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Hidden versatility, competition, and cooperation:
Methanogenesis and nitrification drive unexpected carbon and nitrogen cycle linkages

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Abstract

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Wetlands serve as crucial nodes in the carbon (C) and nitrogen (N) cycles by sequestering ~30% of global soil organic matter (SOM) and contributing to ~20% of annual global methane emissions despite accounting for only ~6% of global land area. The degradation, release, and retention of organic carbon and nitrogen in wetlands is facilitated by the activity of microorganisms that assemble into microbial communities within the pore network of wetland sediment aggregates. However, biogeochemical nutrient gradients will impose selective pressures that control the structure and function of the microbial community responsible for greenhouse gas emissions and SOM storage. The complex feedback between microbial activity and biogeochemistry makes it difficult to predict how increasing anthropogenic activity will influence nutrient cycling in wetland ecosystems. This dissertation aims to address this critical gap by linking single-cell microbial ecophysiology and community structure to ecosystem-scale biogeochemistry while developing predictive understanding of how microbial communities respond to environmental change.

This work aims to investigate both the microbial interactions governing the degradation of organic carbon to methane by methanogenic communities in wetland sediments and the ecophysiology of ammonia-oxidizing microorganisms (AOM)—major contributors to terrestrial nitrous oxide (N₂O)

emissions and the global nitrogen cycle. Methane (CH_4) and N_2O are potent greenhouse gases (GHG) with global warming potentials 27 and 273 times greater than carbon dioxide (CO_2) over a 100-year timescale, respectively. These important environmental processes were studied in laboratory enrichments and isolates with a combination of culture-based, isotopic, multi-omic, and mathematical modeling approaches. Wetland sediment chemostat enrichments were used in the first two sections to investigate spatial community structure, carbon degradation, and links between the carbon and nitrogen cycle. This thesis begins with a comprehensive overview of methanogenesis and nitrification and their connections to global carbon and nitrogen cycles within wetland ecosystems (**Chapter 1**). Synthetic wetland sediment aggregate chemostat enrichments and mathematical models of lactate degrading methanogenic communities demonstrated spatial community structuring and highlighted the importance of spatial proximity for methanogenesis (**Chapter 2**). Aerobic and anaerobic necromass degrading wetland communities reverted to their prior states after oxygen perturbations demonstrating the resiliency of these functionally redundant communities, while emphasizing necromass recycling as an underappreciated link between the carbon and nitrogen cycle (**Chapter 3**). Next, the physiological mechanisms underlying nitrogen removal in a novel partial nitrification-anammox reactor using high affinity AOM were evaluated (**Chapter 4**). This physiological investigation led to the discovery of hydroxylamine-dependent nitrate reduction and the biotic mechanism of N_2O production by the complete ammonia-oxidizing (comammox) bacteria *Nitrospira inopinata* (**Chapter 5**). These results highlight the spatial dimension of metabolic niche partitioning, link the carbon and nitrogen cycles through necromass degradation, and uncover previously unknown nitrogen cycling metabolic versatility.

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Dedication

To my dear wife, Pauli A. Mazzarino, without whom any of this would be possible

Chapter 1.

Introduction

1.1 Wetlands and anthropogenic change

Wetland ecosystems—including marshes, swamps, bogs, and fens—exert a disproportionate influence on biogeochemical cycling relative to their global footprint. Wetlands represent the largest terrestrial reservoir of organic carbon, sequestering an estimated 20-30% of the global soil organic matter (SOM), despite accounting for 5-8% of total land area [1]. However, individual microbes in wetlands sediments produce greenhouse gases (GHGs) while degrading SOM. Approximately 159-200 Tg of methane (CH₄) are produced by wetlands annually [2], representing 25-30% of global CH₄ emissions, and are increasingly recognized as the largest terrestrial source of nitrous oxide (N₂O) [3], especially in drained and warming wetland soils. The emissions of these GHGs can have a profound impact on the global climate, considering CH₄ and N₂O have global warming potentials 27 and 273 times greater than carbon dioxide (CO₂) over a 100-year timescale [4], respectively.

Anthropogenic pressures threaten the balance between carbon sequestration and GHG emission by wetland ecosystems. Agricultural and urban development has led to the widespread drainage of wetlands with an estimated 50% loss in total land area [5]. Undeveloped wetlands are experiencing increased pollution from industrial and agricultural runoff potentially contributing to increased N₂O production, eutrophication, and groundwater contamination[6]. Additionally, global climate change has profoundly altered wetland biogeochemistry, potentially creating feedback loops that

exacerbate climate change [1]. Sea level rise has increased the penetration of brackish water laden with sulfate and nitrate into coastal wetlands, while altered precipitation regimes have increased the frequency of drought and flooding in lacustrine (freshwater) wetlands [1]. These disturbances are reshaping the hydrology and biogeochemistry of wetlands, risking their transformation from carbon sinks to a net source of GHG production. Understanding the feedback between microbial activity and biogeochemistry is essential for predicting future carbon and nitrogen cycling dynamics.

1.2 Predictive microbial ecology

The balance between greenhouse gas release and soil organic carbon sequestration by wetlands is facilitated by the activity of microorganisms that assemble into microbial communities within wetland sediment pore networks [1]. However, predicting the functional outcomes of these diverse microbial communities, including the rates of key biogeochemical transformations, represents a central challenge in microbial ecology. Wetland sediments are highly heterogenous with steep chemical gradients that vary over the millimeter scale [1]. These gradients determine the spatiotemporal distribution of electron donors and acceptors, which determines environmental niches and separates distinct metabolic processes (Figure 1.1). For example, nitrite oxidation to nitrate will occur in the presence of oxygen, but when oxygen is unavailable it can be reduced to N_2O or dinitrogen gas via denitrification. While modern sequencing technology provides insight into the genomic potential and taxonomic composition of wetland microbial communities, predicting *in situ* rates from sequence data alone remains a significant challenge, especially considering the presence or absence of a gene does not always correlate with biogeochemical fluxes.

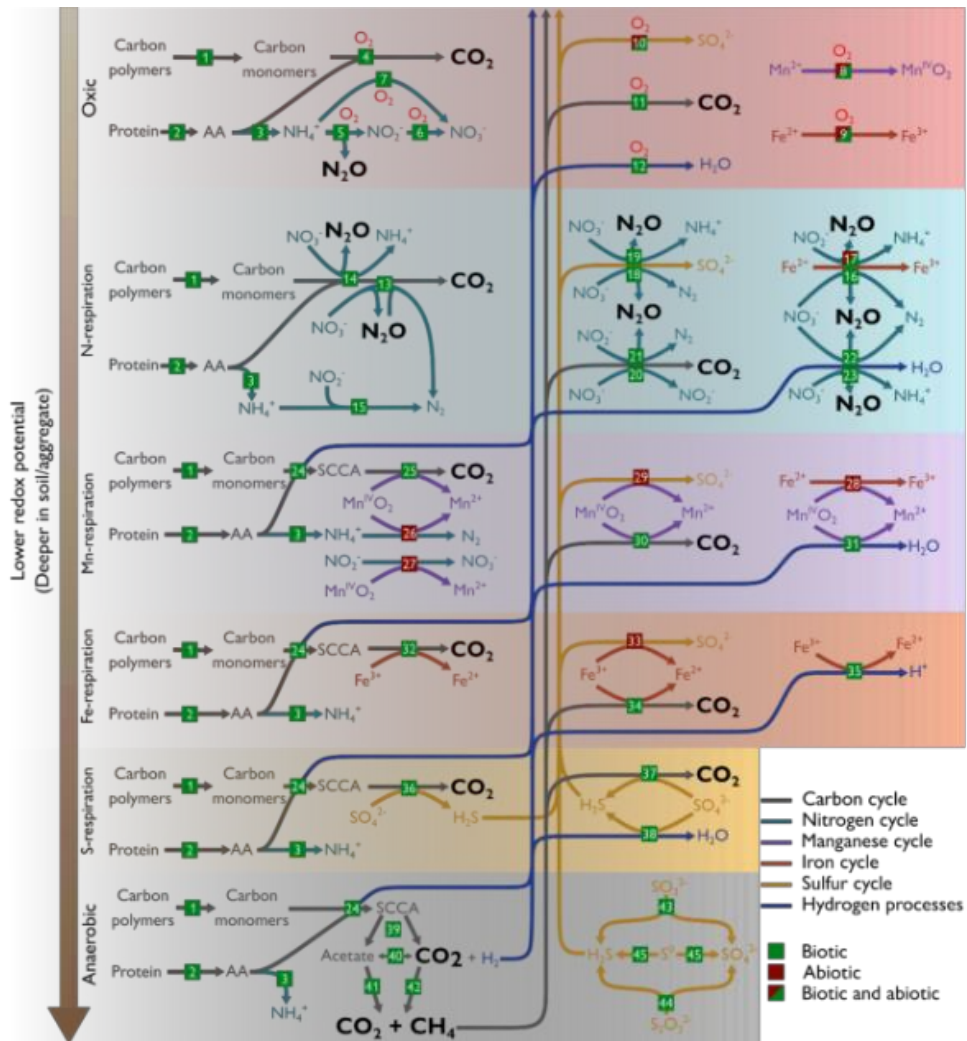


Figure 1.1. Microbial and abiotic processes in wetlands.

Catabolic, biotic, and abiotic redox reactions driving greenhouse gas (GHG) fluxes in wetland ecosystems. Vertical organization of reactions follows the conventional view of the redox gradient in wetland soils, while horizontal organization has no spatial significance in wetlands.

Integrated approaches are needed to develop predictive ecosystem models from sequencing and biogeochemical data [7]. We recently proposed a research approach that combines field observations with controlled experimental systems and mathematical modeling across spatial and temporal scales (Figure 1.2) [1]. Field observations are essential to identify the parameters and taxa driving ecosystem processes. Variables of interest can be isolated with controlled laboratory systems, including chemostat, mesocosm, and pure-culture experiments. These approaches allow researchers to study the microbial response to a given perturbation and precisely measure metabolic rates under defined conditions. Mathematical models then provide a framework to systematically describe microbial process across scales to predict ecosystem outcomes from single-cell measurements.

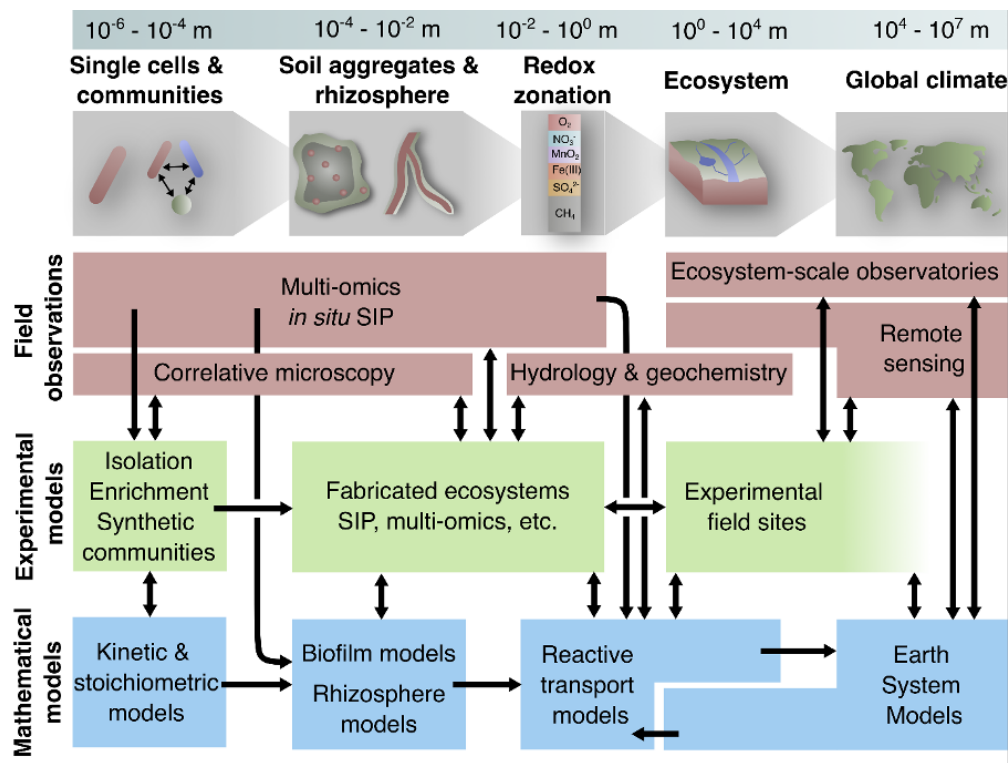


Figure 1.2. Connecting microbial to the global scale measurement and models.

Conceptual overview of framework bridging the gap between microbial and global scale processes by linking field observations, experimental, and mathematical models at appropriate.

Predicting the diversity, function and stability of microbial communities through the development of mathematical models is a guiding principle for this research. Central to quantitative microbial ecology is the determination of biomass-normalized, or cell-specific metabolic, rates that characterize the physiology of microorganisms under defined environmental conditions. Understanding how these rates—expressed in units of mol substrate per unit biomass per time—respond to nutrient limitations is critical to understand environmental niches and develop predictive models.

The Monod model (Equation 1.1), a form of saturation kinetics, provides a widely adopted mathematical description of the cell-specific rate under nutrient limitations [8].

$$R_i = \frac{V_{Max} \cdot S}{K_{m(app)} + S} \quad (1.1)$$

Where R_i is the specific rate ($\text{mol} \cdot \text{biomass}^{-1} \cdot \text{time}^{-1}$), S is the substrate concentration ($\text{mol} \cdot \text{L}^{-1}$), V_{max} is the maximum specific rate achieved with non-limiting substrate ($\text{mol} \cdot \text{biomass}^{-1} \cdot \text{time}^{-1}$), and $K_{m(app)}$ is the half-saturation constant ($\text{mol} \cdot \text{L}^{-1}$).

The model describes how the specific rate depends on substrate concentration (Figure **1.3**). Initially, the specific rate rapidly increases with substrate at low concentrations, reaches half of its maximum rate at an intermediate concentration, and asymptotically approaches the maximum specific rate (V_{max}) as the systems becomes saturated at elevated substrate concentrations.

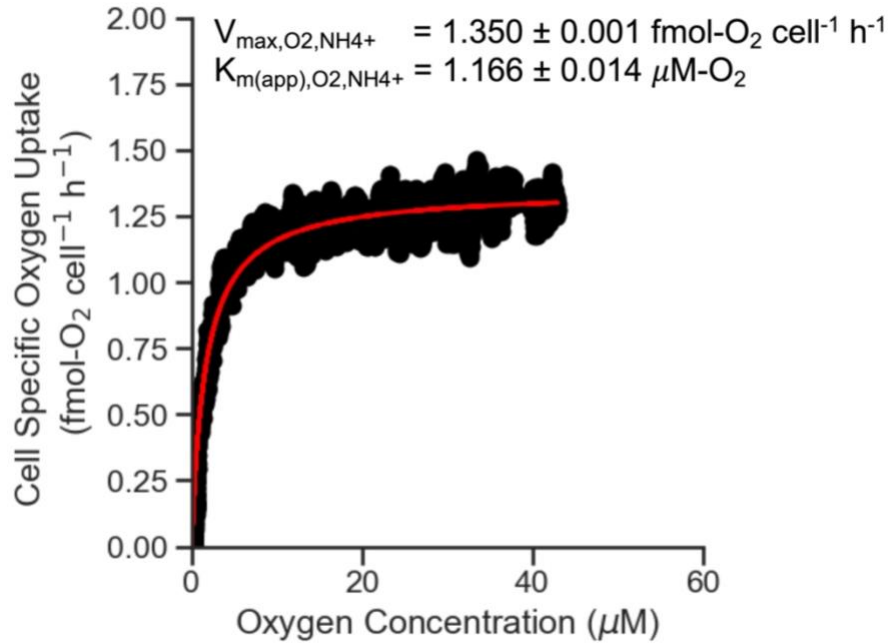


Figure 1.3. Representative Monod curve from oxygen uptake data.

This oxygen uptake rate for the comammox isolate *Nitrospira inopinata* was determined by microrespirometry (MR) upon a 1mM substrate addition of ammonium. Kinetic constants were obtained by fitting a Michaelis-Menten equation (red line) to total cell specific oxygen uptake rates. Image from *Abrahamson et al., In prep.* (Chapter 4).

The half-saturation concentration ($K_{m(app)}$) provides critical insight into environmental niches [9]. A low $K_{m(app)}$ value (high affinity) allows an organism to scavenge resources in oligotrophic (nutrient-deplete) environments, while a high $K_{m(app)}$ suggests a lower affinity or an adaptation to nutrient-rich (copiotroph) environments. However, the half-saturation does not provide information about the ability for an organism to compete for resources [9], which is defined by the specific affinity (Equation 1.2).

$$a_o = \frac{V_{Max}}{K_{m(app)}} \quad (1.2)$$

Where a_o is the specific affinity ($L \cdot \text{biomass}^{-1} \cdot \text{time}^{-1}$).

Organisms with a low affinity typically have a high V_{max} , that allows them to outcompete higher affinity organism when nutrient are available [9–12]. Additionally, the maximum cell-specific rate is correlated with the maximum growth rate through the yield coefficient (Equation 1.3)

$$V_{Max} = \mu_{Max} \cdot Y_{X/S}^{-1} \quad (1.3)$$

where μ_{max} is the maximum specific growth rate (h^{-1}) and $Y_{X/S}$ is the yield coefficient ($\text{Biomass}^{-1} \cdot \text{mols}$)

Yields determine the efficiency of substrate conversion into biomass or other products and, together with rates, dictate the overall stoichiometry and impact of microbial activity on biogeochemical cycles.

Obtaining these parameters provide indicators of ecological strategies and their ability to compete for scarce resources [9]. For example, different groups of ammonia-oxidizing microorganisms (AOMs) exhibit distinct affinities for ammonia[10–12]. Ammonia-oxidizing archaea (AOA) and comammox *Nitrospira* isolates have high ammonia affinities (low $K_{m(app)}$), which confers a competitive advantage in the open ocean, ground water, natural sediments, and other oligotrophic environments [10–12]. Many ammonia-oxidizing bacteria (AOB) have lower ammonia affinities

(higher $K_{m(app)}$) but a higher V_{max} , making them more competitive in agricultural soils or wastewater treatment systems [12]. Understanding this kinetic niche differentiation is crucial for predicting which microbial groups will dominate, how efficiently they compete, the estimate the production of N_2O . Cell-specific kinetics are the foundational traits of mechanistic model that scale microbial activities from the single-cell to ecosystem level [13]. This research strives to quantify yield, cell-specific rates, and other kinetic parameters to connect microbial physiology with ecosystem-scale processes.

1.3 Methanogenesis

Methanogenesis, the biological production of CH_4 , is the terminal step in the anaerobic degradation of organic matter (Figure 1.4) [14]. This defining feature of wetland biogeochemistry is cascade of cross-feeding interactions often operating near thermodynamic equilibrium (Figure 1.4) [14]. Primary degraders hydrolyze organic carbon into sugars, peptides, lipids, and short-chain carboxylic acids (SCCAs) [15]. Syntrophic bacteria subsequently oxidize these hydrolysis byproducts into hydrogen (H_2), carbon dioxide (CO_2), and acetate before subsequent conversion to CH_4 by methanogenic archaea [16].

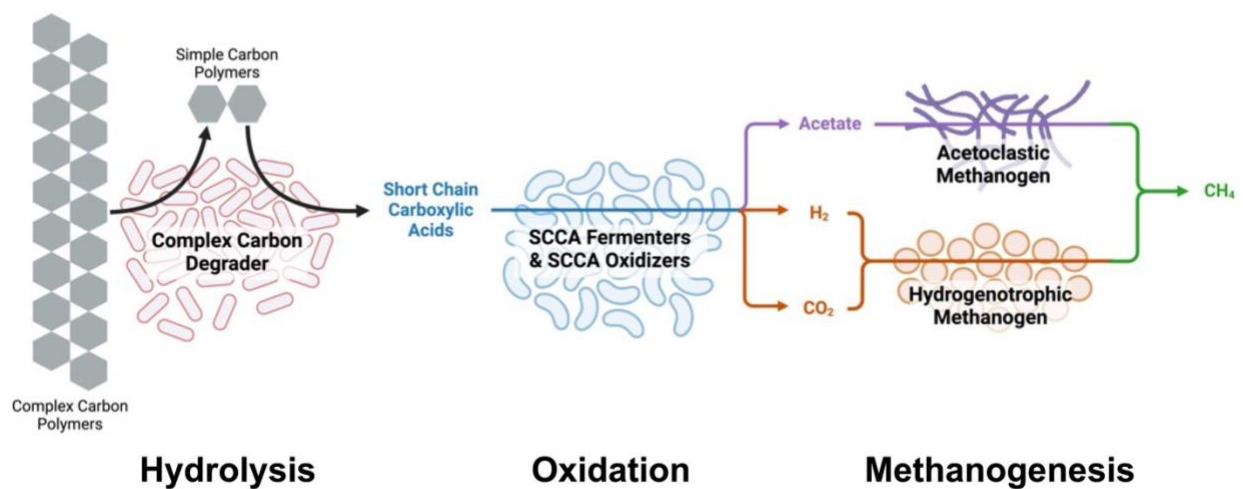


Figure 1.4. Overview of the anaerobic cross-feeding leading to methanogenesis.

A rate-limiting step of methanogenesis involves the syntrophic oxidation of SCCA, which are thermodynamically unfavorable to degrade unless H₂ is maintained at low partial pressures [17–19]. Methanogenic archaea consume the H₂ produced by SCFA-oxidation to form an obligately mutualistic interaction that overcomes thermodynamic constraints and enhances CH₄ production [16]. The efficiency of methanogenesis is influenced by the physical structure of the environment, particularly within sediment aggregates where diffusion limitations and close spatial proximity between partners are crucial for efficient interspecies metabolite transfer [20]. However, methanogens are highly sensitive to biogeochemical perturbations, especially sulfate intrusions and oxygen fluctuations [21, 22].

1.4 Nitrification

Nitrification, the biological oxidation of ammonia to nitrate via nitrite, is a crucial pathway within the global nitrogen cycle. Classically, nitrification was thought to be a stepwise process where ammonia-oxidizing microorganisms (AOM) first oxidize ammonia (NH_3) to nitrite (NO_2^-) with nitrite-oxidizing bacteria (NOB) subsequently oxidizing NO_2^- to nitrate (NO_3^-) [23]. However, the discovery and isolation of a complete ammonia-oxidizing (comammox) bacteria challenged this strict division of labor [12, 24, 25].

Nitrification is also intimately linked to N_2O production. AOB can aerobically convert the ammonia oxidation intermediate hydroxylamine (NH_2OH) and nitric oxide (NO) to N_2O via the *c* cytochrome P460 (*cytL*) and can reduce nitrite (NO_2^-) to N_2O via nitric oxide reductase (NOR) upon the onset of hypoxia [12, 26, 27]. AOA biotically form N_2O via N-nitrosation — a hybrid reaction where hydroxylamine combines with an enzymatically bound nitrosyl intermediate derived from nitrite (NO_2^-) or nitric oxide (NO) to produce N_2O [28–30]. *N. inopinata* aerobically and anaerobically produce N_2O in a proposed abiotic reaction involving accumulated NH_2OH [29].

Reports of nitrite reduction by *Nitrospira* NOB [31], hydrogen oxidation by *Nitrospira* NOB [32, 33], and ammonia repression by ureolytic β -AOB [34] has recent expanded the metabolic versatility of the nitrifying specialists. Understanding and characterizing this versatility is crucial for accurately predicting nitrogen fate in dynamic environments including wetlands, wastewater treatment plants, and marine oxygen minimum zones.

1.5 Organic nitrogen links the carbon and nitrogen cycle

The global carbon and nitrogen cycles are linked through organic nitrogen compounds that release ammonium upon mineralization. Microbial necromass—organic remnants of cellular decay including chitin, peptidoglycan, lipids, and amino sugars—are increasingly being recognized as a significant component of SOM [35]. N-acetylglucosamine (GlcNAc), a key monomer of peptidoglycan and chitin, is an abundant organic nitrogen compound in wetland sediments [35]. The mineralization of GlcNAc releases carbon and inorganic N, typically as NH_4^+ , directly linking the C and N cycles [35]. The dynamics of necromass turnover is influenced by oxygen availability and significantly impacts both SOM stability and biogeochemical cycling in wetlands.

1.6 Thesis aims

This dissertation aims to investigate the metabolic versatility, competitive dynamics, and cooperative interactions that govern carbon and nitrogen cycling in natural engineered environments with the goal of developing a more predictive understanding of how they respond to environmental change.

1.7 Objective and Methods

This dissertation aims to investigate the metabolic versatility, competitive dynamics, and cooperative interactions that govern carbon and nitrogen cycling in natural engineered environments with the goal of developing a more predictive understanding of how they respond to environmental change. This objective will be addressed with the following methods:

- a) **Controlled laboratory chemostat systems** were employed to study microbial communities under defined growth rates and investigate dynamics physiological responses over time (Chapters 2, 3).
- b) **Multi-omic sequencing approaches**, including metagenomics, meta-transcriptomics, and proteomics, demonstrated genomic potential and gene/protein expression (Chapters 2, 3 5).
- c) **Fluorescence *in-situ* hybridization** was used to visualize spatial organization (Chapter 2)
- d) **^{15}N Stable isotope tracing** provided insight into the fate of substrate (Chapters 5).
- e) **Microrespirometry** enabled the direct measurement of cell-specific rates (Chapters 4).
- f) **Mathematical modeling** was applied to simulate complex microbial interactions, spatial dynamics, and diffusion-controlled substrate uptake (Chapters 2, 4).

1.8 Thesis Structure

Chapter 1 introduces the objective for this thesis.

Chapter 2 explores the spatial structuring of methanogenic wetland sediments analogs.

Two synthetic sediment analogs were compared during the chemostat enrichment of lactate-degrading methanogenic communities. The community was visualized with *fluorescence in-situ hybridization* and spatial niches were investigated with a mathematic model.

Chapter 3 investigates N-acetylglucosamine (GlcNAc) mineralization in wetland sediments.

Aerobic and anaerobic GlcNAc-degrading microbial communities were enriched in chemostats subject to periodic oxygen perturbations. GlcNAc mineralization is highlighted as a crucial link between the carbon and nitrogen cycle, while metagenomics revealed the functional resilience of the wetland microbiome.

Chapter 4 evaluates the performance of a MABR system treating mainstream wastewater.

Membrane aerated biofilm reactors (MABRs) were designed to co-culture anammox bacteria with AOA or comammox *Nitrospira* leading to complete nitrate removal. The anaerobic physiology of the comammox isolate *Nitrospira inopinata* was investigated to explain the nitrate removal.

Chapter 5 characterizes hydroxylamine-dependent nitrate reduction by comammox.

The comammox isolate *N. inopinata* can couple the oxidation of hydroxylamine with nitrate reduction to produce nitrite with concomitant ammonium and nitrous oxide production. Stable isotope tracking identified the enzymatic origin of N₂O production offering insight into an overlooked greenhouse gas production pathway in *Nitrospira*.

References

1. Candry P et al. Microbially mediated climate feedbacks from wetland ecosystems. *Glob Chang Biol* 2023;29:5169–5183. <https://doi.org/10.1111/gcb.16850>
2. Saunois M et al. The Global Methane Budget 2000–2017. *Earth Syst Sci Data* 2020;12:1561–1623. <https://doi.org/10.5194/essd-12-1561-2020>
3. Bahram M et al. Structure and function of the soil microbiome underlying N₂O emissions from global wetlands. *Nat Commun* 2022;13:1430. <https://doi.org/10.1038/s41467-022-29161-3>
4. Calvin K et al. IPCC, 2023: Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)]. IPCC, Geneva, Switzerland. 2023.
5. Verhoeven JTA, Setter TL. Agricultural use of wetlands: opportunities and limitations. *Ann Bot* 2010;105:155–163. <https://doi.org/10.1093/aob/mcp172>
6. Nilsson JE, Weisner SEB, Liess A. Wetland nitrogen removal from agricultural runoff in a changing climate. *Science of The Total Environment* 2023;892:164336. <https://doi.org/10.1016/j.scitotenv.2023.164336>
7. Levine NM et al. Microbial Ecology to Ocean Carbon Cycling: From Genomes to Numerical Models. *Annu Rev Earth Planet Sci* 2025. <https://doi.org/10.1146/annurev-earth-040523-020630>
8. Monod J. THE GROWTH OF BACTERIAL CULTURES. *Annu Rev Microbiol* 1949;3:371–394. <https://doi.org/10.1146/annurev.mi.03.100149.002103>
9. Button DK. Kinetics of nutrient-limited transport and microbial growth. *Microbiol Rev* 1985;49:270–297. <https://doi.org/10.1128/mr.49.3.270-297.1985>
10. Jung M-Y et al. Ammonia-oxidizing archaea possess a wide range of cellular ammonia affinities. *ISME J* 2022;16:272–283. <https://doi.org/10.1038/s41396-021-01064-z>
11. Martens-Habbena W et al. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 2009;461:976–979. <https://doi.org/10.1038/nature08465>

12. Dimitri Kits K et al. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 2017;549:269–272. <https://doi.org/10.1038/NATURE23679>
13. Zakem EJ et al. Ecological control of nitrite in the upper ocean. *Nat Commun* 2018;9:1206. <https://doi.org/10.1038/s41467-018-03553-w>
14. Muyzer G, Stams AJM. The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* 2008;6:441–454. <https://doi.org/10.1038/nrmicro1892>
15. Sokol NW et al. Life and death in the soil microbiome: how ecological processes influence biogeochemistry. *Nat Rev Microbiol* 2022;20:415–430. <https://doi.org/10.1038/s41579-022-00695-z>
16. Stams AJM, Plugge CM. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* 2009;7:568–577. <https://doi.org/10.1038/nrmicro2166>
17. Zellner G, Neudörfer F, Diekmann H. Degradation of lactate by an anaerobic mixed culture in a fluidized-bed reactor. *Water Res* 1994;28:1337–1340. [https://doi.org/10.1016/0043-1354\(94\)90299-2](https://doi.org/10.1016/0043-1354(94)90299-2)
18. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* 1997;61:262–280. <https://doi.org/10.1128/mmbr.61.2.262-280.1997>
19. Noguera DR et al. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. *Biotechnol Bioeng* 1998;59:732–746. [https://doi.org/10.1002/\(SICI\)1097-0290\(19980920\)59:6<732::AID-BIT10>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1097-0290(19980920)59:6<732::AID-BIT10>3.0.CO;2-7)
20. Batstone DJ, Picioreanu C, van Loosdrecht MCM. Multidimensional modelling to investigate interspecies hydrogen transfer in anaerobic biofilms. *Water Res* 2006;40:3099–3108. <https://doi.org/10.1016/j.watres.2006.06.014>
21. Noguera DR et al. Simulation of multispecies biofilm development in three dimensions. *Water Science and Technology* 1999;39:123–130. [https://doi.org/10.1016/S0273-1223\(99\)00159-6](https://doi.org/10.1016/S0273-1223(99)00159-6)
22. Raskin L, Rittmann BE, Stahl DA. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Appl Environ Microbiol* 1996;62:3847–3857. <https://doi.org/10.1128/aem.62.10.3847-3857.1996>

23. Winogradsky S. Recherches sur les organismes de la nitrification. *Ann Inst Pasteur* 1891;5:557–616.
24. Van Kessel MAHJ et al. Complete nitrification by a single microorganism. *Nature* 2015;528:555–559. <https://doi.org/10.1038/NATURE16459>
25. Daims H et al. Complete nitrification by *Nitrospira* bacteria. *Nature* 2015;528:504–509. <https://doi.org/10.1038/nature16461>
26. Caranto JD, Lancaster KM. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc Natl Acad Sci U S A* 2017;114:8217–8222. <https://doi.org/10.1073/pnas.1704504114>
27. Kozłowski JA, Kits KD, Stein LY. Comparison of Nitrogen Oxide Metabolism among Diverse Ammonia-Oxidizing Bacteria. *Front Microbiol* 2016;7. <https://doi.org/10.3389/fmicb.2016.01090>
28. Stieglmeier M et al. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J* 2014;8:1135–1146. <https://doi.org/10.1038/ismej.2013.220>
29. Kits KD et al. Low yield and abiotic origin of N₂O formed by the complete nitrifier *Nitrospira inopinata*. *Nat Commun* 2019;10. <https://doi.org/10.1038/s41467-019-09790-x>
30. Kobayashi S et al. Nitric Oxide Production from Nitrite Reduction and Hydroxylamine Oxidation by Copper-containing Dissimilatory Nitrite Reductase (NirK) from the Aerobic Ammonia-oxidizing Archaeon, *Nitrososphaera viennensis*. *Microbes Environ* 2018;33:428–434. <https://doi.org/10.1264/jsme2.ME18058>
31. Koch H et al. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proceedings of the National Academy of Sciences* 2015;112:11371–11376. <https://doi.org/10.1073/pnas.1506533112>
32. Koch H et al. Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. *Science (1979)* 2014;345:1052–1054. <https://doi.org/10.1126/science.1256985>
33. Leung PM et al. A nitrite-oxidising bacterium constitutively consumes atmospheric hydrogen. *ISME J* 2022;16:2213–2219. <https://doi.org/10.1038/s41396-022-01265-0>
34. Qin W et al. Ammonia-oxidizing bacteria and archaea exhibit differential nitrogen source preferences. *Nat Microbiol* 2024;9:524–536. <https://doi.org/10.1038/s41564-023-01593-7>

35. Glaser B, Turrión M-B, Alef K. Amino sugars and muramic acid—biomarkers for soil microbial community structure analysis. *Soil Biol Biochem* 2004;36:399–407. <https://doi.org/10.1016/j.soilbio.2003.10.013>

Chapter 2.

Wetland sediment analogs reveal link between functional convergence, community assembly, and spatial organization in lactate-dependent methanogenesis

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Abstract

Wetlands play a crucial role in the global carbon cycle, serving as the largest natural source of methane (CH₄) emissions and a significant carbon sequestration sink. Within anaerobic wetland sediments, microbial consortia drive CH₄ production through obligate mutualistic interactions between short-chain fatty acid (SCFA) oxidizing bacteria and methanogenic archaea. These communities rely on close spatial organization to facilitate interspecies hydrogen (H₂), formate, and acetate transfer, yet their spatial dynamics in wetlands remain poorly understood due to the complexity of natural sediments. To address this, we investigated the impact of synthetic sediment analogs on the structure and function of lactate-degrading methanogenic communities. Wetland-derived methanogenic communities were enriched in hydrogel aggregates and biofilms growing on glass beads in chemostat reactors for 220 days. Both systems exhibited complete lactate degradation and CH₄ production yet differed in metabolic profiles and community composition. Fluorescence in-situ hybridization (FISH) revealed bacterial-methanogen associations in hydrogel aggregates, suggesting spatial proximity enhanced methanogenesis under the nutrient- and diffusion-limited conditions. A mathematical model predicted long-term microbial interactions under varying biogeochemical conditions. These findings provide new insights into microbial spatial organization and its role in wetland CH₄ cycling, highlighting synthetic sediment analogs as valuable tools for studying anaerobic microbial ecosystems.

2.1 Introduction

Wetlands represent a critical yet vulnerable node with an outsized impact on the global carbon cycle. Despite accounting for 5-8% of land area, wetlands sequester 20-30% of global soil organic carbon and release approximately 159-200 Tg of methane (CH_4) annually, contributing to ~30% of global CH_4 emissions [1]. Microbial communities harbored within anaerobic, waterlogged sediments produce CH_4 from organic carbon through a cascade of cross-feeding interactions often operating near thermodynamic equilibrium [2]. Primary degraders hydrolyze organic carbon — derived from plant detritus and root exudates — into sugars, peptides, lipids, and short-chain fatty acids (SCFAs) [3]. Syntrophic bacteria subsequently oxidize these hydrolysis byproducts into hydrogen (H_2), carbon dioxide (CO_2), and acetate before subsequent conversion to CH_4 by methanogenic archaea, which are physiological specialists that can only utilize hydrogen (H_2), carbon dioxide (CO_2), acetate, and methylated compounds for growth [4].

Lactate is a major byproduct of acidogenesis and a key intermediate within the relatively simple, yet highly cooperative methanogenic metabolic network. Syntrophic bacteria and sulfate-reducing-bacteria (SRB) in methanogenic environments primarily converted lactate to acetate, propionate, and H_2 , but their activity is limited by thermodynamic limitations [5–7]. Methanogenic archaea consume the H_2 produced by SCFA-oxidation to form an obligately mutualistic interaction that overcomes thermodynamic constraints and enhances CH_4 production [4]. However, methanogenic consortia are highly sensitive to biogeochemical perturbations, especially sulfate intrusion into coastal wetland sediments. SRB will oxidize lactate to acetate and H_2 in the absence of sulfate in sulfidogenic environments oxidize lactate while reducing sulfate and compete with methanogens for H_2 and acetate, diverting carbon away from CH_4 production [2, 8, 9]. Therefore,

characterizing the structure, stability, and productivity of lactate degradation microbial communities in wetland sediments and their response to local biogeochemistry is critical to predicting the fate of carbon in wetland ecosystems.

Understanding how metabolic interactions shape obligate mutualism and the spatial organization of methanogenic communities is essential to predict wetland carbon cycling dynamics. SCFA oxidizing bacteria and methanogenic archaea require close spatial proximity to facilitate interspecies H₂ transfer [10]. Diffusion limitations impose distinct spatial structures that sustain metabolic activity, as observed in anaerobic digestion granules [11, 12]. However, the complexity of sediment matrices limits both *in-situ* and *ex-situ* aggregate imaging, because sediment extractions fail to capture spatial organization [1, 13]. To overcome these challenges, synthetic sediment analogs provide a promising alternative for studying microbial spatial dynamics. Synthetic sediment analogs preserve spatial structures while reducing matrix complexity. Glass beads, sand particles, and microfluidic chips replicate sediment pore structures, provide attachment surfaces, and enhance interspecies interactions within biofilms [14–16]. Glass bead have recently been used as a synthetic sediment analog while studying chitin degradation [16]. However, glass cannot be cut with traditional section techniques thereby complicating spatial community analysis with *Fluorescence in situ hybridization* (FISH). Alternatively, hydrogels—water-retaining polymers that can be assembled into spherical aggregates — can rapidly encapsulate microbial communities to create synthetic aggregates that replicate the diffusion limitations of natural sediments while maintaining microscopic, molecular, and isotopic compatibility [17–19]. Despite their potential for studying spatial organization in long-term enrichments, synthetic sediment

analogs have yet to be systematically compared and the extent to which synthetic sediment analogs can replicate spatial organization remains unknown.

To address this gap, wetland-derived methanogenic communities were enriched in hydrogel aggregates and biofilms growing on glass beads in chemostat reactors for 220 days to compare the performance of these synthetic aggregate analogs. We hypothesized that hydrogel-based aggregates replicate the diffusion limitations in natural wetland sediments better than glass beads—a common method to facilitate cell attachment [16]. Both systems completely degraded lactate to CH_4 yet exhibited distinct metabolite and microbial community dynamics. *Fluorescence in situ hybridization* (FISH) was only conducted with hydrogel aggregates and revealed close physical associations between bacteria and methanogenic archaea near the aggregate periphery. We further developed a mathematical model to assess the long-term impact of biogeochemical perturbations on syntrophic SFCA degradation. By integrating synthetic sediment analogs and spatial analysis, this study provides new insights into the role of spatial organization in syntrophic methanogenic communities.

2.2 Material and Methods

2.2.1 *Wetland cell extraction for reactor inoculum*

A complex microbial community was extracted from a wetland soil sample taken from an urban lacustrine wetland (University of Washington Arboretum, Seattle, WA, USA - 47° 38' 31.12'' N, 122° 17' 47.01'' W). Samples were taken using a 2"x4" soil corer (AMS, American Falls, ID, USA) driven down 60 cm. Cores were transported to the lab on blue ice and stored at 4°C until processing. Cells were extracted in an anaerobic chamber (Coy Lab Products, Grass Lake, MI, USA) to minimize potential oxygen toxicity. Soil was first suspended (10% w/v) in modified lacustrine medium, followed by sieving (2 mm) to remove coarse particles. The sieved soil suspension was then paddle blended (Stomacher® 400 Circulator, Seward, UK) for 3 x 1 min at 250 rpm with 1-minute rests between runs. Samples were subsequently centrifuged at 200 g for 2 minutes to remove coarse organics and filtered through a 20 µm filter (Whatman™ Grade 41, Cytiva, Marlborough, MA, USA) to remove eukaryotes and fungal material.

2.2.2 *Synthetic sediment aggregate production*

Synthetic sediment aggregates were produced according to a previously described procedure described below [18]. A copolymer of polyethyleneglycol-dimethacrylate (PEGDMA) and polyvinyl alcohol (PVA), hereafter referred to as PEGDMA-PVA, was chosen as the synthetic sediment aggregate polymer due to its low biodegradability and low cytotoxicity. Nitrogen-sparged PEGDMA-PVA solutions were amended with either ammonium persulfate (APS, 3.64 µM final concentration) or tetramethylethylenediamine (TEMED, 4.93 mM final concentration) as polymerization catalysts. An ElveFlow pressure-based millifluidic flow

controller (Elvesys, France) driven by N₂-gas was used to control flow of both solutions at 700 $\mu\text{L}\cdot\text{min}^{-1}$, combining them in a low-volume static mixer manually inserted in vinyl tubing. Mixed PEGDMA-PVA monomers and catalysts were combined in identical static mixer with the wetland cell extract flowing at 700 $\mu\text{L}\cdot\text{min}^{-1}$. Droplets of cell-PEGDMA-PVA mixture were formed in a homemade co-flow fluidic junction (1.1-mm orifice size) using 10 cSt polydimethylsiloxane polymer (PMX-200 Silicone Fluid, Dow, MI, USA) with 10% cyclopentasiloxane and trimethylsiloxysilicate resin (DOWSIL™ RSN-0749, Dow, MI, USA) as carrier fluid flowing at 1.4 $\text{mL}\cdot\text{min}^{-1}$. Droplets polymerized for 10 minutes in a curing loop (tubing 3-mm inner diameter) before deposition in a flask with a N₂ headspace to prevent O₂ intrusion. Droplets were allowed to fully polymerize into beads overnight before washing.

Bead washing was performed inside an anaerobic chamber to protect the O₂-sensitive microorganisms in the wetland sediment extract. Beads were first strained over a 400- μm sieve, followed by suspension in carbon-free Lacustrine medium at approximately 1:5 bead to liquid volume ratio. Suspensions were centrifuged for 15 minutes at 1000 to separate oil from the aqueous phase. Oil was removed manually before straining beads again. Beads were then washed in carbon-free Lacustrine medium +0.1% Tween 80 as bio-compatible detergent (1:5 bead to liquid volume ratio) for 5 minutes. Beads were strained again, followed by three to four washing steps in carbon-free lacustrine medium, until remaining oil was removed. Beads suspended in carbon-free lacustrine medium in a 250 mL Schott bottle, sealed with a grey bromobutyl rubber stopper, and stored overnight at 37°C.

2.2.3 *Synthetic sediment aggregate and glass bead chemostat design and operation*

Two anaerobic chemostats were operated at 30 °C with lactate (1 mM influent concentration) as the only carbon source and electron donor. Custom 135mL glass, anaerobic, packed-bed chemostats that recirculated media from the bottom of the reactor to the top with an external recirculation pump were designed for this study (Figure S1). The chemostats were filled with 50mL of sterile lactate lacustrine medium and 42mL of sediment analog beads were added above a liquid permeable diffuser plate (P_o Frit: 160–250 µm). Reactor 2 (R₂) was filled with inoculated hydrogel beads. Reactor 1 (R₁) was filled with sterilized 2mm diameter glass beads (Millipore-Sigma, St. Louis, MO, USA) and inoculated with 21 mL of wetland sediment extract. The reactors were filled with medium to an operating volume of 86 mL with a 7mL headspace. Anoxic conditions were maintained by continuous flushing with nitrogen gas through a 0.2-micron Teflon filter (Millipore-Sigma, St. Louis, MO, USA) at a flowrate of 20 mL·min⁻¹ with a mass flow controller (Alicat scientific, Tucson, AZ, USA) maintained between 1-2 psig. After inoculation, the nitrogen gas flowrate was decreased to 0.5 mL·min⁻¹ and the reactors recirculated media at 12 L·h⁻¹ for 24 hours. The chemostats were then fed with lactate lacustrine media using a drip-feed at a rate of 4.3 mL·h⁻¹ (0.05 h⁻¹ dilution rate; 20 h hydraulic retention time). The feed rate was increased to 8.6 mL·h⁻¹ (0.1 h⁻¹ dilution rate; 10 h hydraulic retention time) on day 7 and maintained for the remainder of the experiment. The liquid volume was maintained with an effluent pump. The phosphate buffered media maintained the pH between 7.0 ± 0.1.

The lactate lacustrine media based on DSMZ 503 (anaerobic freshwater medium) with concentrations updated to reflect averaged porewater measurements of the top 60 cm of sediment at the wetland sampling site. The basal medium composition was as follows (in 1000mL MQ

water): 0.29 mL sodium lactate as 60% syrup (1mM), 6.4 mg NH_4Cl (0.12mM), 35.9 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.24mM), 33.2 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.16mM), 2 mL SL-10 trace element solution, 2 mL selenate-tungstate (Se-W) solution. The SL-10 trace element solution consisted of (in 1000 mL MQ water): 10 mL 7.7M HCl , 1.5 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.07 g ZnCl_2 , 0.15 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.006 g H_3BO_3 , 0.19 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.002 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.024 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.036 g $\text{Na}_2\text{MoO}_4 \cdot 4\text{H}_2\text{O}$. The Se-W solution contained (in 1000 mL MQ water): 0.5 g NaOH , 0.003 g $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 0.004 g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. Dissolved oxygen was removed from autoclaved media by bubbling the hot media with nitrogen gas and the media was kept under an oxygen-free atmosphere by flushing the headspace with nitrogen gas. After autoclaving, heat-sensitive media components were aseptically added as follows (in 1000mL MQ water): 0.3 mL 1M KHCO_3 , 0.4 mL 1M NaH_2PO_4 , 1.2 mL 0.5M Na_2HPO_4 , 0.55 mL 0.5% $(\text{NH}_4)_2\text{S}$, and 0.1 mL 7-vitamin solution. The 7-vitamin solution consisted of (in 100 mL MQ water): 0.1 g vitamin B12, 0.08 g p-aminobenzoic acid, 0.02 g D(+)-Biotin, 0.2 g nicotinic acid, 0.1 g Ca-pantothenate, 0.3 g pyridoxine hydrochloride, 0.2 g thiamine- $\text{HCl} \cdot 2\text{H}_2\text{O}$. Sulfide (0.04mM) was added to reduce the media after the addition of all media components.

2.2.4 Analytical methods

Liquid, dissolved gas, and gas samples were collected three times per week. Liquid samples were collected from a three-way sampling port attached to the influent and recirculation lines. Liquid samples were centrifuged at 21500 g for 20 minutes and the supernatant was stored at -20°C . A pH microelectrode (Orion; ThermoScientific) was used to measure pH prior to sample storage. Short-chain carboxylic acids (C2-C6, including C4 & C5 isoforms) were analyzed with ion chromatography (IC) on a Dionex ICS 5000+ equipped with an IonPac AS11HC analytical and

AS11 guard column at 30°C. Dissolved methane was measured using the headspace equilibration technique by injecting a 1 mL liquid sample into a N₂ filled Balch tube at 3 psig and vigorously vortexed for 2 minutes [20]. Volumetric gas flowrates were monitored with a mass flow controller (Alicat scientific, Tucson, AZ, USA). Off-gas was sampled with a flushed, gastight syringe. Methane was measured by gas chromatography (SRI Model 8610 GC) equipped with a flame ionization detector (FID) using nitrogen as carrier gas and a 182.9 cm HayeSep D column (SRI Instruments).

2.2.5 Rate calculations

Consumption and production rates of all dissolved and gaseous carbon compounds were measured throughout the enrichment. Consumption rates are negative and production rates are positive. Overall dissolved carbon rates were calculated from the liquid phase mass balance:

$$R_i = F_{out} \cdot C_{i,out} - F_{in} \cdot C_{i,in} \quad (2.1)$$

with R_i the molar rate ($\mu\text{mol} \cdot \text{h}^{-1}$), F the influent and effluent flow rates ($\text{L} \cdot \text{h}^{-1}$), and C_i the concentration of compound i ($\mu\text{mol} \cdot \text{L}$).

Methane production rates (R_{CH_4}) were calculated from a combined gas and liquid phase mass balance, where the gas phase mass balance was calculated from measurements of the inlet and outlet mole fraction (y_i) and volumetric gas flow ($F_{V,\text{gas}}$).

$$R_{\text{CH}_4} = (y_{\text{CH}_4,out} \cdot F_{G,out} - y_{\text{CH}_4,in} \cdot F_{G,in}) \cdot \left(\frac{P_{\text{atm}}}{RT} \right) + (F_{out} \cdot C_{i,out} - F_{in} \cdot C_{i,in}) \quad (2.2)$$

with P_{atm} the atmospheric pressure (bar), R the universal gas constant ($L \cdot bar \cdot \mu mol^{-1} \cdot K^{-1}$), and T the reactor temperature (K).

The overall carbon and electron balance was calculated from the consumption and production rate of all substrates (R_{in}) and products (R_{out}).

$$C \text{ balance } (\%) = \frac{R_{out}}{R_{in}} = \frac{2 * R_{acetate} + 3 * R_{propionate} + R_{CH_4}}{|3 * R_{lactate}|} \quad (2.3)$$

$$e^- \text{ balance } (\%) = \frac{R_{out}}{R_{in}} = \frac{8 * R_{acetate} + 12 * R_{propionate} + 8 * R_{CH_4}}{|14 * R_{lactate}|} \quad (2.4)$$

2.2.6 DNA extraction and metagenomic sequencing

Hydrogel and glass beads were removed from the reactor and stored in 2mL centrifuge tubes with reactor effluent at $-80^{\circ}C$ on days 29, 59, 101, 134, 168, 204, and 225. DNA was extracted from the beads by homogenization with a bullet blender (Next Advance, Troy, NY) pre-cooled with dry ice for 10 minutes. Filtered reactor effluent was collected on day 168, flash frozen, and stored at $-80^{\circ}C$. The filters were cut with sterile scissors before DNA extraction. DNA was extracted using the Qiagen DNeasy PowerBiofilm Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. DNA concentrations were quantified using the Qubit HS dsDNA (ThermoFisher Scientific, Waltham, MA, USA). DNA samples were sent to the Joint Genome Institute for metagenomic sequencing.

Plate-based DNA library preparation for Illumina sequencing was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Kapa Biosystems library preparation kit. 200 ng of sample DNA was sheared to 500 bp using a Covaris LE220 focused-ultrasonicator. The sheared DNA fragments were size selected by double-SPRI and then the selected fragments were end-repaired, A-tailed, and ligated with Illumina compatible sequencing adaptors from IDT containing a unique molecular index barcode for each sample library. The prepared libraries were quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1.5 reagent kits, S4 flowcell, following a 2x151 indexed run recipe.

2.2.7 Metagenomics data processing

Filtered reads from each chemostat were co-assembled with MetaHipMer 2 version 2.1.0.90-g7529d1f-master [command: mhm2.py -k 21,33,55,77,99 --post-asm-align --post-asm-abd][21] on 1,000 KNL nodes on the NERSC Cori system. Contigs smaller than 500 bp were removed. Alignment information was determined by mapping reads to the assembly reference with BBtools [22] version 38.95 [command: bbmap.sh -Xmx450g nodisk=true interleaved=true ambiguous=random mappedonly=t trimreaddescriptions=t usemodulo=t fast=t]. Coverage was determined by running BBTools version 38.95 [command: pileup.sh]. The IMG/M pipeline [23] was used to annotate the assembled contigs, and prokaryotic bins were predicted using metaBAT [24]. Contig taxonomy was predicted from the best LAST hits of coding sequences [25].

Metagenome bins from each of the four co-assemblies were de-replicated using dRep with default settings [26]. MAG taxonomy was assigned using GTDB-Tk v2.3.2. (29). MAG relative abundance was estimated using CoverM to map sequencing reads from each sample library to the shared MAG set. MAGs were annotated and metabolism was summarized using DRAM [27] v0.1.2 as implemented in KBase [28]. DRAM annotations were downloaded and used for functional analysis.

For gene-based analysis, taxonomic and functional profiles were retrieved from the JGI IMG/M portal using the Abundance Profile tool. For functional profiles, the estimated gene copies of KEGG KOs were used and center-log₂ ratio (clr) normalized on a per sample basis based on the method from ALDEx2 [29]. For taxonomic profiles, the estimated gene copies for class-level GTDB taxonomy at 60+ % identity were used and normalized using relative abundance.

2.2.8 *Statistical analysis*

Differences in abundance were assessed across the reactors and time using the Kruskal-Wallis H-test followed by multiple test correction using the Benjamini-Hochberg method and post-hoc analysis with Dunn's test. Distance-based PERMANOVA and ANOSIM tests were used to evaluate sample groupings based on reactor.

2.2.9 *Fluorescence in-situ hybridization analysis*

Synthetic sediment aggregates were collected on day 101 for fluorescence *in-situ* hybridization (FISH) analysis, fixed in 4% paraformaldehyde (PFA), and stored at 4°C until further processing. Aggregate sectioning, hybridization, and visualization were carried out as previously described

[30, 31] with modifications described in supplementary material. Sections were labelled with the fluorescein-labeled universal archaeal probe ARCH915 and Cy3-labeled universal bacterial probe EUB388 (Table S1).

2.2.10 Modeling

One-dimensional reactive-transport model of the biofilm aggregates was constructed to evaluate the spatial distribution of syntrophic and competitive interactions of a lactate degrading methanogenic community comprised of representative species [9, 32, 33]. Individual aggregates were modeled as a one-dimensional, spherical biofilm assuming microorganisms grew on the surface of glass beads in an ideally mixed chemostat supplied with a constant lactate concentration with a 0.1 h^{-1} dilution rate. Microbial community response to sulfate was simulated by supplying sulfate at a constant concentration on day 100. The numerical model consisted of a biofilm domain and an aqueous domain. The biofilm domain was comprised of particulate biomass components convectively transported by microbial growth and dissolved solute components transported by diffusion. It was assumed that the biofilm domain was in contact with the aqueous domain.

Time-dependent mole balances for all solutes in the biofilm were expressed as a function of solute concentration with source terms for diffusive transport and volumetric reaction rates based on the stoichiometry and kinetics of the wetland microbial community model (Supplemental Tables S2-11). A zero-flux boundary condition for all soluble components was set at the center of the sediment aggregates. Mass-transfer resistance at the aggregate surface was neglected for all solutes, causing solute concentrations at the hydrogel surface to be equivalent to the bulk liquid. It was assumed that the biofilm was comprised of active and inert biomass [33]. Time-dependent mole balances for all biomass component in the biofilm were expressed as a function of biomass

concentration with a source term for biomass growth and advective transport by growth. The biofilm was assumed to have a constant density of 50 mg/L with an initial uniform distribution of biomass [33]. Time dependent solute balances in the bulk liquid were modeled as a zero-dimensional chemostat with a source term for the biofilm exchange flux.

The wetland microbial community model considered the activity 6 representative species—*Desulfovibrio*-like sulfate reducing bacteria (*SRB*), lactate fermenting bacteria (*LF*), propionate oxidizing bacteria (*PO*), *Methanospirillum*-like hydrogenotrophic methanogen (*Met,H₂*), *Methanothrix*-like acetoclastic methanogen (*Met,Ace1*), and *Methanosarcina*-like acetoclastic methanogen (*Met,Ace2*). Microbial growth was modeled with Monod kinetics and a constant biomass decay rate. Sulfate reducing and propionate oxidizing bacteria are sensitive to the partial pressure of hydrogen [7, 11]. Therefore, thermodynamic constraints were implemented to model their activity [7]. The growth of the hydrogenotrophic and acetoclastic methanogens could only be supported by hydrogen and acetate cross-feeding, respectively. All modeling parameters were obtained from literature and reported in Supplemental Tables S2.2-11.

Initial concentrations throughout the synthetic sediment aggregates were assumed to be equivalent. Steady-state behavior was evaluated after simulating biomass growth and decay over 365 days. The model was implemented and evaluated in *COMSOL Multiphysics* (v6.2 Comsol Inc, Burlington, MA). Detailed methods and assumptions along with all model equations, process matrix, and a complete list of parameters are provided in Supplemental Material.

The distribution of syntrophic H₂ cross-feeding throughout the biofilm on day 365 was evaluated with an electron considering H₂ consumption by hydrogenotrophic methanogens and the activity of sulfate reducing, lactate fermenting, and propionate oxidizing bacteria:

$$H_2 \text{ Syntrophy Ratio} = \frac{2 R_{Met,H_2}^H}{12 R_{SRB}^L + 12 R_{SRB}^{LS} + 2.6 R_{SRB}^{H_2} + 12 R_{LF}^{LF} + 14 R_{PO}^P} \quad (5)$$

where R_j^i is the volumetric reaction rate of species j performing process i defined in supplementary Table S2.9.

2.3 Results

2.3.1 Complete lactate oxidation to methane by sediment analog chemostats

Two methanogenic microbial communities were enriched from wetland sediment in chemostats containing synthetic sediment analogs (**Figure 2.1**). The glass beads in reactor 1 (R₁) replicated the attachment of microbial communities to mineral surfaces, while the hydrogel-embedded microbial communities in reactor 2 (R₂) mimicked the porosity of sediment aggregates. Lactate served as the sole carbon source and electron donor, while hydrogen sulfide (H₂S) and ammonium (NH₄⁺) were the sole sulfur and nitrogen sources, respectively. Lactate limiting conditions were imposed to assess the impact of diffusion limitations on community assembly. Biofilm formation on the surface of the glass beads and within the hydrogel aggregates was visible throughout the enrichment (Figure S2.2).

Each chemostat progressed through a distinct enrichment before achieving complete methanogenesis (Figure 1). Following a 7-day start-up, the dilution rate was increased from 0.05 h⁻¹ to 0.1 h⁻¹ (100 μmol-Lactate·h⁻¹) to enhance biofilm formation and wash out the planktonic community. However, it also contributed to acetate and propionate production by the glass-surface attached (R₁) and hydrogel-embedded (R₂) chemostats and their production peaked 2 weeks after increasing the dilution rate. The reactors had contrasting acetate to propionate production ratios (Mann-Whitney p-value = 2.21e-5). Acetate was the primary product in the glass bead chemostat (R₁: 2.04 ± 0.68), while propionate production was favored by the hydrogel chemostat (R₂: 0.67 ± 0.12). Acetate and propionate were consumed as the enrichment progressed and corresponded with increasing CH₄ production. Stead-state was achieved by R₁ and R₂ on day 78 and 95 though the time required to achieve steady-state was negatively correlated to propionate production. Stable

CH₄ production accounted for half of the carbon exiting the reactors (R₁: 48.02 ± 11.673 %; R₂: 47.5 ± 9.552 %) and was produced at a rate 10 times greater than observed at the lacustrine wetland field site. Although CO₂ was not measured in this study, it presumably accounted for the remaining carbon since the electron balances closed within 20% (Figure S3).

2.3.2 Sediment analogs demonstrate taxonomic and functional convergence

Metagenome sequencing was conducted on sediment aggregates collected from the chemostats every 28.0 ± 13.8 days. The chemostats were sampled twice (T₁₄ - T₂₄) before the transition to methanogenesis and five times after achieving steady-state (T₃₆ - T₇₁). Low DNA recovery from the sediment analogs prevented metagenomic sequencing at specific timepoints. Libraries averaged 109.6 ± 77.9 million reads with 88.1 ± 6.8 % of reads being assembled into 15.2 ± 10.9 gigabase assemblies comprised of 608.6 ± 454.2 thousand scaffolds. Detailed sequencing and assembly statistics are available in the Supplemental Data File.

The sediment analogs maintained distinct order-level taxonomic abundance and functional trajectories, as confirmed by both PERMANOVA and ANOSIM analyses ($p < 0.001$; Figure 2). The glass bead demonstrated a high degree of similarity throughout the incubation, whereas the hydrogel chemostat was initially dominated by a single order and became more diverse throughout the enrichment (**Figure 2.2A**). A total of 48 taxonomic orders and 106 KOs were significantly different across the reactors, while 12 orders and 101 KOs were significantly different after steady-state was achieved (Kruskal Wallis H-test: $p < 0.05$). Despite the initial taxonomic and functional divergence, the chemostat communities converged by the end of the enrichment (**Figure 2.2B**).

A diverse community of Gammaproteobacteria, Thermodesulfovibrionia, Bacteroidia, and Desulfurmononadia were initially enriched by the glass bead chemostat (R₁). However, their abundance decreased throughout the enrichment (Figure S4). Hydrogenotrophic methanogens belonging to Methanobacteria, and Anaerolineae became more abundant as the chemostat transitioned to methanogenesis at T₃₆ and maintained their abundance for the remainder of the enrichment. The abundance of acetoclastic methanogens belonging to Methanosarcinia increased throughout the enrichment but exhibited class specific dynamics. Methanosarcinales abundance plateaued at T₃₆ (day 101), while Methanotrichales continued to increase in abundance throughout the enrichment eventually comprising 30% of the community. The only other minor taxa to become more abundant in the methanogenic phase was Alphaproteobacteria. Conversely, the hydrogel chemostat (R₂) initially dominated by Negativicutes and Gammaproteobacteria with the Negativicutes accounting for 43.2 % of the community at T₂₄ (day 59) and decreased in abundance throughout the enrichment. Over the course of the enrichment, Methanobacteria, Thermodesulfovibrio, Bacteroidia, and Desulfuromonadia became increasingly dominant as the chemostat transitioned to methanogenesis. (Figure S2.5). Notably, Methanosarcinia remained relatively stable throughout the enrichment. While chemostats exhibited a high degree of taxonomic and functional divergence at the beginning of the enrichment, the reactors become taxonomically and functionally similar by day 225 (T₇₁) with the hydrogel community resembling the glass bead reactor on day 134 (T₄₅).

Assemblies from each time point were used to create a set of metagenomic bins for analysis. The resulting 134 bins had an average completeness of $84.5 \pm 15.2\%$ and contamination of $1.8 \pm 1.8\%$ with genome sizes of 3.4 ± 1.7 Mbp. Bins were assigned to 31 different phyla with the most

common being Bacteroidota (n=20), Proteobacteria (n=18), Patescibacteria (n=11), Chloroflexota (n=7), Actinobacteriota (n=7), and Desulfobacterota (n=7). The scaffold and bin abundances were similar with several notable differences (Figure S6). Patescibacteria and Eisenbacteria were not detected in the scaffold analysis. Additionally, there were significant differences between *Thermodesulfovibrionia*, *Coriobacteria*, and *Methanosarcinia* abundance.

2.3.3 Differences in metabolic pathway enrichment between sediment analogs reactors

Key genes associated with the SCCA oxidation, methanogenesis, hydrogen oxidation, and sulfur cycling pathways displayed minimal differential abundance between the sediment analog reactor (Figure 2.3). Notable exceptions include the alcohol dehydrogenase and ATP citrate lyase—an enzyme required to fix CO₂ via the reductive TCA cycle—were more abundant in the glass bead reactors. However, the reactors preferentially enriched specific genes within each pathway.

The reactors were fed with a racemic mixture of L- and D-lactate. Lactate racemase—an enzyme required for to interconvert D- and L-lactate—along with the genes required to oxidize both chiral forms of lactate. D-lactate dehydrogenase (*dld*) and lactate racemase had inverse abundance profiles in the glass bead chemostat. Additionally, *lctC*—a gene associated with the conversion of lactate to acetate via the Wood-ljungdahl pathway—was the least abundant type of L-lactate, while *lldG* was the most abundant. Reversible genes associated with the production and consumption of propionate (*prpE*, *epi*, and *mcmA*) were constitutively abundant across all time points.

Methyl-coenzyme M (*mcrA*), a key gene in the methanogenic pathway, became more abundant though out the enrichment. Similar trends were also observed of the archaeal carbon-monoxide dehydrogenase (*cdhB*), F₄₂₀ hydrogenase, and the [NiFe]-hydrogenase *mvhA*. Other abundant

hydrogenases not associated with methanogenesis included the ferredoxin hydrogenase *hoxA* and the [NiFe]-hydrogenase *hyaB*, while the group 2a [NiFe]-hydrogenase *hucL* was significantly less abundant.

A distinct pattern was observed in the sulfur cycling pathway. Sulfide was provided as the primary sulfur source with lower concentrations of sulfur-containing vitamins present in the medium. However, the abundance of the sulfide:quinone oxidoreductase (*sqr*) and the sulfate adenylyltransferase (*sat*) were significantly less abundant than cysteine desulfurase—a cysteine-dependent sulfurtransferase required for sulfide and Fe-S cluster formation. It was among the most highly abundant KOs in both reactors and was associated with the similarly abundant branched-chain amino acid transporter and nitrogenase (*nifK*). However, the gene associated with the production of thiosulfite from serine (*cysM*) was less abundant than *sqr* and *sat*. The high abundance of the branched-chain amino acid transporter across both reactors underscores the importance of amino acid metabolism to the methanogenic communities. Peptidoglycan, a primary component of cell walls, represents a major source of amino compounds. The surfaced attached communities in the glass bead reactor had a greater functional potential for peptidoglycan recycling than the hydrogel embedded communities (**Figure 2.3**).

2.3.4 Bacterial-archaeal associations demonstrated with fluorescent in-situ hybridization

The spatial distribution of the methanogenic community entrapped within a hydrogel aggregate cross-section was evaluated with *fluorescent in-situ hybridization* (**Figure 2.4**). The hydrogel was collected 1 week after transitioning to steady-state (day 109), while glass beads were not imaged considering the difficulty of obtaining glass cross-sections. Using the 16S rRNA targeted oligonucleotide FISH probes EUB 338 (targeting bacteria; red; **Figure 2.4A**) and ARCH 915

(targeting archaea; green; **Figure 2.4B**), the spatial distribution of bacteria and archaea within a synthetic sediment aggregate was successfully visualized. The microbial community extends approximately 40 μM from the aggregate periphery (upper left) to form a synthetic aggregate biofilm with an approximate aggregate volume fraction of 5.88 %. Bacteria were distributed throughout the aggregate biofilm while archaea were confined to the initial 20 μM from the aggregate periphery with individual archaeal cells found in close spatial proximity to bacterial cells (**Figure 2.4D**). Distinct regions of overlap between archaeal and bacterial cells would suggest efficient metabolite cross-feeding over short intracellular distances facilitated the complete methanogenesis observed the hydrogel reactor. The distribution of cells within the hydrogel aligned with images collected after staining with the nucleic acid stain SYBR Green (Figure S2.13).

2.3.5 Reactive transport modeling predicts distinct spatial niches under sulfate perturbations

Under sulfate depletion, SCFA-oxidizing SRB and propionate-oxidizing bacteria depend on the consumption of H_2 by hydrogenotrophic methanogens H_2 to prevent thermodynamic inhibition. However, SRBs will outcompete hydrogenotrophic methanogens in sulfate replete environments due to their higher H_2 affinity. Although this competition has been extensively studied in planktonic culture, the impact of sulfate on these syntrophic and competitive interactions within sediment aggregates remains underexplored. A one-dimensional fixed thickness, spherical hydrogel reactive transport model was developed to assess the impact of sulfate on syntropy and competition in a lactate degrading methanogenic community comprised of 6 representative species—*Desulfovibrio*-like sulfate reducing bacteria, lactate fermenting bacteria, propionate oxidizing bacteria, *Methanospirillum*-like hydrogenotrophic methanogens, *Methanothrix*-like acetoclastic methanogens and *Methanosarcina*-like acetoclastic methanogens. Microbial activity

was modeled with a thermodynamically constrained kinetic model. The SRB model accounted for lactate-oxidation, lactate-based sulfate-reduction, and hydrogen-based sulfate-reduction. Syntrophic regions within the biofilm model were evaluated with an electron balance that compared the rate of H₂ consumption by hydrogenotrophic methanogens to carbon degradation by sulfate reducing, lactate fermenting, and propionate oxidizing bacteria. The long-term steady-state response to sulfate perturbations was simulated by altering the influent sulfate concentration.

Spatial resource gradients within the biofilm promoted hydrogen-based cross-feeding between bacteria and hydrogenotrophic methanogens with a fully syntrophic community was observed in the absence of sulfate (**Figure 2.5A**). The introduction of sulfate resulted in competitive interaction regions at the aggregate periphery and syntrophic H₂ cross-feeding in the biofilm interior. Increasing sulfate concentrations significantly decreased syntrophic interaction and no syntrophic regions were observed at sulfate to lactate molar ratios above 0.48.

Metabolite and biomass distributions within the aggregate were evaluated at sulfate to lactate molar ratios of 0, 0.3, and 0.5 (**Figure 2.5C**). All species were present in the absence of sulfate with a nearly even biomass distribution. A syntrophic-competitive boundary was not observed within the biofilm at a sulfate to lactate ratio of 0.3, however the H₂ syntrophic ratio throughout the biofilm had decreased compared to lower sulfate concentrations. Sulfate diffusing into the aggregate stimulated H₂-dependant sulfate reduction by SRB resulting in competition between hydrogenotrophic methanogens and SRB for H₂ in the sulfate replete biofilm periphery. Despite the competition for H₂, hydrogenotrophic methanogens were able to scavenge H₂ in the presence of sulfate. Presumably, the low sulfate concentration limited the H₂ utilization rate of SRB, which would outcompete the hydrogenotrophic methanogen at elevated sulfate concentrations.

Additionally, hydrogenotrophic methanogen growth in the syntrophic biofilm interior pushed active biomass into the sulfidogenic biofilm region thereby increasing methanogenic activity. At sulfate to lactate molar ratios above 0.48, the SRB completely outcompeted hydrogenotrophic methanogens throughout the entire biofilm (**Figure 2.5C**). However, acetoclastic methanogens continued to produce methane in the presence of sulfate though methane production decreased (**Figure 2.5B**).

2.4 Discussion

Sediment aggregates shape the structure and function of the microbial communities [34]. Individual aggregates and minerals provide a surface for microbial attachment and biofilm formation, while the diffusion through porous sediment matrices limit oxygen and nutrient availability [35]. Despite the influence of sediment aggregates on microbial activity, replicating their spatial structures with reduced complexity matrices remains challenging, especially for anaerobic biofilms where knowledge of spatial-function relationships remains limited [15, 36–38]. Therefore, there is a need to understand the processes governing the spatial assembly of metabolic pathways, community structure, and carbon utilization within sediment aggregates [1, 13, 39].

In this study, we compared the structure, function, and spatial organization of methanogenic wetland communities under lactate limitations using two types of synthetic sediment analogs to replicate sediment porosity. Glass beads—a common method to promote biofilm development [15, 16, 36]—replicated the attachment of microbes to sediment surfaces, while sediment extract was gently entrapped in hydrogel aggregates to replicate aggregate microsites [18, 19]. Despite their distinct trajectories, the sediment analog chemostats functionally and taxonomically converged to achieved stable methanogenesis. However, the hydrogel aggregates allowed for high-resolution visualization of bacterial and archaeal spatial organization. These results highlight hydrogels as tractable model systems to study the influence of spatial organization on community structure and function in complex porous environments.

