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Elucidating Microbial Interactions in Anaerobic Ammonium Oxidizing Systems

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Elucidating Microbial Interactions in Anaerobic Ammonium Oxidizing Systems

By

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Professor Lisa Alvarez-Cohen, Chair
Professor Kara Nelson
Professor Eoin Brodie

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Abstract

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Anthropogenic nitrogen pollution is a global phenomenon that facilitated unprecedented disruptions to the nitrogen cycle distinctly marked by human inputs of reactive nitrogen into the environment. The increased flux of reactive nitrogen has precipitated unchecked discharges into surface waters leading to eutrophication, severely disrupting existing ecological networks. Anaerobic ammonium oxidation (anammox) is a biogeochemical process that is commonly applied for the removal of reactive nitrogen from wastewater. These systems have been demonstrated to be highly efficient at removing nitrogen from wastewater side-streams with a lower carbon and energy footprint than conventional biological nutrient removal technologies. However, they have been plagued by long startup times and process instabilities which can cause reactor performance lapses that can require months of recovery. A critical element for discerning and responding to these performance lapses is a deep understanding of the microbial community within anammox reactors. Anammox reactors create a unique environment that promotes the selection of a distinct assemblage of bacteria that commonly co-occur with anammox bacteria. These bacteria form an assortment of symbiotic and antagonistic relationships with the anammox bacteria that are critical to their growth and activity as well as the performance of anammox based nitrogen removal systems. This dissertation seeks to understand which interactions are most important for community stability and maintenance of ecosystem function and how the circulation of metabolites influences microbial diversity. The results of this research contribute to a robust understanding of community dynamics that can be used to model and manipulate anammox microbial communities.

Chapter 1 seeks to situate the phenomenon of nitrogen pollution and its complementary solutions by contextualizing anthropogenic pollution as a manifestation of settler colonial regimes of power and land relations. This is conducted through a historical grounding of the tenets of Western environmentalism within the broader scope of Western scientific ontology as a means to critically re-examine the motivations of environmental discourse. Historical and political conceptualizations of race, class, materialism, and power structures are utilized to elucidate the

foundations of the current global tenets of capitalism, imperialism, and settler colonialism. Paralleling this analysis is an investigation into the history of human relations with reactive nitrogen and how these relations have developed from Pre-History, to Antiquity, to the Middle Ages, to the modern era and how the conceptualizations of nitrogen have intersected with modes of production, commerce, and state power. This chapter culminates in a re-examination of the work of Haber and Bosch as a coalescence of nitrogen exploitation and imperial power. The implications of this historical moment are used to establish circumstances around modern relations to nitrogen, the utilization of science as a means to materialize state agendas, and the futility of technological solutions to environmental problems.

Chapter 2 of this dissertation seeks to understand how competitive nitrogen dynamics affects the microbial ecology of the system. The widely accepted stoichiometric relationship of anammox dictates a NH_4^+:NO_2^- of 1:1.32, however the anammox bacteria also compete with bacteria performing denitrification and dissimilatory nitrate reduction to ammonia (DNRA) for this nitrite. To understand this dynamic, an analysis was conducted using 16S rRNA and shotgun metagenomic sequencing data from a reactor study where the influent NH_4^+:NO_2^- ratio was changed to 1:1.1. Sequencing data revealed significant decreases in the abundance of anammox bacteria and increased abundance of bacteria capable of utilizing DNRA. Despite significant changes in taxonomic composition, the reactor still maintained high levels of nitrogen removal efficiency. These results would suggest a dynamic relationship between the anammox and DNRA bacteria that can be symbiotic.

Chapter 3 of this dissertation sought to evaluate the effects of a process disturbance on the microbial community in an anammox reactor. To understand these effects, an analysis was conducted using 16S rRNA and shotgun metagenomic sequencing data from a separate reactor study where the solids retention time (SRT) was decreased step wise from 50 days to 28.5 days. Metagenomic and 16S rRNA data revealed a strong deterministic effect of the disturbance that selected bacteria based on growth strategies where K-type strategists seemed to undergo negative selection and r-type strategists underwent positive selection. The results of the study also suggest a more complex relationship between the anammox bacteria and filamentous Chloroflexi bacteria that is critical to biomass aggregation and retention. These results also demonstrated a significantly different microbial community in the reactor recovery period compared to prior stable operation which would suggest high levels of functional redundancy.

Chapter 4 seeks to examine the role of extracellular polymeric substance (EPS) degradation by heterotrophic bacteria in carbon cycling within anammox bioreactors. To investigate this phenomenon EPS was extracted from suspended anammox biomass through an alkaline and heat extraction method and then utilized as a carbon source in batch enrichments utilizing anammox biomass as an inoculum. Shotgun metagenomic and 16S rRNA sequencing were utilized to determine the response of the microbial community to enrichment on the different EPS solutions. TOC and spectroscopic analysis confirmed biological degradation of EPS by the enrichment culture and sequencing analysis demonstrated increased abundance of bacteria classified as Ignavibacteriae in alkaline EPS enrichments, Nitrosomonadaceae and Rhizobiaceae increased in

heat extracted EPS enrichments, and Ruminococcoceae, Commamonadaceae, and Acidimicrobiales insertiae increased in both enrichments. Metagenomic analysis also demonstrated that bacteria increasing in abundance were enriched in extracellular glycoside hydrolases and peptidases that could be utilized to depolymerize complex carbon molecules. These results would suggest the ability to utilize EPS as a carbon source in anammox reactors is broadly phylogenetically distributed across bacteria within the community.

Chapter 5 aims to characterize the functional profiles of anammox bioreactor associated microbial communities through the framework of ecological strategies. Microbial trait-based analyses were conducted on publicly available metagenomes using *microTrait*, a suite of computational tools that annotates microbial functions and maps them to a dimensionally reduced trait space representative of diverse microbial growth strategies. Phylogenomic analysis of retrieved metagenomes demonstrated a strong recurrence of phylogenetically similar organisms in anammox bioreactor systems of various configurations and operational parameters. Results of the trait-based modeling analysis demonstrated that the trait profiles converged closely according to phylogeny for guilds containing specific lineages of Planctomycetes, Chloroflexi, Bacteroidetes, and Iganvibacteriae which had high occurrences of extracellular carbon depolymerization but contained phylogenetically divergent MAGs in guilds enriched in other resource acquisition and stress tolerance traits. Assessing these functional guilds with relation to the Y-A-S (yield, acquisition, stress) ecological framework indicates that some of these guilds possess traits that appear to align with a high yield growth strategy characterized by investments in aerobic respiration and glyoxylate metabolism while others favor specialization in resource acquisition and stress tolerance. Overall, these groupings would indicate that the conditions of anammox bioreactors create a distinct set of ecological niches characterized by various growth and competition strategies.

