Intermittent Perturbation with High-Frequency Aerobic-Anoxic Cycling Enhances N_2O Reductase Activity during Wastewater Treatment

*Xueyang Zhou[1,2], Bharat Manna[1,2], Boyu Lyu[1,2], and Naresh Singhal[1,2] **

[1]Department of Civil and Environmental Engineering, University of Auckland, Auckland 1010, New Zealand

[2]Water Research Centre, University of Auckland, Auckland 1010, New Zealand

KEYWORDS: Greenhouse gas; oxygen modulation; intermittent aeration; metabolic shift; electron availability

ABSTRACT

Nitrous oxide (N_2O) is a potent greenhouse gas, with a global warming potential nearly 300 times that of carbon dioxide, and its emission from wastewater treatment plants is a growing environmental concern. In this study, we investigated the effects of intermittent perturbation with high-frequency aerobic-anoxic cycling on N_2O reduction in activated sludge systems. The study aimed to enhance N_2O reductase (N_2OR) activity and N_2O reduction potential by optimizing redox state cycling. Six bioreactors were operated under different aeration patterns—constant aerobic (CA), continuous perturbation (CP), and intermittent perturbation (IP)—at two dissolved oxygen levels (2 mg/L and 8 mg/L). Results indicated that IP conditions significantly increased N_2OR activity, resulting in higher N_2O consumption rates compared to the other aeration patterns. Metagenomic and metaproteomic analyses revealed *Hyphomicrobium* as a key microorganism contributing to N_2O reduction, with increased enzyme abundance and activity under IP conditions. The frequent aerobic-anoxic transitions were found to enhance electron availability for N_2OR, facilitating more efficient N_2O reduction. These findings suggest that intermittent aeration, particularly with high-frequency cycling, can be an effective strategy for mitigating N_2O emissions in wastewater treatment systems. This approach has the potential to improve the environmental sustainability of wastewater treatment by leveraging microbial dynamics and enzyme activity to reduce greenhouse gas emissions.

1. INTRODUCTION

Nitrous oxide (N_2O) is a potent greenhouse gas, with a global warming potential nearly 300 times that of carbon dioxide, making its reduction in wastewater treatment systems an urgent environmental priority[1,2]. N_2O emissions from wastewater treatment plants (WWTPs) primarily occur through biological nitrogen removal processes, particularly during nitrification and denitrification[3,4]. During nitrification, ammonia-oxidizing bacteria (AOB) can produce N_2O as a byproduct through incomplete oxidation of hydroxylamine (NH□OH) or through nitrifier denitrification. Similarly, during denitrification, N_2O can accumulate as an intermediate when nitrate or nitrite is not fully reduced to nitrogen gas, especially under conditions of limited electron donors. The emission of N_2O not only contributes significantly to global warming but also represents a loss of nitrogen from the wastewater treatment process, potentially affecting treatment efficiency.

Numerous factors influence N_2O emissions in WWTPs, including dissolved oxygen concentration (DO), nitrite accumulation, rapidly changing process conditions, substrate composition, COD/N ratios, pH levels, and temperature[5–7]. Each of these factors can affect the microbial community structure and activity, thereby influencing N_2O production and reduction. For instance, low DO levels can lead to increased N_2O emissions due to incomplete nitrification and denitrification processes, while high nitrite concentrations can inhibit the reduction of N_2O to nitrogen gas. Rapid changes in process conditions, such as fluctuations in ammonia concentration or oxygen availability, can also change microbial metabolism and destabilize microbial communities, leading to increased N_2O production. Furthermore, the composition of substrates and the COD/N ratio influence the availability of electron donors necessary for the complete reduction of N_2O. pH and temperature also play critical roles in modulating enzyme activities involved in nitrogen transformations, further affecting N_2O emissions.

Current strategies to mitigate N_2O emissions during nitrogen removal in WWTPs include optimizing operational parameters (such as DO and carbon supply), adjusting microbial community structures (such as increasing the presence of N_2O-reducing bacteria), and managing dissolved N_2O[8–11]. Achieving the right balance in DO levels is challenging[12,13]—too low, and it increases N_2O emissions during nitrification[14,15]; too high, and it inhibits N_2O reduction during denitrification[16,17]. Moreover, while carbon management is critical, it demands precise control to prevent issues like excessive nitrite accumulation. In the meantime, N_2O-reducing bacteria often struggle to perform optimally due to environmental conditions that are not ideal for their activity[18,19]. However, the biological mechanisms of N_2O production and consumption are not fully understood, particularly at the molecular level[20]. Some studies have examined N_2O metabolism pathways using techniques such as isotope labeling and gene abundance analysis, and these approaches help reveal the actual processes driving N_2O production and consumption. However, incorporating enzyme expression abundance and even actual catalytic activity would be more convincing. This is because N_2O reductase (N_2OR) is an enzyme with relatively weak electron competition and is easily inhibited by environmental factors such as oxygen[21–24].

Despite the challenges of controlling N_2O production in activated sludge systems, studies have shown that the maximum capacity for N_2O reduction far exceeds its production potential[25,26]. Many N_2OR-containing heterotrophic microbes can rapidly switch between aerobic and anaerobic metabolic pathways, allowing them to efficiently utilize the electrons generated under aerobic conditions for nitrate respiration and N_2O reduction under anoxic conditions. Furthermore, this cycling can continuously stimulate the FNR regulon system of N_2O-reducing bacteria under sufficient carbon supply. We hypothesize that a system of rapid

oxic-anoxic switching in a single reactor can leverage the metabolic versatility of N_2O-reducing bacteria to minimize N_2O formation and enhance its reduction. This approach is based on the rapid formation of a large electron pool through oxidation of reduced compounds under aerobic conditions, followed by efficient electron transfer to N_2OR under subsequent anoxic conditions.

To test this hypothesis, we designed an experiment using high-frequency aerobic-anoxic cycling in activated sludge systems. Our study aims to elucidate the underlying mechanisms of N_2O dynamics under these rapidly alternating redox conditions. Through a combination of metagenomics and metaproteomics analyses, we characterize changes in microbial community composition and gene expression patterns in response to high-frequency aerobic-anoxic cycling, focusing on organisms capable of nitrogen transformation. Additionally, we quantify the temporal dynamics of N_2OR enzyme abundance and activity under these conditions. By integrating these multi-omics approaches with process-level N_2O measurements, we aim to provide a comprehensive understanding of the microbial and biochemical factors influencing N_2O formation and reduction under dynamic redox conditions. This research seeks to contribute fundamental insights into the ecological and physiological adaptations of N_2O-reducing microorganisms, potentially informing future strategies for mitigating N_2O emissions in wastewater treatment systems.

2. MATERIALS AND METHODS

2.1 Bioreactor Operation

Activated sludge used in this study was sourced from the Māngere Wastewater Treatment Plant in Auckland, New Zealand. Three identical cylindrical acrylic bioreactors, each with a 1 L working volume, were operated simultaneously under varying aeration conditions at 20□. The same sludge sample was used in triplicate to ensure biological consistency. Each 1 L reactor contained 2.75 g/L of mixed liquor-suspended solids (MLSS) and was supplemented with 3.84 g/L of NaHCO□ as an inorganic carbon source, along with 1 mL of a trace element solution (Table S1). Over a 48-hour period, 50 mL of concentrated artificial wastewater (Table S2) was continuously fed into each reactor at an equal rate using a syringe pump, while magnetic stirring ensured uniform mixing.