The initial metabolic trajectories of the glass bead (R_1) and hydrogel (R_2) chemostats initially diverged. R_1 favoring acetate production whereas propionate was the primary product of R_2 .

Presumably, the sediment analogs applied distinct selective pressures on the wetland extract inoculum, especially the free radical polymerization required to manufacture the hydrogel aggregates [18]. Initial stressor experienced by the hydrogel community might have influenced the early dominance of Negativicutes, a class of gram-negative Bacillota [40]. Their correlation with the propionate production was expected since Bacillota can ferment lactate to acetate and propionate in a 1:2 ratio without H₂ accumulation via the methylmalonyl-CoA or acrylyl-CoA pathways (**Table 2-1**; Reaction 1-2) [41].

Both reactors initially accumulated propionate before transitioning to methanogenesis. Syntrophic propionate oxidation accounts for 27% of total CH₄ emissions in lake sediments [42]. However, it represents a rate-limiting step of anaerobic carbon degradation that operates near the thermodynamic threshold required for ATP synthesis in the absence of hydrogen (**Table 2-1**; Reaction 5; $\Delta G^{o1} = 71.7 \text{ kJ/mol}_{\text{propionate}}$) [6, 43]. Propionate depletion coincided with the increased abundance of Methanobacteria—a class of hydrogen utilizing methanogenic archaea. The reliance of syntrophic propionate oxidation on the low hydrogen partial pressures maintained by hydrogenotrophic methanogens is well established (**Table 2-1**; Reaction 11; $\Delta G^{o1} = -105.45 \text{ kJ/mol}_{\text{propionate}}$) [4, 6] and was a rate-limiting bottleneck for both chemostats. Functional convergence under the prolonged lactate limitations, despite initial divergences, highlights the functional redundancy and resiliency of wetland microbial communities.

Metagenomic analysis provided additional insights into the taxonomic and functional convergence of the sedimental analog chemostat and functional capabilities of the microbial community. The enrichment of hydrogenotrophic Methanobacteria and acetoclastic Methanosarcina from wetland sediment was expected [44]. However, the increasing abundance of Methanotrichales abundance

and plateauing abundance of Methanosarcinales after complete acetate consumption was less expected. Their succession reflects the niche of these acetoclastic orders and the high acetate affinity of Methanotrichales relative to Methanosarcinales [45]. Differential lactate dehydrogenase gene abundance pointed to diverse lactate utilization strategies. However, challenges with interpreting function from metagenomic data was highlighted by the constitutive abundance of the genes *prpE*, *epi*, and *mcmA*, associated with the methylmalonyl-CoA pathway. These reversible enzymes can catalyze formation of propionate by lactate fermentation (**Table 2-1**; Reaction 1) [46] and the oxidation of propionate to acetate (**Table 2-1**; Reaction 5) [41]. Predicting the precise functional of these pathways from metagenomic data alone difficult. Orthogonal measurements from site specific ^{13}C labeling are required to parse the flux through this simplified methanogenic network [46]. Notably, the high cysteine desulfurase abundance and branched-chain amino acid transporters relative to *sat* and *sqr* suggests amino acid catabolism promotes organic sulfur cross-feeding and recycling under sulfur limitations [47] .

Complex cross-feeding within a microbial community creates millimeter-scale nutrient gradients within sediment pores impacting the availability of nutrients required for growth [13, 14, 19, 48, 49]. Synthetic sediment analogs offer a unique approach to investigate ecologically relevant spatial interactions at the single-cell level by overcoming the limitations of visualizing microbes *in-situ* in opaque sediment matrices. The spatial structure of the methanogenic community within the hydrogel aggregate was visualized at the taxonomic-level with *fluorescence in-situ hybridization* (FISH). We observed the development of a 50 μm thick structured biofilm with a high degree of bacteria and archaea intermixing within 20 μm of the aggregate periphery. Intermixing between short-chain carboxylic acid oxidizers and methanogens has been observed in anaerobic digestion

granules and a syntrophic biofilm, where it facilitated efficient interspecies H_2 and formate transfer [11, 37, 50]. Archaea and bacteria intermixing at the aggregate periphery would similarly prevent the thermodynamic inhibition of lactate, propionate, acetate oxidation resulting from H_2 accumulation within the aggregate [6]. Additionally, the aggregate biofilm thickness did not exceed $60\mu m$ throughout this study, reflects the thickness of sediment biofilms and the extent of ^{13}C assimilation by a hydrogel embedded wetland microbial community [19]. Biofilms exceeding these thicknesses would presumably experience lactate diffusion limitations [37]. The inability to perform similar analysis on the glass beads without a confocal scanner laser microscope highlights the advantage of hydrogels as a tool to study the spatial organization of sediment microbial communities.

The role of spatial organization in facilitating syntrophic methanogenesis was demonstrated by the intermixing of bacteria and archaea in the hydrogel aggregates. Our reactive transport model illustrates that diffusion gradients led to the formation of distinct metabolic niches within sediment aggregates. Spatial niches can promote syntrophic and mutualistic interaction within the aggregate interior, while nutrient limitations at the aggregate periphery led to competition [35]. The competition between sulfate reducing bacteria (SRB) and hydrogenic methanogens under varying sulfate concentrations is a well-studied example of niche differentiation [36, 37, 50, 51]. Similar diffusion gradients for lactate, sulfide, and H_2 would shaping the spatial and functional interactions within the sediment analog aggregates studied here. The predicted persistence of syntrophic zones under sulfate stress demonstrates mechanisms that might enable these sensitive archaea to thrive when bulk conditions appear unfavorable [14, 35].

This study highlights the use of synthetic sediment analogs as a tractable tool to study the spatial structure, function, and organization of methanogenic communities. Glass bead attached and hydrogel embedded communities effectively converted lactate to methane, despite initial taxonomic and functional divergence. Notably, the hydrogel aggregates enabled single-cell visualization with FISH, which highlighted spatial proximity as a mechanism unpinning syntrophic interactions. Future studies should use the hydrogel aggregates to and ^{13}C labeled carbon study the influence of substrate diversity on community structure and their measure their assimilation by specific taxa with FISH-NanoSIMS (Nanoscale secondary ion mass spectrometry). Integrating these results into ecosystem scale models will further advance our understanding of sediment porosity on methane emissions from wetland environments [1].

References

1. Candry P et al. Microbially mediated climate feedbacks from wetland ecosystems. *Glob Chang Biol* 2023;**29**:5169–5183. <https://doi.org/10.1111/gcb.16850>
2. Muyzer G, Stams AJM. The ecology and biotechnology of sulphate-reducing bacteria. *Nat Rev Microbiol* 2008;**6**:441–454. <https://doi.org/10.1038/nrmicro1892>
3. Sokol NW et al. Life and death in the soil microbiome: how ecological processes influence biogeochemistry. *Nat Rev Microbiol* 2022;**20**:415–430. <https://doi.org/10.1038/s41579-022-00695-z>
4. Stams AJM, Plugge CM. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. *Nat Rev Microbiol* 2009;**7**:568–577. <https://doi.org/10.1038/nrmicro2166>
5. Zellner G, Neudörfer F, Diekmann H. Degradation of lactate by an anaerobic mixed culture in a fluidized-bed reactor. *Water Res* 1994;**28**:1337–1340. [https://doi.org/10.1016/0043-1354\(94\)90299-2](https://doi.org/10.1016/0043-1354(94)90299-2)
6. Schink B. Energetics of syntrophic cooperation in methanogenic degradation. *Microbiology and Molecular Biology Reviews* 1997;**61**:262–280. <https://doi.org/10.1128/mmbr.61.2.262-280.1997>
7. Noguera DR et al. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. *Biotechnol Bioeng* 1998;**59**:732–746. [https://doi.org/10.1002/\(SICI\)1097-0290\(19980920\)59:6<732::AID-BIT10>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1097-0290(19980920)59:6<732::AID-BIT10>3.0.CO;2-7)
8. Robinson JA, Tiedje JM. Competition between sulfate-reducing and methanogenic bacteria for H₂ under resting and growing conditions. *Arch Microbiol* 1984;**137**:26–32. <https://doi.org/10.1007/BF00425803>
9. Noguera DR et al. Simulation of multispecies biofilm development in three dimensions. *Water Science and Technology* 1999;**39**:123–130. [https://doi.org/10.1016/S0273-1223\(99\)00159-6](https://doi.org/10.1016/S0273-1223(99)00159-6)
10. Boone DR, Johnson RL, Liu Y. Diffusion of the Interspecies Electron Carriers H₂ and Formate in Methanogenic Ecosystems and Its Implications in the Measurement of K_m for H₂ or Formate Uptake. *Appl Environ Microbiol* 1989;**55**:1735–1741. <https://doi.org/10.1128/aem.55.7.1735-1741.1989>
11. Batstone DJ, Picioreanu C, van Loosdrecht MCM. Multidimensional modelling to investigate interspecies hydrogen transfer in anaerobic biofilms. *Water Res* 2006;**40**:3099–3108. <https://doi.org/10.1016/j.watres.2006.06.014>

12. Santegoeds CM et al. Distribution of Sulfate-Reducing and Methanogenic Bacteria in Anaerobic Aggregates Determined by Microsensor and Molecular Analyses. *Appl Environ Microbiol* 1999;**65**:4618–4629. <https://doi.org/10.1128/AEM.65.10.4618-4629.1999>
13. Wilpiseski RL et al. Soil Aggregate Microbial Communities: Towards Understanding Microbiome Interactions at Biologically Relevant Scales. *Appl Environ Microbiol* 2019;**85**. <https://doi.org/10.1128/AEM.00324-19>
14. Borer B, Tecon R, Or D. Spatial organization of bacterial populations in response to oxygen and carbon counter-gradients in pore networks. *Nat Commun* 2018;**9**:769. <https://doi.org/10.1038/s41467-018-03187-y>
15. Valenzuela JJ et al. Origin of biogeographically distinct ecotypes during laboratory evolution. *Nat Commun* 2024;**15**:7451. <https://doi.org/10.1038/s41467-024-51759-y>
16. Rodríguez-Ramos J et al. Environmental matrix and moisture influence soil microbial phenotypes in a simplified porous media incubation. *mSystems* 2025. <https://doi.org/10.1128/msystems.01616-24>
17. Lozano-Andrade CN et al. Establishment of a transparent soil system to study *Bacillus subtilis* chemical ecology. *ISME Communications* 2023;**3**. <https://doi.org/10.1038/s43705-023-00318-5>
18. Candry P, Godfrey BJ, Winkler MK-H. Microbe-cellulose hydrogels as a model system for particulate carbon degradation in soil aggregates. *ISME Communications* 2024;**4**. <https://doi.org/10.1093/ismeco/ycae068>
19. Johnston J et al. Increasing aggregate size reduces single-cell organic carbon incorporation by hydrogel-embedded wetland microbes . *ISME Communications* 2024.
20. Kampbell DH, Vandegrift SA. Analysis of Dissolved Methane, Ethane, and Ethylene in Ground Water by a Standard Gas Chromatographic Technique. *J Chromatogr Sci* 1998;**36**:253–256. <https://doi.org/10.1093/chromsci/36.5.253>
21. Hofmeyr S et al. Terabase-scale metagenome coassembly with MetaHipMer. *Sci Rep* 2020;**10**:10689. <https://doi.org/10.1038/s41598-020-67416-5>
22. Bushnell B. BBMap. sourceforge.net/projects/bbmap/.
23. Chen I-MA et al. The IMG/M data management and analysis system v.7: content updates and new features. *Nucleic Acids Res* 2023;**51**:D723–D732. <https://doi.org/10.1093/nar/gkac976>
24. Kang DD et al. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* 2015;**3**:e1165. <https://doi.org/10.7717/peerj.1165>

25. Clum A et al. DOE JGI Metagenome Workflow. *mSystems* 2021;**6**. <https://doi.org/10.1128/mSystems.00804-20>
26. Olm MR et al. dRep: a tool for fast and accurate genomic comparisons that enables improved genome recovery from metagenomes through de-replication. *ISME J* 2017;**11**:2864–2868. <https://doi.org/10.1038/ismej.2017.126>
27. Aroney STN et al. CoverM: read alignment statistics for metagenomics. *Bioinformatics* 2025;**41**. <https://doi.org/10.1093/bioinformatics/btaf147>
28. Arkin AP et al. KBase: The United States Department of Energy Systems Biology Knowledgebase. *Nat Biotechnol* 2018;**36**:566–569. <https://doi.org/10.1038/nbt.4163>
29. Fernandes AD et al. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2014;**2**:15. <https://doi.org/10.1186/2049-2618-2-15>
30. Gottshall EY et al. Sustained nitrogen loss in a symbiotic association of Comammox Nitrospira and Anammox bacteria. *Water Res* 2021;**202**:117426. <https://doi.org/10.1016/j.watres.2021.117426>
31. Nielsen PH, Daims H, Lemmer H. FISH Handbook for Biological Wastewater Treatment. IWA Publishing, 2009.
32. Wanner O, Gujer W. A multispecies biofilm model. *Biotechnol Bioeng* 1986;**28**:314–328. <https://doi.org/10.1002/bit.260280304>
33. Eberl H et al. Mathematical Modeling of Biofilms. IWA, 2006.
34. Tiedje JM et al. Anaerobic processes in soil. *Plant Soil* 1984;**76**:197–212. <https://doi.org/10.1007/BF02205580>
35. Ebrahimi A, Or D. Hydration and diffusion processes shape microbial community organization and function in model soil aggregates. *Water Resour Res* 2015;**51**:9804–9827. <https://doi.org/10.1002/2015WR017565>
36. Raskin L, Rittmann BE, Stahl DA. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Appl Environ Microbiol* 1996;**62**:3847–3857. <https://doi.org/10.1128/aem.62.10.3847-3857.1996>
37. Brileya KA et al. Biofilm growth mode promotes maximum carrying capacity and community stability during product inhibition syntrophy. *Front Microbiol* 2014;**5**. <https://doi.org/10.3389/fmicb.2014.00693>

38. Nielsen AT et al. Role of commensal relationships on the spatial structure of a surface-attached microbial consortium. *Environ Microbiol* 2000;**2**:59–68. <https://doi.org/10.1046/j.1462-2920.2000.00084.x>
39. Baran R et al. Exometabolite niche partitioning among sympatric soil bacteria. *Nat Commun* 2015;**6**:8289. <https://doi.org/10.1038/ncomms9289>
40. Marchandin H et al. *Negativicoccus succinicivorans* gen. nov., sp. nov., isolated from human clinical samples, emended description of the family Veillonellaceae and description of Negativicutes classis nov., Selenomonadales ord. nov. and Acidaminococcaceae fam. nov. in the bacterial phylum Firmicutes. *Int J Syst Evol Microbiol* 2010;**60**:1271–1279. <https://doi.org/10.1099/ijs.0.013102-0>
41. Seeliger S, Janssen PH, Schink B. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiol Lett* 2002;**211**:65–70. [https://doi.org/10.1016/S0378-1097\(02\)00651-1](https://doi.org/10.1016/S0378-1097(02)00651-1)
42. Rothfuss F, Conrad R. Thermodynamics of methanogenic intermediary metabolism in littoral sediment of Lake Constance. *FEMS Microbiol Ecol* 1993;**12**:265–276. <https://doi.org/10.1111/j.1574-6941.1993.tb00039.x>
43. Jin Y, Lu Y. Syntrophic Propionate Oxidation: One of the Rate-Limiting Steps of Organic Matter Decomposition in Anoxic Environments. *Appl Environ Microbiol* 2023;**89**. <https://doi.org/10.1128/aem.00384-23>
44. Bechtold EK et al. Metabolic interactions underpinning high methane fluxes across terrestrial freshwater wetlands. *Nat Commun* 2025;**16**:944. <https://doi.org/10.1038/s41467-025-56133-0>
45. Jetten MSM, Stams AJM, Zehnder AJB. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Lett* 1992;**88**:181–198. <https://doi.org/10.1111/j.1574-6968.1992.tb04987.x>
46. Stams AJM et al. Contribution of ¹³C-NMR spectroscopy to the elucidation of pathways of propionate formation and degradation in methanogenic environments. *Biodegradation* 1998;**9**:463–473. <https://doi.org/10.1023/A:1008342130938>
47. Schnürer A, Schink B, Svensson BH. *Clostridium ultunense* sp. nov., a Mesophilic Bacterium Oxidizing Acetate in Syntrophic Association with a Hydrogenotrophic Methanogenic Bacterium. *Int J Syst Bacteriol* 1996;**46**:1145–1152. <https://doi.org/10.1099/00207713-46-4-1145>
48. Schlüter S, Eickhorst T, Mueller CW. Correlative Imaging Reveals Holistic View of Soil Microenvironments. *Environ Sci Technol* 2019;**53**:829–837. <https://doi.org/10.1021/acs.est.8b05245>

49. Tecon R, Or D. Cooperation in carbon source degradation shapes spatial self-organization of microbial consortia on hydrated surfaces. *Sci Rep* 2017;**7**:43726. <https://doi.org/10.1038/srep43726>
50. Dar SA et al. Competition and coexistence of sulfate-reducing bacteria, acetogens and methanogens in a lab-scale anaerobic bioreactor as affected by changing substrate to sulfate ratio. *Appl Microbiol Biotechnol* 2008;**78**:1045–1055. <https://doi.org/10.1007/s00253-008-1391-8>
51. Walker CB et al. The Electron Transfer System of Syntrophically Grown *Desulfovibrio vulgaris*. *J Bacteriol* 2009;**191**:5793–5801. <https://doi.org/10.1128/JB.00356-09>
52. Weghoff MC, Bertsch J, Müller V. A novel mode of lactate metabolism in strictly anaerobic bacteria. *Environ Microbiol* 2015;**17**:670–677. <https://doi.org/10.1111/1462-2920.12493>
53. Thauer RK, Jungermann K, Decker K. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol Rev* 1977;**41**:100–180. <https://doi.org/10.1128/br.41.1.100-180.1977>
54. Singh A et al. Syntrophic entanglements for propionate and acetate oxidation under thermophilic and high-ammonia conditions. *ISME J* 2023;**17**:1966–1978. <https://doi.org/10.1038/s41396-023-01504-y>

Figure Legend

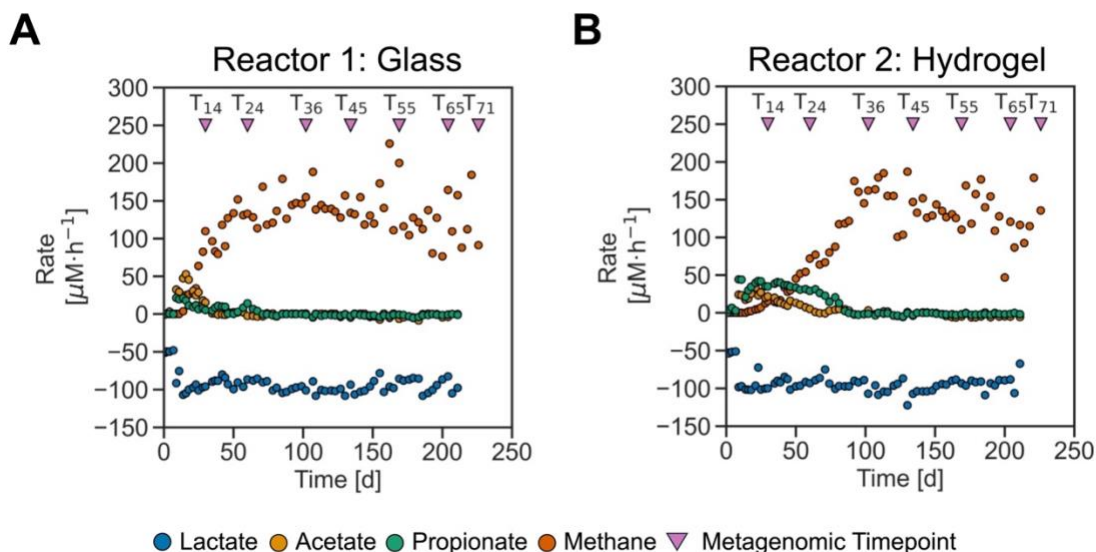


Figure 2.1. The long-term operational performance of the glass bead (reactor 1) and hydrogel (reactor 2) synthetic sediment analog reactors over the entire operational period.

The reactors underwent an initial 22.67 h batch incubation followed by a start-up period with a 0.05 h^{-1} dilution rate. Steady-state was achieved on day 78.19 and 94.90 for the glass bead and hydrogel reactor, respectively. The reactors were maintained for over 130 days after achieving steady-state. Negative rates represent consumption while the production rates are positive. Overall lactate (blue), acetate (yellow), propionate (green), and methane (red) production rates (A,B). Propionate to acetate production ratios (pink) in the glass bead (C) and hydrogel (D) synthetic reactors were determined over the entire operational period. Metagenomic sampling timepoints are shown with purple tringles.

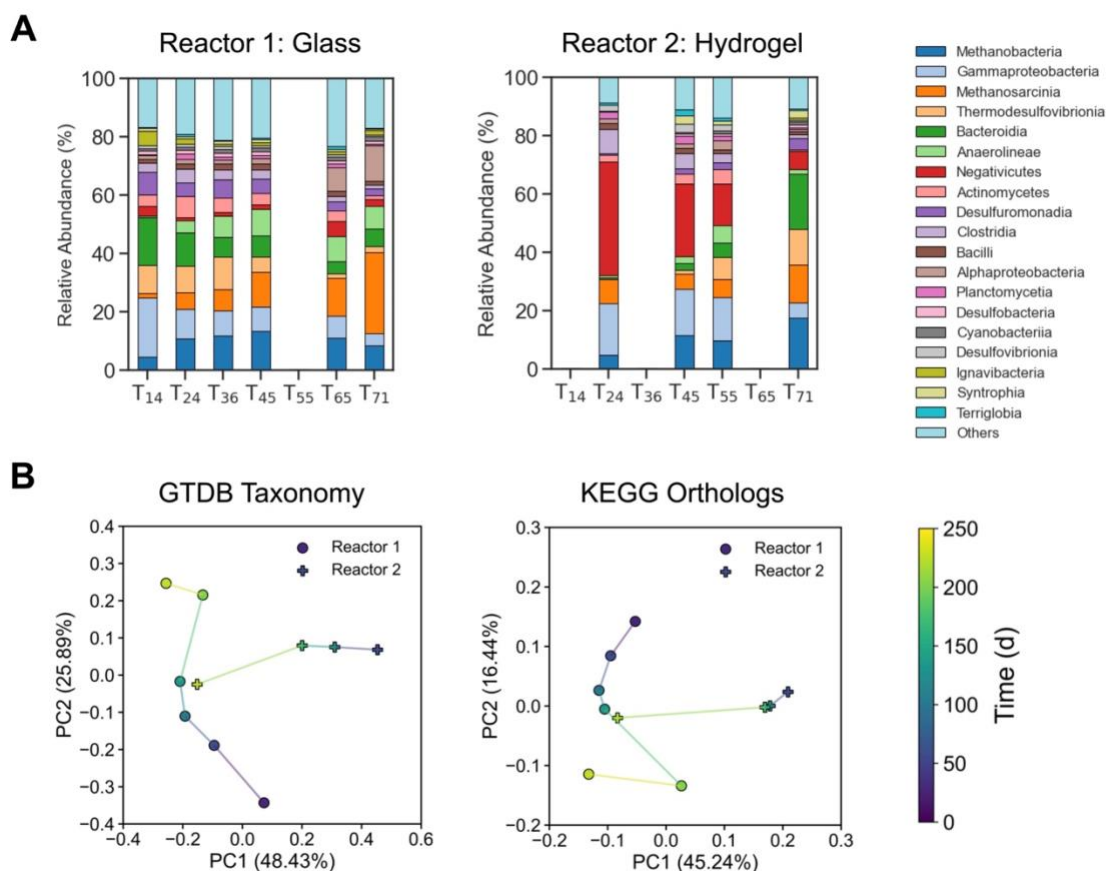


Figure 2.2. Class-level taxonomic abundance across reactors and their enrichment trajectories.

Relative abundance of the top 20 most abundant classes in Reactor 1 (glass beads) metagenomes and Reactor 2 (hydrogel beads) metagenomes (A). Metagenomic sampling timepoints are displayed on the x-axis and the absence of data indicates insufficient DNA was extracted for metagenomic sequencing. Reactor enrichment trajectories of metagenomic taxonomic abundance and functional composition based on KO abundance (B). Bray-Curtis sample dissimilarity was determined after applying a center log transform to the data and visualized with PCoA. Individual markers denote the reactor, and their color indicates the sampling time with time-adjacent samples connected by lines.

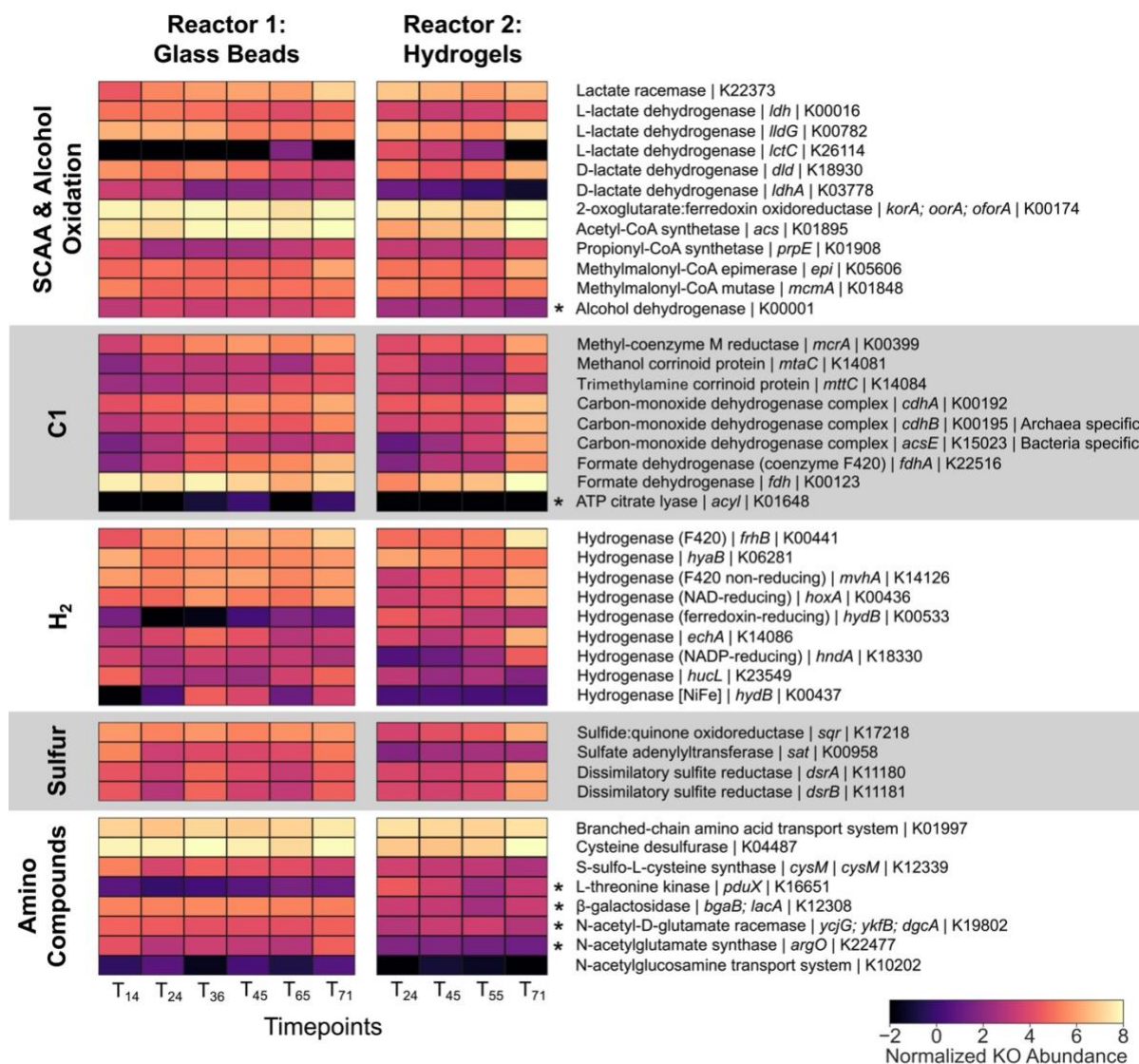


Figure 2.3. Functional gene variation across the synthetic sediment reactors.

Mean abundance of key gene orthologs across reactor metagenomes. Orthologs are organized by metabolism category as denoted by the gray shading. Asterisks next to heatmaps denote gene orthologs with abundances that were significantly different across the reactors throughout the entire enrichment (* : $p < 0.05$, ** : $p < 0.01$, and *** : $p < 0.001$).

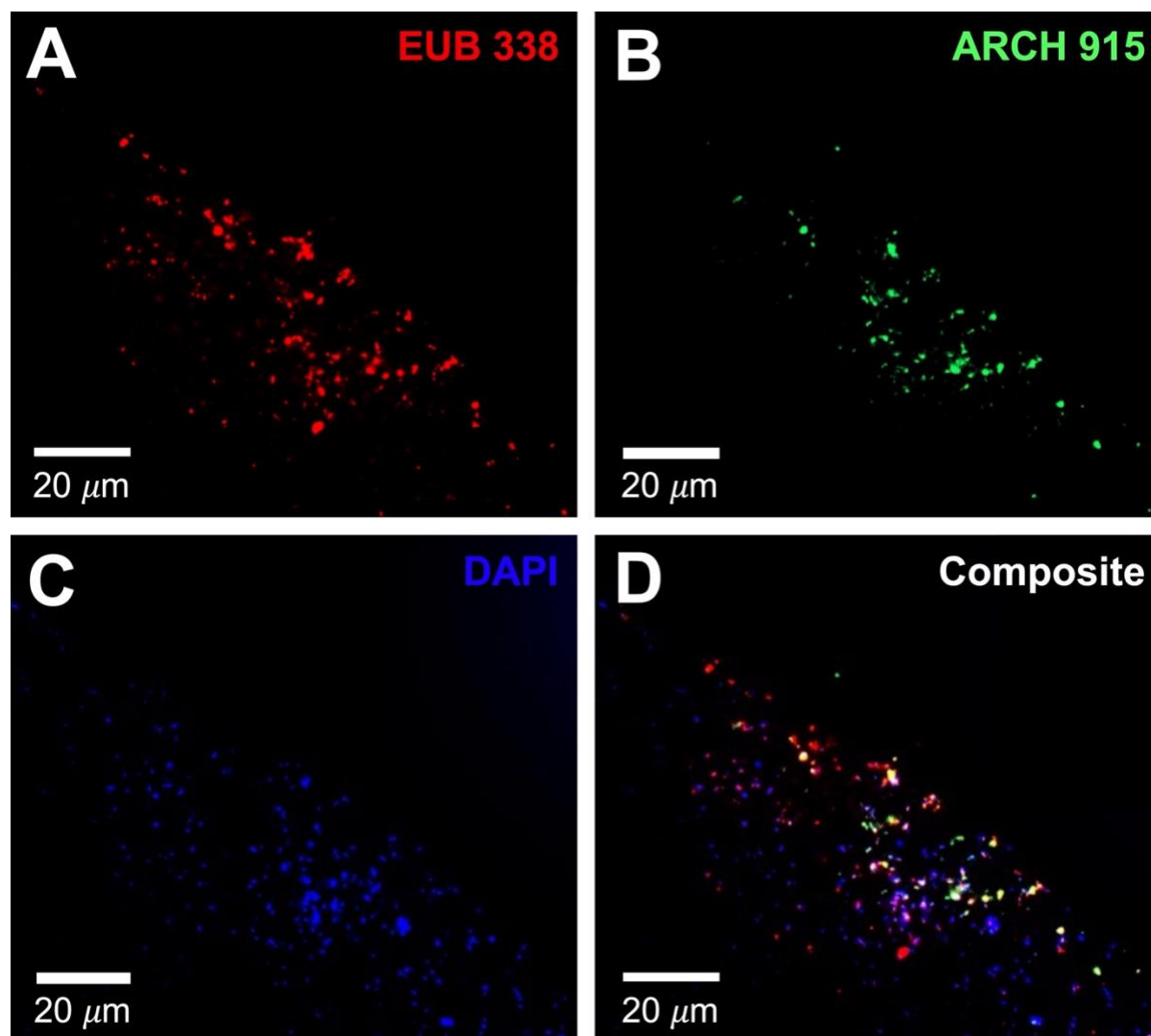


Figure 2.4. Fluorescent *in-situ hybridization* microscopy of the hydrogel synthetic sediment aggregate approximately 1 week after complete SCFA oxidation.

Probes targeting bacteria (EUB 338, red, A), archaea (ARCH 9155, green, B), and nucleic acids (DAPI, blue, C) were used. Images of individual probes were overlayed to obtain composite image (D). The hydrogel synthetic sediment aggregate biofilm can be seen running through the center diagonal of the image. The periphery and interior on the upper right-hand and lower left-hand side of the biofilm, respectively.

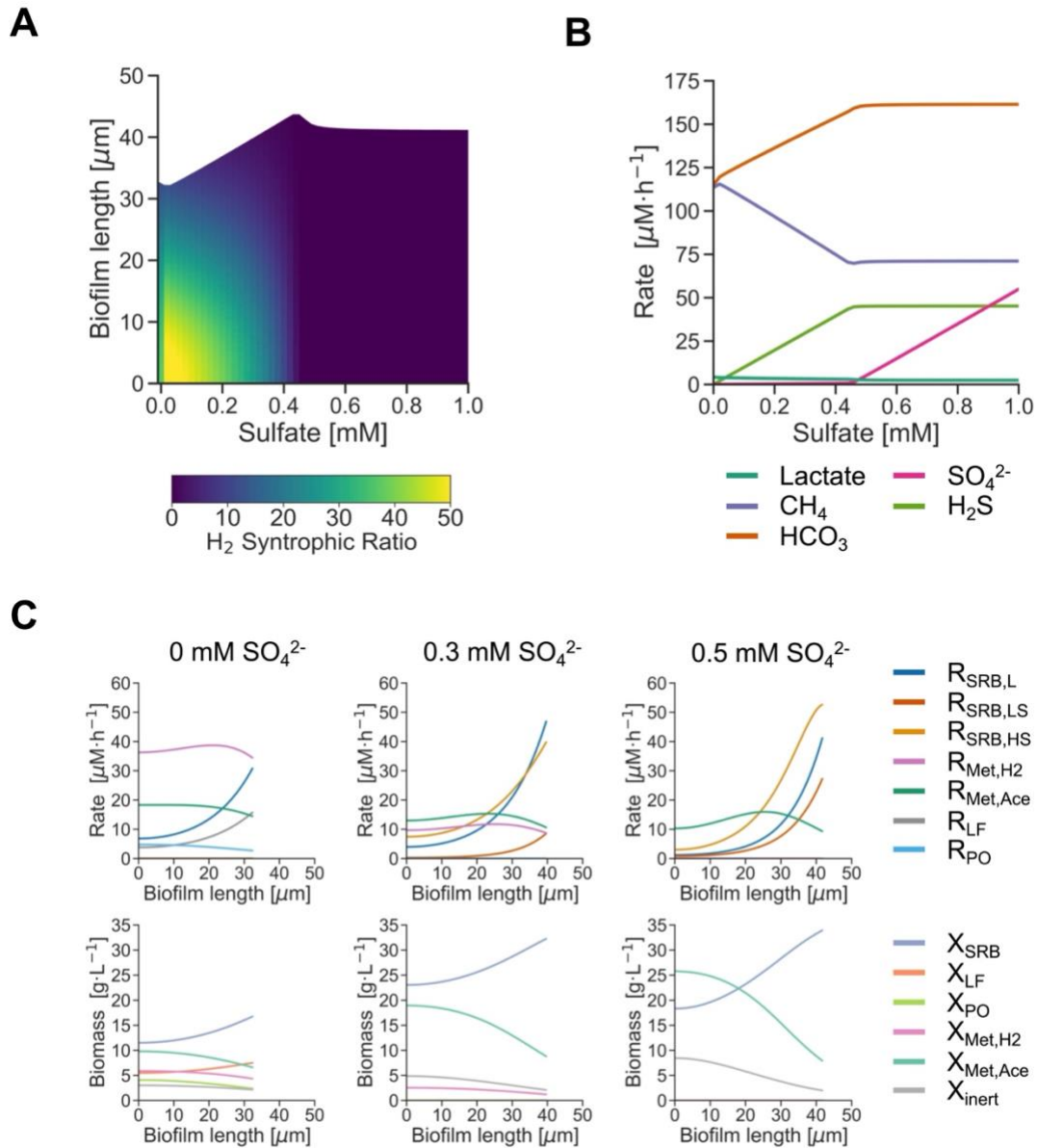


Figure 2.5. One-dimensional biofilm model of a lactate-degrading methanogenic community.

The model simulated a 365-day biofilm enrichment in a lactate-fed chemostat with a continuous supply of sulfate starting on day 100 to determine the biomass and metabolite gradients within synthetic sediment aggregates. The distribution of syntrophic H_2 cross-feeding (A) and effluent metabolites (B) were evaluated at varying sulfate to lactate ratios. The H_2 cross-feeding ratio evaluated with an electron balance by comparing the electron uptake of hydrogenotrophic methanogens to the electron utilization rate of sulfate reducing, lactate fermenting, and propionate oxidizing bacteria. Volumetric reaction rates (top) and biomass (bottom) distribution profiles were determined at lactate to sulfate ratios of 0, 0.3, and 0.5 (B).

Tables

Table 2-1. Overview of metabolic reactions contributing to the anaerobic production of methane from lactate

	Process	Pathway	Reaction	$\Delta G^{\circ 1}$	Ref
1	Lactate fermentation	Methylmalonyl-CoA	3 lactate $\rightarrow$ 1 acetate + 2 propionate + 1 CO ₂ + 1 H ₂ O	-171.90	[41]
2	Lactate fermentation	Acrylyl-CoA	3 lactate $\rightarrow$ 1 acetate + 2 propionate + 1 CO ₂ + 1 H ₂ O	-171.90	[41]
3	Lactate oxidation	Acetogenic	4 lactate $\leftrightarrow$ 6 acetate + 2 H ⁺	-227.87	[51]
4	Lactate oxidation	-	1 lactate $\rightarrow$ 1 acetate + 1 CO ₂ + 2 H ₂	-9.5	[52]
5	Propionate oxidation	Methylmalonyl-CoA	1 propionate + 2 H ₂ O $\rightarrow$ 1 acetate + 1 CO ₂ + 3 H ₂	71.7	[6]
6	Acetate oxidation	-	1 acetate + 1 H ₂ O + 1 H ⁺ $\rightarrow$ 2 CO ₂ + 4 H ₂	94.93	[6]
7	Acetogenesis	Wood-ljungdahl	2 CO ₂ + 4 H ₂ $\leftrightarrow$ 1 acetate + 1 H ⁺ + 2 H ₂ O	-94.93	[53]
8	Methanogenesis	Hydrogenotrophic	4 H ₂ + 1 CO ₂ $\rightarrow$ 1 CH ₄ + 2 H ₂ O	-130.75	[52]
9	Methanogenesis	Acetoclastic	1 acetate + 1 H ⁺ $\rightarrow$ 1 CH ₄ + 1 CO ₂	-35.82	[53]
10	Syntrophic Methanogenesis	Lactate oxidation & hydrogenotrophic	2 lactate $\rightarrow$ 2 acetate + 1 CH ₄ + 1 CO ₂	-149.75	[52]
11	Syntrophic Methanogenesis	Propionate oxidation & hydrogenotrophic	4 propionate + 2 H ₂ O $\rightarrow$ 4 acetate + 3 CH ₄ + 1 CO ₂	-105.45	[41]
12	Syntrophic Methanogenesis	Acetate oxidation & hydrogenotrophic	1 acetate + 1 H ⁺ $\rightarrow$ 1 CH ₄ + 1 CO ₂	-35.82	[54]

Appendix A. Supplemental Material

The following document is intended to support the results presented in the main manuscript.

Supplemental Methods

- S1. *16S rRNA microbial community analysis*
- S2. *Fluorescence in-situ hybridization*
- S3. *Numerical modeling*

Figures

- Figure S2.1.** Configuration of synthetic sediment analog chemostats.
- Figure S2.2.** Biomass growth in the sediment analog reactors.
- Figure S2.3.** Carbon and electron balances in the sediment analog reactors.
- Figure S2.4:** Order-level taxonomic correlations in the glass bead chemostat.
- Figure S2.5.** Order-level taxonomic correlations in the hydrogel chemostat.
- Figure S2.6.** Class-level taxonomic abundance throughout the enrichment.
- Figure S2.7.** Effluent metabolite concentrations in synthetic sediment analog chemostats.
- Figure S2.8.** Class-level relative abundance of planktonic and aggregate phase on day 134.
- Figure S2.9.** Number of assembled metagenomic bins throughout the enrichment.
- Figure S2.10.** Class-level relative abundance and beta-diversity of assembled scaffolds.
- Figure S2.11.** Class-level relative abundance and beta-diversity of sequenced 16S rRNA.
- Figure S2.12.** Order-level metagenomic abundance.
- Figure S2.13.** Distribution of microbial community in hydrogel aggregate on day 59.
- Figure S2.14.** Overview of the lactate fermenting methylmalonyl-CoA pathway.
- Figure S2.15.** Overview of the propionate oxidizing methylmalonyl-CoA pathway.

Tables

- Table S2-2.** FISH Probes used in this study
- Table S2-3.** Catabolic reaction stoichiometry with thermodynamic and equilibrium constants for species in the wetland community model.
- Table S2-4.** Overall reaction stoichiometry for species in the wetland community model.
- Table S2-5.** Kinetic uptake rate expressions for representative species in the wetland microbial community model.
- Table S2-6.** Thermodynamic reaction quotient for species in the wetland community model.
- Table S2-7.** Biomass yields for species in the wetland community model.
- Table S2-8.** Stoichiometric solute yield matrix for species in the wetland community model.
- Table S2-9.** Stoichiometric biomass yield matrix for species in the wetland community model.
- Table S2-10.** Kinetic rate expressions for wetland community model.
- Table S2-11.** Kinetic rate parameters for species in the wetland community model.
- Table S2-12.** Kinetic parameters for lactate species in the wetland community model.
- Table S2-13.** Mass diffusivity of solutes in water at 37°C.
- Table S2-14.** Effective mass diffusivity fractions.
- Table S2-15.** Biofilm reactor model parameters.

Supplemental Methods

S1. 16S amplicon sequencing and analysis

The V4-V5 region of the 16S rRNA gene was amplified by PCR using the 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) primer set [1]. PCR products were purified and sent to Azenta for Amplicon Illumina sequencing. Demultiplexed and adapter-trimmed reads were analyzed with USEARCH v11 [2]. Paired-end reads were merged, primer sequences were removed, and reads were quality filtered. Unique reads and their abundances were computed followed by denoising and chimera removal [3]. Reads were mapped to zero-radius OTUs (zOTUs) and taxonomy was assigned by searching against the RDP 16S database v18 [3, 4].

S2. Fluorescence in-situ hybridization

Fixed hydrogel beads were submerged in OCT Compound (Agar Scientific) or NEG-50 TM (Richard-Allan Scientific TM) overnight and then frozen at -20°C for 3-16 hours prior to sectioning. Thin 20-30 nm sections of hydrogel beads were produced using a micro-cryotome (Cryostar NX50™) at -20°C and mounted on gelatin coated Teflon-covered glass slides. Hybridization was done with 35% formamide hybridization buffer and 80 µM NaCl washing buffer. Oligonucleotide probes (Eurofins, Louisville, KY) were applied in equimolar concentration to cryosections and hybridized at 46°C for 2 hours. Sections were labelled with the fluorescein-labeled universal archaeal probe ARCH915 and Cy3-labeled universal bacterial probe EUB388 (Table S4). A confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss, Germany) equipped

with an Axio 503 mono camera was used to visualize hybridized cryosections with 488, 543, and 633 nm lasers.

S3. Numerical Modeling

A one-dimensional reactive transport model of a biofilm reactor was developed to represent the growth and decay of a lactate degrading methanogenic community [5, 6]. The wetland microbial community model considered the activity of *Desulfovibrio*-like sulfate reducing bacteria (*SRB*), lactate fermenting bacteria (*LF*), propionate oxidizing bacteria (*PO*), *Methanospirillum*-like hydrogenotrophic methanogen (*Met,H₂*), *Methanothrix*-like acetoclastic methanogen (*Met,Ace1*), and *Methanosarcina*-like acetoclastic methanogen (*Met,Ace2*). It was assumed that the microorganisms grew on the surface of glass beads in an ideally mixed chemostat supplied with a constant concentration of lactate ($C_{in,Lac}$) and sulfate (C_{in,SO_4}) concentration at a defined inlet flowrate (Q). The numerical model consisted of a biofilm domain and an aqueous domain. It was assumed that the biofilm domain was in contact with the aqueous domain. The biofilm domain was comprised of particulate biomass components (X) convectively transported by microbial growth and dissolved solute components (C) transported by diffusion. Biofilm thickness was modeled by balancing microbial detachment (d) and the convection of microbial growth (u_F) at the biofilm surface. Material balances for the biofilm and aqueous domains were implemented in *COMSOL Multiphysics* (v6.2, COMSOL Inc., Burlington, MA) and solved with a variable timestep until quasi steady-state was achieved on day 365.

S3.1. Biofilm domain solute balances

Eight solutes, represented by index i , were included in the biofilm model, represented by subscript F , with concentrations $C_{F,i}$ in the biofilm ($\text{mol} \cdot \text{m}_F^{-3}$): lactate (Lac), acetate (Ace), propionate ($Prop$), hydrogen (H_2), sulfate (SO_4^{2-}), hydrogen sulfide (H_2S), carbonate (HCO_3^-), and methane (CH_4)

Time-dependent mole balances for all solutes in the biofilm were expressed as a function of solute concentration with source terms for diffusive transport and volumetric reaction rates:

$$\frac{\partial C_{F,i}}{\partial t} = D_{F,i} \nabla^2 C_{F,i} + R_i \quad (2.5)$$

with $\nabla^2 \equiv \frac{\partial^2}{\partial x^2} + \left(\frac{2}{x}\right) \frac{\partial}{\partial x}$ for the spherical hydrogel geometry. $D_{F,i}$ is the effective diffusion coefficient of solute i in the biofilm matrix, which were chosen based on previously reported values [7]. R_i is the net volumetric reaction rate of component i at position x in the biofilm ($\text{mol} \cdot \text{m}_F^{-3} \cdot \text{h}^{-1}$) based on the stoichiometry and kinetics for each organism. Rates, kinetic expressions, stoichiometry, parameters are detailed in Tables S1.2-13.

A zero-flux boundary condition was set for all solutes at the glass surface ($x = 0$):

$$D_{F,i} \left. \frac{\partial C_{F,i}}{\partial x} \right|_{x=0} = 0 \quad (2.6)$$

The external mass transfer resistance (diffusion boundary layer) was neglected for all solutes. Therefore, the concentration of solute i at the biofilm surface ($x = L_F$) was assumed to be equivalent to the bulk liquid concentration ($\text{mol} \cdot \text{m}_R^3$):

$$C_{F,i}|_{x=L_F} = C_{B,i} \quad (2.7)$$

S3.2. Biofilm domain biomass balances

Seven biomass components, represented by subscript j , were included in the biofilm model with concentrations X_j in the biofilm ($\text{mol} \cdot \text{m}_F^3$): *Desulfovibrio*-like sulfate reducing bacteria (X_{SRB}), lactate fermenting bacteria (X_{LF}), propionate oxidizing bacteria (X_{PO}), *Methanospirillum*-like hydrogenotrophic methanogen ($X_{Met,H2}$), *Methanothrix*-like acetoclastic methanogen ($X_{Met,Ace1}$), and *Methanosarcina*-like acetoclastic methanogen ($X_{Met,Ace2}$), and inert biomass (X_i) formed from microbial decay. Microbial growth was modeled with Monod kinetics and a constant rate of biomass decay (b). Total biomass density (ρ_F) was assumed to be constant throughout the biofilm and biomass was initially assumed to be uniformly distributed throughout the biofilm.

Time-dependent mole balances for all biomass component in the biofilm were expressed as a function of biomass concentration with a source term for biomass growth and advective transport by growth:

$$\frac{\partial X_j}{\partial t} = -u_F \frac{\partial X_j}{\partial x} + R_j \quad (2.8)$$

R_i is the net volumetric reaction rate of component i at position x in the biofilm ($\text{mol} \cdot \text{m}_F^3 \cdot \text{h}^{-1}$) based on the stoichiometry and kinetics for each organism. Rates, kinetic expressions,

stoichiometry, parameters are detailed in Tables S2-13. A zero-flux boundary condition was set for all solutes at the glass surface ($x = 0$):

$$\left. \frac{\partial X_{F,i}}{\partial x} \right|_{x=0} = 0 \quad (2.9)$$

The advective velocity of microbial growth (u_F) at position x was computed from the total biomass balance:

$$\frac{du_F}{dx} = \frac{\sum R_j}{\rho_F} \quad (2.10)$$

with a zero-flux boundary condition at the glass surface ($x = 0$):

$$\left. \frac{du_F}{dx} \right|_{x=0} = 0 \quad (2.11)$$

The boundary condition at the edge of the biofilm ($x=L_F$) was set as an outflow boundary condition, where biomass growing past the edge of the hydrogel was assumed to exit the hydrogel.

Biofilm thickness was expressed as a function of the microbial growth velocity (u_F) at the biofilm surface ($x = L_F$) assuming an initial thickness of $L_{F,0}$:

$$\left. \frac{dL_F}{dt} \right|_{x=L_F} = u_F \quad (2.12)$$

SI.3.3. Aqueous domain solute mass balances in the chemostat

It was assumed the glass bead biofilm chemostat (subscript R) was ideally mixed and supplied with a constant concentration of lactate ($C_{in,Lac}$) at a defined inlet flowrate (Q). Sulfate ($C_{in,SO4}$) was supplied at a constant concentration starting on day 100 ($t = 2400$ h). The contribution of planktonic biomass to substrate conversion in the chemostat was assumed to be negligible. Time dependent solute balances in the bulk liquid were expressed as a function of solute concentration ($C_{in,i}$) and biofilm exchange flux $J_{F,i}$:

$$\frac{dC_{F,i}}{dt} = \frac{Q_R}{V_R} (C_{in,i} - C_{R,i}) + a_F \cdot J_{F,i} \quad (2.13)$$

where Q_R is the volumetric flowrate ($\text{mL} \cdot \text{h}^{-1}$), V_R is the bulk liquid volume in the chemostat (mL), $C_{in,i}$ is the concentration of solute i in the influent ($\text{mol} \cdot \text{m}^{-3}$), a_F is the specific biofilm surface area (m^{-1}), and $J_{F,i}$ is the diffusive flux solute i from the bulk liquid into the biofilm ($\text{mol} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$):

$$J_{i,b} = -D_i \left. \frac{dS_i}{dx} \right|_{t,x=L_f} \quad (2.14)$$

where D_i is the diffusion coefficient of solute i ($\text{m}^2 \cdot \text{h}^{-1}$).

S3.4. Model implementation

The model was implemented in *COMSOL Multiphysics* (v6.2, Comsol Inc., Burlington, MA). All ordinary differential and partial differential equations were solved with variable time step. The biofilm domain was discretized as a moving mesh with a maximum mesh size of 1 mm to implement the variable biofilm thickness. The simulation times were in the order of minutes per case. All reported steady state results were in all conditions obtained after 365 days.

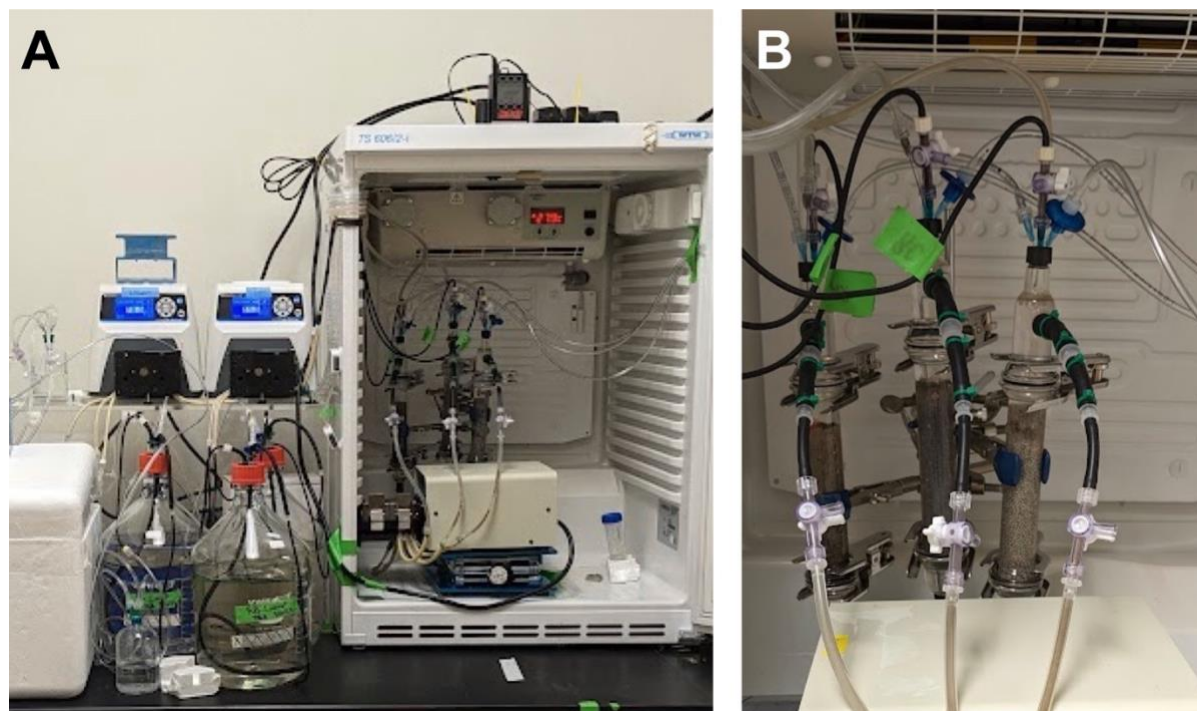


Figure S2.1. Configuration of synthetic sediment analog chemostats.

The complete reactor configuration included anaerobic lactate media, influent pump, effluent pump, recirculation pump, inlet gas mass flow controller, and an off gas line (A). Chemostats were maintained at 30 C with a temperature-controlled incubator. Medium was continuously supplied to the reactors with a drip feed attached to a sampling port (B). Filtered nitrogen gas was supplied to maintain anaerobic conditions. Effluent was directly sampled from the recirculation line.

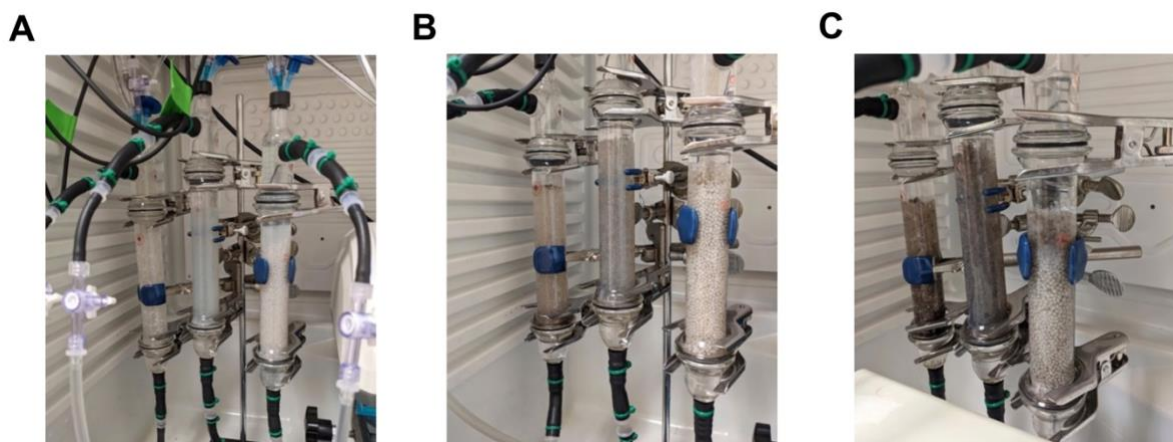


Figure S2.2. Biomass growth in the sediment analog reactors.

Progression of biomass attachment and growth in the glass bead methanogenic synthetic sediment aggregate chemostats on day 0 (A), 30 (B), and 106 (C). Microorganisms attached to the surface of glass beads (left) to form biofilms as can be seen by the increasing bead turbidity from day 0 to 106. The increasing turbidity associated with microbial growth was also observed for the PEG-PVA(middle), and PEGDMA-PVA (right) hydrogel chemostats. Results from the PEG-PVA hydrogel chemostat are not reported in this manuscript due to a high rate of hydrogel shearing and low DNA yields.

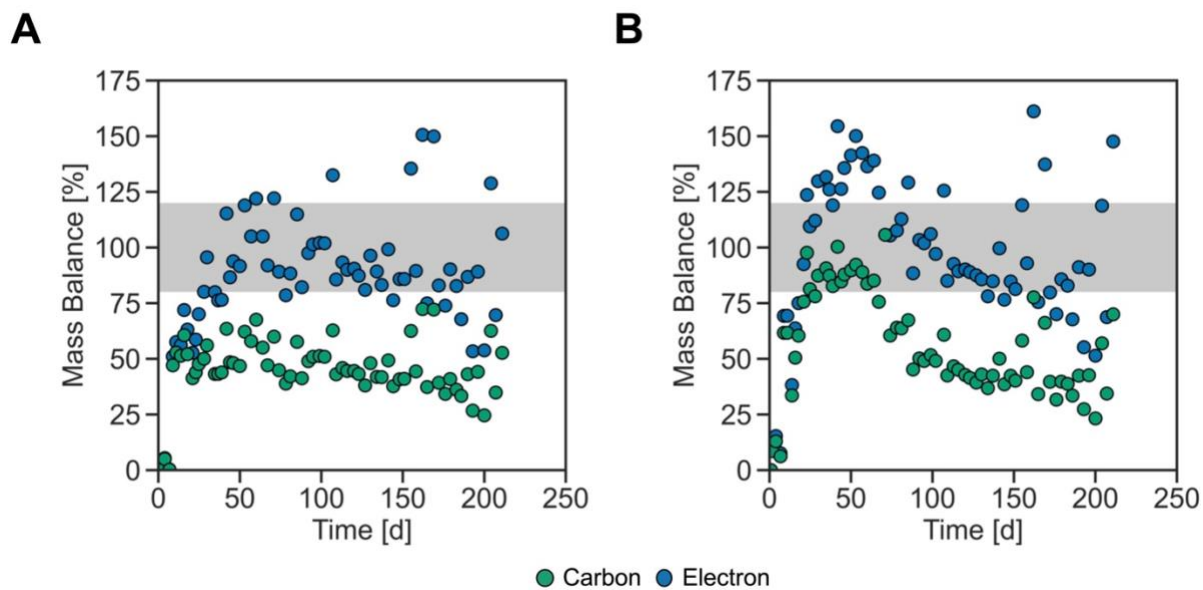


Figure S2.3. Carbon and electron balances in the sediment analog reactors.

Carbon (blue) and electron (green) balances for the glass bead (A) and hydrogel (B) synthetic sediment aggregate chemostats. A grey box highlights the region within 20% of a closed carbon and electron balance.

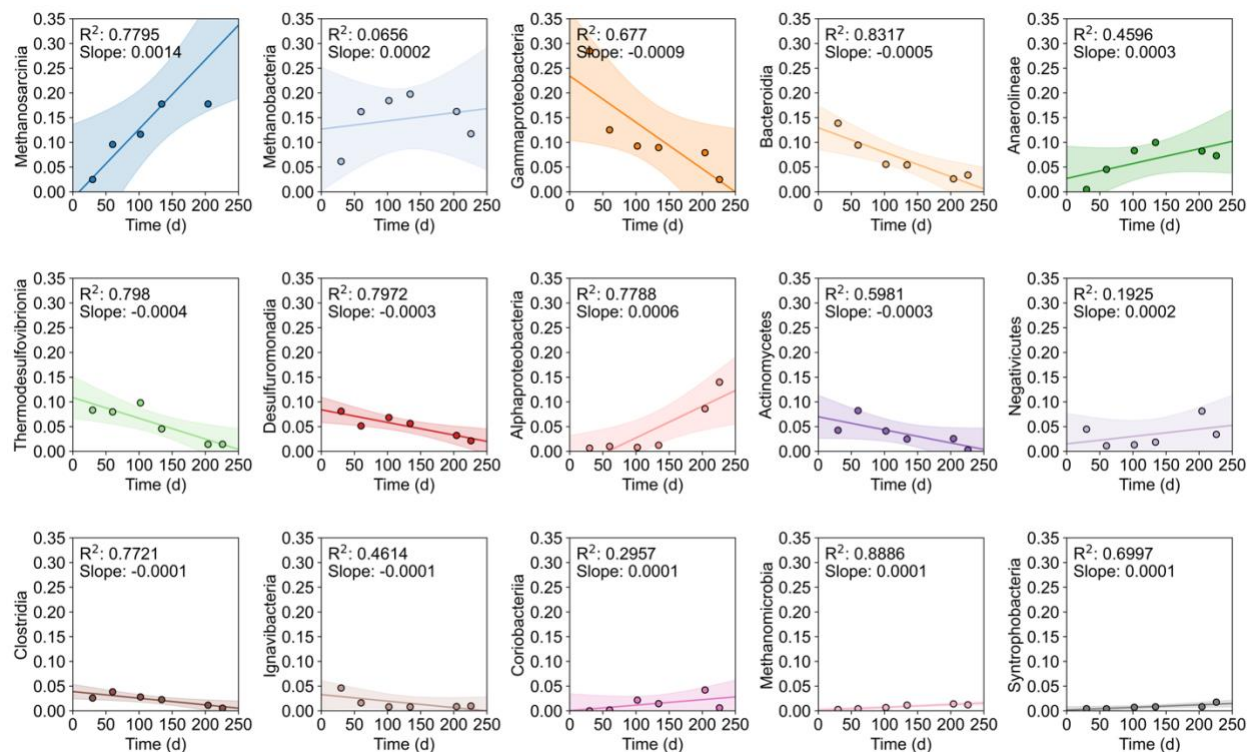


Figure S2.4. Order-level taxonomic correlations in the glass bead chemostat.

Correlation between enrichment time and order-level taxonomic in the glass bead chemostat (R_1) of the top 15 taxa. The correlation was determined by linear correlation with the strength of the correlation determined by the coefficient of determination (R^2). Order-level taxonomic assignments are displayed on the y-axis.

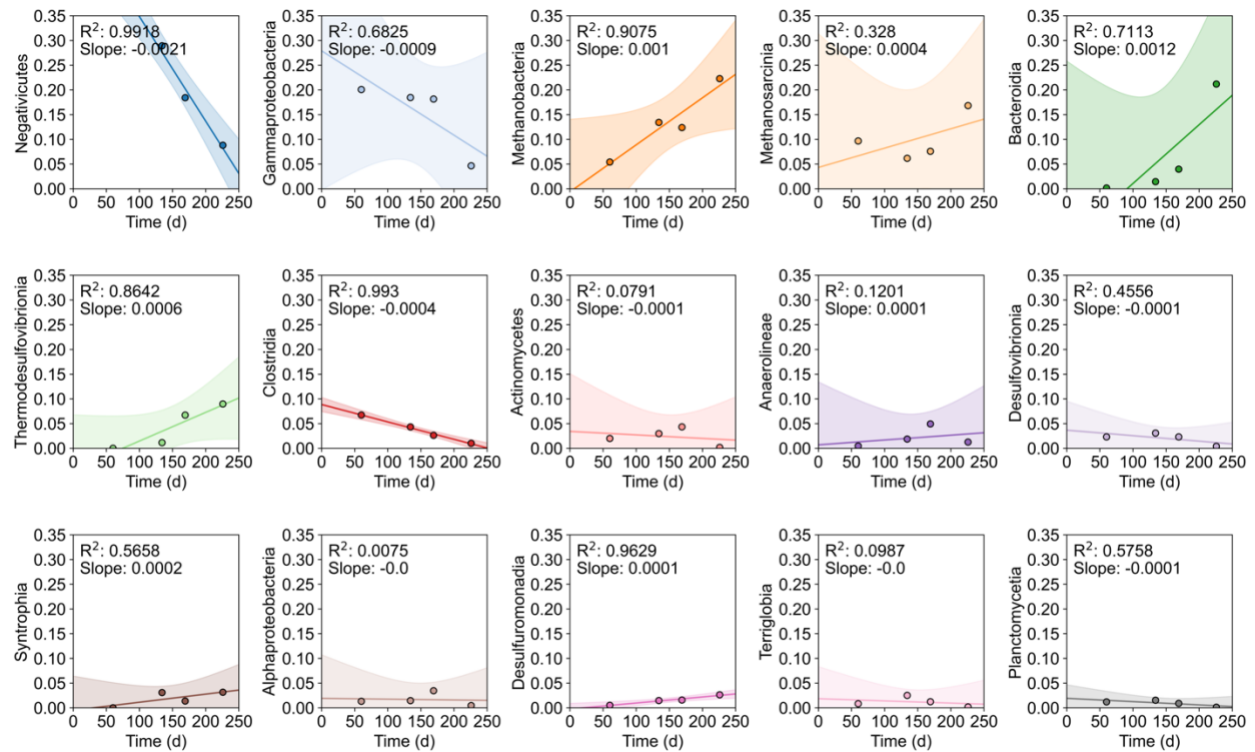


Figure S2.5: Order-level taxonomic correlations in the hydrogel chemostat.

Correlation between enrichment time and order-level taxonomic in the hydrogel chemostat (R_2) of the top 15 taxa. The correlation was determined by linear correlation with the strength of the correlation determined by the coefficient of determination (R^2). Order-level taxonomic assignments are displayed on the y-axis.

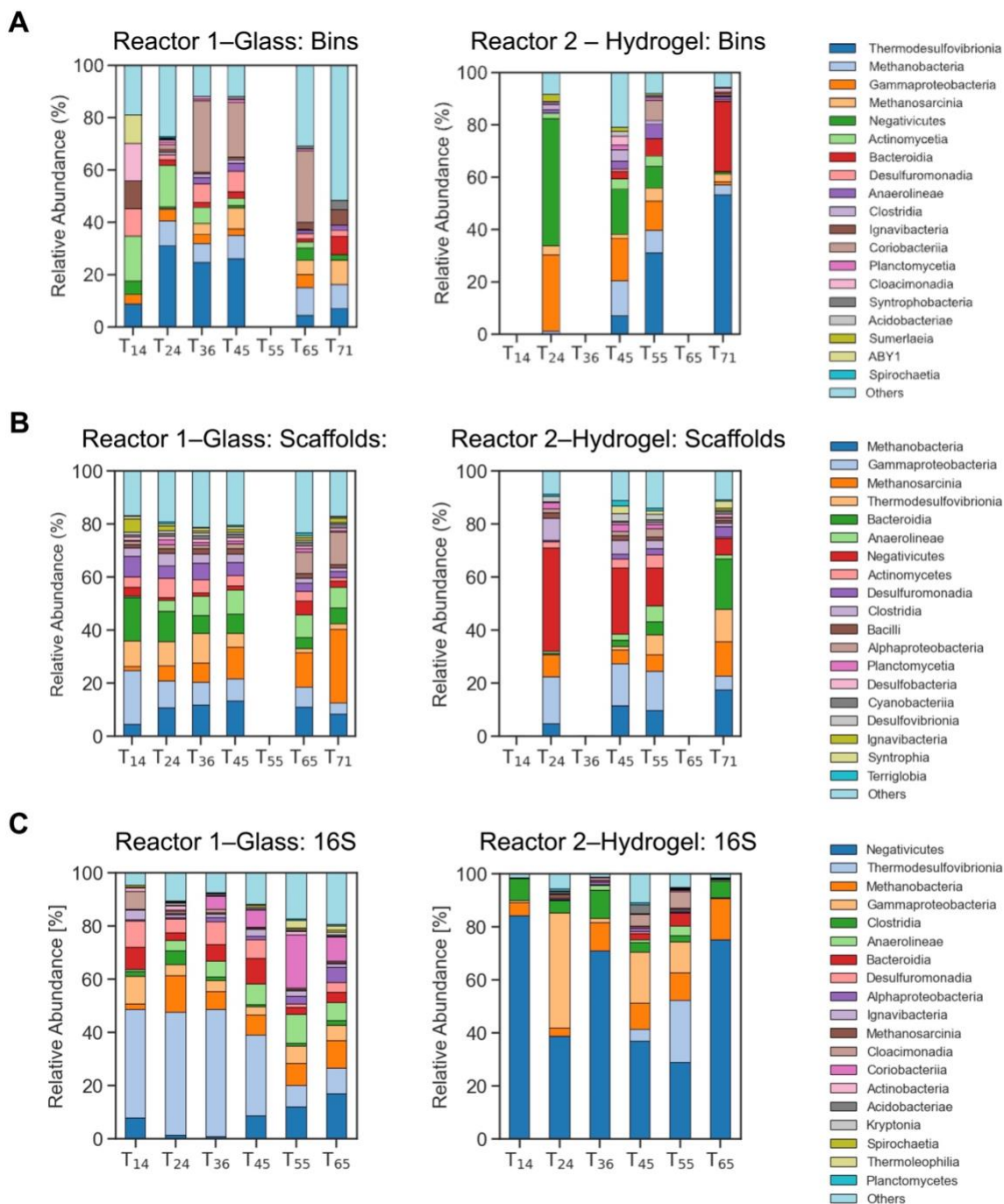


Figure S2.6. Class-level taxonomic abundance throughout the enrichment.

Relative abundance was determined by mapping metagenomic reads to assembled bins (A), mapping metagenomic reads to assembled scaffolds (B), and 16S rRNA sequencing (C).

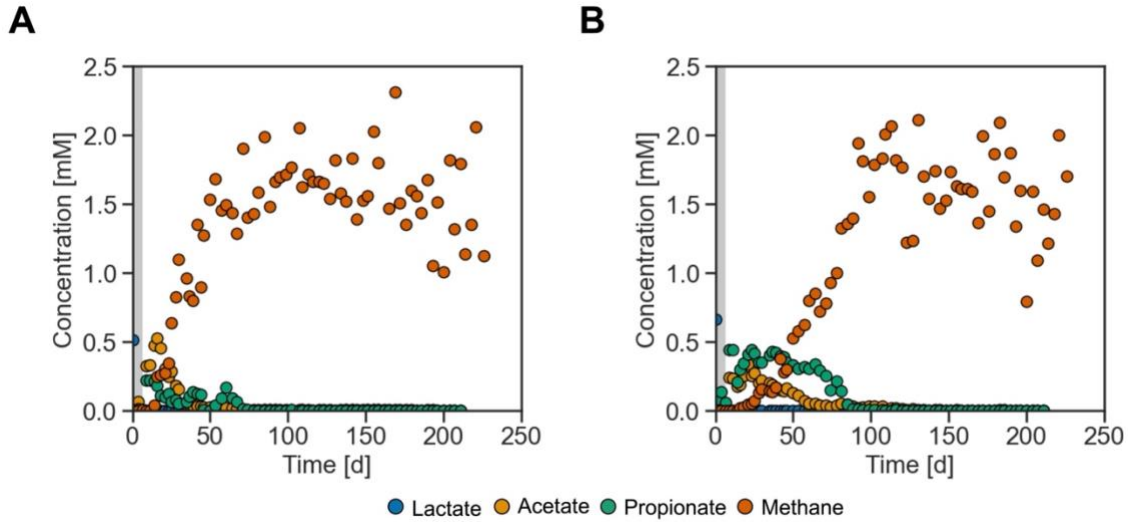


Figure S2.7. Effluent metabolite concentrations in synthetic sediment analog chemostats. Effluent metabolite concentrations in the glass bead (A) and hydrogel (B) synthetic sediment aggregate chemostats. Lactate (blue) was not detected in the effluent after the initial time point. Acetate (yellow) and propionate (green) initially accumulated in the effluent before complete conversion to methane (red). The grey region highlights the start-up period.