Ultimately, the results of this research will support the development of a robust kinetic growth model that can accurately predict the substrate utilization, growth, and microbial interactions in anammox bioreactors. The creation and refinement of these models will promote the design, operation, and maintenance of more resilient anammox bioreactors for municipal and industrial wastewater nitrogen removal. Furthermore, these results will also contribute toward a deeper understanding of the application of ecological theory to complex microbiomes and offer insights into the understudied nuances of microbial metabolic networks and materials exchanges. The contextualization of this biological phenomenon with a sociopolitical analysis of the current state of nitrogen in global commerce is critical to a wholistic understanding of the utility of these technologies, their shortcomings, and opportunities to redirect political momentum towards structural changes to mitigate nitrogen pollution to the environment.

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Acknowledgments

I am of the belief that good scholarship is never done in a vacuum, it is a perpetual homage to those who came before us and an invitation to those after us to pick up the baton and continue the race. Much in the same way I feel that acknowledgments are an invitation to those who have nurtured us, encouraged us, and challenged us to partake in our achievements. In doing so we center the collective body of ideas, perspectives and experiences that have helped shape our thought and the work that we produce. With this in mind, I will fully indulge in this opportunity to give credit where credit is due.

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Chapter 1: Introduction–Nitrogen, Anammox, and Empire

"Engineering is the art of directing the great sources of power in nature for the use and convenience of man." -Thomas Tredgold

"And it is clear that in the colonial countries the peasants alone are revolutionary, for they have nothing to lose and everything to gain. The starving peasant, outside the class system is the first among the exploited to discover that only violence pays. For him there is no compromise, no possible coming to terms; colonization and decolonization is simply a question of relative strength." – Frantz Fanon, The Wretched of the Earth

The progression of human history may be examined through the paradigm of humanity's enduring endeavor to contend with ecological dynamics. Contemporary historical developments have delivered us to the era of technological modernity manifested through the application of engineering praxis aimed at the realization of dominion over the natural world. This praxis engenders transformative changes within natural landscapes, transfigures elements of the land constitutive of cosmological and metaphysical networks of being into fungible assets, and modulates biogeochemical flows that have synchronized over eons of evolutionary accretion. Nitrogen, the subject of this dissertation, constitutes one of these fluxes that has experienced ecologically unfathomable proportions of alteration during the course of these global shifts and represents not a peripheral byproduct of this praxis but an elemental and intrinsic model of its operation. Thus, in this chapter the story of nitrogen will be examined centering its symbiotic role in the codification of racial and colonial epistemology, transmutation of natural amenities for the basis of extraction and accumulation, and the perpetuation of anti-black and anti-indigenous worldviews for the preservation of commercialized violence and oppression. This analysis will culminate towards the development of a critical re-examination of nitrogen in technological modernity embodied in the Haber-Bosch process as well as the depoliticization of anthropogenic pollution and technocratic solutions for ecological restoration.

1.1 The Age of Azotic Empire

1.1.1 You are what you eat

The word nitrogen is an etymological derivative of the Middle English *nitre* derived from the Latin *nitrum*, Greek *nitron*, and Hebrew *nether* is an unfortunate relic of the eponymous alchemical word for saltpeter with roots in its association with saline substances a la the Greek *natron*. The German *stickstoff*, roughly translating to "asphyxiating substance" or the French *azote*, "without life" provide a closer corporeal representation of nitrogen's physical properties. These properties, leading to the formation of the near impermeably strong nitrogen-nitrogen triple bond, imparts a biochemical irony making nitrogen perhaps the most ubiquitous element on the Earth and yet the material most responsible for limiting biological growth and metabolism. Within the biosphere, Smil estimates there to be 10,000 billion tons of dinitrogen, making up 78% of the Earth's atmosphere, while bioavailable pools including ammonia (NH_3), amino acids, and proteins only contribute 10, 10, and one billion tons respectively[1]. Nitrogen, the critical constituent of amino acids forming the basis for proteins used as catalytic agents, structural components of cells, and signaling molecules regulating hormonal systems, comprises

3% of the human body by mass[2]. Given that humans along with most non-diazotrophic organisms lack the capability to utilize dinitrogen as a nitrogen source, we are reliant on organic nitrogen primarily in the form of dietary protein intake. It is estimated that mature adult humans excrete on average around 53 mg of nitrogen/kg of body weight through urine and feces daily, which would necessitate an intake of 0.6-0.7 g of protein per kg of body weight per day to maintain a nitrogen balance[3]. This amount is further constrained by the necessity to ensure adequate intake of the ten essential amino acids that humans lack the biosynthetic pathways for which include lysine, methionine, threonine, and tryptophan that are normally deficient in plant proteins[3]. Thus, it is not an understatement to make the assertion that the story of human history is at its core a quest to obtain sufficient dietary nitrogen. In the process of doing so humans have radically reoriented our relationship with the natural world or as Marx would say the "metabolic interaction between man and the earth." The question that humans must contend with now is how to contextualize the dietary nitrogen needs of over 8 billion people with biogeochemical fluxes that are intricately linked to one another as well as climatic, geohydrological, and atmospheric patterns that facilitate the transposition and conversion of global reservoirs of matter and energy. Nitrogen, in its predominant form a colorless, odorless, nonflammable, nontoxic gas worthy of the German and French etymological descriptions, sits at the intersection of these global processes that demark the threshold of an ecologically hospitable range that is a prerequisite for human civilization from one that could spell its collapse.

1.1.2 Nitrogen here, nitrogen there, nitrogen everywhere

Bioavailable nitrogen species including ammonia (NH_3), ammonium (NH_4), nitrate (NO_3^-), nitrite (NO_2^-), and nitric oxides (NO_x) can be agglomerated into a comprehensive pool of reactive nitrogen (N_r), juxtaposed against the predominantly inaccessible dinitrogen. In light of this juxtaposition and its consequential impact on vital processes, a natural syllogism may posit a nuanced biopolitical and necropolitical dimension to the management of nitrogen, and just as equally its mismanagement. Hence, the forms of nitrogen that we encounter in marine, terrestrial, and atmospheric reservoirs are attributable not solely to natural processes but primarily to functions of politically determined regimes of commerce and transit. To illustrate this phenomenon, we'll use the agricultural sector as an example. Smil estimates that at the dawn of the 21st century the nitrogen mass of the worldwide edible harvest of crops, meat and dairy products, marine and freshwater catches, and aquaculture amounted to 25 Mt N per year which would have resulted in a per capita average of 75g of protein per day[14]. This was enabled by the application of 170 Mt N provided through synthetic fertilizers, bio-fixation, organic recycling, irrigation water, seeds, and atmospheric deposition. Crop phytomass was estimated to recover on average 50% of available nitrogen[2] resulting in 60 Mt N as harvested crops and only 25 Mt N of the harvested total is conserved in extracted residues for edible food products[4]. Consequentially, this results in over 145 Mt of N per year that is "lost" and dispersed to the

[1] This is an aggregate of very different trends in developed and developing countries where affluent countries average about 100 g/day with 55g/day coming from animal protein and developing countries with an average of 70g/day of protein and only 25 of those coming from animal protein.

