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Shift in activated sludge microbiomes associated with nitrite accumulation and high nitrous oxide emissions

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ABSTRACT

Nitrous oxide (N₂O) emissions can constitute over half of the carbon footprint of a wastewater treatment plant (WWTP), and emission peaks frequently correlate with nitrite (NO₂⁻) concentrations. However, connections between the microbiome and high N₂O and NO₂⁻ levels are not well-documented. Here, we characterize the microbiomes in several parallel lines of a WWTP during massive N₂O emissions (20 % of influent nitrogen load) with prolonged NO₂⁻ accumulation in most lines, aiming to identify key differences between communities in lines with high and low NO₂⁻ concentrations. The abundance of nitrite-oxidizing bacteria (NOB) was extremely low in the lines with NO₂⁻ accumulation, which also had slightly lower abundances of ammonia-oxidizing bacteria (AOB). Some incomplete denitrifiers were more abundant in the lines with NO₂⁻ accumulation. Lines without NO₂⁻ had a higher relative abundance of filamentous bacteria and better floc formation. These findings confirmed our hypothesis that loss of NOB caused NO₂⁻ accumulation, inducing increased N₂O emissions. AOB are suspected to be the main source of N₂O during the studied period, with a likely contribution from heterotrophic denitrifiers. A few species were identified as interesting candidates for further study regarding their potential role in increased N₂O emission from WWTPs. Long-term microbiome monitoring is necessary to understand the changes in the microbiome that might initiate NO₂⁻ accumulation and high N₂O emissions.

1. Introduction

Nitrous oxide (N₂O) is one of the three most important greenhouse gases, with a global warming potential nearly 300 times higher than carbon dioxide (IPCC, 2021). Wastewater treatment plants (WWTP) are a marked point source of N₂O, accounting for approximately 3 % of the anthropogenic N₂O emissions (Mannina et al., 2018). In WWTPs, N₂O is generated in the biological nitrogen removal process as a byproduct or intermediate of microbial metabolism during nitrification or denitrification (Vasilaki et al., 2019). Direct N₂O emissions have repeatedly been reported to constitute over half of the carbon footprint of WWTPs (Maktabifard et al., 2022a; Vasilaki et al., 2019). Furthermore, N₂O emissions vary greatly between plants, and emission factors reported from full-scale WWTPs include values from close to 0 % to up to 15 % of the total influent nitrogen load (Daelman et al., 2015; Gruber et al.,

2021b; Kampschreur et al., 2009).

Factors influencing the long-term dynamics of N₂O emissions from wastewater treatment processes have been studied for over a decade, yet the emissions remain partly unexplained (Vasilaki et al., 2019). Previously, different process conditions have been found to affect the level of emissions, including the concentration of dissolved oxygen (DO), aeration pattern, high concentrations of nitrite (NO₂⁻), and the availability of organic carbon (Vasilaki et al., 2019). N₂O emissions of full-scale WWTPs have also been shown to vary seasonally (Daelman et al., 2015; Gruber et al., 2020). The seasonal variability has been correlated with NO₂⁻ concentrations (Daelman et al., 2015) and the trend in temperature change (Chen et al., 2019). However, changes in such process and environmental conditions have not fully explained the variations in N₂O emissions (Vasilaki et al., 2019). Microbial community dynamics have been suggested as another factor contributing to the emission

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dynamics (Daelman et al., 2015; Gruber et al., 2021a).

Similarly to N_2O , microbial communities of activated sludge display seasonal variation, driven to a large extent by changes in temperature (Griffin and Wells, 2017; Ju et al., 2014; Petrovski et al., 2020). These seasonal dynamics have recently been shown to occur at the species level, revealing differing patterns even for species affiliated with the same genus or functional group (Peces et al., 2022). In addition to temperature, examples of proposed drivers of the seasonal dynamics include mass immigration from the influent wastewater (Peces et al., 2022) and changes in influent characteristics (Petrovski et al., 2020). Stochastic processes—such as drift, birth and death, and migration—have been suggested as dominant drivers of activated sludge community compositions at the global level (Wu et al., 2019). However, deterministic factors—such as temperature, sludge retention time, and the influent organic load—also influence the compositions (Wu et al., 2019); many of the same factors drive the microbial community and N_2O emission dynamics.

Correlations between the microbial community structure and N_2O production or emission have been studied mainly in laboratory conditions with synthetic wastewater (e.g., Guo et al., 2019; Vieira et al., 2018; Yan et al., 2024b, 2021; Zheng et al., 2015), with limited applicability in the dynamic and partly uncontrollable conditions of full-scale WWTPs. Furthermore, the community assessment often remains at the level of functional groups or at high taxonomic levels—outlining the dominant phyla, families, and sometimes genera (e.g., Castellano-Hinojosa et al., 2018; Vieira et al., 2019; Yan et al., 2024a)—while the dynamics and responses to environmental conditions can vary even among species of the same genus or functional group. Examples of these species-level differences include the differing effect of DO concentration on N_2O production through AOB denitrification in different AOB species (Ren et al., 2019), underlining the importance of species-level community assessment.

Recently, correlations between the long-term microbial community composition and N_2O production (Valk et al., 2022) or emission (Gruber et al., 2021a) have been investigated more comprehensively in full-scale WWTPs. Gruber et al. (2021a) identified a clear shift in the activated sludge microbiome during seasonal NO_2^- accumulation and high N_2O emissions (up to 30 % of influent nitrogen load), including a decline in filamentous bacteria and nitrite-oxidizing bacteria (NOB). Valk et al. (2022) also reported a negative correlation between NOB and liquid-phase N_2O concentration (but did not measure NO_2^-) and found that more than 50 species correlated with N_2O concentration, despite the lack of an obvious pattern in microbial community dynamics that might explain the N_2O concentration. The findings by Gruber et al. (2021a) in particular suggest that periods of high N_2O emission may involve changes in several functional groups of the microbial community, such as filamentous bacteria important for floc structure, indicating potentially important indirect links between the microbes and N_2O production. Thus, it is important to study the whole microbial community (instead of only the nitrogen-transforming microorganisms) to comprehensively evaluate the links between community composition and N_2O production. If such links can be identified (and previously proposed links further validated in more WWTPs), this could promote the development of new mitigation strategies relying on community optimization in WWTPs.

Seasonal N_2O emission peaks in winter and spring have been found to correlate with NO_2^- concentration in multiple full-scale WWTPs (e.g., Gruber et al., 2021a, 2021b; Sieranen et al., 2024). Due to the lack of online monitoring, we do not fully understand the frequency of these events in WWTPs. However, based on the available data, we assume that seasonal N_2O emission peaks with simultaneous NO_2^- accumulation may be a common phenomenon in WWTPs in temperate climates. As such, the drivers and mitigation of these peaks need to be studied further.

Here, we examine the activated sludge microbial communities in several parallel lines of a municipal WWTP in Finland during an unexpected disturbance event with prolonged high N_2O emissions (up to 20

% of the nitrogen load of the activated sludge influent). In most treatment lines, NO_2^- accumulated, suggesting a failure of the second step of the nitrification process (nitrite oxidation). The abnormal process state could not be attributed to the process conditions. To deepen our understanding of potential causes of the persistent NO_2^- and N_2O accumulation, we investigated the links between the microbial community structures and the high NO_2^- and N_2O levels, as an extension of the previously published study by Kuokkanen et al. (2021), which presents a detailed analysis of the process conditions and performance. The process disturbance was unexpected, and thus anticipatory sampling was not carried out. However, among the parallel lines, two maintained an almost normal nitrogen removal performance (without accumulation of NO_2^- or N_2O), which enabled comparison of microbial communities from lines with low and high NO_2^- and N_2O concentrations. For this purpose, we did the following: 1) analyzed the microbial communities using 16S rRNA gene amplicon sequencing and quantified nitrifying bacteria by quantitative real-time polymerase chain reaction (qPCR); 2) reviewed these results alongside process performance data to allow evaluation of the connections between the community structure (at the species level, when possible) and the nitrogen conversion process disturbance; and 3) focused on microbial taxa previously correlated with N_2O and/or NO_2^- concentrations by Gruber et al. (2021a) or Valk et al. (2022) to synthesize findings from different full-scale WWTPs. In this study, we propose microbes with a possible role in NO_2^- accumulation and/or N_2O emission in WWTPs, which could be targeted in future studies as potential indicators of a nitrogen removal disturbance.