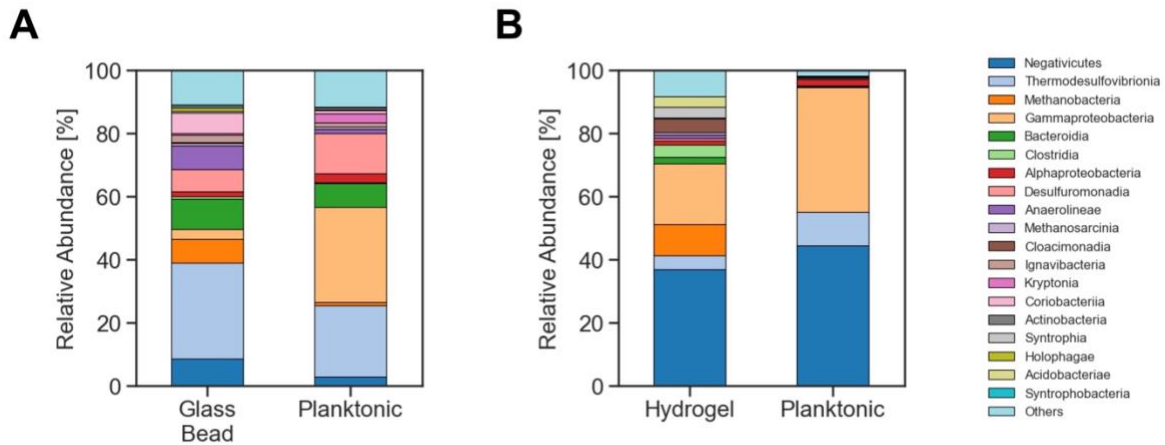


Figure S2.8. Class-level relative abundance of planktonic and aggregate phase on day 134. Class-level taxonomic abundance of sediment analog and planktonic samples obtained on day 134 obtained via 16S rRNA gene amplicon sequencing. Relative abundance of ASVs were determined for the glass bead (A) and hydrogel (B) chemostats.

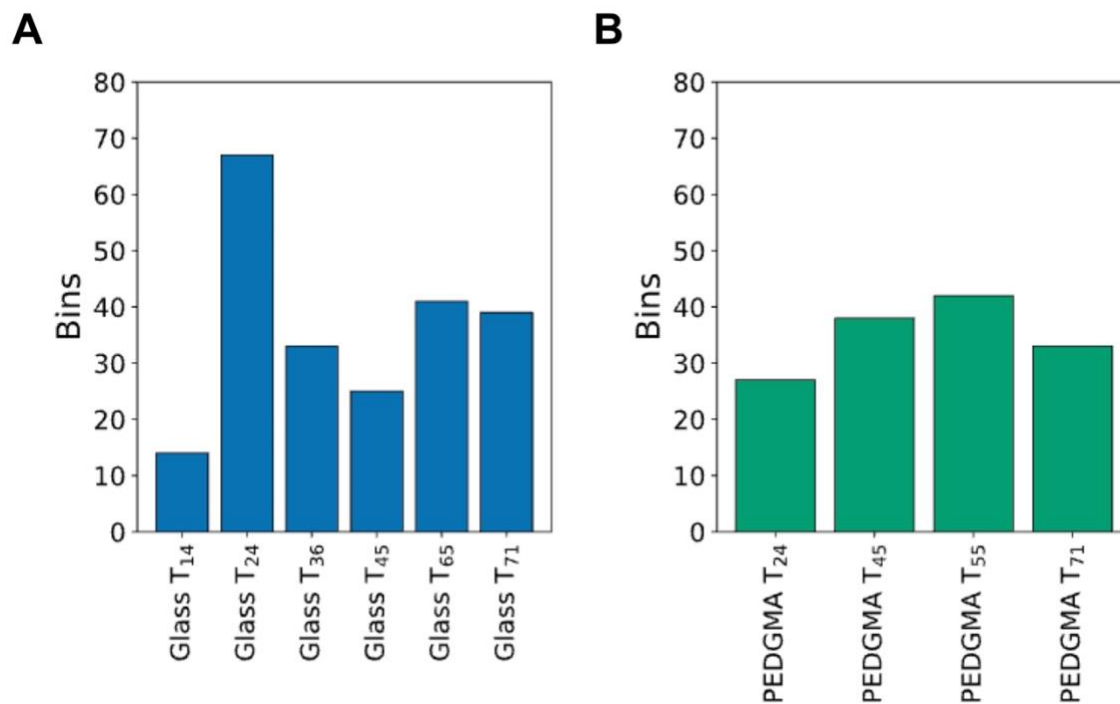


Figure S2.9: Number of assembled metagenomic bins.

Number of assembled metagenomic bins assembled at every time point in the glass bead (A) and hydrogel (B) chemostat.

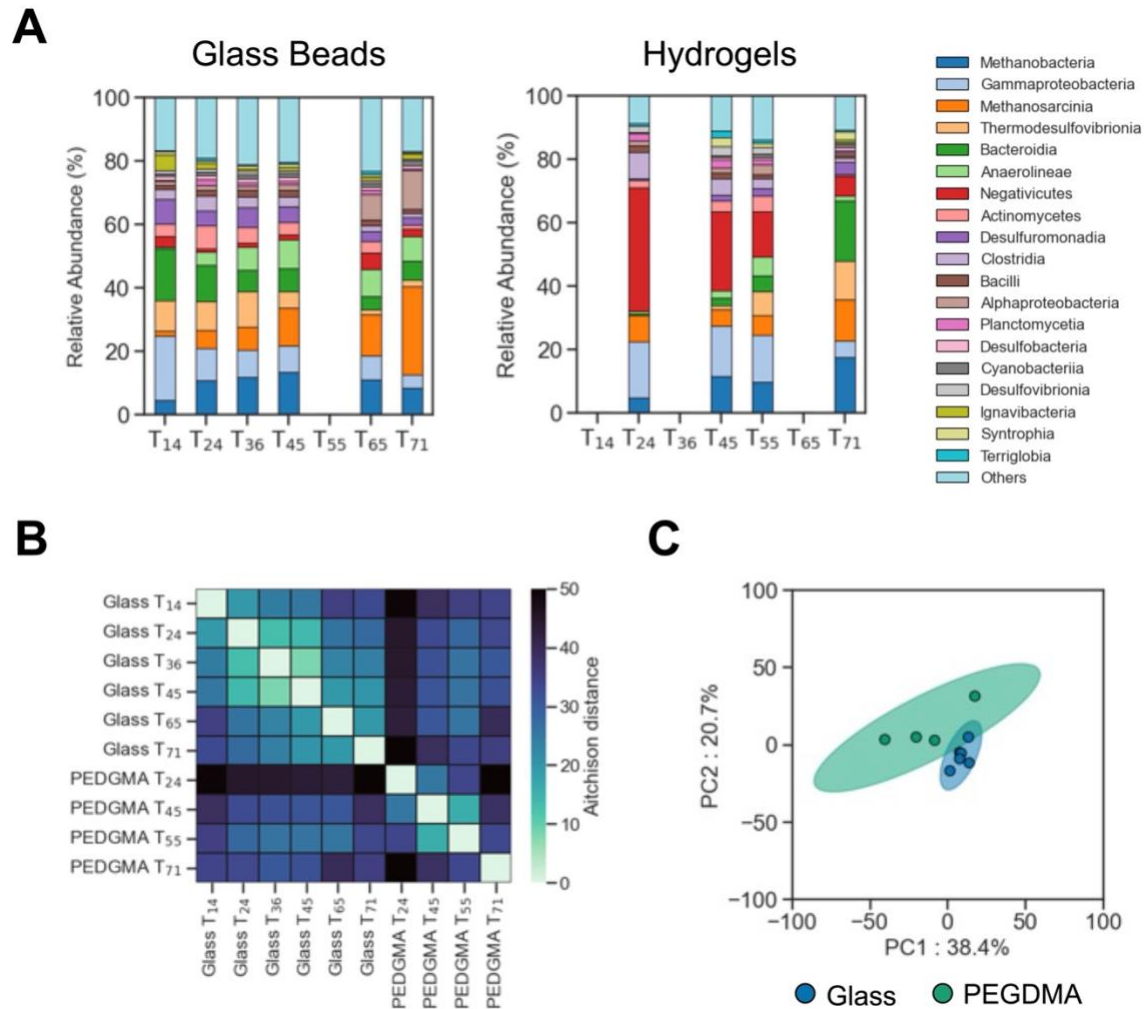


Figure S2.10: Class-level relative abundance and beta-diversity of assembled scaffolds.

Relative abundance of the metagenomic scaffolds from reactor 1 (glass bead) and reactor 2 (hydrogel chemostats) through the incubation (A). Scaffold sample dissimilarity determined with robust center log ratios of taxa relative abundance with lower Aitchison distances indicating a higher degree of sample similarity (B). Robust Aitchison principal coordinate analysis of Aitchison distances between metagenomic samples with 95% confidence interval ellipses.

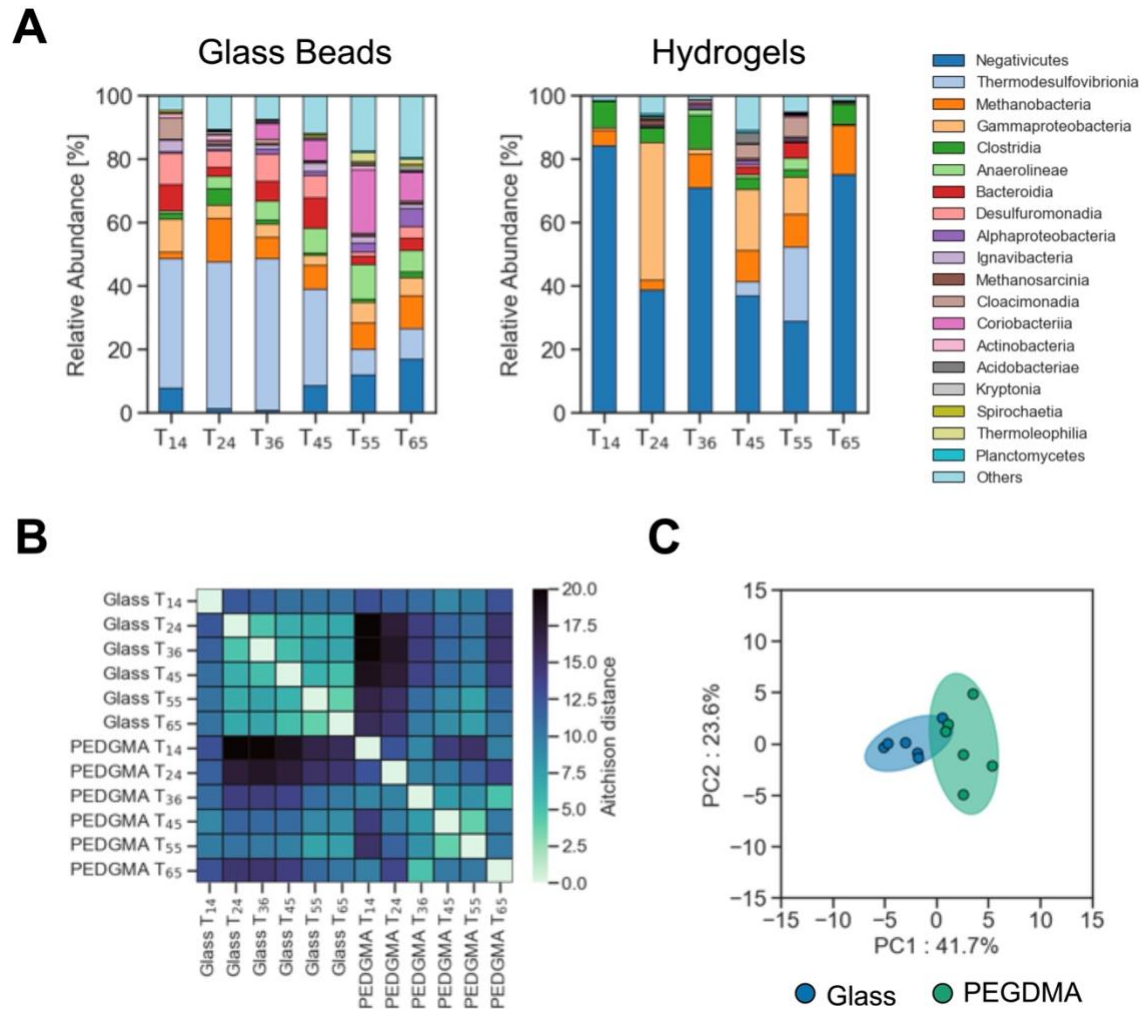


Figure S2.11: Class-level relative abundance and beta-diversity of sequenced 16S rRNA.

Class-level taxonomic abundance and beta-diversity of 16S rRNA samples. Relative abundance of ASVs from the glass bead and hydrogel chemostats (A). Amplicon sample dissimilarity determined with robust center log ratios of taxa relative abundance with lower Aitchison distances indicating a higher degree of sample similarity (B). Robust Aitchison principal coordinate analysis of Aitchison distances between metagenomic samples with 95% confidence interval ellipses.

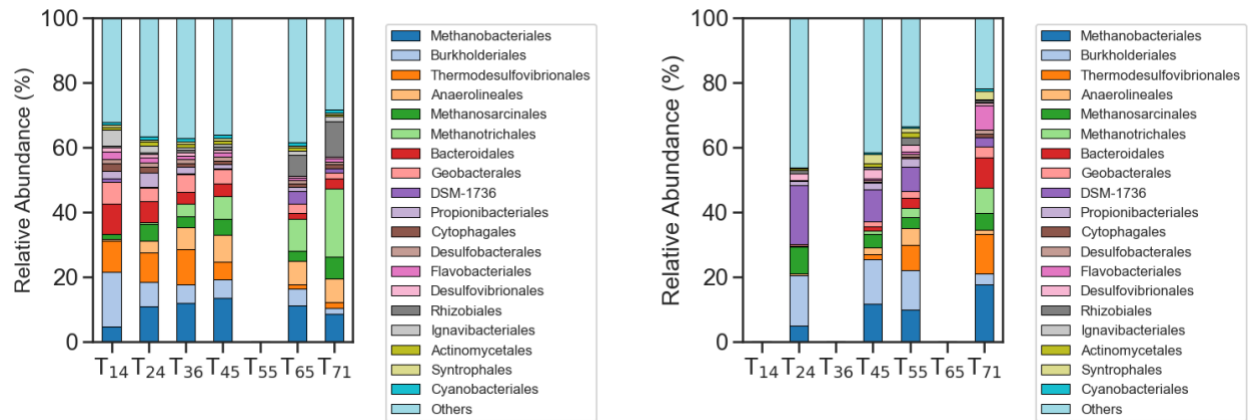


Figure S2.12: Order-level metagenomic abundance.

Relative abundance of scaffolds from the glass bead (left) and hydrogel chemostats (right).

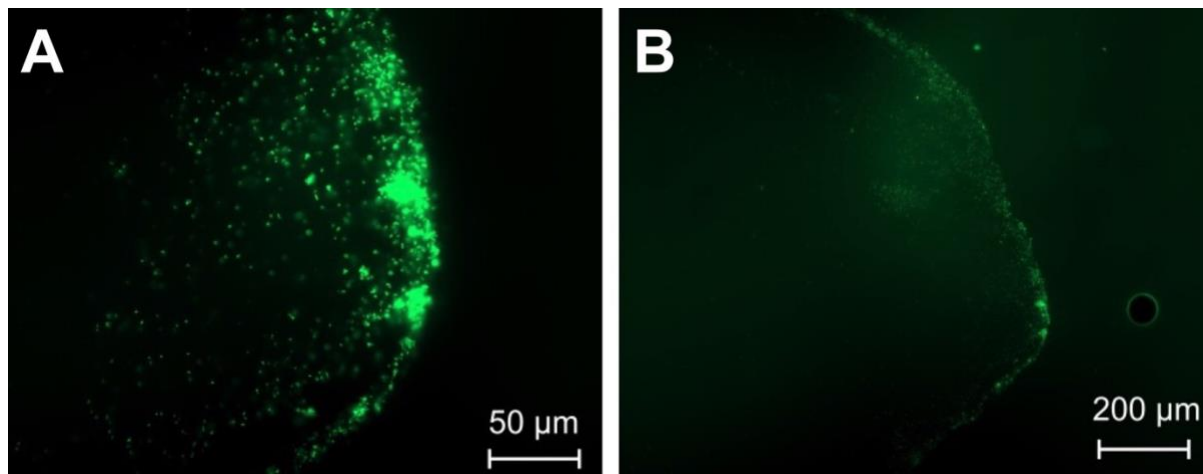


Figure S2.13: Distribution of microbial community in hydrogel aggregate on day 59.

Distribution of microbial community was detected with the SYBR Green nucleic acid stain in a representative hydrogel bead collected on day 59.

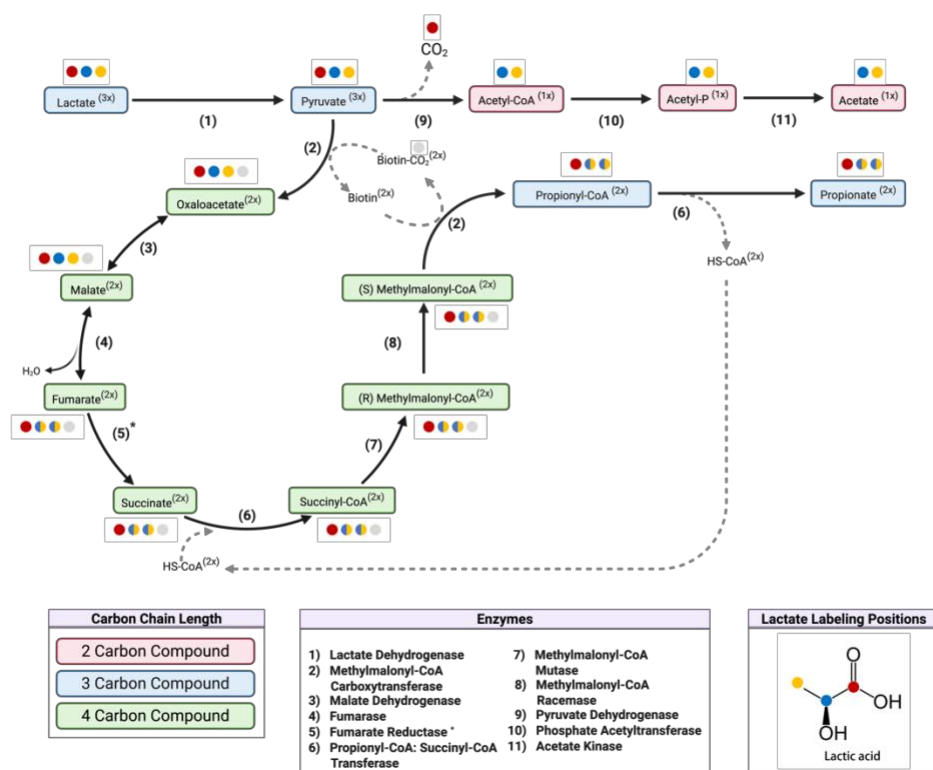


Figure S14. Overview of the lactate fermenting methylmalonyl-CoA pathway.

Schematic overview of the lactate fermenting methylmalonyl-CoA pathway. The carbon chain length of each intermediate is represented by a shaded box, while the position of each carbon derived from lactate is represented by a red, blue, yellow, or grey dot above the intermediate.

Supplemental Tables

Table S2-2: FISH Probes used in this study

Target	Name	Sequence (5'-3')	Probe	Citation
General Bacteria	EUB 338	GCT GCC TCC CGT AGG AGT	Cyanine 3	[8]
General Bacteria	EUB 338 II	GCA GCC ACC CGT AGG TGT	Cyanine 3	[9]
General Bacteria	EUB 338 III	GCT GCC ACC CGT AGG TGT	Cyanine 3	[9]
General Archaea	ARC 915	GTG CTC CCC CGC CAA TTC CT	Fluorescein	[10]

Table S2-3: Catabolic reaction stoichiometry with thermodynamic and equilibrium constants for species in the wetland community model.

	Process	Reaction	$\Delta G^{\circ 1}$ [kJ·mol ⁻¹]	Equilibrium Constant K [-]	Ref
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})					
1	Lactate oxidation	$1 \text{ C}_2\text{H}_4\text{O}_3^- + 2 \text{ H}_2\text{O} \rightarrow 1 \text{ C}_2\text{H}_3\text{O}_2^- + 1 \text{ HCO}_3^- + 2 \text{ H}_2 + 2 \text{ H}^+$	-3.98	$8.81 \cdot 10^{-7}$	[11]
2	Hydrogen dependent sulfate reduction	$4 \text{ H}_2 + 1 \text{ SO}_4^{2-} + 2 \text{ H}^+ \rightarrow 1 \text{ H}_2\text{S} + 4 \text{ H}_2\text{O}$	-157.86	$1.09 \cdot 10^{40}$	[11]
3	Lactate dependent sulfate reduction	$2 \text{ C}_2\text{H}_4\text{O}_3^- + 1 \text{ SO}_4^{2-} \rightarrow 2 \text{ C}_2\text{H}_3\text{O}_2^- + 1 \text{ H}_2\text{S} + 2 \text{ HCO}_3^-$	-165.83	$8.50 \cdot 10^{27}$	[11]
Lactate fermentation (LacFerm; X_{LF})					
4	Lactate fermentation	$3 \text{ C}_3\text{H}_5\text{O}_3^- \rightarrow 2 \text{ C}_3\text{H}_5\text{O}_2^- + 1 \text{ C}_2\text{H}_3\text{O}_2^- + 1 \text{ HCO}_3^- + 1 \text{ H}^+$	-164.92	$1.13 \cdot 10^{21}$	-
Propionate oxidation (PropOx; X_{PO})					
5	Propionate oxidation	$1 \text{ C}_3\text{H}_5\text{O}_2^- + 3 \text{ H}_2\text{O} \rightarrow 1 \text{ C}_2\text{H}_3\text{O}_2^- + 1 \text{ HCO}_3^- + 3 \text{ H}_2 + 1 \text{ H}^+$	76.49	$2.47 \cdot 10^{-20}$	-
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; $X_{Met,H2}$)					
6	Hydrogenotrophic methanogenesis	$4 \text{ H}_2 + 1 \text{ HCO}_3^- + 1 \text{ H}^+ \rightarrow 1 \text{ CH}_4 + 3 \text{ H}_2\text{O}$	-135.57	$3.62 \cdot 10^{29}$	[12]
Acetoclastic methanogen (Met,Ace; $X_{Met,Ace}$)					
7	Acetoclastic methanogenesis	$1 \text{ C}_2\text{H}_3\text{O}_2^- + 1 \text{ H}_2\text{O} \rightarrow 1 \text{ CH}_4 + 1 \text{ HCO}_3^-$	-30.99	$1.66 \cdot 10^5$	-

Table S2-4. Overall reaction stoichiometry for species in the wetland community model.

	Process	Reaction	Ref
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})			
1	Lactate oxidation	$1 \text{ C}_3\text{H}_5\text{O}_3^- + 1.765 \text{ H}_2\text{O} + 0.047 \text{ NH}_3 \rightarrow$ $0.906 \text{ C}_2\text{H}_3\text{O}_2^- + 1.906 \text{ H}_2 + 0.953 \text{ HCO}_3^- + 0.047 \text{ C}_5\text{H}_7\text{O}_2\text{N} + 0.859 \text{ H}^+$	[11]
2	Hydrogen dependent sulfate reduction	$1 \text{ H}_2 + 0.263 \text{ SO}_4^{2-} + 0.05 \text{ C}_3\text{H}_5\text{O}_3^- + 0.025 \text{ NH}_3 + 0.55 \text{ H}^+ \rightarrow$ $0.263 \text{ H}_2\text{S} + 0.025 \text{ HCO}_3^- + 0.025 \text{ C}_5\text{H}_7\text{O}_2\text{N}$	[11]
3	Lactate dependent sulfate reduction	$1 \text{ C}_3\text{H}_5\text{O}_3^- + 0.442 \text{ SO}_4^{2-} + 0.058 \text{ NH}_3 + 0.058 \text{ H}^+ \rightarrow$ $0.884 \text{ C}_2\text{H}_3\text{O}_2^- + 0.116 \text{ H}_2 + 0.442 \text{ H}_2\text{S} + 0.942 \text{ HCO}_3^- + 0.025 \text{ C}_5\text{H}_7\text{O}_2\text{N} + 0.058 \text{ H}_2\text{O}$	[11]
Lactate fermentation (LacFerm; X_{LF})			
4	Lactate fermentation	$1 \text{ C}_3\text{H}_5\text{O}_3^- + 0.013 \text{ NH}_3 \rightarrow 0.324 \text{ C}_2\text{H}_3\text{O}_2^- + 0.649 \text{ C}_3\text{H}_5\text{O}_2^- +$ $0.338 \text{ HCO}_3^- + 0.027 \text{ H}_2 + 0.311 \text{ H}^+ + 0.013 \text{ H}_2\text{O} + 0.013 \text{ C}_5\text{H}_7\text{O}_2\text{N}$	-
Propionate oxidation (PropOx; X_{PO})			
5	Propionate oxidation	$1 \text{ C}_3\text{H}_5\text{O}_2^- + 2.885 \text{ H}_2\text{O} + 0.023 \text{ NH}_3 \rightarrow$ $0.954 \text{ C}_2\text{H}_3\text{O}_2^- + 0.977 \text{ HCO}_3^- + 2.954 \text{ H}_2 + 0.931 \text{ H}^+ + 0.023 \text{ C}_5\text{H}_7\text{O}_2\text{N}$	-
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; $X_{Met,H2}$)			
6	Hydrogenotrophic Methanogenesis	$1 \text{ H}_2 + 0.26 \text{ HCO}_3^- + 0.004 \text{ NH}_3 + 0.26 \text{ H}^+ \rightarrow$ $0.24 \text{ CH}_4 + 0.004 \text{ C}_5\text{H}_7\text{O}_2\text{N} + 0.773 \text{ H}_2\text{O}$	[12]
<i>Methanothrix</i> -like acetoclastic methanogen (Met,Ace1; $X_{Met,Ace1}$)			
7	Acetoclastic methanogenesis	$1 \text{ C}_2\text{H}_3\text{O}_2^- + 0.045 \text{ NH}_3 + 0.755 \text{ H}^+ \rightarrow$ $0.889 \text{ HCO}_3^- + 0.889 \text{ CH}_4 + 0.045 \text{ C}_5\text{H}_7\text{O}_2\text{N}$	-
<i>Methanosarcina</i> -like acetoclastic methanogen (Met,Ace2; $X_{Met,Ace2}$)			
8	Acetoclastic methanogenesis	$1 \text{ C}_2\text{H}_3\text{O}_2^- + 0.064 \text{ NH}_3 + 0.159 \text{ H}^+ \rightarrow$ $0.841 \text{ HCO}_3^- + 0.841 \text{ CH}_4 + 0.064 \text{ C}_5\text{H}_7\text{O}_2\text{N}$	-

Table S2-5. Kinetic uptake rate expressions for representative species in the wetland microbial community model.

	Process	Electron Donor	Electron Acceptor	Uptake rate equation
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})				
1	Lactate oxidation	Lactate	-	$r^L = q^L \cdot \left(\frac{[Lactate]}{K_{Lac, X_{SRB}} + [Lactate]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^L}{\alpha^L K^L} \right) \right)$
2	Hydrogen dependent sulfate reduction	H ₂	SO ₄ ²⁻	$r^{HS} = q^{HS} \cdot \left(\frac{[H_2]}{K_{H_2, X_{SRB}} + [H_2]} \right) \cdot \left(\frac{[SO_4^{2-}]}{K_{SO_4^{2-}, X_{SRB}} + [SO_4^{2-}]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^{HS}}{\alpha^{HS} K^{HS}} \right) \right)$
3	Lactate dependent sulfate reduction	Lactate	SO ₄ ²⁻	$r^{LS} = q^{LS} \cdot \left(\frac{[Lactate]}{K_{Lac, X_{SRB}} + [Lactate]} \right) \cdot \left(\frac{[SO_4^{2-}]}{K_{SO_4^{2-}, X_{SRB}} + [SO_4^{2-}]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^{LS}}{\alpha^{LS} K^{LS}} \right) \right)$
Lactate fermentation (LacFerm; X_{LF})				
4	Lactate fermentation	Lactate	-	$r^{LF} = q^{LF} \cdot \left(\frac{[Lactate]}{K_{Lac, X_{LF}} + [Lactate]} \right) \cdot X_{LF} \cdot \left(1 - \left(\frac{Q^{LF}}{\alpha^{LF} K^{LF}} \right) \right)$
Propionate oxidation (PropOx; X_{PO})				
5	Propionate oxidation	Propionate	-	$r^{PO} = q^{PO} \cdot \left(\frac{[Propionate]}{K_{Prop, X_{PO}} + [Propionate]} \right) \cdot X_{PO} \cdot \left(1 - \left(\frac{Q^{PO}}{\alpha^{PO} K^{PO}} \right) \right)$
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; X_{Met, H_2})				
6	Hydrogenotrophic Methanogenesis	H ₂	-	$r^H = q^H \cdot \left(\frac{[H_2]}{K_{H_2, X_{H_2Meth}} + [H_2]} \right) \cdot X_{H_2Meth} \cdot \left(1 - \left(\frac{Q^H}{\alpha^H K^H} \right) \right)$
<i>Methanothrix</i> -like acetoclastic methanogen (Met,Ace1; $X_{Met, Ace1}$)				
7	Acetoclastic methanogenesis	Acetate	-	$r^{Ac_1} = q^{Ac_1} \cdot \left(\frac{[Acetate]}{K_{Ace, X_{Ac_1}} + [Acetate]} \right) \cdot X_{Ac_1} \cdot \left(1 - \left(\frac{Q^{Ac}}{\alpha^{Ac} K^{Ac}} \right) \right)$
<i>Methanosarcina</i> -like acetoclastic methanogen (Met,Ace2; $X_{Met, Ace2}$)				
8	Acetoclastic methanogenesis	Acetate	-	$r^{Ac_2} = q^{Ac_2} \cdot \left(\frac{[Acetate]}{K_{Ace, X_{Ac_2}} + [Acetate]} \right) \cdot X_{Ac_2} \cdot \left(1 - \left(\frac{Q^{Ac}}{\alpha^{Ac} K^{Ac}} \right) \right)$
Note: Reaction rates are set to 0 when the thermodynamic threshold $\left(\frac{Q^i}{\alpha^i K^i} \right)$ is greater than or equal to 1				

Table S2-6. Thermodynamic reaction quotient for species in the wetland community model.

Process		Thermodynamic Threshold
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})		
1	Lactate fermentation	$Q^L = \frac{[Acetate] \cdot [H^+] \cdot [HCO_3^-] \cdot p_{H_2}^2}{[Lactate]}$
2	Hydrogen dependent sulfate reduction	$Q^{HS} = \frac{[H_2S]}{p_{H_2}^4 \cdot [SO_4^{2-}] \cdot [H^+]^2}$
3	Lactate dependent sulfate reduction	$Q^{LS} = \frac{[Acetate]^2 \cdot [H_2S] \cdot [HCO_3^-]}{[Lactate]^2 \cdot [SO_4^{2-}]}$
Lactate fermentation (LacFerm; X_{LF})		
4	Lactate fermentation	$Q^{LF} = \frac{[Propionate]^2 \cdot [Acetate] \cdot [HCO_3^-]}{[Lactate]^3}$
Propionate oxidation (PropOx; X_{PO})		
	Propionate oxidation	$Q^{PO} = \frac{[Acetate] \cdot [HCO_3^-] \cdot p_{H_2}^3}{[Propionate]}$
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; $X_{Met,H2}$)		
5	Hydrogenotrophic Methanogenesis	$Q^H = \frac{p_{CH_4}}{p_{H_2}^4 \cdot [HCO_3^-] \cdot [H^+]}$
Acetoclastic methanogen (Met,Ace; $X_{Met,Ace}$)		
6	Acetoclastic methanogenesis	$Q^{Ac} = \frac{[HCO_3^-] \cdot p_{CH_4}}{[Acetate]}$

Table S2-7. Biomass yields for species in the wetland community model.

Process	Organism	Symbol	Value	Unit	Reference
Lactate oxidation	SRB	$Y^L_{X/Lactate}$	5.3	$g_x \cdot mol_{Lactate}^{-1}$	[11, 13]
H ₂ dependent sulfate reduction	SRB	Y^{HS}_{X/H_2}	2.8	$g_x \cdot mol_{H_2}^{-1}$	[11, 14–16]
Lactate dependent sulfate reduction	SRB	$Y^{LS}_{X/Lactate}$	6.5	$g_x \cdot mol_{Lactate}^{-1}$	[11, 13, 16–18]
Lactate fermentation	LO	$Y^{LF}_{X/Lactate}$	4.29	$g_x \cdot mol_{Lactate}^{-1}$	[19]
Propionate oxidation	PO	$Y^{PO}_{X/Propionate}$	2.6	$g_x \cdot mol_{Propionate}^{-1}$	[20]
Hydrogenotrophic methanogenesis <i>Methanotrrix</i> -like	Met,H2	Y^H_{X/H_2}	0.482	$g_x \cdot mol_{H_2}^{-1}$	[11, 21]
Acetoclastic methanogenesis <i>Methanosarcina</i> -like	Met,Ace1	$Y^{Ace1}_{X/Acetate}$	1.4	$g_x \cdot mol_{Acetate}^{-1}$	[22]
Acetoclastic methanogenesis	Met,Ace2	$Y^{Ace2}_{X/Acetate}$	2.1	$g_x \cdot mol_{Acetate}^{-1}$	[22]

Table S2-8. Stoichiometric solute yield matrix for species in the wetland community model.

A _{ij} i component → j process ↓	Lactate [mM]	Propionate [mM]	Acetate [mM]	H ₂ [mM]	HCO ₃ ⁻ [mM]	CH ₄ [mM]	SO ₄ ²⁻ [mM]	H ₂ S [mM]	NH ₃ [mM]	H ⁺ [mM]
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})										
Lactate oxidation	-1	0	0.906	0	0.935	0	0	0	-0.047	0.859
H ₂ dependent sulfate reduction	-0.05	0	0	-1	0.025	0	-0.263	0.263	-0.025	-0.55
Lactate dependent sulfate reduction	-1	0	0.884	0.116	0.942	0	-0.442	0.442	-0.058	-0.058
Lactate fermentation (LacFerm; X_{LF})										
Lactate fermentation	-1	0.649	0.324	0.027	0.338	0	0	0	-0.013	0.311
Propionate oxidation (PropOx; X_{PO})										
Propionate oxidation	0	-1	0.954	2.954	0.977	0	0	0	-0.023	0.931
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; $X_{Met,H2}$)										
Hydrogenotrophic Methanogenesis	0	0	0	-1	-0.261	0.239	0	0	-0.004	-0.261
<i>Methanothrix</i> -like acetoclastic methanogen (Met,Ace1; $X_{Met,Ace1}$)										
Acetoclastic methanogenesis	0	0	-1	0	0.889	0.889	0	0	-0.045	-0.112
<i>Methanosarcina</i> -like acetoclastic methanogen (Met,Ace2; $X_{Met,Ace2}$)										
Acetoclastic methanogenesis	0	0	-1	0	0.841	0.841	0	0	-0.064	-0.159

Table S2-9. Stoichiometric biomass yield matrix for species in the wetland community model.

A_{ij} i component → j process ↓	Lactate [mM]	Propionate [mM]	Acetate [mM]	H ₂ [mM]	X _{SRB} [mM]	X _{LO} [mM]	X _{PO} [mM]	X _{H2Meth} [mM]	X _{AcMeth1} [mM]	X _{AcMeth2} [mM]	X _{inert} [mM]
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})											
Lactate oxidation	-1	0	0.906	0	0.047	0	0	0	0	0	0
H ₂ dependent sulfate reduction	-0.05	0	0	-1	0.025	0	0	0	0	0	0
Lactate dependent sulfate reduction	-1	0	0.884	0.116	0.058	0	0	0	0	0	0
Lactate fermentation (LacFerm; X_{LF})											
Lactate fermentation	-1	0.649	0.324	0.027	0	0.013	0	0	0	0	0
Propionate oxidation (PropOx; X_{PO})											
Propionate oxidation	0	-1	0.954	2.954	0	0	0.023	0	0	0	0
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; $X_{Met,H2}$)											
Hydrogenotrophic Methanogenesis	0	0	0	-1	0	0	0	0.004	0	0	0
<i>Methanothrix</i> -like acetoclastic methanogen (Met,Ace1; $X_{Met,Ace1}$)											
Acetoclastic methanogenesis	0	0	-1	0	0	0	0	0	0.045	0	0
<i>Methanosarcina</i> -like acetoclastic methanogen (Met,Ace2; $X_{Met,Ace2}$)											
Acetoclastic methanogenesis	0	0	-1	0	0	0	0	0	0	0.064	0
Biomass decay											
X _{SRB} decay	0	0	0	0	-1	0	0	0	0	0	1
X _{LO} decay	0	0	0	0	0	-1	0	0	0	0	1
X _{PO} decay	0	0	0	0	0	0	-1	0	0	0	1
X _{H2Meth} decay	0	0	0	0	0	0	0	-1	0	0	1
X _{AcMeth1} decay	0	0	0	0	0	0	0	0	-1	0	1
X _{AcMeth2} decay	0	0	0	0	0	0	0	0	0	-1	1

Table S2-10. Kinetic rate expressions for wetland community model.

Process	Equation
<i>Desulfovibrio</i> -like lactate oxidizing SRB (LacOx SRB; X_{SRB})	
1 Lactate oxidation	$R_{SRB}^L = Y_{i/Lactate}^L \cdot r^L = Y_{i/Lactate}^L \cdot q^L \cdot \left(\frac{[Lactate]}{K_{Lac, X_{SRB}} + [Lactate]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^L}{\alpha^L K^L} \right) \right)$
2 Hydrogen dependent sulfate reduction	$R_{SRB}^{HS} = Y_{i/H_2}^{HS} \cdot r^{HS} = Y_{i/H_2}^{HS} \cdot q^{HS} \cdot \left(\frac{[H_2]}{K_{H_2, X_{SRB}} + [H_2]} \right) \cdot \left(\frac{[SO_4^{2-}]}{K_{SO_4^{2-}, X_{SRB}} + [SO_4^{2-}]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^{HS}}{\alpha^{HS} K^{HS}} \right) \right)$
3 Lactate dependent sulfate reduction	$R_{SRB}^{LS} = Y_{i/Lactate}^{LS} \cdot r^{LS} = Y_{i/Lactate}^{LS} \cdot q^{LS} \cdot \left(\frac{[Lactate]}{K_{Lac, X_{SRB}} + [Lactate]} \right) \cdot \left(\frac{[SO_4^{2-}]}{K_{SO_4^{2-}, X_{SRB}} + [SO_4^{2-}]} \right) \cdot X_{SRB} \cdot \left(1 - \left(\frac{Q^{LS}}{\alpha^{LS} K^{LS}} \right) \right)$
Lactate fermentation (LacFerm; X_{LF})	
4 Lactate fermentation	$R_{LF}^{LF} = Y_{i/Lactate}^{LF} \cdot r^{LF} = Y_{i/Lactate}^{LF} \cdot q^{LF} \cdot \left(\frac{[Lactate]}{K_{Lac, X_{LF}} + [Lactate]} \right) \cdot X_{LF} \cdot \left(1 - \left(\frac{Q^{LF}}{\alpha^{LF} K^{LF}} \right) \right)$
Propionate oxidation (PropOx; X_{PO})	
5 Propionate oxidation	$R_{PO}^{PO} = Y_{i/Propionate}^{PO} \cdot r^{PO} = Y_{i/Lactate}^{PO} \cdot q^{PO} \cdot \left(\frac{[Propionate]}{K_{Prop, X_{PO}} + [Propionate]} \right) \cdot X_{PO} \cdot \left(1 - \left(\frac{Q^{PO}}{\alpha^{PO} K^{PO}} \right) \right)$
<i>Methanospirillum</i> -like hydrogenotrophic methanogen (Met,H ₂ ; X_{Met, H_2})	
4 Hydrogenotrophic Methanogenesis	$R_{Met, H_2}^H = Y_{i/H_2}^H \cdot r^H = q^H \cdot \left(\frac{[H_2]}{K_{H_2, X_{H_2}Meth} + [H_2]} \right) \cdot X_{H_2Meth} \cdot \left(1 - \left(\frac{Q^H}{\alpha^H K^H} \right) \right)$
<i>Methanothrix</i> -like acetoclastic methanogen (Met,Ace1; $X_{Met, Ace1}$)	
Acetoclastic methanogenesis	$R_{Met, Ace1}^{Ac1} = Y_{i/Acetate}^{Ac1} \cdot r^{Ac1} = q^{Ac1} \cdot \left(\frac{[Acetate]}{K_{Ace, X_{Ac1}} + [Acetate]} \right) \cdot X_{Ac1} \cdot \left(1 - \left(\frac{Q^{Ac}}{\alpha^{Ac} K^{Ac}} \right) \right)$
<i>Methanosarcina</i> -like acetoclastic methanogen (Met,Ace2; $X_{Met, Ace2}$)	
Acetoclastic methanogenesis	$R_{Met, Ace2}^{Ac1} = Y_{i/Acetate}^{Ac1} \cdot r^{Ac2} = q^{Ac2} \cdot \left(\frac{[Acetate]}{K_{Ace, X_{Ac1}} + [Acetate]} \right) \cdot X_{Ac2} \cdot \left(1 - \left(\frac{Q^{Ac}}{\alpha^{Ac} K^{Ac}} \right) \right)$
Decay	
6 Biomass decay	$R_j^D = -bX_j$

Table S2-11. Kinetic rate parameters for species in the wetland community model.

Process	Organism	Symbol	Value	Unit	Ref
Maximum specific growth rate					
Lactate oxidation	SRB	$\mu^{\text{L}}_{\text{SRB}}$	0.242	h^{-1}	[11]
H ₂ dependent sulfate reduction	SRB	$\mu^{\text{HS}}_{\text{SRB}}$	0.105	h^{-1}	[11]
Lactate dependent sulfate reduction	SRB	$\mu^{\text{LS}}_{\text{SRB}}$	0.271	h^{-1}	[11]
Lactate fermentation	LO	μ^{LF}	0.2	h^{-1}	[19]
Propionate oxidation	PO	μ^{PO}	0.079	h^{-1}	[20]
Hydrogenotrophic methanogenesis	Met,H2	$\mu^{\text{H}}_{\text{Met,H2}}$	0.053	h^{-1}	[12, 21]
<i>Methanotrrix</i> -like Acetoclastic methanogenesis	Met,Ace1	$\mu^{\text{Ac1}}_{\text{Met,Ace1}}$	0.029	h^{-1}	[22]
<i>Methanosarcina</i> -like Acetoclastic methanogenesis	Met,Ace2	$\mu^{\text{Ac2}}_{\text{Met,Ace2}}$	0.058	h^{-1}	[22]
Maximum specific uptake rate					
Lactate oxidation	SRB	$q^{\text{L}}_{\text{SRB}}$	0.0458	$\text{mol}_{\text{Lactate}} \cdot g_{\text{x,SRB}}^{-1} \cdot \text{h}^{-1}$	[11]
H ₂ dependent sulfate reduction	SRB	$q^{\text{HS}}_{\text{SRB}}$	0.0375	$\text{mol}_{\text{H2}} \cdot g_{\text{x,SRB}}^{-1} \cdot \text{h}^{-1}$	[11]
Lactate dependent sulfate reduction	SRB	$q^{\text{LS}}_{\text{SRB}}$	0.0417	$\text{mol}_{\text{Lactate}} \cdot g_{\text{x,SRB}}^{-1} \cdot \text{h}^{-1}$	[11]
Lactate fermentation	LF	q^{LF}	0.0466	$\text{mol}_{\text{Lactate}} \cdot g_{\text{x,LF}}^{-1} \cdot \text{h}^{-1}$	[19]
Propionate oxidation	PO	q^{PO}	0.0304	$\text{mol}_{\text{Propionate}} \cdot g_{\text{x,PO}}^{-1} \cdot \text{h}^{-1}$	[20]
Hydrogenotrophic methanogenesis	H2Meth	$q^{\text{H}}_{\text{Met,H2}}$	0.1100	$\text{mol}_{\text{H2}} \cdot g_{\text{x,H2Met}}^{-1} \cdot \text{h}^{-1}$	[12, 21]
<i>Methanotrrix</i> -like Acetoclastic methanogenesis	Met,Ace1	$q^{\text{Ac1}}_{\text{Met,Ace1}}$	0.0207	$\text{mol}_{\text{Acetate}} \cdot g_{\text{x,AcMeth1}}^{-1} \cdot \text{h}^{-1}$	[22]
<i>Methanosarcina</i> -like Acetoclastic methanogenesis	Met,Ace2	$q^{\text{Ac2}}_{\text{Met,Ace2}}$	0.0276	$\text{mol}_{\text{Acetate}} \cdot g_{\text{x,AcMeth2}}^{-1} \cdot \text{h}^{-1}$	[22]
Endogenous biomass decay rate					
Biomass decay	All	b	0.02	d^{-1}	[23]

Table S2-12. Kinetic parameters for lactate species in the wetland community model.

Metabolite	Organism	Symbol	Value	Unit	Note	Ref
Half-saturation coefficient						
Lactate	SRB	$K_{Lac,X_{SRB}}$	240	μM	-	[24]
Hydrogen	SRB	$K_{H_2,X_{SRB}}$	1.4	μM	-	[11, 21]
Sulfate	SRB	$K_{SO_4^{2-},X_{SRB}}$	18.74	μM	-	[24]
Lactate	LO	$K_{Lac,X_{LF}}$	215	μM	-	[19]
Propionate	PO	$K_{Prop,X_{PO}}$	148	μM	-	[25]
Hydrogen	H2Meth	$K_{H_2,X_{Met,H_2}}$	6.56	μM	-	[12, 21]
Acetate	Met,Ace1	$K_{Ace,X_{Met,Ace1}}$	39	μM	Modified	[22]
Acetate	Met,Ace2	$K_{Ace,X_{Met,Ace2}}$	110	μM	Modified	[22]

Table S2-13. Mass diffusivity of solutes in water at 37°C.

Metabolite	Formula	Phase	Symbol	Value	Unit	Reference
Diffusion coefficients						
Lactate	$\text{C}_3\text{H}_5\text{O}_3^-$	Liquid	D_{Lactate}	$1.07 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Propionate	$\text{C}_3\text{H}_5\text{O}_3^-$	Liquid	$D_{\text{Propionate}}$	$1.06 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Acetate	$\text{C}_2\text{H}_3\text{O}_2^-$	Liquid	D_{Acetate}	$1.13 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Sulfate	SO_4^{2-}	Dissolved gas	D_{Sulfate}	$1.10 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Hydrogen	H_2	Dissolved gas	D_{H_2}	$5.32 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Hydrogen sulfide	H_2S	Dissolved gas	$D_{\text{H}_2\text{S}}$	$1.42 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Bicarbonate	HCO_3^-	Dissolved gas	D_{CO_2}	$1.23 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Methane	CH_4	Dissolved gas	D_{CH_4}	$1.55 \cdot 10^{-9}$	$\text{m} \cdot \text{s}^{-1}$	[7, 26]
Biomass	$\text{C}_5\text{H}_7\text{O}_2\text{N}$	Solid	D_X	0	$\text{m} \cdot \text{s}^{-1}$	Chosen

Effective biofilm diffusions coefficients were determined by multiplying the mass diffusivity by the effective mass diffusivity fraction [7].

Table S2-14. Effective mass diffusivity fractions.

Compounds	Symbol	Value	Unit	Reference
Light gases	$D_{\text{Eff,G}}$	0.25	-	[7]
Organic solutes	$D_{\text{Eff,Org}}$	0.8	-	[7]

Table S2-15. Biofilm reactor model parameters.

Parameter	Symbol	Value	Unit	Ref
Initial metabolite concentrations				
Lactate	$C_{Int,Lac}$	1	mM	Chosen
Propionate	$C_{Int,Prop}$	0	mM	Chosen
Acetate	$C_{Int,Ace}$	0	mM	Chosen
Sulfate	$C_{Int,SO4}$	0	mM	Chosen
Hydrogen	$C_{Int,H2}$	0	mM	Chosen
Hydrogen sulfide	$C_{Int,H2S}$	0	mM	Chosen
Carbonate	$C_{Int,HCO3}$	0	mM	Chosen
Methane	$C_{Int,CH4}$	0	mM	Chosen
Influent metabolite concentrations				
Lactate	$C_{In,Lac}$	1	mM	Chosen
Propionate	$C_{In,Prop}$	0	mM	Chosen
Acetate	$C_{In,Ace}$	0	mM	Chosen
Sulfate	$C_{In,SO4}$	0 – 1 (variable)	mM	Chosen
Hydrogen	$C_{In,H2}$	0	mM	Chosen
Hydrogen sulfide	$C_{In,H2S}$	0	mM	Chosen
Bicarbonate	$C_{In,HCO3}$	0	mM	Chosen
Methane	$C_{In,CH4}$	0	mM	Chosen
Biofilm parameters				
Biomass molar mass	M_X	113	$g \cdot mol^{-1}$	[5]
Biofilm biomass concentration	C_X	353.98	mM	[5]
Initial X_{SRB} conc.	$X_{Int,SRB}$	37.451	mM	Chosen
Initial X_{LF} conc.	$X_{Int,LF}$	37.451	mM	Chosen
Initial X_{PO} conc.	$X_{Int,PO}$	37.451	mM	Chosen
Initial X_{H2Meth} conc.	$X_{Int,MethH2}$	0.215	mM	Chosen
Initial $X_{AcMeth,1}$ conc.	$X_{Int,MetAce1}$	0.215	mM	Chosen
Initial $X_{AcMeth,2}$ conc.	$X_{Int,MetAce2}$	0.215	mM	Chosen
Initial X_{inert} conc.	$X_{Int,inert}$	0	mM	Chosen
Initial biofilm length	$L_{F,int}$	10	μm	Chosen
biofilm concentration				
Biofilm detachment rate	k_{detach}	0.0144	h^{-1}	[5]
Reactor parameters				
Liquid flowrate	Q_R	8.6	$mL \cdot h^{-1}$	Chosen
Reactor liquid volume	V_R	86	mL	Chosen
Dilution rate	Dil	0.1	h^{-1}	Chosen
Specific area of biofilm mass transfer	a_F	39.53	m^{-1}	Chosen

Supplemental References

1. Parada AE, Needham DM, Fuhrman JA. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 2016;**18**:1403–1414. <https://doi.org/10.1111/1462-2920.13023>
2. Edgar RC. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 2010;**26**:2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
3. Edgar RC. UNOISE2: Improved Error-Correction for Illumina 16S and ITS Amplicon Sequencing. *bioRxiv* 2016;081257.
4. Maidak BL et al. The RDP (Ribosomal Database Project). *Nucleic Acids Res* 1997;**25**:109–110. <https://doi.org/10.1093/nar/25.1.109>
5. Eberl H et al. Mathematical Modeling of Biofilms. IWA, 2006.
6. Wanner O, Gujer W. A multispecies biofilm model. *Biotechnol Bioeng* 1986;**28**:314–328. <https://doi.org/10.1002/bit.260280304>
7. Stewart PS. Diffusion in Biofilms. *J Bacteriol* 2003;**185**:1485–1491. <https://doi.org/10.1128/JB.185.5.1485-1491.2003>
8. Amann RI et al. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* 1990;**56**:1919–1925. <https://doi.org/10.1128/aem.56.6.1919-1925.1990>
9. Daims H et al. The Domain-specific Probe EUB338 is Insufficient for the Detection of all Bacteria: Development and Evaluation of a more Comprehensive Probe Set. *Syst Appl Microbiol* 1999;**22**:434–444. [https://doi.org/10.1016/S0723-2020\(99\)80053-8](https://doi.org/10.1016/S0723-2020(99)80053-8)
10. Stahl DA, Amann R. Development and Application of Nucleic Acid Probes in Bacterial Systematics. *Nucleic Acid Techniques in Bacterial Systematics*. 1991, 205–248.
11. Noguera DR et al. A unified model describing the role of hydrogen in the growth of *Desulfovibrio vulgaris* under different environmental conditions. *Biotechnol Bioeng* 1998;**59**:732–746. [https://doi.org/10.1002/\(SICI\)1097-0290\(19980920\)59:6<732::AID-BIT10>3.0.CO;2-7](https://doi.org/10.1002/(SICI)1097-0290(19980920)59:6<732::AID-BIT10>3.0.CO;2-7)
12. Noguera DR et al. Simulation of multispecies biofilm development in three dimensions. *Water Science and Technology* 1999;**39**:123–130. [https://doi.org/10.1016/S0273-1223\(99\)00159-6](https://doi.org/10.1016/S0273-1223(99)00159-6)

13. Traore AS et al. Energetics of Growth of a Defined Mixed Culture of *Desulfovibrio vulgaris* and *Methanosarcina barkeri* : Interspecies Hydrogen Transfer in Batch and Continuous Cultures. *Appl Environ Microbiol* 1983;**46**:1152–1156. <https://doi.org/10.1128/aem.46.5.1152-1156.1983>
14. Badziong W, Thauer RK. Growth yields and growth rates of *Desulfovibrio vulgaris* (Marburg) growing on hydrogen plus sulfate and hydrogen plus thiosulfate as the sole energy sources. *Arch Microbiol* 1978;**117**:209–214. <https://doi.org/10.1007/BF00402310>
15. Brandis A, Thauer RK. Growth of *Desulfovibrio* species on Hydrogen and Sulphate as Sole Energy Source. *Microbiology (N Y)* 1981;**126**:249–252. <https://doi.org/10.1099/00221287-126-1-249>
16. Nethe-Jaenchen R, Thauer RK. Growth yields and saturation constant of *Desulfovibrio vulgaris* in chemostat culture. *Arch Microbiol* 1984;**137**:236–240. <https://doi.org/10.1007/BF00414550>
17. Pankhania IP, Gow LA, Hamilton WA. The Effect of Hydrogen on the Growth of *Desulfovibrio vulgaris* (Hildenborough) on Lactate. *Microbiology (N Y)* 1986;**132**:3349–3356. <https://doi.org/10.1099/00221287-132-12-3349>
18. Ingvorsen K, Zehnder AJB, Jørgensen BB. Kinetics of Sulfate and Acetate Uptake by *Desulfobacter postgatei*. *Appl Environ Microbiol* 1984;**47**:403–408. <https://doi.org/10.1128/aem.47.2.403-408.1984>
19. Seeliger S, Janssen PH, Schink B. Energetics and kinetics of lactate fermentation to acetate and propionate via methylmalonyl-CoA or acrylyl-CoA. *FEMS Microbiol Lett* 2002;**211**:65–70. [https://doi.org/10.1016/S0378-1097\(02\)00651-1](https://doi.org/10.1016/S0378-1097(02)00651-1)
20. Scholten JCM, Conrad R. Energetics of Syntrophic Propionate Oxidation in Defined Batch and Chemostat Cocultures. *Appl Environ Microbiol* 2000;**66**:2934–2942. <https://doi.org/10.1128/AEM.66.7.2934-2942.2000>
21. Robinson JA, Tiedje JM. Competition between sulfate-reducing and methanogenic bacteria for H₂ under resting and growing conditions. *Arch Microbiol* 1984;**137**:26–32. <https://doi.org/10.1007/BF00425803>
22. Jetten MSM, Stams AJM, Zehnder AJB. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanotheroxobacter soehngenii* and *Methanosarcina* spp. *FEMS Microbiol Lett* 1992;**88**:181–198. <https://doi.org/10.1111/j.1574-6968.1992.tb04987.x>
23. Lawrence AW, McCarty PL. Unified Basis for Biological Treatment Design and Operation. *Journal of the Sanitary Engineering Division* 1970;**96**:757–778. <https://doi.org/10.1061/JSEDAI.0001126>

24. Okabe S, Nielsen PH, Characklis WG. Factors affecting microbial sulfate reduction by *Desulfovibrio desulfuricans* in continuous culture: Limiting nutrients and sulfide concentration. *Biotechnol Bioeng* 1992;**40**:725–734. <https://doi.org/10.1002/bit.260400612>
25. Heyes RH, Hall RJ. Kinetics of two subgroups of propionate-using organisms in anaerobic digestion. *Appl Environ Microbiol* 1983;**46**:710–715. <https://doi.org/10.1128/aem.46.3.710-715.1983>
26. Haynes WM. CRC Handbook of Chemistry and Physics. CRC Press, 2014.

Chapter 3.

N-acetylglucosamine degradation by a wetland microbial community under varying oxygen concentrations

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Abstract

N-acetylglucosamine (GlcNAc)—an amino sugar monomer—is a crucial component of the cell wall polymers chitin, peptidoglycan, and necromass making it one of the most abundant organic compounds across domains and a primary component of soil organic carbon (SOC). While much is known about GlcNAc utilization by aerobic isolates, little is known about the assembly of microbial communities around GlcNAc and how GlcNAc recycling—a mineralization process explicitly linking the carbon and nitrogen cycle—influences SOC formation. This study investigated how wetland microbial communities mineralize GlcNAc into nitrogen and carbon and utilize GlcNAc derived metabolites under aerobic and anaerobic conditions. Two low-dilution rate chemostats were used to enrich GlcNAc recycling wetland microbial communities in an oligotrophic environment. Oxygen perturbations were applied throughout the enrichment to simulate periodic drought and flooding events while investigating their impact on biomass-specific rates, pathway selection, and greenhouse gas (methane, nitrous oxide, and carbon dioxide) production. The chemostats demonstrated distinct carbon and nitrogen metabolite dynamics, variations in microbial community composition, and transcriptomic responses. Despite temporary changes, communities ultimately reverted to prior states, highlighting how community resilience and versatility informs necromass turnover dynamics in natural environments. This study also highlights how GlcNAc recycling links the carbon cycle with nitrification and denitrification.

Key words – N-acetylglucosamine, necromass, chemostat, metagenomics, meta-transcriptomics, wetland, microbial communities

3.1 Introduction

Wetlands are critical carbon sinks that sequester 20-30% of global soil organic carbon (SOC) despite occupying 5-8% of land area [1]. To ensure the long-term stability of this carbon reservoir, it is essential to understand how microbially mediated processes contribute to carbon mineralization and SOC formation. Traditionally, SOC formation was thought to arise from the progressive decomposition of complex plant litter to humic substances resistant to decomposition [2]. However, a vital component of SOC has been increasingly recognized as microbial necromass—organic remnants of cellular decay consisting of chitin, peptidoglycan, lipids, and amino sugars [3]. The net rate of cellular decay (production), necromass consumption (recycling), and mineral stabilization contribute to SOC formation through the accumulation of necromass biomarkers [4]. It remains unclear if necromass recycling significantly contributes to carbon and nitrogen mineralization.

The amino sugar N-acetylglucosamine (GlcNAc) is the primary component of the cell wall polymers chitin and peptidoglycan found in fungal and prokaryotic biomass, respectively [3]. Amino sugars have emerged as a preferred necromass biomarker in sediments due to the ubiquity of GlcNAc across microbial domains. Fragmented cell wall residues — including (GlcNAc) — are an enriched component of necromass that comprise approximately 7% of SOC yet are a negligible fraction of active biomass [3–6]. Consequently, the recycling of the necromass biomarker GlcNAc represents an understudied mineralization process explicitly linking the carbon and nitrogen cycle. In our depth-resolved metabolomics study of wetland soils, we observed that GlcNAc and other amino sugars were more abundant in soil layers deeper than 30cm from the surface and another study found amino sugars to comprise an increasing fraction of soil organic

matter in deeper layers [7]. The accumulation of GlcNAc with depth suggests this globally abundant amino sugar may become increasingly recalcitrant under anaerobic conditions, potentially due to reduced microbial metabolic activity and altered decomposition pathways in oxygen-depleted environments.

While knowledge about GlcNAc utilization by complex microbial communities under environmentally relevant conditions remains sparse, the genomic and physiological aspects of GlcNAc utilization in bacterial isolates has been extensively studied. The pathway, facilitated by genes in the *nag* operon, involves four steps: transport into the cell, phosphorylation of GlcNAc to GlcNAc-6P, deacetylation to GlcN-6P, and deamination/isomerization to Fructose-6P which enters glycolysis[8]. This pathway has been studied in bacteria like *E. coli* [9], *Aeromonas* [10, 11], *Shewanella* [8], *Bacteroidota* [12], *Vibrionaceae* [13] and *Candidatus Marinimicrobia* [14]. However, community assembly around GlcNAc, the influence of oxygen on GlcNAc utilization, and whether there are GlcNAc utilizing specialists are less understood.

In this study, two low-dilution rate chemostats were used to enrich GlcNAc recycling microbial communities from wetland sediments under aerobic and anaerobic conditions to mimic oligotrophic wetland environments. Oxygen perturbations were applied throughout the enrichment to simulate periodic drought and flooding events. We hypothesized that periodic oxygen perturbations would select for a resilient microbial community dominated by facultative anaerobes, capable of rapid adaptation to oxygen fluctuations. The chemostats demonstrated distinct carbon and nitrogen metabolite dynamics, variations in microbial community composition, and transcriptomic responses. This study highlighting the importance of necromass recycling as a significant nitrogen and carbon mineralization mechanism in the wetland environments.

3.2 Materials and Methods

3.2.1 *Wetland cell extraction for reactor inoculum*

A microbial community was extracted from a wetland soil sample taken from an urban lacustrine wetland (University of Washington Arboretum, Seattle, WA, USA - 47° 38' 31.12'' N, 122° 17' 47.01'' W). Samples were taken using a 2"x4" soil corer (AMS, American Falls, ID, USA) driven down 60 cm. Cores were transported to the lab on blue ice and stored at 4°C until processing. Cells were extracted in an anaerobic chamber (Coy Lab Products, Grass Lake, MI, USA) to minimize potential oxygen toxicity. Soil was first suspended (10% w/v) in modified lacustrine medium, followed by sieving (2 mm) to remove coarse particles. The sieved soil suspension was then paddle blended (Stomacher® 400 Circulator, Seward, UK) for 3 x 1 min at 250 rpm with 1-minute rests between runs. Samples were subsequently centrifuged at 200 g for 2 minutes to remove coarse organics and filtered through a 20 µm filter (Whatman™ Grade 41, Cytiva, Marlborough, MA, USA) to remove eukaryotes and fungal material.

3.2.2 *Reactor design and operation*

Two chemostats were operated at 20 °C with GlcNAc (5 mM influent concentration) as the sole carbon source. Custom 2.7 L glass chemostats were designed for this study. Chemostats were filled with 1.45 L of sterile GlcNAc lacustrine medium and inoculated with 50 mL of wetland sediment extract. The operating working volume of each chemostat was maintained at 1.5L with a 1.2 L headspace. Anoxic conditions were maintained by continuously sparging the chemostats with nitrogen through a 0.2-micron Teflon filter (Millipore-Sigma, St. Louis, MO, USA) at a flowrate of 20 mL·min⁻¹ with a mass flow controller (Alicat scientific, Tucson, AZ, USA) maintained

between 1-2 psig. Aerobic conditions were maintained by continuously sparging the chemostat with air at a flowrate of $27 \text{ mL} \cdot \text{min}^{-1}$. After inoculation, the chemostats were mixed without for 24 hours.

The chemostats were fed with lacustrine freshwater media containing 5mM GlcNAc using a drip-feed at a rate of $7.5 \text{ mL} \cdot \text{h}^{-1}$ for the remainder of the experiment (0.12 day^{-1} dilution rate; 8.3 day hydraulic retention time). The liquid volume within the reactor was maintained with an effluent pump. The pH was continuously monitored with a pH probe (Hamilton, Reno, NV, USA) and automatically controlled at 7.5 ± 0.1 using 1M HCl and 1M NaOH. Dissolved oxygen probes (Hamilton, Reno, NV, USA) were used to continuously monitor dissolved oxygen concentrations. Reactors were continuously stirred at approximately 500 rpm using magnetic stir bars and stir plates. The walls of the bioreactors were scraped weekly to avoid biofilm accumulation.

The lactate lacustrine media based on DSMZ 503 (anaerobic freshwater medium) with concentrations updated to reflect averaged porewater measurements of the top 60 cm of sediment at the wetland sampling site. The basal medium composition was as follows (in 1000mL MQ water): GlcNAc (5mM), 6.4 mg $(\text{NH}_4)_2\text{SO}_4$ (0.12mM), 35.9 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.24mM), 33.2 mg $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.16mM), 1 mL SL-10 trace element solution, 1 mL selenate-tungstate (Se-W) solution. The SL-10 trace element solution consisted of (in 1000 mL MQ water): 10 mL 7.7M HCl, 1.5 g $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, 0.07 g ZnCl_2 , 0.15 g $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.006 g H_3BO_3 , 0.19 g $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.002 g $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 0.024 g $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.036 g $\text{Na}_2\text{MoO}_4 \cdot 4\text{H}_2\text{O}$. The Se-W solution contained (in 1000 mL MQ water): 0.5 g NaOH, 0.003 g $\text{Na}_2\text{SeO}_3 \cdot 5\text{H}_2\text{O}$, 0.004 g $\text{Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$. After autoclaving, heat-sensitive media components were aseptically added as follows (in 1000mL MQ water): 0.3 mL 1M KHCO_3 , 0.4 mL 1M NaH_2PO_4 , 1.2 mL 0.5M Na_2HPO_4 , 0.55 mL 0.5% $(\text{NH}_4)_2\text{S}$,

and 0.1 mL 7-vitamin solution. The 7-vitamin solution consisted of (in 100 mL MQ water): 0.1 g vitamin B12, 0.08 g p-aminobenzoic acid, 0.02 g D(+)-Biotin, 0.2 g nicotinic acid, 0.1 g Ca-pantothenate, 0.3 g pyridoxine hydrochloride, 0.2 g thiamine-HCl·2H₂O.

Oxygen perturbations occurred on day 67.86 and 187.08 with each perturbation lasting 8.93 and 8.09 days, respectively. The perturbation was induced by switching the inlet sparging gases. Anaerobic conditions were maintained in the previously aerobic chemostat with nitrogen and aerobic conditions were maintained in the previously anaerobic chemostat with air.

3.2.3 Sampling and chemical analysis

Liquid and gas samples were collected two to three times per week. Liquid samples were collected from a three-way sampling port attached to the influent and effluent lines. Cell concentrations were estimated from a biomass using optical density at 600 nm measured with a 96-well plate reader. Samples for metabolite analysis were centrifuged at 21500 g for 20 minutes and stored at -20°C. A pH microelectrode (Orion; ThermoScientific) was used to measure pH prior to sample storage. Ammonium, nitrite, and nitrate concentrations were measured from reactor effluent and influent using a Gallery Discrete Analyzer using off-the-shelf assay kits. Concentrations of GlcNAc, organic acids, sugars, and alcohols were measured by high-performance liquid chromatography (HPLC) with an Aminex column and UV detection. Cell dry weight measurements were performed by filtering 10 mL of effluent onto pre-dried and weighed PES filters which were then dried at 105°C overnight.

Gas production rates were measured using cavity ring-down spectroscopy (Picarro model G2508) by connecting the analyzer to the reactor headspace and recirculating the gas to achieve steady-

state measurements. Gas production rates were then calculated using information about sparging gas flow rate and the volume of the setup.

3.2.4 DNA extraction and metagenomic sequencing

DNA was extracted from biomass pellets stored at -80°C using the DNeasy PowerBiofilm kit (Qiagen) according to the manufacturer's instructions. DNA concentrations were quantified using the Qubit HS dsDNA assay. Samples were sent to the Joint Genome Institute for sequencing.

Plate-based DNA library preparation for Illumina sequencing was performed on the PerkinElmer Sciclone NGS robotic liquid handling system using Kapa Biosystems library preparation kit. 200 ng of sample DNA was sheared to 500 bp using a Covaris LE220 focused-ultrasonicator. The sheared DNA fragments were size selected by double-SPRI and then the selected fragments were end-repaired, A-tailed, and ligated with Illumina compatible sequencing adaptors from IDT containing a unique molecular index barcode for each sample library. The prepared libraries were quantified using KAPA Biosystems' next-generation sequencing library qPCR kit and run on a Roche LightCycler 480 real-time PCR instrument. Sequencing of the flowcell was performed on the Illumina NovaSeq sequencer using NovaSeq XP V1.5 reagent kits, S4 flowcell, following a 2x151 indexed run recipe.

3.2.5 RNA extraction and meta-transcriptomic sequencing

Biomass pellets for RNA extraction were immediately frozen on dry ice and stored at -80°C to prevent degradation. RNA was extracted using the RNeasy PowerBiofilm kit (Qiagen) according to the manufacturer's instructions. An additional DNase treatment step was performed to ensure no residual DNA remained. RNA was quantified using the Qubit BR RNA assay and RNA quality

was assessed using a BioAnalyzer before sending samples to the Joint Genome Institute for library preparation sequencing.

3.2.6 Metagenomics and meta-transcriptomics data analysis

Filtered metagenomic reads were assembled with metaSPAdes v3.15.2 [15] and filtered meta-transcriptomic reads were de-novo assembled with rnaSPAdes v4.0.0 [16]. Contigs smaller than 200 bp were removed. Alignment information was determined by mapping reads to the assembly reference with BBtools [17] version 38.95 [command: `bbmap.sh -Xmx450g nodisk=true interleaved=true ambiguous=random mappedonly=t trimreaddescriptions=t usemodulo=t fast=t`]. Coverage was determined by running BBTools version 38.95 [command: `pileup.sh`]. The IMG/M pipeline [18] was used to annotate the assembled contigs, and prokaryotic bins were predicted using metaBAT [19].

For gene-based analysis, taxonomic and functional profiles were retrieved from the JGI IMG/M portal using the Abundance Profile tool. For functional profiles, the estimated gene copies of KEGG KOs were used and center-log₂ ratio (clr) normalized on a per sample basis based on the method from ALDEx2 [20]. For taxonomic profiles, the estimated gene copies for order-level GTDB taxonomy at 60+ % identity were used and normalized using relative abundance.

3.3 Results

3.3.1 *Carbon and nitrogen cycling dynamics reflect O₂-dependent shifts in community function*

Two planktonic wetland microbial communities were enriched in chemostats under anaerobic and fully aerobic conditions to quantitatively link necromass mineralization rates with community structure in response to fluctuating oxygen availability (Figure 3.1). N-Acetylglucosamine (GlcNAc) served as the sole carbon, nitrogen, and energy source with no dissolved electron supplied. All substrates were continuously provided with a dilution rate of 0.005 h⁻¹ (Table S3-3). Fully anaerobic and aerobic conditions were ensured by continuous nitrogen (N₂) and air sparging, respectively. Dissolved oxygen was maintained above 6.5 mg/L (>75% air saturation) under aerobic conditions, and oxygen (O₂) served as the sole electron acceptor. Two ~8.5-day drought-flooding perturbations were simulated on day 67.9 and 188.8 by switching the air/N₂ supply to the anaerobic and aerobic chemostats. The chemostats were reverted to their prior condition upon terminating the perturbation. These experimental transitions provided insights into how shifting oxygen concentrations influences community function, structure, and stability in wetland-like environments.

