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Exploring the microbial influence on seasonal nitrous oxide concentration in a full-scale wastewater treatment plant using metagenome assembled genomes

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ABSTRACT

Nitrous oxide is a highly potent greenhouse gas and one of the main contributors to the greenhouse gas footprint of wastewater treatment plants (WWTP). Although nitrous oxide can be produced by abiotic reactions in these systems, biological N_2O production resulting from the imbalance of nitrous oxide production and reduction by microbial populations is the dominant cause. The microbial populations responsible for the imbalance have not been clearly identified, yet they are likely responsible for strong seasonal nitrous oxide patterns. Here, we examined the seasonal nitrous oxide concentration pattern in Avedøre WWTP alongside abiotic parameters, the microbial community composition based on 16S rRNA gene sequencing and already available metagenome-assembled genomes (MAGs). We found that the WWTP parameters could not explain the observed pattern. While no distinct community changes between periods of high and low dissolved nitrous oxide concentrations were determined, we found 26 and 28 species with positive and negative correlations to the seasonal N_2O concentrations, respectively. MAGs were identified for 124 species (approximately 31% mean relative abundance of the community), and analysis of their genomic nitrogen transformation potential could explain this correlation for four of the negatively correlated species. Other abundant species were also analysed for their nitrogen transformation potential. Interestingly, only one full-denitrifier (*Candidatus* Dechloromonas phosphorivorans) was identified. 59 species had a *nosZ* gene predicted, with the majority identified as a clade II *nosZ* gene, mainly from the phylum Bacteroidota. A correlation of MAG-derived functional guilds with the N_2O concentration pattern showed that there was a small but significant negative correlation with nitrite oxidizing bacteria and species with a *nosZ* gene (N_2O reducers (DEN)). More research is required, specifically long-term activity measurements in relation to the N_2O concentration to increase the resolution of these findings.

1. Introduction

Nitrous oxide (N_2O) is a highly potent greenhouse gas, with a warming potential 300 times higher than CO_2 (Ravishankara et al., 2009; Terada et al., 2017). N_2O is one of the main contributors to the greenhouse gas footprint of wastewater treatment plants (WWTP) (Daelman et al., 2013; Delre et al., 2019). Consequently, mitigating N_2O-emissions would significantly reduce WWTP carbon footprints, but to create mitigation strategies we need to identify and understand the N_2O sources.

Emission of N_2O in WWTPs is due to an imbalance in biotic and abiotic N_2O production and complete consumption (Conthe et al., 2019). N_2O is an intermediate of denitrification, where heterotrophic denitrifying bacteria (DEN) transform nitrate (NO_3^-) to dinitrogen gas (N_2) (Fig. 1) (Zumft, 1997). The accumulation of N_2O can be due to incomplete denitrification by heterotrophic denitrifying bacteria, where the last transformation of N_2O to dinitrogen gas (N_2) via nitrous oxide reductase (NOS, EC 1.7.2.4; Fig. 1) is not completed (Kampschreur et al.,

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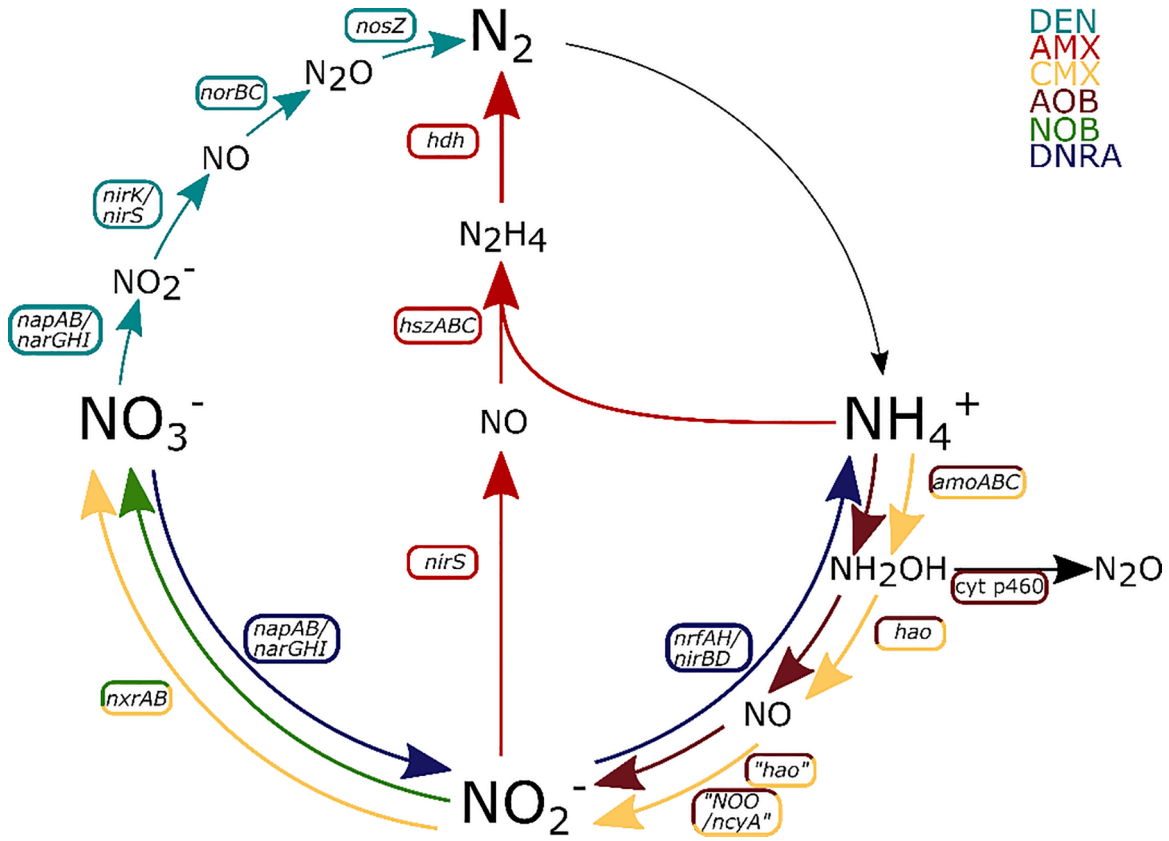

Fig. 1. The nitrogen cycle with the dissimilatory conversions and their associated genes highlighted. The genes with quotation marks have not been included in the KEGG database (release 91, (Kanehisa, 2019)), and have not been included in the annotation. DEN, anoxic heterotrophic denitrifiers; AMX, anammox; CMX, comammox; AOB, ammonium oxidizing bacteria; NOB, nitrite oxidizing bacteria; DNRA, dissimilatory nitrate reduction to ammonium. Nitrifier denitrification was not specifically included for AOB. Adapted from Soler-Jofra et al. (2021).

2009). Additionally, N_2O is a by-product from ammonia oxidizing bacteria (AOB), converting the intermediate hydroxylamine to N_2O using cytochrome P460 (Caranto et al., 2016). Another N_2O-production pathway of AOB is nitrifier denitrification, where the NO_2^- produced by hydroxylamine dehydrogenase (HAO, EC 1.7.2.6) is reduced to N_2O via nitrite reductase (NIR, EC 1.7.2.1) and nitric oxide reductase (NOR, EC 1.7.2.5) (Fig. 1; (Caranto and Lancaster, 2017; Domingo-Félez and Smets, 2019; Wrage-Mönnig et al., 2018). Lastly, N_2O can also be produced via abiotic reactions between intermediates of nitrification and denitrification, or by metal-mediated reactions (Kampschreur et al., 2011; Soler-Jofra et al., 2016).

Although part of the N_2O-emission from WWTPs can originate from truncated-denitrifiers, lacking the nitrous oxide reductase gene (*nosZ*, K00376), there are also many non-denitrifying organisms that have *nosZ* genes predicted in their genome (Conthe et al., 2019; Hallin et al., 2018; Sanford et al., 2012). These organisms have the potential to reduce N_2O, and if their total NOS-activity is higher in the WWTP than the total combined activity of the nitric oxide reductase (NOR) and cytochrome P460, then the WWTP would be a N_2O-sink. The actual NOS-activity within a WWTP will depend on the genetic *nosZ* potential and factors influencing the NOS-activity. Post-translational and transcriptional regulation can cause an imbalance between N_2O-producing (NOR, cytochrome P460) and N_2O-consuming (NOS) enzymes (Liu et al., 2013; Lycus et al., 2017). Other factors that can influence the production-consumption balance are oxygen concentration, NO_2^--accumulation, a low C/N ratio and storage polymer metabolism (Kampschreur et al., 2008; Law et al., 2012; Lu and Chandran, 2010; Otte et al., 1996; Wunderlin et al., 2012).