2. Material and methods

2.1. Study site and process monitoring

The Viikinmäki WWTP (Helsinki, Finland) has a population equivalent of over one million and an average influent flow of 280,000 m^3/d . Industrial wastewater accounts for approximately 4 % of the influent flow and almost 10 % of the organic load (BOD) to the plant. The treatment process (fully described in Kuokkanen et al. (2021)) starts with mechanical pre-treatment, followed by seven primary sedimentation lines. The secondary treatment step is an activated sludge process with a denitrification-nitrification configuration, consisting of nine parallel treatment lines with a volume of 11,500 m^3 each. These plug-flow reactors contain six zones, the first of which is anoxic, the second and third are swing zones, and the last three are always aerated. The principle of operation is the same for each parallel line, and the reactors receive the same influent wastewater (with internal nitrate recycle being 100–300 % of the influent flow). The aeration of the reactors is controlled by an automated system based on the ammonium (NH_4^+) concentration in the last zone, and the DO setpoint is 3 mg/L. Each parallel line has a separate return sludge recirculation, which operates at approximately 70 % of the influent flow rate. The excess sludge from all lines is removed through the primary settlers. Sludge ages in the studied treatment lines ranged from 8.6 to 11 days during May and June 2019, when the biomass samples were collected in this study. The smallest range in an individual line was from 8.6 to 9.3 days, and the largest was from 9.3 to 10.9 days. The average MLVSS values in the studied lines (AS4, 5, 8, and 9) were 2.5, 2.6, 2.3, and 2.2 g/L, respectively.

The Viikinmäki WWTP implements online and laboratory measurements for process monitoring. Because the plant is located underground, gaseous emissions (including N_2O) can be monitored at the exhaust air pipe of the plant. Since 2012, the pipe has been equipped with the Gasmeter CEMS Fourier transfer infrared system to monitor the emissions online from the whole treatment process. In addition to monitoring the N_2O levels in the exhaust air, soluble N_2O concentrations are monitored in the last aerated zone of the activated sludge lines 5 and 9 with the Unisense Wastewater System. Most of the laboratory analyses have been described by Kuokkanen et al. (2021), including the concentrations of

$\text{NH}_4^+\text{-N}$, $\text{NO}_2^-\text{-N}$, and the sum of nitrate ($\text{NO}_3^-\text{-N}$) and $\text{NO}_2^-\text{-N}$ ($\text{NO}_x^-\text{-N}$) in the activated sludge effluents. Concentrations of the nitrogen fractions are not measured from each zone of the reactors at the WWTP, and thus these data were not available for analysis. Sludge volume index (SVI) values were determined according to standard methods (Eaton et al., 2005) from 2-fold diluted samples. Filament index values were determined through microscopy, using reference images for filament abundance on a scale of zero to five.

2.2. Sampling period and process performance

In spring 2019, the Viikinmäki WWTP suffered from its second period of high N_2O emission and/or NO_2^- accumulation since the start of the continuous N_2O monitoring, and that period is assessed in this study. The process conditions are comprehensively described by Kuokkanen et al. (2021). The most important process conditions of the four lines assessed in this study are presented in Fig. 1 and summarized in this section.

Initially, the ammonium removal performance decreased in April in all other lines except AS4 and AS5 (Fig. 1a). After the fast recovery of ammonium removal, NO_2^- accumulated over a prolonged period in all lines except AS4 and AS5 (Fig. 1a). Until April 2019, NO_2^- concentrations were measured only in lines AS1 and AS2, and thus the concentrations from this period are not available for the four lines shown in Fig. 1. As was previously reported by Kuokkanen et al. (2021), NO_2^- -N constituted up to 80 % of $\text{NO}_x^-\text{-N}$ in the effluents of the treatment lines. Furthermore, the N_2O emissions correlated with the effluent NO_2^- -N concentration, with the highest daily N_2O emissions being 20 times higher than the average emissions in 2018, and up to 20 % of the nitrogen load in the activated sludge influent was emitted as N_2O (Kuokkanen et al., 2021). The soluble N_2O concentration in line AS9 followed a similar pattern as the N_2O emissions in the exhaust air (Fig. 1b). In contrast, the soluble N_2O concentration in line AS5 mainly remained at low levels (Fig. 1b). After sludge transfer from well-functioning lines to the lines with impaired performance, the NO_2^- and N_2O concentrations decreased and stabilized to normal levels in the whole process around August 2019. Line AS9 was the last to normalize and the only one left to recover on its own, as the NO_2^- concentrations started to decrease slightly before the sludge transfer was scheduled to begin.

After the onset of impaired nitrogen removal, the filament indices were clearly lower in the treatment lines with NO_2^- accumulation (Fig. 1c). Accordingly, microscopic investigation revealed a lack of filamentous bacteria and visible flocs in sludge samples from these lines, while filaments were abundant in sludge samples from a line without NO_2^- accumulation (see Supplementary Information). As reported by Kuokkanen et al. (2021), this state of the sludge was further reflected in the treatment process as increased turbidity. However, no clear difference was observed for the SVI values (Fig. 1d).

Based on the assessment of the process conditions, the NO_2^- accumulation started after peak flows of cold wastewater entered the WWTP in late March and was considered the cause of increased N_2O production and emission. Otherwise, the process conditions could not explain the causes of the development and maintenance of the abnormal process state. An inhibitory substance was not assumed to have triggered NO_2^- accumulation, as the disturbance did not occur in all treatment lines (receiving the same influent). Furthermore, monthly monitoring of substances that are harmful or dangerous to the aquatic environment did not reveal anything unusual at the beginning of (or before) the disturbance. Thus, in this study, we focus on investigating the microbial community structures of the treatment lines and their links to the persistent NO_2^- and N_2O accumulation. The predominant source and location of N_2O emissions from the process could not be explicitly determined based on the available monitoring data. Thus, the microbial communities related to both nitrification and denitrification were assessed in detail.

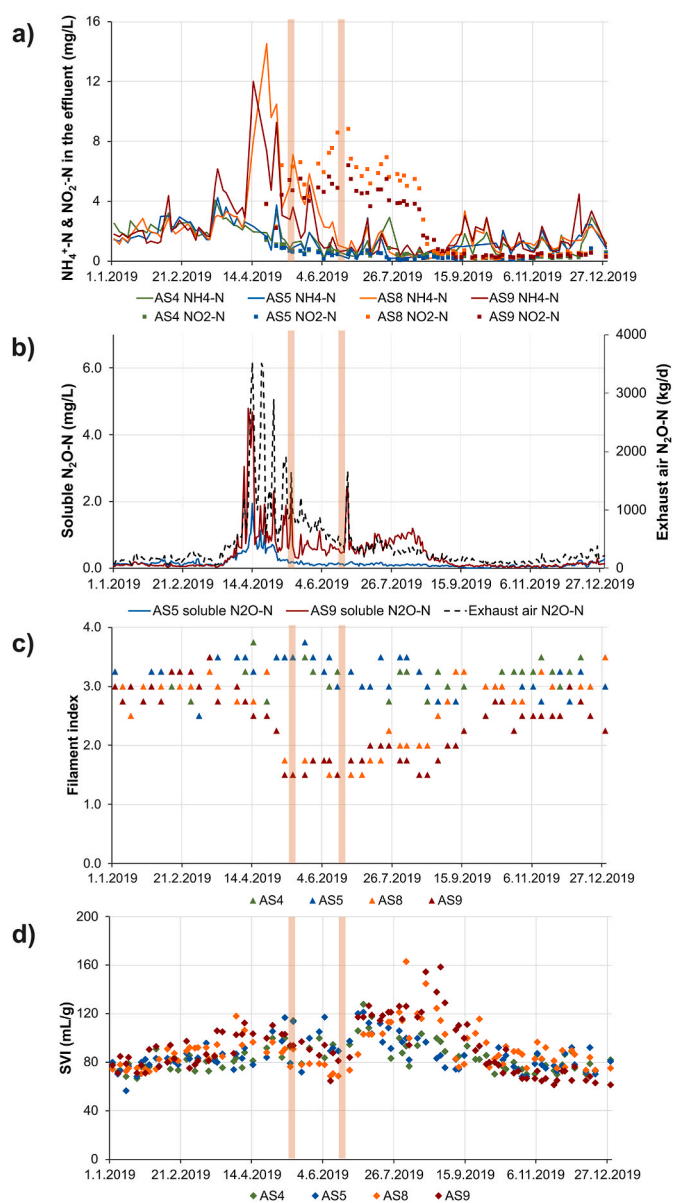


Fig. 1. Performance and conditions of the studied treatment lines in the Viikinmäki WWTP. a) Concentrations of ammonium ($\text{NH}_4^+\text{-N}$) and nitrite ($\text{NO}_2^-\text{-N}$) in the activated sludge (AS) effluents. b) Concentration of soluble nitrous oxide (N_2O -N) in the AS lines and N_2O emissions in the exhaust air of the treatment plant. c) Filament indices of the AS lines. d) Sludge volume index (SVI) values of the AS lines. The sampling times when the AS samples were collected for the microbial community analyses are highlighted with colored bars.