This study's reactor operation exposed activated sludge to various aeration patterns for 48 hours. Six conditions were examined, comprising three aeration modes at two oxygen levels each. The first mode, Intermittent Perturbation with High-Frequency Aerobic-Anoxic Cycling (IP), involved high-frequency alternation between aerobic and anoxic phases, where dissolved oxygen was dynamically regulated to create frequent transitions between oxygen-rich and oxygen-limited conditions. This mode was tested at both 2 mg/L and 8 mg/L DO levels.

The second mode, Continuous Perturbation with High-Frequency Aerobic Cycling (CP), implemented high-frequency aerobic cycling without the introduction of anoxic phases. It was characterized by rapid fluctuations in oxygen supply but without complete depletion of dissolved oxygen. As with the IP mode, CP was also tested at DO levels of 2 mg/L and 8 mg/L.

The third mode, Constant Aerobic Conditions with Stable DO (CA), maintained constant aerobic conditions with stable and continuous dissolved oxygen levels throughout the

treatment process, avoiding significant oxygen fluctuations. This mode was similarly tested at both 2 mg/L and 8 mg/L DO levels.

2.2 N_2O Emission Measurement

In this study, the measurement of gaseous N_2O emissions involved collecting gases released during the reactor operation using multiple gas sampling bags with a maximum volume of 25 L. After collection, the gas samples were analyzed for N_2O concentrations using a Shimadzu GC-BID/FID 2010 plus gas chromatography. The chromatographic column employed was an HP-Plot/molecular sieve (29.8 m × 0.53 mm inner diameter × 40 μm film), and the detection method included an injection port temperature of 150□, oven temperature of 160□, and BID detector temperature of 180□. The N_2O-N content (mg) released into the air from the reactor was calculated based on the N_2O concentration in the gas sample and the volume of gas exiting the reactor.

For the measurement of dissolved N_2O concentrations in the reactor system, we utilized N_2O online sensors (Unisense, Denmark), following the manufacturer's guidelines. This allowed us to determine the N_2O concentrations (mg/L) released into the liquid under different aeration conditions. To ensure accurate and reliable results, the sensors were calibrated and maintained according to the manufacturer's recommendations. Regular checks were performed during the experiments to validate the measurements and ensure proper functioning of the sensors. Moreover, the data collected from the sensors were analyzed and processed using accompanying software, providing valuable insights into the N_2O emissions and their correlation with different aeration strategies.

2.3 In-situ N_2OR Activity Measurement

Following the operation under each aeration condition, 5 mL of a saturated N_2O solution was introduced into the bioreactor to assess the in-situ N_2OR activity. The addition of the saturated N_2O solution aimed to create a high initial concentration of dissolved N_2O in the reaction system, which allowed for the evaluation of the N_2O consumption rate by the microbes present in the activated sludge. To measure the dissolved N_2O concentrations, a Unisense N_2O online sensor was employed. The N_2O concentrations were monitored continuously over time. By analyzing the decrease in dissolved N_2O concentrations as a function of time, the rate of N_2O consumption by in-situ N_2OR activity under the given reaction conditions could be determined. The method provided a reliable and accurate assessment of the N_2O consumption rate in real-time, enabling a better understanding of the effects of various aeration conditions on the in-situ N_2OR activity.

2.4 Metagenomics

For DNA extraction, six biological replicates of seed sludge samples were collected, with 10 mL of sludge used for each replicate. The extraction process was executed according to the manufacturer's protocol of the DNeasy PowerSoil Kit (Qiagen, Germany). The extracted DNA was quantified using a NanoDrop spectrophotometer (if applicable) and stored at -20°C for subsequent analysis.

The extracted DNA samples were then dispatched to the Auckland Genomics Centre for metagenomic analyses. The laboratory procedures included concentrating the DNA samples, performing a quality control (QC) check, and preparing the DNA for Illumina sequencing,

which included polymerase chain reaction (PCR) and indexing. Following the library QC, normalization was performed, and a final pool check was conducted using a bioanalyzer. Finally, sequencing was performed on a HiSeq platform, generating approximately 400 million 2x150bp paired-end reads. The data returned from the sequencing process were analyzed using SqueezeMeta.[27]

2.5 Metaproteomics

Five biological replicates were obtained from the seed sludge samples before the experiment. After 48 hours of treatment, three biological replicates were collected from each of the normal oxygen level conditions (CA2, CP2, and IP2), while two biological replicates were collected from each of the high oxygen level conditions (CA8, CP8, and IP8), resulting in a total of 17 samples. Each replicate sample consisted of 5 ml of activated sludge, which was pelleted, washed, and centrifuged at 6,113 × g for 5 minutes at 4°C. The pellets were rapidly frozen in liquid nitrogen and preserved at -80°C until further processing. For protein extraction, each pellet was resuspended in lysis buffer and sonicated for 20 cycles on ice. The lysate was then collected by centrifugation at 17,467 × g for 30 minutes at 4°C.

Subsequently, the lysate was mixed with 20% trichloroacetic acid in acetone, vortexed, and chilled on ice. Post-centrifugation, the supernatant was removed, and the pellet was rinsed with acetone, centrifuged, and washed once more with 80% acetone in water. The protein pellet was air-dried and dissolved in 50 mM Tris buffer. For protein purification, SpeedBead Carboxylate-Modified E3 and E7 (Sera-Mag, USA) were used, and the EZQ protein assay kit (Invitrogen, USA) was employed for protein concentration quantification. The sample then underwent reduction, alkylation, and trypsin digestion.

After the digestion process, the sample was diluted, passed through a molecular sieve, and exposed to solid-phase extraction. Proteins were eluted and concentrated using a speed vacuum, and a 10 µL aliquot from each sample was analyzed via nano LC-MS/MS. A NanoLC 400 UPLC system (Eksigent, USA) was utilized for sample desalting and separation, while a TripleTOF 6600 Quadrupole-Time-of-Flight mass spectrometer (Sciex, USA) performed mass spectrometry analysis. The data obtained were compared against the Uniref Bacteria database using Protein Pilot version 5.0.

3. RESULTS

3.1 The Effects of Aeration Conditions on N_2O Emissions

We conducted experiments with three different aeration patterns to determine N_2O emissions from activated sludge systems under different aeration conditions and measured gaseous N_2O emissions and dissolved N_2O concentrations. The results (Figure 1) confirmed that N_2O release varied under different oxygen perturbations. CP conditions caused more N_2O emissions than CA, while IP conditions did not increase N_2O emissions.