[2] Many studies have shown N recovery rates of staple crops (corn, rice, wheat) to be well below 50% and can go as low as 30%

environment. Conservation of mass would demand that we account for the mass of nitrogen that is shed during this process and these losses are dominated by volatilization from ammonia-based fertilizers and leaching of nitrate which is estimated to be as high as 57 kg N ha^{-1} for maize and 29 kg N ha^{-1} for wheat[5], two of the most intensively fertilized crops. Notwithstanding these aggregate global figures, the United Nations Food and Agriculture Organization posited in 2013 that an estimated 843 million individuals, constituting approximately 1 in 7 of the global population at the time, experienced chronic hunger and nutrient deficiencies[6]. The discordance between these divergent trends cannot be exclusively understood through the inefficiencies within the global food system, but rather manifests as a symptom of a pecuniary ontology that perceives nature and its services as an inherent prerequisite, obtainable without cost, or as Marx would say "gratis". The sociopolitical ramifications of this discordance merit further examination; however, at present, our focus necessitates an exploration of the ultimate fate and ensuing consequences of this "lost" nitrogen.

Global estimates of anthropogenic reactive nitrogen inputs to terrestrial sources at the turn of the 21st century are in the range of 140-160 Tg N yr^{-1}[7,8]. Of this amount, roughly 60 Tg N yr^{-1} are estimated to be exported to coastal areas through riverine systems[9]. Once reactive nitrogen accumulates in marine ecosystems, severe ecological disruption can occur due to eutrophication. Eutrophication is the process in which photosynthetic organisms (primarily algae), limited by an essential nutrient, proliferate due to the sudden availability of that nutrient in excess and when they die and decay, their necromass is consumed by heterotrophic bacteria resulting in a rapid depletion of oxygen in the water[10,11]. Nitrogen pollution is poised to induce eutrophication within marine ecosystems primarily owing to nitrogen's prevalent status as the limiting nutrient vis-à-vis phosphorus in marine environments[12]. This results in the formation of acute hypoxic zones (<2 mg O_2/L) that can lead to the death of aquatic animals, production of greenhouse gases such as methane and odorous gases such as hydrogen sulfide, and the gradual disruption and degradation of trophic networks[12]. Moreover, eutrophication additionally precipitates the emergence of toxic algal blooms comprising cyanobacteria and dinoflagellates, some of which produce toxins of comparable potency to cobra venom[13]. These blooms render water bodies unsuitable for both consumption and recreational activities, while also exerting direct lethal effects on aquatic fauna such as fish and shellfish[14]. Furthermore, they possess the capacity to bioaccumulate and magnify in toxicity through trophic levels, exacerbating ecological ramifications[15]. Hypoxic dead zones in the world's seas and oceans continue to emerge and expand. The Gulf of Mexico hypoxic zone, largely stimulated by nutrient loading from the Mississippi river, is estimated to encompass an average area of 16,700 km^2 with seasonal zeniths up to 22,000 km^2, an area roughly the size of New Jersey,[16] and continues to grow annually despite pledges to limit its area to 5,000 km^2[17]. Similar dead zones are evident in various marine regions, including the Chesapeake Bay, Baltic Sea, Kattegat Strait, Black Sea, and East China Sea, each posing a spectrum of biogeochemical, ecological, and economic complexities[18].

Reactive nitrogen not transported to marine ecosystems frequently infiltrates groundwater and surface water reservoirs utilized for human consumption. Galloway et al. estimates that up to 25% of nitrogen inputs to agricultural lands ultimately escapes to contaminate water resources[19], while Singh and Caswell estimate that anthropogenic nitrogen inputs to rivers range from 3,100-

14,000 kg N/m^2 with an average of 65.7% originating from agricultural sources[20]. United States Geological Survey data indicates that 22% of domestic wells in the US contain nitrate contamination levels exceeding the maximum contaminant level (MCL) (>10 mg-N/L)[21]. European data show comparable trends[22], while contamination rates in developing countries can soar to as high as 50%[23]. While certain discourses within nitrate epidemiology have endeavored to position the broader adverse nitrate health effects as epiphenomenal to fecal coliform contamination, the consequences of acute nitrate exposure to infants and toddlers often demonstrate profound lethality. Methemoglobinemia or "blue baby syndrome" quite literally asphyxiated many rural infants before the linkages to nitrate contamination in wells was established and this risk persists globally especially for populations residing in water-scarce regions[24,25]. Proposed correlations between nitrate exposure and stomach cancer remains inconclusive, however further research remains to be completed[26]. Nevertheless, beyond its impacts on human health, a comprehensive understanding of nitrate contamination's implications on biogeochemical fluxes, contaminant mobilization, and biodiversity at various spatial and temporal scales remains to be fully elucidated.

While ammonia and nitrate exhibit residence times spanning a few days to several weeks and spatially constrained dispersion under typical baseflow conditions[27], gaseous nitrogen compounds are characterized by considerably lengthier persistence and broader geographical influence. Nitrogen oxide (NO_x) compounds generated from the combustion of fossil fuels can be transported thousands of miles between continents[28] while undergoing photochemical reactions that generate acid rain, deplete ozone, and produce smog detrimental to air quality[29]. Overall, fossil fuel burning leads to the atmospheric deposition of an estimated 25-33 Tg N yr^{-1}[30]. Nitrous oxide (N_2O) generated from fertilized agricultural soils, runoff and leaching from fertilizers, fossil fuels, and wastewater make up approximately 40-50% of the nearly 18 Tg N_2O-N emitted to the atmosphere per year[31]. The predominant proportion of this quantity originates from agricultural activities, 1-2% of nitrogen inputs to agricultural soils ultimately being converted to nitrous oxide[32]. Nitrous oxide, comprising 5-7% of global greenhouse gas emissions, with approximately 300 times the warming potential of CO_2[33] , and an atmospheric residence time spanning decades[34] presages an oracular form of harm that transcends temporal and spatial confines. This is what scholar Rob Nixon would call "slow violence" which defies ontologies of acute and localized regimes of violence[35]: "Violence is customarily conceived as an event or action that is immediate in time, explosive and spectacular in space, and erupting into instant sensational visibility". This paradigm is harrowingly analogous to the violence of carbon dioxide emissions as Malm writes "but there is also a different kind of violence: not rapid but slow motion, not instantaneous but incremental, not body-to-body but playing out over vast stretches of time through the medium of ecosystems and therefore far more difficult to capture between book covers or on-screen than the bullets of a sniper"[36].