2.3. Biomass sampling, sample processing, and DNA extraction

Biomass samples were collected and processed from three to four treatment lines on two days during the N_2O emission period (13.5.2019 and 19.6.2019). It is noteworthy that the first samples were collected approximately a month after the start of the nitrogen conversion process disturbance. Therefore, this microbial community analysis does not include the initial dynamics during the early stages of the disturbance. For each sample, 1 L of sludge was collected from the last aerated zone (approximately 0.5 m below the surface), which can be considered representative of the biomass in the whole line, as the activated sludge flows from one zone to the next. The samples were transported in a cool box to the laboratory at Aalto University (Espoo, Finland) during the sampling day. In the lab, one or two subsamples of 15 mL were

transferred from well-mixed bottles into centrifugation tubes, and the biomass was harvested by centrifuging for 10 min at 10,000 rpm at 4 °C. The biomass samples were stored at –80 °C until shipping to DTU Sustain (formerly DTU Environment; Lyngby, Denmark) for further processing.

DNA was extracted from the biomass (approximately 0.5 g dry weight) with the FastDNA™ Spin Kit for Soil (MP Biomedicals, USA) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were measured and checked by its 260/280 ratio using NanoDrop (ThermoFisher Scientific, Rockwood, USA). DNA was stored at –80 °C until further use.

2.4. 16S rRNA gene amplicon sequencing, bioinformatic analysis, and microbial community analysis

To analyze the microbial communities of the activated sludge samples, the V3-V4 region of the 16S rRNA gene was amplified using the primers 341F and 806R (Yu et al., 2005) and sequenced on an Illumina MiSeq platform (2 × 300 bp) at DTU Multi Assay Core Center (Lyngby, Denmark). On average, 106,120 ± 28,960 reads were obtained per sample. In the bioinformatic analysis, amplicon sequence variants (ASVs) were produced from the reads with the DADA2 pipeline (Callahan et al., 2016) v1.22.0. The sequences were annotated taxonomically using the MiDAS 4 reference database (Dueholm et al., 2022) v4.8.1. More details on the bioinformatic analysis can be found in Supplementary Information.

The microbial community analysis was performed with phyloseq (McMurdie and Holmes, 2013) v1.38.0. Before computing alpha diversity indices, the samples were rarefied to an even depth (corresponding to the sample with the lowest depth), using the function `rarefy_even_depth` with the default `RNGSeed` and without replacement. To explore differences in the microbial communities (beta diversity), principal coordinates analysis (PCoA) was applied based on the Bray-Curtis dissimilarity and the relative abundances of the ASVs. For the PCoA plot and other figures, data visualization was performed using `ggplot2` (Wickham, 2016) v3.3.5, `RColorBrewer` (Neuwirth, 2014) v1.1-2, and `ggrepel` (Slowikowski, 2021) v0.9.1.

For community analysis at lower taxonomic levels (genus and species), the samples were grouped based on NO₂⁻ accumulation in the treatment lines. Samples collected from lines AS4 and AS5 in May and June were grouped together (“No NO₂⁻”, n = 4), and samples collected from lines AS8 and AS9 in May and June were grouped together (“NO₂⁻ accumulation”, n = 6). The maximum NO₂⁻ concentrations in the activated sludge effluents of these four lines were 1.1 mgN/L, 1.0 mgN/L, 8.8 mgN/L, and 6.4 mgN/L, respectively, from the beginning of May to the end of June. With this grouping, the aim was to find differences in the communities potentially related to the differences in process conditions and performance, instead of natural variation over time. To compare the groups, averages (of relative abundance of genera and species) were calculated for each group. Finally, a Student's t-test was used to assess statistically significant differences in alpha diversity indices or the microbial community compositions between the lines with and without NO₂⁻ accumulation (assuming a normal distribution of the data and homogeneity of variances). All the analysis steps were conducted using R (R Core Team, 2021) v4.1.2.

2.5. Quantitative real-time polymerase chain reaction

To quantify the abundances of certain nitrifying bacteria in the biomass samples, we conducted quantitative real-time PCR (qPCR) assays at DTU Sustain (with details presented in Supplementary Information). The target genes were 16S rRNA of the domain Bacteria, 16S rRNA of ammonia-oxidizing bacteria (AOB) from the family *Nitrosomonadaceae*, and 16S rRNA of *Nitrospira*, using the primers and qPCR conditions listed in Table S1. The copy numbers of the target genes of *Nitrosomonadaceae* and *Nitrospira* were normalized with the copy

numbers of the 16S rRNA gene of the domain Bacteria. Finally, a Student's t-test was used to assess statistically significant differences between the lines with and without NO₂⁻ accumulation, using the sample grouping described in Section 2.4.

3. Results and discussion

3.1. The nitrogen conversion process disturbance was accompanied by changes in the microbial community

On average, the 16S rRNA amplicon sequencing produced 68,460 ± 19,273 non-chimeric, filtered, and merged sequences per sample. The final sequence table included a total of 5204 ASVs, of which 2415 were unique species-level ASVs. This ASV table was used in the community analyses.

Microbial communities in the lines with NO₂⁻ accumulation were dissimilar to those in the lines without NO₂⁻. As shown in Fig. 2, samples collected from the lines with NO₂⁻ accumulation and without NO₂⁻ formed separate clusters at opposite ends of the first PCoA axis. This pattern was observed for samples collected in both May and June. The first axis explains nearly 70 % of the variation in the data. It separates the samples based on NO₂⁻ accumulation: the samples collected from different lines with similar conditions (i.e., from AS4 and 5, or AS8 and 9) clustered closely together and thus showed very similar community compositions even if the lines had separate sludges. The clustering pattern in the PCoA plot agrees with previous observations from a full-scale WWTP (Gruber et al., 2021a). In addition, we observed a shift from May to June for both groups, which can be seen in the PCoA plot as a separation of the samples along the second axis based on sampling time (Fig. 2). Furthermore, the lines displaying NO₂⁻ accumulation showed a greater distance, i.e., a greater dissimilarity, between May and June than the line(s) not displaying NO₂⁻ accumulation. This finding also aligns with that of Gruber et al. (2021a), whose study observes a larger temporal shift in the community dissimilarity of reactors with NO₂⁻ accumulation. The results suggest that the community structures were changing, particularly in the lines where NO₂⁻ accumulated.

In addition to dissimilarities between the microbial communities, we assessed diversities within the samples. The estimated Shannon and Simpson (calculated as 1-D) diversity indices suggested a slightly higher diversity in the lines without NO₂⁻ accumulation (see Table 1). Both indices had a higher value for the group without NO₂⁻ accumulation; yet, this difference was statistically significant only for the Shannon index (p < 0.01; for Simpson, p = 0.39). Previously, maintaining the biodiversity of activated sludge has been proposed as a contributor to a stable process performance (Wu et al., 2019), and lower alpha diversity values have been reported during periods of poor nitrification performance. For example, Johnston et al. (2019) observed lower Shannon and Simpson indices in the winter compared to summer in full-scale sequencing batch reactors displaying seasonal nitrification failures. Similarly, Gruber et al. (2021a) observed a lower Shannon index for the communities in reactors with nitrification failure.

