Investigation on Greenhouse Gasses Metabolism by Environmental Microorganisms

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Abstract

Methane (CH4) and nitrous oxide (N2O) are the two most potent greenhouse gases routinely released from wastewater treatment plants, yet they are produced by distinct microbial guilds and are therefore usually targeted by separate mitigation strategies. Whether a single microorganism is able to consume both gases remains unresolved. In this study, wetland sediment, paddy soil and anaerobic–anoxic–oxic (A2O) activated sludge were used to inoculate six bioreactors operated under anaerobic and aerobic mediums with CH4 supplied as the carbon source. After ten months of enrichment, only the sludge reactors showed measurable gas consumption and were submitted for shotgun metagenomic sequencing. Reads were quality-filtered, assembled and binned within the DOE Systems Biology Knowledgebase (KBase), and the resulting metagenome-assembled genomes (MAGs) were functionally annotated to screen for methane- and nitrogen-cycling pathways. The findings however, showed no single MAG encoded both pathways.

Date: August 25, 2026

Contents

1 Introduction **2**

2 Related Work **2**

2.1 Greenhouse Gas Production in Wastewater Treatment 2

2.2 Nitrous Oxide as Terminal Acceptor 2

2.3 Positioning of the Present Study 2

3 Methodology **3**

3.1 Culture medium 3

3.2 Inoculation and enrichment 3

3.3 Metagenomic sequencing and data analysis 4

4 Results **4**

5 Discussion **6**

6 Limitations and Future Work **6**

7 Conclusion **6**

References **7**

1 Introduction

Greenhouse gas (GHG) emissions continue to rise every year [1]. The most abundant GHGs in the atmosphere are Carbon Dioxide (CO_2), Methane, and Nitrous Oxide; despite that fact, CH_4 and N_2O are 28 and 273 times more potent than CO_2, respectively [2] .
Both gases are common in Wastewater Treatment Plants (WWTPs), primarily due to the biological treatment process [3].
Nitrous oxide (N_2O) is mainly generated during biological nitrogen removal when chemical transformations stall midway. Ammonia-oxidizing bacteria (AOB) release it during nitrification—especially when starved for oxygen or exposed to elevated nitrite levels—while heterotrophic denitrifiers produce it if the reduction process fails to convert nitrate all the way into harmless nitrogen gas.
Methane (CH_4), on the other hand, comes from methanogenic archaea breaking down organic waste in anaerobic sludge digesters, as well as in stagnant, oxygen-starved pockets within the sewer pipe network[4].

This issue pushed researchers to explore new approaches focusing on reducing CH_4 and N_2O emissions; however, the existence of a single organism that can metabolize both CH_4 and N_2O remains unknown.
This study aims to cultivate microorganisms that thrive in methane and nitrous oxide-rich environments, as well as utilize metagenomics tools to investigate the microorganisms' ability to process both spontaneously, taking a step towards understanding CH_4 and N_2O metabolism.

2 Related Work

2.1 Greenhouse Gas Production in Wastewater Treatment

Wastewater N_2O emissions stem primarily from incomplete nitrification and denitrification triggered by nitrite accumulation [4, 5], while CH_4 emissions are driven by physical stripping of influent gas during aeration rather than in-tank methanogenesis [6]. Because these gases originate from distinct microbial guilds, they are conventionally mitigated separately. On the sink side, discovery of clade II nosZ in non-denitrifiers demonstrates that N_2O-reducing capacity is phylogenetically widespread in mixed communities [7].

2.2 Nitrous Oxide as Terminal Acceptor

Bulk-activity studies confirm community-level coupling between anaerobic methane oxidation and N_2O reduction in soils and wetlands [8–10], but cannot confirm single-cell execution. Acidophilic methanotrophs carrying nosZ can consume both gases suboxically, but lack full denitrification pathways and fail under strict anoxia [11]. Whether a single organism directly couples both pathways remains unknown.

2.3 Positioning of the Present Study

Existing research relies either on community-level fluxes (which cannot separate dual-capable cells from multi-species consortia) or isolates from niche environments (rather than wastewater). This study combines selective enrichment (N_2O + CH_4) with genome-resolved metagenomics via

KBase [12, 13] to recover metagenome-assembled genomes (MAGs) from activated sludge, testing single-genome metabolic potential directly.