When describing the adverse effects of nitrogen in the environment it is important to make the distinction of harm as opposed to violence. In the context of environmental relationships, harm can be conceptualized as the materialization of an adverse environmental effect on an entity. Violence represents the nexus between harm and the actions of another entity, whereby the latter's conduct precipitates the infliction of such harm. The relationships between these entities

can be connected through structural violence as scholar Max Liboroin writes in conversation with Bandy Lee[37,38]:
"Instead of defining violence as a direct event of force or coercion, the concept of structural violence "directly illustrates a power system wherein social structures or institutions cause harm to people in a way that results in maldevelopment or deprivation… that constrain[s] them from achieving the quality of life that would have otherwise been possible." Structural violence affects different people differently, creating and solidifying social differences and stratification"

Thus, the relegation of violence to harm serves only to obfuscate structural power dynamics, alienate the recipients of harm, and absolve accountability of culpable agents involved. The individuals afflicted by the deleterious health consequences of nitrate exposure, or suffering acute nutritional deficiency from food insecurity, the ecosystems destabilized due to an overabundance of ammonium, and the climate change-induced migrants spurred by nitrous oxide emissions did not merely become victims through spontaneous occurrence. They endure suffering as a consequence of a systemic framework that actively engenders violence, necessitating the presence of a recipient thereof.

1.1.3 Nitrogen and Relationality

The paradoxical duality of reactive nitrogen as both a pollutant and a vital component for life, eludes comprehension through conventional Western paradigms of "waste" or byproduct, as typically applied to other contaminants. Here the Indigenous practice of relationality is critical to properly conceptualize reactive nitrogen in various contexts. Relationality is, as scholar Lauren Tynan describes, "how the world is known and how we, as Peoples, Country, entities, stories and more-than-human kin know ourselves and our responsibilities to one another"[39]. It is a worldview centered in the recognition of all entities existing in a state of relation with one another, intricately interweaving humans, non-humans, and the natural environment within a complex tapestry of memories, narratives, and histories. This epistemological approach hinges upon specificity, which Liboiron refers to as "a methodology of nuanced connection and humility", emphasizing the discernment that the relationships deemed significant within one context may diverge substantially within another. We can understand the effects of nitrogen more appropriately through its relations in any environment, as scholar Emma Cardwell writes:

"Reactive nitrogen defies ontologies of individualism and can only be understood in relation. The reactivity that makes it either a pollutant or a nutrient is relationally determined. The fundamental characteristic of the N_r family is the way it relates. Most of the air around us is nitrogen-about 80 percent of the composition, N_2. Atmospheric nitrogen, however, is stable and unreactive, its chemical bonds are remarkably strong. We breathe it in and breathe it straight out. We pass through it, and it passes through us. It is so inert its early names-azote, mephitic air-literally mean "no life." It does react (does relation require reaction, the response inresponse-ability?). And yet, in its reactive forms-the various nitrogen species in which those strong atmospheric bonds have been broken, and new compounds created that are ready to interrelate with other chemicals to make something new-nitrogen is life. It is key to development and growth in living organisms, a building block of protein and DNA. It provides the energy for

plants and animals to grow, but it is also a powerful explosive, and has played a vital role in human warfare and state-sanctioned murder for centuries."[40]

Thus, the designation of nitrogen as "good" or "bad" relies on systematic binaries that are the cornerstone of Western ontologies of classification and taxonomy. These binaries erode the relational specificity that is paramount to understanding the kinship of nitrogen in various settings. The intricate interrelations governing nitrogen's environmental function are pivotal for comprehending the intricate dynamics between humans and their surrounding milieu. Hence, prioritizing relationality facilitates a departure from paradigms that emphasize categorization and extraction.

1.1.4 A Fix for the Ages

Figure 1.1 from the work of Peñuelas et al. illustrates the anthropogenic inputs of nitrogen and phosphorus to the biosphere since the onset of the industrial revolution, employing the natural fixation of nitrogen in terrestrial and terrestrial and marine ecosystems combined as a comparative benchmark[30].

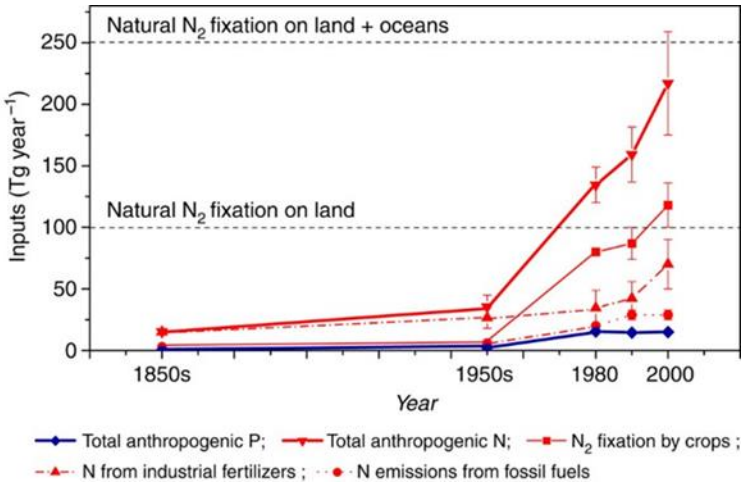

Figure 1.1: Historical inputs of reactive nitrogen into the environment (1850-2000). Acquired from Peñuelas et al. (2013).

Pre-industrial terrestrial biological nitrogen fixation is estimated to be 58 Tg N yr^{-1} and additional 5 Tg N yr^{-1} is contributed from lightning fixation of atmospheric nitrogen[41]. Riverine export from terrestrial sources to coastal waters, marine biological fixation, and atmospheric deposition make up the 140 Tg N yr^{-1} of marine reactive nitrogen inputs[42]. Anthropogenic nitrogen inputs in the 21st century are estimated to be in the range of 210 Tg N yr^{-1}, meaning that anthropogenic nitrogen inputs now make up more than half of the total reactive nitrogen inputs to marine and terrestrial ecosystems[43]. The frenetic pace of increases in anthropogenic reactive nitrogen inputs to the biosphere has essentially made human contributions to reactive nitrogen inputs equal to natural inputs to the environment. Moreover, contextualizing the rate of disruption within the global nitrogen cycle necessitates juxtaposition with human-induced perturbations to the carbon cycle. For instance, while fossil fuel combustion introduces approximately 6 gigatons (Gt) of carbon (C) into the atmosphere annually, this figure pales in comparison to the exchange of

approximately 100 Gt of carbon between terrestrial and atmospheric reservoirs, as well as between marine and atmospheric reservoirs, respectively[44]. What implications does this hold for the natural elemental cycles that have historically governed the conversion and transportation of nitrogen throughout time immemorial?