Under stable oxygen conditions (Figure 1a, 1b), dissolved N_2O concentrations remained relatively constant. At 2 mg/L DO, N_2O-N concentrations were below 0.5 mg/L, while at 8 mg/L DO, they were even lower, below 0.2 mg/L. Gaseous N_2O emissions in the CA2 system increased during the first 8 hours of operation and stabilized afterwards. In contrast, CA8 experienced a decline in N_2O emissions, indicating that stable oxygen conditions provided

sufficient electron acceptors for nitrification and higher oxygen concentrations suppressed denitrification.

Different oxygen perturbation patterns exhibited distinct N_2O release profiles (Figure 1c, 1d, 1e, 1f). Dissolved N_2O concentrations fluctuated with DO levels, increasing during DO declines and escaping the system as gaseous N_2O during aeration. CP conditions resulted in higher dissolved N_2O concentrations and more gaseous N_2O emissions in the first 8 hours compared to IP conditions, with maximum emission rates (mg N_2O-N/hour) of 1.83 and 2.68 for CP2 and CP8, and 1.17 and 1.09 for IP2 and IP8, respectively.

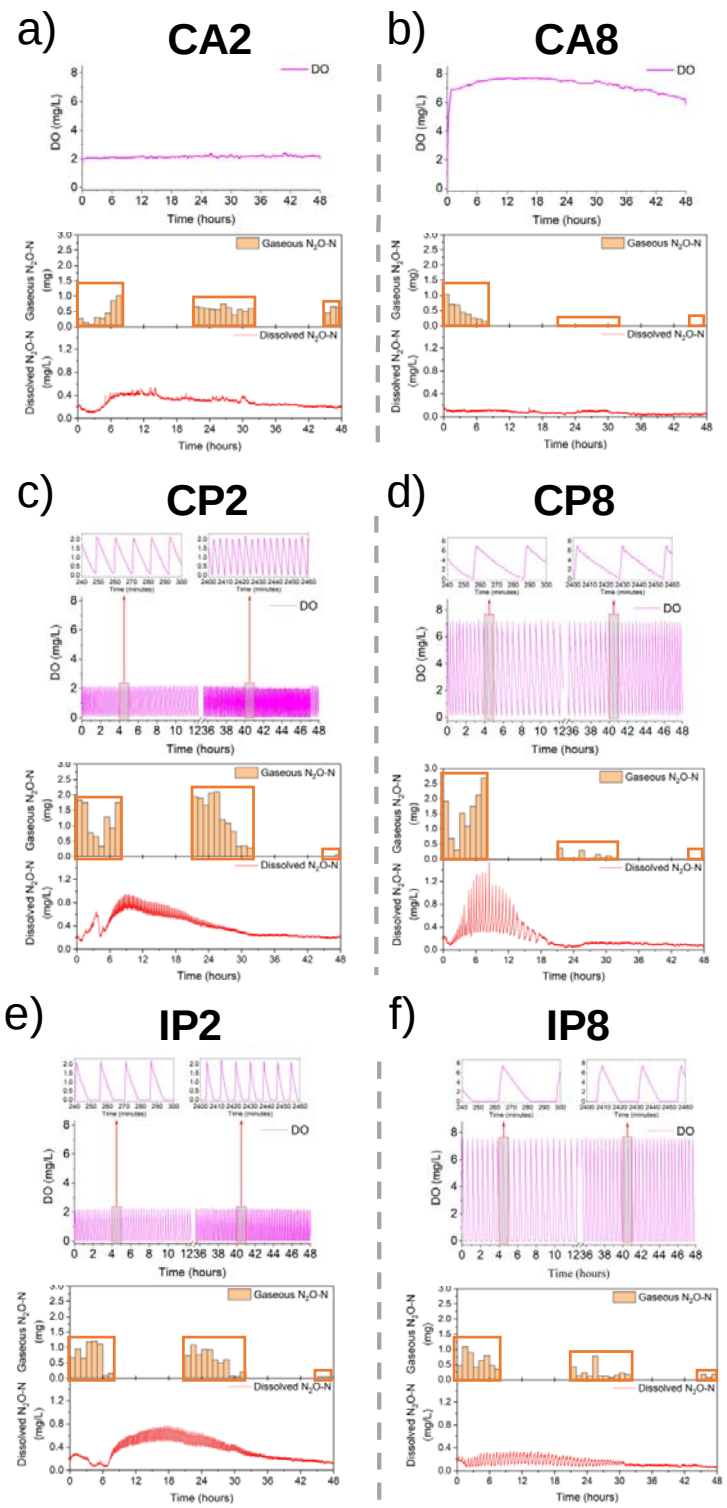

Figure 1. Variations in DO concentrations, gaseous N_2O emissions, and dissolved N_2O concentrations within activated sludge systems under eight aeration conditions: (a-f) Separate plots for the aeration conditions CA2, CA8, CP2, CP8, IP2, and IP8. Owing to measurement constraints, gaseous N_2O was assessed only during the timeframes of 0-8 hours, 22-32 hours, and 46-48 hours.

The capacity of activated sludge systems to consume N_2O depends on factors such as N_2OR abundance, oxygen concentration, and electron availability. To evaluate in-situ N_2OR activity, we maintained the operational state of the system and measured the reduction rate of externally added N_2O concentration. After 48 hours under CA, CP, and IP conditions, IP conditions induced higher N_2OR activity, with IP2 reaching 1.23 mg N_2O-N/L/min and IP8 achieving 4.19 mg N_2O-N/L/min (Figure 2).

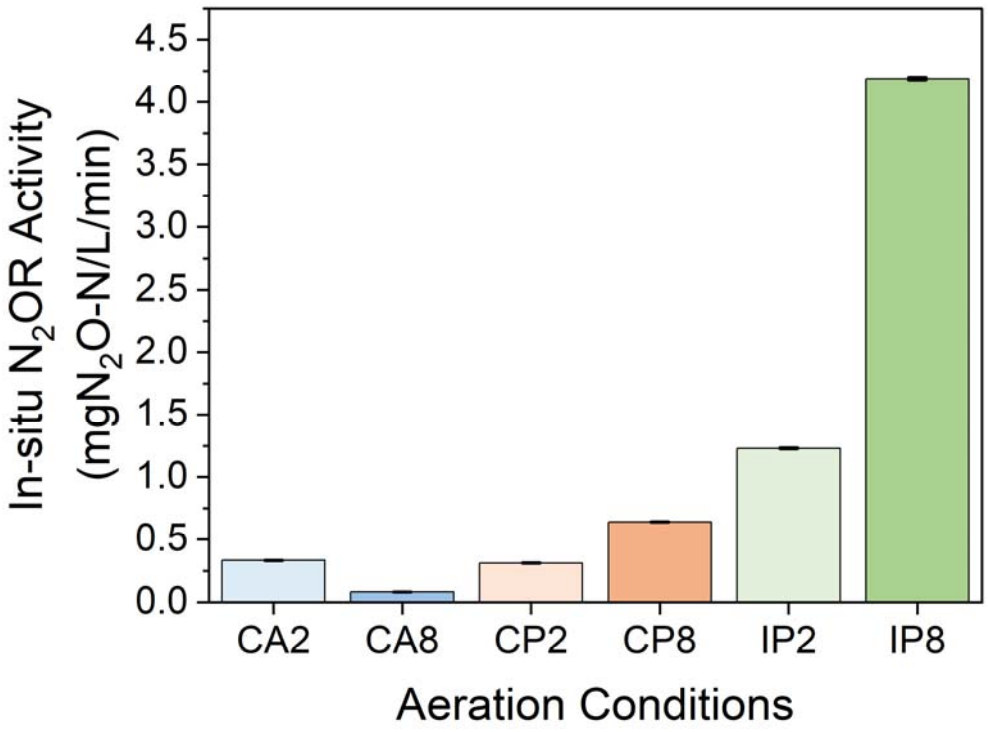

Figure 2. Potential of IP conditions for N_2O consumption optimization based on in-situ N_2OR activity

