 most abundant filamentous genera in the four lines. Values were calculated as average relative read abundance (%) over the three years of sampling in each line.

Microthrix and *Ca.* Amarolinea, as has also been observed in other full-scale WRRF in Denmark and other countries (Wang et al., 2014a; Wágner et al., 2015; Miłobędzka et al., 2016; Jiang et al., 2018; Nierychlo et al., 2020b). Interestingly, the presence of *Ca.* Villigracilis, *Fodinicola* and *Leptothrix* showed a negative correlation to DSVI. In the case of *Ca.* Villigracilis, this could suggest improved settling properties when abundant, by providing a strong backbone in the flocs (Nierychlo et al., 2019). In case of the other two genera, some of their species could be non-filamentous, thus resulting in negative correlation with DSVI.

3.3. Evaluation of microbial seasonal dynamics

In a recent study, we have shown high microbial seasonality at species level in Danish WRRFs, where species belonging to the same functional guild or the same genus did not follow the same seasonal dynamics (Peces et al., 2022). As also observed by Peces et al. (2022), the variation in wastewater temperature could not explain all the different seasonal dynamics, as many species did not show a strong linear correlation with temperature changes (**Figs. S12 and S13, SI**). Independently of the factors contributing to the seasonal dynamics, such variations challenge full-scale mitigating experiments, as it may be difficult to differentiate the effect of the mitigation strategy tested from the normal seasonal variation.

The filamentous microbial community was classified based on their seasonal cohort (i.e., the period during the year where the maximum relative read abundance is typically observed) in each treatment line (Fig. 4). The largest fraction of filamentous bacteria belonged to the autumn and spring cohorts (Fig. 4a). This agrees with other studies, showing that the colder temperatures (or often in the transition between cold and warmer periods) coincide with higher relative abundance of filamentous bacteria and settling problems (Jiang et al., 2016; Petrovski et al., 2020; Nierychlo et al., 2021). The autumn cohort included the highest number of filamentous species (approximately 30 different species) (Fig. 4A). The cumulative relative abundance of the autumn cohort was the highest (mean relative abundance 8–14%), however the relative abundance of the autumn cohort was dominated by *Ca.* Amarolinea midas_s_1 (mean relative abundance 5–9%). The difference observed in line C is related to the cohort classification of *Ca.* Amarolinea midas_s_1, where the estimated maximum abundance was found in week 37 (summer cohort), but very close to the summer and autumn cohort classification threshold (i.e., week 38) (**Table S4, SI**). This species is often found in other Danish WRRFs in the autumn cohort (Peces