The pivotal mechanism linking inert dinitrogen to the diverse array of reactive nitrogen compounds is nitrogen fixation, encompassing the rupture of the triple bond within dinitrogen and subsequent conversion into ammonia. This process, which will be further elucidated in subsequent sections, is orchestrated by a select group of bacteria known as diazotrophs. Many of these organisms engage in symbiotic relationships with leguminous plants, facilitating the conversion of atmospheric nitrogen into ammonia and in return, receive a favorable habitat within root nodules and energy-rich sugars. This biochemical transformation is enabled by oxygen-sensitive enzymes known as nitrogenases, which continue to evade comprehensive elucidation regarding its atomic mechanisms involved in cleaving the nitrogen-nitrogen triple bond[45]. In the absence of human intervention the processing and export of nitrogen in mature ecosystems tends to be local[46]. Organic nitrogen in decaying vegetation is decomposed and mineralized to ammonia flowing back into the soil and is either fixed through cationic interactions with negatively charged soil minerals or converted to nitrate through nitrification. The aerobic process of nitrification converts ammonia into nitrate which is much more bioavailable to plants but is highly soluble and can leach away through groundwater transport. Animals taking up nitrogen compounds through plant consumption deposit nitrogen rich waste and return the organic nitrogen of their bodies to the soil following death. Local nitrogen cycling before anthropogenic influences were to an extent highly regionalized as Gorman explains "All things being equal, growth is balanced by death, and the decomposition of dead material supplies the nitrogen needed for new growth."[46] Nitrate not assimilated by plants was converted into gaseous forms dinitrogen and nitric or nitrous oxide through full or partial denitrification respectively, returning nitrogen to the atmosphere, providing a sink and closing the cycle.

Nitrogen cycling within marine environments exhibits greater variability and remains less comprehensively understood compared to terrestrial cycling. A notable distinction lies in the heightened significance of anaerobic ammonium oxidation in fixed nitrogen removal, contrasting with the predominant role of denitrification in terrestrial systems. Furthermore, nitrogen accumulation within marine sediments presents an additional dynamic, as its mobilization to the surface is contingent upon prevailing currents. Terrestrial and marine nitrogen cycling is only loosely coupled primarily through riverine exports of fixed nitrogen to coastal areas. Overall, the cycling with atmospheric reservoirs tends to follow similar patterns in terrestrial and marine environments. However, the fundamental assertion persists that the exchanges among atmospheric, terrestrial, and marine nitrogen reservoirs engender a predominantly symmetrical elemental flux across diverse Earth systems.

Based on phylogenetic reconstructions of nitrogenase genes, diazotrophy probably emerged 3.6-3.2 billion years ago within the Last Universal Common Ancestor (LUCA) to support growth in nitrogen-poor environments[47]. The comprehensive elucidation of the evolutionary trajectory of denitrification encounters complexity attributed to horizontal gene transfer. However, isotopic

analyses suggest the emergence of aerobic nitrogen metabolisms at least 2.5 billion years ago, indicative of the evolutionary presence of nitrification and denitrification processes by that epoch[48]. The collective body of scientific evidence indicates that the contours of the contemporary nitrogen cycle have persisted for over half of the Earth's 4.6 billion year history, encompassing approximately two-thirds of the entire duration of life on Earth. Humans, in their modern form, have walked the Earth for approximately 100,000 years and have had the capacity to fix nitrogen on an industrial scale through Haber-Bosch for a little more than 100 years amounting to 0.4e-6% of the cumulative history of the modern nitrogen cycle. On a temporal scale conducive to human comprehension, if the historical timeline of nitrogen cycling were compressed into a single day, the human capacity to fix nitrogen would amount to less than four milliseconds. This simple diachronicity underscores the profound disruption wrought by human activities on the equilibrium of nitrogen cycling, a process that took eons upon eons and countless permutations of evolutionary selection to equalize in a proverbial blink of an eye in geological time.

What are the implications of the complete destabilization of a biogeochemical cycle that is critical to life on Earth in a timespan of just over 100 years? Colossal increases in reactive nitrogen through atmospheric deposition and leaching radically reconfigure stoichiometric limitations on primary growth, shifting billions of hectares of land that were conventionally limited by nitrogen to acute phosphorus limitation, completely restructuring soil elemental flows[30]. Reactive nitrogen deposition precipitates the enhancement of terrestrial carbon sinks through increased photosynthetic activity resulting in untold disruptions to the global carbon cycle[49]. Excess ammonium in temperate soils leads to soil acidification, base cation depletion, and toxic metal mobilization[50]. Reactive nitrogen enrichment leads to vertiginous shifts in microbial, plant, and animal diversity at various scales[51]. Thus, the advent of industrial nitrogen fixation through the Haber-Bosch process marks not only human's transcendence of ecological limitations but a fulcrum in the manipulation of biogeochemical fluxes in Earth's biosphere. This juncture does not mark the genesis, nor does it represent the pinnacle of humanity's appropriation of nature; rather, it signifies the apex of centuries-long processes characterized by genocide, dispossession, and exploitation, deeply ingrained within an axiological framework emphasizing commodification, extraction, and accumulation. It embodies the ossification of colonial epistemologies and relational paradigms with the land, etched into the geological substratum and isotopic archives of commerce. It is the imposition of historical materialist perspectives onto the cadaver of pre-Capitalist and pre-Colonial ontologies of subsistence and land stewardship. Haber-Bosch signifies not a "fix" in the conventional sense of transforming dinitrogen to guarantee the production of dietary nitrogen for human sustenance, but rather a "fix" in the most literal connotation of the word "to arrange something in a certain spot or position." It "fixes" colonial land relations that subvert the relationality of all living and non-living beings in favor of a taxonomic ideology that conceptualizes nitrogen not as a gift of the dead to the living, nor a reciprocal exchange between the atmosphere, hydrosphere, biosphere and lithosphere, but rather as a quantifiable input to a unit of production. These relationships are solidified spatially, temporally, and within the cognitive frameworks of humans, who are presently engaged in an enduring endeavor to transform nitrogen into a commodity destined for sale within market economies. How did this fix materialize? How did humans shift the

paradigm of nitrogen as an elusive, amorphous idea of an idea into a modular input for production? How does the use of nitrogen as currently conceptualized perpetuate colonial and racial capitalist frameworks of epistemicide? We will explore and attempt to answer these questions in the subsequent sections.

1.2 Dawn of Azotic Empire

1.2.1 The European Creation of Man

The term "human" up to this point has been utilized liberally with the assumed connotation of the species *Homo sapiens*. Now it is critical to make the distinction between *human* and *Human*. The biocentric state of consciousness and self-awareness attributed to the fundamental essence of human existence necessitates critical deconstruction, revealing it as a socially constructed concept originating distinctly from European intellectual traditions. The Human as scholar Derrick White writes in engagement with Wynter's Theory of the Human is "a transcultural space that is not bound by science or superstition, it is a narratively created identity in which people are socialized."[52] in response to Wynter's assertion "[T]he phenomenon of culture rather than 'nature' or 'history' … provides the ground of all human existential reality or actuality."[53] Wynter, whose Theory of the Human builds on the work of Mikhail Epstein adopts a similar theoretical approach of "transculture" meaning "a space in, or among, cultures, which is open to all of them. Culture frees us from nature; transculture frees us from culture, any one culture."[54] Wynter extends this methodology to its logical conclusion, contending that various societies and cultures engender a dialectical framework concerning cultural and humanistic understandings of existence, which serve to delineate a concept of "Self" or "man" juxtaposed against an "Other"[53]. This conceptual framework of self-awareness, contextualized within socially constructed notions of a unified cultural identity juxtaposed with the perception of the outsider, is pivotal for elucidating the Greco-Roman, Judeo-Christian European conceptualization of "Man".