3.2 Nitrogen transformation mechanism

Metagenomic analysis of the initial samples from the reactors identified key functional genera involved in nitrogen transformation (Figure 3). *Nitrosomonas* was confirmed as the dominant ammonia-oxidizing genus, responsible for the oxidation of ammonia (NH_3) to NH_2OH and subsequently to nitrite (NO_2^-). This process is fundamental in nitrification, setting the stage for subsequent nitrogen removal steps. *Nitrospira* was identified as the primary nitrite-oxidizing genus, facilitating the oxidation of nitrite to nitrate (NO_3^-).

However, further analysis revealed that *Nitrospira* harbors a set of genes related to denitrification, though not sufficient for complete denitrification to nitrogen gas (N_2). Similarly, *Nitrosomonas* was found to possess genes associated with nitrifier denitrification, a process wherein ammonia-oxidizing bacteria produce N_2O as a byproduct. These genes include those involved in the reduction of nitrite to nitric oxide and further to nitrous oxide. The presence of these nitrifier denitrification pathways in

both *Nitrosomonas* and *Nitrospira* indicates that they are potential sources of N_2O during the nitrification process.

Additionally, the system was found to harbor a diverse array of denitrifying genera, which are essential for the reduction of nitrate to dinitrogen gas (N_2), thus completing the nitrogen removal cycle. Because these denitrifiers basically contain genes for producing NO, they can all be regarded as potential sources of N_2O. In the entire nitrogen cycle system, *Hyphomicrobium* emerged as a major contributor to N_2O reduction. This genus is known for its ability to perform complete denitrification, including the reduction of N_2O to N_2 via the enzyme N_2OR. The identification of *Hyphomicrobium* as a key player suggests its potential role in mitigating N_2O emissions in the system.

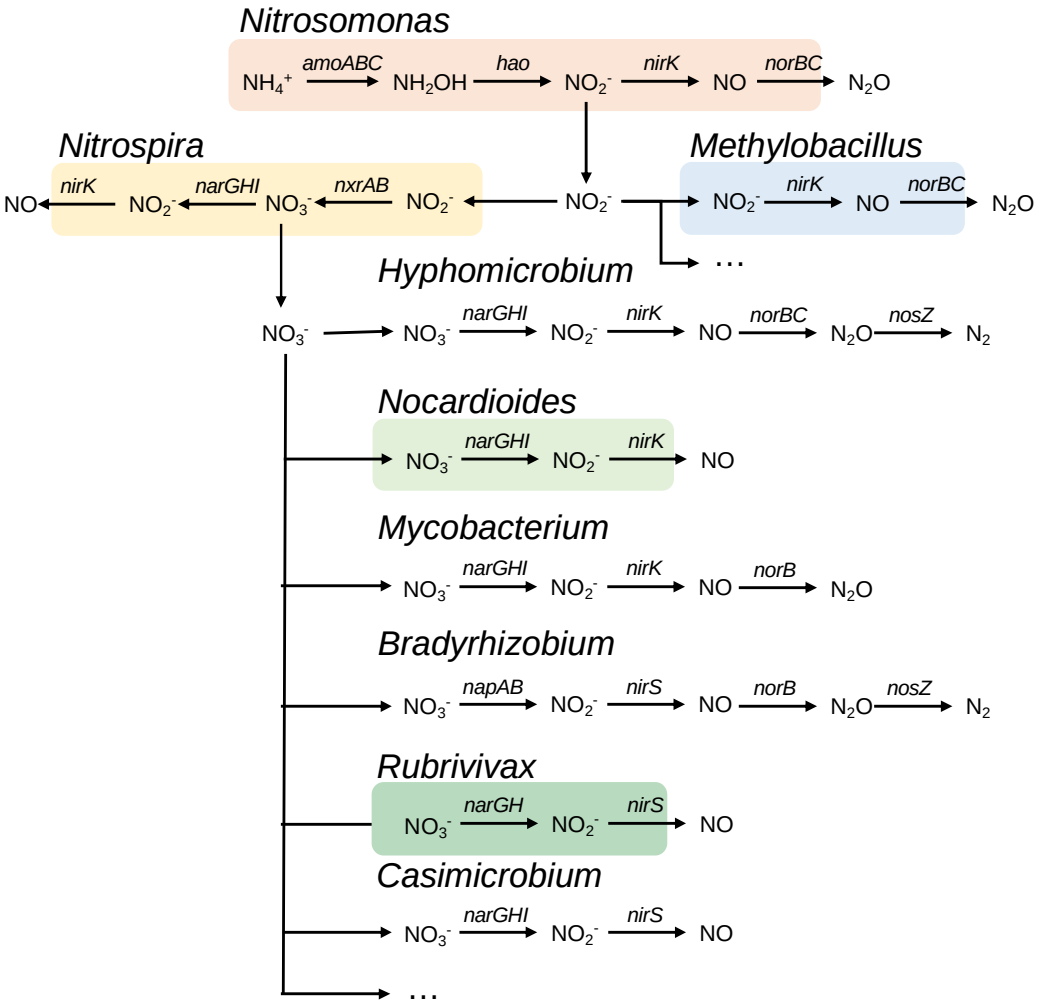

Figure 3. Identification of genera with nitrogen transformation potential in activated sludge system based on metagenomics analysis

Metaproteomic analysis was employed to examine the abundance of enzymes involved in nitrogen transformation under various aeration conditions. The results indicated that the expression of enzymes associated with denitrification was more significantly affected by oxygen modulation than those involved in nitrification. Specifically, the conversion of

NO□□ to NO and the reduction of N_2O to N□ were the processes most influenced by oxygen perturbations(Figure 4).

Under IP conditions, there was a marked increase in the abundance of N_2OR, particularly within *Hyphomicrobium* (Figure S4). This suggests that the IP conditions create an environment conducive to the expression and activity of N_2OR, thereby enhancing the system's capacity to reduce N_2O. The observed increase in N_2OR expression under IP conditions could be attributed to the frequent transitions between aerobic and anoxic phases, which may stimulate the metabolic pathways responsible for N_2O reduction.

The fluctuating oxygen levels in IP conditions likely provide optimal conditions for N_2OR activity by preventing the accumulation of intermediates such as NO and maintaining a balance between the supply of electron donors and acceptors. This dynamic environment may promote the rapid consumption of N_2O, thereby reducing its emission into the atmosphere.