Short-term influences are not the only factors influencing N_2O-emissions. Seasonal abiotic parameters have been suggested to influence long-term N_2O-emissions, such as temperature (for WWTPs located in temperate climates), accumulation of nitrogen-species, e.g., NO_2^-, NO_3^-, NH_4^+ and influent flow rate (Chen et al., 2019; Daelman et al., 2015; Gruber et al., 2021; Vasilaki et al., 2018). Seasonal community composition variation and dynamics have also been suggested to influence N_2O-emissions in full-scale WWTPs (Daelman et al., 2015; Gruber et al., 2021). However, most of the abundant organisms in WWTPs are undescribed and have unknown functions. A comprehensive database of WWTP-specific high quality metagenome-assembled genomes (HQ MAGs) was made available only recently; the MiDAS genome database (Singleton et al., 2021). This novel database might provide new insights into the (until now unknown) potential N_2O producers and consumers responsible for N_2O emissions.

Here we examine the N_2O production pattern and microbial community data of a full-scale Danish WWTP with nutrient removal spanning multiple years, combined with the MAG-derived nitrogen-transformation potential of the community. We searched for correlations between the relative abundance of specific species and the N_2O-production pattern. Species showing a statistically significant correlation were cross-referenced with the species representatives of the previously constructed MiDAS genome database of HQ MAGs (Singleton et al., 2021), and positive identity-hits were investigated for nitrogen-transformation potential. Community members with a substantial abundance (> 0.1% relative abundance) in the WWTP and microorganisms of special interest were also cross-referenced to the species representatives of the HQ MAGs, to ensure no key N_2O-producer or -consumer were omitted. The genomic N_2O reduction potential was investigated in more detail, based on clade-type and phylogeny. Additionally, the cumulative relative abundance of the species that were categorised into functional guilds, using metagenome-based

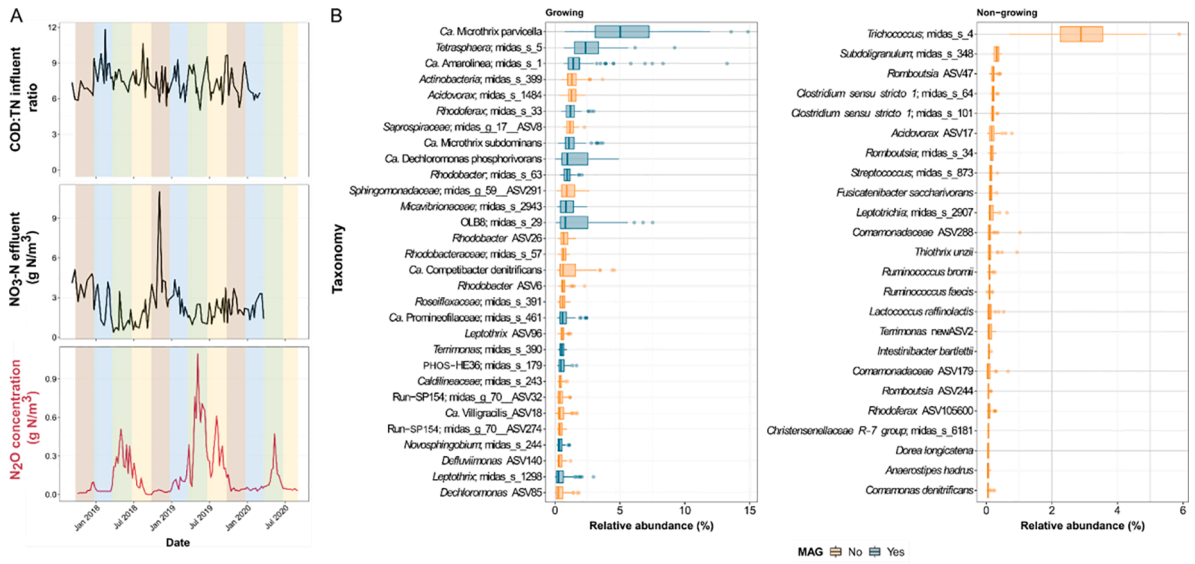

Fig. 2. The chemical and community characteristics of the Avedøre WWTP during the sampling period. A) The COD:N ratio, the concentration of nitrate in the effluent (g N/m^3) and the N_2O concentration (g N/m^3) of the Avedøre WWTP during the measured period. B) The growing population and the non-growing population. Species in blue have an identity-hit with a species in the MiDAS genome database, species in orange have no identity-hit.

classification, was compared to the N_2O concentration pattern over three years. This combined approach provided novel insights into the microbiology of nitrogen transformations in full-scale WWTPs, although a direct correlation to the N_2O-production pattern could not be established.

2. Methods

2.1. Plant process design and data collection

The Avedøre WWTP (Copenhagen, Denmark) has biological nitrogen removal and enhanced biological phosphate removal (EBPR) with a capacity to treat approximately 70,000 m^3 of wastewater daily, under dry weather conditions, serving approximately 350,000 population equivalents (PE). Key parameters measured during the studied period are shown in Fig. 2A and other operational parameters are shown in Supplemental Data S1. Mainstream treatment consists of screening, grit removal, and primary settlement before biological treatment with activated sludge. Four lines, with two carousel reactors per line, work in parallel and have alternating aeration and anoxic periods. Aeration is controlled by STAR control and is based on the ammonia concentration. The average effluent total N was 4.7 ± 2.1 g N/m^3 during the period 2017 - 2020 (Supplemental Data S1). Nitrous oxide was measured in the liquid phase in Carrousel reactor 1 and 3 using two dissolved N_2O sensors (Unisense Environment A/S, Denmark) per line. Only line 3 was considered for this study, as line 1 changed conditions during the period studied.

2.2. 16S rRNA gene amplicon sequencing and bioinformatic analysis

Activated sludge biomass was sampled within the MiDAS project as previously described (McIlroy et al., 2015; Nierychlo et al., 2020). Briefly, the activated sludge was sampled every one-two weeks in the aerobic phase. DNA was extracted, prepared and amplified as previously described (Stokholm-Bjerregaard et al., 2017). The V1-V3 16S rRNA gene region was amplified using the 27F (AGAGTTTGATCCTGGCTCAG) (Lane, 1991) and 534R (ATTACCGCGGCTGCTGG) (Muyzer et al., 1993) primers. Forward reads were processed using usearch v11.0.667 (Edgar, 2010) and raw fastq files were filtered for phiX sequences using usearch -filter_phix, trimmed to 250 bp using usearch -fastx_truncate -trunclen 250, and quality filtered using usearch -fastq_filter with -fastq_-maxee 1.0. Dereplication of the sequences was done using usearch -fastx_uniques with -sizeout. -unoise3 (Edgar, 2017), with standard settings to generate exact amplicon sequence variants (ASVs). The MiDAS 4 reference database (available at https://www.midasfieldguide.org/guide/downloads), combined with the SINTAX classifier with a confidence threshold of 0.8 (Edgar, 2018), was used to assign taxonomy at the species level (Nierychlo et al., 2020). For ASVs without species-level classification, the taxonomy was assigned at the lowest available taxonomic level (e.g. genus), and these ASVs were treated as separate species.

2.3. Classification of growing and non-growing species in activated sludge

The majority of species in activated sludge are found in the influent wastewater (Dottorini et al., 2021). Therefore, by means of mass-balances, it is possible to identify whether these species are expected to grow or die in the activated sludge reactors. In this study, we assigned the species into growing and non-growing groups based on the classification obtained by Dottorini et al., 2021. In Dottorini et al., 2021, the classification list is based on a mass-balance between paired influent and activated sludge samples collected from 11 municipal WWTPs across Denmark. Although Avedøre WWTP was not among the 11 WWTPs used to obtain the classification list, Avedøre WWTP has very similar taxa, process design and geographical location. Consequently, it can be assumed that species will follow the same growth groups as described in Dottorini et al., 2021. Species in WWTPs with different process designs may not follow the same growth groups, and mass-balances should be conducted between paired influent and activated sludge samples. The detailed mass-balance methodology can be found elsewhere (Dottorini et al., 2021).