The European conception of the Human is grounded in epistemic origins stemming from Judeo-Christian tradition. Following the Gregorian reform era, this ideology delineated the Human as "sinful by nature", subsequent to the Adamic fall, and thus "primarily the religious subject of the Church". This belief system is rooted in the Greco-Roman astronomical tradition which Wynters explains through scholar E.C. Krupp's words to have "mapped their "descriptive statements" or governing master codes on the heavens, on their stable periodicities and regular recurring movements"[53,55] and thus:

"mapped their specific criterion of being human, of what is was "to be a good man and woman of one's kind", onto the physical cosmos, thereby absolutizing each such criterion; and with this enabling them to be experienced by each order's subjects as if they had been supernaturally (and, as such, extrahumanly) determined criteria, their respective truths had necessarily come to function as an "objective set of "facts" for the people of that society-seeing that such truths were now the indispensable condition of their existence as such a society, as such people, as such a mode of being human."[53,56]

Put differently, the Greco-Roman presuppositions regarding celestial stationarity functioned to agglutinate conceptions of a purportedly "natural" social hierarchy with notions of "celestial

perfection" juxtaposed against tellurian "imperfection", thereby evoking notions of an "objective truth" or "universalism." Hence, pre-Renaissance conceptualizations of the Human were closely intertwined with the ecclesiastical framework of Christendom which anointed the European populace as "God's chosen people" with a divine right to salvation. These constructs were intricately linked with perceptions of individuals adhering to alternative religious traditions, notably Islam, delineated as diametrically opposed to the Christian Man, thus emblematic of the quintessential "Other". This sentiment underwent a significant escalation catalyzed by the military campaigns of the Crusades during the 11th to 13th centuries and the ensuing Reconquista orchestrated by the consolidated Castilian-Aragonese monarchy. With this idea of the Human, religion served as the axiological paradigm defining society as scholar, Ramón Grosfoguel describes in Reconquista era Spain the "purity of blood" discourse was used to commit genocide and epistemicide of Jews and Muslims in the conquest of Al-Andalus[57]. The aforementioned rationale was operationalized to burn Indo-European women as "witches" in the sixteenth century whose communal land relations and oral traditions of "astronomy, medicine biology, ethics, etc." were perceived as a direct challenge to the prevailing "Christian-centric patriarchy"[57].

During the Renaissance period, marked by significant influences such as the Copernican heliocentric model—initially deemed heretical—, the discovery of the Americas, and the Protestant Reformation, there occurred a notable shift in the animating axis of cultural cohesiveness and thus the conception of the Human. This transition witnessed a departure from an ecumenical religiosity towards an ideological framework rooted in rational humanism. This transformation finds its most poignant expression in the advent of Cartesian dualism, encapsulated in the axiom, "I think, therefore I am," signifying a profound reorientation of the locus of knowledge and authority away from the omnipotent God of Christendom towards the individual "I"[57]. Through Cartesian philosophy Descartes "claims that the mind is of a different substance from the body" and thus "the mind is similar to the Christian God" marginalizing knowledge generated in "particular social relations" or "from a particular space in the world", favoring an epistemological stance of "objectivity" or "neutrality" that aspires to universalize the process of knowledge production[57]. This process played a pivotal role in the epistemicide which was symbiotically coupled to the genocide of Indigenous peoples in the Americas, and to dismiss alternative forms of knowledge deemed "inferior", while concurrently perpetuating discourses of objectivity within Western academic establishments. Therefore, the Renaissance-era comprehension of the Human, underpinned by the discourse of European conquest and colonization, elevated Man to the role of paramount arbiter and transmitter of universal truth, supplanting the divine as the primary source, and interpreter of epistemic superiority[58]. Hence, the maxim "I think, therefore I am" morphed into "I conquer, therefore I am", serving to justify the dispossession of Indigenous territories, finally evolving into "I exterminate, therefore I am", a rationale employed to justify acts of genocide.[57,58] Moreover, the invocation of rationalism during this period served to diminish the primacy of the Human's allegiance to the Church, instead highlighting the importance of socially constructed norms of reason, as defined by the state[53]. Consequently, the Renaissance-era concept of the Human transitioned from being primarily aligned with the interest of the Church to becoming a biopolitical entity subject to the

authority of the state[53]. This shift not only eroded the Church's hegemony but also bolstered the authority of burgeoning nation-states.

Amidst the frenetically expanding pace of colonization in the Americas and the simultaneous religious upheaval of the Reformation, which engendered a palpable schism between Protestants and Catholics, the antecedent perceptions of the Human functionalized by pre-Renaissance religious frameworks encountered a profound existential quandary. Catholic Spain, the dominant force in the initial phase of European colonization in the Americas, significantly overextended its resources in the pursuit of gold[59]. Its reliance on the transatlantic slave trade and Indigenous encomiendas to meet labor demands paradoxically hindered its capacity to quell incessant slave revolts, as its stringent adherence to principles of blood purity limited colonization efforts to individuals of pure Catholic lineage[59]. Thus, race slowly replaced religion as the dominant axiological framework to separate the Human from the "Other" as scholar Garald Horne writes:

"Another analyst, looking back from the vantage point of 1933, found that Spain was overstretched as early as the mid-sixteenth century, creating an opening for England; besides, said Henry Wilkinson, Madrid's "financing was from Lombardy, since in their religious frenzy, Spain had extruded not only the Moors but the Jews also," a policy inimical to a viable colonialism. Yet by the 1560s, he says, Africans "greatly outnumbered the Spaniards" in the colonies, creating inherent instability and numerous arbitrage opportunities for the enslaved to exploit. London's ability to create Pan-Europeanism in settlements served to forestall what had debilitated Madrid."[59]

The English's stringent racialism of non European peoples, refined and optimized through their brutal colonization of Ireland, served to materialize the codification of "whiteness". The concept of race pre-dates the development of Capitalism in European feudal societies as the late scholar Cedric Robinson explains in his outline of racial capitalism in *Black Marxism* "Racism… was not simply a convention for ordering the relations of European to non-European peoples but has its genesis in the 'internal' relations of European peoples. The development, organization, and expansion of capitalist society pursued essentially racial directions, so too did social ideology. As a material force, then it could be expected that racialism would inevitably permeate the social structures emergent from capitalism"[60]. Nevertheless, the emergence of the pan-European construct of whiteness did not manifest until the imperative of European colonization compelled its formulation to facilitate the subjugation of Africans and the eradication of Indigenous populations. Thus, the English expedited the course of colonization by operationalizing the concept of whiteness, employing it as a tool to escalate the genocidal practices against Indigenous populations and the enslavement of Africans to apocalyptic levels, exemplified by the English axiom, "the only good Indians are dead Indians."[59]. Against the backdrop of European colonial terror and the fissure of the Reformation, the renaissance era Human sublimated into Colonial Man that was driven by rationalism imbued with divine authority, a biocentric superiority complex, and relentless dominance of the "Other" facilitated by racial constructs inherent in the ideology of whiteness.