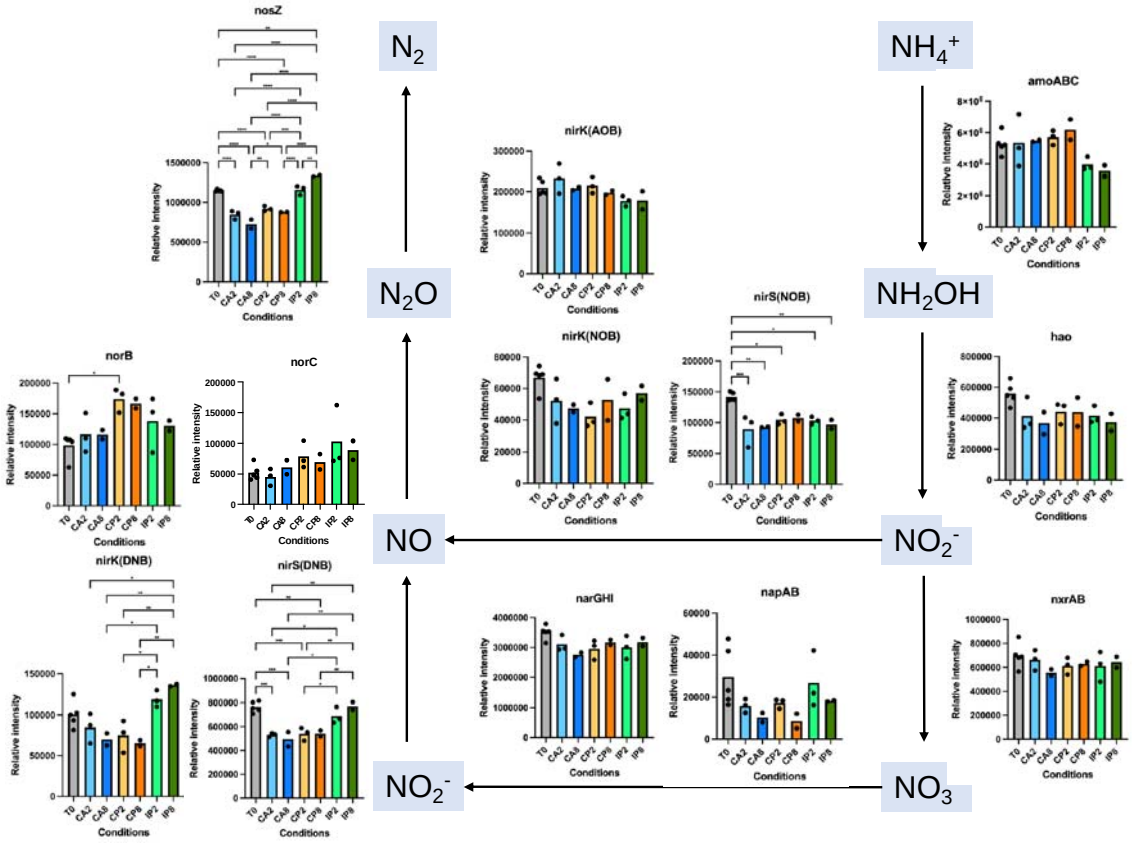

Figure 4. The enzyme abundance related to nitrogen conversion, including ammonia monooxygenase (amoABC), hydroxylamine oxidoreductase (hao), nitrite oxidoreductase (nxrAB), nitrate reductase (narGHI), periplasmic nitrate reductase (napAB), copper-containing nitrite reductase (nirK), cytochrome cd1-containing nitrite reductase (NirS), nitric-oxide reductase (norBC), and nitrous-oxide reductase (nosZ). The bar charts show the average value of the same condition (CA2, CP2, and IP2 have biological triplicates; n=3. CA8, CP8, and IP8 have biological duplicates; n=2)

3.3 Metabolic regulation of *Hyphomicrobium*

Metaproteomic analysis indicated that the genus *Hyphomicrobium* exhibited a distinct response to IP conditions when compared to other genera within the activated sludge system. Interaction effect analysis based on the two-way ANOVA showed no significant interaction effect between oxygen level (normal vs. high) and aeration pattern on enzyme expression. Additionally, oxygen level alone did not have a significant impact on enzyme expression. However, the aeration pattern (CA, CP, IP) had a significant effect, with *Hyphomicrobium* displaying a marked increase in overall enzyme expression under IP conditions (Figure S17). This increase was particularly evident in enzymes associated with N_2O reduction (Figure 5). The enhanced enzymatic activity under IP conditions suggests that *Hyphomicrobium* plays a crucial role in mitigating N_2O emissions under fluctuating oxygen conditions.

The results showed that the IP conditions induced a metabolic state in *Hyphomicrobium* that favored the activation of the CRP/FNR (cyclic AMP receptor protein/fumarate and nitrate reduction regulator) family transcriptional regulators. These regulators are known to be involved in the bacterial response to changes in oxygen levels and redox states. Specifically, they can activate genes associated with anaerobic metabolism and nitrate respiration, which are crucial for N_2O reduction.

The CRP/FNR family regulators are widely present in bacteria and are involved in sensing and responding to environmental cues such as oxygen availability and carbon source presence. In *Hyphomicrobium*, the upregulation of CRP/FNR under IP conditions appears to enhance the metabolic pathways responsible for methanol oxidation, NADH dehydrogenation, and various reductive processes. This metabolic shift ensures a continuous supply of reducing equivalents, such as NADH, which are essential for N_2OR activity.

Figure 5 shows the distinct enzyme expression profiles in *Hyphomicrobium* under IP conditions. It indicated an upregulation of key enzymes associated with carbon metabolism and anaerobic respiration pathways. Specifically, there was a notable increase in the expression of methanol dehydrogenase (MDH), an enzyme central to the methanol oxidation pathway. This pathway plays a critical role in generating reducing equivalents, which are essential for the subsequent steps in the denitrification process, including the reduction of N_2O.

In addition to methanol oxidation, enzymes involved in redox balance and energy metabolism, such as NADH dehydrogenase, were also upregulated. The increased expression of these enzymes suggests that IP conditions may enhance the efficiency of the electron transport chain in *Hyphomicrobium*, thereby facilitating a more robust supply of reducing power needed for N_2O reduction.

Under low-oxygen or anoxic conditions induced by IP, FNR activates genes involved in anaerobic respiration, including those for nitrate reductase (narGHJI), nitrite reductase (nir), and N_2OR (nos). The increase in N_2OR expression under IP conditions facilitates the rapid reduction of N_2O, effectively mitigating its emission.