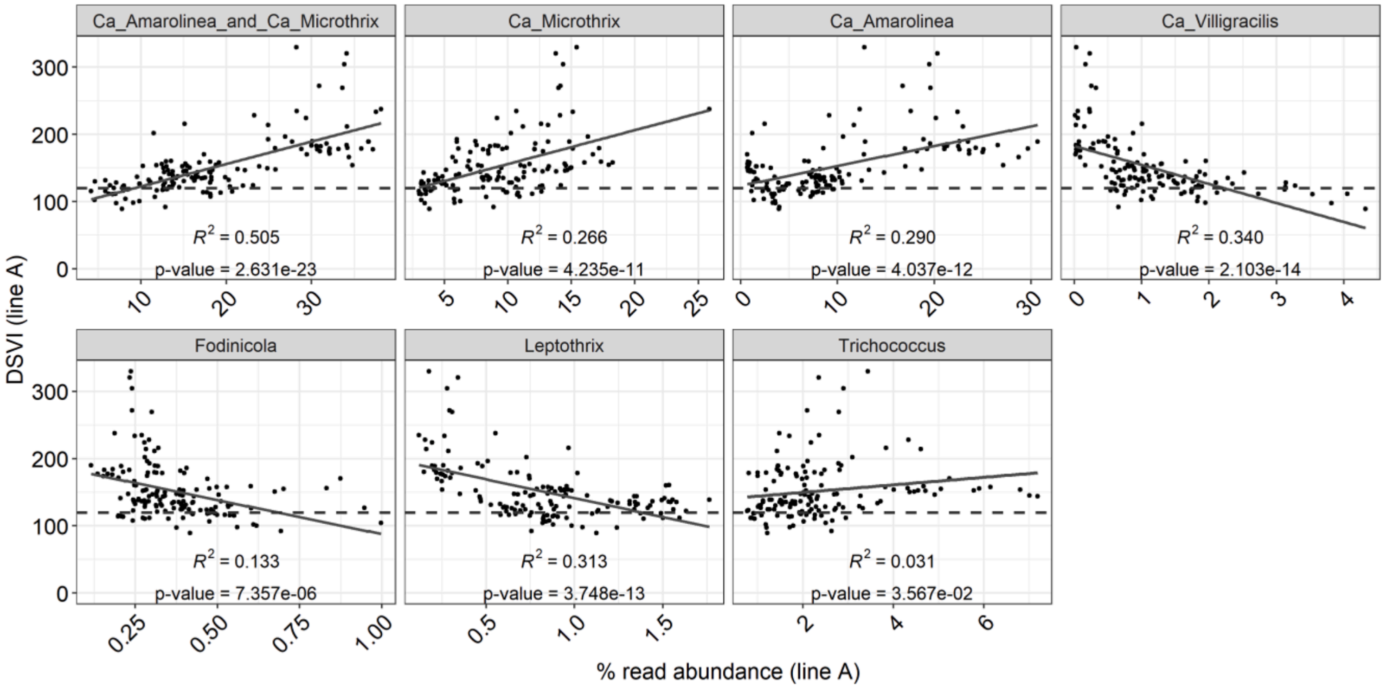


Fig. 3.. Correlation analysis between sludge settleability and abundance of filamentous bacteria. The correlation analysis was carried out between DSVI and the relative abundance of the top six filamentous genera in line A. Additionally, the sum of *Ca.* Microthrix and *Ca.* Amarolinea, was included in the correlation analysis. The dashed horizontal line shows the threshold of 120 mL/g between good and poor sludge settleability.

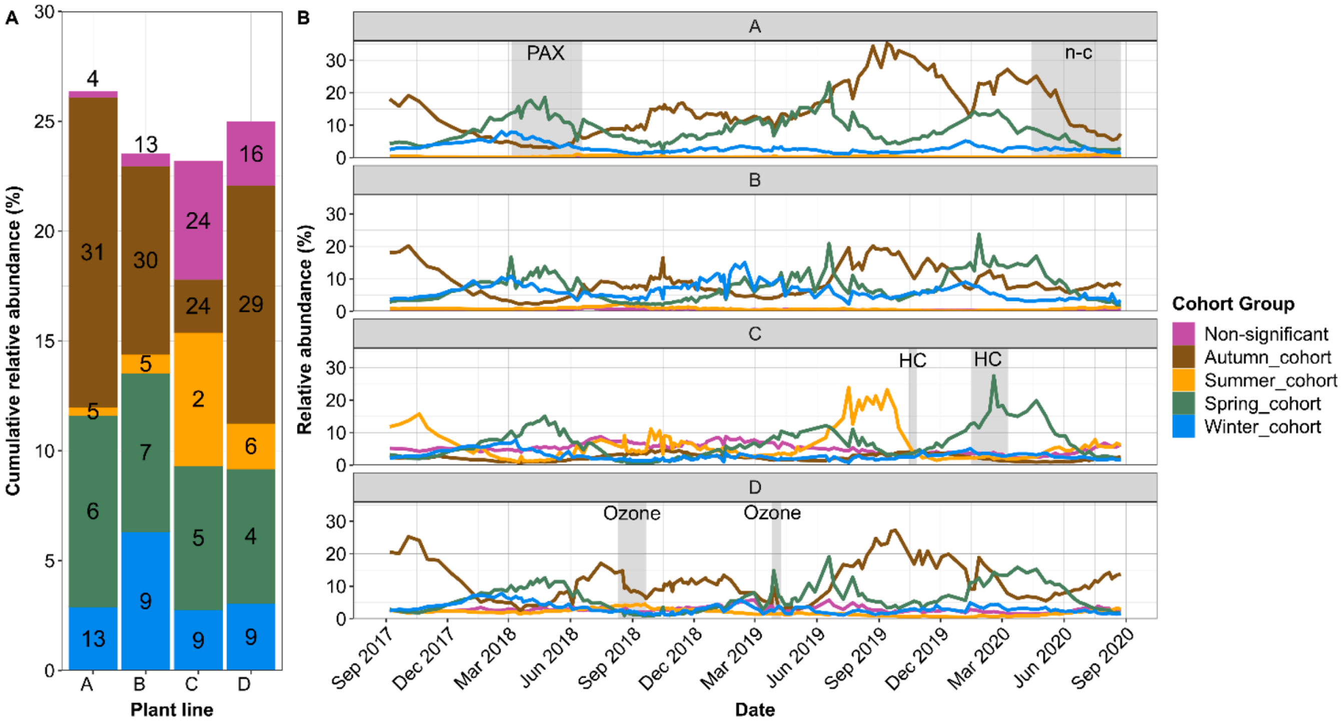


Fig. 4.. Filamentous microbial community grouped based on their seasonality. (A) Distribution of filamentous species grouped into their seasonal cohorts in each of the four treatment lines. Numbers inside the bars correspond to the total number of filamentous species in each seasonal cohort and line. (B) Dynamics of filamentous species grouped by their seasonal cohorts in each treatment line. The periods with different treatments are marked with grey shading.