2.4. MAG identification, annotation, and metabolic reconstruction

A list of the abundant species (relative read abundance of the 16S rRNA gene of >0.1% at least twice in the sampling period) in the Avedøre WWTP was made, resulting in 367 16S rRNA gene defined species. This species list was cross-referenced with the 581 HQ MAGs species representatives previously recovered from Danish WWTPs ((Singleton et al., 2021); Supplemental Data S2). The 581 HQ MAG species representatives are defined as species at the whole genome level (95% ANI), and in cases where multiple genomes were recovered with

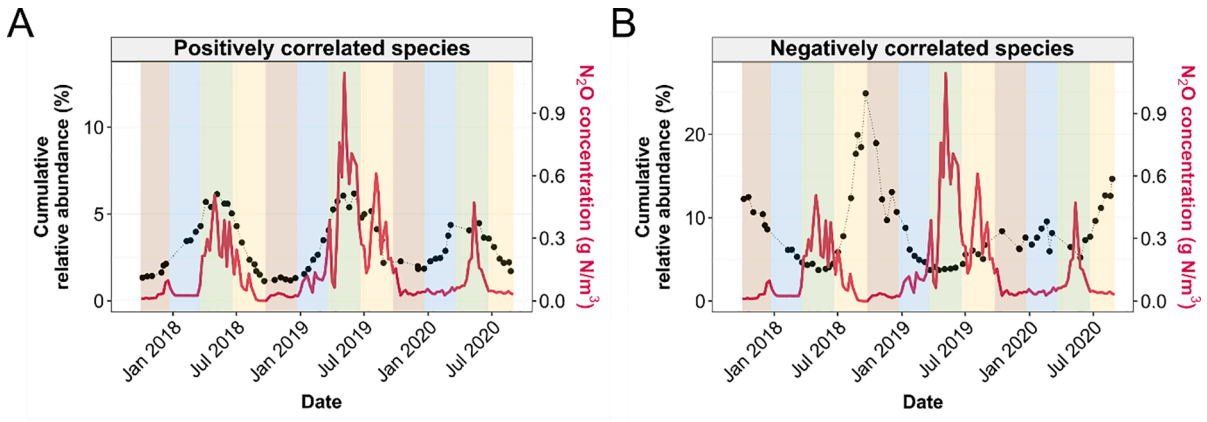

Fig. 3. Correlation of the relative read abundance (%) of species from the Avedøre WWTP with the seasonal N_2O concentration (g N/m^3). The cumulative relative read abundance of A) positively correlated species, B) negatively correlated species.

>95% ANI to each other, the best genome (most complete, and least contaminated) was selected as the representative genome for the species group (Singleton et al., 2021). HQ MAGs that represented an abundant 16S rRNA gene defined species in Avedøre WWTP, with >98.6% average nucleotide identity (Yarza et al., 2014) across the full-length 16S rRNA gene, were annotated using EnrichM v0.5.0 (https://github.com/geronimp/enrichM) annotate with the EnrichM v10 database, incorporating a KO-annotated uniref100 database (Suzek et al., 2015). In some cases, one 16S rRNA gene defined MiDAS species is associated with multiple MAGs, for example, *Tetrasphaera* midas_5 is associated with 6 species representative MAGs. This occurs due to the discrepancy between 16S rRNA gene based and full-genome based species definitions (Singleton et al., 2022). In these instances we examined all the MAGs with a >98.6% identity-hit of their 16S rRNA genes to the 16S rRNA gene defined MiDAS species. R v4.0.5 (R Core Team, 2019, https://www.R-project.org/) and R-studio (Wickham et al., 2019) (RStudio Team, 2015) were used for downstream processing. Tidyverse (Wickham et al., 2019) was used to filter the KO-ids associated with nitrogen transformations and RColorBrewer v1.1–2 (https://cran.r-project.org/web/packages/RColorBrewer/index.html) was used to make Fig. 6. To reduce the probability of false positive annotations (Mise et al., 2021), a gene was assumed present if all KO-ids of the subunits were identified. Re-annotation of certain HQ MAGs (*Rhodanobacteraceae* midas_s_2835) was done with blastp (https://blast.ncbi.nlm.nih.gov/Blast.cgi). All HQ MAGs with predicted genes associated with ammonia oxidizing bacteria were additionally analysed for the presence of cytochrome p460 using HMMER v3.3.2 (http://hmmer.org/) hmmbuild and hmmsearch with the PF16694 (seed sequence) from the Pfam database (El-Gebali et al., 2019). Sequences predicted by HMM with a sequence E-value of at least 1 * 10^{-8} were identified as positive hits. The key gene for nitrite oxidation, *nxrAB*, was predicted in five species (Fig. 4B, Supplemental Data S5). As the KEGG database does not distinguish between *narGH* and *nxrAB* (K00370, K00371), a protein tree was made (Supplemental Figure S3) comparing the putative nitrite oxidoreductase (NXR-A) sequence predicted in the HQ MAGs (Supplemental Data S5) with publicly available NXR-A and NAR-G sequences (Supplemental Data S3).

2.5. Nitrous oxide reductase and nitrite oxidoreductase protein trees

Fxtract v2.3 (https://github.com/ctSkennerton/fxtract) extracted all predicted NOS sequences (K00376) or NXR-A (K00370) from the HQ MAG species representatives. MUSCLE v3.8.31 (Edgar, 2004) was used to make a multisequence alignment as input for IQ-TREE version 2.0.6 (Minh et al., 2020). The best models for the NOS and NXR-A/NAR-G tree, LG + R7 and LG + *F* + R10 respectively, were identified using ModelFinder (Kalyaanamoorthy et al., 2017) and 1000 iterations were made using ultrafast bootstrapping with UFBoot (Hoang et al., 2018). The NOS sequences were classified by clade type using GraftM v0.13.1 graft (Boyd et al., 2018) and the nitrous oxide reductase package (https://github.com/geronimp/graftm_gpkgs). iTol v5 (Letunic and Bork, 2021) was used to visualize and refine the tree. The positive control sequences for NOS were obtained from the uniprot database (www.uniprot.org) Supplemental Table S1; A4IP44_GEOTN, B8FZJ4_DESHD, NOSZ_PARDE, A0A3B7A9B0_9RHOO, Q12M27_SHEDO, I0AHV3_IGNAJ, A0A0C5BY41_METNJ, A0A3G6WI33_RHOSH, NOSZ_PSEST, NOSZ_ACHCY, Q3SJ28_THIDA, NOSZ_BRADU, A0A069Q756_PSEAI, Q5NZ01_AROAE, Q6ZXZ8_WOLSC, C6×281_FLAB3. The positive control sequences for the NXR-A tree were obtained from the GraftM nitrite oxidoreductase and nitrate reductase package (https://github.com/geronimp/graftm_gpkgs) Supplemental Data S3.

2.6. Data processing of 16S rRNA gene amplicon time-series

Downstream processing of 16S rRNA gene time-series was carried out in R v4.0.5 (R Core Team, 2019, https://www.R-project.org/) and R-studio (RStudio Team, 2015) using the following packages: ampvis2 v.2.6.1 (Andersen et al., 2018), tidyverse v.1.3.0 (Wickham et al., 2019) and Hmisc v. 4.5.0 (https://CRAN.R-project.org/package=Hmisc). Prior to data analysis, samples with less than 10,000 reads were discarded and duplicate samples from the same sampling point were combined by averaging the relative abundance of each ASV. The total reads per sample ranged from 32,481 to 124,155. The relative abundance of species was visualised with boxplots using the mean relative abundance for growing and non-growing species. Differences in microbial community beta-diversity were explored by non-metric multidimensional scaling (NMDS), using Bray-Curtis dissimilarity distance in ampvis2. Based on the N_2O concentration profile during the 2017–2020 period, two grouping periods have been described *ad-hoc* using the mean N_2O concentration as a threshold value. The two groups are defined as "high" (N_2O concentration was higher than 0.138 g N/m^3) and "low" (N_2O concentration was lower than 0.138 g N/m^3). Pearson correlations between species time-series and N_2O concentration were assessed with the Hmisc package. To approximate the data to a normal distribution, species time-series were transformed using a robust-centered log-ratio transformation and N_2O concentration was squared-root transformed prior correlation. P-values of the correlations were adjusted using the Benjamini & Hochberg method to control the false discovery rate in multiple comparisons (Benjamini and Hochberg, 1995).