Each chemostat progressed through distinct operational phases, including start-up, steady-state, transient disturbances, and recovery. Fermentation dominated the two steady-states (Phase 2 & 4) in the anaerobic chemostat, as indicated by their high biomass-specific production rate of acetate and propionate (Figure 3.1A; Table S3-1). Acetate was the primary GlcNAc mineralization product and gradually decreased throughout the initial 5 phases of the enrichment. Following the second O₂ perturbation (Phase 6), a sharp increase in CH₄ production marked the transition to methanogenesis (Phase 7), which coincided with the rapid consumption of acetate and propionate.

Biomass-specific CO₂ and biomass production increased during the O₂ perturbation (Phase 3 & 5) suggesting the anoxic microbial community enhanced respiration and growth in response to O₂ availability. Transient anaerobic features emerged in the aerobic chemostat during N₂ perturbations (Figure 3.1B). Notably, acetate accumulated in response to the two N₂ pulses (Phase 3 & 5) demonstrating the community shifted from heterotrophic respiration to fermentative metabolisms. Surprisingly, CH₄ was detected throughout the first 4 phases of the aerobic GlcNAc degrading enrichment—albeit at rates below $0.02 \pm 0.018 \mu\text{mol-C}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ —and was no longer observed at the beginning of the second steady-state. As observed in the anaerobic chemostat, CO₂ production increased with O₂ availability. However, the biomass production rates remained consistently higher in the aerobic chemostat, underscoring the increased yield of heterotrophic respiration relative to fermentation.

While both chemostats initially mineralized GlcNAc to ammonium (NH₄⁺), the trajectory of nitrogen transformation significantly differed between the anaerobic and aerobic microbial communities. Nitrogen cycling was largely inactive in the anaerobic chemostat (Figure 3.1A), as indicated by the constant NH₄⁺ production rate and absence of other nitrogen species. In contrast, the aerobic chemostat displayed dynamic nitrogen transformations throughout the enrichment (Figure 3.1B). Before the first N₂ perturbation, NH₄⁺ was stoichiometrically oxidized to nitrite (NO₂⁻) with a steady-state biomass-specific ammonia-oxidation rate of $3.612 \pm 0.601 \mu\text{mol-N}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$ and transitioned to complete nitrification after the N₂ fluctuation. This transition was characterized by increasing nitrate (NO₃⁻) production as NO₂⁻ production decreased in Phase 5 and 7. Nitrous oxide (N₂O), a potent greenhouse gas, was produced at higher rates in the aerobic chemostat at steady-state and exhibited significant spikes immediately after all O₂ fluctuations.

Together, this suggests constitutive N₂O production by the nitrifying population that significantly responds to changes in oxygen availability. The largest N₂O pulse occurred immediately after the onset of second N₂ perturbation, when nitrate was the predominant nitrogen species. Nitrate-reduction by facultative partial denitrifiers likely contributed to this massive spike in N₂O. The different N₂O dynamics reflects the expression of distinct N₂O production pathways in the aerobic and anaerobic chemostat.

3.3.2 *Microbial community response*

Microbial cell densities were higher in the aerobic chemostat compared to the anaerobic chemostat throughout the study period (Figure 3.1). In general, biomass-specific biomass production rates were more stable in the anaerobic chemostat, while the aerobic chemostat experienced fluctuations particularly around regular cleaning of biofilms from the reactor vessel walls. The perturbations caused an increase in biomass production in the anaerobic chemostat and a decrease in the aerobic chemostat.

The two reactors maintained distinct taxonomic and functional compositions throughout the experiment, as confirmed by both PERMANOVA and ANOSIM analyses ($p < 0.001$; Figure 3.2). Prior to perturbation, the anaerobic reactor was primarily dominated by Gammaproteobacteria, Clostridia, and Bacteroidia, whereas the aerobic reactor was enriched in Alphaproteobacteria, Gammaproteobacteria, and Bacteroidia (Figure 3.2A, C). Minor taxa also differed between reactors: the anaerobic reactor harbored Fusobacteriia, Bacilli, Desulfuromonadia, and Desulfovibrionia, while the aerobic reactor contained low-abundance populations of Verrucomicrobia and Fimbriimonadia. At the family level, Rhodocyclaceae (Gammaproteobacteria) steadily increased up to 60% abundance in the anaerobic reactor, while

Devosiaceae (Alphaproteobacteria) was the dominant family in the aerobic reactor though its abundance declined in the second half of the experiment (Figure S3.5).

During the pre-perturbation phase, the anaerobic reactor exhibited significantly higher taxonomic diversity compared to the aerobic reactor (Figure S3.4A, B). Both systems, however, experienced pronounced shifts in microbial composition in response to their respective oxygen perturbations. In the anaerobic reactor, perturbations led to increases in Alphaproteobacteria and Bacteroidia and decreases in Clostridia and Fusobacteriia (Figure 3.2A, B). Conversely, the aerobic reactor responded to perturbation with declines in Alphaproteobacteria and increases in Clostridia and Gammaproteobacteria; notably, Bacilli became more prominent during the second perturbation (Figure 3.2C, D).

Following the first perturbation, the anaerobic reactor showed a decline in both taxonomic and functional diversity, while diversity in the aerobic reactor remained stable or increased. This contrast became especially pronounced during the second perturbation, during which the aerobic reactor exhibited significantly higher diversity than the anaerobic counterpart.

Recovery trajectories also differed between reactors. In the aerobic reactor, the taxonomic composition of transcripts rapidly returned to pre-perturbation states, while the anaerobic reactor failed to revert to its original community structure (Figure 3.3B). Despite these taxonomic differences, both reactors exhibited similar trends in the recovery of functional composition (Figure 3.3D).

Over the course of the experiment, Gammaproteobacteria became increasingly dominant in the anaerobic reactor, while Verrucomicrobiae steadily increased in the aerobic reactor. Notably,

Bacteroidia remained relatively stable in both systems across all operational phases. Ultimately, both reactors underwent substantial shifts in taxonomic and functional composition, culminating in distinct endpoint communities. While the aerobic reactor experienced a more pronounced shift in taxonomic structure (Figure 3.3A), both reactors showed comparable changes in functional composition (Figure 3.3C).

3.3.3 Differences in metabolic pathways between reactors and across operational phases

Clear differences in amino sugar metabolic pathways were observed between the two reactors. The *ngcE* ABC transporter for GlcNAc was significantly more abundant in the aerobic reactor, while the *nagE* PTS transport system was more abundant in the anaerobic reactor (Figure 3.4). Similarly, *gspK* (glucosamine kinase) and *dac* (GlcNAc deacetylase) were enriched in the aerobic reactor, whereas *nagB* (glucosamine-6-phosphate deaminase) was more abundant in the anaerobic reactor. Both systems exhibited active transcription of chitinases, notably given that chitin is a polymer of GlcNAc. A schematic representation of GlcNAc pathway differences is shown in Figure 3.5, highlighting that enzymes favored in the anaerobic system require less ATP. Specifically, the PTS transport system utilizes phosphoenolpyruvate (PEP) to simultaneously transport and phosphorylate GlcNAc, while the *ngcE* ABC transporter consumes one ATP for transport and another for subsequent phosphorylation.

Distinct patterns also emerged in one-carbon (C1) metabolism. As expected, genes involved in methanogenesis were significantly enriched in the anaerobic reactor metagenomes and meta-transcriptomes, including *mcr*, *mvh*, *cdh*, *mta*, and *mtt*, suggesting methane production from hydrogen, acetate, methanol, and methylamines. Surprisingly, methanogenesis-related transcripts were also detected in the aerobic reactor during the perturbation phase. Methanol dehydrogenases

(*xox* and *mxo*) were enriched in the aerobic reactor, although their abundance increased in the anaerobic reactor following the oxygen perturbation.

Utilization of organic acids and alcohols also varied by reactor. Genes for acetate, propionate, and butyrate kinases, as well as ethanolamine utilization, were more abundant in the anaerobic reactor metagenomes, with corresponding transcript levels increasing in the aerobic reactor during the N₂ perturbation. Conversely, a propanol-preferring alcohol dehydrogenase, L-lactate dehydrogenase, and acetoacetate decarboxylase were more prevalent in the aerobic reactor, with their expression also stimulated by oxygen perturbation in the anaerobic reactor. Numerous other alcohol dehydrogenases were expressed with reactor-specific preferences.

Cytochrome terminal oxidases were generally more abundant in the aerobic reactor (Figure 3.4) and were upregulated in response to the N₂ perturbation (Figure 3.1B). In contrast, sodium-pumping respiratory complexes (*rnf*, *nqr*) and hydrogenases (*hnd*, *ech*, *mvh*) were significantly more abundant in the anaerobic reactor.

The key ammonia oxidation gene, *amo*, was more abundant in the aerobic reactor, although its overall abundance was low due to its specialist function. Transcripts for *nar* (nitrate reductase)/*nxr* (nitrite oxidoreductase) and *nirK* (nitrite reductase) were significantly more abundant in the aerobic reactor, whereas *nrf* (associated with dissimilatory nitrate reduction to ammonia) and *nas* (assimilatory nitrate reductase) transcripts were enriched in the anaerobic reactor. *Nor* (nitric oxide reductase) was more abundant in the anaerobic reactor metagenomes. Notably, denitrification genes showed little transcriptional response to perturbations, except for a decrease in *nap* (nitrate reductase) expression in the aerobic reactor and an increase in *nos* (nitrous oxide reductase) expression in the anaerobic reactor.

3.4 Discussion

This study examined how the ubiquitous structural amino sugar N-acetylglucosamine (GlcNAc) was mineralized by wetland microbial communities in aerobic and anaerobic low-dilution-rate chemostats, to better understand the turnover of GlcNAc—a significant component of necromass—in wetlands. Transient perturbations were used to probe community responses to shifts in oxygen availability—an analog for natural drought and flooding events. GlcNAc was readily mineralized under all conditions, but downstream metabolite dynamics and microbial community composition differed markedly between the two reactors. The anaerobic reactor, dominated by Gammaproteobacteria, showed steadily increasing methane concentrations and declining levels of organic acids, suggesting the gradual establishment of acetoclastic methanogenesis and resilience to transient oxygen exposure. In contrast, the aerobic reactor, dominated by Alphaproteobacteria, mineralized GlcNAc completely to carbon dioxide. However, under nitrogen gas (N₂) perturbations, organic acids rapidly accumulated and oxidized nitrogen was depleted, indicating a metabolic shift from aerobic respiration to fermentation and denitrification. Over time, the aerobic system developed complete nitrification, while the anaerobic reactor consistently retained residual ammonium in the effluent, highlighting a tight coupling between carbon and nitrogen cycling.

This study highlights how amino sugars, such as GlcNAc, serve as important links between the carbon and nitrogen cycles, with their breakdown feeding into nitrification, denitrification, dissimilatory nitrate reduction to ammonium (DNRA), and assimilation pathways. We detected transcripts for ammonia monooxygenase from *Nitrosomonas oligotropha*, an ammonia-oxidizing bacterium known to be enriched under low-ammonia conditions [21, 22]. In the aerobic reactor,

nitrite and nitrate produced via nitrification were rapidly denitrified during the N₂ perturbation, resulting in sharp spikes in nitrous oxide emissions. Elevated N₂O emissions were also consistently observed during aerobic phases, potentially due to ammonia oxidation [23] or aerobic denitrification—a process recently reported in nitrification-inhibited chemostats exposed to frequent oxygen fluctuations [24]. Supporting the possibility of aerobic denitrification, we observed high expression of denitrification-related gene transcripts across all oxygen conditions.

We observed oxygen-dependent variation in the preferred gene systems for GlcNAc utilization. Under anaerobic conditions, GlcNAc transport was primarily mediated by the *nagE* phosphotransferase system (PTS), whereas the *ngc* ABC transporter was more prevalent under aerobic conditions. The PTS system couples GlcNAc transport with phosphorylation, driven by phosphoenolpyruvate, offering an energetically efficient mechanism [25]. In contrast, the ABC transporter relies on ATP for transport and does not confer simultaneous phosphorylation [26]. However, it provides a key advantage in its higher affinity for GlcNAc ($K_M = 0.5 \mu\text{M}$ vs. $5 \mu\text{M}$ for the PTS system) and its dual activity for transporting chitobiose (GlcNAc₂) [27, 28]. Thus, the PTS system may be favored in anaerobic, energy-limited environments due to its efficiency, while the ATP-dependent, high-affinity ABC transporter may be advantageous under aerobic conditions, where organisms can afford greater energy expenditure to outcompete others for substrate, thereby reducing residual GlcNAc concentrations. Indeed, this dichotomy between ABC and PTS-mediated transport has been explored in the context of marine bacteria, suggesting that ABC transporters allow for oligotrophic organisms to attain nano-molar half-saturation constants [29].

The aerobic and anaerobic reactors followed distinct microbial succession trajectories, despite being inoculated with the same wetland soil extract. This underscores the powerful selective

pressure oxygen exerts on microbial communities. We hypothesized that alternating oxygen conditions might drive convergence between systems; however, despite some functional and taxonomic overlap, the communities remained distinct. Both reactors favored facultative anaerobes, but only the anaerobic system could support aero-tolerant or transiently surviving strict anaerobes, which may explain the observed decline in Shannon diversity. Still, the rapid return of methanogenic activity suggests that some strict anaerobes endured transient oxygen exposure, as has been demonstrated in wetland sediments [30]. Methane and low levels of *mcr* transcripts detected in the aerobic reactor during the N₂ phase further suggest the persistence of methanogens, likely protected in biofilms or microsites.

Fluctuating redox conditions, common in wetland environments, shape microbial activity and carbon cycling. Our chemostat systems serve as valuable models for probing these dynamics. While GlcNAc was readily mineralized under all conditions, aerobic systems had higher energy availability that likely supported greater investment in high-affinity transporters and hydrolytic enzymes. Although we studied the GlcNAc monomer, it is typically present in complex polymers such as chitin or peptidoglycan, which requires additional enzymatic breakdown—an energy-intensive process that is prone to scavenging [31, 32] and may be limited under anaerobic conditions. This energetic constraint along with limits on transporter affinity could help explain the accumulation of necromass compounds and their amino sugar monomers in deeper, anoxic wetland soils.

The recognition that microbial necromass forms a substantial component of stable soil organic matter (SOM) has reshaped our understanding of microbial contributions to long-term carbon storage [33]. These findings extend beyond wetlands, with implications for marine systems,

wastewater treatment, and anaerobic digestion [34, 35]. Ultimately, understanding how microbial biomass is recycled—and under what conditions carbon is retained or lost—requires both taxonomic insight and physiological characterization, which chemostat systems are well-suited to deliver.

Future work should expand on these findings by investigating how the hydrolysis of the necromass biopolymer chitin influences turnover, community assembly, and biogeochemical cycling. Together, these efforts can move us closer to a predictive understanding of microbial contributions to SOM formation and persistence. Such insight is critical for designing strategies to enhance soil carbon sequestration and support climate-resilient ecosystem management.

References

1. Candry P et al. Microbially mediated climate feedbacks from wetland ecosystems. *Glob Chang Biol* 2023;**29**:5169–5183. <https://doi.org/10.1111/gcb.16850>
2. Lehmann J, Kleber M. The contentious nature of soil organic matter. *Nature* 2015;**528**:60–68. <https://doi.org/10.1038/nature16069>
3. Glaser B, Turrión M-B, Alef K. Amino sugars and muramic acid—biomarkers for soil microbial community structure analysis. *Soil Biol Biochem* 2004;**36**:399–407. <https://doi.org/10.1016/j.soilbio.2003.10.013>
4. Buckeridge KM, Creamer C, Whitaker J. Deconstructing the microbial necromass continuum to inform soil carbon sequestration. *Funct Ecol* 2022;**36**:1396–1410. <https://doi.org/10.1111/1365-2435.14014>
5. Salas E et al. Reevaluation and novel insights into amino sugar and neutral sugar necromass biomarkers in archaea, bacteria, fungi, and plants. *Science of The Total Environment* 2024;**906**:167463. <https://doi.org/10.1016/j.scitotenv.2023.167463>
6. Ni X et al. The vertical distribution and control of microbial necromass carbon in forest soils. *Global Ecology and Biogeography* 2020;**29**:1829–1839. <https://doi.org/10.1111/geb.13159>
7. Ni X et al. A quantitative assessment of amino sugars in soil profiles. *Soil Biol Biochem* 2020;**143**. <https://doi.org/10.1016/j.soilbio.2020.107762>
8. Yang C et al. Comparative genomics and experimental characterization of N-acetylglucosamine utilization pathway of *Shewanella oneidensis*. *Journal of Biological Chemistry* 2006;**281**:29872–29885. <https://doi.org/10.1074/jbc.M605052200>
9. Plumbridge JA. Repression and induction of the *nag* regulon of *Escherichia coli* K-12: the roles of *nagC* and *nagA* in maintenance of the uninduced state. *Mol Microbiol* 1991;**5**:2053–2062. <https://doi.org/10.1111/j.1365-2958.1991.tb00828.x>
10. Li SW et al. Chitin degradation and electricity generation by *Aeromonas hydrophila* in microbial fuel cells. *Chemosphere* 2017;**168**:293–299. <https://doi.org/10.1016/j.chemosphere.2016.10.080>

11. Stumpf AK et al. Identification of a novel chitinase from *Aeromonas hydrophila* AH-1N for the degradation of chitin within fungal mycelium. *FEMS Microbiol Lett* 2019;**366**. <https://doi.org/10.1093/femsle/fny294>
12. Cottrell MT, Kirchman DL. Natural Assemblages of Marine Proteobacteria and Members of the Cytophaga-Flavobacter Cluster Consuming Low-and High-Molecular-Weight Dissolved Organic Matter. *APPLIED AND ENVIRONMENTAL MICROBIOLOGY* . 2000.
13. Riemann L, Azam F. Widespread N-acetyl-D-glucosamine uptake among pelagic marine bacteria and its ecological implications. *Appl Environ Microbiol* 2002;**68**:5554–5562. <https://doi.org/10.1128/AEM.68.11.5554-5562.2002>
14. Katayama T et al. A Marine Group A isolate relies on other growing bacteria for cell wall formation. *Nat Microbiol* 2024;**9**:1954–1963. <https://doi.org/10.1038/s41564-024-01717-7>
15. Nurk S et al. MetaSPAdes: A new versatile metagenomic assembler. *Genome Res* 2017;**27**:824–834. <https://doi.org/10.1101/gr.213959.116>
16. Bushmanova E et al. RnaSPAdes: A de novo transcriptome assembler and its application to RNA-Seq data. *Gigascience* 2019;**8**. <https://doi.org/10.1093/gigascience/giz100>
17. Bushnell B. BBTools. <https://jgi.doe.gov/data-and-tools/software-tools/bbtools/>. .
18. Chen IMA et al. The IMG/M data management and analysis system v.7: content updates and new features. *Nucleic Acids Res* 2023;**51**:D723–D732. <https://doi.org/10.1093/nar/gkac976>
19. Kang DD et al. MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* 2015;**2015**. <https://doi.org/10.7717/peerj.1165>
20. Fernandes AD et al. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. *Microbiome* 2014;**2**:15. <https://doi.org/10.1186/2049-2618-2-15>
21. Bollmann A, Laanbroek HJ. Continuous culture enrichments of ammonia-oxidizing bacteria at low ammonium concentrations. *FEMS Microbiol Ecol* 2006;**37**:211–221. <https://doi.org/10.1111/j.1574-6941.2001.tb00868.x>

22. Limpiyakorn T et al. Effects of ammonium and nitrite on communities and populations of ammonia-oxidizing bacteria in laboratory-scale continuous-flow reactors. *FEMS Microbiol Ecol* 2007;**60**:501–512. <https://doi.org/10.1111/j.1574-6941.2007.00307.x>
23. Prosser JI et al. Nitrous oxide production by ammonia oxidizers: Physiological diversity, niche differentiation and potential mitigation strategies. *Glob Chang Biol* . 2020. Blackwell Publishing Ltd, 2020. , **26**: 103–118
24. Roothans N et al. Aerobic denitrification as an N₂O source from microbial communities. *ISME Journal* 2024;**18**. <https://doi.org/10.1093/ismejo/wrae116>
25. McCoy JG, Levin EJ, Zhou M. Structural insight into the PTS sugar transporter EIIC. *Biochimica et Biophysica Acta (BBA) - General Subjects* 2015;**1850**:577–585. <https://doi.org/10.1016/j.bbagen.2014.03.013>
26. Thomas C, Tampé R. Structural and Mechanistic Principles of ABC Transporters. *Annu Rev Biochem* 2020;**89**:605–636. <https://doi.org/10.1146/annurev-biochem-011520-105201>
27. Wang F et al. *Streptomyces olivaceoviridis* possesses a phosphotransferase system that mediates specific, phosphoenolpyruvate-dependent uptake of N-acetylglucosamine. *Molecular Genetics and Genomics* 2002;**268**:344–351. <https://doi.org/10.1007/s00438-002-0749-3>
28. Saito A, Schrempf H. Mutational analysis of the binding affinity and transport activity for N-acetylglucosamine of the novel ABC transporter Ngc in the chitin-degrader *Streptomyces olivaceoviridis*. *Molecular Genetics and Genomics* 2004;**271**:545–553. <https://doi.org/10.1007/s00438-004-0981-0>
29. Norris N et al. Mechanistic model of nutrient uptake explains dichotomy between marine oligotrophic and copiotrophic bacteria. *PLoS Comput Biol* 2021;**17**. <https://doi.org/10.1371/journal.pcbi.1009023>
30. Angle JC et al. Methanogenesis in oxygenated soils is a substantial fraction of wetland methane emissions. *Nat Commun* 2017;**8**. <https://doi.org/10.1038/s41467-017-01753-4>
31. Allison SD. Cheaters, diffusion and nutrients constrain decomposition by microbial enzymes in spatially structured environments. *Ecol Lett* 2005;**8**:626–635. <https://doi.org/10.1111/j.1461-0248.2005.00756.x>
32. Frankena J, Van Verseveld HW, Stouthamer AH. Substrate and energy costs of the production of exocellular enzymes by *Bacillus licheniformis*. *Biotechnol Bioeng* 1988;**32**:803–812. <https://doi.org/10.1002/bit.260320612>

33. Lehmann J, Kleber M. The contentious nature of soil organic matter. *Nature* . 2015. Nature Publishing Group, 2015. , **528**: 60–68
34. Guo H et al. Reconsidering hydrolysis kinetics for anaerobic digestion of waste activated sludge applying cascade reactors with ultra-short residence times. *Water Res* 2021;**202**. <https://doi.org/10.1016/j.watres.2021.117398>
35. Datta MS et al. Microbial interactions lead to rapid micro-scale successions on model marine particles. *Nat Commun* 2016;**7**. <https://doi.org/10.1038/ncomms11965>

Figure legends

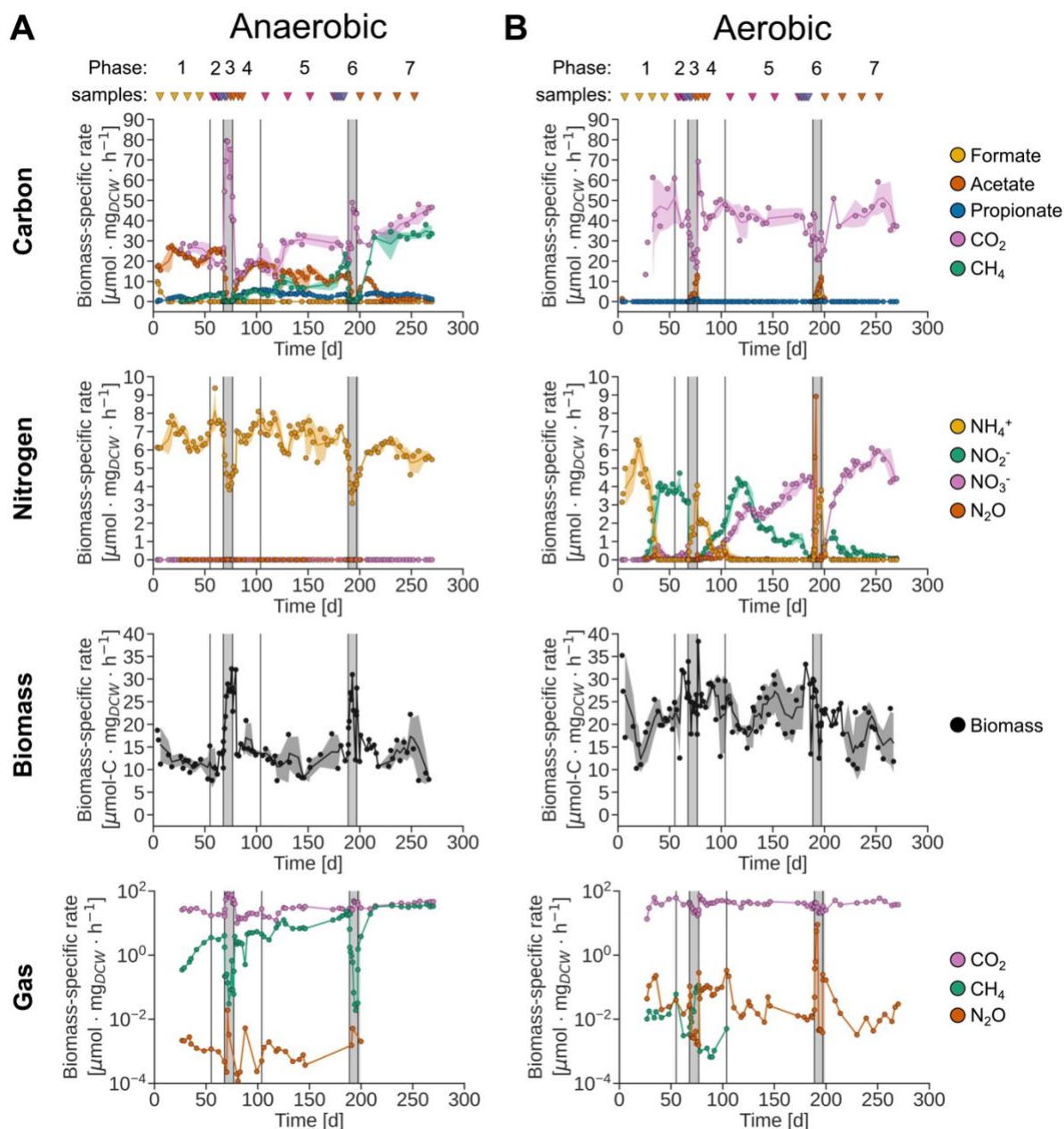


Figure 3.1. Biomass-specific production rates ($\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$) GlcNAc degrading, wetland sediment chemostats over the entire operational period.

The anaerobic (A) and aerobic (B) GlcNAc degrading, wetland sediment chemostats were started under carbon limitations for 55 days (Phase 1). Steady-state was achieved by the anaerobic and aerobic chemostat on day 14.78 and 38.8, respectively. The chemostats were maintained at steady-state (Phase 2) for over 29 days (>5 generation times) and were perturbed with O₂/N₂ for 8.9 days

on day 67.9 (Phase 3; grey). Afterwards, the reactors underwent a 27.1 day transition (Phase 4) and achieved a new steady-state (Phase 5) on day 103.9. The chemostats were perturbed again for 8.2 days on day 188.8 (Phase 6; grey) and transitioned to a final steady-state (Phase 7). Biomass-specific production rates of formate, acetate, propionate, biomass, CO₂, CH₄, NH₄⁺, NO₂⁻, NO₃⁻, and N₂O production were measured throughout the enrichment by dividing the molar production rate by dry cell weight. Rates are separated into carbon, nitrogen, biomass, and gas production rates with points represent individual production rate measurements. Lines denote the 3-point rolling average production rate with shading representing the standard deviation. Phases and sequencing timepoints (triangles) are marked above each figure. Samples for metagenomic (Phase 1-7) and meta-transcriptomic (Phase 1-4) sequencing were collected before (pink), during (purple), and after (orange) each O₂/N₂ perturbation. Additional samples were collected during start-up (yellow).

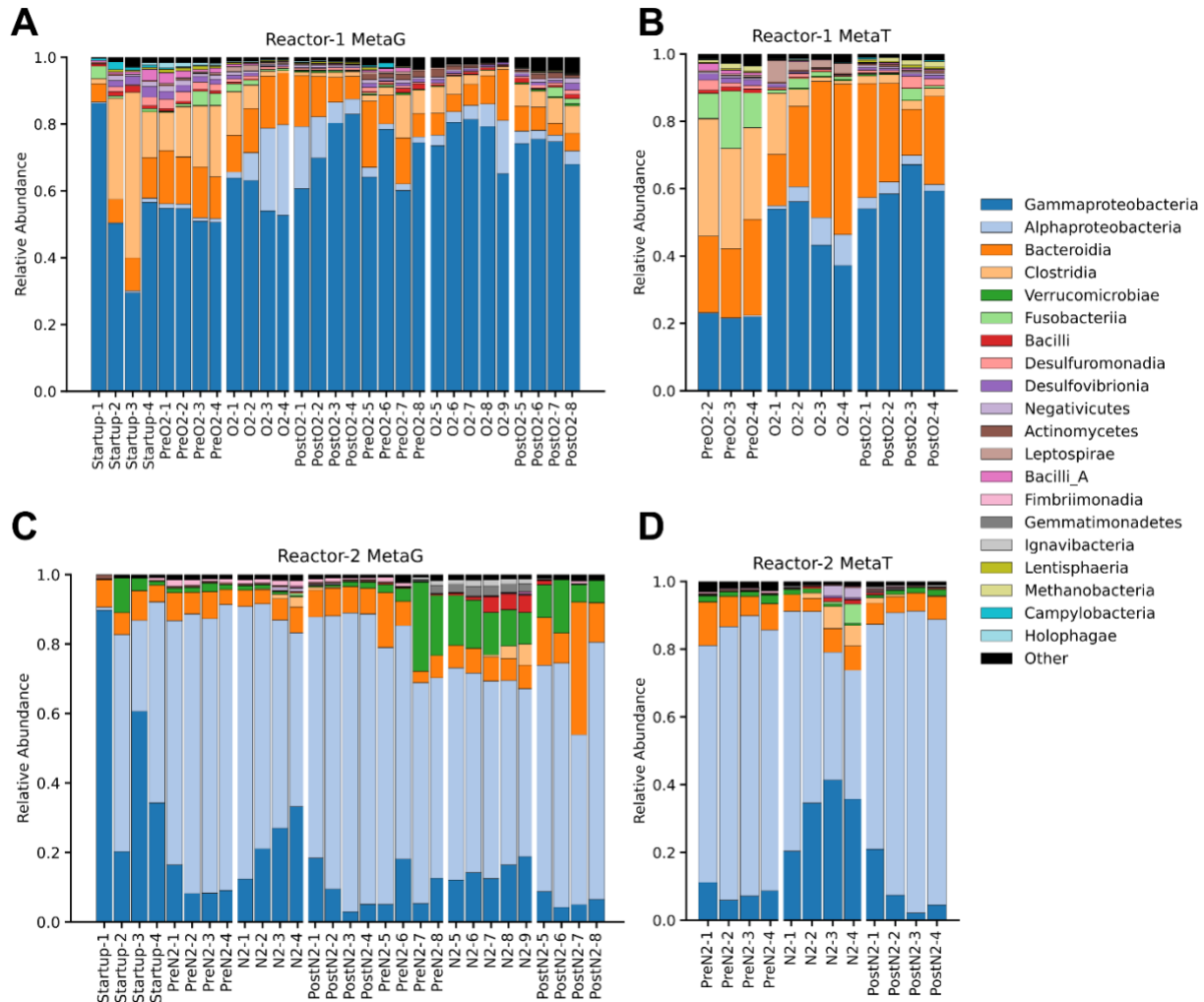


Figure 3.2. Class-level taxonomic abundance across reactors and operational phase.

Relative abundance of the top 20 most abundant classes in Reactor 1 (Anaerobic) metagenomes, i.e. MetaG (A) and meta-transcriptomes, i.e. MetaT (B) and Reactor 2 (Aerobic) metagenomes (C) and meta-transcriptomes (D). Operational phase is detailed on the x-axis labels. Perturbation phases (O_2 for Reactor 1 and N_2 for Reactor 2) are highlighted by the vertical white lines.

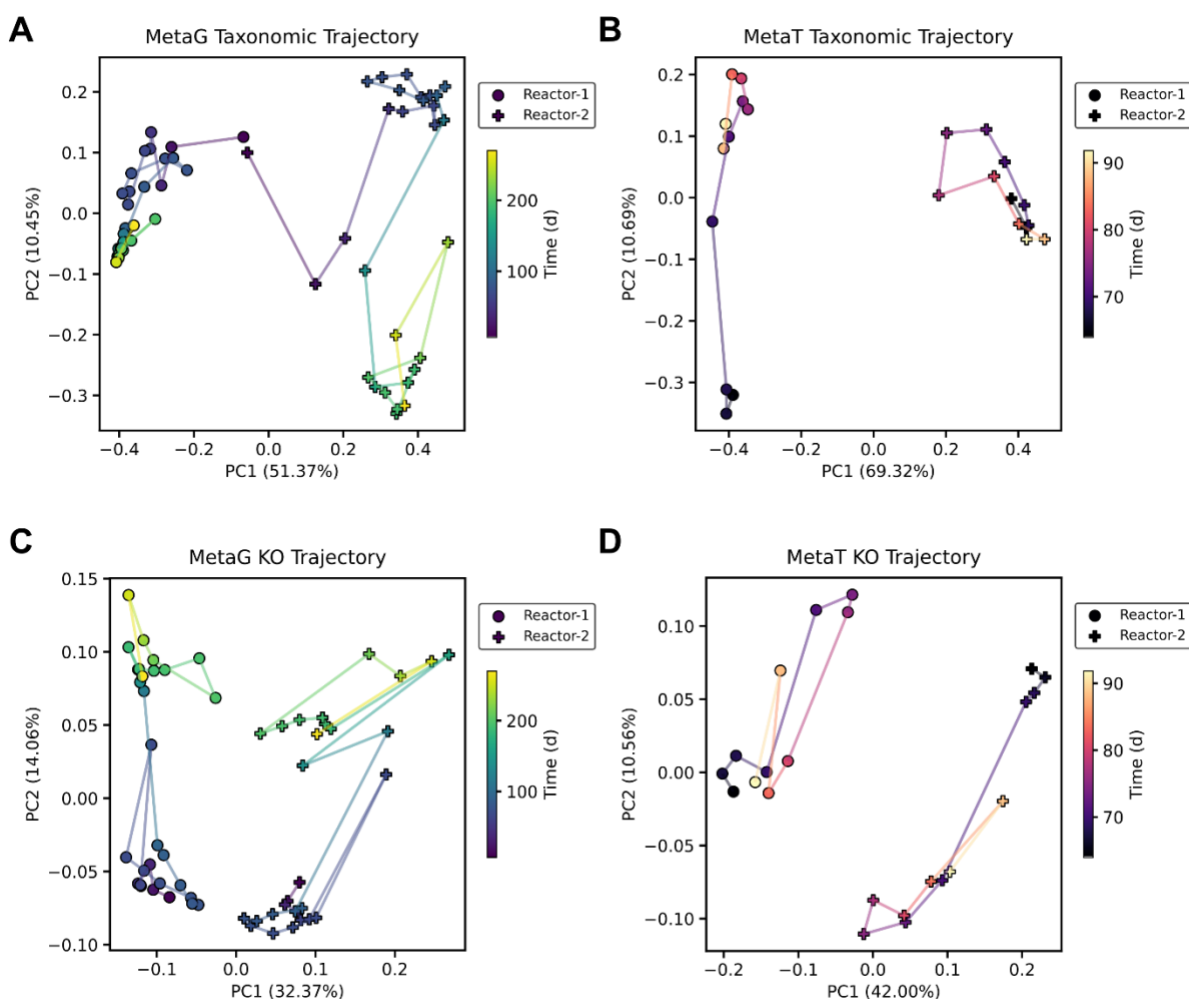


Figure 3.3. Trajectory of taxonomic and functional composition.

PCoA plots of family-level taxonomic composition of metagenomes, i.e. MetaG (A) and meta-transcriptomes, i.e. MetaT (B). PCoA plots of functional composition based on KO abundance for metagenomes (C) and meta-transcriptomes (D). Plots are separated by reactor and colored based on sample date with time-adjacent samples connected with lines.

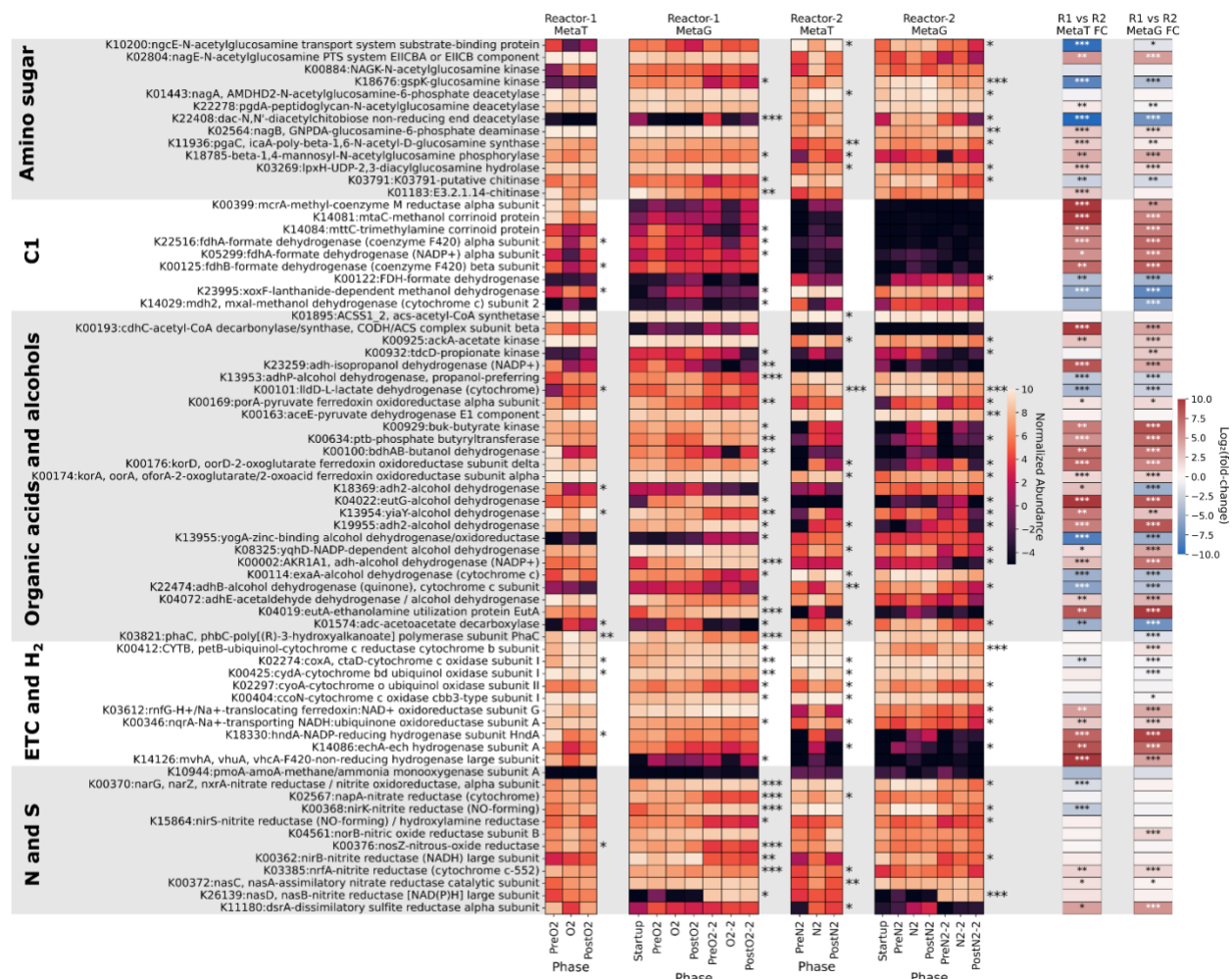


Figure 3.4. Functional gene variation across reactors and phases.

Mean abundance of key gene orthologs in each operational phase across reactor metagenomes (MetaG) and meta-transcriptomes (MetaT). Orthologs are organized by metabolism category as denoted by the gray shading. Asterisks next to heatmaps denote orthologs with abundances that were significantly different across phases in that reactor. Mean fold-change between Reactors 1 and 2 is shown in the right two columns, with asterisks denoting significantly different gene orthologs (* : $p < 0.05$, ** : $p < 0.01$, and *** : $p < 0.001$).

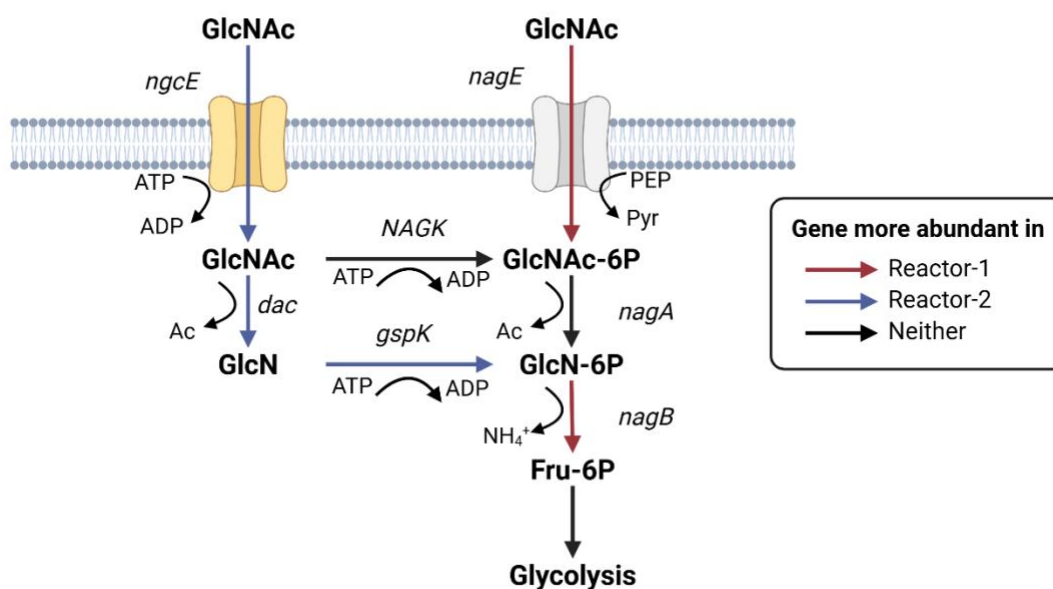


Figure 3.5. N-acetylglucosamine utilization pathways.

Schematic representation of different N-acetylglucosamine utilization pathways with gene abbreviations shown in italics. Pathway arrows are colored based on observed differences in metagenomic and meta-transcriptomic abundance. Abbreviations: N-acetylglucosamine (GlcNAc), N-acetylglucosamine-6-phosphate (GlcNAc-6P), glucosamine (GlcN), glucosamine-6-phosphate (GlcN-6P), fructose-6-phosphate (Fru-6P), acetate (Ac), phosphoenolpyruvate (PEP), pyruvate (Pyr).

Appendix A. Supplemental Material

The following document is intended to support the results presented in the main manuscript.

Figures

- Figure S1. Biomass-specific carbon production rates
- Figure S2. Biomass-specific nitrogen production rates
- Figure S3. Taxonomic and functional diversity
- Figure S4. Order-level taxonomic composition across reactors and operational phases
- Figure S5. Family-level taxonomic composition across reactors and operational phases
- Figure S6. Relationship between the biomass and CO₂ biomass-specific production rates in the anaerobic chemostat (R₁)
- Figure S7. Relationship between the acetate and CO₂/CH₄ biomass-specific production rates in the anaerobic chemostat (R₁)

Tables

- Table S1. Average biomass-specific carbon and nitrogen production rates in each phase
- Table S2. Modified lacustrine media composition

S1. Supplemental figure legends

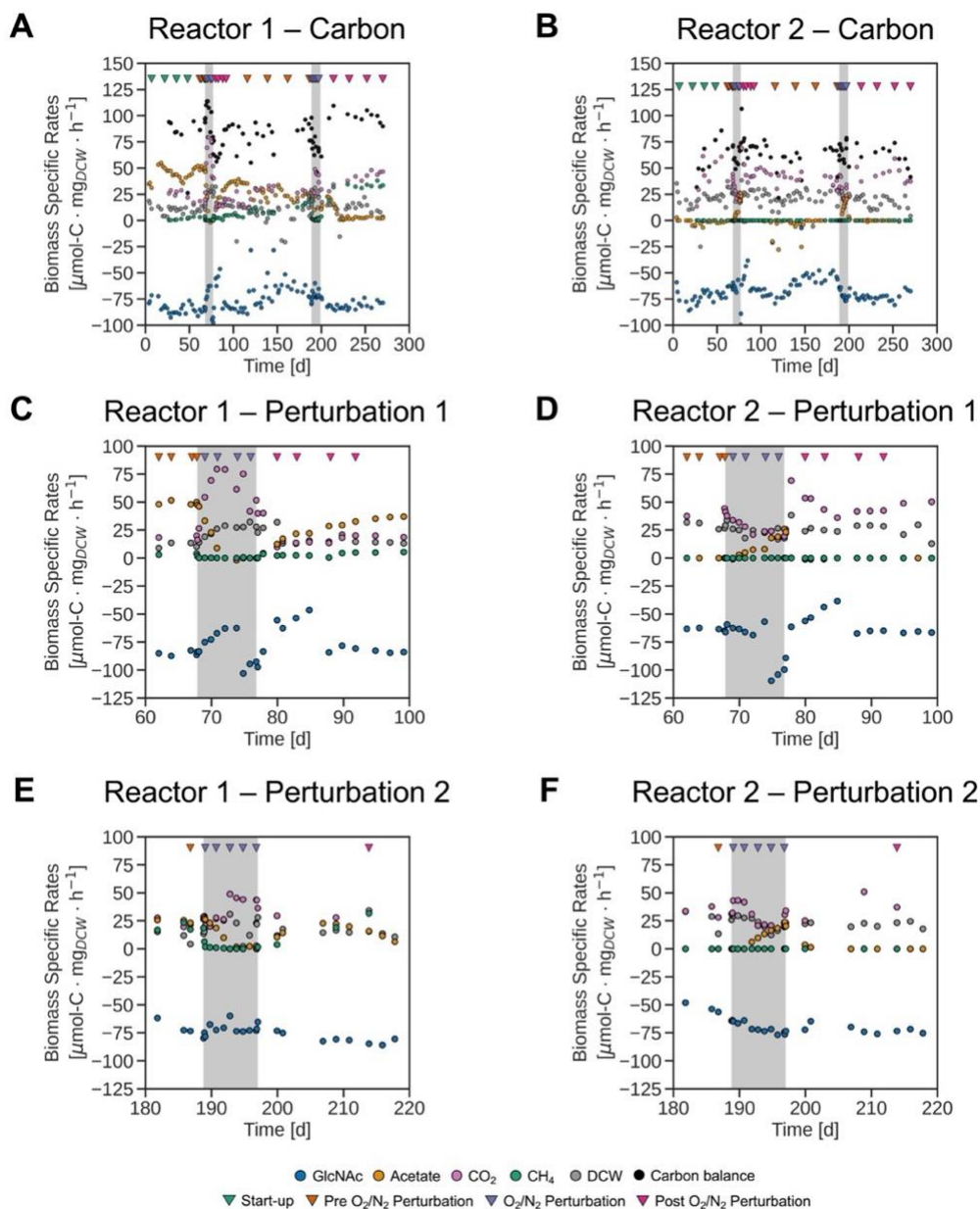


Figure S3.1. Biomass-specific carbon production rates.

Biomass-specific carbon production rates ($\mu\text{mol-C} \cdot \text{mg}_{\text{DCW}}^{-1} \cdot \text{h}^{-1}$) were measured over the entire operational period for the anaerobic (A) and aerobic (B) GlcNAc degrading, wetland sediment chemostats. Steady-state was achieved by the anaerobic and aerobic chemostat on day 14.78 and 38.8, respectively. The chemostats were maintained at steady-state for over 29 days (>5 generation

times) and were perturbed with O₂/N₂ for 8.9 days on day 67.9 (C-D; grey;). Afterwards, the reactors underwent a 27.1 day transition and achieved a new steady-state on day 103.9. The chemostats were perturbed again for 8.2 days on day 188.8 (E-F; grey) and transitioned to a final steady-state. Biomass-specific production rates were measured throughout the enrichment by dividing the molar production rate by dry cell weight. Samples for metagenomic and meta-transcriptomic sequencing (triangles) were collected before (pink), during (purple), and after (orange) each O₂/N₂ perturbation. Additional samples were collected during start-up (yellow).

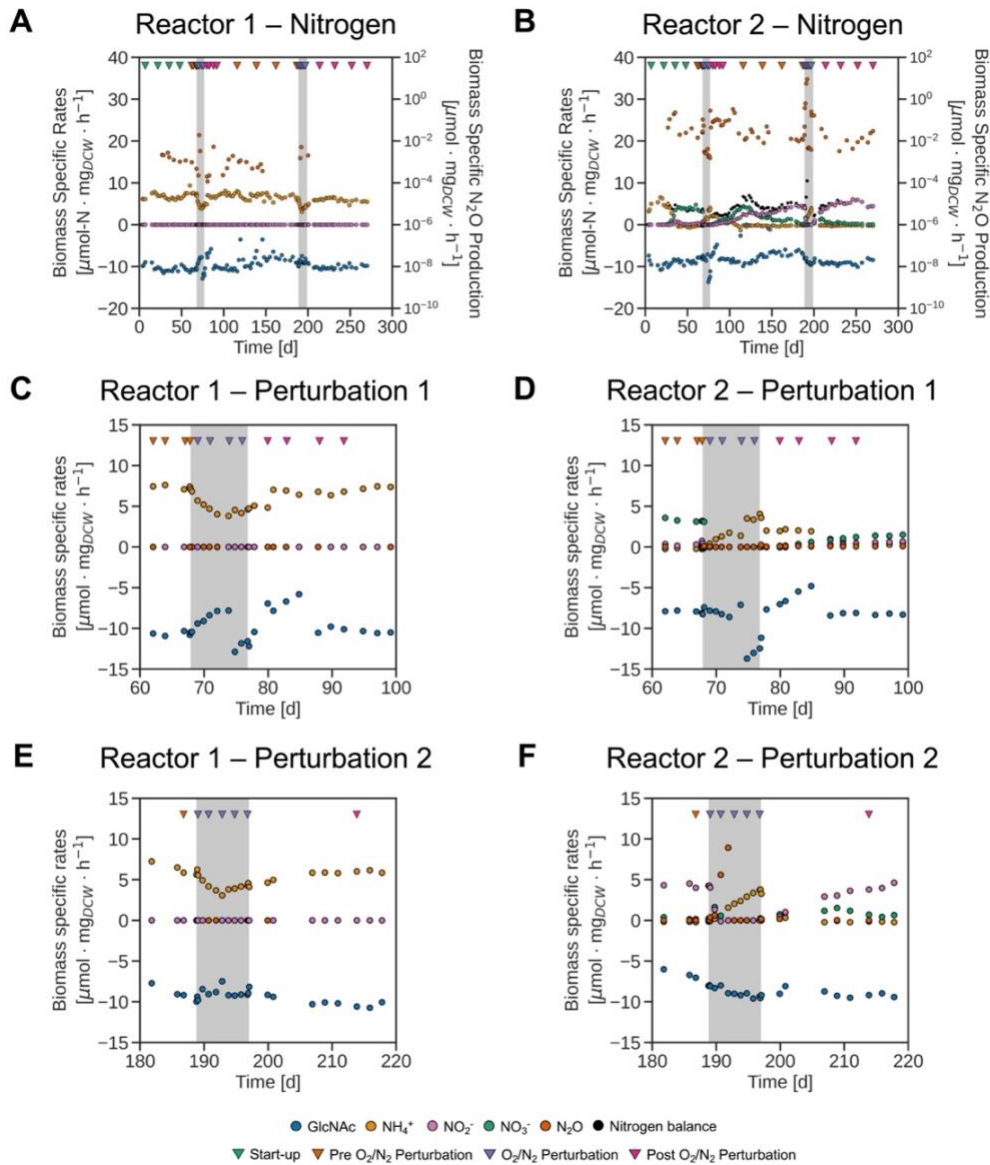


Figure S3.2. Biomass-specific nitrogen production rates.

Biomass-specific nitrogen production rates ($\mu\text{mol} \cdot \text{mg}_{\text{DCW}}^{-1} \cdot \text{h}^{-1}$) were measured over the entire operational period for the anaerobic (A) and aerobic (B) GlcNAc degrading, wetland sediment chemostats. Steady-state was achieved by the anaerobic and aerobic chemostat on day 14.78 and 38.8, respectively. The chemostats were maintained at steady-state for over 29 days (>5 generation times) and were perturbed with O_2/N_2 for 8.9 days on day 67.9 (C-D; grey;). Afterwards, the reactors underwent a 27.1 day transition and achieved a new steady-state on day 103.9. The chemostats were perturbed again for 8.2 days on day 188.8 (E-F; grey) and transitioned to a final

steady-state. Biomass-specific production rates were measured throughout the enrichment by dividing the molar production rate by dry cell weight. Samples for metagenomic and meta-transcriptomic sequencing (triangles) were collected before (pink), during (purple), and after (orange) each O₂/N₂ perturbation. Additional samples were collected during start-up (yellow).

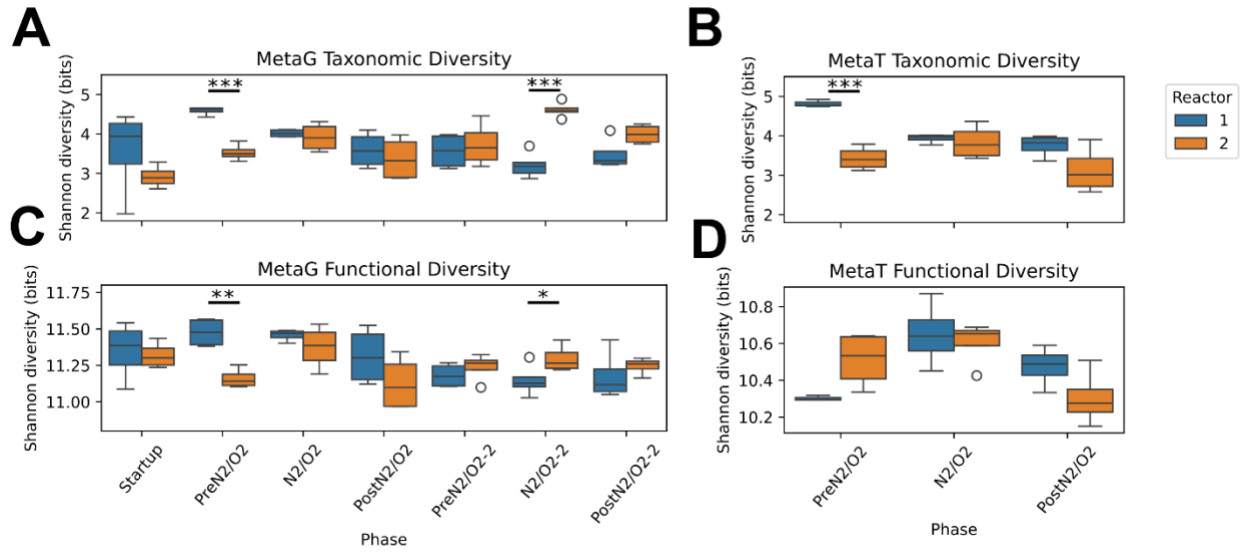


Figure S3.3. Taxonomic and functional diversity.

Shannon diversity of reactor metagenomes (A, C) and meta-transcriptomes (B, D) based on family-level taxonomic composition (A, B) and KEGG ortholog abundance (C, D). Statistical comparisons were made between reactors in each phase with significant differences noted with asterisks (* : $p < 0.05$, ** : $p < 0.01$, and *** : $p < 0.001$).

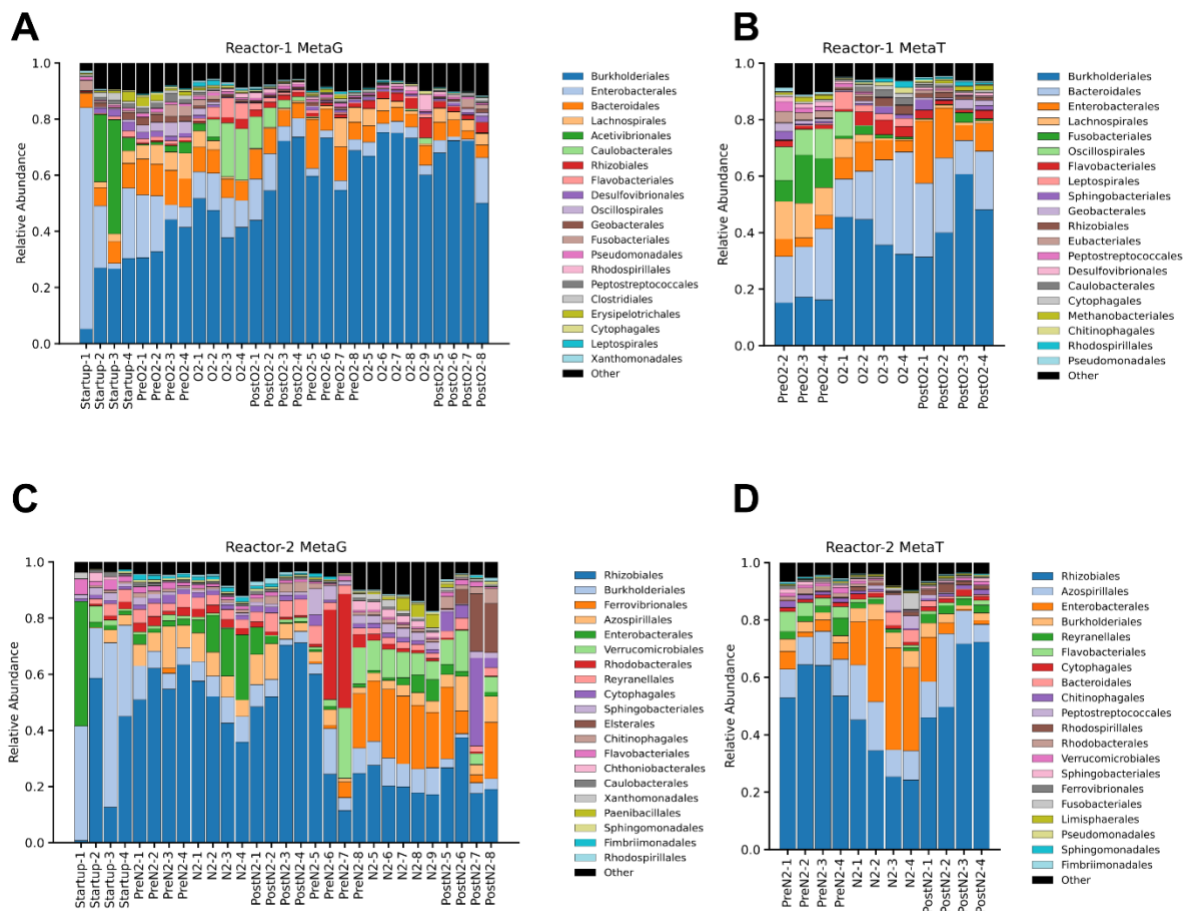


Figure S3.4. Order-level taxonomic composition across reactors and operational phases.

Relative abundance of the top 20 most abundant orders in Reactor 1 metagenomes, i.e. MetaG (A) and meta-transcriptomes, i.e. MetaT (B) and Reactor 2 metagenomes (C) and meta-transcriptomes (D). Operational phase is detailed on the x-axis labels.

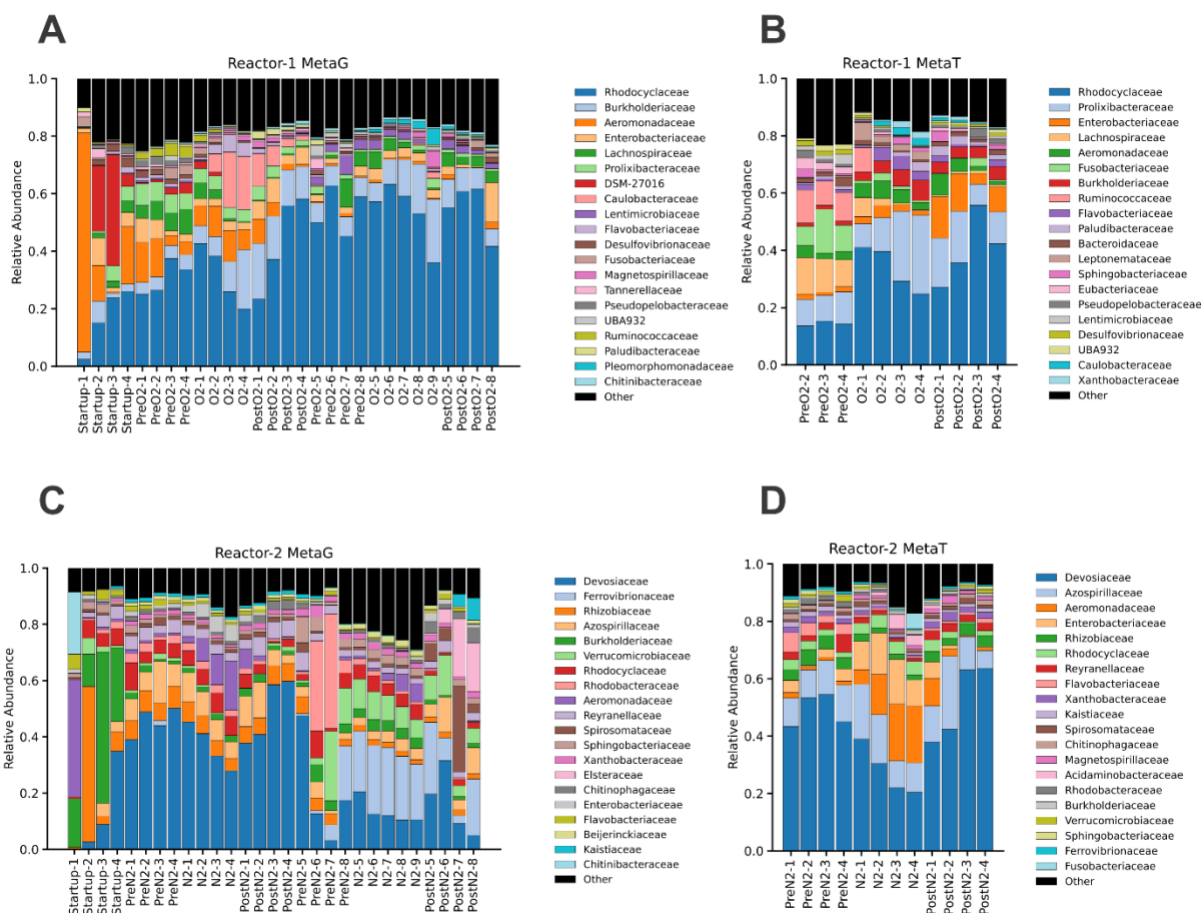


Figure S3.5 Family-level taxonomic composition across reactors and operational phases.

Relative abundance of the top 20 most abundant families in Reactor 1 metagenomes, i.e. MetaG (A) and meta-transcriptomes, i.e. MetaT (B) and Reactor 2 metagenomes (C) and meta-transcriptomes (D). Operational phase is detailed on the x-axis labels.

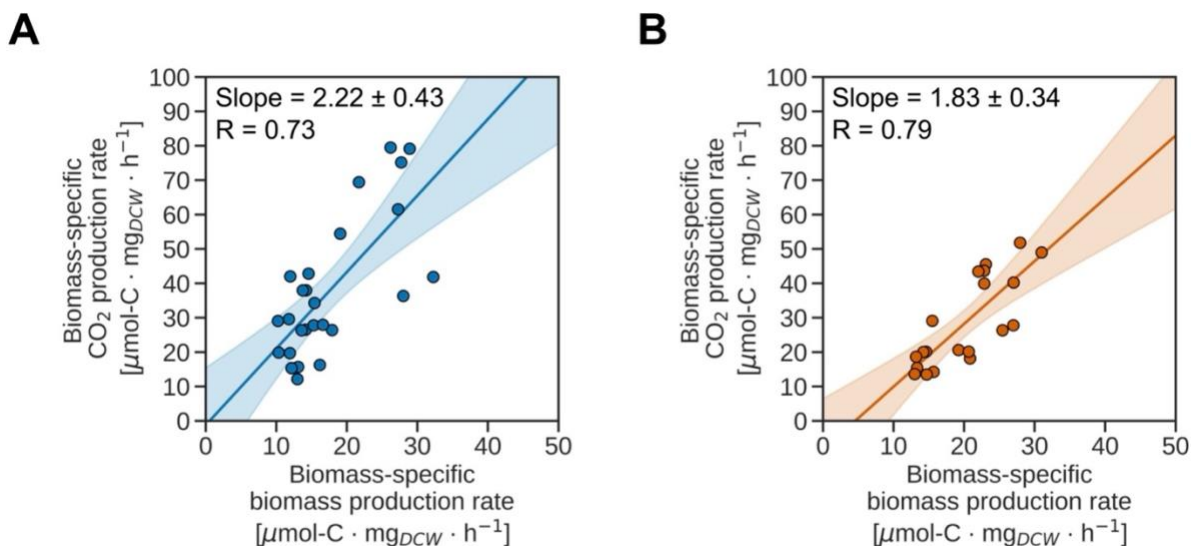


Figure S3.6. Relationship between the biomass and CO_2 biomass-specific production rates in the anaerobic chemostat (R_1).

Biomass-specific CO_2 production rates were either collected during start-up, steady-states, and O_2 perturbations (A; $n=26$) or during the transitions (B; $n=21$). Linear correlations were determined by fitting an ordinary least squares regression model. Solid lines represent the linear regression while the shaded area represents the 95% confidence interval.

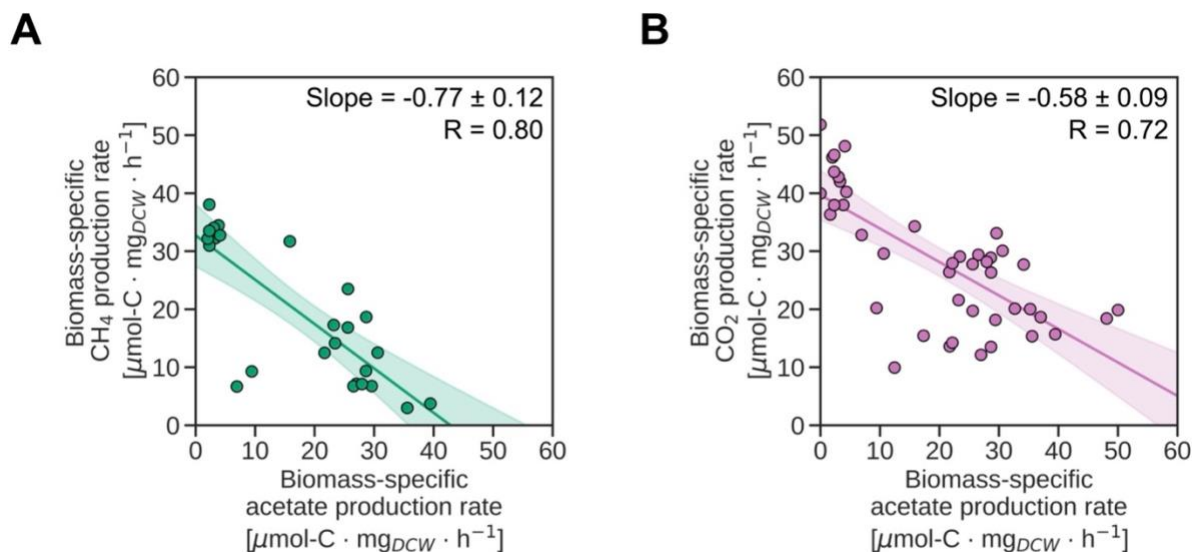


Figure S3.7. Relationship between the acetate and CO_2/CH_4 biomass-specific production rates in the anaerobic chemostat (R_1).

Biomass-specific CH_4 production rates were collected during the second and third steady-state (A; $n=25$), while the CO_2 rates were collected during the steady-states, and transitions (B, $n=40$). Linear correlations were determined by fitting an ordinary least squares regression model. Solid lines represent the linear regression while the shaded area represents the 95% confidence interval.