As outlined in Section 2.2 for the differences in process performance, the initial trigger for differences in community development between the different lines remains unresolved (although peak flows of cold wastewater are suspected among the triggers). However, free nitrous acid may have affected the community development during the prolonged NO₂⁻ accumulation because of its inhibitory effects (see Supplementary Information). In the following sections, we scrutinize the differences between communities in the lines with prolonged NO₂⁻ accumulation—a state normally not maintained in the nitrification-denitrification process—and communities in the lines without NO₂⁻. As NO₂⁻ and N₂O were the process (performance) parameters that distinguished the disturbed lines from the undisturbed ones, the discussion about the microbial communities and their differences between the lines is centered around NO₂⁻ and N₂O.

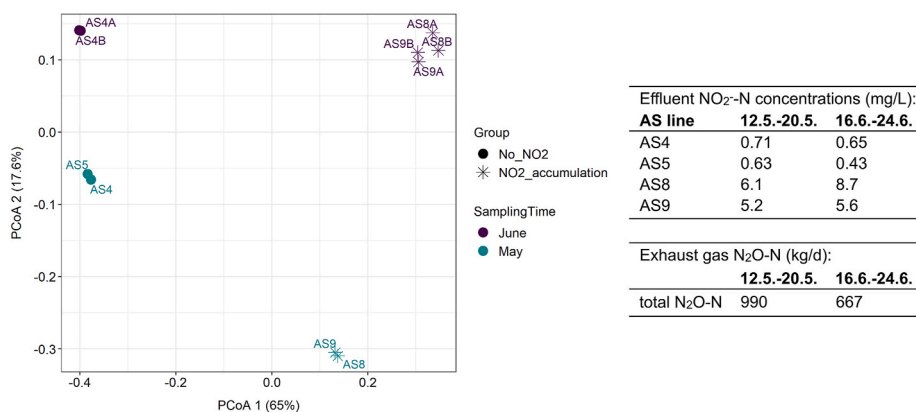


Fig. 2. Principal coordinates analysis (PCoA) plot based on the Bray-Curtis dissimilarity matrix. The activated sludge (AS) lines are visualized as dots (without nitrite (NO₂⁻) accumulation) or asterisks (with NO₂⁻ accumulation). The samples collected in May are marked in turquoise, and those collected in June are marked in violet. The contributions of the first and second axes to explaining the variation in the data are given as percentages following the axis names. The average concentration of NO₂⁻ in the effluent of each AS line (during nine days around the sampling day) is given on the right side of the plot, alongside the average N₂O emission in the exhaust gas. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Alpha diversity indices of activated sludge samples. The Simpson index was calculated as 1-D. NO₂⁻ accumulated in treatment lines AS8 and AS9 but not in AS4 and AS5.

Sampling time	Treatment line	Shannon	Simpson
May	AS4	5.505	0.9867
	AS5	5.395	0.9863
	AS8	5.374	0.9881
	AS9	5.263	0.9851
June	AS4	5.443 ± 0.111	0.9853 ± 0.0007
	AS8	5.191 ± 0.043	0.9829 ± 0.0008
	AS9	5.285 ± 0.064	0.9852 ± 0.0007
	Average	Lines without NO ₂ ⁻	5.447 ± 0.078
	Lines with NO ₂ ⁻ accumulation	5.265 ± 0.077	0.9849 ± 0.0020

3.2. Most abundant phyla and classes differed in association with nitrite accumulation

We compared the most abundant taxa at the phylum and class levels in the lines with and without NO₂⁻ accumulation. Fig. 3a and b illustrate the top 10 most abundant phyla and the top 20 most abundant classes,

respectively. *Proteobacteria* was the most abundant phylum in both the lines with and without NO₂⁻ accumulation (with relative abundances in the samples ranging from 40.2 % to 63.8 %). In the lines without NO₂⁻ accumulation, it was followed by *Actinobacteriota* (12.6–14.6 %), *Chloroflexota* (formerly *Chloroflexi*; 10.1–13.6 %), *Bacteroidota* (6.8–10.9 %), and *Myxococcota* (4.5–7.9 %). In the lines with NO₂⁻ accumulation, it was followed by *Bacteroidota* (12.2–23.2 %), *Patescibacteria* (3.9–5.8 %), *Actinobacteriota* (3.1–7.1 %), and *Firmicutes* (3.9–5.9 %). These phyla have previously been identified as dominant phyla in WWTPs (Hu et al., 2024; Xia et al., 2018).

Among the phyla that were in the top five in the lines without (but not in the lines with) NO₂⁻ accumulation, *Chloroflexota* had substantially lower relative abundance in the lines with NO₂⁻ (Fig. 3a). They are filamentous bacteria with an important role in floc formation (Speirs et al., 2019). Furthermore, *Chloroflexota* are facultatively anaerobic fermenters, and some of them may also have a role in nitrogen removal (Petriglieri et al., 2023). Interestingly, Gruber et al. (2021a) reported a decreased abundance of *Chloroflexota* during NO₂⁻ accumulation and high N₂O emissions from a WWTP, and the authors suggested this dynamic as a key factor initiating changes in the microbial community and loss of floc integrity, leading to loss of NOB and accumulation of NO₂⁻. In the Viikinmäki WWTP, the NO₂⁻ concentrations seem to have started increasing already before the filament index values decreased (see Fig. 1). Thus, the decline in filaments and the weak floc structures may have been initiated by the increased NO₂⁻ concentrations or by an uncharacterized inhibitory effect of the influent wastewater. A thorough

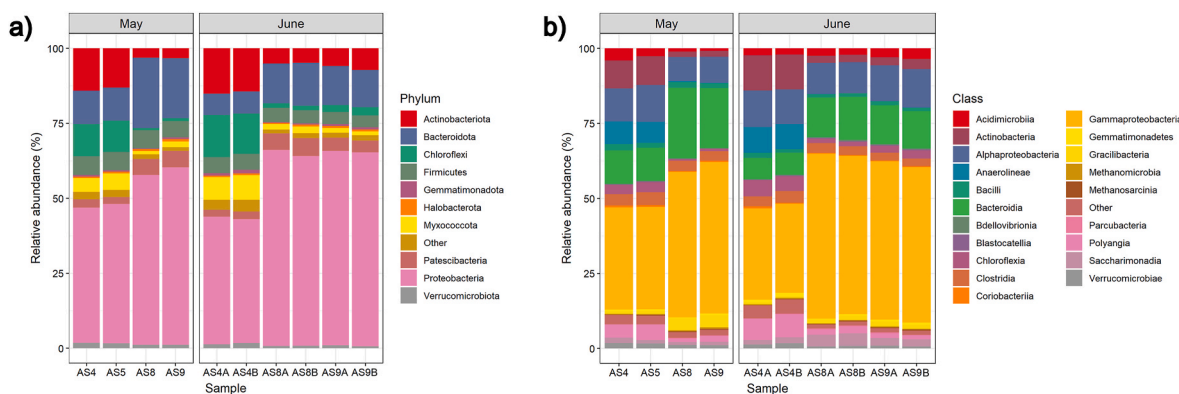


Fig. 3. Relative abundances of most abundant phyla and classes in the activated sludge samples. a) Top 10 phyla with the highest relative abundances. The taxonomic database included the former name (*Chloroflexi*) of the phylum *Chloroflexota*. b) Top 20 classes with the highest relative abundances. The phylum/class “Other” includes all taxa with lower relative abundances. In June, samples A and B for each line represent duplicates.

analysis of the process conditions did not reveal an evident cause (Kuokkanen et al., 2021). Previously, high concentrations of NO_2^- have been reported to impair floc structures through disintegration and lack of filaments (Philips and Verstraete, 2001) or through bulking caused by excessive growth of filamentous bacteria (Philips and Verstraete, 2001; Yang et al., 2016).

Among the classes shown in Fig. 3b, we assessed the ones with $\geq 1\%$ relative abundance in May and/or June in the lines with and/or without NO_2^- accumulation (see Table S2 for details). We specifically looked at the classes displaying a ≥ 1.5 -fold difference in relative abundance between the lines with and without NO_2^- accumulation in May and/or June. We could see that *Actinobacteria*, *Anaerolineae*, *Chloroflexia*, *Polyangia*, and *Verrucomicrobiae* had consistently and statistically significantly higher relative abundances in the lines without NO_2^- accumulation, while *Bacteroidia* and *Gammaproteobacteria* had consistently and statistically significantly higher relative abundances in the lines with NO_2^- accumulation (see Table S2 for abundances and Supplementary Information for a brief description of known functions of these classes in activated sludge processes). These classes showing

significant differences were selected for further exploration.