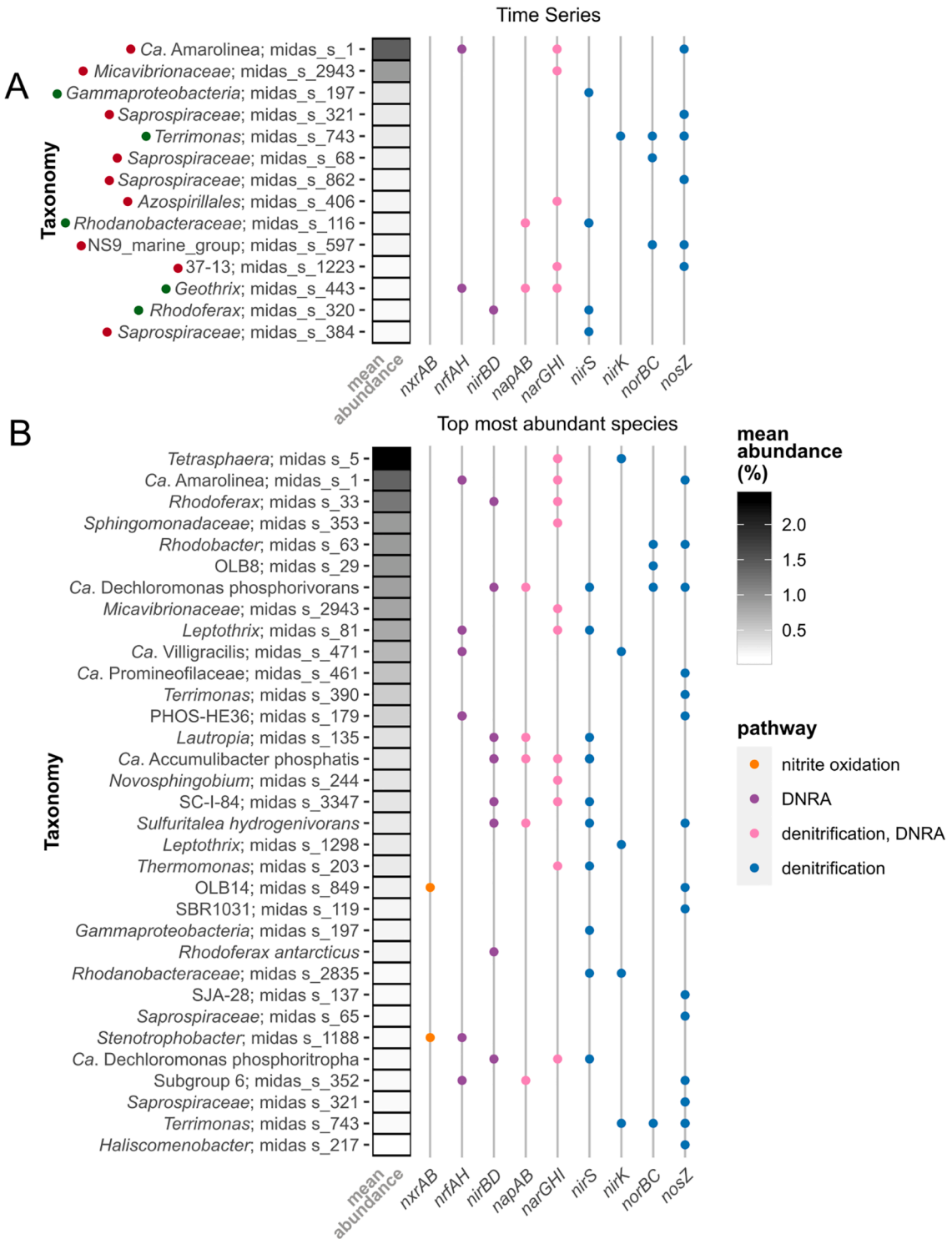

Fig. 4. The mean abundance (%) and genetic nitrogen transformation potential of bacterial species with a corresponding HQ MAG (Singleton et al., 2021) in the Avedøre WWTP. A) the positive (green dot) and negative (red dot) associated species with N_2O-production pattern and B) the top 33 most abundant species with a corresponding HQ MAG. All species had a relative abundance of at least 0.1% twice in the sampling period. Heatmap strips indicate the mean relative abundance (%). Genes were considered present if all the subunits of the gene were predicted. (Supplemental Data S2).

3. Results

3.1. Avedøre WWTP showed a distinct seasonal N_2O concentration pattern

The full-scale Avedøre WWTP with biological nitrogen and phosphorus removal showed a distinct seasonal N_2O-production pattern with a recurring peak in the period between March and June (Fig. 2A). This was not concurrent with other operational parameters, as the COD:N ratio in the influent and nitrate in the effluent (g N/m^3) were relatively constant over time (Fig. 2A). Although there was a nitrate peak in the effluent in the winter of 2018/2019, it did not appear to influence the N_2O concentration during this period. The contrast between the relatively stable abiotic parameters and the strong N_2O-production pattern

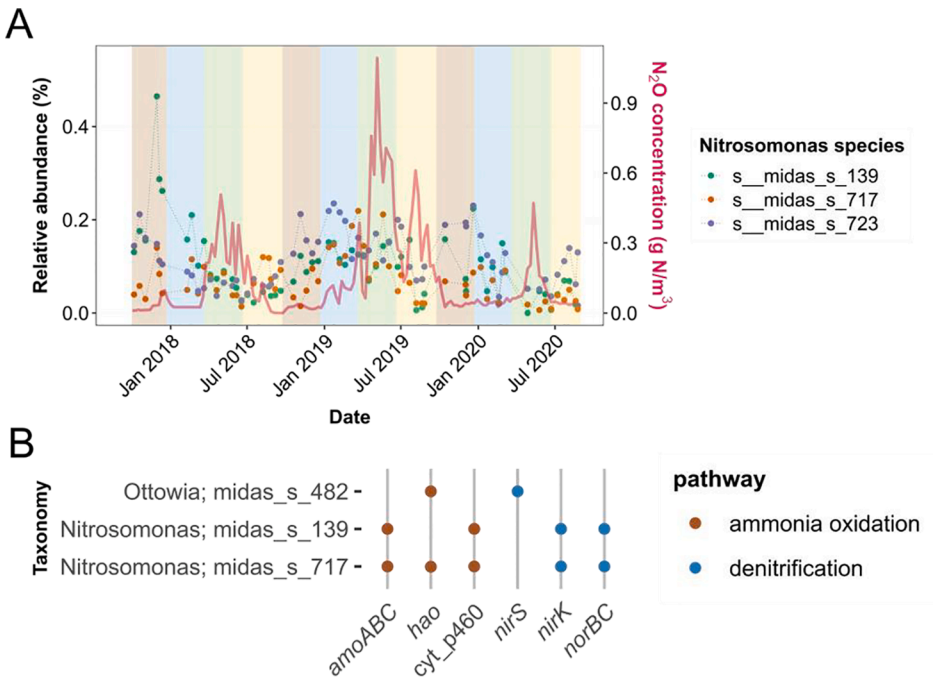

Fig. 5. Relative read abundance and nitrogen transformation potential of the ammonia oxidizing bacteria present in the Avedøre WWTP. A) The relative abundance (%) of all *Nitrosomonas* species over time, with the N_2O-concentration (g N/m^3) depicted in red. B) Predicted nitrogen transformation genes associated with ammonium oxidation and nitrifier denitrification identified in the HQ MAGs of the Avedøre WWTP. No HQ MAG is available for *Nitrosomonas* midas_s_723.

could indicate that the trend was caused by the microbial community present in the WWTP. However, the top 25 growing and non-growing species (Fig. 2B) did not show many known N_2O-consumers or -producers. The growing species mainly consisted of known process-critical species in WWTPs with nutrient removal, including biological P-removal, and many undescribed species (Nierychlo et al., 2020). The non-growing species were only present because of continuous addition via the influent wastewater (Dottorini et al., 2021) and likely not very active in the transformation processes.

3.2. The relative abundance of a subset of species was significantly positively or negatively correlated to the N_2O concentration pattern

We hypothesized that the strong N_2O-production pattern would be reflected by the relative read abundance of microbial community members of the Avedøre WWTP. We identified a total of 54 species (8.7% mean cumulative relative read abundance), 39 growing-species and 15 ambiguous-species, with a significant correlation to the seasonal N_2O concentration pattern (Supplemental data S4). 26 species had a significant positive correlation ($\rho > 0.4$, p-value < 0.01 (Supplemental Data S4, Fig. 3)). The most abundant positively correlated species was SJA-25 midas_s_31 (Bacteroidota), with an average relative abundance between 0.1 and 0.4%, but two peaks around 1.2% relative abundance, (Supplemental Figure S1). The mean relative abundance of all other positively correlated species was below 1%, with most species around 0.2% (Supplemental Figure S1). Five of the 26 species (0.8% mean cumulative relative read abundance) had an identity-hit (>98.6% nucleotide identity) with a species representative in the MiDAS genome database, and were subsequently investigated for their nitrogen transformation potential. While all five species had nitrogen transformation genes predicted (Fig. 4A, green dot), only *Terrimonas* midas_s_743 encoded denitrification genes directly associated with N_2O production and reduction, *norBC* (K04561, K02305) and *nosZ* (K00376), respectively. Interestingly, two other positively correlated species, *Geothrix* midas_s_443 and *Rhodoferax* midas_s_320, also had the predicted genes *nrfAH* and *nirBD*, respectively, which have been suggested to be involved in N_2O production (Heo et al., 2020). 28 species were identified with a significant negative correlation ($\rho < -0.4$, p-value < 0.01) (Supplemental Data S4), with *Ca.* Amarolinea midas_s_1 (Supplemental Figure S2) showing the highest relative read abundance. *Ca.* Amarolinea midas_s_1 had a read abundance of approximately 2.5% throughout the sampling period, except for a peak of 15% around the end of September 2018 and a peak of 5% around February 2020 (Supplemental Figure S2). Of the 23 species, nine (2.9% mean cumulative relative read abundance) had an identity-hit with a species representative of the MiDAS genome database. Analysis of these genomes showed that all species had nitrogen transformation genes predicted (Fig. 4A, red dot). Four species, *Ca.* Amarolinea midas_s_1, *Saprospiraceae* midas_s_321, *Saprospiraceae* midas_s_862 and 37–13 midas_s_1223 (Bacteroidota), had a *nosZ* gene predicted without the *norBC* genes, indicating those species as potential N_2O reducers. Of those four species, only *Ca.* Amarolinea midas_s_1 had denitrification gene predicted (*narGHI*) in combination with the *nosZ*.