S2. Supplemental Tables

Table S3-1. Averaged biomass-specific rates of in each reactor phase for all measured carbon compounds

Compound	Reactor	Phase							Unit
		1	2	3	4	5	6	7	
Formate	R1	1.17 ±2.99	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	0.0 ±0.0	0.0 ±0.0	0.13 ±0.27	0.0 ±0.0	0.0 ±0.0	1.51 ±1.71	0.0 ±0.0	
Acetate	R1	43.78 ±6.52	50.10 ±1.70	13.9 ±18.12	23.31 ±13.6	26.15 ±7.34	10.16 ±11.4	6.47 ±7.08	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	0.15 ±0.65	0.0 ±0.0	7.56 ±7.43	3.29 ±8.66	0.0 ±0.0	10.14 ±9.6	1.01 ±4.10	
Propionate	R1	5.36 ±2.72	10.77 ±1.75	1.73 ±3.70	10.51 ±6.44	11.63 ±3.15	1.92 ± 3.58	9.38 ±3.21	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	0.0 ± 0.0	0.0 ± 0.0	1.47 ±1.73	0.51 ±1.37	0.0 ±0.0	0.63 ±0.90	0.05 ± 0.27	
CO ₂	R1	24.78 ±4.25	19.13 ±1.03	60.94 ±19.1	23.33 ±12.8	24.79 ±6.49	34.96 ±11.2	39.45 ±6.59	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	43.00 ±17.9	40.92 ±4.81	27.15 ±7.01	44.15 ±12.6	39.32 ±5.76	30.79 ±9.28	41.58 ±8.99	
CH ₄	R1	1.37 ±1.21	3.54 ±0.71	0.21 ±0.20	2.95 ±1.87	10.95 ±5.83	1.15 ±1.95	27.02 ±12.2	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	0.02 ±0.02	0.003 ±0.0	0.042 ±0.04	0.01 ±0.03	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	
Biomass-C	R1	12.84 ±2.53	11.93 ±1.87	24.67 ±5.91	18.73 ±6.65	13.08 ±2.22	21.89 ±5.46	15.3 ± 4.20	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	20.65 ±5.74	25.28 ±7.08	25.15 ±4.46	25.42 ±5.92	22.02 ±6.16	23.34 ±6.02	18.49 ±5.13	
GlcNAc-C	R1	-83.23 ±5.8	-86.56 ±2.9	-77.7 ±14.9	-76.6 ±14.9	-68.9 ±14.6	-71.66 ±4.7	-80.21 ±4.8	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	-73.41 ±6.6	-66.12 ±4.5	-73.8 ±20.8	-65.0 ±15.2	-56.5 ±17.8	-70.49 ±4.9	-72.74 ±3.9	
C-balance	R1	-14.0 ±16.3	-10.2 ±12.8	23.80 ±24.1	-3.72 ±15.0	-6.29 ±20.4	-6.86 ±21.2	-21.4 ±34.4	μmol-C·mg _{DCW} ⁻¹ ·h ⁻¹
	R2	-36.0 ±36.3	-27.2 ±27.8	-12.2 ±18.3	2.47 ±25.97	-16.2 ±29.6	-6.90 ±13.6	-34.7 ±25.6	

Table S3-2. Averaged biomass-specific rates of in each reactor phase for all measured nitrogen compounds

Compound	Reactor	Phase							Unit
		1	2	3	4	5	6	7	
NH₄⁺	R1	6.65 ±0.58	7.75 ± 0.82	4.88 ± 1.00	6.44 ±1.10	6.74 ±0.70	4.40 ±0.90	5.75 ±0.62	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	2.49 ±2.43	0.0 ±0.0	1.59 ±1.27	1.50 ±1.18	0.0 ±0.016	1.87 ±1.51	0.17 ±0.66	
NO₂⁻	R1	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	0.0 ±0.0	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	1.83 ±1.85	3.61 ±0.60	0.39 ±1.10	0.85 ±0.72	2.02 ±1.32	0.24 ±0.50	0.50 ± 0.40	
NO₃⁻	R1	0.0 ±0.0	0.0 ± 0.0	0.0 ± 0.002	0.0 ±0.001	0.0 ±0.002	0.0 ±0.001	0.0 ±0.001	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	0.13 ±0.22	0.34 ±0.22	0.0 ±0.0	0.34 ±0.36	2.96 ±0.92	0.87 ±1.66	4.22 ±1.63	
N₂O	R1	0.0 ±0.001	0.001 ±0.0	0.01 ±0.01	0.0 ±0.002	0.001 ±0.0	0.0 ±0.003	0.002	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	0.09 ±0.09	0.02 ±0.01	0.02 ±0.04	0.11 ±0.01	0.03 ±0.05	1.58 ±3.10	0.04 ±0.06	
Biomass-N	R1	2.57 ±0.51	2.39 ±0.37	4.93 ±1.18	3.75 ±1.33	2.62 ±0.45	4.38 ±1.10	3.06 ±0.84	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	4.13 ±1.15	5.06 ±1.42	5.03 ±0.90	5.08 ±1.19	4.40 ±1.23	4.67 ±1.20	3.70 ±1.03	
GlcNAc-N	R1	-10.4 ±0.73	-10.8 ±0.36	-9.71 ±1.86	-9.57±1.87	-8.61±1.83	-8.96±0.60	-10.0±0.60	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$
	R2	-9.18 ±0.82	-8.27 ±0.56	-9.22 ±2.59	-8.13 ±1.90	-7.06 ±2.23	-8.81 ±0.62	-9.09 ±0.49	
N-balance	R1	-1.05 ±3.54	-2.24 ±1.33	1.35 ±0.79	0.83 ± 2.63	-0.48±2.80	0.25±0.40	-2.155	$\mu\text{mol}\cdot\text{mg}_{\text{DCW}}^{-1}\cdot\text{h}^{-1}$

1 **Table S3-3.** Modified lacustrine media composition

Compound	Concentration (mM)
GlcNAc	5
(NH ₄) ₂ SO ₄	0.05
NaH ₂ PO ₄	0.04
Na ₂ HPO ₄	0.06
KHCO ₃	0.3
CaCl ₂ * H ₂ O	0.244
MgCl ₂ * 6 H ₂ O	0.16
SL-10	1x
Se-W	1x
7-Vit	1x
HEPES	10

2

Chapter 4.

Pairing anammox with high affinity ammonia-oxidizing microorganisms reduces effluent nitrate accumulation during mainstream treatment

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Abstract

Implementing anaerobic ammonium-oxidizing bacteria (anammox) in mainstream wastewater treatment could significantly decrease aeration, carbon, and volume requirements. However, efficient mainstream nitrogen removal with anammox has not yet been achieved, because the low ammonium concentrations, ambient temperatures, and strict oxygen control—required to suppress nitrite-oxidizing bacteria (NOB)—throttle the activity of ammonia-oxidizing bacteria (AOB). The high ammonia and oxygen affinities of ammonia-oxidizing archaea (AOA) and complete ammonia-oxidizing bacteria (comammox) make them ideal candidates for mainstream partial nitrification/anammox (PNA) treatment. We paired anammox with AOA and comammox in two laboratory-scale membrane aerated biofilm reactors (MABRs). Both systems achieved high nitrogen removal efficiencies without the nitrate accumulation associated with anammox anabolism. Complete nitrate removal was associated with the enrichment of NOB and comammox *Nitrospira* in anoxic regions of the MABR biofilms. Anoxic comammox activity was supported by measuring the oxygen-affinity of *Nitrospira inopinata* and demonstrating the ability for it to carry out formate-dependent nitrate reduction. We find anoxic *Nitrospira* activity enabled total-nitrogen removal during mainstream PNA treatment by reducing anammox-derived nitrate to nitrite, which anammox subsequently reduced to dinitrogen gas. The cooperation between ammonia oxidizers, anammox, and anoxic *Nitrospira* reported here provides stable mainstream PNA treatment and upends conventional NOB suppression strategies.

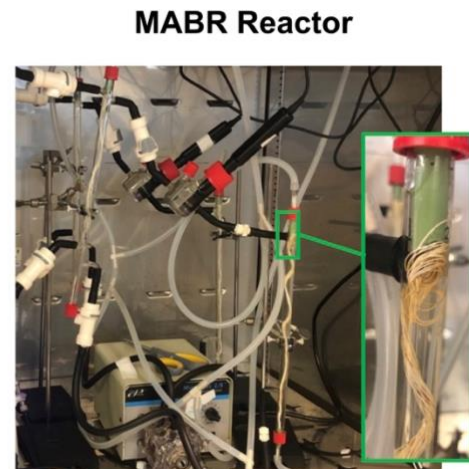
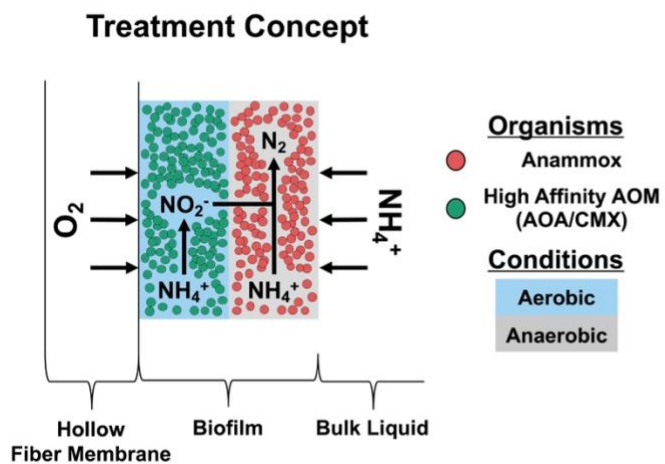
Keywords:

Comammox, AOA, anammox, mainstream partial nitrification/anammox treatment, comammox oxygen affinity, formate-dependent nitrate reduction

Highlights:

- Ammonia-oxidizing, *Nitrospira*, and anammox bacteria provided stable mainstream PNA
- A high relative abundance of *Nitrospira* was detected in mainstream PNA MABRs
- High *Nitrospira* oxygen affinities enable proliferation in anoxic environments
- Formate-dependent nitrate reduction was demonstrated by a comammox isolate
- Nitrate reduction by *Nitrospira* led to total-N removal in two PNA systems

Graphical Abstract:



4.1 Introduction

Implementing anaerobic ammonium oxidation (anammox) into single-stage, partial nitrification/anammox (PNA) treatment—aerobic granular sludge (AGS)—has decreased the cost of treating warm, ammonium-replete side streams by decreasing aeration, carbon supplementation, and tank volume requirements (Abma et al., 2007; van der Star et al., 2007). Co-diffusion of ammonium and oxygen into spherical AGS biofilms selects for the cooperation between ammonia-oxidizing bacteria (AOB) and anaerobic ammonium oxidizing (anammox) bacteria (Straka et al., 2019). AOB in the aerobic periphery consume oxygen while oxidizing part of the incoming ammonium to nitrite (i.e., partial nitrification) (Winkler et al., 2012). Residual ammonium and nitrite then co-diffuse into the anoxic biofilm interior, where anammox bacteria convert the ammonium and nitrite to dinitrogen gas (N_2) (Speth et al., 2016). Despite the success of AGS, mainstream PNA treatment with AGS configurations has not yet been widely implemented. The combination of low mainstream ammonium concentrations, ambient temperatures, and the need for strict dissolved oxygen (DO) control—required to suppress nitrite-oxidizing bacteria (NOB)—limits AOB activity and the accompanying anammox nitrogen removal (Wang et al., 2022). While increasing DO might increase nitrite production by AOB, it would stimulate NOB that compete with anammox for nitrite (Winkler et al., 2012). Therefore, new PNA configurations are needed to provide a reliable nitrite supply to anammox under mainstream conditions.

The counter-diffusive ammonia and oxygen gradients promoted by membrane aerated biofilm reactor (MABR) configurations are well suited for stable mainstream wastewater treatment with high-affinity ammonia-oxidizing microorganisms (AOM) and anammox (Li et al., 2023a; Straka et al., 2019). In this configuration, oxygen supplied by a hollow-fiber membrane diffuses towards

an anoxic bulk-liquid, while ammonia in the bulk-liquid diffuses toward the membrane. The ammonia-limited environment near the aerobic biofilm base creates a niche suitable for ammonia-oxidizing archaea (AOA) and complete ammonia-oxidizing bacteria (comammox), both high-affinity AOM, to outcompete low-affinity AOB (Kits et al., 2017). High nitrite production rates by high-affinity AOM could provide a continuous nitrite supply to anammox in the anaerobic biofilm region thereby enabling stable mainstream PNA treatment.

A mainstream PNA pilot system harboring comammox and anammox recently reported complete ammonium removal (Li et al., 2023a)—presumably facilitated by comammox-anammox cross-feeding. Surprisingly, the system also completely removed nitrate—a product of anammox anabolism (Speth et al., 2016)—without the carbon supplementation. Yet, the physiological mechanism enabling complete nitrogen removal from this system remains unclear, leaving a major gap in the engineering of mainstream PNA systems with long-term process stability. Anaerobic formate-dependent nitrate reduction has been demonstrated for *Nitrospira moscoviensis*, a lineage II *Nitrospira* NOB (Koch et al., 2015). The reduction of anammox-derived nitrate to nitrite by *Nitrospira* may facilitate complete nitrogen removal via anaerobic nitrite cross-feeding. However, nitrate reduction has not yet been demonstrated by a comammox species and the comammox isolate *N. inopinata* does not encode a canonical formate dehydrogenase required for formate-oxidation. A demonstration of nitrate reduction by a comammox *Nitrospira* could help us understand why selectively pairing comammox with anammox has previously enabled complete nitrate removal (Li et al., 2023a).

Here, we explored the microbial community composition and nitrogen removal in two lab-scale membrane aerated biofilm reactors (MABRs) co-cultured with anammox and either AOA strains

or the comammox isolate *N. inopinata*. Oxygen was delivered through hollow-fiber membranes submerged in synthetic wastewater, resulting in ammonium and oxygen counter-diffusing across an artificial biofilm providing a low ammonia niche near the aerated biofilm base for the high affinity AOM. As we now report, physiological and kinetic studies of *N. inopinata* showed that oxygen affinity and a capacity for formate-dependent nitrate reduction both likely contribute to complete nitrogen removal by *Nitrospira*.

4.2 Methods

4.2.1 Hollow-fiber membrane coating procedure

We bypassed the startup time associated with establishing naturally occurring biofilm by coating hollow-fiber membranes (HFMs) with AOM grown as described in Supplemental Material. Cells were concentrated by filtration on 0.2 μm track-etched polycarbonate membrane filters (MilliporeSigma, Burlington, MA) and resuspended in 50 ml of synthetic wastewater. Anammox granular sludge was obtained from a 2-stage centrate-treatment plant in Sluisjesdijk, Rotterdam, NL (van der Star et al., 2007) and maintained for six months anaerobically at 30°C and pH 7 in a sequencing batch reactor supplied with a mineral media containing 1 mM ammonium and 1.3 mM nitrite (van de Graaf et al., 1996). Anammox granules (25 mL) were homogenized with a Pro200 Electronic Tissue Grinder (Bio-Gen, Cambridge, MA USA) at 50% power for 1-2 minutes to create a dense dispersed culture.

Composite hollow-fiber membranes (STERAPORE™ Model No. 20M3400A, Mitsubishi Chemical, Tokyo, Japan) were potted in 1-cm diameter tubes with epoxy (1838 B/A green Scotch-Weld TM, 3M, Saint Paul, MN USA) to create membrane modules. Cell attachment was enhanced

by coating the fibers with ten applications of 0.01% poly-L-lysine and waiting 3 hours before coating with biomass. Once the poly-L-lysine layers fully dried, membranes were submerged in medium containing either concentrated comammox culture (*N. inopinata*) or mixture of AOA strains (Straka et al., 2019) and incubated on a low RPM shaker for 30 minutes. Subsequently, the membrane fibers were submerged in homogenized anammox biomass and incubated for 30 minutes on a low RPM shaker.

4.2.2 Counter-diffusion membrane aerated biofilm reactor systems

The MABRs had a total volume of 400 mL and consisted of two potted membrane modules connected to a recirculation pump, N₂-sparging bottle, and DO measurement bottle, as illustrated in **Figure S1**. Each membrane module contained 40 biomass-coated hollow fiber membranes. Compressed air supplied the coated biofilm with oxygen via a mass flow controller (Alicat scientific, Tucson, AZ, USA) maintained between 1.07-1.14 bar. An anoxic bulk liquid was maintained by continuously sparging with N₂ gas via a mass flow controller (Alicat scientific, Tucson, AZ, USA) maintained between 1.07-1.14 bar. The DO was continuously measured with an optical DO probe (Vernier Optical DO Probe, Beaverton, OR USA), and all DO measurements were < 6 µM-O₂, the limit of detection. A synthetic mainstream wastewater medium (Table S4-1) with 1 mM NH₄⁺-N was continuously supplied at a feed rate of 15 mL/h (1.1-day HRT). Homogeneous MABR bulk liquid conditions were maintained with a recirculation pump operating at a rate of 12L/h. All pumps were manually calibrated prior to reactor operation. The reactors were sampled three times a week using the three-way sampling port.

4.2.3 Chemical analysis

Ammonium, nitrite, and total oxidized nitrogen (TON) assays were conducted and measured with a Gallery™ Discrete Analyzer (Thermo Fisher Scientific, Waltham, MA, USA). Nitrate concentrations were determined by subtracting the nitrite concentration from the TON concentration. Influent and effluent chemical oxygen demand (COD) measurements were analyzed with Hach™ Low Range COD Digestion Vials (Hach, Loveland, CO USA) and formate concentrations were measured via ion chromatography (Dionex™ ICS-5000+, Thermo Fisher Scientific, Waltham, MA, USA).

4.2.4 DNA Extraction, 16S rRNA analysis, qPCR, and FISH

Membrane fibers were removed from the reactor at the end of the experiment, cut into 3 cm sections with sterilized scissors, and stored in 1.5 mL Eppendorf tubes at 4°C. This destructive sampling procedure prevented time course sampling. DNA was extracted from the membrane fibers and the initial anammox granular sludge with the DNeasy PowerSoil Pro kit (Qiagen, Hilden, Germany) according to the manufacturer protocol. Extracted DNA concentrations were quantified with the Qubit™ dsDNA High Sensitivity Assay (Thermo Fisher Scientific, Waltham, MA, USA). The V4-V5 region of the 16S rRNA gene was amplified by PCR using the 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) primer set (Parada et al., 2016). PCR products were purified and sent to Molecular Research Laboratories (Shallowater, TX, USA) for Amplicon-EZ Illumina sequencing with 20k reads per assay. Demultiplexed and adapter-trimmed reads were analyzed with USEARCH v11 (Edgar, 2010). Paired-end reads were merged, primer sequences were removed, and reads were quality filtered. Unique reads and their abundances were computed followed by denoising and chimera removal

(Edgar, 2016). Reads were mapped to zero-radius OTUs (zOTUs) and taxonomy was assigned by searching against the RDP 16S database v18 (Edgar, 2016; Maidak et al., 1997).

The absolute abundance of AOA, comammox, and anammox in the membrane biofilms was quantified with qPCR. Custom AOA *amoA* primers targeting the strains used in this study (described in Supplemental Material), comammox *amoB* primers (148F and 485R; Cotto et al., 2020) and anammox 16S primers (818F and 1040R; Hamersley et al., 2007) were used in this study. All qPCR reactions were performed in triplicate with a LightCycler® system (Roche, Rotkreuz, Switzerland) with the primers and reaction conditions listed in Supplemental Material (Table S4-2).

Membrane samples for fluorescence *in-situ* hybridization (FISH) analysis were fixed and hybridized according to previously described procedures (Nielsen et al., 2009) with modifications described in Supplementary Material.

4.2.5 Evaluating formate-dependent nitrate reduction activity in *N. inopinata*

Substrate depleted *N. inopinata* biomass (100-mL aliquots) was transferred to sterile, acid washed 240-mL serum bottles ($\sim 3 \cdot 10^7$ cells·mL⁻¹). Formate, nitrate, and ammonium stock solutions were supplemented to achieve the desired experimental concentrations (0.4mM-formate with 1mM-nitrate or 5mM-formate with 5mM-nitrate and 1mM-ammonium). The serum bottles were made anoxic by sealing with butyl rubber stoppers and aluminum crimps, sparging with N₂ gas for 45 minutes with periodic shaking, and setting the headspace pressure to 1.55 bar with N₂ gas. Anoxic sodium bicarbonate was added to each serum bottle to achieve a final concentration of 2mM. Serum bottles were incubated at 37 °C in the dark without shaking. Liquid samples were collected

using anaerobic technique with N₂ flushed syringes and filtered with 0.2 µm PES syringe-filter membranes. Cell concentrations were determined with flow cytometry as described in Supplemental Material (Feng et al., 2014).

4.2.6 Oxygen affinity measurements in *N. inopinata*

Substrate oxidation kinetics were measured using a microrespirometry (MR) system equipped with a 4.5-mL glass MR chamber, glass-coated magnetic stir bar, and an OX-MR oxygen microsensor with 500-µm tip diameter (Unisense, Aarhus, Denmark), as described previously (Martens-Habben et al., 2009). Oxygen sensors were continuously polarized before use and calibrated using air-equilibrated culture media and oxygen-free water. Measurements were conducted in a recirculated water bath maintained at 37°C. Data was continuously logged via the microsensor multimeter using the SensorTrace Logger software (Unisense, Aarhus, Denmark).

Experiments were conducted with concentrated ($15.3 - 19.3 \cdot 10^7$ cells·mL⁻¹; NO₂⁻) and unconcentrated ($3.54 - 4.81 \cdot 10^7$ cells·mL⁻¹; NH₄⁺) *N. inopinata* biomass after substrate depletion. Concentrated cells were collected by gentle centrifugation (6000g, 6 min). Unconcentrated cells were transferred directly into the MR chamber. Cell concentrations were determined with microscopy as described previously (Lunau et al., 2005). Chambers were immersed in the recirculating water bath, low stirring at 400 – 500 rpm was applied, and background sensor signal drift was measured for 30 – 60 min before initial substrate injections. Concentrated NH₄Cl or NaNO₂ stock solutions were injected into the chamber to achieve an initial substrate concentration of 1 mM. Oxygen uptake was recorded until at least 30 min after oxygen depletion. All measurements were carried out with a minimum of three replicates per condition.

Oxygen-concentration traces were used to determine the kinetic constants of *N. inopinata*. Each trace was manually inspected and subjected to downstream kinetic analysis. Cell specific oxygen consumption rates were calculated using a 1-minute time averaging algorithm to reduce measurement noise. Kinetic constants were obtained by fitting a Michaelis–Menten equation to the measured oxygen consumption rates using equation 4.1.

$$V = \frac{V_{max} * S}{K_{m(app)} + S} \quad (4.1)$$

where V refers to the oxygen uptake rate (in $\text{fmol-O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), $V_{\max}$ is the maximum oxygen uptake rate ($\text{fmol-O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), S is the O_2 concentration (μM) and $K_{m(app)}$ is the apparent half-saturation constant (μM).

where V refers to the oxygen uptake rate ($\text{fmol-O}_2 \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$), $V_{\max}$ is the maximum oxygen uptake rate, S is the O_2 concentration (μM), and $K_{m(app)}$ is the apparent half-saturation constant (μM). Specific oxygen affinities were calculated from kinetic constants using equation 4.2:

$$a^o = \frac{V_{max}}{K_{m(app)}} \quad (4.2)$$

where a^o is specific affinity (in $\text{L} \cdot \text{g}_{\text{wcw}}^{-1} \cdot \text{h}^{-1}$), $V_{\max}$ values were normalized to protein concentration ($\mu\text{mol-N} \cdot \text{mg}_{\text{protein}}^{-1} \cdot \text{h}^{-1}$) and $K_{m(app)}$ is the apparent half-saturation constant (μM). The factor of 5.7 g wet weight per g protein was used to estimate wet cell weights from protein content (Martens-Habbena et al., 2009). Time averaging, curve fitting, and statical analysis were performed in Python v3.9.12 with NumPy v 1.26.4, Scikit-Learn v1.4.2, and Scipy v1.14.1.

4.3 Results

4.3.1 Nitrogen Removal

Effluent nitrogen species and influent ammonium concentrations, monitored throughout the operation of the MABR reactors, are reported in **Figure 4.1-B**. Each reactor demonstrated stable performance after a three-week start-up and reaching an average total inorganic nitrogen (TIN) removal efficiency of $88.0 \pm 6.8 \%$ and $89.5 \pm 4.8 \%$ for the comammox-anammox and AOA-anammox MABR reactor, respectively. Nitrogen was removed at a rate of $1.0 \pm 0.3 \text{ mM-N} \cdot \text{d}^{-1}$ (comammox-anammox) and $0.9 \pm 0.1 \text{ mM-N} \cdot \text{d}^{-1}$ (AOA-anammox) with residual nitrogen primarily leaving the MABRs as ammonium, instead of nitrate. Minimal effluent nitrate accumulation was observed in either MABR with average concentrations of $0.05 \pm 0.06 \text{ mM-NO}_3^-$ and $0.01 \pm 0.062 \text{ mM-NO}_3^-$ for the comammox-anammox and AOA-anammox MABR, respectively. The high ammonia-removal efficiencies and near absence of effluent nitrate indicates that the MABR configuration could achieve stable PNA performance and total-nitrogen removal under mainstream conditions.

4.3.2 Fluorescent in-situ hybridization microscopy of the MABR biofilms

The spatial distribution of nitrifying populations was evaluated with FISH on a cross-section of the MABR biofilm at the end of operation; the micrographs are in reported **Figure 4.2**. A distinct layer of AOB was observed throughout the middle of the comammox-anammox MABR biofilm (blue; **Figure 4.2A**), potentially denoting an aerobic-anaerobic interface. The AOA distribution in the AOA-anammox MABR biofilm (purple; **Figure 4.2B**) was not as distinct as the AOB distribution, as AOA were distributed throughout the aerobic and anoxic biofilm regions.

Nitrospira (green; **Figure 4.2**) and anammox (red; **Figure 4.2**) co-localized near the anoxic biofilm periphery in both biofilms (top; **Figure 2**). However, *Nitrospira* were also distributed throughout the aerobic and anoxic biofilm regions, which was unexpected considering comammox and NOB *Nitrospira* utilize oxygen as a terminal electron acceptor during aerobic oxidation. Moreover, viability staining (**Figure S4.2**) demonstrated most cells in the biofilm were in an unstressed state (green).

4.3.3 Microbial community analysis via 16S rRNA amplicon sequencing and qPCR

The relative abundances of taxa in the MABR biofilms were estimated with 16S rRNA amplicon sequencing — reported in **Figure 4.3A-C**, **Figure S4.4**, and **Figure S4.5**— while the absolute abundance of nitrifying population was further quantified with qPCR targeting the AOA *amoA*, comammox *amoB*, and anammox 16S rRNA genes, reported in **Figure 4.3D-E**. Nitrifying populations accounted for approximately 20% of the total microbial community in both MABRs. Over 60% of taxa belonged to the Proteobacteria, Chloroflexi, Ignavibacteria, and Acidobacteria phyla, as reported in (**Figure S4.3A**). The abundance of these heterotrophic taxa is consistent with previous studies utilizing the same anammox inoculum to autotrophically treat low-strength wastewater with high affinity nitrifiers (Li et al., 2023a, 2023b).

A co-existing population of *Nitrosomonas* AOB, *Nitrospira*, and anammox bacteria were detected in both MABR biofilms, while a single AOA zero-radius OTU (zOTU), closely related to *N. viennensis*, was primarily detected in the AOA-anammox MABR. Despite the use of high-affinity nitrifier coatings, sequencing confirmed the presence of *Nitrosomonas* AOB in both MABR systems, suggesting that the nitrifier coating did not fully suppress the AOB population originating from the anammox inoculum. Anammox bacteria were present in both MABRs with relative

abundances less than 2%. However, these low abundances are contradicted by independent analysis with FISH, which indicate the anammox abundance aligned with AOB, AOA, and *Nitrospira* in both MABR reactors as reported in **Figure 4.2**. Discrepancy between FISH and PCR amplification techniques (16S rRNA gene sequencing and qPCR) has been attributed to variations in DNA extraction efficiency (Van Teeseling et al., 2015), primer biases (Orschler et al., 2019), and variable 16S rRNA-gene copy numbers per cell across microbial lineages (Louca et al., 2018).

An uncharacterized lineage II *Nitrospira* zOTU closely related to *Nitrospira* sp. OLB3—a *Nitrospira* NOB commonly detected in PNA systems (Speth et al., 2016)—had a relative abundance greater than 14% in both MABR reactors. However, a zOTU related to the comammox isolate *N. inopinata* had a relative abundance below 1%, reported in **Figure 4.3B-C**. Amplicon sequencing cannot distinguish between comammox and NOB *Nitrospira*. Therefore, we evaluated the abundance of comammox *Nitrospira* with qPCR using primers targeting comammox *amoB* genes (Cotto et al., 2020). Comammox *Nitrospira* were detected in both reactors, which was unexpected considering comammox was not inoculated into the AOA-anammox MABR.

Absolute comammox abundances were compared against the AOA abundance in the AOA-anammox MABR using primers targeting the *N. viennensis amoA* gene to determine the ratio of comammox and NOB *Nitrospira*. The abundance of AOA *amoA* copies detected in the AOA-anammox MABR was 7-fold and 53-fold time higher than the copies of comammox *amoB* detected in the comammox-anammox and AOA-anammox MABRs, respectively. Despite the amplicon sequencing results suggesting *Nitrospira* were more abundant than AOA, the qPCR abundances suggest the majority of the *Nitrospira* population was only capable of nitrite oxidation, reported in **Figure 4.3B-E**. While the primers might not have amplified all comammox species (Blom et al.,

2024), the magnitude of this measured disparity cannot be ignored. A pilot-scale study about mainstream PNA has linked the presence of comammox and NOB *Nitrospira* with complete nitrate removal from PNA (Li et al., 2023a). Our results further support that NOB and comammox *Nitrospira* are associated with complete nitrate removal in mainstream PNA systems.

4.3.4 Oxygen affinity of *Nitrospira inopinata*

The ammonia- and nitrite-dependent oxygen affinity of the comammox isolate *N. inopinata* was measured with microrespirometry, results presented in **Figure 4.4**, **Figure S4.5**, and **Figure S4.6**. Oxygen consumption followed Michaelis-Menten-type kinetics. The ammonia-dependent ($K_{m(\text{app}),\text{O}_2,\text{NH}_4^+} = 1.49 \pm 0.38 \mu\text{M-O}_2$, $n = 3$) and nitrite-dependent ($K_{m(\text{app}),\text{O}_2,\text{NO}_2^-} = 2.27 \pm 0.98 \mu\text{M-O}_2$, $n = 3$) apparent half-saturation constants for oxygen, reported in **Figure 4.4A**, were statically similar (ANOVA p-value = 0.355). However, the ammonia- and nitrite-dependent specific oxygen affinities reported in **Figure 4.4B** significantly differed (ANOVA p-value = 0.0026), presumably due to differences between the maximum specific rate (V_{max}) of ammonia- and nitrite-oxidation by *N. inopinata*. The oxygen affinity of *N. inopinata* aligns with previously reported *Nitrospira* NOB and AOA oxygen affinities, yet are statically different from AOB and *Nitrobacter* NOB, reported in **Figure 4.4C**.

4.3.5 Anaerobic formate-dependent nitrate reduction by *Nitrospira inopinata*

We hypothesized that *Nitrospira* lacking an annotated formate dehydrogenase (*fdh*) and formate transporters can utilize formate as an electron donor for nitrate reduction and tested this hypothesis with *N. inopinata*. Anoxic incubations with formate and nitrate confirmed *N. inopinata* can couple formate oxidation with nitrate reduction, results presented in **Figure 4.5**. Formate-dependent

nitrate reduction by *N. inopinata*, reported in **Figure 4.5A**, occurred at an equimolar stoichiometric ratio (1 formate oxidized : 1 nitrate reduced : 1 nitrite produced) with an average cell-specific nitrate reduction rate of $0.022 \pm 0.016 \text{ fmol-NO}_3^- \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$ in a high-density culture ($2.76 \cdot 10^7 \pm 0.24 \text{ cells} \cdot \text{mL}^{-1}$). Low-density incubations (estimated: $3 \cdot 10^6 \text{ cells} \cdot \text{mL}^{-1}$), reported in **Figure 5B**, experienced a week-long lag phase before the onset of formate-dependent nitrate reduction. Formate and nitrate were consumed at a 0.8:1 stoichiometric molar ratio, comparable to the 0.9:1 ratio previously observed in the NOB *N. moscoviensis* (Koch et al., 2015). *N. inopinata* was unable to reduce nitrate in the absence of formate and formate-dependent nitrate reduction was not observed in heat-killed controls, reported in **Figure 4.5C** and **Figure 4.5D** respectively. Together, these results indicate that *N. inopinata* performed anaerobic formate-dependent nitrate reduction despite lacking an annotated formate dehydrogenase and formate transporter.

4.4 Discussion

4.4.1 Low oxygen adaptations of *Nitrospira inopinata*

Combining high-affinity AOM with anammox for mainstream treatment offers a promising pathway towards mainstream PNA with minimal nitrogen effluent discharge, yet a fundamental understanding of nitrifier physiology under oxygen limitations is required to harness this partnership.

Oxygen has been suggested as an environmental parameter driving the niche differentiation between nitrifying lineages harboring different terminal oxidases (Martinez-Rabert et al., 2022). Comparative genomic analysis of *Nitrospira* indicates that all comammox and NOB *Nitrospira*

species harbor a yet to be biochemically characterized, putatively high-affinity cytochrome *bd*-like terminal oxidase (Lawson and Lucker, 2018), while AOA, AOB, and *Nitrobacter* NOB harbor the low-affinity *aa*₃-type terminal oxidase (Martinez-Rabert et al., 2022). Despite harboring *bd*-like terminal oxidase, apparent oxygen affinity is comparable to the affinity of lineage II *Nitrospira* NOB (Ushiki et al., 2017) and AOA (Qin et al., 2017; Straka et al., 2019), which respectively harbor *bd*-like and *aa*₃-type (low affinity) terminal oxidases. Considering large variations in nitrifier substrate affinity have been reported under different physiological conditions (Martens-Habben and Stahl, 2011), higher comammox oxygen affinities may be achieved after growth under prolonged oxygen-limitations. However, the similarity between oxygen affinity of comammox and AOA suggests oxygen does not differentiate their metabolic niche.

Nitrospira have traditionally been considered obligate aerobes, yet comammox and NOB *Nitrospira* are widely distributed in oxygen limited and microaerophilic environments (Koch et al., 2019; Park and Noguera, 2007). While nitrite oxidation by NOB in anaerobic or microaerophilic conditions is atypical, recent studies have demonstrated that the nitrite-oxidizing enzyme – nitrite oxidoreductase (NXR) – may be reversible under anaerobic conditions. *Nitrospira moscoviensis*, a lineage II *Nitrospira* NOB, can anaerobically reduce nitrate to nitrite while oxidizing formate via FDH (Koch et al., 2015). It has been proposed that *N. inopinata* and other comammox *Nitrospira* might also reduce nitrate reduction through reverse NXR activity. A meta-transcriptomic analysis of municipal WWT facility reported increased transcription of comammox *nxrA* and *nxrB* with decreasing DO, suggesting comammox might reduce nitrate in oxygen limited mainstream WWT (Johnston et al., 2024). Although clade A comammox – including *N. inopinata* – lack a formate dehydrogenase and formate transporter (Lawson and Lucker, 2018), this study provides evidence

that *N. inopinata* can couple anaerobic formate oxidation to the stoichiometric reduction of nitrate to nitrite as reported in **Figure 4.5**, corroborating previous observations of aerobic and anaerobic formate oxidation by *N. inopinata* (Pjevac, 2017). Formate oxidation might be catalyzed by the NXR enzyme as previously reported for a *Nitrobacter* NOB isolate lacking formate dehydrogenase (Peck Jr et al., 1983). Whether this mechanism is present in *N. inopinata* requires further investigation. The ability for *N. inopinata* to conduct formate-dependent nitrate reduction provides a mechanistic explanation for the distribution of comammox in oxygen limited environments thereby broadening their environmental niche. Harnessing the nitrate reducing capacity of comammox and NOB *Nitrospira* for PNA treatment could reduce the energy, carbon, and footprint requirements for mainstream nitrogen removal.

4.4.2 *Nitrospira* and anammox are associated with near complete nitrate removal

In this study, anammox and high affinity nitrifiers (AOA or comammox) were coated onto MABR hollow-fiber membranes and demonstrated efficient autotrophic nitrogen removal without nitrate accumulation from synthetic mainstream wastewater. The counter-diffusion environment provided by the MABR promoted the enrichment of high affinity nitrifiers, which enabled ammonium removal. While both reactors maintained anammox, AOB, NOB *Nitrospira*, and comammox populations, AOA confined to the MABR system initially coated with AOA suggesting membrane coatings offer a path towards integrating high affinity AOM strains into existing wastewater treatment configuration. The low abundances of AOB and high abundance of *Nitrospira* in both systems was unexpected considering anammox requires a nitrite-supplying population and NOB suppression has been implemented for all successful PNA reactor configurations (Wang et al., 2022). *Nitrospira* and AOA were distributed throughout the aerobic and anoxic biofilm regions in

both reactors as reported in **Figure 4.2** with comammox *Nitrospira* comprising a small fraction of the overall *Nitrospira* population reported in **Figure 4.3**. Comammox *Nitrospira* (this study), *Nitrospira* NOB (Ushiki et al., 2017), and AOA (Qin et al., 2017; Straka et al., 2019) all have oxygen affinities near the oxygen inhibition constant of anammox ($IC_{50} = 2.7\text{--}4.2\ \mu\text{M-O}_2$) (Okabe et al., 2023), while *Nitrosomonas* AOB have significantly higher oxygen affinities ($6.11\text{--}20.42\ \mu\text{M-O}_2$) (Laanbroek and Gerards, 1993). The comparatively low AOB oxygen affinity suggests comammox *Nitrospira*, NOB *Nitrospira*, and AOA will outcompete AOB under oxygen limitations as observed by the proliferation of *Nitrospira* and AOA detected throughout the MABR biofilms with FISH in **Figure 4.2**. The counter-diffusive MABR biofilms likely enabled one-stage mainstream PNA treatment by promoting the scavenging of oxygen by *Nitrospira* and AOA while simultaneously cross-feeding nitrite to anammox. However, oxygen scavenging alone cannot explain the near absence of effluent nitrate.

Mainstream PNA systems report unwanted nitrate accumulation and require organic carbon supplementation to stimulate the removal of nitrate through heterotrophic denitrification to avoid effluent threshold violations (Cao et al., 2017). Known facultative, heterotrophic denitrifiers were detected with 16S rRNA amplicon sequencing in both MABR reactors as reported in **Figure S4.3** and **Figure S4.4** despite the absence of carbon dosing. Heterotrophic denitrification might have been stimulated by the poly-L-lysine facilitating cell attachment, microbial necromass degradation, or autotrophic organic acid excretion (Okabe et al., 2005; Rittmann et al., 1994). Although denitrification cannot be ruled out, denitrification appears unlikely considering an AOA-anammox MABR reactor utilizing the same anammox granular sludge inoculum and AOA strains completely removed nitrogen from mainstream municipal wastewater without significant evidence of

heterotrophic denitrification (Li et al., 2023). Similarities between this study and ours suggests counter-diffusive MABR biofilms supported an autotrophic microbial community capable of near-complete nitrogen removal with minimal effluent nitrate accumulation.

The anoxic distribution of *Nitrospira* in both MABR biofilms and high relative abundance detected with 16S rRNA amplicon sequencing led us to hypothesize that the *Nitrospira* populations might contribute to nitrate removal in mainstream PNA. Lineage II NOB *Nitrospira* and comammox *Nitrospira* can catalyze formate-dependent nitrate reduction (Koch et al., 2015; Pjevac, 2017; this study). However, carbon must be supplied by a community member to support this metabolism. Influent and effluent chemical oxygen demand (COD) measurements indicate the absence of organic carbon production by either MABR (**Table S4-4**). A theoretical model was developed to determine if endogenous biomass decay alone could support the observed nitrate reduction assuming all nitrate was reduced by *Nitrospira* performing formate-dependent nitrate reduction. The model indicates additional carbon supplementation would be required to completely remove nitrate (Tables S5-8). Therefore, the COD deficit in the model leads us to consider the possibility of cryptic organic acid cycling within the MABR biofilms.

A recent metabolic-network model of anammox granular sludge based on meta-transcriptomic data suggests anammox can excrete organic substrates and nitrate (Lawson et al., 2017). The genome of the anammox species *Ca. Brocadia sinica* contains a formate dehydrogenase enzyme, which has been observed to produce formate in DNRA microorganisms by acting in the reductive direction (Oshiki et al., 2016; Yu et al., 2019). Therefore, anammox species might produce formate or other organic acids during carbon fixation via the reductive acetyl-CoA pathway. These organic carbon compounds may be utilized by comammox *Nitrospira*, NOB *Nitrospira*, or heterotrophs

while reducing the nitrate produced by anammox to nitrite. We hypothesize, anammox and *Nitrospira* (or heterotrophs) could establish a mutualistic metabolic interaction where high affinity AOM supply nitrite to anammox, anammox supplies nitrate and formate to nitrate reducers, and nitrate reducers provides additional nitrite to anammox to enable complete nitrogen removal as represented in **Figure 4.6**. Future research, possibly utilizing flow-through stable isotope probing (Flow-SIP), proteomics, and/or transcriptomics, is required to confirm this mutualistic metabolic interaction. Our data along with previous studies (Gottshall et al., 2021; Li et al., 2023a; Van Kessel et al., 2015) suggest autotrophic nitrate removal could be accomplished by a high affinity AOM-*Nitrospira*-anammox consortium to treat mainstream wastewater without additional carbon supplementation. Ultimately, our study reveals that comammox and NOB *Nitrospira* may enhance mainstream PNA treatment through autotrophic nitrate removal. This enhances our understanding about the role of *Nitrospira* in conventional AOB-anammox PNA treatment and raises questions about the role of NOB suppression. The mutualistic interactions between high affinity AOM-*Nitrospira*-anammox enables a path towards developing mainstream PNA treatment systems capable of providing better effluent quality than existing solutions.

4.5 Conclusions

Here, we coated high affinity nitrifying strains (AOA and comammox *Nitrospira*) and anammox sludge onto MABR hollow-fiber membranes to develop a one-stage mainstream PNA system. Both systems efficiently removed nitrogen from synthetic mainstream wastewater without accumulating effluent nitrate. *Nitrospira* were highly abundant and comammox *Nitrospira* were detected in both MABR reactors. Surprisingly, *Nitrospira* were distributed throughout both aerobic and anaerobic regions of the MABR biofilms. We tested the hypothesis that this

distribution and complete nitrate removal may be due to either the high oxygen affinity of *Nitrospira*, or to their capacity to perform formate-dependent nitrate reduction. The oxygen affinity of *N. inopinata* was similar to the oxygen affinity of lineage II *Nitrospira* NOB and AOA species, indicating this hypothesis cannot account for the *Nitrospira* distribution in anoxic biofilm regions. However, it was demonstrated that *N. inopinata* can catalyze formate-dependent nitrate reduction, which might explain the observed *Nitrospira* distribution and near-complete nitrate removal.

Our data along with previous studies suggest a mutualistic metabolic interaction between high affinity AOM, nitrate-reducing *Nitrospira* (comammox and NOB), and anammox can completely remove nitrate during mainstream PNA (Gottshall et al., 2021; Li et al., 2023a, 2023b; Van Kessel et al., 2015). This significantly differs from conventional AOB-anammox PNA treatment that suppress *Nitrospira* and accumulate effluent nitrate. This work explores the mechanisms of high affinity AOM-*Nitrospira*-anammox cooperation and how their interactions may enable high-performance, low-energy nitrogen removal from mainstream municipal wastewater.

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References

- Blom, P., Smith, G.J., van Kessel, M.A.H.J., Koch, H., Lückner, S., 2024. Comprehensive evaluation of primer pairs targeting the ammonia monooxygenase subunit A gene of complete ammonia-oxidizing *Nitrospira*. *Microbiol Spectr* 12. <https://doi.org/10.1128/spectrum.00516-24>
- Cao, Y., van Loosdrecht, M.C.M., Daigger, G.T., 2017. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. *Appl Microbiol Biotechnol* 101, 1365–1383. <https://doi.org/10.1007/s00253-016-8058-7>
- Cotto, I., Dai, Z., Huo, L., Anderson, C.L., Vilardi, K.J., Ijaz, U., Khunjar, W., Wilson, C., De Clippeleir, H., Gilmore, K., Bailey, E., Pinto, A.J., 2020. Long solids retention times and attached growth phase favor prevalence of comammox bacteria in nitrogen removal systems. *Water Res* 169. <https://doi.org/10.1016/j.watres.2019.115268>
- Edgar, R.C., 2016. UNOISE2: Improved Error-Correction for Illumina 16S and ITS Amplicon Sequencing. *bioRxiv* 081257.
- Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* 26, 2460–2461. <https://doi.org/10.1093/bioinformatics/btq461>
- Feng, J., Wang, T., Zhang, S., Shi, W., Zhang, Y., 2014. An Optimized SYBR Green I/PI Assay for Rapid Viability Assessment and Antibiotic Susceptibility Testing for *Borrelia burgdorferi*. *PLoS One* 9, e111809. <https://doi.org/10.1371/journal.pone.0111809>
- Gottshall, E.Y., Bryson, S.J., Cogert, K.I., Landreau, M., Sedlacek, C.J., Stahl, D.A., Daims, H., Winkler, M., 2021. Sustained nitrogen loss in a symbiotic association of Comammox *Nitrospira* and Anammox bacteria. *Water Res* 202, 117426. <https://doi.org/10.1016/J.WATRES.2021.117426>
- Hamersley, M.R., Lavik, G., Woebken, D., Rattray, J.E., Lam, P., Hopmans, E.C., Sinninghe Damsté, J.S., Krüger, S., Graco, M., Gutiérrez, D., Kuypers, M.M.M., 2007. Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnol Oceanogr*. <https://doi.org/10.4319/lo.2007.52.3.0923>
- Johnston, J., Vilardi, K., Cotto, I., Sudarshan, A., Bian, K., Klaus, S., Bachmann, M., Parsons, M., Wilson, C., Bott, C., Pinto, A., 2024. Metatranscriptomic Analysis Reveals Synergistic Activities of Comammox and Anammox Bacteria in Full-Scale Attached Growth Nitrogen Removal System. *Environ Sci Technol* 58, 13023–13034. <https://doi.org/10.1021/acs.est.4c04375>

- Kits, K.D., Sedlacek, C.J., Lebedeva, E. V., Han, P., Bulaev, A., Pjevac, P., Daebeler, A., Romano, S., Albertsen, M., Stein, L.Y., Daims, H., Wagner, M., 2017. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 549, 269–272. <https://doi.org/10.1038/nature23679>
- Koch, H., Lücker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., Nielsen, P.H., Wagner, M., Daims, H., 2015. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proc Natl Acad Sci U S A* 112, 11371–11376. <https://doi.org/10.1073/pnas.1506533112>
- Koch, H., van Kessel, M.A.H.J., Lücker, S., 2019. Complete nitrification: insights into the ecophysiology of comammox *Nitrospira*. *Appl Microbiol Biotechnol* 103, 177–189. <https://doi.org/10.1007/s00253-018-9486-3>
- Laanbroek, H.J., Gerards, S., 1993. Competition for limiting amounts of oxygen between *Nitrosomonas europaea* and *Nitrobacter winogradskyi* grown in mixed continuous cultures. *Arch Microbiol* 159, 453–459. <https://doi.org/10.1007/BF00288593>
- Lawson, C.E., Lücker, S., 2018. Complete ammonia oxidation: an important control on nitrification in engineered ecosystems? *Curr Opin Biotechnol* 50, 158–165. <https://doi.org/10.1016/J.COPBIO.2018.01.015>
- Lawson, C.E., Wu, S., Bhattacharjee, A.S., Hamilton, J.J., McMahon, K.D., Goel, R., Noguera, D.R., 2017. Metabolic network analysis reveals microbial community interactions in anammox granules. *Nat Commun* 8, 15416. <https://doi.org/10.1038/ncomms15416>
- Li, B., Godfrey, B.J., RedCorn, R., Candry, P., Abrahamson, B., Wang, Z., Goel, R., Winkler, M.K.H., 2023a. Mainstream nitrogen removal from low temperature and low ammonium strength municipal wastewater using hydrogel-encapsulated comammox and anammox. *Water Res* 242. <https://doi.org/10.1016/j.watres.2023.120303>
- Li, B., Godfrey, B.J., RedCorn, R., Wang, Z., Goel, R., Winkler, M.K.H., 2023b. Simultaneous anaerobic carbon and nitrogen removal from primary municipal wastewater with hydrogel encapsulated anaerobic digestion sludge and AOA-anammox coated hollow fiber membrane. *Science of the Total Environment* 883. <https://doi.org/10.1016/j.scitotenv.2023.163696>
- Louca, S., Doebeli, M., Parfrey, L.W., 2018. Correcting for 16S rRNA gene copy numbers in microbiome surveys remains an unsolved problem. *Microbiome* 6, 41. <https://doi.org/10.1186/s40168-018-0420-9>

- Lunau, M., Lemke, A., Walther, K., Martens-Habbena, W., Simon, M., 2005. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* 7, 961–968. <https://doi.org/10.1111/j.1462-2920.2005.00767.x>
- Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., Woese, C.R., 1997. The RDP (Ribosomal Database Project). *Nucleic Acids Res* 25, 109–110. <https://doi.org/10.1093/nar/25.1.109>
- Martens-Habbena, W., Berube, P.M., Urakawa, H., de la Torre, J.R., Stahl, D.A., 2009. Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. *Nature* 461, 976–979. <https://doi.org/10.1038/nature08465>
- Martens-Habbena, W., Stahl, D.A., 2011. Nitrogen Metabolism and Kinetics of Ammonia-Oxidizing Archaea, in: *Methods in Enzymology*. Academic Press Inc., pp. 465–487. <https://doi.org/10.1016/B978-0-12-386489-5.00019-1>
- Martinez-Rabert, E., Smith, C.J., Sloan, W.T., González-Cabaleiro, R., 2022. Biochemistry shapes growth kinetics of nitrifiers and defines their activity under specific environmental conditions. *Biotechnol Bioeng* 119, 1290–1300. <https://doi.org/10.1002/bit.28045>
- Nielsen, P.H., Daims, H., Lemmer, H., 2009. FISH Handbook for Biological Wastewater Treatment. IWA Publishing. <https://doi.org/10.2166/9781780401775>
- Okabe, S., Kindaichi, T., Ito, T., 2005. Fate of ¹⁴ C-Labeled Microbial Products Derived from Nitrifying Bacteria in Autotrophic Nitrifying Biofilms. *Appl Environ Microbiol* 71, 3987–3994. <https://doi.org/10.1128/AEM.71.7.3987-3994.2005>
- Okabe, S., Ye, S., Lan, X., Nukada, K., Zhang, H., Kobayashi, K., Oshiki, M., 2023. Oxygen tolerance and detoxification mechanisms of highly enriched planktonic anaerobic ammonium-oxidizing (anammox) bacteria. *ISME Communications* 3. <https://doi.org/10.1038/s43705-023-00251-7>
- Orschler, L., Agrawal, S., Lackner, S., 2019. On resolving ambiguities in microbial community analysis of partial nitritation anammox reactors. *Sci Rep* 9, 6954. <https://doi.org/10.1038/s41598-019-42882-8>
- Oshiki, M., Shinyako-Hata, K., Satoh, H., Okabe, S., 2016. Draft genome sequence of an anaerobic ammonium-oxidizing bacterium, “*Candidatus Brocadia sinica*.” *Genome Announc* 3. <https://doi.org/10.1128/genomeA.00267-15>

- Parada, A.E., Needham, D.M., Fuhrman, J.A., 2016. Every base matters: assessing small subunit rRNA primers for marine microbiomes with mock communities, time series and global field samples. *Environ Microbiol* 18, 1403–1414. <https://doi.org/10.1111/1462-2920.13023>
- Park, H.-D., Noguera, D.R., 2007. Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. *J Appl Microbiol* 102, 1401–1417. <https://doi.org/10.1111/j.1365-2672.2006.03176.x>
- Peck Jr, H.D., Strohl, W.R., Tuorinen, O.H., 1983. *Microbial Chemoautotrophy*. Ohio State University Press, Columbus.
- Pjevac, P., 2017. News on Comammox ... environmental distribution and metabolic flexibility., in: 5th International Conference on Nitrification and Related Processes. Vienna, Austria.
- Qin, W., Meinhardt, K.A., Moffett, J.W., Devol, A.H., Virginia Armbrust, E., Ingalls, A.E., Stahl, D.A., 2017. Influence of oxygen availability on the activities of ammonia-oxidizing archaea. *Environ Microbiol Rep* 9, 250–256. <https://doi.org/10.1111/1758-2229.12525>
- Rittmann, B.E., Regan, J.M., Stahl, D.A., 1994. Nitrification as a source of soluble organic substrate in biological treatment. *Water Science and Technology* 30, 1–8. <https://doi.org/10.2166/wst.1994.0247>
- Speth, D.R., in 't Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M., 2016. Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. *Nat Commun* 7, 11172. <https://doi.org/10.1038/ncomms11172>
- Straka, L.L., Meinhardt, K.A., Bollmann, A., Stahl, D.A., Winkler, M.-K.H., 2019. Affinity informs environmental cooperation between ammonia-oxidizing archaea (AOA) and anaerobic ammonia-oxidizing (Anammox) bacteria. *ISME J* 13, 1997–2004. <https://doi.org/10.1038/s41396-019-0408-x>
- Ushiki, N., Jinno, M., Fujitani, H., Suenaga, T., Terada, A., Tsuneda, S., 2017. Nitrite oxidation kinetics of two *Nitrospira* strains: The quest for competition and ecological niche differentiation. *J Biosci Bioeng* 123, 581–589. <https://doi.org/10.1016/J.JBIOSEC.2016.12.016>
- van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology (N Y)* 142, 2187–2196. <https://doi.org/10.1099/13500872-142-8-2187>

- van der Star, W.R.L., Abma, W.R., Blommers, D., Mulder, J.-W., Tokutomi, T., Strous, M., Picioreanu, C., van Loosdrecht, M.C.M., 2007. Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam. *Water Res* 41, 4149–4163.
- Van Kessel, M.A.H.J., Speth, D.R., Albertsen, M., Nielsen, P.H., Op Den Camp, H.J.M., Kartal, B., Jetten, M.S.M., Lückner, S., 2015. Complete nitrification by a single microorganism. *Nature* 528, 555–559. <https://doi.org/10.1038/NATURE16459>
- Van Teeseling, M.C.F., Mesman, R.J., Kuru, E., Espaillet, A., Cava, F., Brun, Y. V., Vannieuwenhze, M.S., Kartal, B., Van Niftrik, L., 2015. Anammox Planctomycetes have a peptidoglycan cell wall. *Nat Commun* 6, 1–6. <https://doi.org/10.1038/ncomms7878>
- Wang, Z., Zheng, M., Duan, H., Yuan, Z., Hu, S., 2022. A 20-Year Journey of Partial Nitrification and Anammox (PN/A): from Sidestream toward Mainstream. *Environ Sci Technol* 56, 7522–7531. <https://doi.org/10.1021/acs.est.1c06107>
- Winkler, M.K.H., Bassin, J.P., Kleerebezem, R., Sorokin, D.Y., van Loosdrecht, M.C.M., 2012. Unravelling the reasons for disproportion in the ratio of AOB and NOB in aerobic granular sludge. *Appl Microbiol Biotechnol* 94, 1657–1666. <https://doi.org/10.1007/s00253-012-4126-9>
- Winkler, M. K.H., Kleerebezem, R., Van Loosdrecht, M.C.M., 2012. Integration of anammox into the aerobic granular sludge process for main stream wastewater treatment at ambient temperatures. *Water Res* 46, 136–144. <https://doi.org/10.1016/J.WATRES.2011.10.034>
- Yu, X., Niks, D., Ge, X., Liu, H., Hille, R., Mulchandani, A., 2019. Synthesis of Formate from CO₂ Gas Catalyzed by an O₂-Tolerant NAD-Dependent Formate Dehydrogenase and Glucose Dehydrogenase. *Biochemistry* 58, 1861–1868. <https://doi.org/10.1021/acs.biochem.8b01301>

Figure legends

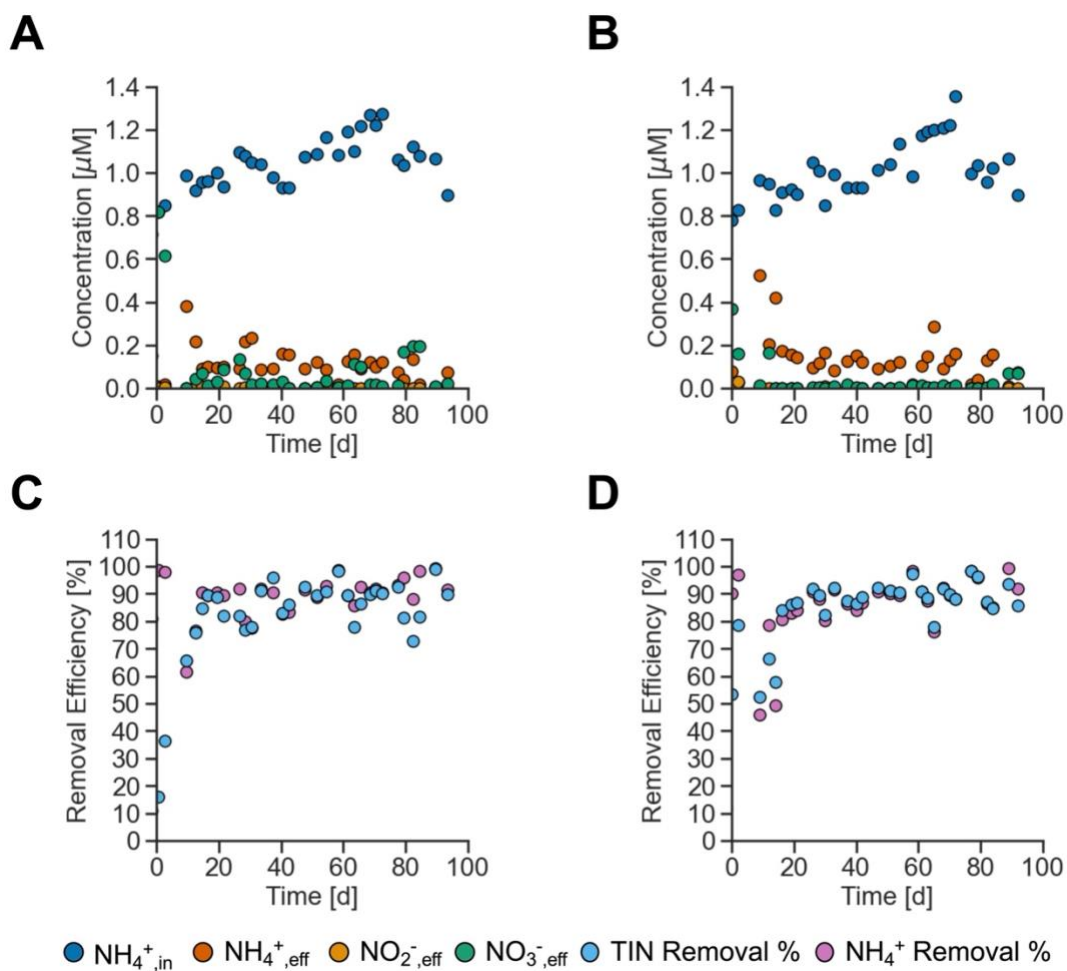


Figure 4.1. Nitrifying MABR reactor performance.

The long-term operational performance of the comammox-anammox MABR (A, C) and AOA-anammox MABR (B, D). Metabolite concentrations (A, B) and reactor performance (C, D) were monitored throughout the reactor experiments. Reactor performance was based on total inorganic nitrogen (TIN) and total ammonium removal.

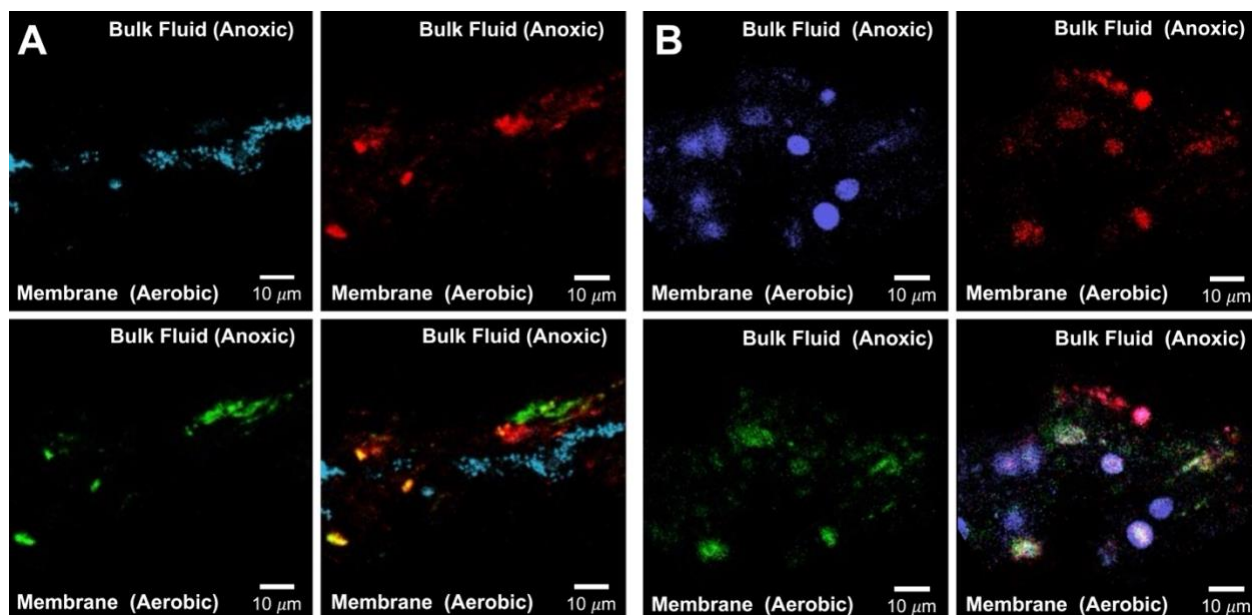


Figure 4.2. Spatial distribution of the nitrifying community.

FISH microscopy of membrane biofilms from the (A) comammox-anammox MABR and (B) AOA-anammox MABR at the end of operation. Probes targeting anammox (Cy 3, red), AOB (Cy 5, blue-right), AOA (Cy 5, purple-left), and *Nitrospira* (Fluorescein, green) were used. The bulk fluid side of the biofilm (top) was anoxic, while the membrane side (bottom) was supplied with air to maintain an aerobic environment.

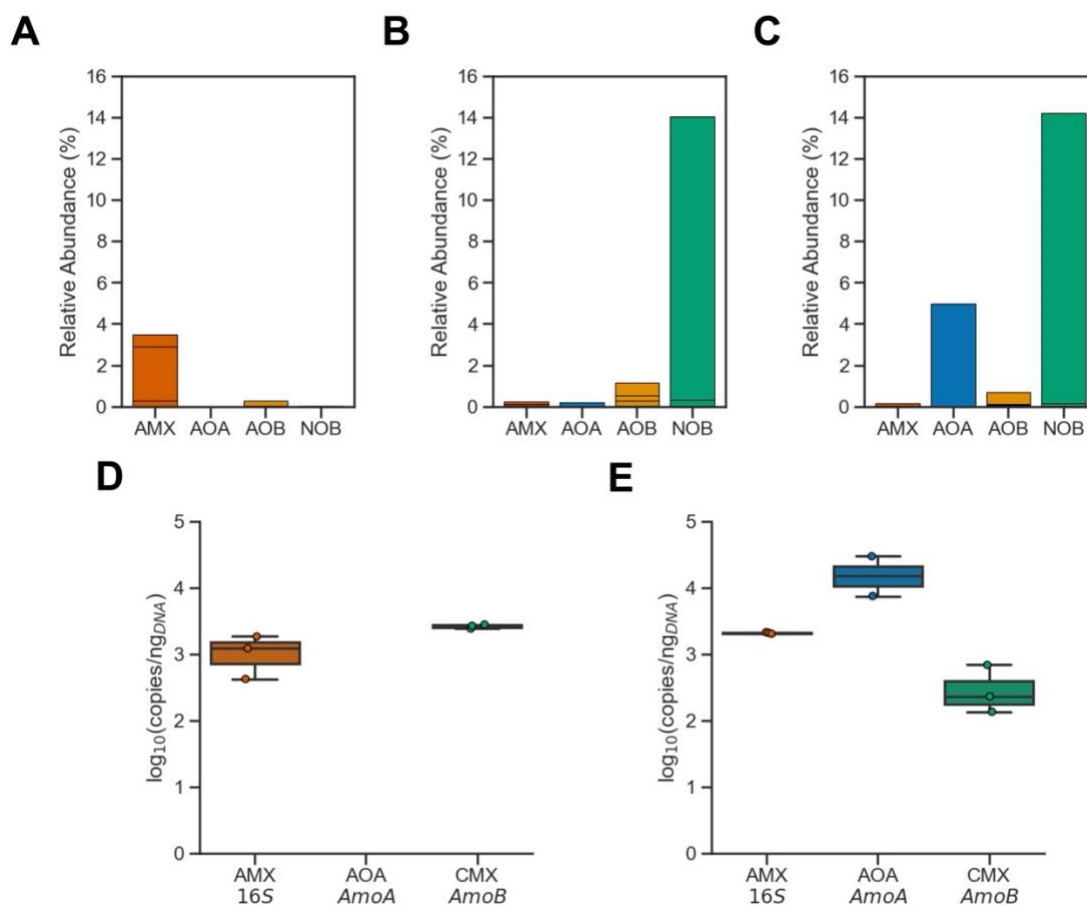


Figure 4.3. Microbial community analysis of the nitrifying population.

The relative abundances of the nitrifying populations in the anammox granular sludge inoculum (3A; n = 1), comammox-anammox MABR (3B; n=1), and the AOA-anammox MABR (3C; n = 1). The relative abundance of nitrifying zero-radius OTUs were detected with by 16S rRNA amplicon sequencing. The zOTUs comprising each nitrifying lineage have been clustered by family with individual zOTUs represented by bars within the nitrifying lineage (3A-B). The absolute abundance of anammox, AOA, and comammox in the comammox-anammox MABR (3D; n = 3) and AOA-anammox MABR (3E; n = 2-3) were measured by gene copies using qPCR primers targeting specific nitrifying populations after reactor termination. AOA *amoA* primers were not used for AOA quantification in the comammox-anammox MABR.

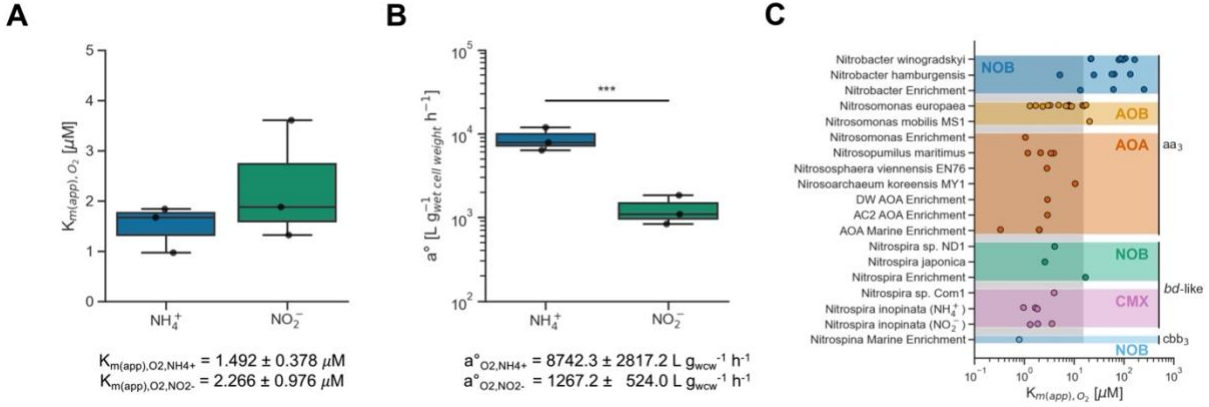


Figure 4.4. Ammonia and nitrite dependent oxygen uptake kinetics of *N. inopinata* measured under oxygen-limited conditions.

Ammonia-dependent (blue) and nitrite-dependent (green) oxygen affinity (A) and specific oxygen affinity (B) of *N. inopinata*. $K_{m(app)}$ for O_2 of *N. inopinata* (purple), *Nitrobacter* NOB (dark blue), AOB (yellow), AOA (orange), *Nitrospira* NOB (green), and *Nitrospira* NOB (light blue) (C). Boxplots depict the 25–75% quantile range, with the center line depicting the median (50% quantile); whiskers encompass data points within 1.5x the interquartile range. Data of each measured rate are represented as individual points. *N. inopinata* has a significantly higher ammonia-dependent specific oxygen affinity as indicated with stars (ANOVA p-value = 0.00264).

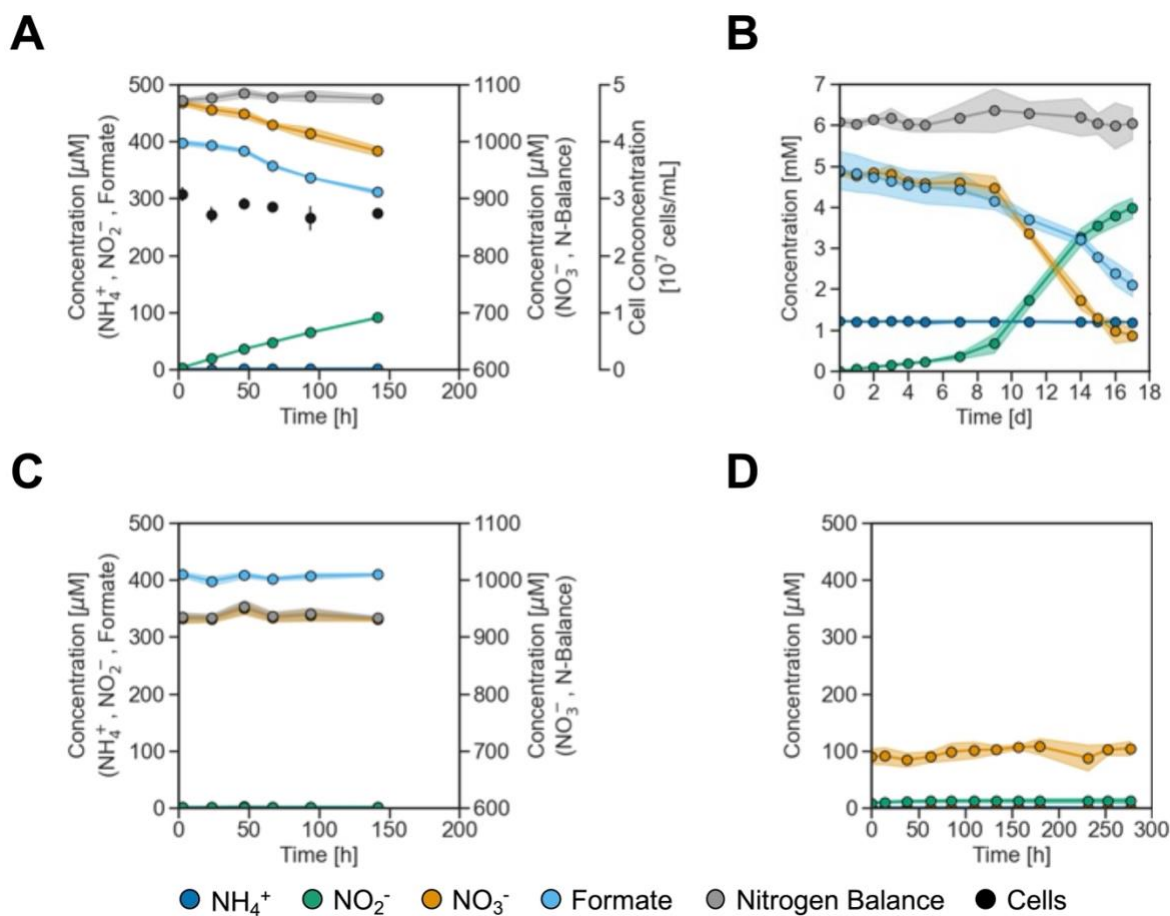


Figure 4.5. Anaerobic oxidation of formate coupled to the reduction of nitrate to nitrite by the complete ammonia oxidizing bacteria *N. inopinata*.

Shaded regions and error bars indicate standard deviation of biological replicates. Experiments were conducted with (5A) a fully grown comammox culture with formate and nitrate, (5B) a 10% comammox subculture with formate, nitrate and ammonium, (5C) a fully grown comammox culture with nitrate, and (5D) an autoclaved comammox culture with formate and nitrate. All experiments were conducted in 240mL serum bottles with a 1.55 bar N_2 headspace supplemented with sodium bicarbonate and grown at 37 °C.