3.3. Filamentous bacteria and some denitrifiers were more abundant in the lines without nitrite accumulation

Among the classes with significant differences between the lines, we assessed the genera with an average relative abundance of $\geq 0.1\%$ in the lines with and/or without NO_2^- accumulation and ≥ 1.5 -fold difference in relative abundance between the lines (Fig. 4; see Table S3 for details), aiming to identify taxa that could serve as indicators of nitrogen removal disturbance.

From the class *Actinobacteria*, for example, the genus *Tetrasphaera* had a markedly higher relative abundance in the lines without NO_2^- accumulation (on average 7.9 %; highlighted with red text in Fig. 4) than in the lines with NO_2^- (0.65 %). Bacteria affiliated with *Tetrasphaera* have been found to have the genomic potential for some of the denitrification steps. For example, many of the *Tetrasphaera* genomes studied by Singleton et al. (2022) had the genes encoding the nitrate and nitrite reductases while lacking the genes encoding the nitric oxide and nitrous

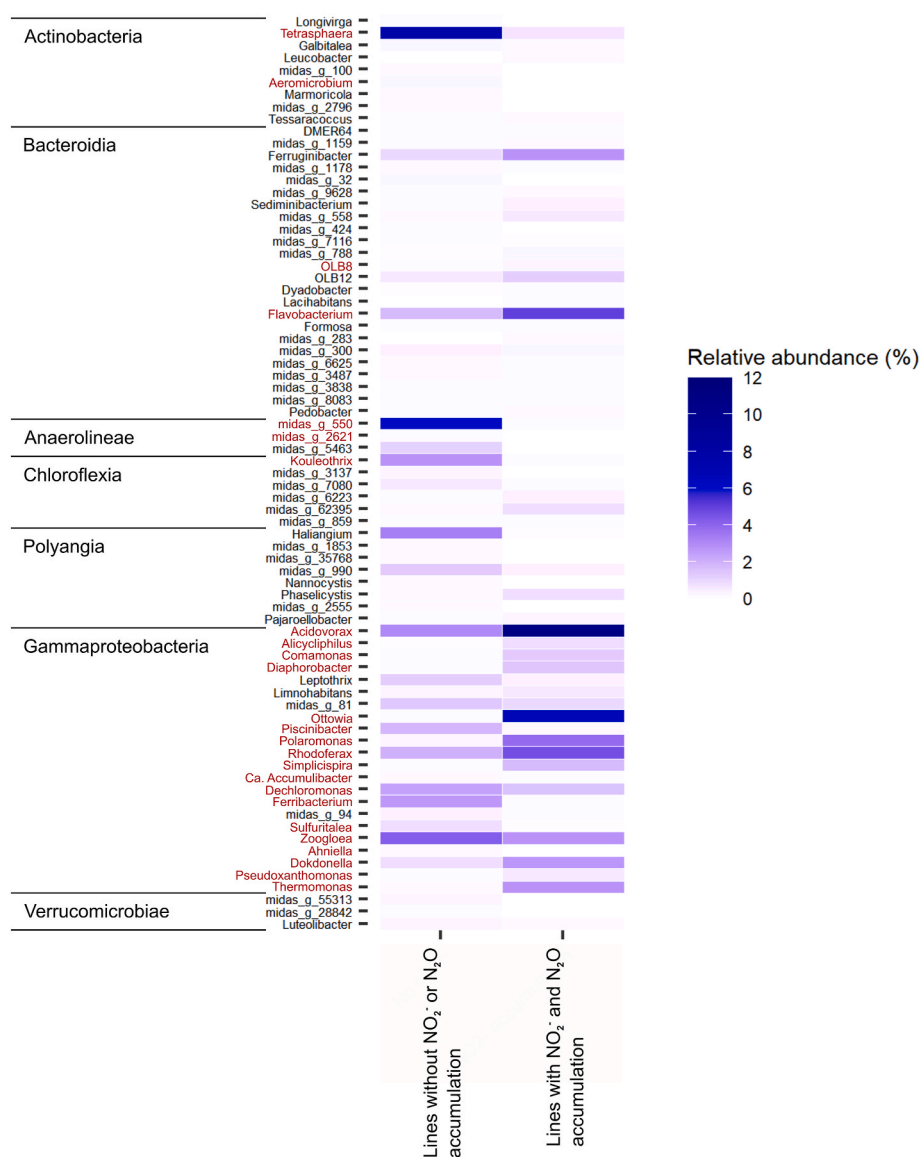


Fig. 4. Heatmap of genus abundance in the lines with and without NO_2^- accumulation. Shown are the genera fulfilling the following criteria: 1) belong to the classes that displayed significant differences between the lines (discussed in Section 3.2), 2) have $\geq 0.1\%$ average relative abundance, and 3) display ≥ 1.5 -fold difference between the lines. Genera written in red are discussed in Sections 3.3 and 3.4. The names of the classes are shown on the left of the genus names. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

oxide reductases. However, a *Tetrasphaera*-enriched culture (constituting >80 % of the whole community) was shown to denitrify without N₂O accumulation when N₂O was provided to the culture simultaneously with another electron acceptor (NO₂⁻ and/or NO₃⁻) (Marques et al., 2018). In the present study, midas_s_328 was the dominant species affiliated with *Tetrasphaera* in the lines without NO₂⁻ accumulation, followed by *Tetrasphaera vanveenii* (see Table S4 for species abundances). In the lines with NO₂⁻ accumulation, midas_s_5 (recently reclassified into the genus *Candidatus Phosphoribacter* (Singleton et al., 2022)) and *Tetrasphaera jenkinsii* were the dominant species from this genus. Among these species, midas_s_328, *T. vanveenii* and *T. jenkinsii* can form filaments (McKenzie et al., 2006; Singleton et al., 2022), and thus they (especially midas_s_328, which had a relative abundance of 5.9–7.9 % in the samples collected from lines without NO₂⁻) may have contributed to the formation of stable flocs in the lines without NO₂⁻. Also, the genus *Aeromicrobium* from Actinobacteria displayed higher relative abundances in the lines without NO₂⁻ accumulation (Fig. 4). Valk et al. (2022) recently identified a positive correlation between a species affiliated with this genus (*Aeromicrobium choanae*) and seasonal N₂O concentration in a WWTP. However, in the Viikinmäki WWTP, the lines with a higher relative abundance of *Aeromicrobium* did not produce high N₂O levels, and the sequences affiliated with this genus were annotated as a different species (midas_s_841).

From the class *Chloroflexia*, we detected higher relative abundances of the genus *Kouleothrix* in the lines without NO₂⁻ accumulation (on average 2.8 % compared to 0.040 % in the lines with NO₂⁻ accumulation; Fig. 4). In previous studies, the filamentous bacteria *Kouleothrix* (Eikelbloom Type 1851) have been associated with bulking in WWTPs in Japan (Nittami et al., 2019, 2020) and negatively correlated with the mixed liquor temperature (Nittami et al., 2020). However, the lines without NO₂⁻ accumulation displayed good floc-forming abilities in the Viikinmäki WWTP, and the *Kouleothrix* filaments may possibly have contributed to the floc formation.

From the class *Anaerolineae*, the genus midas_g_2621 had higher relative abundances in the lines without NO₂⁻ accumulation (Fig. 4). Valk et al. (2022) identified the species midas_s_2621 from this genus among the taxa that were negatively correlated with seasonal N₂O production in a WWTP. The sequences affiliated with this genus were annotated as a different species (midas_s_8909) in our samples. Nevertheless, this genus had a higher relative abundance in the lines that did not produce high levels of N₂O. Furthermore, the genus midas_g_550 affiliated with *Anaerolineae* displayed a substantially higher relative abundance in the lines without NO₂⁻ accumulation (on average 6.2 %; see Fig. 4) than the lines with NO₂⁻ (0.064 %). This genus has not yet been functionally characterized, but it was recently proposed with the name *Candidatus Pachofilum* (Petriglieri et al., 2023). The high relative abundance observed in the lines without NO₂⁻ suggests that this genus may have had an important role in the community.