The aforementioned definitions of the Human posit three critical implications for our examination of the anthropogenic nitrogen cycle. The first is that the Human is inherently

separate from nature. This concept emanates directly from the theocentric ideology of creation influenced by Platonic philosophy which asserts that "God, having completed his Creation and wanting someone to admire His works, had created Man on a model unique to him, then placed him at the center/midpoint of the hierarchy of this creation, commanding him to "make of himself" what he willed to be—to decide for himself whether to fall to the level of the beasts by giving into his passions, or, through the use of his reason, to rise to the level of the angels"[61]. According to this rationale, the Human is imbued with a divinely sanctioned entitlement to dominion over the natural world by virtue of the act of creation. Wynter further expounds on this distinction: "as a creature standing between "the physical world of nature" and the "spiritual world of the angels of God" as balanced between "natural" and "supernatural" order. It was in the context of this syncretized reinscription that the new criterion of Reason would come to take the place of the medieval criterion of the Redeemed Spirit as its transumed form—that the master code of symbolic life and death would now become that of reason/sensuality". This view of nature is in direct opposition to Indigenous cosmologies that emphasize relationality as embodied in the work of scholar Enrique Sálmon: "When [Indigenous] people speak of the land, the religious and romantic overtones so prevalent in Western environmental conversation are absent. To us, the land exists in the same manner as do our families, chickens, the river, and the sky. No hierarchy of privilege places one above or below another. Everything is woven into a managed, interconnected tapestry. Within this web, there are particular ways that living things relate to one another"[62]. The European concept of the Human is by definition external to the land, devoid of relation, unbounded by an obligation to the other human, non-human and other physical and spiritual elements that constitute it. In the eyes of the Human, nature is perceived merely as an untamed reservoir, divisible into physical units of "standing reserve" for the unidirectional extraction and utilization of resources[63]. This process is facilitated by Western ontologies characterized by principles of separation, classification, and quantification, which seek to transform nature into an inexhaustible repository of discrete units primed for production.

The second critical implication is that the Human has an inalienable right to property. In the liberal framework, property inherently constitutes a system of rights delineated by social relations, fundamentally characterized, as elucidated by legal scholar Cheryl Harris, as a "right to exclude"[64]. This delineates what we might conceptualize within the realm of classical political economy as bourgeois private property, distinct from alternative forms such as communal property or commons[65]. Property in this sense being coterminous with individual ownership is philosophically and politically imbricated with the dichotomy between the conditions of freedom and enslavement. As the philosopher John Locke famously asserts, "every man has a 'property' in his own 'person,'"[66] signifying the inherent bodily autonomy of the Human, which is manifested through the right of individuals to exercise ownership over themselves. The teleological implication of this assertion is that individuals who are enslaved are deprived of both the right to bodily autonomy and the right to property. In the context of Colonial Man and the European endeavor of Indigenous genocide and African enslavement, property rights establish a metonymic association with the nascent construct of whiteness, wherein freedom was equated with whiteness and concomitant property entitlements, while enslavement was correlated with blackness and the absence of such rights. Harris adopts this ideological framework and extends its ramifications by asserting that: "Whiteness defined the legal status of a person as slave or

free. White identity conferred tangible and economically valuable benefits and was jealously guarded as a valued possession, allowed only to those who met a strict standard of proof. Whiteness—the right to white identity as embraced by the law—is property if by property one means all of a person's legal rights" or in others word whiteness in itself constitutes a property right. This same ideological framework was also weaponized for the dehistoricization of indigenous land stewardship and concurrently Indigenous land dispossession. This is rooted in Locke's labor theory of property: "The labour of his body, and the work of his hands, we may say, are properly his. Whatsoever then he removes out of the state that nature hath provided, and left it in, he hath mixed his labour with, and joined to it something that is his own, and thereby makes it his property."[66] In light of these premises, the attribution of property rights to land is predicated upon an owner's capacity to enhance the land, a concept rooted in European frameworks governing land commodification and administration. Indigenous methods of land stewardship, often communal in nature and divergent from these standards, were deemed inferior by Colonial Man. Consequently, Indigenous practices fostered an impression of lands perceived as unaltered and untended, a perspective endorsed by colonial authorities and subsequently adopted in the nascent American context[67]. This perception served to undermine Indigenous land rights, relegating them to mere usufructs. Moreover, this rationale constituted the legal foundation for Indigenous land dispossession and concurrently shaped the framework for the valorization of property rights within early American legal systems[68]. Hence, concomitant with the Human's right to property is a biopolitical agenda of the state, which dictates the legal safeguards afforded to property and consequently facilitates the transformation of nature, irrespective of prior patterns of habitation or governance, into private property intended as a material basis for production.

In the framework of our analysis of anthropogenic nitrogen pollution, the initial two implications of the Human are pivotal for elucidating the modalities underlying the social relations that engender the transfiguration of nitrogen into a modular input for production. The recognition of the Human as inherently distinct from nature, coupled with the assertion of property rights enabling the transition of communal resources into private ownership, forms the foundational tenet that underpins the historical materialism evident in agriscience and biochemistry. This transition delineates our movement from the relational paradigms inherent in Indigenous cosmology and land stewardship towards the ontological underpinnings that facilitate the quantification and commodification of nature. The evolving understanding of nitrogen resides within the interstices of the historical progression of nation states embodied by liberal interpretations of bourgeois property rights, political economy, and market orientated modes of production. The ultimate and paramount implication of the Human, which will establish the framework for the subsequent discussions in this chapter, will be expounded upon in due course.

1.2.2 The Relational Paradigm of the Human and the Inhuman

The third and most critical implication of the world-building framework engendered by the Human is the conception of what scholar Kathryn Yusoff outlines as the "Inhuman"[69] or alternatively articulated by scholar Frank Wilderson III as the "Slave"[70]. Considering every designation of a state of existence it becomes evident that an antithesis is indispensable for