3.3. The most abundant microorganisms had a highly diverse nitrogen-transformation potential

The nitrogen transformation potential of all abundant species with an identity hit with a HQ MAG from the MiDAS genome database (Singleton et al., 2021) was additionally investigated. Species considered significantly abundant had a relative read abundance of at least 0.1% twice a year. Of the 367 abundant species (80.3% of the total cumulative relative read abundance), 124 species had an identity-hit with a species representative of the MiDAS genome database. Together the species represented by a HQ MAG had a mean cumulative relative abundance of 31.4%. Of the 156 species representatives, 108 had one or more nitrogen transformation genes (Supplemental Data S5). An overview of the predicted nitrogen-transformation genes of the top 33 most abundant species with an associated HQ MAG, based on the mean relative abundance, showed a varied nitrogen transformation potential (Fig. 4B). Interestingly, only one potential full-denitrifier was predicted in the Avedøre WWTP community, *Ca.* Dechloromonas phosphorivorans (Fig. 4B). Although full denitrification potential was rare, partial-denitrification was not. There were 81 species with at least

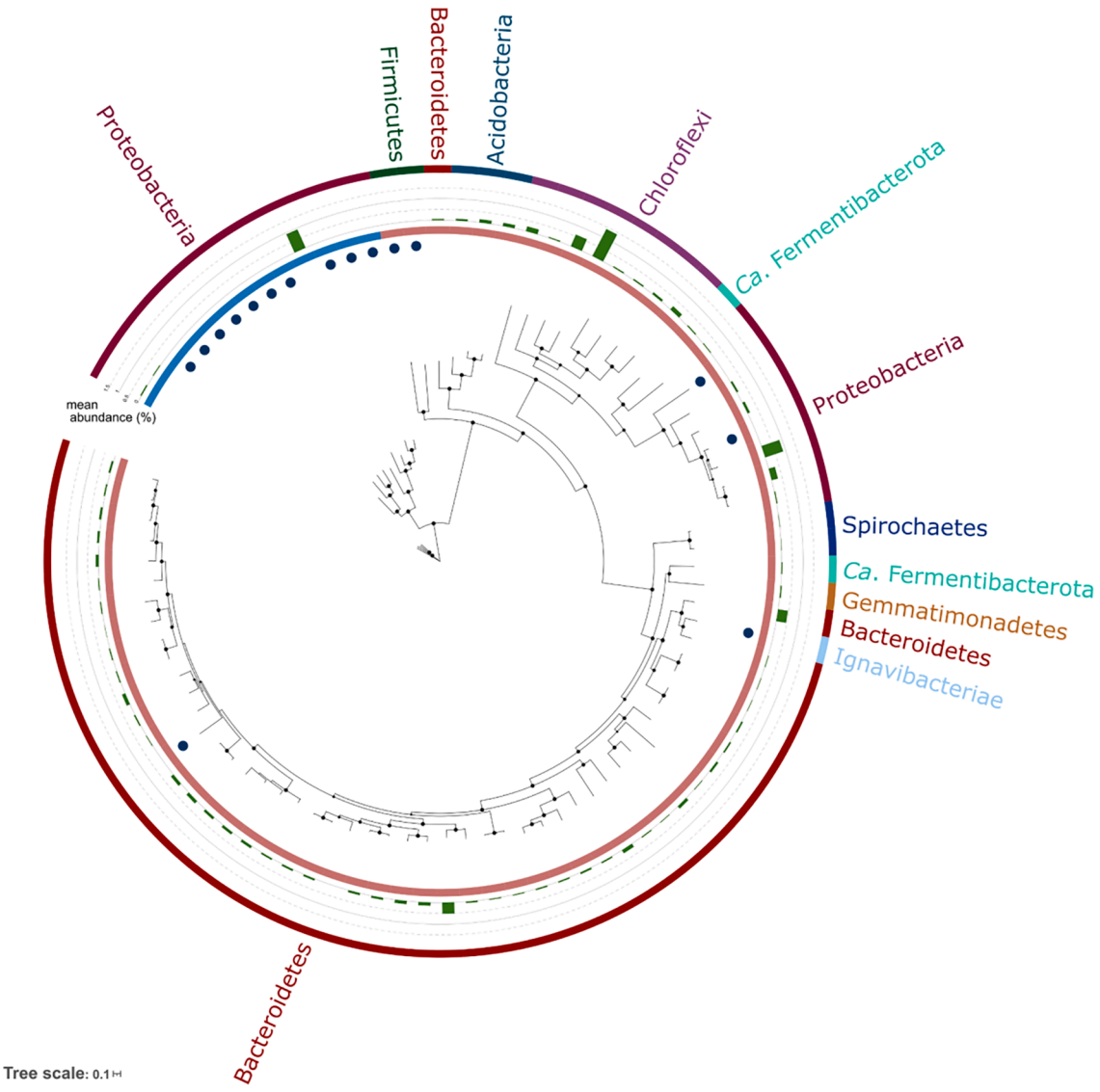

Fig. 6. Bootstrapped phylogenetic nitrous oxide reductase (NOS, EC 1.7.2.4) tree. The tree represents 88 NOS sequences, showing the diversity of the predicted NOS protein sequences in the de-replicated HQ MAGs of the most abundant species in the Avedøre WWTP. Branches with bootstraps >70% are indicated with black circles. Experimentally confirmed and database-derived NOS sequences are indicated by blue dots (Supplemental Table S1). The different NOS clades are depicted with light blue (clade I) or pink (clade II). The mean abundance (%), based on the 16S rRNA gene amplicon sequencing, of the species that had reached a relative abundance of at least 0.1% twice in the sampling period in the Avedøre WWTP, is indicated by the bar chart. The scale bar at the lower left corresponds to the mean number of amino acid substitutions per site.

one of the denitrification genes predicted, including 11 potential N_2O producers (*norBC* predicted, but no *nosZ*) and 59 potential N_2O reducers (*nosZ* predicted, but no *norBC*). An interesting observation was that the HQ MAG of *Rhodanobacteraceae* midas_s_2835 had both a *nirK* and a *nirS* predicted, suggested to be mutually exclusive (Jones et al., 2013). Indeed, re-annotation of the *nirK* and *nirS* genes with BLAST could only replicate this predicted function for *nirK*. The closest hit for *nirS* was a c-type cytochrome (Supplemental Data S6). Further study is required to identify the function of this predicted gene.

To investigate if the potential NOB were correctly predicted, a NXR-A protein tree was made (Supplementary Figure S3). *Nitrotoga* species are well-known NOB (Boddicker and Mosier, 2018), but the HQ-MAG of *Nitrotoga* midas_s_181 had only one of the two subunits predicted, *nxrB* (K00371) (Supplemental Data S5), and was therefore not included in the protein tree. The NXR-A sequences of the two NS9 marine group species clustered with the cytoplasmic NXR-A and NAR-G sequences. OLB14 midas_s_849 clustered most closely to the periplasmic nitrite oxidoreductase sequences, and *Nitrospira defluvii* clustered most closely to NXR-A sequences of other *Nitrospira* species. Lastly, *Stenotrophobacter* midas_s_1188 clustered near branches with a variety of functions. Although most sequences clustered near potential NXR-A proteins, experimental confirmation is required to functionally characterize these predicted proteins and determine if these species are actual NOB.