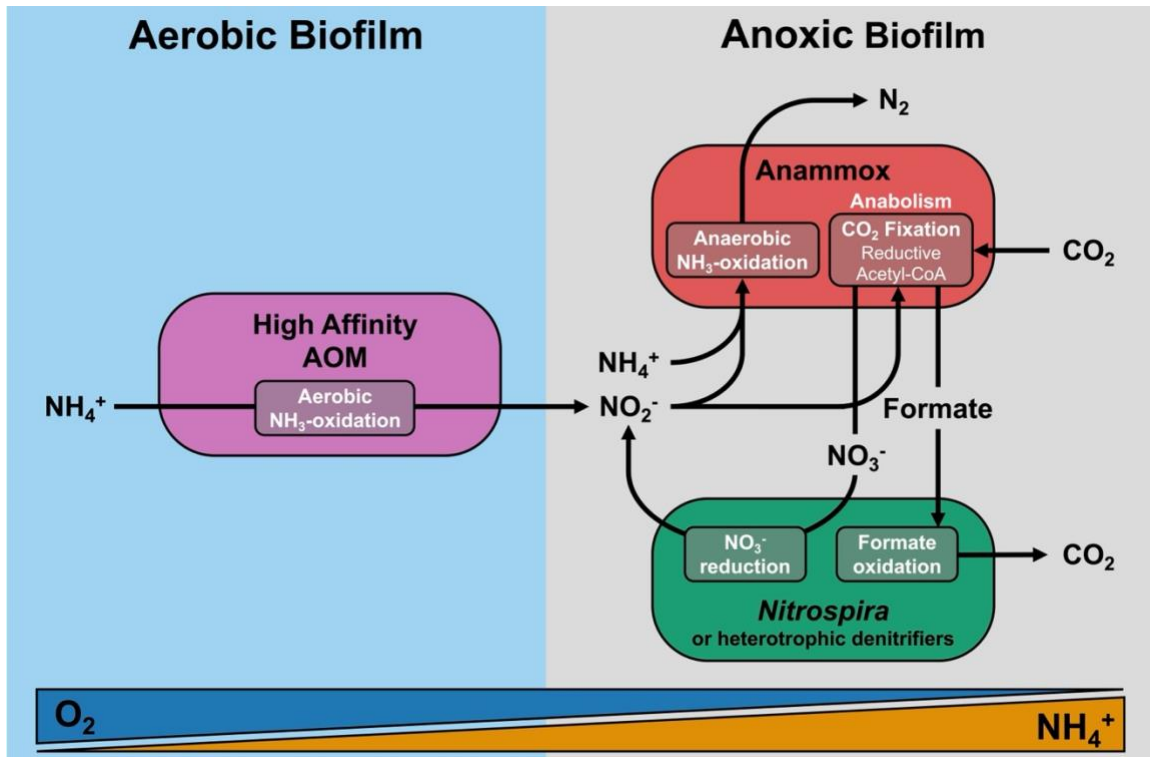


Figure 4.6. Hypothesized co-operation between high affinity AOM, *Nitrospira*, and anammox.

High affinity AOM aerobically oxidize ammonia to NO_2^- in the aerobic biofilm region. Anammox in the anoxic biofilm region utilize the produced NO_2^- and supplied NH_4^+ to catabolically produce N_2 via anaerobic ammonia-oxidation. Anammox also utilizes carbon dioxide (CO_2) and NO_2^- to produce NO_3^- and formate ($CHOO^-$) via the reductive acetyl-CoA pathway. *Nitrospira* or heterotrophic denitrifiers can then anaerobically reduce NO_3^- to NO_2^- to enable near-complete Nitrogen removal.

Appendix A. Supplemental Material

The following document is intended to support the results presented in the main manuscript.

Supplemental Methods

- S1. *Laboratory strains*
- S2. *Cultivation of ammonia-oxidizing microorganisms*
- S3. *16S rRNA microbial community analysis*
- S4. *qPCR and custom amoA primers*
- S5. *Fluorescence in-situ hybridization*
- S6. *Cell counts with Guava easyCyte cytometer*

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- Figure S4.2. Live/dead staining microscopy of AOA-anammox MABR cross-section
- Figure S4.3. Phylum-level 16S rRNA relative abundance of MABR inoculum and biofilm swabs after operation ceased
- Figure S4.4. Order-level 16S rRNA relative abundance of MABR inoculum and biofilm swabs after operation ceased
- Figure S4.5. Instantaneous measurement of oxygen uptake from microrespirometry used to determine the oxygen affinity of *N. inopinata*
- Figure S4.6. Oxygen oxidation kinetics of *N. inopinata*

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- Table S4.4. Influent and effluent COD measurements from both reactors.
- Table S4.5. Theoretical model to determine if the formate COD required completely remove nitrate via formate-dependent nitrate reduction by *Nitrospira* could have been supplied via endogenous decay.
- Table S4.6. Constants used to calculate the percent COD deficit (Tables S7 & S8) using the theoretical model (Tables S5)
- Table S4.7. Comparison of theoretical formate required to COD supplied via endogenous decay reveals a COD deficit for nitrate reduction in the AOA-anammox MABR
- Table S4.8. Comparison of theoretical formate required to COD supplied via endogenous decay reveals a COD deficit for nitrate reduction in the CMX-anammox MABR

Supplemental methods

S1. Laboratory strains

The complete ammonia-oxidizing bacteria *Nitrospira inopinata* strain ENR4 was kindly provided to the University of Washington by Dr. Holger Daims (University of Vienna). The ammonia-oxidizing archaea *Nitrososphaera viennensis* strain EN76 (DSM 26422) was purchased from the German Type and Culture collection (DSMZ, Braunschweig, Germany). Ammonia oxidizing archaea strains originating from shoreline sediment of Lake Acton (AC2) and Lake Delaware (DW1) were provided by Annette Bollmann (Miami University).

S2. Cultivation of ammonia-oxidizing microorganism (AOM) strains

All media pH were 7.6–7.8. Cultures were routinely cultivated in 250 ml acid washed bottles containing 100 ml medium supplemented with ammonia. Cultures were incubated in the dark to avoid light inhibition. All AOA strains were supplemented with 100 μ M sodium pyruvate as a scavenger for hydrogen peroxide (H_2O_2) or other reactive oxygen species. *N. inopinata*, *N. viennensis*, AC2, and DW1 were incubated at 37 °C, 37 °C, 30 °C, and 30 °C, respectively. Growth was monitored by ammonia consumption as well as nitrite or nitrate accumulation. When mid- to late-exponential phase was reached, a 0.2–2% inoculum was transferred to fresh media. Culture purity was confirmed monthly by microscopic evaluation and absence of growth on R2A agar. Culture purity was confirmed every 6 months with full length 16S rRNA sanger sequencing.

S3. Cultivation of ammonia-oxidizing microorganism (AOM) strains

The V4-V5 region of the 16S rRNA gene was amplified by PCR using primer 515F-Y (5'-GTGYCAGCMGCCGCGGTAA) and 926R (5'-CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016) by mixing the following ingredients to a final volume of 25 μ L : 12.5 μ L of LongAmp® Taq 2x master mix (New England Biolabs, Ipswich, MA, USA), 1 μ L of 10 μ M stocks of 515F and 926R primers with partial Illumina adapter sequences, 2 μ L of DNA sample, and 8.5 μ L of nuclease-free water (Parada et al., 2016). Samples were amplified according to the following process: denaturation at 94 °C for 30 s, 50 °C for 45 s, and 65 °C for 30 s for 30 cycles, with a final extension at 65 °C for 10 min. Agarose gel electrophoresis was performed to confirm the size and purity of PCR products. Paired-end reads were merged using fastq_mergepairs, primer sequences were removed using fastx_truncate, and reads were quality filtered using fastq_filter with the fastq_maxee option set to 1.0 to remove reads with greater than one expected error. Unique reads and their abundances were computed using fastx_uniques followed by denoising and chimera removal using the unoise3 command (R. C. Edgar, 2016a). Reads were mapped to the resulting zero-radius OTUs using the otutab command. The taxonomy of the zero-radius OTUs (zOTU) was assigned using the syntax command to search against the RDP 16S database v18 (R. Edgar, 2016; Edgar, 2010; R. C. Edgar, 2016b; Maidak et al., 1997).

S4. qPCR and custom AOA amoA primers

All qPCR reactions were performed in triplicate with a LightCycler® system (Roche, Rotkreuz, Switzerland) using the primers and reaction conditions listed in the supplemental material (Table S1). Each reaction contained 10 μ L Master Mix (DreamTaq Green PCR Master Mix, Thermo Fisher Scientific, Waltham, MA, USA), 6 μ L nuclease free water, 2 μ L sample DNA, 1 μ L of each

PCR primers (10 μ M) added to a final volume of 20 μ L per reaction. Denaturation was performed at 95°C for 5 min followed by 45 cycles of 15 s denaturation at 95°C, 15 sec annealing at target gene temperature (Table S1), and 13 sec extensions at 72°C. The specificity of the amplification was tested with melting curve analysis at the end of qPCR. Calibration curves for each target gene were constructed by qPCR with serial dilution of DNA standards in the concentration range of 10^1 - 10^7 gene copy/reaction. Amplification efficiencies of the qPCR were in the range of 87%-113% with R^2 higher than 0.98%. qPCR was run in duplicate for each reaction.

A standard for the anammox 16S rRNA gene was constructed by cloning the amplicon for the 808 to 1066 gene region using 808F(Hamersley et al., 2007) and 1066R(Tsushima et al., 2007) into the pCRTM4-TOPO[®] TA vector (TOPO[®] TA Cloning[®] Kit, InvitrogenTM, Carlsbad, CA USA) and validating the construct by sequencing (Eurofins Scientific, Luxembourg). For quantification of the comammox amoB gene, a plasmid containing the targeted sequence in *Nitrospira inopinata* was used as a standard.(Daims et al., 2015)

Primers to identify the *N. viennensis* AmoA gene were selected by identifying conserved regions in the *amoA* gene after alignment of sequences with two other AOA strains, AC2 and DW1. Sequence regions that contained at least two mismatches from the other two *amoA* sequences were selected for primer sequences (Table S2). For each of the three *amoA* sequences, the complete gene sequence was synthesized and cloned within the pUC18 plasmid (BIOBASIC[®]). qPCR tests with all three plasmids showed specific amplification of the *N. viennensis amoA* gene by the primers. For quantification the pUC18-*N.viennensis*, an *amoA* construct was used as a standard. For quantification of the comammox amoB gene, a plasmid containing the targeted sequence in *Nitrospira inopinata* was used as a standard (Cotto et al., 2020). A standard for the anammox 16S

rRNA gene was constructed by cloning the amplicon for the 808 to 1066 gene region using 808F(Hamersley et al., 2007) and 1066R(Tsushima et al., 2007) into the pCR™4-TOPO® TA vector (TOPO® TA Cloning® Kit, Invitrogen™, Carlsbad, CA USA) and validating the construct by sequencing (Eurofins Scientific, Luxembourg).

S5. Fluorescence in situ hybridization (FISH)

Membrane fiber samples for fluorescence in situ hybridization (FISH) analysis were fixed and hybridized according to previously described procedures (Nielsen et al., 2009) with the following modifications. The membrane samples were washed in phosphate buffered solution pH 7.0 (PBS) and incubated in 4% paraformaldehyde solution for 2 hours before being stored in a mixture of 98% ethanol and PBS at -20°C. Membrane samples were dried (46°C for 10 minutes), sequentially dehydrated in 50, 80, 95% ethanol, air dried, and stored at -20°C. Hybridization was done with 35% formamide hybridization buffer and 80 µM NaCl washing buffer. Oligonucleotide probes (Eurofins, Louisville, KY) are summarized in the supplementary material (Table S2) and were applied in equimolar concentration to membranes and hybridized at 46°C for 2 hours. A confocal laser scanning microscope (Zeiss LSM700, Carl Zeiss, Germany) equipped with an Axio 503 mono camera was used to visualize hybridized membrane cross-sections with 488, 543, and 633 nm lasers. All images were collected and processed in the Zen Blue software.

S6. Cell counts with Guava easyCyte cytometer

Cell concentrations were determined via cell counts with a Guava easyCyte Flow Cytometry system as described previously(Feng et al., 2014). Briefly, cells were diluted in FWM to a concentration of approximately 10^5 cells per mL (990µL final volume) and stained with 10µL

SYBR Green solution containing 1 μ L SYBR Green I Nucleic Acid Gel Stain (10,000 X stock, Thermo Fisher Scientific, Waltham, MA, USA) and 29 μ L FWM. Stained cells were incubated for 30 minutes in the dark at 37 °C. Fluorescence intensities at 535nm (green emission) and 635nm (red emission) were measured for each sample using a Guava easyCyte Flow Cytometry system. After counting 5000 total events, cell concentrations were determined by counting the number of events on a forward scatter (FSC) - green fluorescent intensity scatterplot occurring within a predefined gate for AOM. Blank measurements were conducted with FWM.

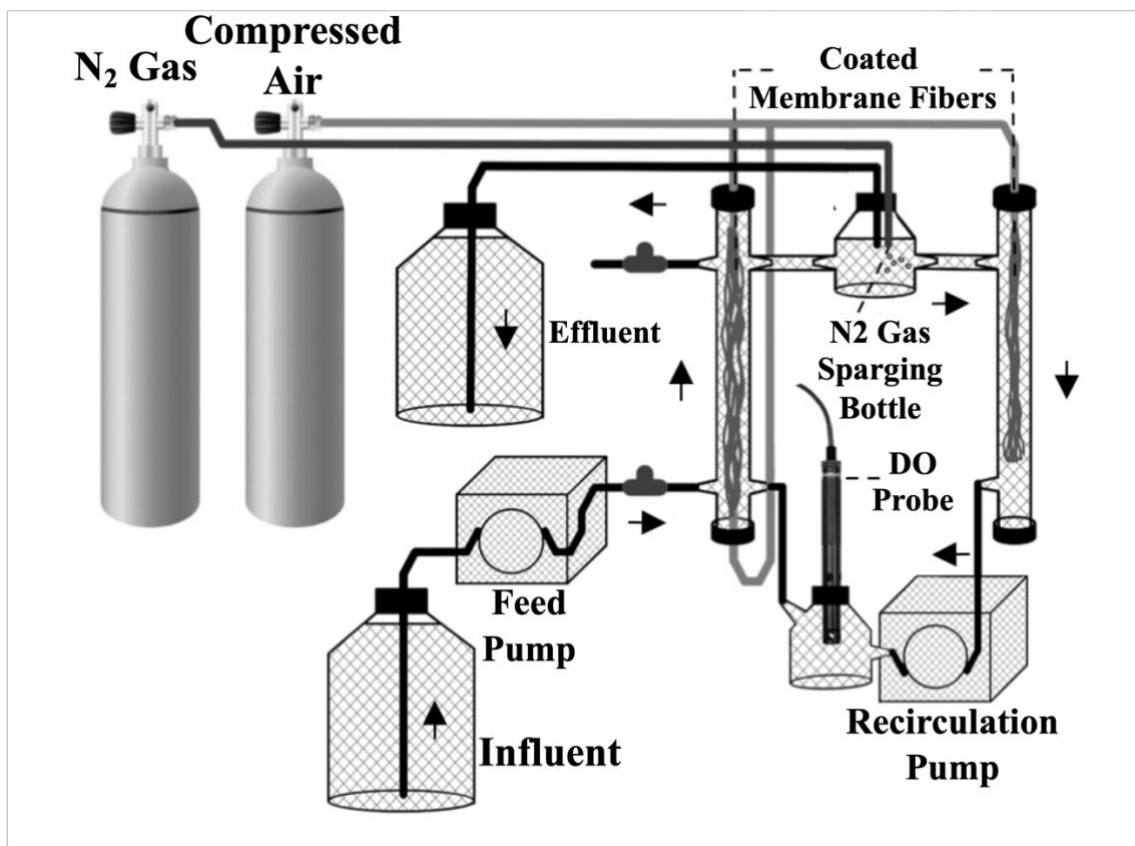


Figure S4.1. Schematic representation of the MABR set-up.

Air was supplied to the hallow-fiber membranes and N₂ gas was directly added to bulk liquid.

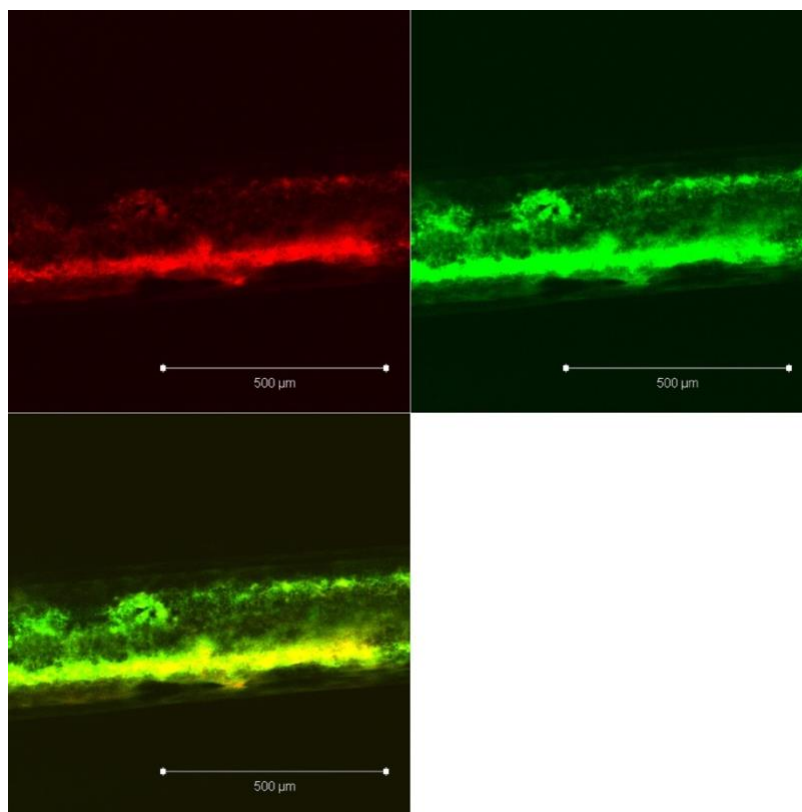


Figure S4.2. Live/dead stain performed on a segment of the membrane fibers present in the AOA.

Dead cells are stained with both propidium iodide (red) and SYTO 9 (green). Live cells were only stained with SYTO 9 (green). Propidium iodide only stains dead cells, while Syto9 permeates live cells and competitively binds DNA with propidium iodide. The areas in the overlay containing more green than red or only green suggests the MABR biofilm contained a mixture of live and dead cells.

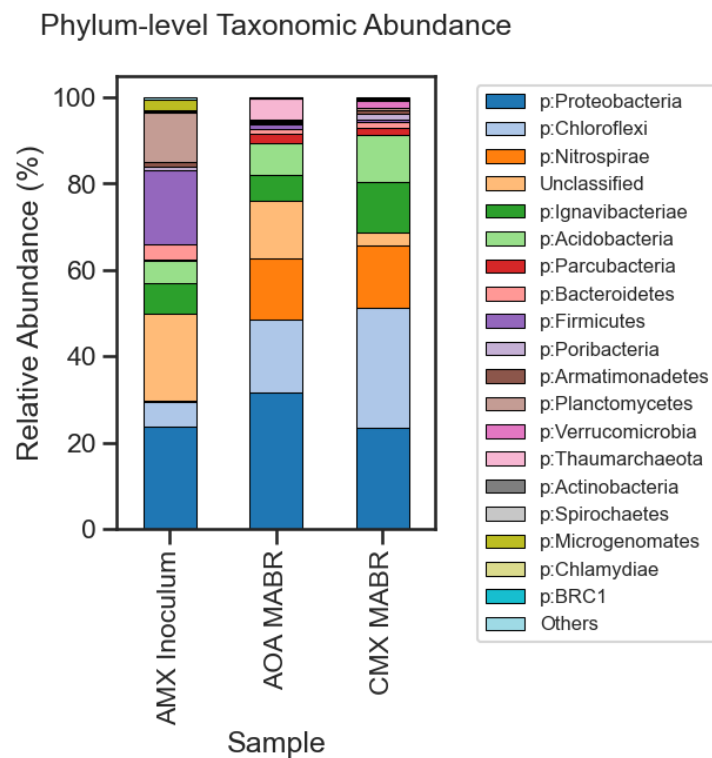


Figure S4.3. Phylum-level relative abundance

Phylum-level relative abundance of zOTUs detected by 16S sequencing from the anammox inoculum along with endpoint samples from the AOA and CMX MABR.

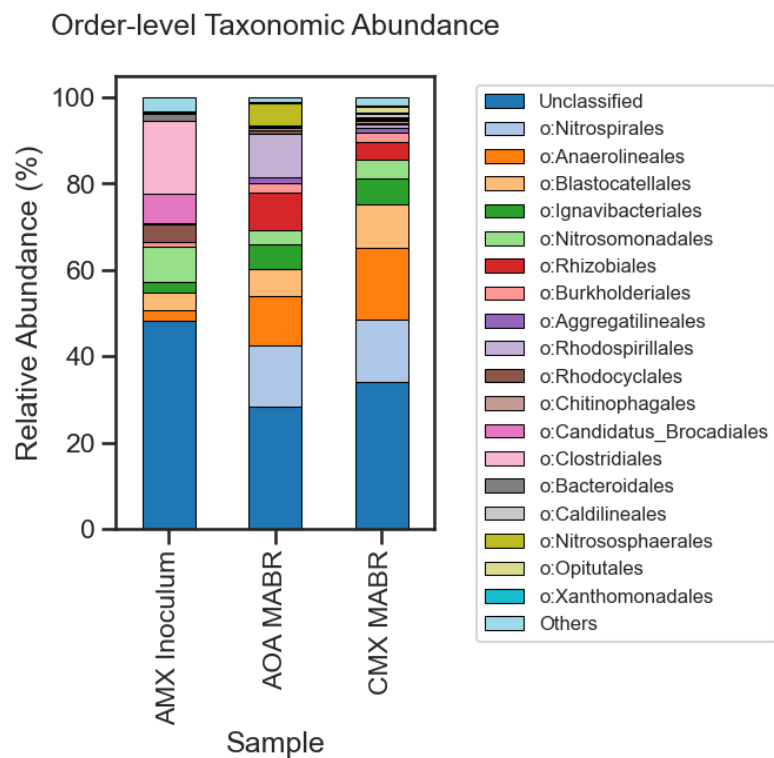


Figure S4.4. The order-level relative abundance.

The order-level relative abundance of zOTUs detected by 16S sequencing from the anammox inoculum along with endpoint samples from the AOA and CMX MABR.

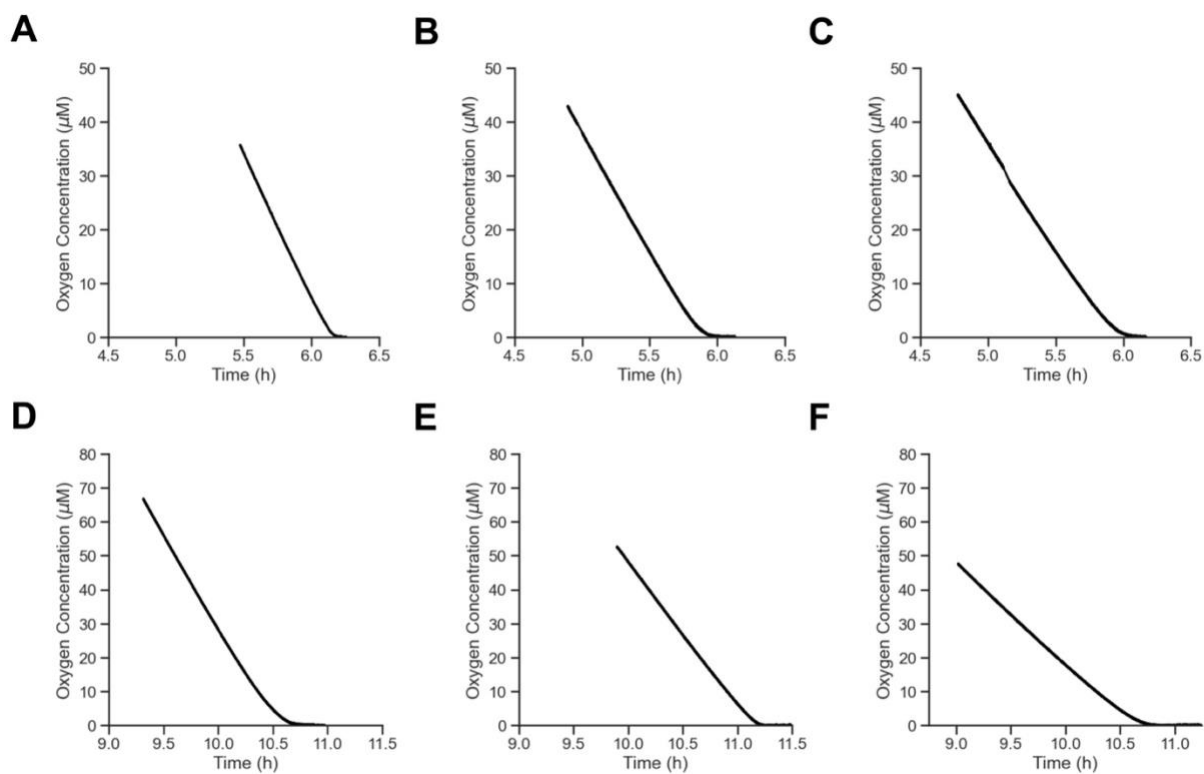


Figure S4.5. Oxygen consumption profiles by *N. inopinata* (1 minute time averaged).

Oxygen consumption was measured by microrespirometry (MR) upon a 1mM substrate addition of ammonium (A-C) or nitrite (D-E).

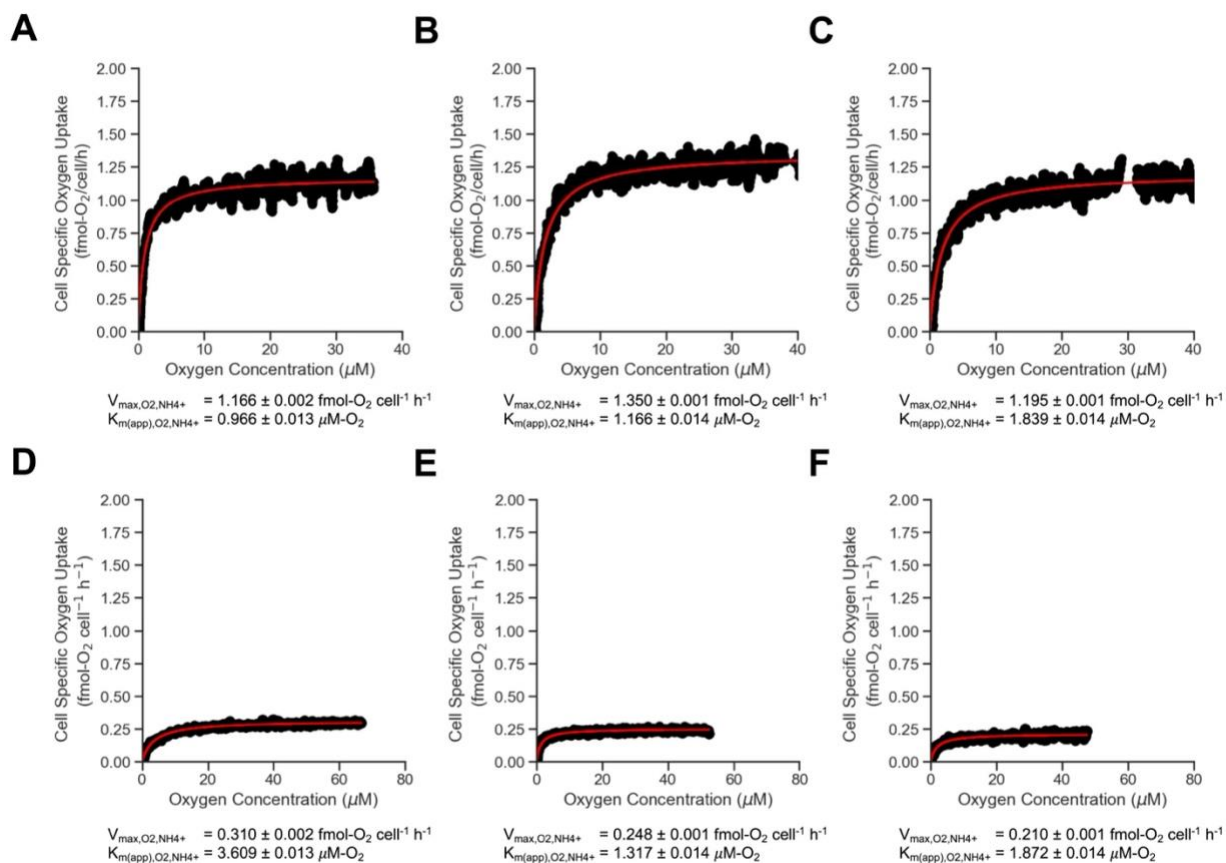


Figure S4.6. Oxygen oxidation kinetics of *N. inopinata*.

Each graph represents a single 1 minute time averaged trace of experimental replicates (black). Oxygen uptake rates were determined by microrespirometry (MR) upon a 1mM substrate addition of ammonium (A-C) or nitrite (D-E). Kinetic constants were obtained by fitting a Michaelis-Menten equation (red line) to total cell specific oxygen uptake rates.

Supplemental Tables

Table S4-1. Influent Medium Composition

<i>Constituent</i>	<i>Concentration</i>
NH ₄ Cl	1 mM
NaHCO ₃	2 mM
NaCl	10 mM
KCl	1 mM
CaCl ₂ • 2 H ₂ O	1 mM
MgSO ₄ • 7 H ₂ O	0.2 mM
HEPES	4 mM
KH ₂ PO ₄	2 µM
Ferric Sodium EDTA	7.5 µM
Trace Element Solution (Könneke et al., 2005)	1 mL/L

Table S4-2. qPCR Primers and conditions

<i>Name</i>	<i>5'-3' Sequence</i>	<i>Annealing Temp/# Cycles</i>	<i>Citation</i>
cmx amoB-148F	TGG TAY GAY ACN GAA TGG G	52 °C / 40	(Cotto et al., 2020)
cmx amoB 485R	CCCGTG ATR TCC ATC CA		
AMX-808-F	ARC YGT AAA CGA TGG GCA CTA A	58 °C /40	Hamersley et al., 2007)
AMX-1040-R	CAG CCA TGC AAC ACC TGT RAT A		
nvs amoA-F	AGTATTCTCCGTGTCACAGT	63 °C / 45	This study
nvs amoA-R	GTCCCTGACGAGCAAC		

Table S4-3. FISH probes used in this study

<i>Organism</i>	<i>Name</i>	<i>Sequence (5'-3')</i>	<i>Probe</i>	<i>Citation</i>
Nitrospira	Ntsp662	GGA ATT CCG CGC TCC TCT	Fluorescein	(Daims et al., 2001)
General Archaea	ARC915	GTG CTC CCC CGC CAA TTC CT	Cyanine 5	(Stahl and Amann, 1991)
Anammox	AMX368	CCT TTC GGG CAT TGC GAA	Cyanine 3	(Schmid et al., 2003)
AOB	NSO190	CGA TCC CCT GCT TTT CTC C	Cyanine 5	(Mobarry et al., 1996)
AOB	NSO1225	CGC GAT TGT ATT ACG TGT GA	Cyanine 5	(Mobarry et al., 1996)

Table S4-4. Influent and effluent COD measurements.

<i>Reactor</i>	<i>Influent (mgCOD/L)</i>	<i>Effluent (mgCOD/L)</i>
AOA MABR	1010	1010
CMX MABR	1010	1010

Table S4-5. Theoretical nitrate reduction model.

Theoretical model to determine if the formate COD required completely remove nitrate via formate-dependent nitrate reduction by *Nitrospira* could have been supplied via endogenous decay.

<i>Description</i>	<i>Variable Name</i>	<i>Calculation</i>
Days	D_i	None
TA influent, mM*	TA_{in}	Measured Value
TA effluent, mM	TA_{out}	Measured Value
TON** effluent, mM	TON_{out}	Measured Value
Nitrogen Removed, mmol†	N_{rem}	$\left[TA_{in,avg} - (NH_{3,out} + TON_{out})_{avg} \right] \times (D_i - D_{i-1}) \times Q$
Anammox Produced NO_3^- , mmol	$NO_{3,AMX}$	$N_{rem} \times S_{NO3,AMX}$
Theoretical Formate Required, gCOD	$COD_{Req,NOB}$	$NO_{3,AMX} \times S_{NO3,NOB} \times COD_{HCOO^-}$
Anammox Growth, mgVSS	$X_{G,AMX}$	$N_{rem} \times S_{NH3,AMX} \times Y_{AMX} \times 14 \frac{mgN}{mmol}$
AOA/CMX Growth, mgVSS	$X_{G,AOA}$	$N_{rem} \times (1 - S_{NH3,AMX}) \times Y_{AOA} \times 14 \frac{mgN}{mmol}$
Total Anammox, gVSS	$X_{Tot,AMX}$	$X_{Tot,AMX,i-1} + X_{G,AMX,i} - X_{D,AMX,i}$
Total AOA/CMX, gVSS	$X_{Tot,AOA}$	$X_{Tot,AOA,i-1} + X_{G,AOA,i} - X_{D,AOA,i}$
Anammox Decay, gVSS	$X_{D,AMX}$	$X_{Tot,AMX} \times b_{AMX} \times (D_i - D_{i-1})$
AOA/CMX Decay, gVSS	$X_{D,AOA}$	$X_{Tot,AOA} \times b_{AOA} \times (D_i - D_{i-1})$
COD Produced via decay, gCOD	COD_{Decay}	$(X_{Tot,AOA} + X_{Tot,AMX}) \times n$
% COD Deficit		$\frac{COD_{Decay} - COD_{Req,NOB}}{COD_{Req,NOB}} \times 100\%$
* Total Ammonium ($NH_3 + NH_4^+$) ** TON = Total oxidized nitrogen, Nitrite + Nitrate † avg indicates the average of these values at Day = D_i and Day = D_{i-1}		

Table S4-6. Theoretical nitrate reduction model constants.

Constants used to calculate the percent COD deficit (Tables S7 & S8)

<i>Description</i>	<i>Value</i>	<i>Variable</i>	<i>Units</i>	<i>Source</i>
Counter-Diffusing Mesocosm Flowrate	4.5	Q	ml/h	This Study
	0.108	Q	L/d	
Anammox Stoichiometric Ratios	0.13	$S_{NO3,AMX}$	mole NO_3^- /mole N consumed	(Straka et al., 2019)
	0.43	$S_{NH3,AMX}$	mole NH_3 /mole N consumed	
Nitrospira Formate Stoichiometry	0.9	$S_{NO3,NOB}$	mol NO_3^- : mol $HCOO^-$	This study
COD of Formate	8	COD_{HCOO^-}	g O_2 : mol formate	Calculated
Anammox Decay Rate	0.022	b_{AMX}	gVSS/gVSS.d	(Hellings et al., 1999)
AOA Decay Rate	0.05	b_{AOA}		
Anammox Yield	0.07	Y_{AMX}	gVSS/g NH_3	(Hao et al., 2002)
AOA Yield	0.12	Y_{AOA}	gVSS/gN	
COD to Biomass Conversion Rate	1.4	n	gCOD/gVSS	(Lotti et al., 2012)

Day	TON effluent mM	TA effluent mM	TA influent mM	Nitrogen Removed mmol	Anammox Produced NO ₃ ⁻ mmol	Theoretical Formate Required mmol	Anammox Growth mgVSS	AOA Growth mgVSS	Total Anammox gVSS	Total AOA gVSS	Anammox Decay gVSS	AOA Decay gVSS	COD Produced via decay mgCOD	% COD Deficit
0	0.03	0.19	0.83											
7	0.52	0.02	0.97	0.39	0.05	0.37	0.16	0.37	0.16	0.37	0.03	0.13	0.16	-57%
10	0.20	0.17	0.95	0.16	0.02	0.15	0.07	0.16	0.07	0.16	0.00	0.02	0.03	-82%
12	0.42	0.00	0.83	0.11	0.01	0.10	0.04	0.10	0.04	0.10	0.00	0.01	0.01	-88%
14	0.18	0.00	0.91	0.12	0.02	0.11	0.05	0.12	0.05	0.12	0.00	0.01	0.01	-88%
17	0.16	0.00	0.92	0.24	0.03	0.23	0.10	0.23	0.10	0.23	0.01	0.03	0.04	-82%
19	0.14	0.00	0.90	0.16	0.02	0.15	0.07	0.16	0.07	0.16	0.00	0.02	0.02	-88%
24	0.10	0.00	1.05	0.46	0.06	0.43	0.19	0.44	0.19	0.44	0.02	0.11	0.13	-69%
26	0.12	0.01	1.01	0.20	0.03	0.19	0.08	0.19	0.08	0.19	0.00	0.02	0.02	-88%
28	0.17	0.01	0.85	0.17	0.02	0.16	0.07	0.16	0.07	0.16	0.00	0.02	0.02	-88%
31	0.08	0.01	0.99	0.26	0.03	0.24	0.11	0.24	0.11	0.24	0.01	0.04	0.04	-82%
35	0.13	0.02	0.93	0.37	0.05	0.34	0.15	0.35	0.15	0.35	0.01	0.07	0.08	-76%
38	0.15	0.00	0.93	0.25	0.03	0.24	0.11	0.24	0.11	0.24	0.01	0.04	0.04	-82%
40	0.12	0.00	0.93	0.17	0.02	0.16	0.07	0.16	0.07	0.16	0.00	0.02	0.02	-88%
45	0.09	0.00	1.01	0.47	0.06	0.44	0.20	0.45	0.20	0.45	0.02	0.11	0.13	-69%
49	0.10	0.00	1.04	0.40	0.05	0.38	0.17	0.38	0.17	0.38	0.01	0.08	0.09	-76%
52	0.12	0.00	1.13	0.32	0.04	0.30	0.13	0.30	0.13	0.30	0.01	0.05	0.05	-82%
56	0.02	0.01	0.98	0.42	0.06	0.40	0.18	0.41	0.18	0.41	0.02	0.08	0.10	-76%
59	0.11	0.01	1.17	0.33	0.04	0.30	0.14	0.31	0.14	0.31	0.01	0.05	0.06	-82%
61	0.15	0.00	1.19	0.23	0.03	0.21	0.10	0.22	0.10	0.22	0.00	0.02	0.03	-88%
63	0.28	0.01	1.20	0.21	0.03	0.20	0.09	0.20	0.09	0.20	0.00	0.02	0.02	-88%
66	0.09	0.01	1.21	0.33	0.04	0.30	0.14	0.31	0.14	0.31	0.01	0.05	0.06	-82%
68	0.13	0.00	1.22	0.24	0.03	0.22	0.10	0.23	0.10	0.23	0.00	0.02	0.03	-88%
70	0.16	0.01	1.36	0.25	0.03	0.23	0.10	0.24	0.10	0.24	0.00	0.02	0.03	-88%
75	0.02	0.00	1.00	0.58	0.08	0.55	0.25	0.56	0.25	0.56	0.03	0.14	0.17	-69%
77	0.04	0.00	1.03	0.21	0.03	0.20	0.09	0.20	0.09	0.20	0.00	0.02	0.02	-88%
80	0.13	0.00	0.96	0.29	0.04	0.28	0.12	0.28	0.12	0.28	0.01	0.04	0.05	-82%
82	0.15	0.02	1.02	0.18	0.02	0.17	0.08	0.17	0.08	0.17	0.00	0.02	0.02	-88%
87	0.01	0.07	1.06	0.50	0.06	0.47	0.21	0.48	0.21	0.48	0.02	0.12	0.14	-69%
0	0.07	0.07	0.90	0.28	0.04	0.26	0.12	0.27	0.12	0.27	0.01	0.04	0.05	-82%

*TON = Total Oxidized Nitrogen (Nitrite + Nitrate); **Negative % COD deficit suggests that there is not enough carbon produced by endogenous decay to support denitrification of anammox produced nitrate

Day	TON* effluent, mgN/L	NH ₄ effluent, mgN/L	NH ₄ influent, mgN/L	Nitrogen Removed, mmol	Anammox Produced NO ₃ ⁻ , mmol	Theoretical Formate Required, mmol	Anammox Growth, mgVSS	AOA Growth, mgVSS	Total Anammox, gVSS	Total AOA, gVSS	Anammox Decay, gVSS	AOA Decay, gVSS	COD Produced via decay, mgCOD	% COD Deficit **
0	11.472	0.1498	11.472											
2	8.6764	0.2547	11.858	0.30	0.04	0.28	0.13	0.29	0.13	0.29	0.01	0.03	0.05	-83%
9	0.0156	5.3342	13.8276	4.31	0.56	4.04	1.82	4.13	1.82	4.13	0.28	1.44	2.45	-39%
12	0.6932	3.0121	12.8409	2.85	0.37	2.67	1.20	2.73	1.20	2.73	0.08	0.41	0.69	-74%
14	1.1131	1.2844	13.4194	2.18	0.28	2.04	0.92	2.08	0.92	2.08	0.04	0.21	0.35	-83%
16	0.231	1.4024	13.4467	2.47	0.32	2.31	1.04	2.36	1.04	2.36	0.05	0.24	0.40	-83%
19	0.499	1.3381	13.9819	3.88	0.50	3.63	1.64	3.72	1.64	3.72	0.11	0.56	0.94	-74%
21	1.3394	1.3877	13.0692	2.43	0.32	2.27	1.02	2.33	1.02	2.33	0.05	0.23	0.39	-83%
26	1.8794	1.2557	15.3305	6.09	0.79	5.70	2.56	5.83	2.56	5.83	0.28	1.46	2.47	-57%
28	0.985	3.0194	15.1101	2.52	0.33	2.36	1.06	2.41	1.06	2.41	0.05	0.24	0.41	-83%
30	0.389	3.2925	14.6558	2.38	0.31	2.23	1.00	2.28	1.00	2.28	0.04	0.23	0.39	-83%
33	0.3066	1.1856	14.5477	3.89	0.51	3.64	1.64	3.73	1.64	3.73	0.11	0.56	0.95	-74%
37	0.2177	1.3011	13.7135	5.45	0.71	5.10	2.30	5.22	2.30	5.22	0.20	1.04	1.77	-65%
40	0.4293	2.2433	13.037	3.65	0.48	3.42	1.54	3.50	1.54	3.50	0.10	0.52	0.89	-74%
42	0.0306	2.1596	13.037	2.29	0.30	2.14	0.97	2.19	0.97	2.19	0.04	0.22	0.37	-83%
47	0.0248	1.2548	15.0208	6.64	0.86	6.21	2.80	6.36	2.80	6.36	0.31	1.59	2.69	-57%
51	0.1289	1.6832	15.2486	5.87	0.76	5.49	2.47	5.62	2.47	5.62	0.22	1.12	1.91	-65%
54	0.4947	1.1861	16.3342	4.55	0.59	4.26	1.92	4.36	1.92	4.36	0.13	0.65	1.11	-74%
58	0.0685	0.2161	15.1323	6.37	0.83	5.96	2.69	6.10	2.69	6.10	0.24	1.22	2.07	-65%
61	0.183	1.7658	16.6971	4.79	0.62	4.49	2.02	4.59	2.02	4.59	0.13	0.69	1.17	-74%
63	1.6117	2.1878	15.3962	2.85	0.37	2.66	1.20	2.72	1.20	2.72	0.05	0.27	0.46	-83%
65	1.424	1.2652	17.0568	2.80	0.36	2.62	1.18	2.69	1.18	2.69	0.05	0.27	0.46	-83%
68	0.2179	1.6855	17.7809	4.90	0.64	4.59	2.06	4.69	2.06	4.69	0.14	0.70	1.19	-74%
70	0.219	1.3808	17.1087	3.39	0.44	3.17	1.43	3.25	1.43	3.25	0.06	0.32	0.55	-83%
72	0.1646	1.6859	17.809	3.40	0.44	3.18	1.43	3.25	1.43	3.25	0.06	0.33	0.55	-83%
77	0.178	1.019	14.8755	8.00	1.04	7.49	3.37	7.66	3.37	7.66	0.37	1.92	3.25	-57%
79	2.352	0.5675	14.4665	2.72	0.35	2.55	1.15	2.61	1.15	2.61	0.05	0.26	0.44	-83%
82	2.7332	1.8697	15.7306	3.67	0.48	3.44	1.55	3.52	1.55	3.52	0.10	0.53	0.89	-74%
84	2.7372	0.2473	15.1031	2.51	0.33	2.35	1.06	2.40	1.06	2.40	0.05	0.24	0.41	-83%
89	0.0982	0.0829	14.9046	7.25	0.94	6.78	3.05	6.94	3.05	6.94	0.34	1.74	2.94	-57%
3	0.3253	1.0534	12.5525	5.59	0.73	5.24	2.36	5.36	2.36	5.36	0.21	1.07	1.82	-65%

Chapter 5.

Hydroxylamine-dependent nitrate reduction by the complete ammonia oxidizing bacteria *Nitrospira inopinata*

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Abstract

Genomic evidence predicts comammox *Nitrospira* can perform hydroxylamine-dependent nitrate reduction—an anaerobic nitrite production pathway coupling hydroxylamine oxidation with nitrate reduction. However, this pathway lacks experimental validation. We demonstrate the comammox isolate *Nitrospira inopinata* can perform hydroxylamine-dependent nitrate reduction under hydroxylamine-replete conditions to produce nitrite with concomitant ammonium and nitrous oxide (N₂O) production. All nitrification proteins were constitutively abundant compared to aerobic ammonia-oxidizing conditions, suggesting *N. inopinata* will rapidly respond to nutrient availability. However, ammonia oxidation was inhibited when *N. inopinata* underwent an anaerobic-to-aerobic transition. Stable isotope tracking identified the enzymatic origin of N₂O production by *N. inopinata*, which increasing hydroxylamine concentrations shifted product formation from nitrite towards N₂O with a constant relative contribution of biotic N₂O production. Together, these results offer mechanistic insight into an overlooked nitrogen cycling and greenhouse gas production pathways, highlighting the expanded role of comammox and hydroxylamine beyond complete nitrification.

Keywords – Comammox, hydroxylamine-dependent nitrate reduction, stable isotope tracing, hybrid nitrous oxide production, N-nitrosation, branched-chain amino acid catabolism

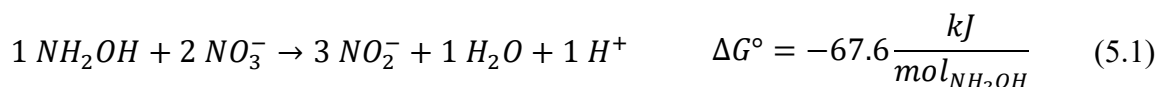
5.1 Introduction

Nitrification, the biological oxidation of ammonia to nitrate via nitrite, is a crucial pathway within the global nitrogen cycle. Anthropogenic activity has significantly perturbed nitrification contributing to increased nitrogen loss from agricultural soils, eutrophication, groundwater contamination, and nitrous oxide (N_2O) emissions. Classically, nitrification was thought to be a stepwise process where ammonia-oxidizing microorganisms (AOM) first oxidize ammonia (NH_3) to nitrite (NO_2^-) with nitrite-oxidizing bacteria (NOB) subsequently oxidizing NO_2^- to nitrate (NO_3^-) [1]. However, the discovery and isolation of a complete ammonia-oxidizing (comammox) bacteria challenged this strict division of labor [2–4].

To date, all described comammox organisms belong to the *Nitrospira* genus, known primarily for hosting diverse group of NOB [5–11], and specifically affiliate with sublineage II [2, 3, 12]. While *Nitrospira* are recognized for their contribution to nitrite oxidation, the known metabolic versatility within this genus has been expanding. Anaerobic nitrate reduction coupled to formate oxidation was recently demonstrated by *Nitrospira moscoviensis*, a sublineage II NOB [13]. Considering genome of *N. moscoviensis* does not contain the periplasmic nitrate reductase (*nap*) nor the membrane-bound nitrate reductase (*nar*), it was hypothesized that the observed nitrate reduction was catalyzed by the nitrite oxidoreductase (*nxr*) operating in the reverse direction [13], similar to *Nitrobacter* NOB [14]. Nitrate reduction by *Nitrospira* suggests their contribution to the nitrogen cycle is broader than expected.

All comammox *Nitrospira* encode the genetic inventory required to oxidize hydroxylamine to nitrite—hydroxylamine oxidoreductase (*haoAB*); cytochrome *c554* and *cm552*; and respiratory complex III (*Rieske/cytochrome b complex*)—and reduce nitrate to nitrite—*nxrABC* and

cytochrome *cycAB* [4]. Therefore, comammox *Nitrospira* may catalyze hydroxylamine-dependent nitrate reduction (Equation 5.1) , an anaerobic nitrite comproportionating pathway coupling hydroxylamine oxidation with nitrate reduction (Extended Figure 5.1). This thermodynamically favorable reaction would represent an unexpected link between hydroxylamine (NH₂OH), a reactive ammonia oxidation intermediate, and NO₃⁻ in an aerobic nitrifying lineage.



Hydroxylamine is also a precursor of N₂O production across all AOM lineages (Extended Figure 5.2) [15]. Ammonia-oxidizing bacteria (AOB) can aerobically convert NH₂OH and NO to N₂O via the *c* cytochrome P460 (*cytL*) or reduce NO₂⁻ to N₂O via nitric oxide reductase (NOR) upon the onset of hypoxia [4, 16, 17]. The ammonia-oxidizing archaea (AOA) *Nitrososphaera viennensis* biotically forms N₂O via N-nitrosation—a hybrid reaction where hydroxylamine combines with an enzymatically bound nitrosyl intermediate derived from nitrite (NO₂⁻) or nitric oxide (NO) to produce N₂O [15, 18, 19]. Despite the genomes of comammox *Nitrospira* lacking a characterized NO reductase, *N. inopinata* can aerobically and anaerobically produce N₂O in a proposed abiotic reaction involving accumulated NH₂OH [15]. However, the contribution of nitrate reduction to N₂O production by *Nitrospira* has not yet been explored and may represent an unidentified source of anaerobic nitrogen loss.

Here we use a combination of stable isotope tracing, stoichiometric modeling, and proteomics to characterize the hydroxylamine-dependent nitrate reduction pathway with the comammox isolate *N. inopinata*. By revealing a strong biotic contribution to hybrid N₂O production by *N. inopinata*, these analyses provide mechanistic insight into an overlooked source of nitrogen loss that may

improve wastewater treatment processes, agricultural practices, and nitrogen cycle models. Together, these findings expand understanding of nitrogen cycling processes and comammox ecophysiology.

5.2 Methods

5.2.1 Laboratory strains and cell maintenance

N. inopinata was maintained in artificial freshwater medium (FWM). The artificial freshwater basal medium contained (per L Milli-Q water) 0.584 g of NaCl, 0.147 g of CaCl₂·2H₂O, 0.074 g of KCl, 0.049 g of MgSO₄·7H₂O, and 12.0 g of HEPES (pH 7.5) [45]. After autoclaving, the basal medium was supplemented with sterile stock solutions of 2.0 mL of 1 M NaHCO₃, 0.1 mL of 7.5 mM FeNaEDTA, 5.0 mL of KH₂PO₄ (0.4 g/L), 1 mL of 1 M NH₄Cl (1 mM NH₄Cl in final medium), and 1.0 mL of *Nitrosomonas oligotropha* trace element solution (100 mM HCl, 0.5 mM H₃BO₃, 0.5 mM MnCl₂·4H₂O, 0.8 mM CoCl₂·6H₂O, 0.1 mM NiCl₂·6H₂O, 0.01 mM CuCl₂·2H₂O, 0.5 mM ZnSO₄·7H₂O, 0.15 mM Na₂MoO₄·2H₂O) [46].

All cultures were grown in 250 mL Pyrex bottles with GL45 screw caps containing 100 mL of medium in the dark without agitation at 37 °C to avoid light inhibition. The medium was pre-warmed to culturing temperature before inoculation to avoid lower growth rates induced by temperature shock. Growth was monitored by total oxidized nitrogen (nitrite + nitrate) accumulation as well as ammonium depletion. When mid- to late-exponential phase was reached, 0.2 – 2% inoculum was transferred to fresh media. Culture purity was confirmed monthly by microscopic investigation and the absence of growth on R2A agar. Heterotrophic contamination was tested regularly by inoculating 100 µL of culture onto nutrient agar, 5% R2A agar and monitoring for two weeks. Contaminants were not detected in these organic media or through light

microscopy of *N. inopinata* cultures. Sanger sequencing of full length 16S ribosomal RNA gene was conducted every 6 months to confirm strain purity.

5.2.2 *Hydroxylamine dependent nitrate reduction experiments*

5.2.2.1 *Anaerobic hydroxylamine dependent nitrate reduction activity experiments*

The ability of *N. inopinata* to perform nitrate reduction in the presence of hydroxylamine was determined with hydroxylamine-replete and hydroxylamine-deplete cultures along with non-biotic controls. 1M Nitrate (NaNO_3), and 1M bicarbonate (NaHCO_3) stock solutions were prepared in MQ- H_2O in serum bottles and were made anaerobic by flushing with N_2 and CO_2 respectively for 30 minutes. Quadruplicate cell cultures were harvested by centrifugation ($4800 \times g$, 30 min) from an ammonia-oxidizing culture fed with 1 mM ammonia within 24 hours of N substrate depletion. Cell pellets were resuspended, washed twice by centrifuging as described above, and resuspended in 240-mL serum bottles with 100 mL of nitrate-free FWM. A fresh 1M hydroxylamine ($\text{NH}_2\text{OH} \cdot \text{HCl}$ pH 2.0) stock solution was prepared and filter sterilized. Hydroxylamine and NaNO_3 were added to serum bottles to achieve a final concentration of 0.2 mM- NH_2OH and 1 mM NO_3^- . Serum bottles were crimp-sealed with blue butyl rubber stoppers and the cultures were flushed with argon (Ar) for 45 minutes. Afterwards the headspace pressure was set to 1.55 bar. A 1M NaHCO_3 stock solution was anaerobically added to the serum bottles as a carbon source. Serum bottles were incubated at 37 °C in the dark to avoid light inhibition. Control incubations were performed with filter-sterilized (0.2um PES filter) and heat-sterilized (121 °C, 15 psig for 30 min) *N. inopinata* maintenance cultures within 24 hours of N substrate depletion.

Serum bottles were monitored daily until hydroxylamine depletion from the biotic condition and weekly until near hydroxylamine depletion in the control incubations. At each timepoint, 3 mL of liquid was anaerobically collected using Ar flushed syringe. A 2 mL aliquot was filter sterilized using a 0.2 μ m syringe-driven PES filter (VWR 28145-499) for chemical analysis and a 1mL aliquot was retained for cell count measurements. Additionally, 500 μ L of headspace gas was sampled using an argon (Ar) flushed, gas-tight syringe and injected into an Ar filled Balch tube for subsequent analysis with gas chromatography (GC). Prior to gas sampling, Balch tubes crimp-sealed with blue butyl rubber were made anoxic by sequential application of vacuum and flushing with Ar, the Balch tube headspace was set to 1.18-1.25 bar, and the serum bottle headspace pressure was recorded. Gas concentrations were mathematically corrected for liquid and gas sampling.

5.2.2.2 Influence of initial hydroxylamine concentrations

The impact of hydroxylamine concentration on the endpoint metabolite distribution was determined with triplicate cultures, set up and incubated as described above. Serum bottles were supplemented with a hydroxylamine stock solution (1M $\text{NH}_2\text{OH}\cdot\text{HCl}$ pH 2.0) to achieve initial NH_2OH concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 mM NH_2OH . Serum bottles were flushed with N_2 gas for 45 minutes and monitored daily until hydroxylamine depletion. Cell concentrations were not measured. Gas samples were collected at the final timepoint to measure N_2O production.

5.2.2.3 Cell counts

Cell concentrations were determined via cell counts with a Guava easyCyte Flow Cytometry system as described previously [47]. Briefly, cells were diluted in FWM to a concentration of

approximately 10^5 cells per mL (990 μ L final volume) and stained with a 10 μ L SYBR Green solution containing 1 μ L SYBR Green I Nucleic Acid Gel Stain (10,000 X stock, Thermo Fisher Scientific, Waltham, MA, USA) and 29 μ L FWM. Stained cells were incubated for 30 minutes in the dark at 37 °C. Fluorescence intensities at 535nm (green emission) and 635nm (red emission) were measured for each sample using a Guava easyCyte Flow Cytometry system. After counting 5000 total events, cell concentrations were determined by counting the number of events on a forward scatter (FSC) - green fluorescent intensity scatterplot occurring within a predefined gate for *N. inopinata* by the flow volume and correcting for the sample dilution. Blank measurements were conducted with FWM. The cell concentration of endpoint samples measured via cytometry were verified with microscopy as described previously [48]. No discernible large cell aggregates were observed under the tested conditions in this study.

5.2.2.4 Kinetic analysis

The impact of hydroxylamine concentration on the rate of hydroxylamine-dependent nitrate reduction was determined with quadruplicate cultures, independently incubated as described above with the following modifications. Serum bottles were monitored daily until hydroxylamine depletion. Due to the abiotic decay of hydroxylamine, cell specific reaction rates of hydroxylamine-dependent nitrate reduction were calculated by dividing the nitrate concentration change between the previous and current timepoint by the cell concentration. Kinetic constants were obtained by fitting a Michaelis–Menten equation to the cell specific rate of hydroxylamine-dependent nitrate reduction according to equation 5.2:

$$V = \frac{V_{max} * S}{K_{m(app)} + S} \quad (5.2)$$

where V refers to the rate cell specific rate of hydroxylamine-dependent nitrate reduction (in fmol-NO₃⁻ cell⁻¹ h⁻¹), V_{max} is the maximum cell specific rate of hydroxylamine-dependent nitrate reduction (fmol-NO₃⁻ cell⁻¹ h⁻¹), S is the concentration of hydroxylamine (μM-NH₂OH), and K_{m(app)} is the hydroxylamine concentration at which half of the maximum rate is achieved (μM). Curve fitting was conducted using the *curve_fit* function from the Scikit-Learn package v1.4.2 in Python v3.9.12.

5.2.2.5 Anaerobic to aerobic transition

The influence of hydroxylamine-dependent nitrate reduction activity on the recovery of ammonia- and nitrite-oxidation upon transitioning from an anaerobic to aerobic environment was determined with triplicate cultures, incubated independently as described above with the following modifications. 1M Hydroxylamine, 1M NaNO₃, 1M NaNO₂, and 1M NH₄Cl stock solutions were added to the following final concentrations (A: 200 μM-NH₂OH; B: 200 μM-NH₂OH + 200 μM-NH₄⁺; C: 175 μM-NH₄⁺; D: 175 μM-NO₂⁻). After the observation of near-complete hydroxylamine depletion, all serum bottles were transitioned from anaerobic to aerobic conditions by depressurizing serum bottles with a 21-gauge needle and transferring the cultures to sterile, acid washed Pyrex bottles with GL45 screw caps. The cultures were monitored daily for 96 hours after the aerobic transition. Samples were collected for cell counting before and after the aerobic transition to calculate cell specific ammonia and nitrite oxidation rates. Cell specific oxidation rates were calculated by dividing the ammonium or nitrite concentration change between the

current and previous timepoint by the cell concentration. Aerobic cell specific oxidation rates were obtained from an aerobic culture grown in FWM with 1mM NH_4^+ .

5.2.3 *Tracking the fate of nitrate with ^{15}N labeling*

5.2.3.1 *Incubation with ^{15}N labeled nitrate*

Due to the abiotic decay of hydroxylamine, the distribution of metabolites produced by hydroxylamine-dependent nitrate reduction was determined with cells, sterile FWM, and heat-sterilized cells (121 °C, 15 psig for 30 min), incubated independently with $^{15}\text{N}\text{-NO}_3^-$ as described above with the following modifications. The labeling incubations were conducted using a 1M NaNO_3 stock solution comprised of 5% ^{15}N -nitrate (Sigma-Aldrich #31432-45-8) and 95% unlabeled nitrate. Four parallel incubations were set up for replication and nutrient measurements. At each timepoint, 4 mL of culture was anaerobically collected using N_2 flushed syringe. with 3.5 mL being filter-sterilized using a 0.2 μm syringe-driven PES filter (VWR 28145-499). 1.75 mL of filtered culture were used for nutrient measurements with remaining 1.75 mL being transferred into a microcentrifuge tube and immediately frozen at -80°C for isotope analysis. The unfiltered 0.5mL aliquot was retained for cell count measurements.

5.2.3.2 *$\delta^{15}\text{N}$ of N_2O , NO_2^- , NO_3^- , and NH_4^+*

Concentrations and isotopes of N_2O were measured using a modified GC-IRMS (Thermo Finnigan Gasbench interfaced to a Delta V^{PLUS} isotopic ratio mass spectrometer) with large volume purge and trap system[49]. Briefly, two needles were used for sample transfer and He pressurization; sample was transferred into a sparging flask (Pyrex, USA) using ultra-high purity He (>99.999%) and purged with He. Samples were purged for 30 min at a flow rate of 50 mL min⁻¹. The extracted

gases were passed through an ethanol trap with dry ice and a chemical trap filled with magnesium perchlorate and Ascarite to remove H₂O and CO₂. N₂O was trapped by liquid nitrogen twice for purification and concentration and then injected into the GC-IRMS with He as carrier gas. N₂O concentrations were determined by ion peak area ($m/z = 44$). Calibration of N₂O concentration was calculated from ion peak areas [$m/z = 44$] with standard gases of 199.6 and 501.0 ppmv N₂O/He (Research Institute of China National Standard Materials), which were run at ~10 sample intervals. The serum bottle was weighed before and after transfer to calculate the amount of water transferred. The precision of this method was estimated to be better than $\pm 3\%$ [49]. $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ were calibrated against two reference tanks (R1: 199.6 ppmv N₂O/He, $\delta^{15}\text{N} = -3.2 \pm 0.1\text{‰}$, $\delta^{18}\text{O} = 36.6 \pm 0.1\text{‰}$; R2: 501.0 ppmv N₂O/He, $\delta^{15}\text{N} = -1.6 \pm 0.1\text{‰}$, $\delta^{18}\text{O} = 36.6 \pm 0.3\text{‰}$). The precision of $\delta^{15}\text{N}$ and $\delta^{18}\text{O}$ measurements with 2 nmol N₂O reference gas was better than 0.3‰ and 0.4‰, respectively ($n = 20$) [50].

$\delta^{15}\text{N}$ of NO₂⁻ was measured by chemical conversion (sodium azide, Sigma-Aldrich) of NO₂⁻ to N₂O [51]. For $\delta^{15}\text{N}$ of NO₃⁻ determination, the NO₂⁻ was initially removed from samples by adding sulfamic acid ($\geq 99\%$ sulfamic acid, Sigma-Aldrich) [52] and the $\delta^{15}\text{N}$ of NO₃⁻ was determined using the bacterial denitrifier method [53]. The $\delta^{15}\text{N}$ of the converted N₂O either through chemical conversion or denitrifier reduction was quantified using a modified GC-IRMS system (Thermo Finnigan Gasbench system with cryogenic extraction and purification system interfaced to a Delta V^{PLUS} isotopic ratio mass spectrometer) [53]. $\delta^{15}\text{N}$ of NO₂⁻ was calibrated against five in-house NO₂⁻ standards ($\delta^{15}\text{N}$ of the five in-house NO₂⁻ standards were 5.19‰, 126.07‰, 336.54‰, 671.78‰, 1343.28‰, respectively). $\delta^{15}\text{N}$ of N NO₃⁻ values were calibrated

against NO_3^- isotope standards USGS 34, IAEA N3 and USGS 32. Standard curves were run at the beginning and end of sample analysis and at ten sample intervals.

For $\delta^{15}\text{N}$ of NH_4^+ , the samples were measured using hypobromite oxidation coupled to azide conversion method [54] with slight modification [55]. Before the NH_4^+ was oxidized to NO_2^- , the pre-existing NO_2^- was removed from samples using sulfamic acid ($\geq 99\%$ sulfamic acid, Sigma-Aldrich) [52]. The removal efficiency of NO_2^- was tested by measuring the NO_2^- concentration before and after adding the sulfamic acid to $10 \mu\text{mol L}^{-1}$ of NO_2^- , and the oxidation efficiency of NH_4^+ to NO_2^- was tested by comparing the NH_4^+ and NO_2^- concentrations before and after hypobromite oxidation with initial NH_4^+ concentration of $2 \mu\text{mol L}^{-1}$. The NO_2^- removal and NH_4^+ oxidation efficiencies in our experiments were $>99\%$ and $>90\%$, respectively. All the samples were diluted to the NH_4^+ concentration of $2 \mu\text{mol L}^{-1}$ for hypobromite oxidation. $\delta^{15}\text{N}$ of NH_4^+ was calibrated against five in-house NH_4^+ standards ($\delta^{15}\text{N}$ of the five in-house NH_4^+ standards were 0.31‰ , 135.04‰ , 326.07‰ , 588.08‰ , 1324.84‰ , respectively). Standard curves were run at the beginning and end of sample analysis and at ten sample intervals.

5.2.3.3 Calculating contribution of NH_4^+ , NO_2^- , and N_2O from NO_3^-

The contribution of NO_3^- to NH_4^+ , NO_2^- , and N_2O production was calculated based on the isotopic mass balance model at each time point. Briefly, $\delta^{15}\text{N}$ of the net produced NH_4^+ , NO_2^- , or N_2O between two consecutive time points during the time course incubation was calculated using a two-end member mixing model (Equation 5.3).

$$\delta^{15}\text{N} = \frac{C_{t2} \cdot \delta^{15}\text{N}_{t2} - C_{t1} \cdot \delta^{15}\text{N}_{t1}}{C_{t2} - C_{t1}} \quad (5.3)$$

where $\delta^{15}\text{N}_{t1}$ and $\delta^{15}\text{N}_{t2}$ denote the $\delta^{15}\text{N}$ value of the net produced NH_4^+ , NO_2^- , or N_2O during the $^{15}\text{NO}_3^-$ labelling incubation at two consecutive time points, respectively. C_{t2} and C_{t1} denote the measured NH_4^+ , NO_2^- , or N_2O concentration at the two time points, respectively.

The fraction contribution of NO_3^- to the net produced NH_4^+ , NO_2^- , or N_2O by NO_3^- between two consecutive time points () were derived using equation 5.4.

$$AT^{15}\text{N} = AT^{15}\text{N}_{\text{NO}_3^-} \cdot a\% + AT^{15}\text{N}_{\text{NH}_2\text{OH}} \cdot (100\% - a\%) \quad (5.4)$$

where $AT^{15}\text{N}$ is the fraction of ^{15}N atom (%) of the net produced NH_4^+ , NO_2^- , or N_2O between two time points, which is calculated by the $\delta^{15}\text{N}$ in Equation 1. $AT^{15}\text{N}_{\text{NO}_3^-}$ and $AT^{15}\text{N}_{\text{NH}_2\text{OH}}$ is the fraction of ^{15}N atoms (%) in the NO_3^- and NH_2OH pool of the corresponding time point (refer to $t1$ in Equation 5.3). $a\%$ denotes the fractional contribution of NO_3^- to the net produced NH_4^+ , NO_2^- , or N_2O at the corresponding time point.

5.2.4 Chemical analysis

All samples were filtered through a 0.2 μm pore-size PES syringe filters (VWR 28145-499) prior to chemical analysis. Ammonium, nitrite, and hydroxylamine were analyzed immediately after sample collection. Ammonia concentrations were determined colorimetrically from the monochloramine generated by the reaction with alkaline hydrolysis of sodium dichloroisocyanurate (ISO 15923-1). Nitrite was measured spectrophotometrically using the sulphanilamide and N-a-naphthylethylenediamine dihydrochloride method (EPA Method 354.1). Hydroxylamine concentrations were determined colorimetrically as previously described by Frear and Burrell [56]. Briefly, liquid samples were mixed with 8-quinolinol solution (0.48% (w/v)

trichloroacetic acid, 0.2% (w/v) 8-hydroxyquinoline and 0.2 M Na₂CO₃), and heated at 100 °C for 1 min. After cooling down for 15 min, absorbance was measured at 705 nm.

Nitrate concentrations were analyzed with ion chromatography (IC) on a Dionex ICS 5000+ equipped with an IonPac AS11HC analytical and AS11 guard column at 30°C. Eluent consisted of generated KOH (Dionex EG-5 & Dionex EGCIH KOH, Thermo Fisher Scientific) at a flow rate of 1.5 mL·min⁻¹. An eluent concentration gradient of 1-60 mM KOH was applied by first running 7 minutes at 1 mM, increasing at (i) 1.56 mM·min⁻¹ for 9 minutes, then (ii) 1.67 mM·min⁻¹ for 9 minutes, and eventually (iii) 3.75 mM·min⁻¹ for 8 minutes to 60 mM, where concentration was held for 90 seconds. The concentration was then decreased as a step function back to 1 mM KOH for 2 minutes before the end of the run. Nitrate concentrations were externally calibrated and validated every analytical run.

Concentrations of nitrous oxide (N₂O) were measured by gas chromatography (SRI Model 8610 GC) equipped with an electron capture detector (ECD) using nitrogen as carrier gas and a 182.9 cm HayeSep D column (SRI Instruments). Briefly, the gas sample storage Balch tube pressure was recorded and 500 µL of gas was injected into the GC using a nitrogen flushed, gas-tight syringe. N₂O concentrations were determined by ECD peak areas. Internal reference standards with known N₂O concentrations were prepared with N₂O (Air Liquide) and pressurized nitrogen (Air Liquide). Dissolved N₂O concentrations were calculated according to Henry's law as previously described [57] with constants for N₂O adapted from Sander [58]. Overall N₂O production concentrations were calculated using Equation 5.5 assuming all headspace N₂O was dissolved.

$$c_{N_2O} = p_{N_2O} \cdot k_{H,N_2O} + \frac{\left(\frac{p_{N_2O} \cdot V_g}{RT}\right)}{V_L} \quad (5.5)$$

where c_{N_2O} is the overall concentration of N_2O in water (in mol L^{-1}), p_{N_2O} is the partial pressure of the gas in the headspace (in atm), k_{H,N_2O} is the temperature adjusted Henry's coefficient for N_2O (in $\text{mol L}^{-1} \text{ atm}^{-1}$; 37 °C), V_g is the headspace volume (in L), R is the universal gas constant ($0.082057 \text{ L atm mol}^{-1} \text{ K}^{-1}$), and T is the incubation temperature (310.15 K), and V_L is the liquid volume (in L).

5.2.5 Proteomic analysis

5.2.5.1 Proteomic incubations and sample collection

Differential protein abundance of *N. inopinata* cultures performing anaerobic hydroxylamine-dependent nitrate reduction (H), anaerobic formate-dependent nitrate reduction (F), and aerobic ammonia-oxidation (A) were determined by proteomic analysis of triplicate (H) and quadruplicate (F & A) cultures, incubated independently. The hydroxylamine-dependent nitrate reduction and formate-dependent nitrate reduction incubations were incubated as described above with the following modifications. Formate and NaNO_3 were added to the formate-dependent nitrate reduction serum bottles to a final concentration of 0.4 mM-formate and 1 mM- NO_3^- . The incubations were made anaerobic by flushing with nitrogen (N_2) gas. The aerobic ammonia-oxidation incubations were incubated as described in *Laboratory culture and cell maintenance* with the following modifications. Cultures were grown in 2 L Pyrex bottles with GL45 screw caps containing 400 mL of FWM after a 1% inoculum was transferred to the fresh media. Samples were collected at mid-exponential phase (H & A) or after 96 hours of incubation (F) by filtration onto a

sterile 0.2 μ m SUPOR filter membrane (Thermo Fisher Scientific, Waltham, MA) using an acid washed filtering apparatus. All samples were immediately flash frozen and stored at -80°C until protein extraction.