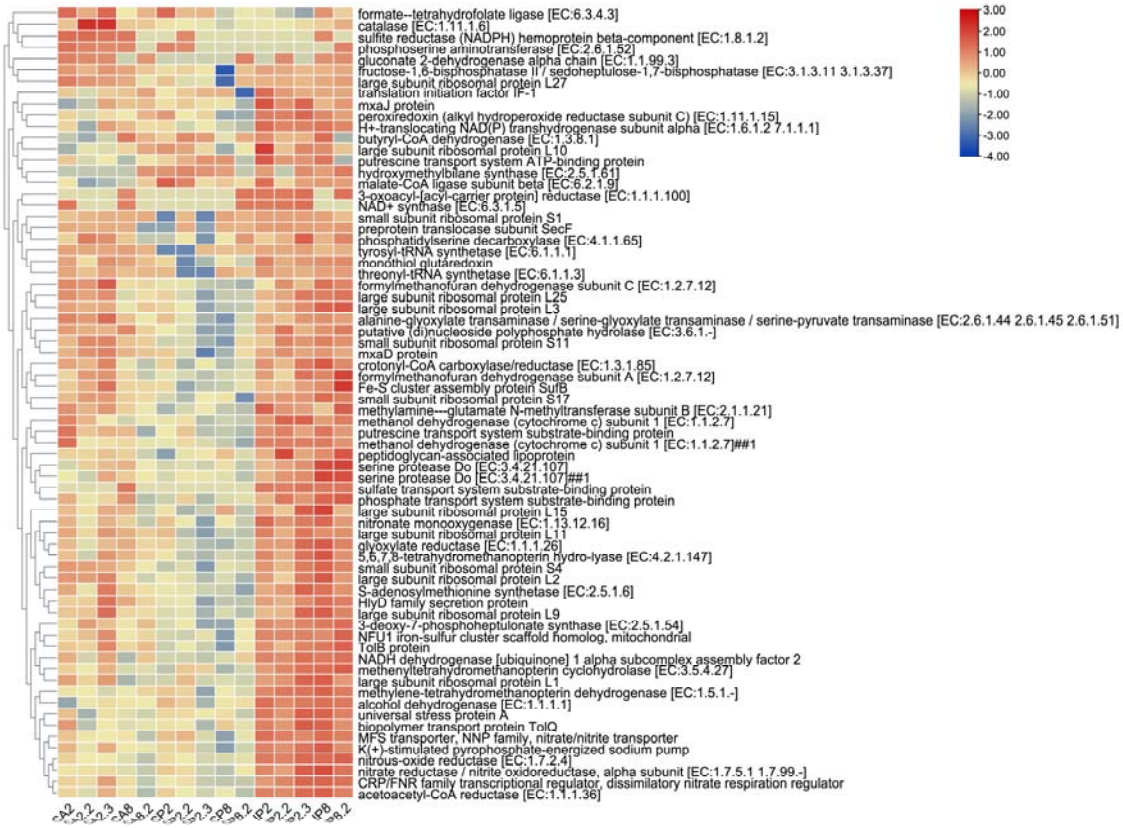

Figure 5. Enzymes from *Hyphomicrobium* with significantly different abundances under different aeration conditions (The numbers in the sample names in the heat map represent the number of biological replicates: CA2, CP2, and IP2 have biological triplicates; n=3. CA8, CP8, and IP8 have biological duplicates; n=2)

4. DISCUSSION

This study demonstrates that intermittent perturbation with high-frequency aerobic-anoxic cycling (IP conditions) significantly enhances the N_2O removal capacity of activated sludge systems. The results indicate that IP conditions promote an optimal environment for key nitrogen-transforming functional microorganisms, particularly *Hyphomicrobium*, to thrive and efficiently reduce N_2O emissions. The enhanced activity of *Hyphomicrobium* under IP conditions, as revealed by metaproteomic analysis, highlights the potential of intermittent aeration as a strategic approach for mitigating N_2O emissions in wastewater treatment processes. The lack of a significant interaction effect between oxygen level and aeration pattern, as revealed by the two-way ANOVA, suggests that the aeration pattern itself is a critical factor influencing the microbial response rather than the absolute oxygen concentration. This finding is critical because it implies that managing the aeration pattern could be more effective for controlling N_2O emissions than simply adjusting oxygen levels. The fact that *Hyphomicrobium*'s enzyme expression is strongly affected by the aeration pattern, resulting in distinct metabolic properties, underscores the importance of redox state cycling in modulating microbial activity for N_2O reduction.

The study's metaproteomic analysis provides mechanistic insights into how IP conditions enhance N_2O reduction in *Hyphomicrobium*. The increased expression of enzymes involved

in carbon metabolism, such as methanol dehydrogenase, under IP conditions suggests that *Hyphomicrobium* optimizes its metabolic pathways to generate a robust pool of reducing equivalents. This metabolic shift, facilitated by the CRP/FNR regulatory system, ensures a continuous supply of electrons required for the activity of N_2OR. Furthermore, the upregulation of N_2OR under IP conditions indicates that *Hyphomicrobium* is effectively utilizing the frequent redox cycling to enhance its N_2O reduction capacity. The dynamic environment created by IP conditions likely triggers the activation of the FNR regulon, promoting anaerobic respiration and N_2O reduction even in the presence of fluctuating oxygen levels. This aligns with previous studies suggesting that intermittent aeration can induce favorable conditions for N_2O-reducing bacteria, but our study provides a more in-depth understanding of the molecular mechanisms involved.

Existing literature demonstrates that intermittent aeration can significantly reduce N_2O emissions in wastewater treatment systems. For instance, Béline and Martinez observed that intermittent aeration facilitates an optimal redox state, promoting complete denitrification and thereby minimizing N_2O emissions[28]. Their study emphasized that maintaining an appropriate aerobic-to-anoxic ratio, such as 0.375, can lead to complete nitrogen transformation without accumulating N_2O. Similarly, Mello et al. found that while N_2O emissions occur during both aerated and non-aerated stages in an intermittently aerated system, the ability to control the conditions and timing of N_2O release resulted in an overall reduction of emissions to about 0.10% of the influent total nitrogen load[29]. We found that the reduction in N_2O emissions in our system was primarily due to the enhanced capacity of the microbial community to reduce N_2O. This shifts the focus from simply minimizing N_2O production to efficiently managing its removal. Our finding differs from traditional approaches that aim to optimize oxygen concentrations or reduce N_2O production; instead, it emphasizes the need to fully harness N_2OR activity to mitigate N_2O emissions during wastewater treatment.

In examining the microbial communities in various activated sludge systems, it is evident that a diverse array of N_2OR-containing organisms contributes to N_2O reduction. Our study identified *Hyphomicrobium* as a significant contributor, which aligns with findings in other systems where it coexists with a variety of denitrifiers[30]. Notably, genera such as *Flavobacterium*, *Dechloromonas*, and *Comamonas* have been reported in other studies as containing the nosZ gene and participating in N_2O reduction[30–32]. These genera include denitrifying strains that are facultative anaerobes, capable of switching between aerobic and anaerobic metabolism. *Dechloromonas* and *Comamonas* are well-documented for their facultative anaerobic nature, with *Dechloromonas* particularly known for its ability to reduce nitrate and other compounds under anoxic conditions. While *Flavobacterium* is primarily aerobic, certain denitrifying strains within this genus have demonstrated facultative anaerobic capabilities, performing denitrification in oxygen-limited environments. This metabolic flexibility suggests that these denitrifying organisms could possess regulatory mechanisms that enable them to adapt to changing redox conditions, such as those imposed by intermittent aeration.