Many genera from *Gammaproteobacteria* that had higher relative abundances in the lines without NO₂⁻ accumulation include denitrifying members. Examples of such genera that have been found to be important denitrifiers in activated sludge processes include *Dechloromonas*, *Sulfuritalea*, and *Zoogloea* (Huibin Chen et al., 2020; Hagman et al., 2008; McIlroy et al., 2016; see Fig. 4 for abundances). The genus *Zoogloea* further contributes to floc formation (Shin et al., 1993). In activated sludge WWTPs, *Dechloromonas*-like clade II *nosZ* sequences have been identified among the most abundant *nosZ* groups (Kim et al., 2020). Furthermore, a sequence affiliated with *Dechloromonas* was found to be negatively correlated with N₂O concentration in a WWTP (Valk et al., 2022). Additionally, the genus *Candidatus Accumilibacter* (see Fig. 4 for abundances) has been shown to carry out complete denitrification without N₂O accumulation also when multiple electron acceptors are available (Roy et al., 2021). Furthermore, *Ferribacterium* (see Fig. 4 for abundances) has been reported among the dominant bacteria carrying out heterotrophic nitrification and aerobic denitrification in a pilot-scale constructed wetland (Tan et al., 2021). As such, all these genera may

have contributed to the efficient reduction of NO₂⁻ and N₂O in the lines without NO₂⁻ accumulation. Furthermore, *Zoogloea* may have played a role in the better floc-formation abilities of the sludge in these lines. From these genera, we detected some previously characterized species among the dominant ones in the studied samples. Among these species, for example, *Candidatus Dechloromonas phosphorivorans* has been found to have the genomic potential for complete denitrification (Petriglieri et al., 2021). Furthermore, an isolate of *Ferribacterium limneticum* studied by Duffner et al. (2022) harbored a clade II *nosZ* gene, which was thought to have decreased the N₂O accumulation in the denitrification reactions by this isolate due to the higher affinity of organisms with clade II *nosZ* to N₂O compared to those with clade I. Finally, the genera *Ahniella* and *Piscinibacter* from *Gammaproteobacteria* also displayed higher relative abundances in the lines without NO₂⁻ accumulation (Fig. 4). Valk et al. (2022) identified positive correlations between the N₂O concentration in a WWTP and the species *Ahniella* midas_s_116 as well as a sequence affiliated with the genus *Piscinibacter*. However, the lines without NO₂⁻ accumulation did not produce high levels of N₂O in the Viikinmäki WWTP.

3.4. Genera with incomplete denitrification capabilities were more abundant in the lines with nitrite accumulation

From the class *Bacteroidia*, we observed a higher relative abundance of the genus *OLB8* in the lines with NO₂⁻ accumulation (on average 0.29 % compared to 0.072 % in the lines without NO₂⁻; see Fig. 4). Some *OLB8* genomes have been found to contain nitrite, nitric oxide, or nitrous oxide reductases among the denitrification genes (Kondrotaitė et al., 2022; Speth et al., 2016). *OLB8* was also shown to contribute to NO₂⁻ accumulation in a partially denitrifying reactor upon a low C/N ratio (Hou et al., 2023). In the studied samples, midas_s_29 (recently proposed with the name *Candidatus Brachybacter algidus* (Kondrotaitė et al., 2022)) was among the dominant *OLB8* species. Based on studied *Ca. B. algidus* genomes, this species is capable of partial denitrification with the nitrite and nitric oxide reductase genes found in most of the genomes (Kondrotaitė et al., 2022), and thus it has the genomic potential for net N₂O production. Also, Valk et al. (2022) reported a similar pattern in the seasonal N₂O concentration and the relative abundance of N₂O producers, dominated by *OLB8* midas_s_29, but the correlation was not supported by statistical analysis. Nevertheless, our findings further propose this species as an interesting candidate of potential N₂O sources in WWTPs (even if AOB are considered to have been a major source of N₂O during the studied period, as discussed in Section 3.6).

Like in the lines without NO₂⁻ accumulation, many genera from *Gammaproteobacteria* that had higher relative abundances in the lines with NO₂⁻ accumulation include denitrifying members. Examples of genera that have been identified as predominant denitrifiers in wastewater treatment systems include *Acidovorax*, *Alicyclophilus*, *Comamonas*, *Diaphorobacter*, *Rhodoferrax*, *Simplicispira*, and *Thermomonas* (Huibin Chen et al., 2020; McIlroy et al., 2016; Wang and Chu, 2016; Zhang et al., 2019; see Fig. 4 for abundances). Furthermore, *Polaromonas* (see Fig. 4 for abundances) has been found to be an important denitrifier in cold temperatures (Jang et al., 2019; Rodriguez-Sanchez et al., 2020). The genera *Pseudoxanthomonas* and *Dokdonella* (see Fig. 4 for abundances) also contain denitrifying species. Interestingly, members of *Pseudoxanthomonas* have been shown to reduce NO₂⁻ with N₂O as the main or only product (Finkmann et al., 2000; Lee et al., 2008; Thierry et al., 2004). As such, *Pseudoxanthomonas* (which had an average relative abundance of 0.56 % in the lines with NO₂⁻ and 0.053 % in the lines without NO₂⁻) may have contributed to the higher N₂O production in the lines with NO₂⁻ accumulation. Furthermore, the genus *Ottowia* (see Fig. 4 for abundances) and many of the aforementioned genera also include species that have the other denitrification genes in their genomes except *nosZ* (Ehsani et al., 2015; Jin et al., 2020; Spring et al., 2004; Wu et al., 2018), thus having the genomic potential for net N₂O production. For these genera, most of the dominant species identified in this study were

annotated with MiDAS placeholder names and thus lacked functional descriptions (but also their relative abundances in the lines with high and low NO_2^- concentrations are reported in Table S4, providing information that may be interesting in the future when these species will have been characterized and renamed). Additionally, the dominant species that have been described did not include potential net N_2O producers, according to the literature. Furthermore, Valk et al. (2022) reported a positive correlation between the *Rhodoferax* species *midas_s_320* and N_2O . Even if, in our samples, this genus had a higher average relative abundance in the lines with NO_2^- accumulation, the species *midas_s_320* had a slightly higher relative abundance in the lines without NO_2^- accumulation (0.44 % compared to 0.35 % in the lines with NO_2^- accumulation), which did not produce high levels of N_2O .

Some of these genera from *Gammaproteobacteria* that had higher relative abundances in the lines with NO_2^- accumulation and high N_2O production have actually been suggested to contribute to N_2O reduction. For example, *Dokdonella* has been detected among the dominant bacteria in activated sludge removing N_2O in the presence of 2 % oxygen (Figuerola-González et al., 2016). Furthermore, *Alicyclophilus denitrificans* (which we detected as the second most abundant species from this genus at an average relative abundance of 0.13 % in the treatment lines with NO_2^- accumulation) is a complete denitrifier, and a strain of this species has been characterized as a net N_2O sink in wastewater treatment systems (Zhou et al., 2022). Similarly, for example, *Flavobacterium* from *Bacteroidia* had a higher relative abundance in the lines with NO_2^- and N_2O accumulation (Fig. 4), while *Flavobacterium*-like clade II *nosZ* genes have previously been identified among the dominant *nosZ* groups in activated sludge WWTPs (Kim et al., 2020).

Finally, the species *Terrimonas midas_s_743* and *Rhodobacter midas_s_24* had higher relative abundances in the lines with NO_2^- accumulation in the Viikiniemi WWTP (see Table S5) and were reported by Valk et al. (2022) to have a positive correlation with N_2O . The genus *Terrimonas* has been detected among the dominant genera in partially denitrifying reactors with NO_2^- accumulation (Zhang et al., 2021, 2022), and it was also proposed as one of the genera associated with N_2O emission potential in a lab-scale rain garden for stormwater management (H. Wang et al., 2021). Furthermore, some strains of *Rhodobacter* have the ability to perform denitrification (Girija et al., 2010; Li et al., 2022). This genus has previously been associated with both high (Vieira et al., 2019) and low (Song et al., 2014) N_2O emissions in full-scale WWTPs. Thus, these two species might be interesting candidates with a potential link to the N_2O production in the lines with NO_2^- accumulation. A comparison with additional species identified with a correlation to N_2O by Valk et al. (2022) is provided in Supplementary Information.