5.2.5.2 *Protein identification and quantification*

The protein extraction was carried out according to a previously reported method [59–62]. Briefly, harvested cell were rinsed off the filter membrane with lysis buffer followed by resuspension in lysis buffer (containing 10 mM Tris-HCl, 1% SDS, and 0.1 M dithiothreitol [DTT]), and incubated at 60°C for 1 h, and then the supernatant was collected after centrifugation. Proteins were then precipitated by trichloroacetic acid overnight at 4°C and pelleted by centrifugation. Protein pellets were washed with ice-cold acetone three times and resuspended in guanidine buffer. Protein concentrations were quantified by bicinchoninic acid assay [63]. One hundred-milligram samples of proteins were processed using filter-aided sample preparation and digested using trypsin-LysC mixture [62]. The resulting peptide samples were purified using Pierce C18 Spin Columns (Thermo Fisher Scientific, Waltham, MA) and eluted in 20 μ L elution buffer. Each sample was analyzed using one-dimensional liquid chromatography-tandem mass spectrometry (1D-LC-MS/MS) on an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, USA) at the IDeA National Resource for Quantitative Proteomics. Tryptic peptides were separated by reverse phase XSelect CSH C₁₈ 2.5- μ m resin (Waters) on an in-line 150 by 0.075-mm column using an UltiMate 3000 RSLCnano system (Thermo Fisher Scientific, USA). Peptides were eluted using a 120 minute gradient from 98:2 to 65:35 buffer A:B ratio (Buffer A, 0.1% formic acid, 0.5% acetonitrile; Buffer B, 0.1% formic acid, 99.9% acetonitrile). Eluted peptides were ionized by electrospray (2.4 kV) followed by mass spectrometric analysis on an Orbitrap Fusion Tribrid mass

spectrometer (Thermo Fisher Scientific, USA). MS data were acquired using a Fourier transform MS (FTMS) analyzer in profile mode at a resolution of 240,000 over a range of 375 to 1,500 m/z . Following high-energy collisional dissociation (HCD) activation, MS/MS data were acquired using the ion trap analyzer in centroid mode and normal mass range with normalized collision energy of 28 to 31% depending on charge state and precursor selection range. Mass spectrometry spectra were searched using Sipsos Ensemble against a matched protein database constructed from the genome of *Nitrospira inopinata* [64, 65]. Raw search results were filtered to achieve a 1% false-discovery rate (FDR) at the peptide level, estimated by the target-decoy approach. Peptide identifications are assigned to proteins or protein groups following the parsimonious rule [64]. A protein group included multiple proteins sharing a set of common peptides, at least one of which cannot be attributed to any other protein or protein group. A minimum of one unique peptide was required for each identified protein or protein group. To avoid ambiguity in data analysis, only proteins were used for biological analysis, while protein groups were not evaluated. Intensity-based label-free quantification for protein was performed using ProRata [66, 67].

Protein abundance, represented by the total peak height of all quantified unique peptides from a protein, across the samples was median normalized [28, 68, 69]. Proteins were grouped by condition and proteins quantified in less than 2/3 of the samples were removed for differential abundance analysis [28]. Missing values from the normalized and filtered protein intensity dataset were imputed by performing a log₂ transformation and applying the ‘Missing not At Random’ (MNAR) assumption in label-free proteomics, which uses random draws from a left-shifted Gaussian distribution of 1.8 StDev (standard deviation) apart with a width of 0.3, as previously described [28, 60, 70, 71]. The DESeq2 package from R Bioconductor was used to generate a list

of differentially abundant proteins for each pairwise comparison between the hydroxylamine-dependent nitrate reduction, formate-dependent nitrate reduction, and aerobic ammonia-oxidation conditions [62, 72, 73]. A cutoff of the ‘adjusted p value’ of 0.05 (Benjamini-Hochberg method) along with a $|\log_2 \text{fold change}|$ of 1 was applied to determine significantly differentially abundant proteins in each pairwise comparison [28, 62].

5.3 Results

5.3.1 Hydroxylamine-dependent nitrate reduction by *N. inopinata*

To investigate hydroxylamine-dependent nitrate reduction, *N. inopinata* strain EN76 was incubated in sealed serum bottles containing anaerobic AOM medium supplemented with hydroxylamine, nitrate, and bicarbonate. Consistent with the hypothesized pathway, *N. inopinata* simultaneously oxidized NH_2OH ($176.35 \pm 1.93 \mu\text{M NH}_2\text{OH}$) and reduced NO_3^- ($197.35 \pm 4.09 \mu\text{M NO}_3^-$) until NH_2OH depletion after 192 hours (Figure 5.1A). The reaction yielded nitrite ($270.89 \pm 5.94 \mu\text{M NO}_2^-$), nitrous oxide ($25.03 \pm 1.50 \mu\text{M N}_2\text{O}$), and ammonium ($22.99 \pm 1.11 \mu\text{M NH}_4^+$), which did not continue to react after NH_2OH depletion. Appreciable growth was not detected despite the accumulation of NH_4^+ , a known assimilatory nitrogen source. NH_4^+ supplementation did not induce nitrate reduction (Figure S 5.1), growth (Figure S 5.1), or influence hydroxylamine-dependent nitrate reduction (Figure S 5.2). Nitrate reduction was not observed in the heat-killed, filtrate, and hydroxylamine-deplete controls (Figure 5.1B-D and Figure S 5.3B-C). Therefore, hydroxylamine-dependent nitrate reduction can be attributed to the biological activity of *N. inopinata*.

Stoichiometric analysis indicated the ratio of nitrate reduced to nitrite produced ($2.75 \pm 0.10 \text{ NO}_2^- : 2 \text{ NO}_3^-$) was lower than the proposed hydroxylamine-dependent nitrate reduction stoichiometry ($3 \text{ NO}_2^- : 2 \text{ NO}_3^-$). The discrepancy suggests nitrite might have been involved in a secondary reaction. However, an abiotic reaction between NO_2^- and NH_2OH or a reactive intermediate of NH_2OH decay was not observed in abiotic controls (Figure S 5.4). Additionally, gaps in the nitrogen and electron balances after NH_2OH depletion were very small, at just $2.28 \pm 0.83 \%$ and $0.76 \pm 0.58 \%$ respectively. This suggests all species—except dinitrogen gas (N_2), a known product of

hydroxylamine decay [20]—were accounted for. Therefore, a fraction of the produced NO_2^- must have been further reduced to nitric oxide (NO) by *N. inopinata*.

5.3.2 ^{15}N stable isotope tracing

Stable isotope tracing experiments were conducted with ^{15}N labeled NO_3^- to distinguish abiotic NH_2OH decay from biotic reactions in the biotic incubation (Figure 5.2). The contribution of NO_3^- reduction to N_2O , NO_2^- , and NH_4^+ production was monitored throughout the biotic, heat-killed, and filtrate incubation (Figure S 5.5). Endpoint isotopologue ratios of N_2O and ^{15}N relative abundance of NO_2^- and NH_4^+ were compared against the controls to determine if metabolite production could be attributed to biological activity (Figure 5.2).

5.3.2.1 Role of NO_3^- in NO_2^- and NH_4^+ production

$^{15}\text{NO}_3^-$ did not contribute to NH_4^+ and N_2O in the controls demonstrating their production was exclusively attributed to NH_2OH decay (Figure S 5.5). Biotic $^{15}\text{NH}_4^+$ production was predominantly sourced from NH_2OH with over 98% of the N atoms originating from NH_2OH (Figure 5.2). However, the $^{15}\text{NH}_4^+$ relative abundance increased from 0.18 ± 0.13 % at hour 3.5 to 0.82 ± 0.31 % upon hydroxylamine depletion at hour 260 (Figure 2A). No increase in $^{15}\text{NH}_4^+$ was observed in the controls and the endpoint $^{15}\text{NH}_4^+$ relative abundance was statistically greater than the controls ($p < 0.005$; Tukey HSD). Overall, the majority of NH_4^+ was produced by NH_2OH decay in the biotic incubation, yet the minor contribution of dissimilatory nitrate reduction to ammonia (DNRA) cannot be ruled out.

$^{15}\text{NO}_3^-$ significantly contributed to NO_2^- production throughout the biotic incubation with the contribution increasing from 41.62 ± 3.28 % at hour 3.5 to 71.64 ± 0.37 % at hour 260 and

plateauing upon NH_2OH depletion (Figure 5.2). The endpoint $^{15}\text{NO}_3^-$ contribution was significantly higher than expected from stoichiometry (66.6 %) even after correcting for the reduction of NO_2^- to N_2O formation (69.87 ± 0.59 %). While a constant $^{15}\text{NO}_3^-$ contribution was expected, the increasing contribution suggests unexpected dynamics within the extracellular NO_2^- pool. The dynamic $^{15}\text{NO}_3^-$ contribution cannot be attributed to NO_2^- reduction given NO_2^- depletion could not concentration $\delta^{15}\text{N}-\text{NO}_2^-$ under the conditions tested here. However, increasing the ratio of NO_3^- reduction relative to NH_2OH oxidation or the preferential sourcing of periplasmic NO_2^- by *nirK* would concentrate the $\delta^{15}\text{N}-\text{NO}_2^-$ of the extracellular NO_2^- pool. Nevertheless, the unexpected increase in the $\delta^{15}\text{N}$ of extracellular NO_2^- suggests complex reaction dynamics that warrant further investigation.

5.3.2.2 NO_3^- reduction by *N. inopinata* contributes to N_2O production

The contribution of NO_3^- reduction to N_2O was measured at select timepoints (Figure S 5.6). Throughout the biotic incubation, the contribution of NO_3^- was constant with 25.53 ± 1.61 % of N atom in N_2O sourced from NO_3^- (Figure 5.2B), while the contribution was $<0.75\%$ in the controls (Figure S 5.6). This clearly demonstrates NO_3^- reduction by *N. inopinata* during hydroxylamine-dependent nitrate reduction contributed to N_2O formation.

Nitrous oxide isotopologues ($^{44}\text{N}_2\text{O}$, $^{45}\text{N}_2\text{O}$, and $^{46}\text{N}_2\text{O}$) provide valuable insight into the precursors of N_2O production. The ratio of single labeled N_2O to unlabeled N_2O ($^{45}\text{N}_2\text{O}:^{44}\text{N}_2\text{O}$) and double labeled N_2O to unlabeled N_2O ($^{46}\text{N}_2\text{O}:^{44}\text{N}_2\text{O}$) were analyzed to determine if N_2O was produced from a hybrid pathway or derived from nitric oxide (NO) reduction (Figure S 5.7). The endpoint $^{45}\text{N}_2\text{O}:^{44}\text{N}_2\text{O}$ ratio was significantly elevated in the biotic incubation compared to the control incubations and N_2O reference standard ($p < 0.001$; Tukey HSD; Figure 5.2E). No

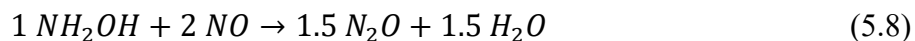
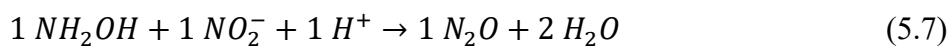
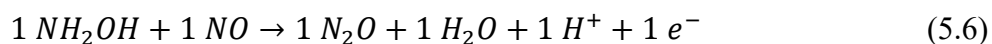
statistical differences in $^{46}\text{N}_2\text{O}$: $^{44}\text{N}_2\text{O}$ was detected between the biotic, control, and reference samples (Figure S 5.8). While enrichment of $^{45}\text{N}_2\text{O}$ and absence of $^{46}\text{N}_2\text{O}$ by *N. inopinata* suggests a hybrid pathway where one N atom from NH_2OH combines with one N atom from a $^{15}\text{N}\text{-NO}_3^-$ reduction intermediate, a nitrogen isotope probability model demonstrated the maximum $^{46}\text{N}_2\text{O}$ concentration would have been below the limit of detection (Supplementary Material). Therefore, N_2O production from nitric oxide reduction, instead of the hybrid pathway, reduction cannot be dismissed based on the absence of $^{46}\text{N}_2\text{O}$. However, the lack of a characterized nitric oxide reductase in genome of *N. inopinata* and the consistent enrichment of $^{45}\text{N}_2\text{O}$ suggest a hybrid pathway.

5.3.2.3 N_2O is produced by a N-nitrosating hybrid N_2O reaction

The $^{45}\text{N}_2\text{O}$ isotopologue ratios demonstrate NO_3^- reduction by *N. inopinata* contributes to N_2O production with 25.53 ± 1.61 % of N atom in N_2O produced by *N. inopinata* sourced from NO_3^- . However, NH_2OH decay to N_2O was observed in the abiotic controls and likely contributed to N_2O production (Figure S 5.3). A bulk metabolite and isotope model were developed to calculate the contribution of NO_3^- to N_2O production. There was no statistical difference between the models ($p=0.68$; Mann-Whitney) with both reporting NO_3^- reduction by *N. inopinata* produced approximately 50 % of the N_2O (bulk model: 54.14 ± 3.03 %; isotope model: 52.89 ± 2.65 %). The contribution of $^{15}\text{NO}_3^-$ to biotic N_2O production was recalculated with the output of the isotope model and to $^{15}\text{NO}_3^-$ accounted for $51.56 \pm 5.02\%$ of the N atoms in biotically derived N_2O . Collectively, the 1:1 stoichiometric contribution of $^{15}\text{NO}_3^-$ and NH_2OH and enrichment of $^{45}\text{N}_2\text{O}$ provides unequivocal evidence the nitrate reduced by *N. inopinata* contributed to hybrid pathway

where one N atom from NH₂OH combines with one N atom from a ¹⁵N-NO₃⁻ reduction intermediate.

No mechanism for N₂O production by *N. inopinata* has been proposed aside from NH₂OH decay has been proposed [4, 21]. The ¹⁵NO₃⁻ contribution to biotic N₂O production and the enriched ⁴⁵N₂O:⁴⁴N₂O indicates biotic N₂O production proceeds via a N-nitrosating hybrid N₂O reaction. The candidate hybrid N₂O mechanisms include two N-nitrosation reactions involving an enzyme bound nitroxyl (NO⁺) intermediate (Figure 5.2F; Extended Figure 5.2; Equation 5.6-8) and the abiotic reaction between NO and NH₂OH (Equation 5.8).



The expected contribution of NO₃⁻ reduction to the candidate mechanisms (Equation 5.6-8) would be 51.56 ± 5.02% given ⁴⁶N₂O was below the limit of detection. There was no statistical difference between the theoretical and actual contribution of ¹⁵NO₃⁻ to ⁴⁵N₂O production. However, dynamics in the extracellular ¹⁵NO₂⁻ pool were not observed in ⁴⁵N₂O (Figure 5.2; Figure S 5.7) and the ¹⁵NO₂⁻ contribution after NH₂OH depletion (71.34 ± 7.01 %) was significantly greater than the theoretical stoichiometry of NO₂⁻ dependent N-nitrosation (p-value < 0.01; t-test; Equation 5.6). Therefore, extracellular NO₂⁻ was not involved in the biotic production of N₂O by *N. inopinata*.

Instead, the N₂O production mechanism must proceed through the reduction of NO₃⁻ to NO₂⁻ and presumably NO.

5.3.3 *Ratio of biotic N₂O production remains constant across NH₂OH concentrations*

The influence of NH₂OH on the endpoint metabolite dynamics of hydroxylamine-dependent nitrate reduction was investigated by varying the initial NH₂OH concentration (Figure 5.3A; Figure S 5.9). All cultures simultaneously oxidized NH₂OH and reduced NO₃⁻ until NH₂OH depletion with near complete nitrogen (99.0 ± 1.3 %) and electron ($101.8 \pm 2.7\%$) recovery across all conditions (Figure S 5.9). Nitrite was initially produced at a similar rate across all NH₂OH concentrations (Figure S 5.10), however endpoint NO₂⁻ concentrations approached asymptotic saturation at NH₂OH concentrations above 400 μ M (Figure 3A). Deviations between the theoretical NO₂⁻ production from nitrate reduction (grey) and actual NO₂⁻ production (green) coincided with NO₂⁻ saturation (Figure 5.3A). While N₂O production increased with NH₂OH concentration, the relative contribution of biotic hybrid N₂O formation remained constant (Figure 5.3B) with an average contribution of 72.22 ± 5.30 % (ANOVA p-value = 0.295). The fixed biotic contribution is consistent with saturated enzymatic activity though it was unexpected. Presumably the difference between the endpoint and theoretical NO₂⁻ production can be attributed to increased hybrid N₂O formation.

5.3.4 *NH₂OH affinity*

The whole-cell kinetic properties of hydroxylamine-dependent nitrate reduction by *N. inopinata* was characterized (Figure 5.4A). The NH₂OH affinity was calculated from the cell specific NO₃⁻ reduction rate to avoid any confounding factors arising from NH₂OH decay. Nitrate reduction

followed Michaelis–Menten kinetics ($R^2 = 0.89$), with a mean apparent half-saturation constant ($K_{m(app)}$) of $33.09 \pm 8.74 \mu\text{M NH}_2\text{OH}$ and a mean maximum cell-specific nitrate reduction rate ($V_{max(app)}$) of $0.047 \pm 0.003 \text{ fmol-NO}_3^- \cdot \text{cell}^{-1} \cdot \text{h}^{-1}$.

The whole-cell NH_2OH affinity was compared against previously measured NH_2OH affinities (Figure 5.4B). The measured half-saturation constant is over an order of magnitude higher than NH_2OH concentration measured during aerobic ammonia oxidation by *N. inopinata* [21] and *in-vitro* Hao activity assays [22–24]. However, it is four times lower than the whole-cell affinity of the AOB *Nitrosomonas europaea* [25] and aligns with the *in-vitro* Hao affinity of the anaerobic ammonia-oxidizing bacteria *Ca. Brocadia anammoxidans* [26]. Differences between the whole-cell affinity and *in-vitro* Hao activity assays may reflect NH_2OH transport limitations and the mutagenic effects of hydroxylamine on *N. inopinata*.

5.3.5 Proteomic response of *Nitrospira inopinata* to anaerobic nitrate reduction

The proteomic response of *N. inopinata* during hydroxylamine-dependent nitrate reduction was compared to aerobic ammonia oxidation and formate-dependent nitrate reduction, an anaerobic control (Figure S 5.11). A total of X, Y, Z proteins were identified in the respective conditions and accounted for X, Y, Z % of proteins encoded in the *N. inopinata* genome. Proteins central to the complete ammonia oxidation pathway including the ammonia monooxygenase (AmoABC), Hao, NxrABC, and the copper-containing nitrite reductase (NirK)—were constitutively abundant across all conditions (Figure 5.5). These proteins were among the most highly abundant proteins accounting for X, Y, Z, percent of detected proteins in the tested conditions, respectively. Only 33 proteins showed statistically significant differences between hydroxylamine-dependent and formate-dependent nitrate reduction. Therefore, variations between hydroxylamine-dependent

nitrate reduction and aerobic ammonia oxidation can be attributed to anaerobic stress rather than hydroxylamine toxicity.

Two NO_3^- reduction proteins were detected in the proteome of *N. inopinata*. The nitrite oxidoreductase (NxrABC) exhibited high, constitutive abundance. Additionally, the putative periplasmic nitrate reductase (NapA) abundance significantly increased in response to hydroxylamine-dependent nitrate reduction compared to the aerobic control (16.3-fold, $q < 0.001$), suggesting a role in nitrate reduction. However, NapA was 37.4 ± 10.2 and 71.1 ± 25.8 times less abundant than NxrA and NxrB during hydroxylamine-dependent nitrate reduction, respectively. While the upregulation of NapA indicates a role in NO_3^- reduction, the magnitude of Nxr abundance cannot be ignored.

Proteins involved in cell division—FtsA (21.1-fold; $q = 2.3\text{e-}15$) and FtsZ (not detected)—significantly decreased in response to anaerobic nitrate reduction, consistent with the observed lack of growth (Figure 5.1A; Figure S 5.1). It is unclear if growth was limited by the oxygen or nitrogen considering $15.38 \pm 1.13 \mu\text{M-NH}_4^+$ was present at the time of sampling. Assimilatory nitrogen limitations were further suggested by the increased abundance of the urea transporter (UrtA; 61.8-fold; $q < 0.001$) and putative outer-membrane short short-chain amide/urea porin (SAP; 4-fold; $q < 0.001$). However, the constitutive abundance of glutamine synthase, the small subunit of glutamate synthase, the global nitrogen regulation P-II protein contradict this observation.

A gene cluster consisting of twenty-two protein coding domain was only detected during anaerobic nitrate reduction, but not ammonia-oxidation (Extended Figure 5.3). The proteins exhibited high sequence similarity to the leucine and isoleucine branched-chain amino acid catabolism pathway

and the methylcitrate cycle—a TCA cycle modification that incorporates the propionyl-CoA produced by isoleucine catabolism via oxaloacetate into the TCA cycle as succinate (Figure 5C). The BCAA catabolism operon maintains a high degree of synteny among members of the *Nitrospira* genus, however it is not maintained by manganese-oxidizing *Nitrospirota*, *Nitrobacter* NOB, and *Nitrotoga* NOB (Figure S 5.12Figure S 5.13). Catabolizing branched-chain amino acids and reduced cell division may be part of a broader response that shifts resources from oxidation and growth to cell maintenance by recycling existing cell material for energy conservation. However, further work is required to confirm this hypothesis.

5.3.6 Loss of ammonia oxidation activity following anaerobic perturbations

To investigate the impact of hydroxylamine-dependent nitrate reduction on the recovery of aerobic nitrification, *N. inopinata* was transitioned from an anaerobic to an aerobic environment (). Nitrite-oxidation was immediately detected upon oxygen exposure (Figure 5.6). Notably, the cell-specific rate of cell previously performing hydroxylamine-dependent nitrate reduction was comparable to the maximum aerobic nitrite oxidation rate ($p > 0.05$; Tukey HSD). Cells incubated with nitrite had significantly lower nitrite-oxidation rates ($p < 0.001$; Tukey HSD), even after applying a Michaelis-Menten correction ($p < 0.001$; Tukey HSD).

Ammonia oxidation was detected upon exposure to oxygen (Figure 5.6). However, the rate of ammonia oxidation was linear, and ammonia was not depleted 93 hours after initial oxygen exposure. The cell-specific rates were significantly lower than the aerobic ammonia oxidation ($p > 0.001$) with a maximum recovery of ~5% observed in hydroxylamine-deplete condition. NH_2OH did not influence the recovered ammonia-oxidation rates ($p > 0.05$), which were all significantly lower than the nitrite-oxidation rates ($p < 0.001$). The difference between the ammonia and nitrite

oxidation rate indicates a differential recovery of nitrification by *N. inopinata* with hypoxia having a significant influence on ammonia oxidation.

5.4 Discussion

This study provides compelling evidence for hydroxylamine-dependent nitrate reduction with *Nitrospira inopinata*—a comammox isolate with the genomic inventory required to catalyze this pathway. Comammox are prevalent in oxygen limited environments with the noted enrichment of *Ca. Nitrospira nitrosa* and *Ca. Nitrospira nitrificans* in hypoxic bioreactors ($\leq 3.1 \mu\text{M-O}_2$) [2, 12, 27, 28]. However, the mechanism allowing comammox to outcompete ammonia oxidizing archaea (AOA) and bacteria (AOB) under these conditions remains unclear [29]. Comparing terminal oxidase type suggests comammox *Nitrospira* would have the highest whole-cell oxygen affinity, but we recently demonstrated it does not significantly differ across AOM lineages [ref]. Complete ammonia oxidation—a pathway with a higher oxygen demand than ammonia oxidation—must confer a competitive advantage under oxygen limitations. While combining aerobic ammonia oxidation with nitrate reduction could allow comammox to minimize oxygen uptake [29], oxygen consumption was completely avoided with hydroxylamine-dependent and formate-dependent nitrate reduction (Figure 5.1; Figure S 5.11), a novel nitrite comproportionating pathway coupling hydroxylamine oxidation with nitrate reduction.

We demonstrated hydroxylamine-dependent nitrate reduction is a biotic pathway that reduces nitrate to nitrite in the presence of active *N. inopinata* biomass (Figure 5.1A). Activity was detected upon the onset of anoxia, yet this energy conserving pathway did not yield discernable growth (Figure 5.1; Figure S 5.1). Abiotic hydroxylamine decay to ammonium, nitrous oxide, and presumably dinitrogen was observed in the biotic and control incubations highlighting the instability of this reactive ammonia oxidation intermediate (Figure 5.1; Figure S 5.3).

AOM tightly regulate their ammonia oxidation pathway to avoid hydroxylamine cytotoxicity. While *Alcaligenes faecalis* and other heterotrophic nitrifiers can tolerate excessive hydroxylamine concentrations [30], nitrifiers exhibit high sensitivity to hydroxylamine with hydroxylamine inhibiting *Nitrospira* more than other nitrifying lineages [31]. AOM manage this toxicity with the rapid oxidation of hydroxylamine to nitric oxide by *hao*. Imbalanced between hydroxylamine and ammonia oxidation rates will trigger NH_2OH accumulation, especially in response to anaerobic-aerobic transitions or excessive ammonia oxidation rates [21, 31, 32]. Comammox frequently encounter these conditions at oxic-anoxic interfaces in sediment, subsurface, freshwater, geothermal, and engineered environments [33]. Excess hydroxylamine produced by aerobic nitrifiers will diffuse into anoxic zones and accumulate [34]. Hydroxylamine-dependent nitrate reduction may represent an adaptation allowing comammox *Nitrospira* to conserve energy and mitigate hydroxylamine toxicity while conditions are not suitable for complete ammonia oxidation. The constitutive abundance of nitrification proteins (Figure 5.5) and the recovery of nitrite oxidation after an anaerobic-to-aerobic transition (Figure 5.6) we observed support this hypothesis. However, the inhibited ammonia-oxidation rate after the transitions (Figure 5.6F) was unexpected considering the enrichment of comammox in hypoxic bioreactors [2, 12]. The response we observed might decrease the competitiveness of comammox in fluctuating ecosystems, especially if the oxidation of ammonia is coupled to growth. Future work should determine if the inhibition of ammonia oxidation after oxygen exposure is a universal comammox trait or a physiological response unique to *N. inopinata*.

Dissimilatory nitrate reduction to nitrite is increasingly recognized as a conserved trait across NOB lineages that promotes metabolic [33]. *Nitrobacter*, *Nitrococcus*, and *Nitrospira* NOB isolates

have exhibited the capacity to perform acetate- and formate-dependent nitrate reduction [13, 35–37]. Nitrate reduction by NOB is attributed to the reversible activity of NXR—a biochemical activity characterized with a purified *Nitrobacter hamburgensis* NXR [37]. Nitrate reduction in our study corresponded with the high abundance of NXR proteins and increased abundance of the periplasmic nitrate reductase (NapA) (Figure 5.5). While the upregulation of NapA is noteworthy, NxrA was 2.5 times more abundance than NapA, suggesting the NXR was primarily responsible for nitrate reduction. However, the contrasting proteomic response may confer *N. inopinata* with greater flexibility, distinguishing it from the majority of *Nitrospira* that rely exclusively on reverse NXR activity.

Differential proteomic analysis revealed a coordinated restructuring of the *N. inopinata* proteome in the absence of ammonia oxidation. Resources were shifted from growth towards cell maintenance while reducing nitrate that is reflected by the downregulation of cell division proteins (*ftsAZ*) and upregulation of universal stress proteins (Figure 5.5). Central carbon metabolism was restructured to recycle cellular components and conserve energy through branched-chain amino acid (BCAA) catabolism (Extended Figure 5.3; Figure S 5.12Figure S 5.13), a strategy mirroring the nitrite-deplete response of *Nitrospira moscoviensis* [38]. Conservation of the unique BCAA operon within the *Nitrospira* indicates BCAA catabolism may be a general feature that allows members to this genus to conserve energy and persist during periods of nutrient limitation.

Demonstrating the biological activity of *N. inopinata* directly contributes to N₂O production was one of the most important findings of our study. N₂O production by *N. inopinata* has been measured under NH₃-limited and hypoxic conditions despite the absence of known NO reductases (i.e. *nor* and *cytL*) from the genome of most comammox species [15, 21]. The consistently positive

N₂O site preference values (NH₃-limited: $34.2 \pm 1.4\text{‰}$; O₂-limited: $34.2 \pm 1.4\text{‰}$) from *N. inopinata* suggested N₂O originates from an abiotic NH₂OH conversion [15]. However, site preference reflects the intermediate undergoing the N–O bond cleavage, rather than the N₂O production process [39]. Stable isotope tracing with ¹⁵N labeled nitrate allowed us to demonstrate nitrate reduction by *N. inopinata* directly contributes N₂O formation through the enrichment of ⁴⁵N₂O compared to our controls (Figure 5.2E; Figure S 5.6Figure S 5.8). We determined abiotic hydroxylamine decay and biotic N₂O formation contribute equally to the observed N₂O production (biotic contribution: $54.14 \pm 3.03\%$). ¹⁵N derived from the labeled nitrate accounted for $52.89 \pm 2.65\%$ of the nitrogen atoms in the biotically derived N₂O with the remaining nitrogen coming from unlabeled hydroxylamine. We also observed the ratio of biologically derived N₂O did not vary in response to initial hydroxylamine concentration (Figure 5.3B). The equal contribution of the labeled nitrate and unvarying biologically derived N₂O ratio suggests *N. inopinata* biologically produces N₂O by N-nitrosation, a hybrid reaction where the nitrogen atom from NH₂OH reacts with the nitrosyl (NO⁺) intermediate from nitrite reduction to form N₂O [40].

Biologically mediated N₂O from N-nitrosation was shown with *Nitrososphaera viennensis*—an AOA isolate without an annotated NO reductase [18]—where ammonium and nitrite equally contributed to N₂O formation in ¹⁵N-labeling experiments. The N₂O site preference from AOA also has a positive value (13.1–30.8‰) [18, 41] suggesting comammox might produce N₂O with this mechanisms. Hybrid N₂O production from hydroxylamine has been extensively studied with denitrifying isolates in a process called co-metabolic denitrification [40]. Characterization of a purified copper-containing nitrite reductase (NirK) from *Achromobacter cycloclastes* revealed N₂O is produced by N-nitrosation when the enzyme-bound nitrosyl intermediate (NO⁺) from nitrite

reduction reacts with hydroxylamine (Figure 5.2F) [42]. This mechanism was later verified with a recombinant NirK from *N. viennensis* provided direct evidence of enzymatic N₂O production from N-nitrosation in a nitrifying lineage [19]. Both AOA and comammox encodes NirK and the mechanism is likely independent of lineage. This newly identified enzymatic mechanism of N₂O production in comammox *Nitrospira* has significant implications. It represents an overlooked source of N₂O production, especially in the Yangtze Estuary where comammox contributes to 28.5% to total N₂O emissions [43].

In conclusion, this study provides compelling evidence for hydroxylamine-dependent nitrate reduction by *Nitrospira inopinata*. We provide mechanistic insight into an overlooked source of nitrogen loss from the *Nitrospira* genus and how comammox conserve energy under fluctuating oxygen limitations. Our discovery highlights the significance of extracellular hydroxylamine in the global nitrogen cycle and has major implications for how comammox and hydroxylamine are modeled. Incorporating dissimilatory nitrate reduction by comammox and NOB *Nitrospira* is important for the refinement of biogeochemical and engineering models that aim to characterize nitrogen cycling and the environmental niche of comammox.

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5.5 References

1. Winogradsky S. Recherches sur les organismes de la nitrification. *Ann Inst Pasteur* 1891;**5**:557–616.
2. Van Kessel MAHJ et al. Complete nitrification by a single microorganism. *Nature* 2015;**528**:555–559. <https://doi.org/10.1038/NATURE16459>
3. Daims H et al. Complete nitrification by *Nitrospira* bacteria. *Nature* 2015;**528**:504–509. <https://doi.org/10.1038/nature16461>
4. Dimitri Kits K et al. Kinetic analysis of a complete nitrifier reveals an oligotrophic lifestyle. *Nature* 2017;**549**:269–272. <https://doi.org/10.1038/NATURE23679>
5. Daims H et al. In Situ Characterization of *Nitrospira* -Like Nitrite-Oxidizing Bacteria Active in Wastewater Treatment Plants. *Appl Environ Microbiol* 2001;**67**:5273–5284. <https://doi.org/10.1128/AEM.67.11.5273-5284.2001>
6. Schramm A et al. Identification and Activities In Situ of *Nitrosospira* and *Nitrospira* spp. as Dominant Populations in a Nitrifying Fluidized Bed Reactor. *Appl Environ Microbiol* 1998;**64**:3480–3485. <https://doi.org/10.1128/AEM.64.9.3480-3485.1998>
7. Martiny AC et al. Identification of Bacteria in Biofilm and Bulk Water Samples from a Nonchlorinated Model Drinking Water Distribution System: Detection of a Large Nitrite-Oxidizing Population Associated with *Nitrospira* spp. *Appl Environ Microbiol* 2005;**71**:8611–8617. <https://doi.org/10.1128/AEM.71.12.8611-8617.2005>
8. Lebedeva E V. et al. Isolation and characterization of a moderately thermophilic nitrite-oxidizing bacterium from a geothermal spring. *FEMS Microbiol Ecol* 2011;**75**:195–204. <https://doi.org/10.1111/j.1574-6941.2010.01006.x>
9. Watson SW et al. *Nitrospira marina* gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Arch Microbiol* 1986;**144**:1–7. <https://doi.org/10.1007/BF00454947>
10. Hovanec TA et al. *Nitrospira*-Like Bacteria Associated with Nitrite Oxidation in Freshwater Aquaria. *Appl Environ Microbiol* 1998;**64**:258–264. <https://doi.org/10.1128/AEM.64.1.258-264.1998>
11. Pester M et al. *NxrB* encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic marker for nitrite-oxidizing *Nitrospira*. *Environ Microbiol* 2014;**16**:3055–3071. <https://doi.org/10.1111/1462-2920.12300>

12. Sakoula D et al. Enrichment and physiological characterization of a novel comammox *Nitrospira* indicates ammonium inhibition of complete nitrification. *ISME J* 2021;**15**:1010–1024. <https://doi.org/10.1038/s41396-020-00827-4>
13. Koch H et al. Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proc Natl Acad Sci U S A* 2015;**112**:11371–11376. <https://doi.org/10.1073/pnas.1506533112>
14. Peck Jr HD, Strohl WR, Tuorinen OH. Microbial Chemoautotrophy. Ohio State University Press, Columbus, 1983.
15. Kits KD et al. Low yield and abiotic origin of N₂O formed by the complete nitrifier *Nitrospira inopinata*. *Nat Commun* 2019;**10**. <https://doi.org/10.1038/s41467-019-09790-x>
16. Caranto JD, Lancaster KM. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. *Proc Natl Acad Sci U S A* 2017;**114**:8217–8222. <https://doi.org/10.1073/pnas.1704504114>
17. Kozlowski JA, Kits KD, Stein LY. Comparison of Nitrogen Oxide Metabolism among Diverse Ammonia-Oxidizing Bacteria. *Front Microbiol* 2016;**7**. <https://doi.org/10.3389/fmicb.2016.01090>
18. Stieglmeier M et al. Aerobic nitrous oxide production through N-nitrosating hybrid formation in ammonia-oxidizing archaea. *ISME J* 2014;**8**:1135–1146. <https://doi.org/10.1038/ismej.2013.220>
19. Kobayashi S et al. Nitric Oxide Production from Nitrite Reduction and Hydroxylamine Oxidation by Copper-containing Dissimilatory Nitrite Reductase (NirK) from the Aerobic Ammonia-oxidizing Archaeon, *Nitrososphaera viennensis*. *Microbes Environ* 2018;**33**:428–434. <https://doi.org/10.1264/jsme2.ME18058>
20. Alluisetti GE et al. Metal-catalyzed anaerobic disproportionation of hydroxylamine. Role of diazene and nitroxyl intermediates in the formation of N₂, N₂O, NO⁺, and NH₃. *J Am Chem Soc* 2004;**126**:13432–13442. <https://doi.org/10.1021/ja046724i>
21. Liu S et al. Abiotic Conversion of Extracellular NH₂OH Contributes to N₂O Emission during Ammonia Oxidation. *Environ Sci Technol* 2017;**51**:13122–13132. <https://doi.org/10.1021/acs.est.7b02360>
22. Maalcke WJ et al. Structural Basis of Biological NO Generation by Octaheme Oxidoreductases. *Journal of Biological Chemistry* 2014;**289**:1228–1242. <https://doi.org/10.1074/jbc.M113.525147>

23. Hooper AB, Nason A. Characterization of Hydroxylamine-Cytochrome c Reductase from the Chemoautotrophs *Nitrosomonas europaea* and *Nitrosocystis oceanus*. *Journal of Biological Chemistry* 1965;**240**:4044–4057. [https://doi.org/10.1016/S0021-9258\(18\)97148-6](https://doi.org/10.1016/S0021-9258(18)97148-6)
24. Versantvoort W et al. Multiheme hydroxylamine oxidoreductases produce NO during ammonia oxidation in methanotrophs. *Proceedings of the National Academy of Sciences* 2020;**117**:24459–24463. <https://doi.org/10.1073/pnas.2011299117>
25. Bruijn P et al. Growth of *Nitrosomonas europaea* on hydroxylamine. *FEMS Microbiol Lett* 1995;**125**:179–184. <https://doi.org/10.1111/j.1574-6968.1995.tb07355.x>
26. Shimamura M et al. Another multiheme protein, hydroxylamine oxidoreductase, abundantly produced in an anammox bacterium besides the hydrazine-oxidizing enzyme. *J Biosci Bioeng* 2008;**105**:243–248. <https://doi.org/10.1263/jbb.105.243>
27. Camejo PY et al. Genome-Enabled Insights into the Ecophysiology of the Comammox Bacterium “*Candidatus Nitrospira nitrosa*”. *mSystems* 2017;**2**. <https://doi.org/10.1128/mSystems.00059-17>
28. Roots P et al. Comammox *Nitrospira* are the dominant ammonia oxidizers in a mainstream low dissolved oxygen nitrification reactor. *Water Res* 2019;**157**:396–405. <https://doi.org/10.1016/j.watres.2019.03.060>
29. Kleerebezem R, Lücker S. Cyclic Conversions in the Nitrogen Cycle. *Front Microbiol* 2021;**12**. <https://doi.org/10.3389/fmicb.2021.622504>
30. Castignetti D, Gunner HB. Differential tolerance of hydroxylamine by an *Alcaligenes* sp., a heterotrophic nitrifier, and by *Nitrobacter agilis*. *Can J Microbiol* 1982;**28**:148–150. <https://doi.org/10.1139/m82-018>
31. Soler-Jofra A, Pérez J, van Loosdrecht MCM. Hydroxylamine and the nitrogen cycle: A review. *Water Res* 2021;**190**:116723. <https://doi.org/10.1016/J.WATRES.2020.116723>
32. Yu R et al. *Nitrosomonas europaea* adaptation to anoxic-oxic cycling: Insights from transcription analysis, proteomics and metabolic network modeling. *Science of The Total Environment* 2018;**615**:1566–1573. <https://doi.org/10.1016/J.SCITOTENV.2017.09.142>
33. Daims H, Lücker S, Wagner M. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. *Trends Microbiol* 2016;**24**:699–712. <https://doi.org/10.1016/j.tim.2016.05.004>

34. Sabba F et al. Hydroxylamine Diffusion Can Enhance N₂O Emissions in Nitrifying Biofilms: A Modeling Study. *Environ Sci Technol* 2015;**49**:1486–1494. <https://doi.org/10.1021/es5046919>
35. Füssel J et al. Adaptability as the key to success for the ubiquitous marine nitrite oxidizer *Nitrococcus*. *Sci Adv* 2017;**3**. <https://doi.org/10.1126/sciadv.1700807>
36. Freitag A, Rudert M, Bock E. Growth of *Nitrobacter* by dissimilatoric nitrate reduction. *FEMS Microbiol Lett* 1987;**48**:105–109. <https://doi.org/10.1111/j.1574-6968.1987.tb02524.x>
37. Sundermeyer-Klinger H et al. Membrane-bound nitrite oxidoreductase of *Nitrobacter*: evidence for a nitrate reductase system. *Arch Microbiol* 1984;**140**:153–158. <https://doi.org/10.1007/BF00454918>
38. Leung PM et al. A nitrite-oxidising bacterium constitutively consumes atmospheric hydrogen. *ISME Journal* 2022;**16**:2213–2219. <https://doi.org/10.1038/s41396-022-01265-0>
39. Heil J et al. Site-specific ¹⁵N isotopic signatures of abiotically produced N₂O. *Geochim Cosmochim Acta* 2014;**139**:72–82. <https://doi.org/10.1016/j.gca.2014.04.037>
40. Spott O, Russow R, Stange CF. Formation of hybrid N₂O and hybrid N₂ due to codenitrification: First review of a barely considered process of microbially mediated N-nitrosation. *Soil Biol Biochem* 2011;**43**:1995–2011. <https://doi.org/10.1016/j.soilbio.2011.06.014>
41. Jung M-Y et al. Isotopic signatures of N₂O produced by ammonia-oxidizing archaea from soils. *ISME J* 2014;**8**:1115–1125. <https://doi.org/10.1038/ismej.2013.205>
42. Hulse CL, Tiedje JM, Averill BA. Evidence for a copper-nitrosyl intermediate in denitrification by the copper-containing nitrite reductase of *Achromobacter cycloclastes*. *J Am Chem Soc* 1989;**111**:2322–2323. <https://doi.org/10.1021/ja00188a067>
43. Sun D et al. Chlorate as a comammox *Nitrospira* specific inhibitor reveals nitrification and N₂O production activity in coastal wetland. *Soil Biol Biochem* 2022;**173**:108782. <https://doi.org/10.1016/j.soilbio.2022.108782>
44. Stieglmeier M et al. *Nitrososphaera viennensis* gen. nov., sp. nov., an aerobic and mesophilic, ammonia-oxidizing archaeon from soil and a member of the archaeal phylum Thaumarchaeota. *Int J Syst Evol Microbiol* 2014;**64**:2738–2752. <https://doi.org/10.1099/ij.s.0.063172-0>

45. Martens-Habbena W et al. The production of nitric oxide by marine ammonia-oxidizing archaea and inhibition of archaeal ammonia oxidation by a nitric oxide scavenger. *Environ Microbiol* 2015;**17**:2261–2274. <https://doi.org/10.1111/1462-2920.12677>
46. Feng J et al. An Optimized SYBR Green I/PI Assay for Rapid Viability Assessment and Antibiotic Susceptibility Testing for *Borrelia burgdorferi*. *PLoS One* 2014;**9**:e111809. <https://doi.org/10.1371/journal.pone.0111809>
47. Lunau M et al. An improved method for counting bacteria from sediments and turbid environments by epifluorescence microscopy. *Environ Microbiol* 2005;**7**:961–968. <https://doi.org/10.1111/j.1462-2920.2005.00767.x>
48. McIlvin MR, Casciotti KL. Fully automated system for stable isotopic analyses of dissolved nitrous oxide at natural abundance levels. *Limnol Oceanogr Methods* 2010;**8**:54–66. <https://doi.org/10.4319/lom.2010.8.54>
49. Wan XS et al. Epipelagic nitrous oxide production offsets carbon sequestration by the biological pump. *Nat Geosci* 2023;**16**:29–36. <https://doi.org/10.1038/s41561-022-01090-2>
50. McIlvin MR, Altabet MA. Chemical Conversion of Nitrate and Nitrite to Nitrous Oxide for Nitrogen and Oxygen Isotopic Analysis in Freshwater and Seawater. *Anal Chem* 2005;**77**:5589–5595. <https://doi.org/10.1021/ac050528s>
51. Granger J, Sigman DM. Removal of nitrite with sulfamic acid for nitrate N and O isotope analysis with the denitrifier method. *Rapid Communications in Mass Spectrometry* 2009;**23**:3753–3762. <https://doi.org/10.1002/rcm.4307>
52. Weigand MA et al. Updates to instrumentation and protocols for isotopic analysis of nitrate by the denitrifier method. *Rapid Communications in Mass Spectrometry* 2016;**30**:1365–1383. <https://doi.org/10.1002/rcm.7570>
53. Zhang L et al. Sensitive Measurement of $\text{NH}_4^+^{15}\text{N}/^{14}\text{N}$ ($\delta^{15}\text{NH}_4^+$) at Natural Abundance Levels in Fresh and Saltwaters. *Anal Chem* 2007;**79**:5297–5303. <https://doi.org/10.1021/ac070106d>
54. Peng X et al. Long-term fertilization alters the relative importance of nitrate reduction pathways in salt marsh sediments. *J Geophys Res Biogeosci* 2016;**121**:2082–2095. <https://doi.org/10.1002/2016JG003484>
55. Frear DS, Burrell RC. Spectrophotometric Method for Determining Hydroxylamine Reductase Activity in Higher Plants. *Anal Chem* 1955;**27**:1664–1665. <https://doi.org/10.1021/ac60106a054>

56. Koch H et al. Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. *Science (1979)* 2014;**345**:1052–1054. <https://doi.org/10.1126/science.1256985>
57. Sander R. Compilation of Henry’s law constants (version 4.0) for water as solvent. *Atmos Chem Phys* 2015;**15**:4399–4981. <https://doi.org/10.5194/acp-15-4399-2015>
58. Li Z et al. Genome-Resolved Proteomic Stable Isotope Probing of Soil Microbial Communities Using ¹³CO₂ and ¹³C-Methanol. *Front Microbiol* 2019;**10**. <https://doi.org/10.3389/fmicb.2019.02706>
59. Yao Q et al. Community proteogenomics reveals the systemic impact of phosphorus availability on microbial functions in tropical soil. *Nat Ecol Evol* 2018;**2**:499–509. <https://doi.org/10.1038/s41559-017-0463-5>
60. Tao X et al. Polyethylene Degradation by a Rhodococcous Strain Isolated from Naturally Weathered Plastic Waste Enrichment. *Environ Sci Technol* 2023;**57**:13901–13911. <https://doi.org/10.1021/acs.est.3c03778>
61. Wang D et al. Cross-Feedings, Competition, and Positive and Negative Synergies in a Four-Species Synthetic Community for Anaerobic Degradation of Cellulose to Methane. *mBio* 2023;**14**. <https://doi.org/10.1128/mbio.03189-22>
62. Walker JM. The Bicinchoninic Acid (BCA) Assay for Protein Quantitation.
63. Guo X et al. Sipros Ensemble improves database searching and filtering for complex metaproteomics. *Bioinformatics* 2018;**34**:795–802. <https://doi.org/10.1093/bioinformatics/btx601>
64. Wang Y et al. Sipros/ProRata: A versatile informatics system for quantitative community proteomics. *Bioinformatics* 2013;**29**:2064–2065. <https://doi.org/10.1093/bioinformatics/btt329>
65. Pan C et al. ProRata: A quantitative proteomics program for accurate protein abundance ratio estimation with confidence interval evaluation. *Anal Chem* 2006;**78**:7121–7131. <https://doi.org/10.1021/ac060654b>
66. Li Z et al. Diverse and divergent protein post-translational modifications in two growth stages of a natural microbial community. *Nat Commun* 2014;**5**. <https://doi.org/10.1038/ncomms5405>

67. Li Z et al. Integrated proteomics and metabolomics suggests symbiotic metabolism and multimodal regulation in a fungal-endobacterial system. *Environ Microbiol* 2017;**19**:1041–1053. <https://doi.org/10.1111/1462-2920.13605>
68. Välikangas T, Suomi T, Elo LL. A systematic evaluation of normalization methods in quantitative label-free proteomics. *Brief Bioinform* 2018;**19**:1–11. <https://doi.org/10.1093/bib/bbw095>
69. Lazar C et al. Accounting for the Multiple Natures of Missing Values in Label-Free Quantitative Proteomics Data Sets to Compare Imputation Strategies. *J Proteome Res* 2016;**15**:1116–1125. <https://doi.org/10.1021/acs.jproteome.5b00981>
70. Shah AD et al. LFQ-Analyst: An easy-To-use interactive web platform to analyze and visualize label-free proteomics data preprocessed with maxquant. *J Proteome Res* 2019;204–211. <https://doi.org/10.1021/acs.jproteome.9b00496>
71. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 2014;**15**:550. <https://doi.org/10.1186/s13059-014-0550-8>
72. Bayer B et al. Metabolic versatility of the nitrite-oxidizing bacterium *Nitrospira marina* and its proteomic response to oxygen-limited conditions. *ISME Journal* 2021;**15**:1025–1039. <https://doi.org/10.1038/s41396-020-00828-3>

Figure legends

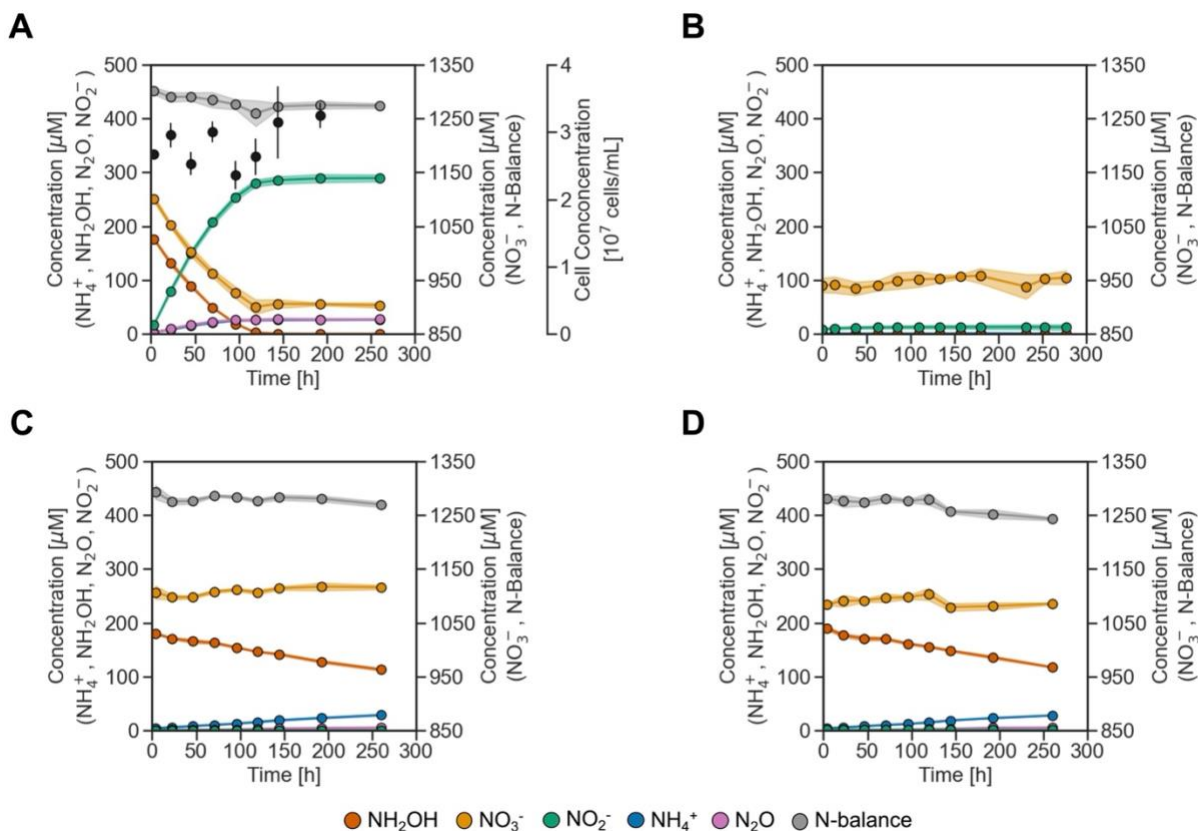


Figure 5.1. Hydroxylamine-dependent nitrate reduction by *N. inopinata*.

Hydroxylamine-dependent nitrate reduction was observed in biotic incubations under hydroxylamine replete conditions (A). Biotic nitrate reduction did not occur in the absence of the electron donor hydroxylamine (B). Abiotic hydroxylamine decay was observed in a filtrate (C) and heat-killed biomass controls (D). All incubations were monitored until the biotic depletion of hydroxylamine at 259.8 h. Nitrous oxide and ammonium were biotically produced in a near stoichiometric ratio in the hydroxylamine-replete incubation with *N. inopinata* causing nitrous oxide (pink) to mask ammonium (blue) in Figure 1A. Cell concentrations in the biotic incubation were monitored until hydroxylamine depletion at 192.1 h (A) and cell concentrations were not monitored for the biotic hydroxylamine-deplete incubation (B). Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), nitrous oxide (pink), ammonia (blue), nitrogen balance (grey), *N. inopinata* cell (black) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations.

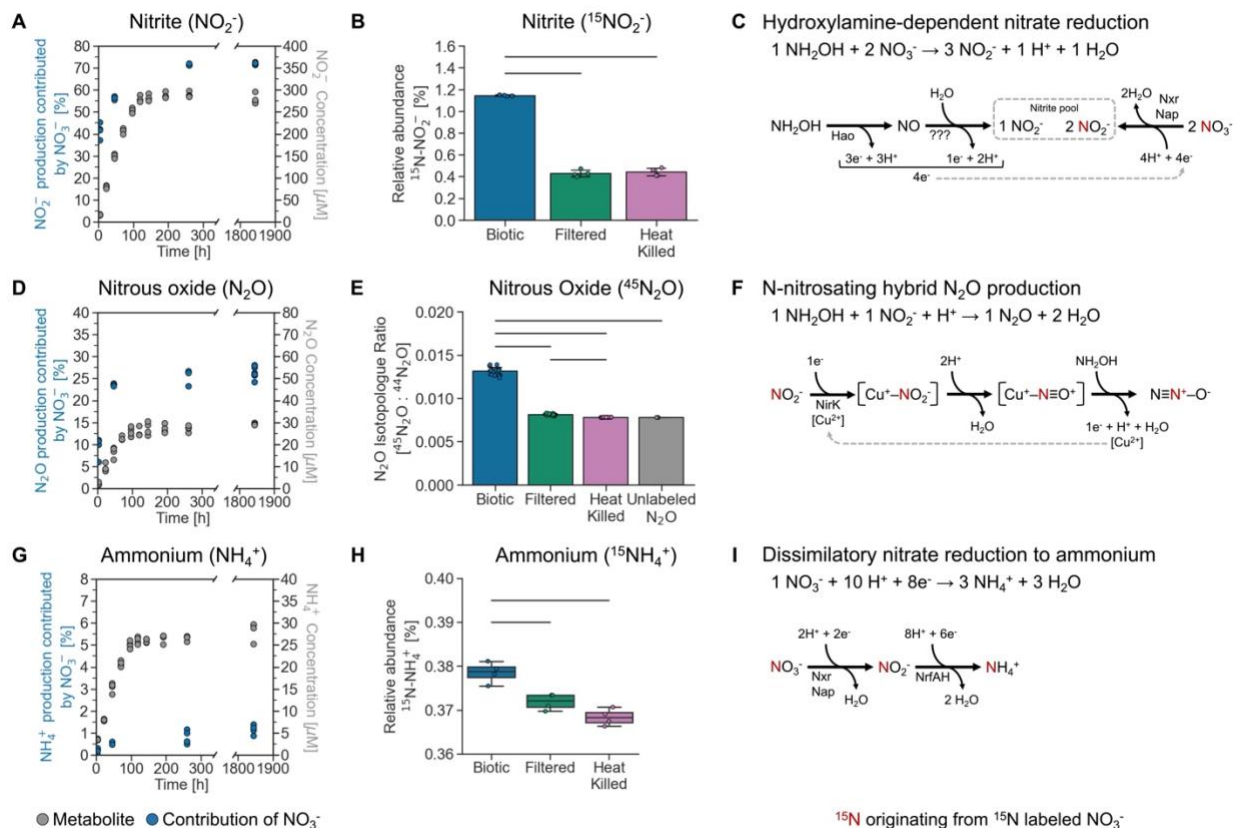


Figure 5.2. Contribution of NO_3^- to hydroxylamine-dependent nitrate reduction products

The percent contribution of nitrate to the production of nitrite (A), nitrous oxide (D), and ammonium (E) during hydroxylamine dependent nitrate reduction by *N. inopinata*. The nitrate contribution percentage was determined with stable isotope tracing. Percent contribution is presented in blue and the bulk metabolite concentration is presented in grey. Endpoint $^{45}\text{N}_2\text{O}$: $^{44}\text{N}_2\text{O}$ isotopologue ratios (E) and ^{15}N relative abundance of NO_2^- (B) and NH_4^+ (H) for the biotic (blue), filtrate (green), and heat-killed (pink) incubation. Bars indicates statistical difference between the samples ($p < 0.01$; Tukey HSD). Proposed pathways leading the enrichment of ^{15}N in the biotic incubation (C, F, I).

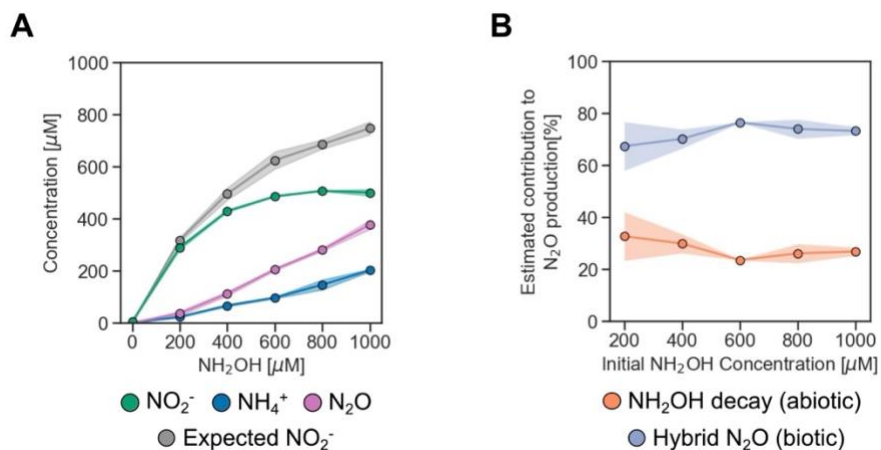


Figure 5.3. Impact of hydroxylamine on hydroxylamine-dependent nitrate reduction.

Endpoint concentrations of nitrite (green), nitrous oxide (pink), and ammonium (blue) when incubated with hydroxylamine (x-axis) and nitrate (A). The expected nitrite concentration (grey) from hydroxylamine dependent nitrate reduction was calculated with the total concentration of nitrate reduced and the theoretical reaction stoichiometry. The estimated contribution of hybrid N₂O formation (yellow) and NH₂OH decay (light blue) to nitrous oxide production across hydroxylamine concentrations (B). N₂O produced from NH₂OH decay was estimated with NH₄⁺ and the expected reaction stoichiometry. All remaining N₂O was assumed to be produced from hybrid N₂O formation. Shaded areas in all figures represent 95% confidence intervals.

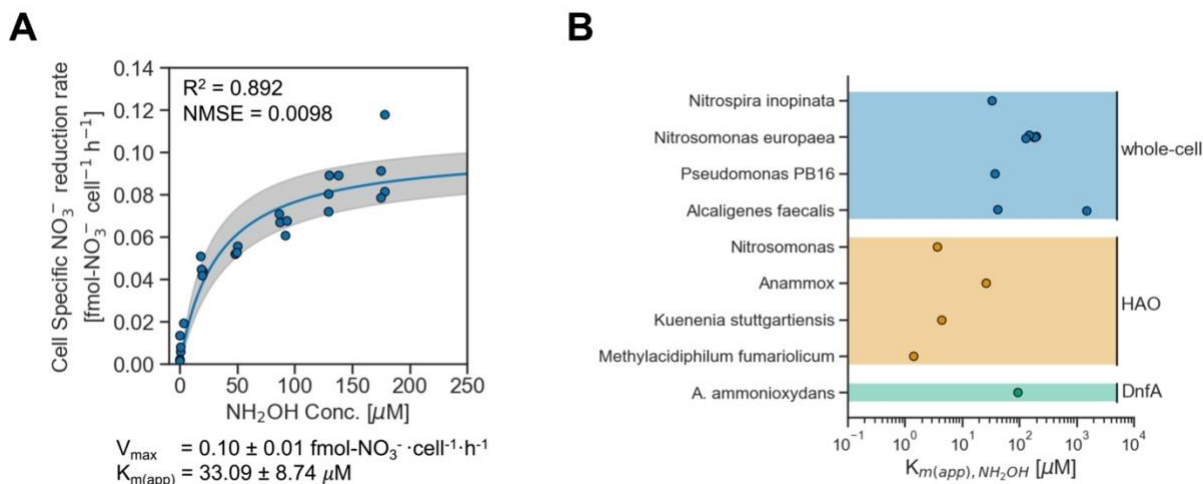


Figure 5.4. Whole-cell NH_2OH oxidation kinetics.

Whole-cell NH_2OH oxidation kinetics of *N. inopinata* during hydroxylamine dependent nitrate reduction (A). Kinetic constants were obtained by fitting a Michaelis-Menten equation (blue line) to total cell specific nitrate reduction rate with shaded areas represent the 95% confidence intervals. The measured whole-cell hydroxylamine affinity was compared to other hydroxylamine affinities (B) from whole-cell incubations (blue), *in vitro* HAO enzyme assay (yellow), and an *in vitro* DnfA enzyme assay (green).

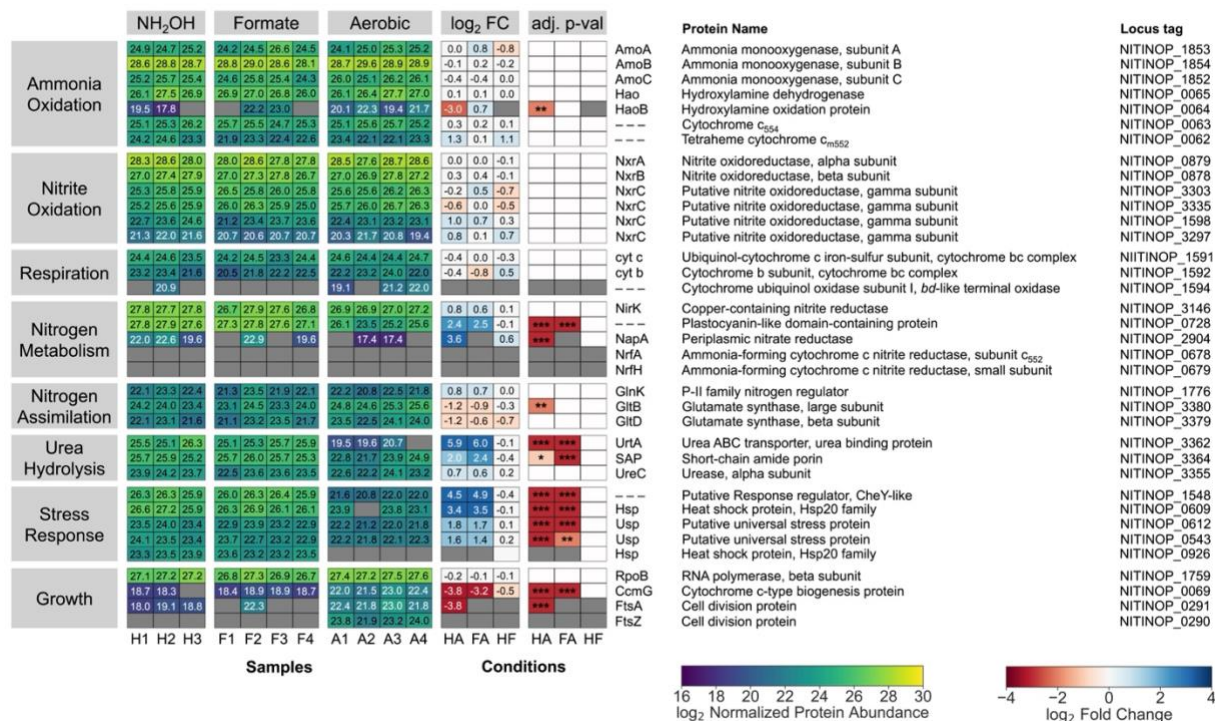


Figure 5.5. Heat map of selected *N. inopinata* proteins abundance.

Normalized protein abundance values were log₂-transformed and grey color indicates absence from the proteome. The complete set of untransformed values is listed in Table S1. Biological replicates under anaerobic hydroxylamine-dependent nitrate reducing (H1-3), anaerobic formate-dependent nitrate reducing (F1-4), and aerobic ammonia-oxidizing conditions (A1-4) were collected to compare protein abundance. The log₂-fold changes between each condition (HA, HF, and FA) and the corresponding significance values (-log₁₀-transformed adjusted *p* values) are shown to the right of the protein abundances. Red and blue color indicates a significant positive and negative fold changes, respectively. Grey indicates statistical comparisons could not be made due to low protein recovery. Select proteins of interest were included despite not being statistically significant. Proteins are clustered by functional category (left) and labeled with annotations and locus tag numbers (right).

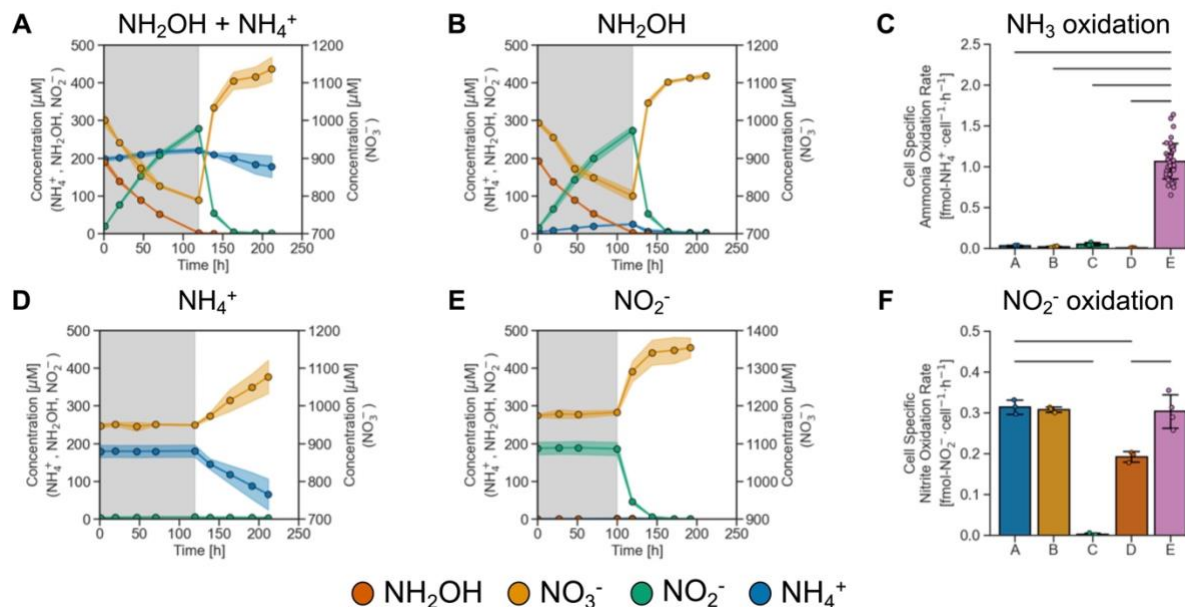
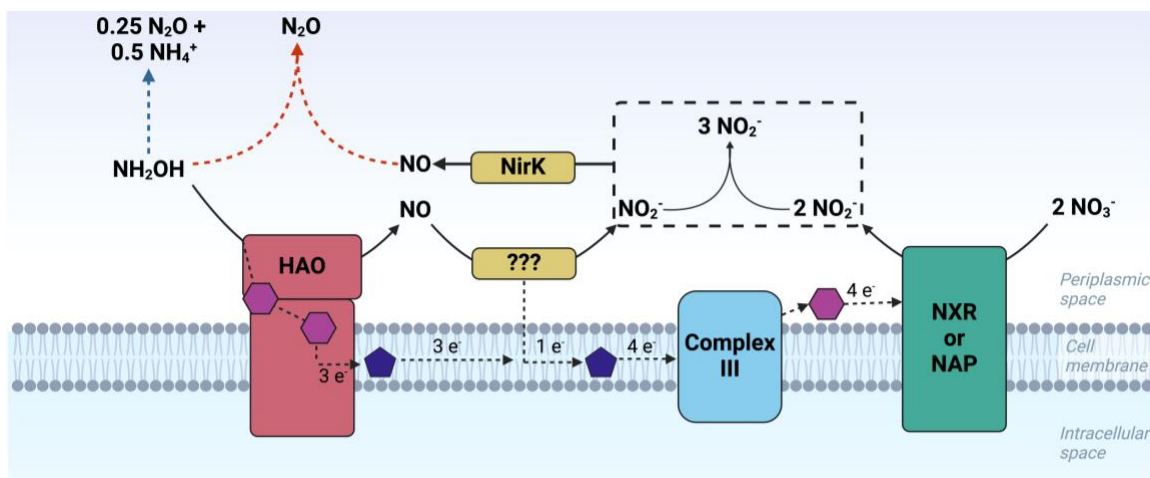


Figure 5.6. Recovery of ammonia and nitrite oxidation after an anaerobic-aerobic transition.

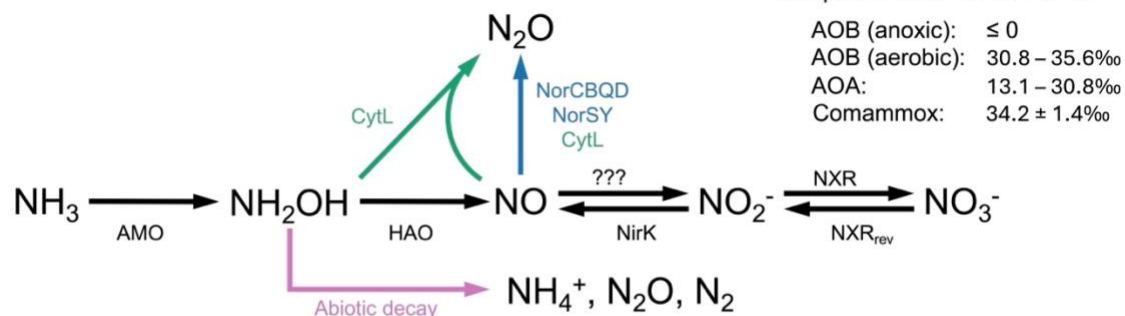
The recovery of ammonia and nitrite oxidation by *N. inopinata* after being exposed to anaerobic conditions for 100 hours (A-D). *N. inopinata* was anaerobically incubated with NH₂OH and NH₄⁺ (A); NH₂OH (B); NH₄⁺ (D); and NO₂⁻ (E). The cultures were transitioned to aerobic conditions after 100 hours and the cell-specific ammonia and nitrite oxidation rates were measured. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), and ammonia (blue) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations. Cell-specific rates were compared to rates measured under aerobic conditions (E-F). The x-axis corresponds to the figure label with E represent the aerobic rate. Bars above bars indicates a significant difference between rates (p < 0.01; Tukey HSD).



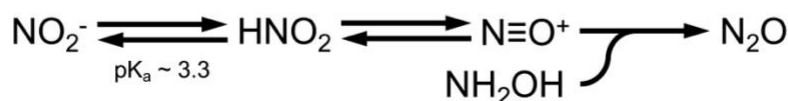
Extended Figure 5.1. Genome-based model of hydroxylamine-dependent nitrate reduction.