The findings of this study have practical relevance for wastewater treatment plants aiming to minimize greenhouse gas emissions. Implementing intermittent high-frequency aerobic-anoxic cycling can enhance the N_2O reduction potential of the system without requiring major infrastructure changes. This approach could be integrated into existing systems, offering a cost-effective strategy for reducing N_2O emissions, particularly in regions with stringent environmental regulations. By focusing on the aeration pattern rather than merely adjusting oxygen levels, treatment plants can achieve more efficient N_2O mitigation while

maintaining overall process stability and performance. However, further research is needed to optimize the cycling parameters for various operational contexts. Understanding the broader community dynamics under IP conditions, including the potential contributions of other microbial populations to N_2O reduction, will be essential for developing comprehensive strategies for greenhouse gas mitigation in wastewater treatment. Additionally, exploring the long-term stability and adaptability of activated sludge systems under intermittent aeration will be crucial for assessing the practicality of this approach in full-scale operations. Future investigations could also focus on the interplay between carbon source availability and redox state cycling to further enhance N_2O reduction potential.

5. CONCLUSION

This study highlights the potential of intermittent perturbation with high-frequency aerobic-anoxic cycling as an effective strategy for enhancing N_2O reduction in activated sludge systems. By elucidating the mechanistic role of *Hyphomicrobium* and the regulatory processes involved, this research contributes to a deeper understanding of how aeration patterns can be used to enhance microbial N_2O removal capacity. The findings provide insights for developing more sustainable and environmentally friendly treatment processes, emphasizing the importance of dynamic aeration control in achieving these goals.

Associated content

Supporting information

Detailed materials and methods, the abundance of denitrification-related enzymes, the performance of biosystems, N_2O consumption rates, and two-way ANOVA analysis(PDF)

Author information

Corresponding authors

*Phone: +64 9 923 4512; fax: +64 9 373 7462; e-mail: n.singhal@auckland.ac.nz.

Funding sources

The study was funded by the Marsden Award from the Royal Society of New Zealand [grant number MFP-UOA2018].

Acknowledgments

We thank Nikki Freed for the assistance with metagenomic sequencing. We thank Martin Middleditch and George Guo for their assistance with metaproteomics analysis. We also thank Watercare Services Limited for providing the activated sludge culture from the Māngere Wastewater Treatment Plant. The authors acknowledge assistance from the New

Zealand eScience Infrastructure (NeSI) high-performance computing facilities and the Centre for eResearch at the University of Auckland.

REFERENCES

(1) Cantarel, A. A. M.; Bloor, J. M. G.; Pommier, T.; Guillaumaud, N.; Moirot, C.; Soussana, J.-F.; Poly, F. Four Years of Experimental Climate Change Modifies the Microbial Drivers of N_2O Fluxes in an Upland Grassland Ecosystem. *Global Change Biology* **2012**, *18* (8), 2520–2531. https://doi.org/10.1111/j.1365-2486.2012.02692.x.

(2) Galloway, J. N.; Aber, J. D.; Erisman, J. W.; Seitzinger, S. P.; Howarth, R. W.; Cowling, E. B.; Cosby, B. J. The Nitrogen Cascade. *BioScience* **2003**, *53* (4), 341–356. https://doi.org/10.1641/0006-3568(2003)053[0341:TNC]2.0.CO;2.

(3) Gustavsson, D. J. I.; la Cour Jansen, J. Dynamics of Nitrogen Oxides Emission from a Full-Scale Sludge Liquor Treatment Plant with Nitritation. *Water Science and Technology* **2011**, *63* (12), 2838–2845. https://doi.org/10.2166/wst.2011.487.

(4) Plouviez, M.; Guieysse, B. Nitrous Oxide Emissions during Microalgae-Based Wastewater Treatment: Current State of the Art and Implication for Greenhouse Gases Budgeting. *Water Science and Technology* **2020**, *82* (6), 1025–1030. https://doi.org/10.2166/wst.2020.304.

(5) Liu, T.; Liu, S.; He, S.; Tian, Z.; Zheng, M. Minimization of N_2O Emission through Intermittent Aeration in a Sequencing Batch Reactor (SBR): Main Behavior and Mechanism. *Water* **2021**, *13* (2), 210. https://doi.org/10.3390/w13020210.

(6) Mannina, G.; Chandran, K.; Capodici, M.; Cosenza, A.; Di Trapani, D.; van Loosdrecht, M. C. M. Greenhouse Gas Emissions from Membrane Bioreactors: Analysis of a Two-Year Survey on Different MBR Configurations. *Water Science and Technology* **2018**, *78* (4), 896–903. https://doi.org/10.2166/wst.2018.366.

(7) Kemmou, L.; Amanatidou, E. Factors Affecting Nitrous Oxide Emissions from Activated Sludge Wastewater Treatment Plants—A Review. *Resources* **2023**, *12* (10), 114. https://doi.org/10.3390/resources12100114.

(8) Al-Hazmi, H. E.; Lu, X.; Majtacz, J.; Kowal, P.; Xie, L.; Makinia, J. Optimization of the Aeration Strategies in a Deammonification Sequencing Batch Reactor for Efficient Nitrogen Removal and Mitigation of N_2O Production. *Environ. Sci. Technol.* **2021**, *55* (2), 1218–1230. https://doi.org/10.1021/acs.est.0c04229.

(9) Domingo-Félez, C.; Mutlu, A. G.; Jensen, M. M.; Smets, B. F. Aeration Strategies To Mitigate Nitrous Oxide Emissions from Single-Stage Nitritation/Anammox Reactors. *Environ. Sci. Technol.* **2014**, *48* (15), 8679–8687. https://doi.org/10.1021/es501819n.

(10) Sun, Y.; Pang, J.; Wang, S.; Tao, T.; Fu, X.; Zhang, Y.; Sun, B.; Pan, J. Confirmation the Optimal Aeration Parameters for Nitrogen Removal and Nitrous Oxide Emission in Wastewater Ecological Soil Infiltration Systems with Brown Earth. *Water Science and Technology* **2019**, *80* (1), 144–152. https://doi.org/10.2166/wst.2019.260.

(11) An, Z.; Zhang, Q.; Gao, X.; Ding, J.; Shao, B.; Peng, Y. Nitrous Oxide Emissions in Novel Wastewater Treatment Processes: A Comprehensive Review. *Bioresource Technology* **2024**, *391*, 129950. https://doi.org/10.1016/j.biortech.2023.129950.

(12) Kampschreur, M. J.; Temmink, H.; Kleerebezem, R.; Jetten, M. S. M.; van Loosdrecht, M. C. M. Nitrous Oxide Emission during Wastewater Treatment. *Water Research* **2009**, *43* (17), 4093–4103. https://doi.org/10.1016/j.watres.2009.03.001.

(13) Sun, S.; Cheng, X.; Li, S.; Qi, F.; Liu, Y.; Sun, D. N_2O Emission from Full-Scale Urban Wastewater Treatment Plants: A Comparison between A2O and SBR. *Water Science and Technology* **2013**, *67* (9), 1887–1893. https://doi.org/10.2166/wst.2013.066.