et al., 2022) and was a main contributing filament to poor settling in this treatment plant (Fig. 3) and other Danish WRRFs (Nierychlo et al., 2020b).

The spring cohort included only a few filamentous species (4-7 different species), but their cumulative average relative abundance was surprisingly high (6 – 8%), due to the second-most abundant filamentous species, *Ca.* M. parvicella. *Ca.* M. parvicella is present mainly in the northern hemisphere and has a very strong seasonal pattern (Nierychlo et al., 2021; Peces et al., 2022). Not all the filamentous species showed a significant seasonal variation, for example *Ca.* Microthrix subdominans, as shown in this study (Fig. 5a) and in other Danish WRRFs (Nierychlo et al., 2021; Peces et al., 2022). We observed some differences in the seasonal dynamics among the different treatment lines, but the maximum abundance peaks for each species were present at similar time points in each line (Fig. 4b). This clearly shows that understanding the seasonal dynamics of specific species is important, and may be confounded with relative read abundance variations caused by the tested mitigation strategy.

3.4. *Evaluation of filamentous bulking mitigation strategies*

Four mitigation strategies were tested at the treatment plant in periods ranging from a few weeks to a few months. The effect of the mitigation strategy was evaluated by including both the behavior in all four lines and the seasonal variation in microbial community structure. Dosage of PAX was assumed to control *Ca.* Microthrix. If we used only one line (line A) to assess the results, we would conclude that the PAX dosing was very successful in reducing the abundance of *Ca.* M. parvicella from 15% to 8%, and still decreasing by end of the PAX dosing (Fig. 5a). However, by comparing to the other lines, we could see that *Ca.* M. parvicella decreased in all lines, also in those without PAX dosage. This suggested that this decrease was most probably related to its normal seasonal variation, a result that was confirmed by time-series analysis of the 3-year long experimental period showing a recurrent strong seasonal variation of *Ca.* M. parvicella (Fig. 5a).

Some positive effects of two of the mitigation strategies could be observed when analyzing them in combination with time-series analysis and reference lines (Fig. 5a). To do so, the raw relative read abundance time-series were deseasonalized to evaluate if the effect of the mitigation strategy was still observable (Fig. 5b). During the PAX dosing period abundance of *Ca.* Amarolinea midas_s_1 increased in line A, similarly to the reference lines, while *Ca.* M. parvicella was stable in this period in all lines (Fig. 5b). Despite the abundance of *Ca.* M. subdominans was consistently lower in line A than the rest of the lines, it showed a decreasing trend in line A during the PAX dosing, as opposed to the reference lines in both the raw time-series and the deseasonalized time-series (Fig. 5a and b). This suggests that this species might be affected by the PAX dosing in contrast to *Ca.* M. parvicella. Data collected from other Danish WRRFs during PAX dosing periods (these periods are unrelated to the experiments at Damhusåen WRRF and were recorded during standard dosing periods targeted against filamentous bulking) also show that PAX treatment was overall ineffective for *Ca.* M. parvicella, but in some cases effective against *Ca.* M. subdominans (**Figs. S14 and S15, SI**). The typical values for PAX dosing in full scale WRRFs are reported to be between 1.5 – 4.5 g Al/kg SS (Roels et al., 2002; Fan et al., 2020). This corresponds with the dosage applied in this study in the first 37 days of dosing, which was then reduced to half dosage based on the recommendation by the supplier. Since species resolution was not possible in previous studies, the differences in observed outcomes of PAX dosages may be due to differences in the species composition in the WRRFs.