3.4. Two novel Nitrosomonas species might be important N_2O producers, with multiple N_2O production pathways predicted

Although no AOB were identified within the top 33 most abundant species represented by a HQ MAG (Singleton et al., 2021) (Fig. 4B), they were present in lower abundance. Three *Nitrosomonas* species were identified, *Nitrosomonas* midas_s_139, *Nitrosomonas* midas_s_717 and *Nitrosomonas* midas_s_723, with a maximum relative abundance of 0.45%, 0.21% and 0.23%, respectively (Fig. 5). HQ MAGs are available for *Nitrosomonas* midas_s_139 and *Nitrosomonas* midas_s_717. Both species had the genes predicted of the canonical ammonia oxidation pathway, ammonia monooxygenase (*amoABC*; K10944, K10945,

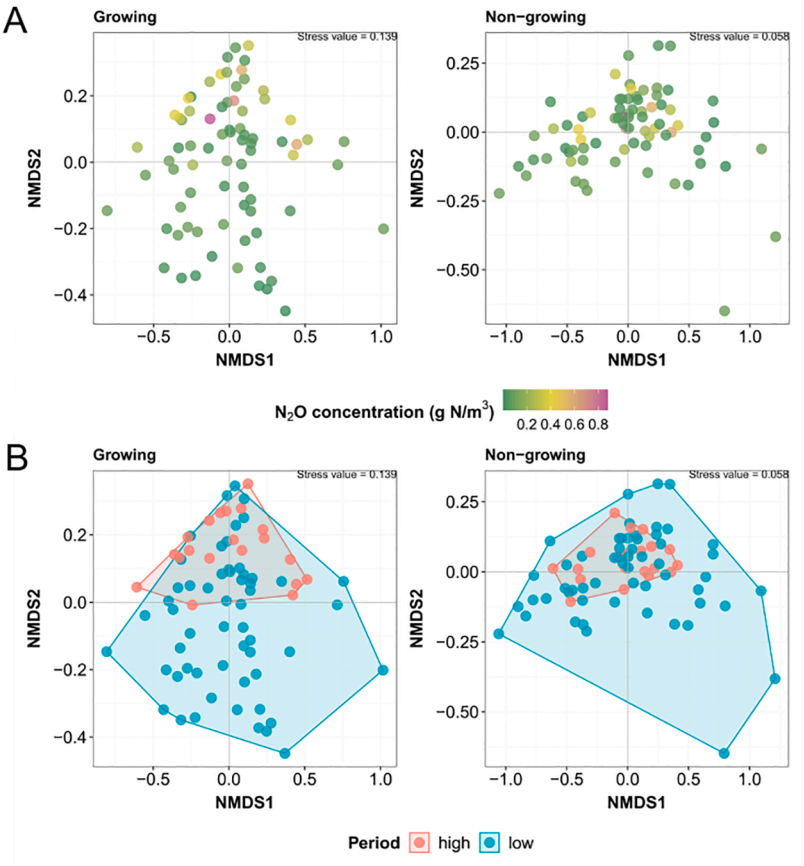

Fig. 7. Non-metric multidimensional scaling analysis (NMDS) between growing and non-growing species, and the N_2O-concentration measured (g N/m^3). The analysis was based on the Bray-Curtis dissimilarity, with A) the individual community members labelled according to their correlation with the N_2O concentration (g N/m^3), and B) grouped according to the period in which they correlate, with red the high N_2O-concentration period and blue the low N_2O-concentration period.

K10946) and hydroxylamine dehydrogenase (*hao*; K10535). Additionally, nitrifier denitrification was predicted in both AOB, with *nirS/K* and *norBC* identified. One other species, *Ottowia* midas_s_482 (family *Comamonadaceae*), had hydroxylamine dehydrogenase, but no ammonia monooxygenase, predicted. All three species were subsequently investigated for the potential to produce N_2O from hydroxylamine with cytochrome p460 (Caranto et al., 2016; Kozlowski et al., 2014). Only the two *Nitrosomonas* species were predicted to encode the cytochrome p460 (Fig. 5). Based on the genomic prediction of *Ottowia* midas_s_482, it appears that although this bacteria might be capable of converting hydroxylamine and/or nitric oxide to nitrite via *hao*, and nitrite to nitric oxide via *nirS*, it does not appear to have the potential to produce N_2O (Fig. 1 and 5B).

3.5. The majority of the nosZ genes were predicted in HQ MAGs from the Bacteroidota phylum

Of the 124 species with an associated HQ MAG, 59 had a *nosZ* gene predicted in their genome (Supplemental Data S5). However, comparing the predicted NOS sequences with NOS sequences from known N_2O reducers (Fig. 6), we found a large evolutionary distance between the NOS sequences of known N_2O reducers (blue dots) and the predicted NOS sequences from the species in the Avedøre WWTP. The known N_2O reducers were predominantly species ($n = 16$) from the Proteobacteria phylum, with 12 out of 16 clustering together and all encoding a clade I NOS. It appeared that the protein tree was dominated by NOS clade II sequences, with the majority of sequences from Bacteroidota species (53 out of the 76). Only one species (*Rhodobacter* midas_s_63) clustered with *nosZ* sequences of known N_2O reducers. To support the annotation of the HQ MAGs, a NOS sequence from *Ignavibacterium album* strain DSM 19864 (Sanford et al., 2012), and from a *Flavobacterium* species (Kim et al., 2020) from the Bacteroidota phylum was included in the tree. These sequences clustered with the other sequences from Bacteroidota, showing that the *nosZ* sequences of the Bacteroidota were closely related to the characterised NOS of *I. album* DSM 19864.

Of the bacteria predicted to have a *nosZ* gene, only five had a mean abundance higher than 0.5% during the period sampled (Fig. 6). The microorganisms with the highest abundance were a *Ca.* Amarolinea midas_s_1 (Chloroflexota), *Rhodobacter* midas_s_63 (Proteobacteria), *Ca.* Dechloromonas phosphorivorans (Proteobacteria), *Ca.* Promineofilaceae midas_s_461 (Chloroflexota) and *Terrimonas* midas_s_390 (Bacteroidota), with 1.4%, 0.9%, 0.9%, 0.6% and 0.5% mean abundance, respectively.

3.6. The N_2O-production pattern could not be explained by the relative abundance of species, individually or grouped into functional guilds

No individual member seemed to be solely responsible for the seasonal N_2O concentration pattern (Fig. 3). Non-growing species are not expected to take an active role in the process. As expected, there was a large overlap of the community between high and low N_2O-production periods (Fig. 7B) and an absence of a correlation between the non-growing microbial community and the N_2O concentration (g N/m^3) (Fig. 7A). Although there was an overlap between high and low N_2O-production periods for the growing communities (Fig. 7B), a slight gradient was observed between the beta-diversity of growing microbial communities and N_2O concentration (Fig. 7A). Additionally, as shown in Section 3.2, a number of individual species were positively correlated with N_2O concentration-level.

However, it was possible that the cumulative relative abundance of a functional guild of microorganisms could show a better correlation to the N_2O-production pattern. The functional guilds were based on the presence of key nitrogen-transforming or N_2O-associated genes predicted in the HQ MAGs. The three AOB and the full denitrifier (*Ca.*

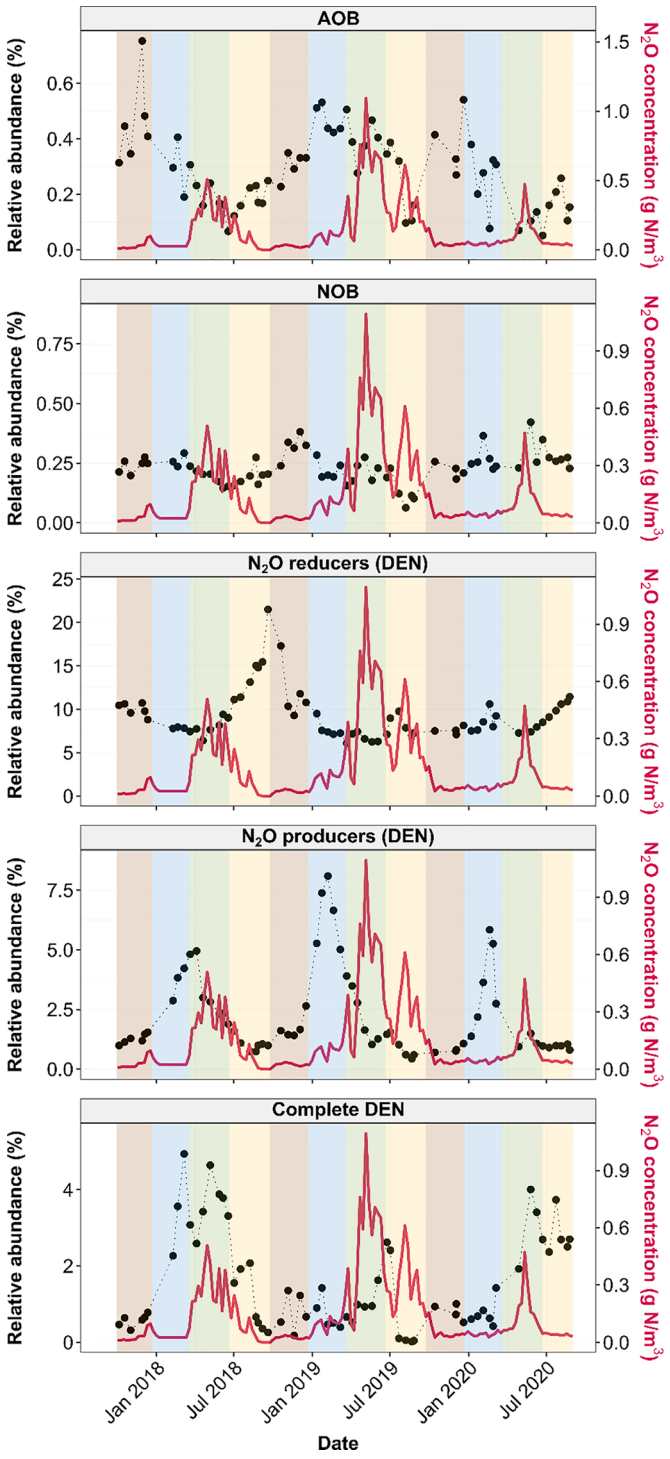

Fig. 8. Time-series of the cumulative relative read abundance (%) of the 16S rRNA gene amplicons of the HQ MAG-determined functional guilds in Avedøre WWTP. The line shows the N_2O-concentration (g N/m^3). AOB (*amoABC* and *hao* predicted), NOB (*nxrAB* predicted), N_2O-producers (DEN) (*norBC* predicted, but no *nosZ*), N_2O-reducers (DEN) (*nosZ,* but no *norBC)* predicted and full-denitrifiers (complete DEN) (*narGHI* or *napAB, nirK* or *nirS, norBC* and *nosZ* predicted).