Hypothesized hydroxylamine-dependent nitrate reduction pathway in *N. inopinata*. Hydroxylamine is oxidized to nitrite via the hydroxylamine oxidoreductase (*hao*) and an unknown enzyme oxidizing nitric oxide. Electrons are transferred from hydroxylamine to cytochromes (pink hexagon). Electrons are donated to quinones (purple pentagons) that transfer electron to a soluble cytochrome via the proton pumping complex III. The electrons can be accepted by the nitrite oxidoreductase (*nxr*) or the periplasmic nitrate reductase (*nap*) to reduce nitrate to nitrite. Nitrite can be reduced to nitric oxide via *nirK*, which can react with hydroxylamine to produce nitrous oxide (N_2O). Alternatively, the hydroxylamine could decay to ammonium (NH_4^+) and N_2O . Solid black arrows represent enzymatic reactions, dashed black lines represent the flow of electrons and electron carriers, red dashed lines represent the hybrid N_2O formation pathway, and the blue dashed lines represent abiotic hydroxylamine decay.

Nitrifier-denitrification

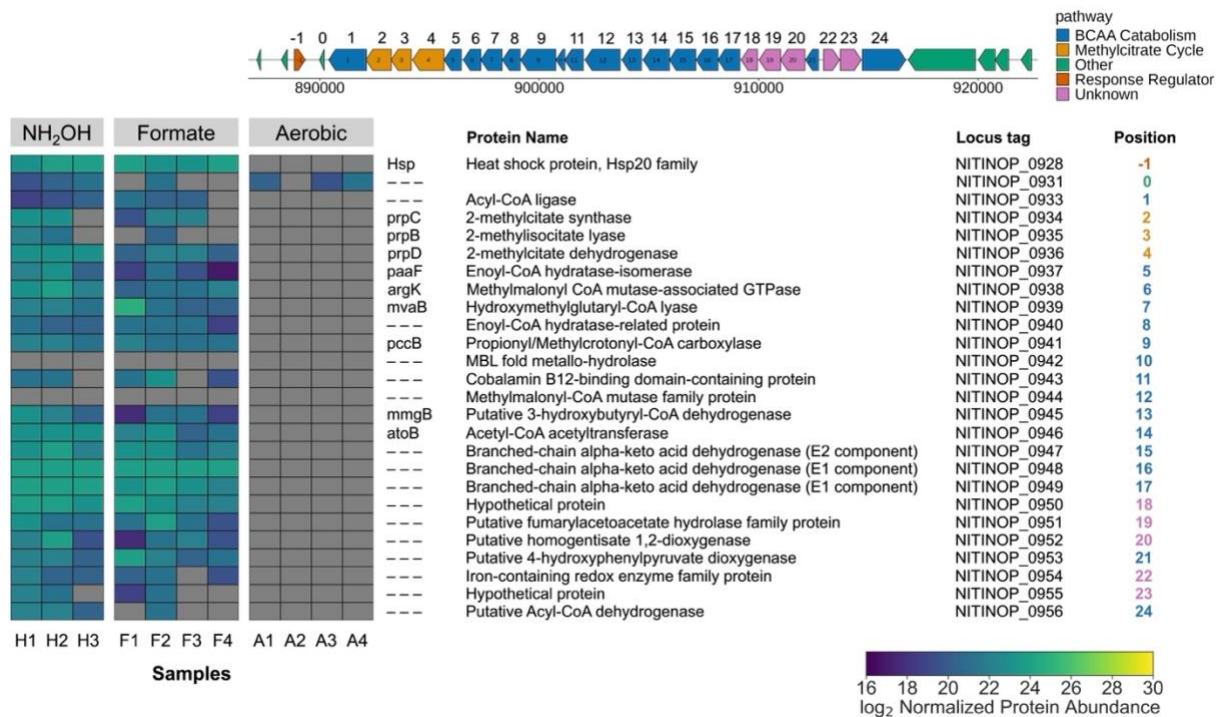


N-nitrosating hybrid N_2O



Extended Figure 5.2. N_2O production pathways by ammonia-oxidizing microorganisms.

Schematic overview of pathway and the enzymes involved in nitrifier-denitrification (AOB) and N-nitrosating hybrid N_2O production (AOA). Nitrifier-denitrification by AOB can aerobically produce N_2O with *cytL* or anaerobically reduce nitric oxide with *nor* or *cytL*. N-nitrosating hybrid N_2O production by AOA can form a nitroxyl intermediate (NO^+) that reacts with hydroxylamine to produce N_2O .



Extended Figure 5.3. BCAA catabolism operon expressed during anaerobic nitrate reduction.

Heat map of protein expression of the BCAA catabolism operon during hydroxylamine-dependent nitrate reduction (H1-3), formate-dependent nitrate reduction (F1-4), and aerobic ammonia oxidation (A1-4). Each protein is individually labeled with a number corresponding to the gene in the BCAA operon. Color boxes next to the protein signifying BCAA catabolism (blue), methylcitrate cycle (orange), response regulators (red), unknown (pink), and other (green).

Appendix A. Supplemental Material

The following document is intended to support the results presented in the main manuscript.

Figures

- Figure S1.** *N. inopinata* is unable to couple the oxidation of ammonia with nitrate reduction
Figure S2. NH_2OH does not influence hydroxylamine dependent nitrate reduction
Figure S3. Hydroxylamine-dependent nitrate reduction by *N. inopinata* and control incubations
Figure S4. Absence of an abiotic reaction between NH_2OH and NO_2^-
Figure S5. ^{15}N relative abundance across the biotic, filtrate, and heat-killed incubations
Figure S6. Contribution of $^{15}\text{NO}_3^-$ to N_2O production
Figure S7. Nitrous oxide isotopologue ratios
Figure S8. Endpoint nitrous oxide isotopologue ratios
Figure S9. Influence of NH_2OH on hydroxylamine-dependent nitrate reduction
Figure S10. Influence of NH_2OH on NO_2^- production
Figure S11. Proteomic experiment incubation data
Figure S12. Synteny of the BCAA operon within *Nitrospira* isolates
Figure S13. Synteny of the BCAA operon within the *Nitrospira* genus
Figure S14. Schematic overview of BCAA catabolism pathway in the *Nitrospira* genus.

Tables

- Table S1.** Theoretical and actual contribution of nitrate to the production of nitrite, nitrous oxide, and ammonium.
Table S2. Standard and actual Gibbs free energy of hydroxylamine-dependent nitrate reduction and hypothesized secondary reactions

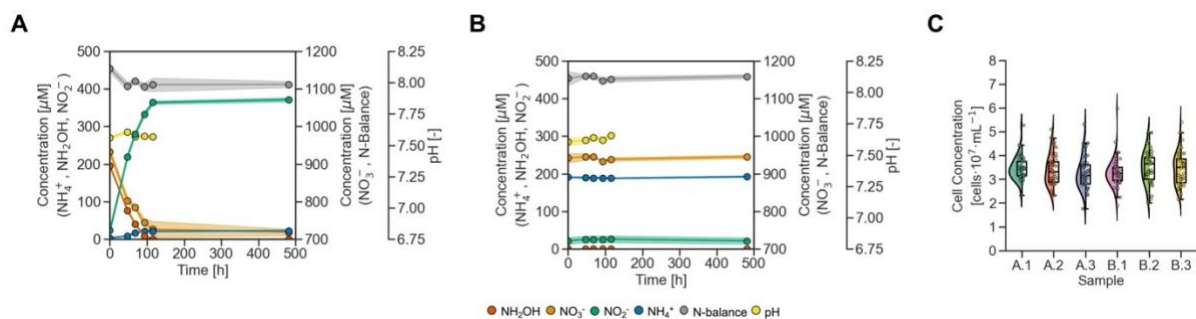


Figure S 5.1. *N. inopinata* is unable to couple the oxidation of ammonia with nitrate reduction.

Hydroxylamine-dependent nitrate reduction by *N. inopinata* (A), anaerobic incubation with ammonium (B) and nitrate, and endpoint cell counts (C). Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), nitrous oxide (pink), ammonia (blue), and nitrogen balance (grey). Shaded areas in all figures represent the standard deviations.

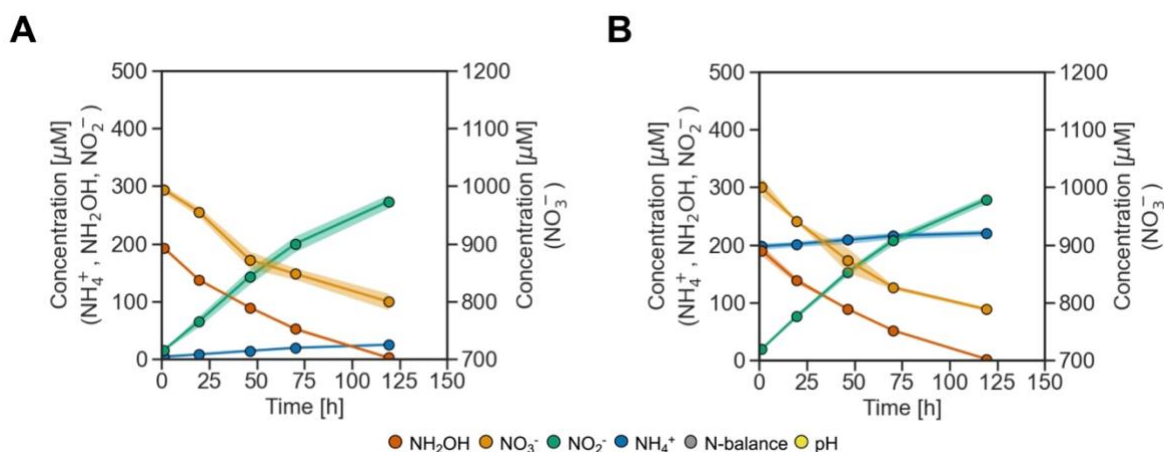


Figure S 5.2. NH_2OH does not influence hydroxylamine dependent nitrate reduction.

Hydroxylamine-dependent nitrate reduction by *N. inopinata* under ammonia-deplete (A) and ammonia-replete (B0 conditions. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), ammonia (blue), and nitrogen balance (grey). Shaded areas in all figures represent the standard deviations.

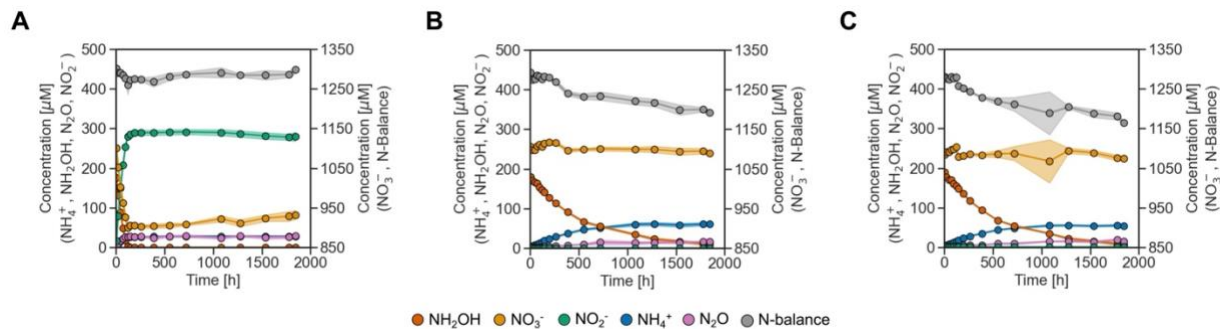


Figure S 5.3. Hydroxylamine-dependent nitrate reduction by *N. inopinata* and control incubations.

Hydroxylamine-dependent nitrate reduction by *N. inopinata* (A) and abiotic hydroxylamine decay in a filtrate (B) and heat-killed biomass controls (C). Results are reported until the near abiotic depletion of hydroxylamine at 1843.6 h. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), nitrous oxide (pink), ammonia (blue), nitrogen balance (grey), *N. inopinata* cell (black) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations.

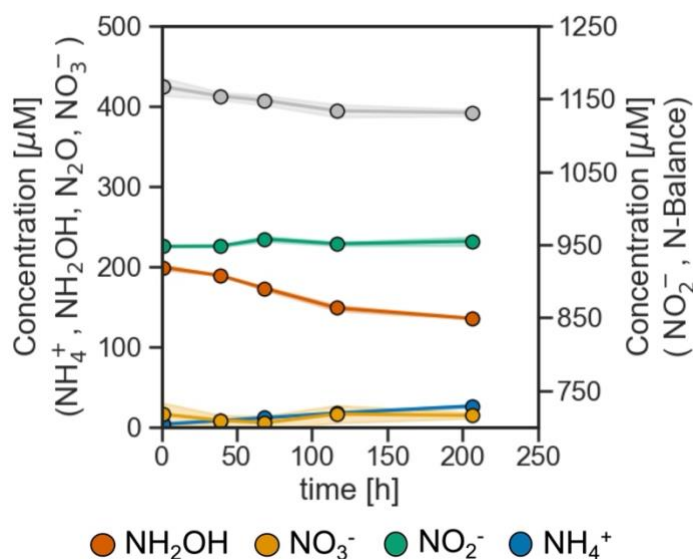


Figure S 5.4. Absence of an abiotic reaction between NH_2OH and NO_2^- .

Abiotic incubation in anaerobic media containing NH_2OH and NO_2^- . Results are reported until 200 h. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), ammonia (blue), and nitrogen balance (grey) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations.

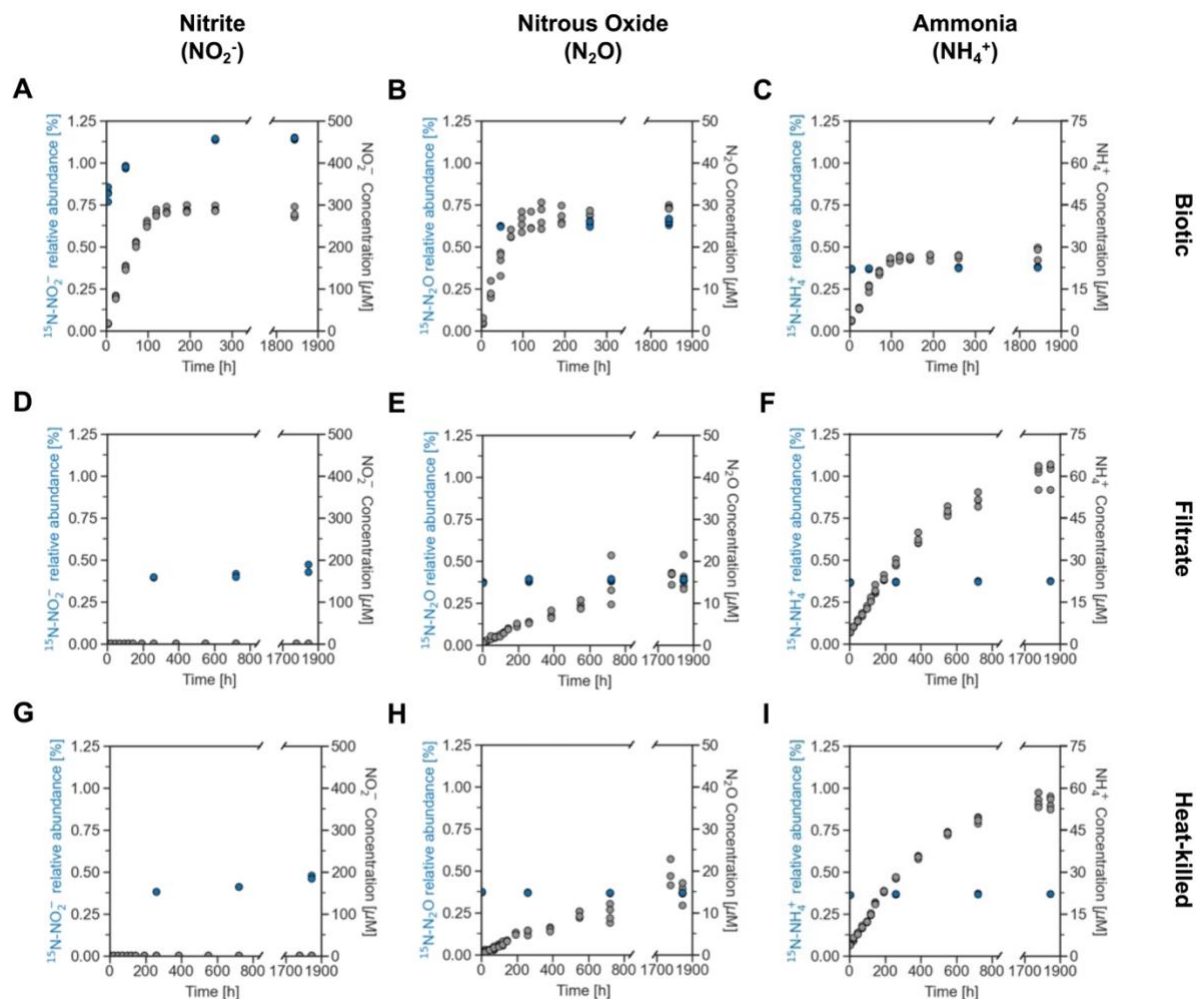


Figure S 5.5. ^{15}N relative abundance across the biotic, filtrate, and heat-killed incubations

The relative abundance of ^{15}N (blue) and bulk NO_2^- , N_2O , and NH_4^+ concentrations (grey) produced by *N. inopinata* during hydroxylamine-dependent nitrate reduction (A-C) and abiotic hydroxylamine decay in filtrate (D-F) and heat-killed biomass (G-I) controls. The relative abundance of $^{15}\text{N}\text{-NO}_2^-$ throughout the hydroxylamine-dependent nitrate reduction incubation, suggesting complex reaction dynamics, and remained stable upon hydroxylamine depletion (A). There were minimal variations in the $^{15}\text{N}\text{-N}_2\text{O}$ and $^{15}\text{N}\text{-NH}_4^+$ relative abundance was observed in the biotic incubation (B,C), however, the relative abundance was significantly higher than the in the filtrate (E,F) and heat-killed controls (H,I).

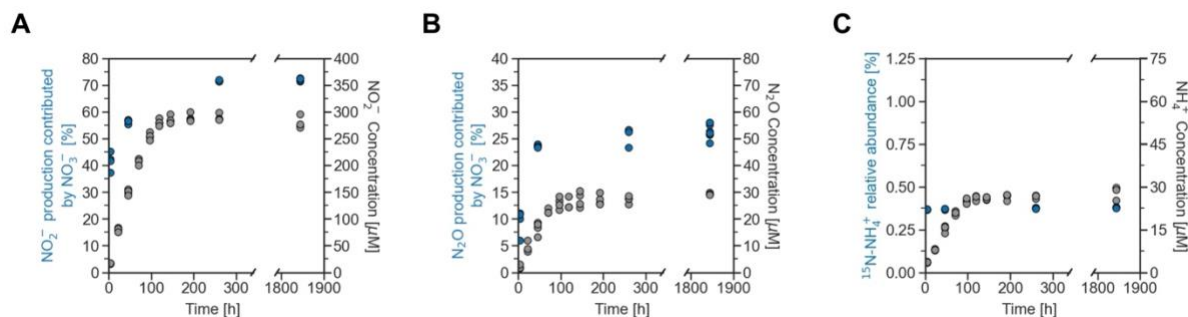


Figure S 5.6. Contribution of $^{15}\text{NO}_3^-$ to N_2O production

Percent contribution of nitrate towards nitrite (A) and nitrous oxide (B) production during hydroxylamine dependent nitrate reduction by *N. inopinata*. Percent contribution (blue), bulk metabolite concentration (grey).

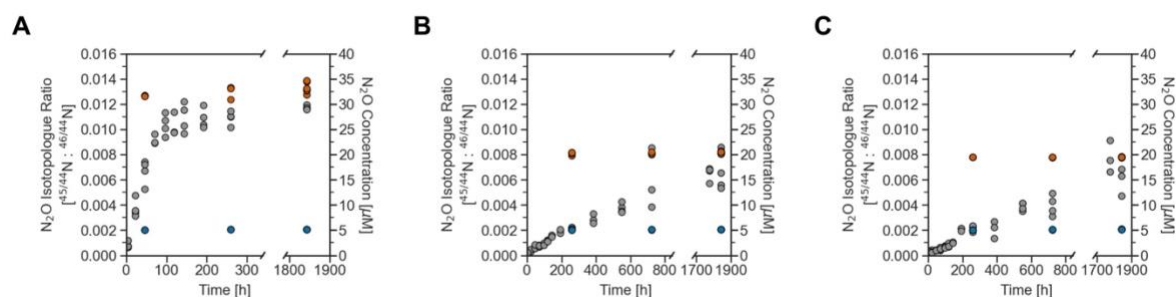


Figure S 5.7. Nitrous oxide isotopologue ratios

Nitrous oxide isotopologue ratios of single labeled to unlabeled N_2O ($^{45}\text{N}_2\text{O} : ^{44}\text{N}$ - red/vermillion) and double labeled to unlabeled N_2O ($^{46}\text{N}_2\text{O} : ^{44}\text{N}$ - blue) throughout (A) the hydroxylamine-dependent nitrate reduction (biotic) and (B) the abiotic hydroxylamine decay controls (filtrate and heat-killed biomass). Bulk N_2O concentrations are represented by grey circles. All isotopologue ratios remain constant throughout each incubation, however the $^{45}/^{44}\text{N}-\text{N}_2\text{O}$ ratio is significantly higher in the biotic incubation compared to the abiotic controls.

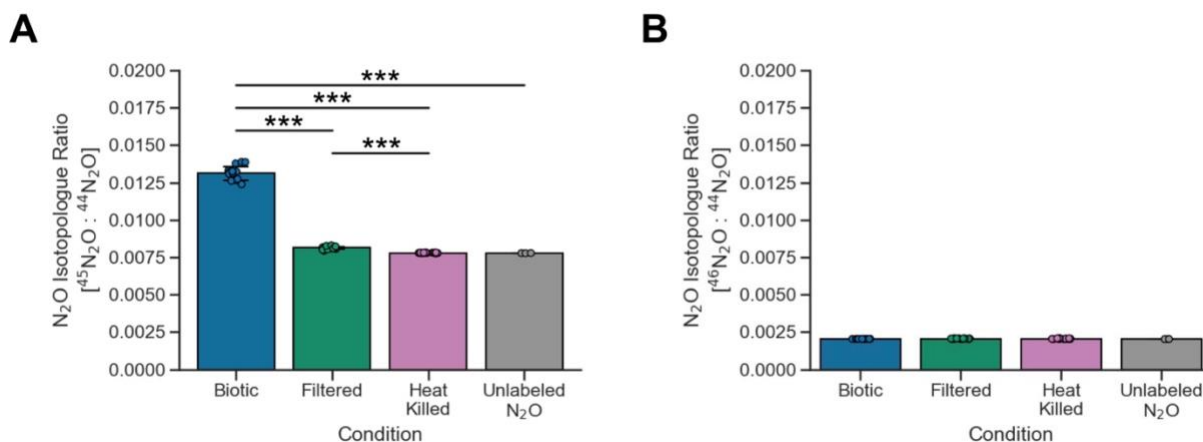


Figure S 5.8. Endpoint nitrous oxide isotopologue ratios

Endpoint isotopologue ratios of (A) $^{45}/^{44}\text{N}$ and (B) $^{46}/^{44}\text{N}$ measured for the biotic incubation (blue), hydroxylamine decay controls (green and purple), and unlabeled N₂O (grey). Nitrous oxide produced by hydroxylamine-dependent nitrate reduction was significantly enriched in $^{45}/^{44}\text{N}$ -N₂O compared to the abiotic controls and unlabeled N₂O (A), while there was no statical difference in $^{46}/^{44}\text{N}$ -N₂O between samples (B). These results suggest nitrate and hydroxylamine equally contributed to nitrous oxide production presumably via the abiotic reaction between hydroxylamine and nitric oxide.

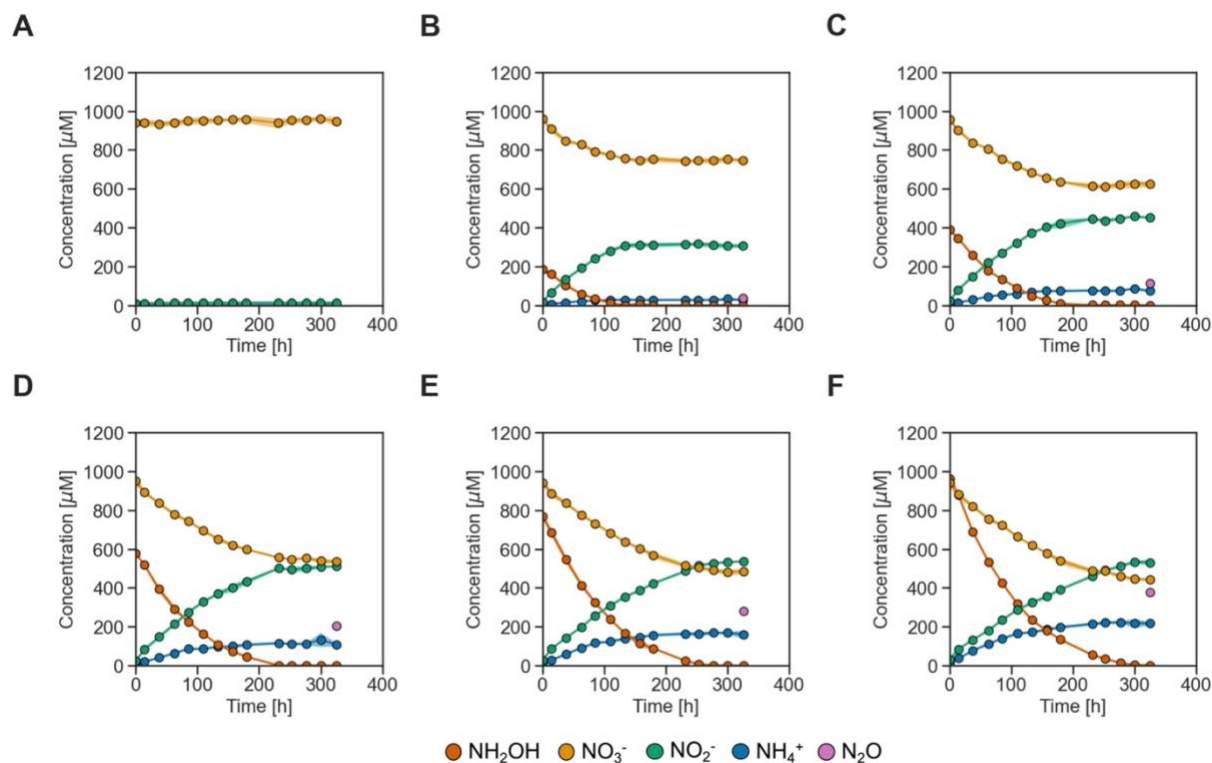


Figure S 5.9. Influence of NH_2OH on hydroxylamine-dependent nitrate reduction

Influence of initial hydroxylamine concentration on hydroxylamine-dependent nitrate reduction and product distribution. Incubation containing 950 μM nitrate and hydroxylamine concentrations ranging between 0 and 1000 μM in intervals of 200 μM were set up in triplicate and monitored for 325 h. Mean metabolite concentrations are represented with a marker and the standard deviation between replicates is represented by the shaded lines. Nitrous oxide was only measured at the final timepoint. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), nitrous oxide (pink) and, ammonia (blue) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations.

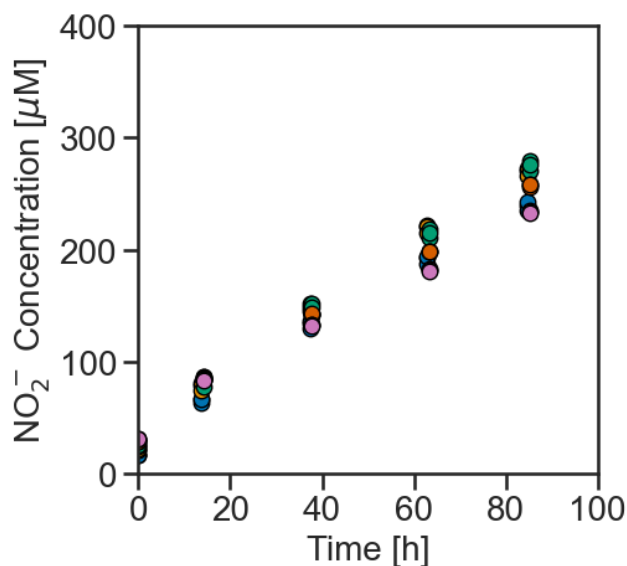


Figure S 5.10. Influence of NH_2OH on NO_2^- production.

Influence of initial hydroxylamine concentration on **NO_2^- production** by hydroxylamine-dependent nitrate reduction. Incubation containing 950 μM nitrate and hydroxylamine concentrations ranging between 0 and 1000 μM in intervals of 200 μM were set up in triplicate and monitored for 325 h.

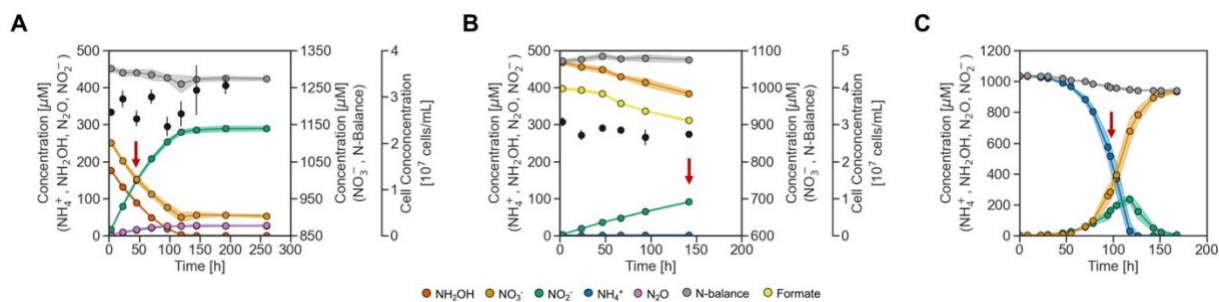


Figure S 5.11. Proteomic experiment incubation data

Activities: anaerobic hydroxylamine-dependent nitrate reduction (A), formate-dependent nitrate reduction (B), aerobic ammonia oxidation (C). Cells were harvested at the timepoints indicated by the red arrow. Hydroxylamine (red/vermillion), nitrate (orange), nitrite (green), nitrous oxide (pink), ammonia (blue), nitrogen balance (grey), *N. inopinata* cell (black) concentrations are reported according to the colors in parentheses. Shaded areas in all figures represent the standard deviations.

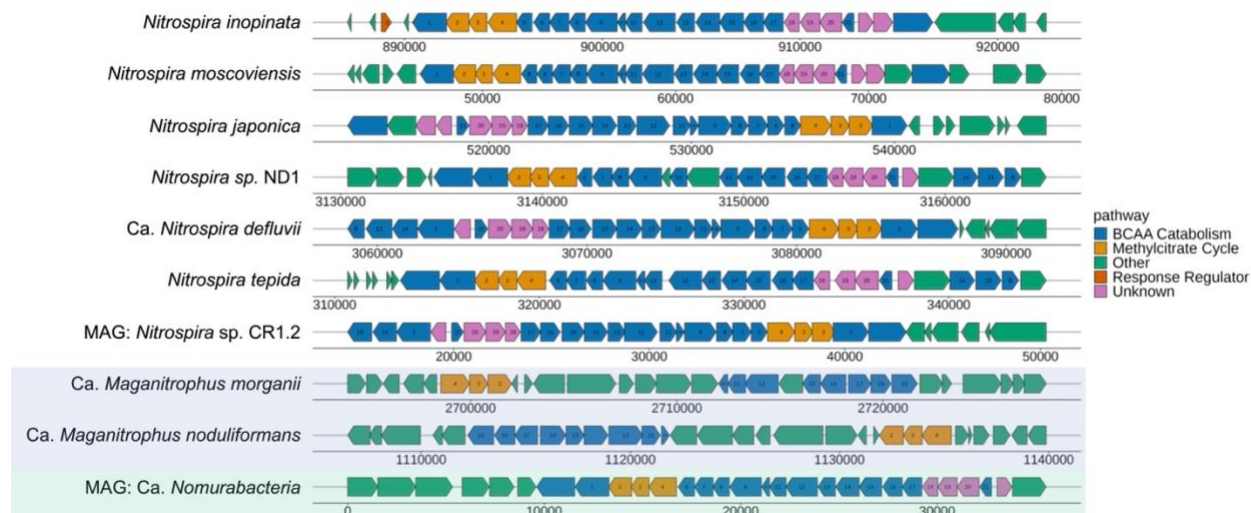


Figure S 5.12. Synteny of the BCAA operon within *Nitrospira* isolates.

Highly conserved synteny of the BCAA operon was observed within the *Nitrospira* genus, but that synteny did not extend of manganese oxidizing *Nitrospirota* (light blue). Similar synteny was found in a CPR bacteria MAG (light green).

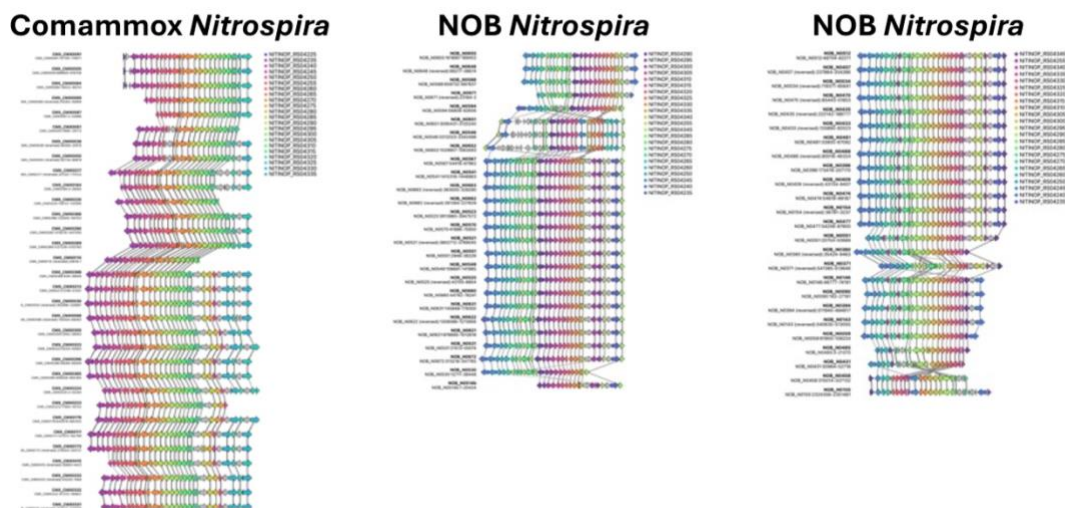


Figure S 5.13. Synteny of the BCAA operon within the *Nitrospira* genus.

Highly conserved synteny of the BCAA operon was observed within the *Nitrospira* genus across comammox *Nitrospira* and NOB *Nitrospira* genomes and metagenomes.

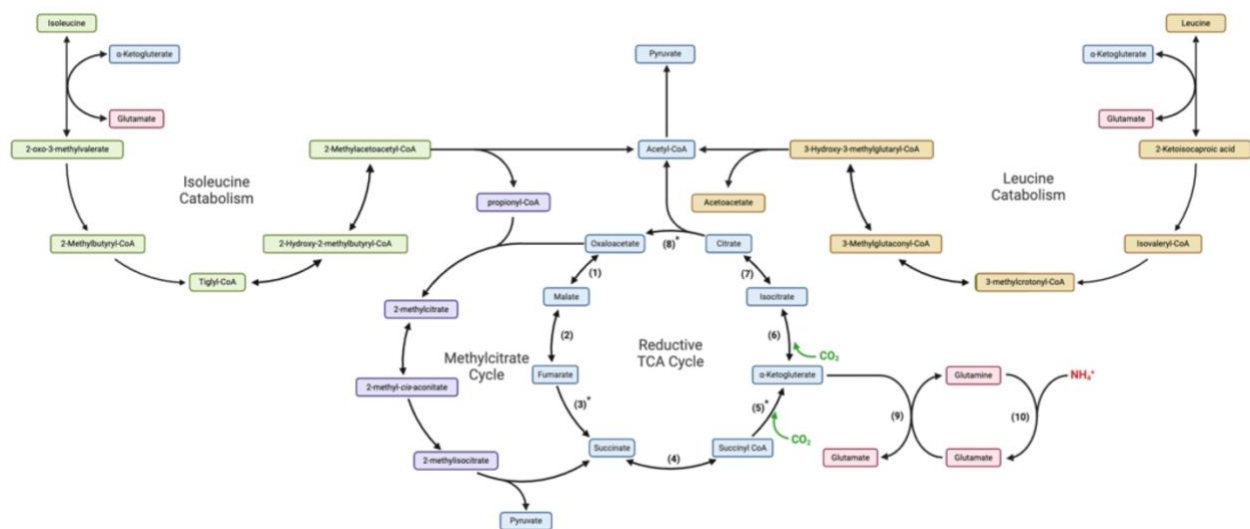


Figure S 5.14. Schematic overview of BCAA catabolism pathway in the *Nitrospira* genus.

Schematic overview of BCAA catabolism pathway in the *Nitrospira* genus.

Table S5-1: Theoretical and actual contribution of nitrate to the production of nitrite, nitrous oxide, and ammonium.

Metabolite	Reaction Name	Reaction	Theoretical NO ₃ ⁻ contribution [%]	Actual NO ₃ ⁻ contribution [%]	
NO ₂ ⁻	NH ₂ OH-dependent NO ₃ ⁻ reduction	$NH_2OH + 2 NO_3^- \rightarrow 3 NO_2^- + H_2O + H^+$	66.67 %	Upper-bound ^c	Lower-bound ^{b,c}
				72.05 ± 0.56 %	69%
NH ₄ ⁺	NH ₂ OH-dependent DNRA	$NH_2OH + \frac{1}{2} NO_3^- \rightarrow NO_2^- + \frac{1}{2} NH_4^+ + \frac{1}{2} H_2O$	100 %	1.16 ± 0.22 %	
	NH ₂ OH decay	$4 NH_2OH + H^+ \rightarrow 2 NH_4^+ + N_2O + 3 H_2O$	0 %		
N ₂ O	NH ₂ OH decay	$4 NH_2OH + H^+ \rightarrow 2 NH_4^+ + N_2O + 3 H_2O$	0 %	25.67 ± 1.58 %	
	N-nitrosating hybrid N ₂ O formation	NH₂OH + 2 NO → 1.5 N₂O + 1.5 H₂O Isotope Half Reactions: $^{14}NH_2OH + ^{15}NO \rightarrow ^{45}N_2O + H_2O + H^+ + e^-$ $^{14}NO + ^{15}NO + 2H^+ + 2e^- \rightarrow 2 ^{45}N_2O + H_2O$ $^{15}NO + ^{15}NO + 2H^+ + 2e^- \rightarrow 2 ^{46}N_2O + H_2O$	24.06 ± 1.35 % ^a		
^a Calculation assumed N ₂ O produced by NH ₂ OH decay was proportional to NH ₄ ⁺ accumulation.					
^b Calculation accounted for the reduction of NO ₂ ⁻ to NO					
^c Calculated using isotopic data					

Table S5-1: Theoretical and actual contribution of nitrate to the production of nitrite, nitrous oxide, and ammonium (cont.).

Metabolite	Reaction Name	p-value		NO ₃ ⁻ reduction contribution [μM-N]	NO ₃ ⁻ reduction contribution [%]
NO ₂ ⁻	NH ₂ OH-dependent NO ₃ ⁻ reduction	1.1e-4	0.62	194.3 ± 4.00 ^c	98.26 ± 4.67 % ^c
NH ₄ ⁺	NH ₂ OH-dependent DNRA	8.2e-9		0.21 ± 0.07 ^c	0.11 ± 0.04 % ^c
	NH ₂ OH decay	0.013			
N ₂ O	NH ₂ OH decay	6.4e-5		13.63 ± 0.85 ^c	6.89 ± 0.63 % ^c
	N-nitrosating hybrid N ₂ O formation	0.17			
^{a,b,c} Same as above					

Table S5-2: Gibbs free energy of hydroxylamine-dependent nitrate reduction and hypothesized secondary reactions

Reaction Name	Reaction	Standard Gibbs energy (ΔG^0) [kJ/mol _{NH₂OH}]	Actual Gibbs energy (ΔG) [kJ/mol _{NH₂OH}]
NH ₂ OH-dependent NO ₃ ⁻ reduction	$NH_2OH + 2 NO_3^- \rightarrow 3 NO_2^- + H_2O + H^+$	-67.60	-137.11
NH ₂ OH-dependent DNRA	$NH_2OH + \frac{1}{2} NO_3^- \rightarrow NO_2^- + \frac{1}{2} NH_4^+ + \frac{1}{2} H_2O$	-91.25	-103.84
NH ₂ OH decay	$4 NH_2OH + H^+ \rightarrow 2 NH_4^+ + N_2O + 3 H_2O$	-147.95	-128.03
N ₂ O production	$NH_2OH + 2 NO \rightarrow 1.5 N_2O + 1.5 H_2O$	-329.10	-262.44 *
Hybrid N ₂ O production	$NH_2OH + NO \rightarrow N_2O + H_2O + H^+ + e^-$	-176.00	-184.47 *
* Gibbs free energy calculation assumed 10nM NO.			

Chapter 6.

Other scientific contributions

6.1 Photoinhibition of comammox reaction in *Nitrospira inopinata* in a dose-and wavelength-dependent manner

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Abstract. Apparent contribution of complete ammonia-oxidizing organisms (comammox) to the global nitrogen cycle highlights the necessity for understanding niche differentiation of comammox bacteria among other ammonia oxidizers. While the high affinity for ammonia of the comammox species *Nitrospira inopinata* suggests their niche partitioning is expected to be centered in oligotrophic environments, their absence in nutrient-depleted environments (such as the oceans) suggests that other (abiotic) factors might control their distribution and spatial localization within microbial communities. Many ammonia- and nitrite-oxidizing organisms are sensitive to light; however, the photosensitivity of comammox has not been explored. Since comammox bacteria encode enzymatic machinery homologous to canonical ammonia- and nitrite-

oxidizers, we hypothesized that comammox *N. inopinata*, the only available pure culture of this group of microorganisms, may be inhibited by illumination in a similar manner. We evaluated the impact of light intensity, wavelength, and duration on the degree of photoinhibition for cultures of the comammox species *N. inopinata* and the soil ammonia-oxidizing archaea *Nitrososphaera viennensis*. Both species were highly sensitive to light. Interestingly, mimicking diurnal light exposure caused an uncoupling of ammonia and nitrite oxidation in *N. inopinata*, indicating nitrite oxidation might be more sensitive to light exposure than ammonia oxidation. It is likely that light influences comammox spatial distribution in natural environments such as surface fresh waters according to diurnal cycles, light attenuation coefficients, and the light penetration depths. Our findings therefore provide ecophysiological insights for further studies on comammox both in field and laboratory settings.

6.2 Mainstream nitrogen removal from low temperature and low ammonium strength municipal wastewater using hydrogel-encapsulated comammox and anammox

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Abstract. Application of partial nitrification (PN)-anammox to mainstream wastewater treatment faces challenges in low water temperature and low ammonium strength. In this study, a continuous flow PN-anammox reactor with hydrogel-encapsulated comammox and anammox was designed and operated for nitrogen removal from mainstream wastewater with low temperature. Long-term operation with synthetic and real wastewater as the feed demonstrated nearly complete ammonium and total inorganic nitrogen (TIN) removal by the reactor at temperatures as low as 10 °C. A significantly decreased nitrogen removal performance and biomass activity was observed in the reactor at 4 °C before a selective heating strategy was employed. A novel heating technology using radiation to heat carbon black co-encapsulated in the hydrogel matrix with biomass was used to

selectively heat biomass but not water in the treatment system. This selective heating technology enabled nearly complete ammonium removal and 89.4 ± 4.3 % TIN removal at influent temperature of 4 °C and reactor temperature 5 °C. Activity tests suggested selective heating brought the biomass activity at influent temperatures of 4 °C and reactor temperature 5 °C to a level comparable to that at 10 °C. Comammox and anammox were consistently present in the system and spatially organized in the hydrogel beads as revealed by qPCR and fluorescence in-situ hybridization (FISH). The abundance of comammox largely decreased by 3 orders of magnitude during the operation at 4 °C, and rapidly recovered after the application of selective heating. The anammox-comammox technology tested in this study essentially enabled mainstream shortcut nitrogen removal, and the selective heating ensured good performance of the technology at temperature as low as 5 °C.

6.3 Microbially mediated climate feedbacks from wetland ecosystems

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Abstract. Wetlands are crucial nodes in the carbon cycle, emitting approximately 20% of global CH₄ while also sequestering 20%–30% of all soil carbon. Both greenhouse gas fluxes and carbon storage are driven by microbial communities in wetland soils. However, these key players are often overlooked or overly simplified in current global climate models. Here, we first integrate microbial metabolisms with biological, chemical, and physical processes occurring at scales from individual microbial cells to ecosystems. This conceptual scale-bridging framework guides the development of feedback loops describing how wetland-specific climate impacts (i.e., sea level rise in estuarine wetlands, droughts and floods in inland wetlands) will affect future climate trajectories. These feedback loops highlight knowledge gaps that need to be addressed to develop predictive models of future climates capturing microbial contributions. We propose a roadmap connecting environmental scientific disciplines to address these knowledge gaps and improve the representation of microbial processes in climate models. Together, this paves the way to understand how microbially mediated climate feedbacks from wetlands will impact future climate change.

6.4 Co-immobilization of AOA strains with anammox bacteria in 3 different synthetic biogranules maintained under two substrate-level conditions

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Abstract. Hydrogel encapsulation of ammonium oxidizing archaea (AOA) along with anammox bacteria holds potential to enable mainstream partial nitrification (PN)-anammox process attributing to AOA's high affinity to ammonia and oxygen. This study explored the growth of AOA and anammox in hydrogel-based synthetic biogranules by testing two AOA strains, three types of hydrogel beads and two substrate levels, to identify the optimal combination favoring the concomitant growth of AOA and anammox. The AOA *Nitrososphaera viennensis* (AOA-NV) exhibited higher abundance ($10^{-2.3 \pm 0.6}$ AOA/16S) than the AOA-DW ($10^{-4.7 \pm 0.8}$ AOA/16S) during the entire experimental period. Amongst the three types of hydrogel beads, the PVA-SA-BaCl₂ (140 days) and PVA-SA-H₃BO₃ beads (>180 days) exhibited better long-term structural stability than the PEGDMA-SA-CaCl₂ beads. The PVA-SA-H₃BO₃ beads exhibited the best long-term stability and both the PVA-SA-BaCl₂ and PVA-SA-H₃BO₃ beads had comparable ability to retain AOA, anammox and the overall microbial community. Substrate conditions rather than the bead type primarily controlled the microbial community structure. Modest substrate concentrations (1mM NH₄⁺-N in the feed and 0.8 mg/L dissolved oxygen (DO) in the reactor during aeration

phase) followed by low substrate conditions (0.1 mM $\text{NH}_4^+\text{-N}$ and 0.2 mg DO/L) both supported the growth of AOA and anammox, while the low substrate condition also suppressed the growth of ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB), with AOA /AOB and anammox/NOB ratio of 0.7 and 0.4 at moderate substrate condition and 16.5 and 2.6 at low substrate condition.

6.5 Ammonia-oxidizing bacteria and archaea exhibit differential nitrogen source preferences

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Abstract. Ammonia-oxidizing microorganisms (AOM) contribute to one of the largest nitrogen fluxes in the global nitrogen budget. Four distinct lineages of AOM: ammonia-oxidizing archaea (AOA), beta- and gamma-proteobacterial ammonia-oxidizing bacteria (β -AOB and γ -AOB) and complete ammonia oxidizers (comammox), are thought to compete for ammonia as their primary nitrogen substrate. In addition, many AOM species can utilize urea as an alternative energy and nitrogen source through hydrolysis to ammonia. How the coordination of ammonia and urea metabolism in AOM influences their ecology remains poorly understood. Here we use stable isotope tracing, kinetics and transcriptomics experiments to show that representatives of the AOM lineages employ distinct regulatory strategies for ammonia or urea utilization, thereby minimizing direct substrate competition. The tested AOA and comammox species preferentially used ammonia over urea, while β -AOB favored urea utilization, repressed ammonia transport in the presence of urea and showed higher affinity for urea than for ammonia. Characterized γ -AOB co-utilized both substrates. These results reveal contrasting niche adaptation and coexistence patterns among the major AOM lineages.

6.6 Increasing aggregate size reduces single-cell organic carbon incorporation by hydrogel-embedded wetland microbes

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Abstract. Microbial degradation of organic carbon in sediments is impacted by the availability of oxygen and substrates for growth. To better understand how particle size and redox zonation impact microbial organic carbon incorporation, techniques that maintain spatial information are necessary to quantify elemental cycling at the microscale. In this study, we produced hydrogel microspheres of various diameters (100, 250, and 500 μm) and inoculated them with an aerobic heterotrophic bacterium isolated from a freshwater wetland (*Flavobacterium* sp.), and in a second experiment with a microbial community from an urban lacustrine wetland. The hydrogel-embedded microbial populations were incubated with ^{13}C -labeled substrates to quantify organic carbon incorporation into biomass via NanoSIMS. Additionally, luminescent nanosensors enabled spatially explicit measurements of oxygen concentrations inside the microspheres. The experimental data were then incorporated into a reactive-transport model to project long-term

steady-state conditions. Smaller (100 μm) particles exhibited the highest microbial cell-specific growth per volume, but also showed higher absolute activity near the surface compared to the larger particles (250 and 500 μm). The experimental results and computational models demonstrate that organic carbon availability was not high enough to allow steep oxygen gradients and as a result, all particle sizes remained well-oxygenated. Our study provides a foundational framework for future studies investigating spatially dependent microbial activity in aggregates using isotopically labeled substrates to quantify growth.

6.7 Toward an effective delivery system of a microbial sink of the uremic toxin, p-cresol; an *in vitro* study with *Thauera aminoaromatica* S2

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Abstract: Individuals with chronic kidney disease (CKD) suffer from uremia, a condition characterized by the accumulation of uremic toxin in the blood. The aromatic uremic toxin p-cresol, a byproduct of tyrosine fermentation in the gut, binds to plasma albumin and cannot be removed with dialysis. However, the ingestion of densified p-cresol degrading microorganisms encapsulated in protective hydrogel beads could provide a therapeutic benefit by removing p-cresol from the colon. In this study the p-cresol degradation capacity of a known anaerobic, p-cresol degrading microorganism, *T. aminoaromatica* S2, encapsulated in polyvinyl alcohol and sodium alginate (PVA/SA) hydrogels was evaluated as a potential oral delivery method for intestinal p-cresol removal. Planktonic degradation was induced through prior p-cresol exposure, yielding a 100% removal efficiency at a rate of 92 nmol (Log CFU)⁻¹ h⁻¹ when exposed to 1.2 mM of p-cresol at 37°C. Increasing p-cresol concentrations inhibited p-cresol degradation.

Hydrogel encapsulation of the bacteria supported high cell density packaging at 2.5 Log CFU (mL hydrogel)⁻¹ and high activity right after hydrogel production, and more rapid activity than the planktonic cells, providing a powerful p-cresol-consuming microbial sink. Our experimental design mimicked distal colon conditions with an initial p-cresol level of 0.60 mM and at a pH 7 where the p-cresol degradation capacity of encapsulated culture was 2.3×10^3 nmol (Log CFU)⁻¹ h⁻¹. The encapsulation of 10-fold increased cell concentrations resulted in more than 2-fold increased degradation rates. With the cell densification, the estimated daily hydrogel intake could be reduced from 134 mL to 58 mL to match daily exposure, thereby achieving mass balance. The effective removal rates were due to well distribution of bacteria cells within the hydrogels. The hydrogels with p-cresol pre-induced biomass showed immediate p-cresol removal even at p-cresol higher than 1.0 mM concentration. The current study demonstrated the potential application of encapsulated *T. aminoaromatica* S2 for the removal of colon p-cresol hence offloading the kidney from processing protein-bound uremic toxins. Further research in hydrogel design could yield efficient removal as well as cell encapsulation.

6.8 Higher-order microbial interactions revealed by comparative metabolic modeling of synthetic communities with varying species composition

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Abstract: Understanding how microbial interactions scale with community complexity is key for microbiome engineering and ecological theory. This study investigates emergent metabolic behaviors in controlled in vitro synthetic anaerobic communities of 2-, 3-, or 4-species: cellulolytic bacterium (*Ruminiclostridium cellulolyticum*), a hydrogenotrophic methanogen (*Methanospirillum hungatei*), an acetoclastic methanogen (*Methanosaeta concilii*), and a sulfate-reducing bacterium (*Desulfovibrio vulgaris*), representing core metabolic guilds in cellulose degradation and carbon conversion. We applied a systems biology framework combining proteogenomics, stoichiometric flux modeling, and SMETANA (Species Metabolic Coupling Analysis) to quantify syntrophic cooperation and competition across configurations. Cooperation peaked in tri-cultures and declined nonlinearly in more complex assemblies. Species roles shifted contextually. *R. cellulolyticum* was the dominant donor, adjusting cellulase and hydrogenase

expression by partner. *M. concilii* became fully metabolite-dependent while enhancing methanogenesis. *D. vulgaris* improved syntrophic efficiency via redox and hydrogen turnover. In contrast, *M. hungatei*'s metabolic centrality declined despite higher CH₄ output, suggesting interaction strength depends more on compatibility than richness. Reduced interactions in the 4-species community stemmed from a single configuration and need further validation. This study moves beyond descriptive work by quantitatively resolving how metabolic networks rewire across defined communities. By characterizing context-dependent flux shifts at multiple layers, we provide a framework for interpreting and engineering stable, functionally interdependent microbial ecosystems.

6.9 Soil depth shapes microbial community composition and function in a freshwater wetland

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In prep.

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Abstract. Wetland microbiomes play a crucial role in the global carbon cycle by modulating soil organic carbon (SOC) and greenhouse gas (GHG) emissions. Understanding how microbial communities respond to environmental changes is essential for predicting wetland carbon fluxes under future climate scenarios. Here, we investigated the biogeochemistry of a temperate lacustrine wetland across four seasons and three soil depths, integrating greenhouse gas flux measurements, porewater metabolite profiles, metagenomics, metabolomics, and metaproteomics. While seasonal shifts in GHG fluxes and porewater chemistry were evident, microbial community composition and function were primarily structured by soil depth, suggesting resilience to short-term seasonal fluctuations. Depth-correlated microbial taxa and metabolic pathways revealed distinct stratification: surface soils were enriched in metabolically versatile Gammaproteobacteria

capable of oxygen and nitrate respiration, as well as methane and sulfur oxidation, whereas deeper layers favored strict anaerobic metabolism, with increasing abundances of *Anaerolinea* and *Methanomicrobia*. Metabolomics showed an enrichment of purine nucleotides and amino acids at the surface, while deeper soils accumulated amino sugars and phenolic compounds, highlighting differences in carbon processing. Metaproteomics confirmed active metabolic pathways, linking functional potential to microbial activity. By integrating multi-omics with biogeochemical measurements, this study provides a system-level view of wetland microbial function and resilience, contributing to predictive models of wetland carbon cycling under future climate change.

6.10 Comparative physiological and genomic characterization of a novel *Nitrobacter vulgaris* strain from a nitrate-contaminated subsurface

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Abstract. Nitrite-oxidizing bacteria (NOB) represent a crucial node in global nitrogen cycle. By catalyzing the second step of nitrification—the oxidation of nitrite to nitrate—NOB activity controls the fate of nitrite (NO_2^-) in aerobic environments while generating energy for growth. Despite thriving in diverse environments, including soils, freshwater, marine ecosystems, subsurface habitats, and water treatment systems, NOB are confined to *Nitrobacter*, *Nitrospina*, *Nitrobacter*, *Nitrotoga*, and other specific lineages. The genus *Nitrobacter*, recognized for its facultative heterotrophic metabolism, is often associated with high-nitrogen environments. Here, we report the isolation and physiological characterization of a novel strain, *Nitrobacter vulgaris* MLSD-S22, from a nitrate- and heavy-metal-contaminated subsurface. Growth inhibition experiments revealed that *N. vulgaris* MLSD-S22 and the type strain Z exhibited similar sensitivities to nitrite, nitrate, and chloride, with nitrite being the most inhibitory. Microrespirometry demonstrated that the *N. vulgaris* strains and *Nitrobacter winogradskyi* Nb-255 possessed higher affinities for nitrite and oxygen than previously reported for *Nitrobacter* NOB, suggesting some potential to compete in low-substrate environments. Long-read DNA

sequencing provided a complete genome for MLSD-S22, revealing two plasmids and a fully intact nitrous oxide reduction operon—an unexpected feature for *Nitrobacter*. This discovery points towards an unrecognized mechanism of nitrogen loss in this genus and raises questions about the contribution of *Nitrobacter* NOB to denitrification. These findings broaden the physiological and genomic diversity of *Nitrobacter*, offering new insights into their adaptation strategies and potential contributions to nitrogen loss.

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Publications

1. Johnston J, Nguyen Quoc B, **Abrahamson B**, Candry P, Ramon C, Cash K, Saccomano SC, Samo TY, Ye C, Weber PK, Winkler M-KH, Mayali X. Increasing aggregate size reduces single-cell organic carbon incorporation by hydrogel-embedded wetland microbes. *ISME Commun.* **4**: ycae086 (2024).
2. Qin W, Wei S, Zheng Y, Choi E, Li X, Johnston J, Wan X, **Abrahamson B**, Flinkstrom Z, Wang B, Li H, Hou L, Sun X, Wells M, Ngo L, Hunt K, Urakawa H, Tao X, Wang D, Pan C, Weber P, Jiang J, Zhou J, Zhang Y, Stahl DA, Ward BB, Mayali X, Martens-Habben W, Winkler M-KH. Ammonia-oxidizing bacteria and archaea exhibit differential nitrogen source preferences. *Nat. Microbiol.* **9**: 524-536 (2024).
3. Godfrey BJ, Li B, Gotshall EY, Bryson S, **Abrahamson B**, Winkler M-KH. Co-immobilization of AOA strains with anammox bacteria in three different synthetic bio-granules maintained under two substrate-level conditions. *Chemosphere* **342**: 140192 (2023).
4. Candry P, **Abrahamson B**, Stahl DA, Winkler M-KH. Microbial mediated climate feedbacks from wetland ecosystems. *Glob. Chang. Biol.* **29**, 5169-5183 (2023).
5. Li B, Godfrey BJ, RedCorn R, Candry P, **Abrahamson B**, Wang Z, Goel R, Winkler M-KH. Mainstream nitrogen removal from low temperature and low ammonium strength municipal wastewater using hydrogel-encapsulated comammox and anammox. *Water Res.* **242**, 120303 (2023).
6. Gotshall EY, Godfrey B, Li B, **Abrahamson B**, Qin W, Winkler M-KH. Photoinhibition of Comammox reaction in *Nitrospira ionipinata* in a dose and wavelength dependent manner. *Front. Microbiol.* **13**: 1022899 (2022).
7. Puiman L, **Abrahamson B**, van der Lans RGJM, Haringa C, Noorman HJ, Picioreanu C. Alleviating mass transfer limitations in industrial external-loop syngas-to-ethanol fermentation. *Chem. Eng. Sci.* **259**: 117770 (2022).
8. Tanger P, Vega-Sánchez ME, Fleming M, Tran K, Singh S, **Abrahamson B**, Jahn CE, Santoro N, Naredo EB, Baraoidan M, Danku JMC, Salt DE, McNally KL, Simmons BA, Ronald PC, Leung H, Bush DR, McKay JK, Leach JE. Cell wall composition and bioenergy potential of rice straw tissues are influenced by environment, tissue type, and genotype. *Bioenergy Resources.* **8**: 1165 (2015).

Articles In review & preparation

1. Wang D, Hunt K.A, Tao X, **Abrahamson B**, Flinkstrom Z, Tanner RS, DeLeón KB, Zhou A, Zhou J, McInerney MJ, Winkler MKH, Stahl DA, Candry P, Pan C Emergent higher-order microbial interactions revealed by comparative metabolic modeling of synthetic communities with varying species compositions. ISME. In review
2. **Abrahamson B**, Cogert KI, Shapiro M, Tran, K, Hunt KA, Gottshall E, Bryson S, Flinkstrom Z, Li B, Candry P, Qin W, Rittmann B, Stahl D, Winkler M-KH. Counter-diffusing membrane aerated biofilm reactors promote cooperation between Anammox and high affinity nitrifiers for mainstream wastewater treatment. Water Research. In prep
3. **Abrahamson B**, Wan X, Candry P, Hunt KA, Wang D, Flinkstrom Z, Lückner S, Qin W, Pan C, Ward BB, Winkler M-KH. Hydroxylamine-dependent nitrate reduction by the complete ammonia oxidizing organism *Nitrospira inopinata*. Nature Microbiology, In prep
4. **Abrahamson B**, Candry P, Wang PH, Larson Tevis AA, Godfrey B, Flinkstrom Z, Hunt KA, Winkler M.-K. H. Synthetic wetland sediment analogs provide insight into the spatial organization and function of syntrophic methanogenesis. Environmental Microbiology. In prep
5. Flinkstrom Z, **Abrahamson B**, Teodosiadis D, Brunzell F, Mafune K, Winkler MKH. Influence of oxygen perturbations on aerobic and anaerobic N-acetylglucosamine degradation by wetland microbial communities. ISME. In prep
6. **Abrahamson B**, Hunt KA, Candry P, Flinkstrom Z, Hou L, Zhang Z, Vuono D, Wang D, Mafune K, Sun X, Zakem, Qin W, Winkler MKH. Nitrite oxidation by the complete ammonia oxidizing bacteria *Nitrospira inopinata*. In prep
7. Flinkstrom Z, Candry P, Godfrey B, **Abrahamson B**, Wang D, Louie KB, Bowen BP, Northen TR, Pan C, Winkler MKH. Soil depth shapes microbial community composition and function in a freshwater wetland. ISME. In prep

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Conference and Workshop Participation

Invited Talks and Presentations (presenter) Poster Presentations (presenter)

1. **Abrahamson B**, Wan X, Candry P, Hunt KA, Wang D, Flinkstrom Z, Lückner S, Qin W, Pan C, Ward BB, Winkler M-KH. Hydroxylamine-dependent nitrate reduction by the complete ammonia oxidizing organism *Nitrospira inopinata*. Center for Environmental Microbial Interactions Seminar, Caltech, Pasadena, CA, USA, 5/2025.
2. **Abrahamson B**, Department of Civil and Environmental Engineering Water Seminar, University of Washington, Seattle, WA, USA, 4/2025.
3. **Abrahamson B**, Candry P, Hunt K, Winkler M-KH. Modeling the spatial syntrophic and competitive interactions between a sulfate reducing bacteria and hydrogenotrophic methanogen. Modeling Ecological Dynamics of Microbial Communities, NSF-Simmons NITMB Workshop, Chicago, IL USA 5/2024.
4. **Abrahamson B**, Department of Civil and Environmental Engineering Water Seminar, University of Washington, Seattle, WA, USA, 5/2024.
5. **Abrahamson B**, Wan X, Candry P, Wang D, Hunt KA, Flinkstrom Z, Lückner S, Qin W, Pan C, Ward BB, Winkler M-KH. Hydroxylamine driven nitrate reduction: A novel anoxic pathway in the complete ammonia oxidizing bacteria *Nitrospira inopinata*. The 8th International Conference on Nitrification and Related Processes, Princeton, NJ USA, 8/2023.
6. **Abrahamson B**, Cogert KI, Shapiro M, Tran K, Bryson S, Gottshall E, Redcorn R, Qin W, Candry P, Picioreanu C, Rittmann B, Stahl DA, Winkler M-KH. Comammox and AOA cooperate with anammox in counter-diffusing membrane aerated biofilm reactors, IWA Biofilms Conference, Phuket, Thailand, 11/2022, accepted as podium presentation by Abrahamson but presented by Candry due to injury sustained by Abrahamson prior to conference.

Poster Presentations (presenter)

1. **Abrahamson B**, Wan X, Candry P, Wang D, Hunt KA, Flinkstrom Z, Lückner S, Qin W, Pan C, Ward BB, Winkler M-KH. Anaerobic hydroxylamine-dependent nitrate reduction by the complete ammonia oxidizing organism *Nitrospira inopinata*. The 19th International Symposium on Microbial Ecology (ISME19), declined poster presentation, 8/2024.
2. Qin W, Wei S, Zheng Y, Choi E, Li X, Johnston J, Wan X, **Abrahamson B**, Flinkstrom Z, Wang B, Li H, Hou L, Sun X, Wells M, Ngo L, Hunt KA, Urakawa H, Tao X, Wang D, Wang D, Pan C, Weber P, Jiang J, Zhou J, Zhang Y, Stahl DA, Ward BB, Mayali X, Martens-Habbena W, Winkler M-KH. Ammonia-oxidizing microorganisms employ distinct strategies coordinating ammonia and urea metabolism, Joint-Genome Institute Microbial Genomics & Metagenomics Workshop 31 (JGI-MGM31), 10/2023.
3. Qin W, Wei S, Zheng Y, Choi E, Li X, Johnston J, Wan X, **Abrahamson B**, Flinkstrom Z, Wang B, Li H, Hou L, Sun X, Wells M, Ngo L, Hunt KA, Urakawa H, Tao X, Wang D, Wang D, Pan C, Weber P, Jiang J, Zhou J, Zhang Y, Stahl DA, Ward BB, Mayali X, Martens-Habbena W, Winkler M-KH. Ammonia-oxidizing microorganisms employ distinct strategies coordinating ammonia and urea metabolism. The 8th International Conference on Nitrification and Related Processes, Princeton, NJ USA, 8/2023.
4. Vuono DC, Flinkstrom Z, **Abrahamson B**, Staub C, Winkler M-KH. Continuous measurements of nitrite-oxidizing bacteria growth through the application of photoconduction in a Microbial Growth Intervalometer (MAGI). The 8th International Conference on Nitrification and Related Processes, Princeton, NJ USA, 8/2023.
5. Wang D, Flinkstrom Z, Candry P, Godfrey B, **Abrahamson B**, Johnston J, Bryson S, Chistoserdova L, Stahl DA, Pan C, Mayali X, and Winkler M-KH. Experimental models bridging single cell-to-ecosystem scales to evaluate climate-wetland feedback mechanisms, DOE-GSP-PI Conference, virtual due to pandemic, 02/2022.
6. Johnston J, **Abrahamson B**, Flinkstrom Z, Wei S, Li X, Li H, Wells M, Ngo L, Choi E, Wan X, Sun X, Stahl DA, Weber PK, Mayali X, Pan C, Qin W, Martens-Habbena W, Ward BB, Winkler M-KH. Nitrous oxide formation and organic nitrogen utilization in wetland microbial communities, DOE-GSP-PI Conference, virtual due to pandemic, 02/2022.
7. Johnston J, Godfrey B, Ye C, Candry P, **Abrahamson B**, Bryson S, Stahl DA, Chistoserdova L, Mayali X, Winkler M-KH. Hydrogel beads to encapsulate sediment microbes as a strategy to quantify climate impacts on microscale biogeochemical activity, DOE-GSP-PI Conference, virtual due to the pandemic, 02/2021.
8. Candry P, Flinkstrom Z, Xiong Y, Bryson S, Johnston J, **Abrahamson B**, Chistoserdova L, Pan C, Mayali X, Stahl DA, Winkler M-KH. Bridging Scales: Conceptualizing microbe-climate links in wetland ecosystems, DOE-GSP-PI Conference, virtual due to pandemic, 02/2021.
9. Wang D, **Abrahamson B**, Tao X, Wofford NQ, Zhou A, Stahl DA, Zhou J, McInerney MJ, Tanner RS, Mayali X, Winkler M-KH., Pan C. Metabolic modeling of synthetic estuarine wetlands microbial communities in response to climate change, DOE-GSP-PI Conference, virtual due to the pandemic, 02/2021.

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10. Bryson S, Candry P, **Abrahamson B**, Wang D, Chistoserdova L, Pan C, Mayali X, Stahl DA, Winkler M-KH. Integrating single-cell wetland microbiome structure, function, and activity to ecosystem-scale biogeochemical fluxes, DOE-GSP-PI Conference, Washington D.C., USA, 02/2020.
11. Delaire C, van Genuchten CM, **Abrahamson B**, Amrose SE, Gadgil A. Concurrent microbe and arsenic removal from groundwater with Fe electrocoagulation. AGU Fall Meeting, San Francisco, CA, USA, 12/2013.

Conference and Workshop Participation

Strategies and Techniques for Analyzing Microbial Population Structures, MBL Course, Woods Hole, MA.	July 2024
Modeling Ecological Dynamics of Microbial Communities, NSF-Simmons NITMB Workshop, Chicago, IL.	April 2024
Joint-Genome Institute Microbial Genomics & Metagenomics Workshop 31, Berkeley, CA.	October 2023
International Conference on Nitrification 8, Early Career Workshop, Princeton, NJ.	July 2023
American Geophysical Union Fall Meeting, virtual due to pandemic.	December 2020 & 2021
International Water Association Biofilms Conference, virtual due to pandemic.	October 2020
Biofilms, TU Delft Course, Delft, Netherlands.	September 2019
Multi-scale Modelling of Microorganisms in Bioprocesses, TU Delft Course, Delft, Netherlands.	November 2018
Society for Industrial Microbiology and Biotechnology Annual Meeting, Chicago, USA.	July 2018

Academic Activities

Honors and Awards

Holland Scholarship, Dutch Ministry of Education, Culture, and Science	2017-2019
Dean's List, UC Berkeley College of Chemistry	Spring 2015

Teaching

CEWA 540: Microbiological Process Fundamentals, UW, Seattle, WA, Invited Lecturer.	Fall 2021 – 2022
CEWA 582: Wastewater Reuse and Resource Recovery, UW, Seattle, WA, Invited Lecturer.	Winter 2020
Biofilms: Course, TU Delft, Delft, Netherlands, Course Assistant.	September 2019
ChemE 3061: Multiscale Reactor Engineering, TU Delft, Delft, Netherlands, Teaching Assistant.	Winter 2019

Outreach

Organizer, Benjamin Hall Research Building Symposium, University of Washington, Seattle.	Spring 2024
Invited lecturer and Curriculum development, Skyline High School Chemistry Class, Oakland, CA.	2022 – Present

Supervising Undergraduate Students

Lillian Crossley, Undergraduate Research Assistant, University of Washington.	November 2021 – July 2022
Anders Larson Tevis, Undergraduate Research Assistant, University of Washington.	January 2023 – June 2023

Journal Peer Reviewer

Nature Communications, Environmental Science & Technology, Water Research

Professional Affiliations

International Society of Microbial Ecology (ISME), International Water Association (IWA)
American Society of Microbiology (ASM), American Association for the Advancement of Science (AAAS)

Industry Experience

DSM Delft, NL <i>Biochemical Engineering Intern</i>	2018
Zymergen Emeryville, CA <i>Research Associate II, Fermentation</i>	2016 – 2017
AbbVie Biotherapeutics Redwood City, CA <i>Associate Scientist</i>	2015

Accomplishments

Eagle Scout Award, Boy Scouts of America, Troop 799 Morgan Hill, CA	2008
Certified Emergency Medical Technician	2012 – 2015
Certified Wilderness First Responder, National Outdoor Leadership School	2011 – 2015

Interests

Microbial ecology and ecophysiology, mathematical modeling, surfing, cycling, live music, reality TV, and baking.