3 Methodology

3.1 Culture medium

Aerobic (Table 1) and anaerobic (Table 2) culture mediums were formulated for the microorganisms' enrichment; both solutions were continuously bubbled with N_2 for 20 minutes to ensure anoxic conditions.

Table 1 Composition of aerobic medium

Chemical	Concentration (per liter)
$MgSO_4 \cdot 7H_2O$	0.4929g
KH_2PO_4	0.1361g
Na_2HPO_4	0.0284g
$CaCl_2 \cdot 2H_2O$	0.0294g
Trace elements solution	1mL
Vitamins solution	1mL

Table 2 Anaerobic medium composition

Chemical	Concentration (per liter)
KH_2PO_4	0.075g
$CaCl_2 \cdot 2H_2O$	0.3g
$MgSO_4 \cdot 7H_2O$	0.2g
Alkaline trace element solution	0.2mL
Acidic trace element solution	0.5mL

3.2 Inoculation and enrichment

Three distinct biomass sources were harvested for microbial enrichment: wetland sediment (0–10 cm depth), paddy soil (0–10 cm depth), and anaerobic–anoxic–oxic (A^2O) activated sludge. Biomass samples were suspended in both aerobic and anaerobic culture media separately and inoculated into bioreactor bottles, resulting in six distinct bioreactor conditions. Reactors consisted of 500 mL total volume containing 175 mL of inoculum–medium suspension and 325 mL of headspace. Initial headspace gas composition was set to 5% CH_4, 5% CO_2, and 90% N_2. The gas

supply regimes were divided as follows:

- Anaerobic bioreactors: Supplied via gas bags filled with 100% N_2O.
- Microaerobic bioreactors: Supplied via gas bags filled with 75% N_2O and 25% O_2.

All bioreactors were incubated at 30 °C with continuous shaking at 150 rpm. Routine reactor maintenance followed a fixed timeline:

- Gas bag replacement: Weekly
- Headspace gas flushing: Bi-weekly (every 2 weeks)
- Biomass sampling and archival storage: Every 2 months
- Culture medium replacement: Every 4 months

Headspace gas composition (CH_4, N_2O, and O_2) was monitored routinely. Reactor pH was measured regularly and adjusted to remain within 6.8–7.2 by supplying CH_4 gas via different time intervals.

3.3 Metagenomic sequencing and data analysis

Within 10-month of enrichment, the bioreactors containing sludge showed significant gas combustion, therefore, 10 mL of sludge was collected and sent to a laboratory for metagenomic sequencing. To evaluate the metabolic potential for methane and nitrous oxide utilization, raw metagenomic sequencing reads were processed through a custom bioinformatics pipeline in the DOE Systems Biology Knowledgebase (KBase). Initial read quality was assessed using

FastQC, followed by quality filtering and adapter removal via Trimmomatic. Cleaned reads were assembled into contigs using MEGAHIT. Metagenome-assembled genomes (MAGs) were recovered by binning contigs using three independent algorithms: MaxBin2, CONCOCT, and MetaBAT2. Bin predictions were integrated and optimized using DAS Tool to generate a consensus

set of non-redundant MAGs. Bin completeness and contamination were evaluated using CheckM, and high-quality MAGs were filtered for downstream analysis. Taxonomic classification of the recovered MAGs was assigned using GTDB-Tk based on the Genome Taxonomy Database, and phylogenetic placement was established using the KBase SpeciesTree tool Finally, high-quality

MAGs were extracted as individual assemblies and functionally annotated using RASTtk and DRAM (Distillation and Annotation of Genomes) to profile specific metabolic pathways and identify key enzymes involved in methane oxidation and N_2O reduction.

4 Results

Figure 1 and Figure 2 display per-base sequence quality profile of raw Illumina metagenomic sequencing reads. Box plots display the distribution of Phred quality scores across each base position (1–150 bp). Yellow boxes represent the interquartile range (25th–75th percentiles), red lines denote median values, and the blue line tracks the mean quality score.
The heatmaps (Figure 3 and Figure 4) demonstrate the functional potential of the recovered aerobic and anaerobic MAGS, a green pixel indicates that the gene sequence is present in the genome of the organism.