Dechloromonas phosphorivorans) were grouped according to their functionality, AOB and complete DEN, respectively. The other species were grouped according to the presence of the *nosZ* gene (N_2O-reducers (DEN)) or the presence of the *norBC* gene without a *nosZ* gene (N_2O-producers (DEN)). The NOB without a *nosZ* gene, but with a *nxrAB*, were grouped together as well (NOB). There was a small but significant negative correlation ($\rho = -0.37$, p-value $= 2.3 * 10^{-3}$, Fig. 8, Supplemental Data S7) with species grouped as (potential) NOB ($n = 3$, *nxrAB*, but no *nosZ*, Supplemental Data S7). Additionally, species grouped as DEN—N_2O-reducers ($n = 59$) ($\rho = -0.48$, p-value $= 5.6 * 10^{-5}$, Fig. 8, Supplemental Data S7; encoding *nosZ*) had a significant negative correlation with the N_2O concentration. The relative read abundance of the DEN—N_2O producers (encoding *norBC*, but not *nosZ, n* = 8) was dominated by one species from the *Saprospiraceae* family, OLB8 midas_s_29 (Fig. 8, Supplemental Data S7). While at first glance there was a similar pattern in relative read abundance of this specific group and the seasonal N_2O concentration, the large time difference makes a direct metabolic correlation unlikely. This was also reflected in the statistical analysis as no correlation ($\rho = 3.0 * 10^{-3}$, p-value $= 0.98$, Fig. 8, Supplemental Data S7) in relative read abundance of the N_2O-producers was identified. The full-denitrifiers ($n = 1$), consisting only of the *Ca.* D. phosphorivorans (Fig. 8, Supplemental Data S7), showed an increased relative abundance during high N_2O periods. However, the specific pattern in increase was not reflected in the N_2O concentration pattern as it was not significantly correlated ($\rho = 3.2 * 10^{-3}$, p-value $= 0.98$, Supplemental Data S7). Lastly, the relative read abundance of the other functional group able to produce N_2O, the AOB (*amoABC* and *hao* predicted, $n = 3$), had no significant correlation to the N_2O concentration pattern ($\rho = -9.6 * 10–2$, p-value $= 0.45$, Supplemental Data S7) (Fig. 8).

4. Discussion

4.1. The N_2O concentration pattern appeared to be microbially mediated by several candidate species

The abiotic and biotic influences on the N_2O-emissions in full-scale WWTPs have been studied at multiple locations across Europe (Chen et al., 2019; Daelman et al., 2015; Gruber et al., 2020, 2021). These four studies also report the highest N_2O-emission between March and May and additionally indicated a correlation between N_2O, seasonality and accumulation of nitrite and/or nitrate. These studies also acknowledge the relationship between the accumulation of intermediates and the microbial conversions. Such annual patterns in microbial conversions may also be determined by variations in the abundance of specific bacterial taxa, a change in the activity of certain taxa due to environmental variations, or a combination of the two. We identified 54 species showing a strong recurrent seasonality and a significant correlation to the N_2O concentration, indicating that the abundance of certain taxa could be important. The majority (39) of these taxa also belonged to the growing fraction, which is assumed to be metabolically active and not only present due to continuous transportation by the influent wastewater (Dottorini et al., 2021). Of these growing species, 15 had an identity-hit with a HQ MAG, so it was possible to investigate their genomic potential in greater detail.

Terrimonas midas_s_743 was the only positively correlated species with a HQ MAG that had denitrification genes predicted directly associated with both N_2O production (*norBC*) and reduction (*nosZ*). Although species of this genus have been identified in (partial) denitrification communities, it has not been directly associated with N_2O emissions (Zhang et al., 2021). Four of the negatively associated species with a HQ MAG had the potential to reduce N_2O. The filamentous bacteria *Ca.* Amarolinea midas_s_1, the most abundant of the species, has previously been suggested to have the potential for N_2O reduction (Andersen et al., 2019). Additionally, other species from the *Ca.* Amarolinea genus have been reported to actively transcribe *nosZ* in an anammox bioreactor community (Suenaga et al., 2021). The other three species were Bacteroidota (*Saprospiraceae* midas_s_321, *Saprospiraceae* midas_s_862 and 37–13 midas_s_1223). While *Saprospiraceae* has been associated with N_2O emissions in soils and anammox bioreactors (Jones et al., 2013, 2014; Speth et al., 2016), this is the first instance that

members of the *Saprospiraceae* family have been predicted as N_2O-reducers in a full-scale WWTP community. Another closely related genus within the Bacteroidota, *Flavobacterium*, has also been associated with N_2O production in WWTP, suggesting that Bacteroidota species might have a more important role than previously thought (Kim et al., 2020). All four species are interesting candidates to investigate in more detail for their N_2O-reduction potential.

The majority (39 of the 54) of the significantly correlated species did not have an identity-hit with a HQ MAG in the MiDAS genome database (Singleton et al., 2021). It is difficult to investigate the possible genetic traits these species had to explain a significant correlation and possible effect on the seasonal N_2O concentration. It also shows that many of the species in the WWTP remain functionally elusive and that we need to increase the number of HQ MAGs to improve the resolution and increase our insight into species functionality.

However, some species correlating with the seasonal N_2O concentration, but without a HQ MAG available, had a genus-level association with known (partial) denitrifiers. Although functional traits are often species-specific (Petriglieri et al., 2021), these species could be of special interest to study in more detail. Among the species with a significant correlation to the seasonal N_2O concentration was a *Rhodobacter*, a genus harbouring known denitrifiers and with species previously correlated with N_2O emissions in WWTPs (Vieira et al., 2019). Other microbial lineages that significantly correlated with N_2O emission (*Ca.* Competibacter, *Ca.* Accumulibacter and *Dechloromonas* species) have been shown to produce and reduce N_2O in lab-scale enrichment experiments (Roy et al., 2021; Vieira et al., 2018; Yoon et al., 2016). While multiple *Ca.* Accumulibacter and *Dechloromonas* species had a HQ MAG available, only *Ca.* D. phosphorivorans had genes predicted directly associated with N_2O production and reduction. *Dechloromonas* species have not been attributed with high N_2O emissions, under optimal growth conditions, due to their high affinity to N_2O (Suenaga et al., 2019; Yoon et al., 2016). However, non-ideal conditions, e.g. salt or alkaline stress, may stimulate N_2O-production by this species (Han et al., 2019).

The species grouped as NOB consisting of *Nitrospira defluvii*¸ *Nitrotoga* midas_s_181 and two predicted species with at least one copy of the *nxrAB* gene, had a statistically significant negative correlation with the seasonal N_2O concentration. This correlation was also observed by Gruber et al. (2021). Although NOB have no direct genes able to produce or reduce N_2O, they can be involved in complex symbiosis with AOB or heterotrophic bacteria (Daims et al., 2016) potentially affecting seasonal N_2O concentration. More research is required to elucidate the relationship between abundance of NOB and the seasonal N_2O concentration.

AOB have been suggested as mainly responsible for N_2O production in WWTPs in several studies (Dias et al., 2022; Law et al., 2012; Wunderlin et al., 2012). Although the AOB in the Avedøre WWTP had the potential to produce N_2O via both the hydroxylamine pathway and nitrifier denitrification (Caranto et al., 2016; Wrage-Mönnig et al., 2018), no significant correlation was identified between their abundance and the N_2O concentration peak. Such stable abundance, in contrast to strongly changing seasonal N_2O concentration, was also observed by Gruber et al. (2021). More detailed research is required to determine if this discrepancy demonstrates that AOB do not significantly contribute to the seasonal N_2O-production peak.