(14) Shiskowski, D. M.; Mavinic, D. S. Missing Aerobic-Phase Nitrogen: The Potential for Heterotrophic Reduction of Autotrophically Generated Nitrous Oxide in a Sequencing Batch Reactor Wastewater Treatment System. *Environmental Technology* **2005**, *26* (8), 843–856. https://doi.org/10.1080/09593332608618501.
(15) Wunderlin, P.; Mohn, J.; Joss, A.; Emmenegger, L.; Siegrist, H. Mechanisms of N_2O Production in Biological Wastewater Treatment under Nitrifying and Denitrifying Conditions. *Water Res* **2012**, *46* (4), 1027–1037. https://doi.org/10.1016/j.watres.2011.11.080.
(16) Jia, W.; Liang, S.; Zhang, J.; Ngo, H. H.; Guo, W.; Yan, Y.; Zou, Y. Nitrous Oxide Emission in Low-Oxygen Simultaneous Nitrification and Denitrification Process: Sources and Mechanisms. *Bioresource Technology* **2013**, *136*, 444–451. https://doi.org/10.1016/j.biortech.2013.02.117.
(17) Otte, S.; Grobben, N. G.; Robertson, L. A.; Jetten, M. S.; Kuenen, J. G. Nitrous Oxide Production by Alcaligenes Faecalis under Transient and Dynamic Aerobic and Anaerobic Conditions. *Applied and Environmental Microbiology* **1996**, *62* (7), 2421–2426. https://doi.org/10.1128/aem.62.7.2421-2426.1996.
(18) Freyschmidt, A.; Beier, M. Model-Based Identification and Testing of Appropriate Strategies to Minimize N_2O Emissions from Biofilm Deammonification. *Water Science and Technology* **2022**, *86* (7), 1810–1820. https://doi.org/10.2166/wst.2022.307.
(19) Yu, R.; Kampschreur, M. J.; Loosdrecht, M. C. M. van; Chandran, K. Mechanisms and Specific Directionality of Autotrophic Nitrous Oxide and Nitric Oxide Generation during Transient Anoxia. *Environ. Sci. Technol.* **2010**, *44* (4), 1313–1319. https://doi.org/10.1021/es902794a.
(20) Yang, R.; Yuan, L. Generation, Emission Reduction/Utilization, and Challenges of Greenhouse Gas Nitrous Oxide in Wastewater Treatment Plants – A Review. *Journal of Water Process Engineering* **2023**, *53*, 103871. https://doi.org/10.1016/j.jwpe.2023.103871.
(21) Chen, M.; Zhou, X.; Chen, X.; Cai, Q.; Zeng, R. J.; Zhou, S. Mechanisms of Nitrous Oxide Emission during Photoelectrotrophic Denitrification by Self-Photosensitized Thiobacillus Denitrificans. *Water Research* **2020**, *172*, 115501. https://doi.org/10.1016/j.watres.2020.115501.
(22) Pauleta, S. R.; Dell'Acqua, S.; Moura, I. Nitrous Oxide Reductase. *Coordination Chemistry Reviews* **2013**, *257* (2), 332–349. https://doi.org/10.1016/j.ccr.2012.05.026.
(23) Qu, Z.; Bakken, L. R.; Molstad, L.; Frostegård, Å.; Bergaust, L. L. Transcriptional and Metabolic Regulation of Denitrification in Paracoccus Denitrificans Allows Low but Significant Activity of Nitrous Oxide Reductase under Oxic Conditions. *Environmental Microbiology* **2016**, *18* (9), 2951–2963. https://doi.org/10.1111/1462-2920.13128.
(24) Zumft, W. G.; Kroneck, P. M. H. Respiratory Transformation of Nitrous Oxide (N_2O) to Dinitrogen by Bacteria and Archaea. In *Advances in Microbial Physiology*; Poole, R. K., Ed.; Academic Press, 2006; Vol. 52, pp 107–227. https://doi.org/10.1016/S0065-2911(06)52003-X.
(25) Conthe, M.; Lycus, P.; Arntzen, M. Ø.; Ramos da Silva, A.; Frostegård, Å.; Bakken, L. R.; Kleerebezem, R.; van Loosdrecht, M. C. M. Denitrification as an N_2O Sink. *Water Research* **2019**, *151*, 381–387. https://doi.org/10.1016/j.watres.2018.11.087.
(26) Hu, L.; Wang, X.; Chen, C.; Chen, J.; Wang, Z.; Chen, J.; Hrynshpan, D.; Savitskaya, T. NosZ Gene Cloning, Reduction Performance and Structure of Pseudomonas Citronellolis WXP-4 Nitrous Oxide Reductase. *RSC Adv.* **2022**, *12* (5), 2549–2557. https://doi.org/10.1039/D1RA09008A.
(27) Tamames, J.; Puente-Sánchez, F. SqueezeMeta, A Highly Portable, Fully Automatic Metagenomic Analysis Pipeline. *Frontiers in Microbiology* **2019**, *9*.

(28) Béline, F. Nitrogen Transformations during Biological Aerobic Treatment of Pig Slurry: Effect of Intermittent Aeration on Nitrous Oxide Emissions. *Bioresource Technology* **2002**, *83* (3), 225–228. https://doi.org/10.1016/S0960-8524(01)00219-X.

(29) Mello, W. Z. D.; Ribeiro, R. P.; Brotto, A. C.; Kligerman, D. C.; Piccoli, A. D. S.; Oliveira, J. L. M. Nitrous Oxide Emissions from an Intermittent Aeration Activated Sludge System of an Urban Wastewater Treatment Plant. *Quím. Nova* **2013**, *36* (1), 16–20. https://doi.org/10.1590/S0100-40422013000100004.

(30) Ye, J.; Mark Jensen, M.; Goonesekera, E. M.; Yu, R.; Smets, B. F.; Valverde-Pérez, B.; Domingo-Félez, C. Denitrifying Communities Enriched with Mixed Nitrogen Oxides Preferentially Reduce N_2O under Conditions of Electron Competition in Wastewater. *Chemical Engineering Journal* **2024**, *498*, 155292. https://doi.org/10.1016/j.cej.2024.155292.

(31) Sun, Y.; Xin, L.; Wu, G.; Guan, Y. Nitrogen Removal, Nitrous Oxide Emission and Microbial Community in Sequencing Batch and Continuous-Flow Intermittent Aeration Processes. *Environmental Engineering Research* **2018**, *24* (1), 107–116. https://doi.org/10.4491/eer.2018.141.

(32) Gabarró, J.; Hernández-del Amo, E.; Gich, F.; Ruscalleda, M.; Balaguer, M. D.; Colprim, J. Nitrous Oxide Reduction Genetic Potential from the Microbial Community of an Intermittently Aerated Partial Nitritation SBR Treating Mature Landfill Leachate. *Water Research* **2013**, *47* (19), 7066–7077. https://doi.org/10.1016/j.watres.2013.07.057.