Ozone was dosed during two periods in line D. Ozone treatment

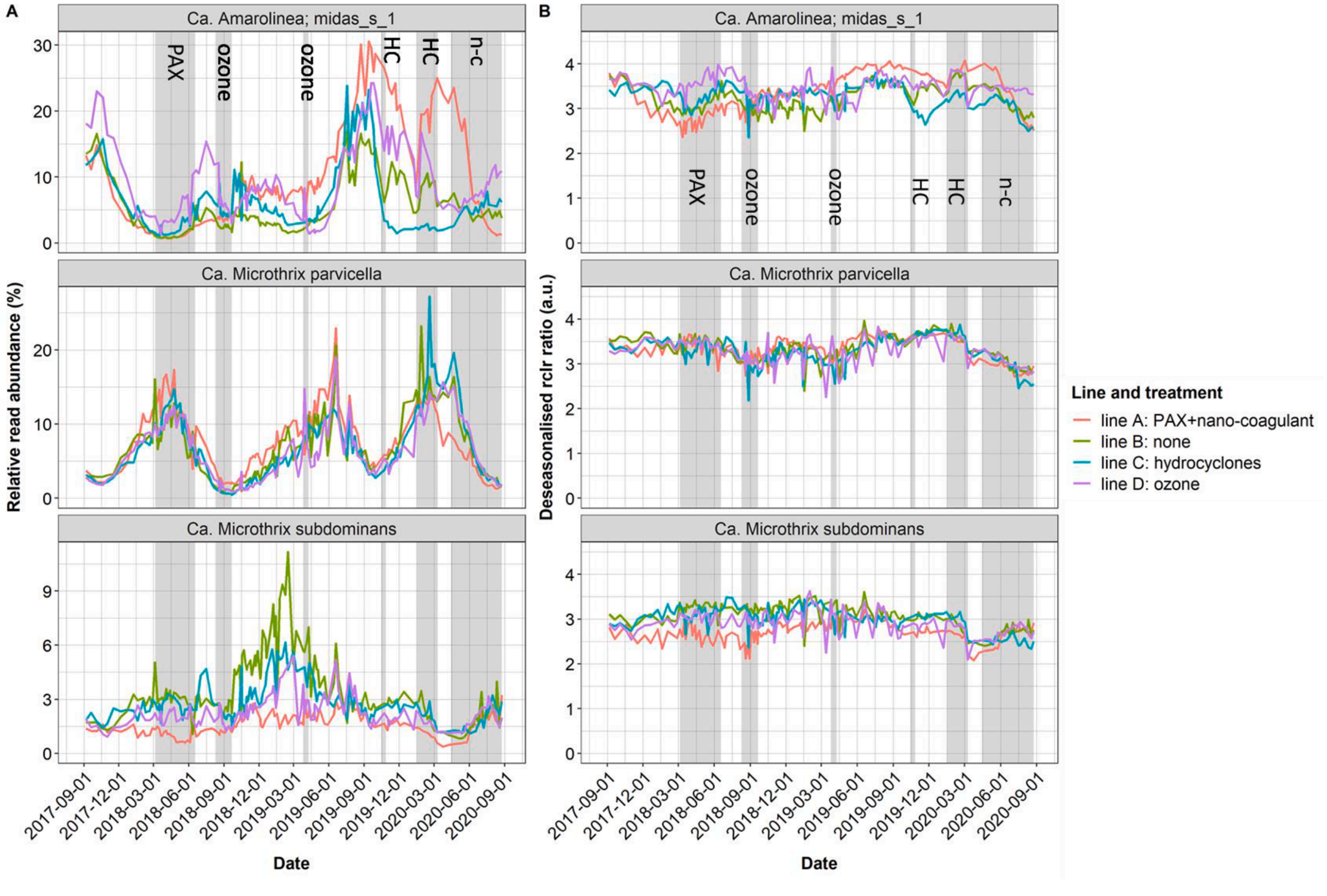


Fig. 5.. Time-series of the top three most abundant filamentous species in Damhusåen WRRF. (A) Raw time-series plot of the three most abundant filamentous species. (B) Deseasonalized time-series of robust-centered log-ratio transformed data of the same three filamentous species. The periods with different treatments are marked with grey shading. Line A (red) - PAX and the nano-coagulant (n-c) was dosed. Line B (green) did not receive any treatments. Line C (blue) - hydrocyclone (HC) was implemented. Line D (purple) - ozone treatment was implemented. The y-axis of the (A) figure are set to be figure specific, to make the assessment of the abundance of *Ca.* M. subdominans easier.