4.2. Denitrification seemed dominated by partial denitrifiers

Full-denitrifiers are often assumed to dominate the denitrification process in WWTP (Nielsen and McMahon, 2014). However, in our study of Avedøre WWTP we only identified one full-denitrifier (NO_3^- to N_2, (Lu et al., 2014; Zumft, 1997)), *Ca.* D. phosphorivorans. This is also reflected in the MiDAS genome database of species from WWTPs all over Denmark. While Singleton et al. (2021) identified 21 full-denitrifiers, they identified manifold more HQ MAGs with at least one gene of the pathway predicted. The variety in denitrification gene combinations is well established and seen in many natural and engineered environments (Baker et al., 2015; Graf et al., 2014; Lu et al., 2014). Division of labor between different species has additionally been shown in denitrifying enrichment cultures (Vieira et al., 2018; Wang et al., 2021), suggesting that the denitrifying potential, and as such the N_2O production or reduction potential, is likely impacted by the community structure.

4.3. The high N_2O reduction potential, mainly identified in Bacteroidota species, was dominated by clade II nosZ

The majority of the *nosZ* genes were predicted in members of the Bacteroidota. The importance of this phylum in N_2O reduction has been observed in soils (Jones et al., 2014)). While functionally verified N_2O-reducers are predominantly Proteobacteria (Conthe et al., 2018; Hallin et al., 2018; Suenaga et al., 2018), the many undiscovered N_2O-reducers from other phyla might play an important role in natural and engineered environments (Jones et al., 2013; Sanford et al., 2012; Singleton et al., 2021).

The majority of clade I *nosZ* gene sequences have been identified in Proteobacteria, and are normally associated with denitrifying organisms (Hallin et al., 2018; Sanford et al., 2012; Yoon et al., 2016). Interestingly, this was not observed in our analysis. Although there were three HQ MAGs with a denitrifier clade I *nosZ* gene predicted, none of these HQ MAGs had the full denitrification pathway predicted. Additionally, none of these species had a significant correlation with the seasonal N_2O concentration. The majority (69 of the 72) of the *nosZ* sequences predicted within the Avedøre WWTP community were clade II. Although this has been observed in other environments (Jones et al., 2014; Kim et al., 2020; Orellana et al., 2014; Suenaga et al., 2021), this is the first study showing this overwhelming dominance of clade II over clade I (ratio = 25.7). It could be that the proposed higher oxygen tolerance and affinity for N_2O of the clade II NOS was selective for this community (Yoon et al., 2016).

4.4. Other factors than the genomic N_2O production or reduction potential predict the N_2O concentration in the Avedøre WWTP

The community in Avedøre WWTP had a large genetic potential to reduce N_2O during the period studied. Other studies have shown that genomic N_2O reduction potential and N_2O emission level are correlated (Jones et al., 2014; Vieira et al., 2018), with high *nosZ* values relating to low N_2O emissions. In our dataset there were a high number of HQ MAGs with a *nosZ* predicted (approximately 45% of the HQ MAGs available), suggesting a high genomic potential to reduce N_2O. This would imply that other factors than the genetic potential determine the N_2O concentration in the WWTP. However, as we do not cover the entire community with the MiDAS genome database, long term monitoring of the *nosZ* gene in the WWTP will be required to examine the *nosZ* potential of the community without a HQ MAG.

Many factors can influence the actual N_2O reduction activity in full-scale WWTPs, with dissolved oxygen concentrations often suggested as one of the key-inhibitors for N_2O reduction (Law et al., 2012; Pan et al., 2013; Suenaga et al., 2018). The seasonal change in temperature can affect the dissolved oxygen concentration in the WWTP, as well as the microbial conversion rates, but its effect should be expected to be observed in both spring and fall. Additionally, previous studies have identified only weak to no correlation between temperature as a parameter and N_2O emissions (Ahn et al., 2010; Chen et al., 2019), suggesting that temperature was not the dominant influencing factor. Another key-parameter in N_2O reduction is the electron availability, as all enzymatic conversions in the denitrification pathway require electrons, electron competition within the pathway can decrease in N_2O reduction activity (Conthe et al., 2018; Felgate et al., 2012). However, the COD:N ratio in the influent and the COD quality in the effluent remained stable throughout the studied period (Supplementary Data S1). This does not exclude the possibility that the type of electron donor

Table 1
KEGG orthology identifier (KO-id) used in this study. Functional guilds, the three-letter abbreviation of their associated genes, the KEGG orthology identifier (KO-id), and the nitrogen-transformation performed by the protein encoded by the gene. DEN; denitrifier, AOB; ammonia oxidizing bacteria, NOB; nitrite oxidizing bacteria, CMX; comammox, DNRA; dissimilatory nitrite reducing bacteria, AMX; anammox bacteria.

Functional guild	Gene	KO-id	Transformation
DEN	*narGHI*[a]	K00370, K00371, K00374	$NO_3^- > NO_2^-$
	napAB	K02567, K02568	$NO_3^- > NO_2^-$
	nirK	K00368	$NO_2^- > NO$
	nirS	K15864	$NO_2^- > NO$
	norBC	K04561, K02305	$NO > N_2O$
	nosZ	K00376	$N_2O > N_2$
AOB	*amoABC*	K10944, K10945, K10946	$NH_3^+ > NH_2OH$
	hao	K10535	$NH_2OH > NO$
	*		$NO > NO_2^-$
	Cyt-p460		$NH_2OH > N_2O$
NOB	*nrxAB*[a]	K00370, K00371	$NO_2^- > NO_3^-$
CMX	*amoABC*	K10944, K10945, K10946	$NH_3^+ > NH_2OH$
	hao	K10535	$NH_2OH > NO$
	*		$NO > NO_2^-$
	nxrAB[a]	K00370, K00371	$NO_2^- > NO_3^-$
DNRA	*narGHI*[a]	K00370, K00371, K00374	$NO_3^- > NO_2^-$
	napAB	K02567, K02568	$NO_3^- > NO_2^-$
	nrfAH	K03385, K15876	$NO_2^- > NH_4^+$
	nirBD	K00362, K00363	$NO_2^- > NH_4^+$
AMX	*nirS*	K15864	$NO_2^- > NO$
	hszABC	K20932, K20933, K20934	$NO + NH_4^+ > N_2H_4$
	hdh	K20935	$N_2H_4 > N_2$

*No gene has been conclusively identified for this conversion (Kuypers et al., 2018).
[a] The NXR and NAR are homologs and cannot be distinguished by KEGG IDs. A protein tree is used to resolve potential function as NXR or NAR.

or carbon source, shown to severely impact the N_2O reductase activity (Itokawa et al., 2001; Pan et al., 2013; Qi et al., 2022; Ribera-Guardia et al., 2014, 2016), changed over the year. Table 1

N_2O production can occur in both the aerobic and in the anoxic phase, by AOB and (truncated) denitrifiers (Song et al., 2020). As N_2O reduction only occurs during the anoxic phase (Kampschreur et al., 2009; Suenaga et al., 2018), part of the produced N_2O will directly be sparged out of the liquid during the aerobic phase. Although aerobic denitrification may occur (Robertson et al., 1989, 1995) and produce N_2O, the majority of the aerobically produced N_2O is produced by AOB (Tallec et al., 2008). Low dissolved oxygen concentrations and high nitrite concentrations have been shown to stimulate N_2O production by AOB (Kampschreur et al., 2009; Kozlowski et al., 2016). As stated above, if the seasonal temperature effect on dissolved oxygen affected N_2O production by AOB, this would be expected to occur in both spring and fall. While no nitrite concentrations were measured in the period studied, it might be an interesting additional parameter to measure in future studies.

Future work is required to elucidate the factors influencing seasonal (under-)utilization of the high genetic N_2O reduction potential of the community. Although this work provides a list of interesting candidates that could act as a N_2O sink within Avedøre WWTP, many species are novel with little to no knowledge of their (potential) function. Metaomics data, combined with N_2O reductase activity assays measured with activated sludge throughout the year(s), could provide more insight into the physiology of N_2O sink species. This could result in mitigation strategies controlling species composition and N_2O production and reduction activity.

5. Conclusions

- We found a recurrent strong seasonal N_2O concentration pattern, with spring peaks and autumn lows, from Avedøre WWTP over 3 years.
- The relative read abundance of 54 species was significantly correlated to the N_2O concentrations, either positively or negatively.
- Partial denitrifiers, instead of complete denitrifiers, appeared to dominate the genetic denitrification potential of the community.
- The cumulative abundances of N_2O reducers and NOB had a significant negative correlation to N_2O concentrations.
- Clade II *nosZ* genes, primarily found in Bacteroidota, were more common than clade I *nosZ* genes in the HQ MAGs.
- Multiple novel potential N_2O sink species have been identified for further study.
- Overall, there was some correlation between community structure and N_2O concentration, but activity measurements of specific bacteria are needed to understand the correlation in greater detail.

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.2022.118563.

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