3.5. Abundances of ammonia oxidizers and nitrite oxidizers differed in relation to nitrite accumulation

Finally, we inspected the abundances of nitrifying bacteria to evaluate their connection to the NO_2^- accumulation. Ammonia-oxidizing bacteria (AOB) were detected in all samples, while the abundance of NOB was very low in the lines where NO_2^- accumulated. The total abundance of the family *Nitrosomonadaceae* (with known ammonia-oxidizing genera) was lower in the lines with NO_2^- accumulation compared to the lines without NO_2^- based on both amplicon sequencing (0.34 % and 0.89 % average relative abundances in the lines with and without NO_2^- , respectively, $p < 0.001$; see Fig. 5a) and qPCR (the average normalized 16S rRNA gene copy numbers being $1.7 \cdot 10^{-2}$ and $2.3 \cdot 10^{-2}$, $p < 0.05$; see Fig. 5b). However, we could observe clear differences between the genera affiliated with this family based on amplicon sequencing. As shown in Fig. 5a, the genus *Nitrosomonas* had rather similar relative abundances in all treatment lines, with average values of 0.33 % and 0.35 % in the lines with and without NO_2^- accumulation, respectively. On the other hand, the genus *Ellin6067* had a clearly higher relative abundance in the lines without NO_2^- accumulation (on average 0.47 %) compared to the lines with NO_2^- accumulation

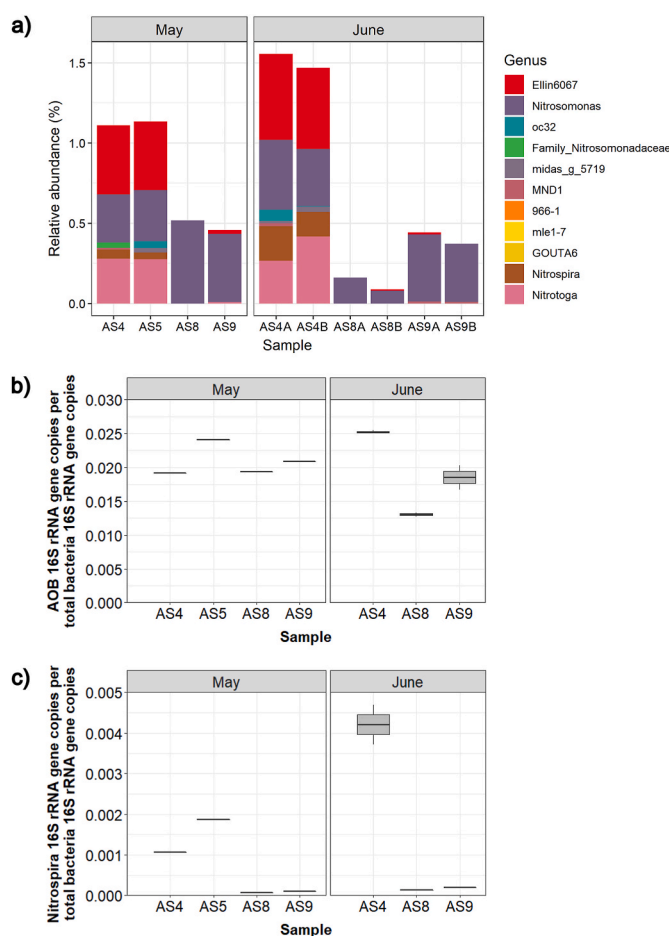


Fig. 5. Nitrifying bacteria in the activated sludge samples. a) Relative abundances of ammonia-oxidizing bacteria from the family *Nitrosomonadaceae* (first nine genera on the list) and nitrite-oxidizing bacteria from the families *Nitrospiraceae* (genus *Nitrospira*) and *Gallionellaceae* (genus *Nitrotoga*) based on the 16S rRNA gene amplicon sequencing. In June, samples A and B for each line represent duplicates. b) Normalized abundance of the 16S rRNA gene of the family *Nitrosomonadaceae* based on qPCR. c) Normalized abundance of the 16S rRNA gene of *Nitrospira* based on qPCR.

(0.0076 %, $p < 0.001$; see Fig. 5a). This genus has been suggested to be an ammonia oxidizer (L. Wang et al., 2021; Xia et al., 2005), although its physiology has not been studied in a culture. Based on our observations, *Ellin6067* might be more sensitive to NO_2^- accumulation than *Nitrosomonas*. However, previous studies have reported tolerance of *Ellin6067* to environmental stress, including cyanobacterial blooms (Lezcano et al., 2017) and light irradiation (L. Wang et al., 2021). Finally, the genera *oc32*, *MND1* and *midas_g_5719* were only detected in the treatment lines without NO_2^- accumulation (at < 0.1 % relative abundances). The species identified from the *Nitrosomonadaceae* family had MiDAS placeholder names, and thus none of them were previously characterized bacteria. From *Ellin6067*, the dominant species we detected was *midas_s_379* (with an average relative abundance of 0.45 % in the lines without NO_2^- accumulation). From *Nitrosomonas*, the dominant species was *midas_s_139* (with average relative abundances of 0.33 % and 0.35 % with and without NO_2^- accumulation, respectively), which has been identified as a dominant nitrifier also in Danish WWTPs (Peces et al., 2022). Furthermore, *Nitrosomonas midas_s_139* was reported to have the genomic potential for N_2O production through the AOB denitrification and hydroxylamine pathways (Valk et al., 2022). This species may thus have been involved in the higher N_2O production in the lines where NO_2^- accumulated.

Among known NOB, the dominant genus in this study was *Nitrotoga*,

which had average relative abundances of 0.31 % and 0.0015 % in the lines without and with NO_2^- accumulation, respectively ($p < 0.001$; Fig. 5a). The sequences were annotated as the species *midas_s_181*, which has also been reported among the dominant nitrifiers in Danish WWTPs (Peces et al., 2022). Based on amplicon sequencing, the genus *Nitrospira* was only detected in the lines without NO_2^- accumulation at an average relative abundance of 0.12 % (Fig. 5a), and all the sequences were annotated as *Nitrospira defluvii*. Similarly, based on qPCR, *Nitrospira* was detected at a very low abundance in the lines with NO_2^- accumulation (with average normalized 16S rRNA gene copy numbers being $1.4 \cdot 10^{-4}$ in the lines with NO_2^- and $2.8 \cdot 10^{-3}$ in the lines without NO_2^- , $p < 0.01$; see Fig. 5c). These extremely low abundances of NOB in some of the lines are assumed to have led to the NO_2^- accumulation. Even in the treatment lines without NO_2^- accumulation, *Nitrospira* had a clearly lower abundance in May (on average 0.049 % relative abundance based on amplicon sequencing and $1.5 \cdot 10^{-3}$ normalized gene abundance based on qPCR) than in June (0.18 % and $4.2 \cdot 10^{-3}$). However, the average relative abundance of *Nitrotoga* in these lines was 0.28 % in May, and thus this NOB may have played a role in the more efficient removal of NO_2^- in these lines. As *Nitrotoga* was not included in the qPCR analysis, the abundance pattern of this genus could not be confirmed. However, as the other qPCR results agree with the observations based on sequencing, we can assume that the nitrifier abundances generally followed the pattern seen in the sequencing results. Interestingly, previous studies have observed associations between *Nitrotoga* and high N_2O emissions. Gruber et al. (2021a) reported a transient dominance of *Nitrotoga* (instead of *Nitrospira*) following a period of NO_2^- accumulation and high N_2O emission, and Vieira et al. (2019) reported a positive correlation between *Nitrotoga* abundance and N_2O emissions from a conventional activated sludge WWTP. Even if we detected *Nitrotoga* as the dominant NOB in the lines that did not show high NO_2^- accumulation and N_2O production, this genus may originally have been the dominant NOB also in the lines where NO_2^- accumulated. However, as we had not collected samples before the NO_2^- accumulation and high N_2O production, we do not have data on the microbial community prior to this period.

Our observations on nitrifier abundances mostly agree with recent studies from WWTPs exhibiting seasonal NO_2^- accumulation and/or high N_2O production/emission. Gruber et al. (2021a) and Valk et al. (2022) reported negative correlations between NOB and seasonal N_2O emission or concentration in WWTPs, while both studies detected stable AOB abundances. Interestingly, in our samples, AOB actually had a lower total relative abundance in the lines with NO_2^- accumulation and higher N_2O production. Based on our study and recently published studies, seasonal decreases in NOB abundance (while AOB remain fairly abundant) appear to be an important factor leading to NO_2^- accumulation and inducing higher N_2O production.