seemed to rapidly decrease the abundance of *Ca.* Amarolinea midas_s_1 after the first dosing period, while after the second dosing period, it seemed to delay the growth compared to the other lines (**Fig. 5a**). However, as opposed to the raw time-series data, we did not observe a difference in the trend of any of the bacteria in this treatment line compared to the reference lines in the deseasonalized time-series (**Fig. 5b**), thus the effect of ozone could not be conclusively resolved. Barbarroja et al. (2019) found that different filamentous morphotypes were affected in different ways by ozone addition. Levén et al. (2016) found that ozone dosing had an effect on the *Ca.* Microthrix abundance in the treated line compared to the control line already after 21 days of dosing. However, in this period their ozone dosage was higher (6.6 g O_3/kg MLSS) than in our study, potentially making it more efficient to target filaments. They have found, however, that even at a dose of 4.4 g O_3/kg MLSS (comparable to this study), the SVI remained low compared to the control line. There could be discrepancies inSRT between the two studies, resulting in differences in the effectiveness of the ozone treatment. Lyko et al. (2012) reported the usage of low ozone dosage (i.e., 1.6 g O_3/ kg MLSS) for a long time period (for 8 months, ozone applied only 1 week a month) to be successful in lowering the SVI at a full-scale WRRF. Thus, higher ozone dosage and/or longer application times should be tested in order to properly evaluate the effect of ozone dosing on the filamentous bacteria abundance.

The implementation of hydrocyclones in line C also showed discrepancies between raw time-series data and deseasonalized time-series data. For example, during the second hydrocyclone run the increase of *Ca.* Amarolinea midas_s_1 was delayed compared to the other lines, but this observation could not be confirmed with the deseasonalized time-series (Fig. 5b). The SRT in the system is relatively high, approximately 25 days. Thus, our experimental hydrocyclone trials were probably too short (12 and 23 days) to observe any substantial change. Although not many full-scale studies are reported in literature, likely several months would be needed to change the sludge characteristics (Kent et al., 2018). Therefore, a longer hydrocyclone operation time should be tested to properly evaluate the effect of hydrocyclone implementation on the filamentous bacteria.

Similarly, during the nano-coagulant dosing period in line A, we did not observe differences compared to the reference lines in the top three filamentous bacteria (Fig. 5b). However, the plant operators reported that during wet weather conditions, line A was able to handle the hydraulic load (the wet weather flow to the four biological lines is 10,000 m^3/h) of the plant better than the other lines. The effluent TSS (**Fig. S16, SI**) was only measured in the combined effluent from the WRRF, thus any quantitative evaluation could not be made. This could potentially be due to improved floc structure in line A. Thus, although changes in the filamentous bacteria composition did not occur, structural changes might have occurred in the sludge flocs due to the coagulant dosing.

Overall, we found that none of the treatment strategies successfully controlled the main filamentous bacteria associated with poor settling characteristics at the WRRF. However, these results could only be interpreted properly when removing the seasonal component of the time-series data and by comparing with parallel lines in the same WRRF. In light of our results, it is important to plan such experiments with knowledge about the occurrence of the targeted bacteria and their seasonal dynamics. For example, knowing that *Ca.* M. parvicella shows a strong recurrent seasonal pattern increasing during late autumn until spring, experiments can be planned to target the period of rising abundance. However, seasonal dynamics may be species- and WRRF-specific (Peces et al., 2022), so long-term monitoring of microbial communities prior to full-scale experiments is important to understand the normal seasonal pattern of the target species in each WRRF, and to effectively choose and implement mitigation strategies.

4. Conclusions

- Different mitigation strategies to reduce filamentous bulking in full-scale WRRF commonly experiencing settling problems were tested in four parallel independent treatment lines: PAX dosing, ozone addition, hydrocyclone implementation, and the addition of nano-coagulants. The sludge settleability, measured as DSVI, was not affected by any of the mitigation strategies at the tested dose and length of the test period.
- *Ca.* Microthrix and *Ca.* Amarolinea were the two main filamentous bacterial genera causing bulking. PAX, ozone, and nano-coagulant addition appeared to have a strong mitigating effect on these species, however more detailed investigations showed that seasonal dynamics of these bacteria masked the real results and that no mitigation method showed convincing results.
- The effect of the seasonal variation was removed by using a time-series decomposition approach developed using a 3-years long community survey. PAX dosing was possibly successful in controlling *Ca.* Microthrix subdominans, but not the more common *Ca.* Microthrix parvicella. Overall, it is important to monitor the microbial communities at species level to understand the normal seasonal dynamics in order to effectively plan and execute experiments.
- The results highlight the difficulties and biases of drawing conclusions based on testing in a single full-scale treatment line. Long time-series and/or parallel reference lines are needed to properly evaluate the effect of mitigation strategies in full-scale WRRFs.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.118340.

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