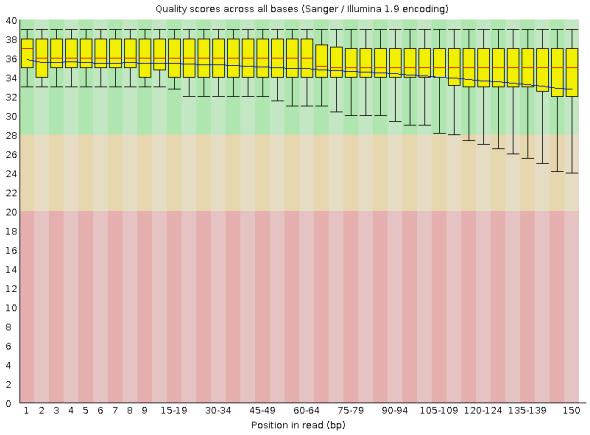


Figure 1 Aerobic quality profile of raw metagenomic reads

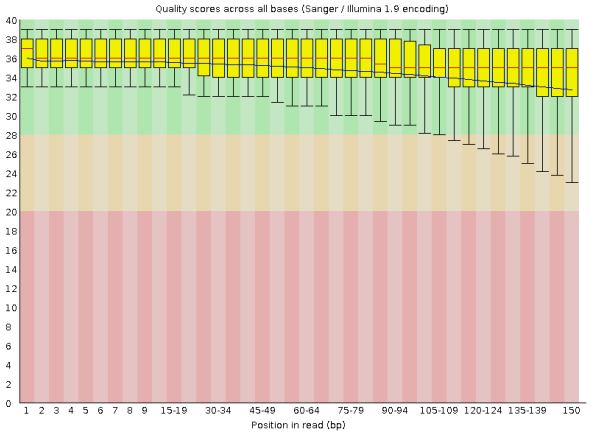


Figure 2 Anaerobic quality profile of raw metagenomic reads

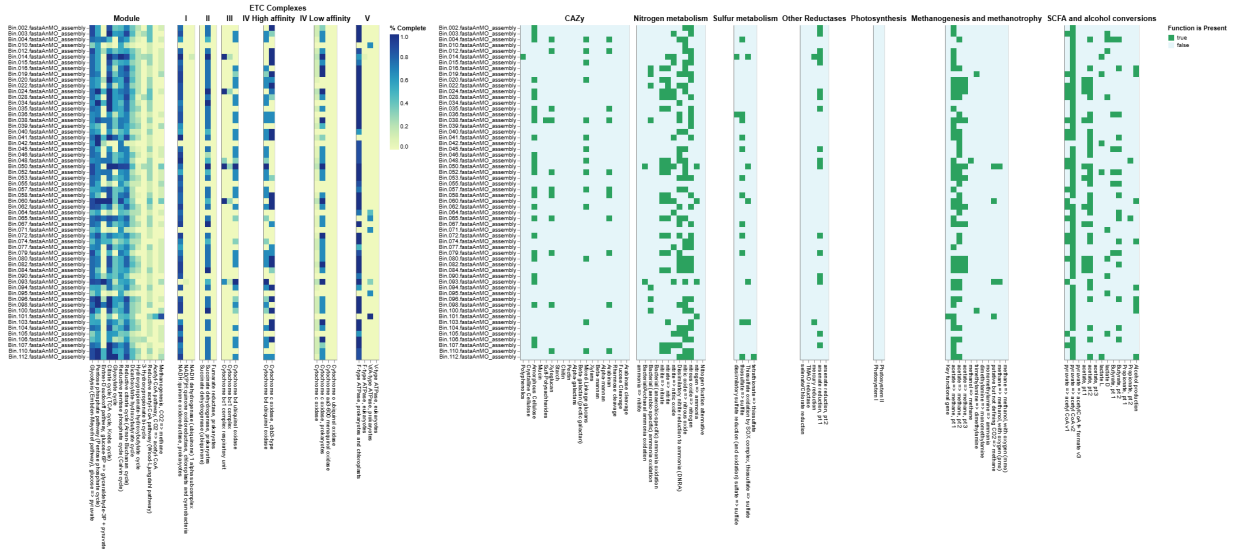


Figure 3 Anaerobic functional potential and metabolic pathway profiling

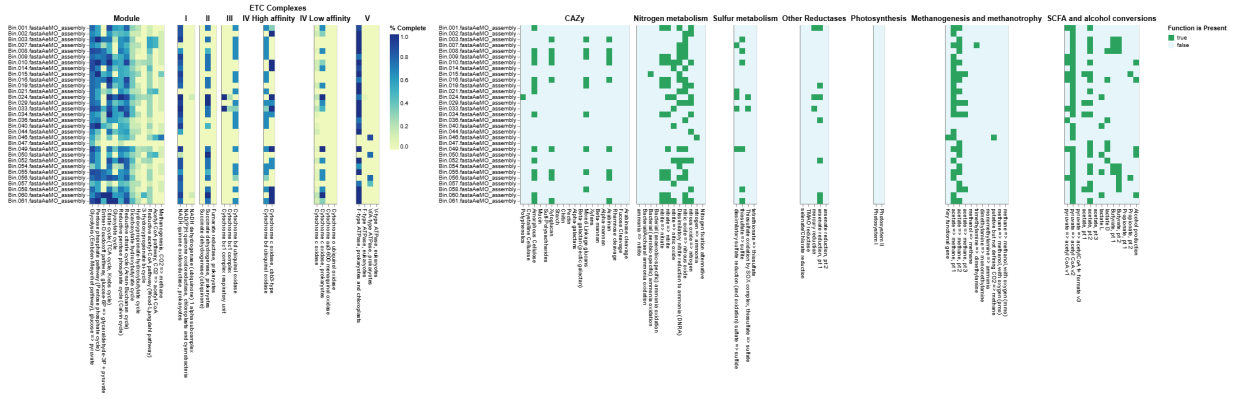


Figure 4 Aerobic functional potential and metabolic pathway profiling

5 Discussion

The high quality of reads retrieved from both anaerobic and aerobic biomass (Figures 1 and 2) supported further metagenomic investigation of the samples. Most notably, functional profiling revealed that several bins encoded genes involved in N_2O metabolism (Figures 3 and 4). However, methane-metabolizing genes were strictly restricted to two specific assemblies: Bin.093 and Bin.050. Crucially, no single bin harbored both N_2O and CH_4 metabolizing pathways simultaneously, indicating an absence of individual organisms capable of dual digestion within the evaluated microbial community.

It is important to note that these observations were limited to the sludge bioreactor. Consequently, the possibility remains that organisms capable of dual digestion could be identified in the other bioreactors upon complete biomass enrichment.

6 Limitations and Future Work

A primary limitation of this study was the lack of experimental replication; due to spatial constraints, each condition was evaluated using a single bioreactor ($n = 1$) for both aerobic and anaerobic systems.
Furthermore, time constraints precluded batch testing and direct gas consumption comparisons, as only the sludge bioreactor yielded measurable gas consumption within the study period, while the remaining bioreactors required additional time for biomass enrichment.
To build upon these findings, future research should employ at least three replicates ($n \geq 3$) to mitigate potential operational errors during anaerobic gas supplementation. Extending the experimental duration will also be essential to allow adequate enrichment across all biomass sources.

7 Conclusion

This study investigated whether a single organism can simultaneously metabolize methane (CH_4) and nitrous oxide (N_2O), addressing a fundamental gap in greenhouse gas mitigation during wastewater treatment. By combining a 10-month selective enrichment regime ($CH_4 + N_2O$) with genome-resolved metagenomics, metagenome-assembled genomes (MAGs) were successfully recovered from activated sludge biomass to test for single-genome metabolic co-occurrence.
Metagenomic functional profiling revealed widespread capacity for N_2O metabolism across multiple microbial bins, whereas genes involved in CH_4 utilization were strictly confined to two specific assemblies (Bin.093 and Bin.050). Crucially, no single recovered bin harbored both CH_4 and N_2O metabolic pathways simultaneously. For future research, extended enrichment periods across wetland and paddy soil is encouraged.

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