3.6. Both ammonia oxidizers and denitrifiers were potential sources of nitrous oxide

Taken together, the characteristics of the microbial communities in the Viikinmäki WWTP suggest that both AOB and denitrifiers may have been potential sources of N_2O during the period of nitrogen conversion process disturbance. Furthermore, neither of these microbial groups could be ruled out as potential initial sources of NO_2^- and N_2O accumulation in light of the previous findings from modeling studies of the Viikinmäki WWTP (Blomberg et al., 2018; Maktabifard et al., 2022b).

AOB are typically considered the main source of N_2O in WWTPs, for example, in oxygen-limiting conditions or during NO_2^- accumulation, which can induce N_2O production through AOB denitrification (Hongbo Chen et al., 2020). NO_2^- has been proposed as the predominant trigger of this pathway even in aerobic conditions (Schneider et al., 2014). Even if oxygen-limiting conditions were not observed in the aerobic compartment during the studied period based on the monitoring data, the bacteria may have been exposed to low DO concentrations inside the flocs

or during transition between anoxic and aerobic conditions. Furthermore, NO_2^- accumulation likely induced the AOB denitrification pathway as a major source of N_2O , even in aerobic conditions (Schneider et al., 2014; Wunderlin et al., 2012). As the abundance of AOB was lower in the lines with NO_2^- accumulation, the lower ratio of AOB abundance to the nitrogen load (assuming that the incoming nitrogen load was divided approximately uniformly between the different treatment lines) may also have increased N_2O production through the hydroxylamine pathway (Aboobakar et al., 2013; Law et al., 2012). Furthermore, the contribution of this pathway to N_2O production from nitrification-anammox biomass has been found to be at least equally important as AOB denitrification under varying concentrations of DO, NO_2^- , and NH_4^+ , apart from conditions with simultaneously low DO and high NO_2^- levels (Ma et al., 2017). However, Ma et al. (2017) reported DO and NH_4^+ as the main parameters controlling the hydroxylamine pathway activity and NO_2^- as an important driver of AOB denitrification. As the NO_2^- concentration, rather than DO or NH_4^+ , differed between the lines with high and low N_2O production in the present study, AOB denitrification is assumed to have been the main AOB-related pathway leading to differences in the N_2O levels.

Additionally, the substantial differences observed in the microbial community compositions included many denitrifying bacteria. Interestingly, in the lines with NO_2^- accumulation that also produced high levels of N_2O , we detected higher relative abundances of many genera that have members with incomplete denitrification capabilities, such as *Acidovorax*, *Comamonas*, *OLB8*, *Ottowia*, and *Pseudoxanthomonas*. This observation suggests that incomplete heterotrophic denitrification may also have contributed to the high N_2O concentration in these lines. However, apart from *OLB8* *midas_s_29* and *Pseudoxanthomonas yeongjuensis*, we were not able to identify specific species affiliated with these genera that would likely be net N_2O producers and would thus have contributed to the high N_2O production in the Viikinmäki WWTP. The total nitrogen removal in the activated sludge process was higher in the lines with NO_2^- accumulation (in agreement with the lower carbon requirements of denitrification compared to denitrification), but this was at least partly attributed to the N_2O stripping. Some of this N_2O may also have originated from incomplete denitrification, possibly triggered by the NO_2^- accumulation. Overall, denitrification functioned as a NO_2^- and N_2O sink even during the studied period, as an increase of the anoxic volume (which was manually limited to 33 % so as not to compromise ammonium removal) helped to decrease the NO_2^- levels and N_2O emission to a certain extent (Kuokkanen et al., 2021). As the NO_2^- accumulation persisted in the process, the anoxic volume seems to have been insufficient for complete removal of NO_2^- by heterotrophic denitrification.

Furthermore, among the species that Valk et al. (2022) reported with correlations to N_2O concentration in a Danish WWTP, a few displayed similar patterns in our samples—having higher relative abundances in the lines with NO_2^- accumulation if a positive correlation had been reported and vice versa for negative correlations. For example, *Terrimonas* *midas_s_743* and *Rhodobacter* *midas_s_24* had higher relative abundances in the lines with NO_2^- accumulation and showed a positive correlation to N_2O in Valk et al. (2022). These species represent particularly interesting candidates that may be associated with variations in N_2O production in WWTPs.

3.7. Engineering value of the study and limitations of the approach

Monitoring and understanding the microbial community dynamics could provide valuable information for troubleshooting and preventing nitrogen removal disturbances in WWTPs. In the Viikinmäki WWTP, process conditions during the studied period (including DO concentration, carbon availability, pH, and alkalinity) were comparable to periods with stable process performance according to the monitoring data. Similarly to our study, Gruber et al. (2021a) found sludge transfer as the only active measure that successfully recovered the process performance

after a period of NO_2^- accumulation and high N_2O emission (although one line recovered by itself after a few months in the Viikinkmäki WWTP). Thus, the activated sludge microbiome appears critical for a well-functioning nitrogen removal process, which would be important to consider in the regular monitoring of wastewater treatment process performance. For this purpose, long-term and frequent monitoring is needed to determine the variations that occur in the microbiome under normal process conditions in a well-functioning process. Furthermore, this long-term monitoring would allow investigating the key taxa that are involved in both the development and maintenance stages of a process disturbance with high N_2O emission and NO_2^- accumulation. Then we could determine the specific operational conditions that could be used to try to control these taxa. With this knowledge, we could aim to restore healthy activated sludge microbiomes already during early indications of a potential nitrogen removal disturbance and thus prevent a prolonged period of process failure and environmental pollution.

It is important to note that this study was conducted in the context of a sudden process disturbance in a full-scale WWTP. Consequently, the sampling campaign, monitoring, and analytical methods were constrained by the resources available at the plant during the disturbance. As a result, this study illustrates the insights that can be gained from the available data, supplemented by the existing literature. However, additional measurements of the concentrations of various nitrogen fractions in each zone of the different treatment lines, as well as targeting functional genes involved in nitrogen conversions (such as *nirK*, *nirS* and *nosZ*), would be beneficial for understanding the roles of nitrifiers and denitrifiers in the accumulation of NO_2^- and N_2O . Furthermore, to identify the dominant N_2O production pathways, techniques like isotope tracing would enhance future microbial analyses (Duan et al., 2017).

4. Conclusions

We measured substantial differences in the microbial community structures of parallel activated sludge lines with and without prolonged NO_2^- accumulation in the Viikinkmäki WWTP. Filamentous bacteria had lower relative abundances in the lines with NO_2^- accumulation, which contributed to impaired floc formation in these lines. Furthermore, many denitrifying genera displayed differing relative abundances in the different lines. Some of the genera with higher relative abundances in the lines with NO_2^- accumulation and high N_2O production include members with a genomic potential for net N_2O production, which are interesting candidate species to investigate further related to high N_2O production in full-scale WWTPs. AOB had slightly lower abundances in the lines with NO_2^- accumulation, with further differences in the dominant genus. Furthermore, NOB abundance decreased dramatically in these lines, causing the NO_2^- accumulation. The high NO_2^- concentration is assumed to have induced N_2O production mainly through the AOB denitrification pathway. Furthermore, the differences observed in the denitrifying communities suggest that denitrifiers may also have played a role in the massive N_2O emission. Taken together, this microbiome study provided a more comprehensive view of the state of the activated sludge process than regular process monitoring alone. Extensive monitoring of the microbiome is recommended for further exploring the connections between the microbiome and N_2O emissions.

CRedit authorship contribution statement

Oona Kinnunen: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonina Kruglova:** Writing – review & editing, Validation, Investigation. **Marlene Mark Jensen:** Writing – review & editing, Methodology, Data curation. **Anna Kuokkanen:** Writing – review & editing, Resources. **Barth F. Smets:** Writing – review & editing, Conceptualization. **Anna Mikola:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envres.2025.121591>.

Data availability

The raw 16S rRNA gene amplicon sequencing data can be found on the NCBI Sequence Read Archive under the repository number PRJNA1120795.

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