delineating its material implications, the status of being alive necessitates an awareness of death, the perception of light is contingent upon the existence of darkness, and heat cannot be understood without the absence of heat—cold. Thus, the corporeal manifestation of the Human as a biocentric actant within a narrative of conquest and domination cannot be fully conceptualized without juxtaposing it against its antithesis, the "Inhuman". The "transcultural space" theorized by Epstein[54] and Wynter produces culturally contextualized understandings of the "Self" necessarily in relation to the "Other"[53] which lies beyond the confines of any communal framework of self-awareness. It is imperative to clarify that within the hierarchical construct of living and autonomous entities as delineated by the Human, the notion of the "Other" assumes a crucial role as an ontological marginalization. However, it is more accurately designated as "Sub-Human," as it exists external to the parameters of the Human but does not inherently constitute its essential reciprocal negation[70]. The Inhuman or the Slave is the agglomeration of everything that is antithetical to the Human who has autonomy, biocentric superiority, and inherent property rights. It represents an object that is inert, devoid of will, agency, or consciousness functionalized through the concept of blackness[69]. The Slave according to scholar Saidiya Hartman is "the object or the ground that makes possible the existence of the bourgeois subject and, by negation or contradistinction, defines liberty, citizenship, and the enclosures of the social body"[71] meaning the personhood, rights, and agency of the Human is necessarily defined by the negation of those things in the Slave. The transfiguration of the enslaved African into the Inhuman is facilitated by what the esteemed scholar Orlando Patterson terms "social death"[72] and further elucidated by scholar Hortense Spiller through the notion of "historical stillness"[73] that fully alienates and removes the enslaved from the social tapestry of the world, rendering them ahistorical and inherently static and undynamic. Within this framework, it becomes apparent that the Inhuman does not embody life in the manner comprehended by the Human, characterized by the unrestricted prerogatives of dominion and appropriation. This designation of the Inhuman as nonliving is what Yusoff describes as "geopolitics" that is a praxis utilized to relegate objects to the status of living or non-living, resonant with geological taxonomies utilized in mineral extraction as Yusoff explicates "The biopolitical category of nonbeing is established through slaves being exchanged for and as gold. Slavery was a geologic axiom of the inhuman in which nonbeing was made, reproduced, and circulated as flesh."[69] In essence, the Human, asserting property rights and converting nature into private property for extraction, necessitates the designation of the Inhuman as a non-living entity, thereby positioning it as a subject for extraction. Without the Inhuman, the Human forfeits biopolitical autonomy essential for the assertion of freedom, leading to the obfuscation of distinctions between states of enslavement and liberty as Wilderson explains "What civil society needs from Black people is confirmation of Human existence."[70]

Although the imbrication of slave and blackness constitutes the fundamental axiom shaping the contemporary global order, the European derivations of the "Other" in relation to the Human and constructs of race and blackness, have established intertwined yet distinct evolutionary trajectories before coalescing. In the pre-Renaissance conceptualization of the Human, defined primarily through Christendom and the hegemony of the Church, the "Other" was manifested through Islam exemplified by the presence of the Moors in Al-Andalus and the Arab kingdoms of the Middle East and later Ottoman Empire at the peripheries of Christendom. Therefore,

preceding the complete crystallization of race as a social construct, Medieval Europe associated Africa with a representation of satanic witchcraft, symbolized by the figures of the "black moore" or the "Ethiope"[360]. Nevertheless, the intricate entanglements of Islam with notions of blackness and demonology were not inherently racialized during this period. Consequently, the designation of the "Other" was typically correlated with individuals perceived as adherents of a "lesser god"[57]. The construct of race emerged in Medieval Europe to delineate various ethnic groups that today would all be considered "white" in order to rationalize the exploitation of specific caste as Robinson explains "The tendency of European civilization through capitalism was thus not to homogenize but to differentiate—to exaggerate regional, subcultural, and dialectical differences into 'racial' ones." The nascent stages of capitalism and the emergence of a modern bourgeoisie, crucial components in shaping the contemporary global structure, were inherently intertwined with the widespread and aggressive racialization of diverse ethnic groups within Europe. It was not until the dissolution of feudalism and emergence of mercantilism that this praxis was extended to other non-European peoples.

Slavery had been entrenched in European societies since Antiquity, with historical records indicating Greek and Roman economies to be heavily dependent on the extensive utilization of slave labor[74,75]. The rationalizations of slavery as a necessary condition for society were deeply ingrained within Greek law and philosophy and were subsequently extended to its evolutionary derivate in Judeo-Christian ideology[76]. By the Medieval Era in Europe slave labor was concentrated primarily around the Mediterranean with slave domestic work in urban centers being quite common[77]. Notably, in early industrial and mercantile metropoles Genoa and Venice, enslaved individuals constituted a significant portion of the population, reaching levels as high as 10%[78]. Thus, the Mediterranean developed as a principal conduit for the trafficking of slaves from various ethnic and racial origins to major slave ports including Genoa, Venice, Barcelona, Valencia, Seville, and Malta in Europe, Algiers, Tunis, Tripoli, and Alexandria in North Africa, Istanbul in Anatolia, and Cyprus (notably for sugar production)[79]. As a result, the Mediterranean slave trade is estimated to be one of the most lucrative trades in all of Medieval Europe and especially critical to the development of mercantile capitalism in Italian city states[80]. Religiously motivated enslavement proliferated with papal proclamations sanctioning the subjugation of non-Christians consequently propelling enslavement of Muslims within Christian territories, as well as the enslavement of Christians in Al-Andalus and North Africa to become commonplace[81]. Through the Mediterranean slave trades Europeans encountered North African Moors through commerce with Morocco and sub-Saharan African slaves through the Trans-Saharan slave trade[59]. However, the most commonly enslaved ethnic group in Europe for the majority of the medieval period were Slavs from Eastern Europe and the Black Sea and hence the etymological introduction of "slave" into the English language[80].

Two historical occurrences were paramount to the increasing popularization of African slave labor and the consequent homogenization of diverse sub-Saharan African peoples into the "Negro" and the conflation of blackness with slaveness. The first is the fallout of the War of

[3] Europeans had a general awareness of Ethiopia since Greek and Roman antiquity and had colloquially associated Ethiopians with blackness and thus Medieval discourse often generalized blacks to Ethiopes.

Chioggia in 1381 between Genoa and Venice that alienated Genoa from Eastern Mediterranean spice routes and the lucrative sugar trade based in Cyprus, and second is the fall of Constantinople to the Ottoman Empire in 1453 severing Mediterranean connections to the Black Sea and its reservoirs of slave labor[82]. These historical developments compelled Genoa to turn West to the Iberian peninsula to leverage burgeoning relationships with Spain and Portugal both of which were looking to expand into nascent frontiers in the Atlantic and Africa. Consequently, the disruption of traditional slave routes in the Black Sea spurred an increased demand for other sources of slave labor[59]. Genoese capital and financing were critical to the initial slaving expeditions of the Portuguese into West Africa culminating in the documented maiden slaving voyage in 1444 into what is now Sierra Leone that delivered 300-400 Africans into bondage in Lisbon[83]. Throughout the remainder of the 15th century Lisbon dominated the emerging West African slave trade reaching Senegal and Cape Verde by 1446, Benin and Biafra by 1474 and Congo by 1483[59]. The incipient prevalence of African slavery in the Iberian peninsula demanded an ideological companion as an accoutrement of the mercantile capitalism developing simultaneously. Propagations of the inherent inferiority and bestiality of individuals of African descent were formulated to justify paternalistic attitudes towards their enslavement, portraying it as a requisite endeavor for their supposed civilizing and Christianizing as royal Portuguese Chronicler Gomes Eanes de Zurara reports on Lisbon's first slaving voyage of 1444 in 1450:

"for amongst them were some white enough, fair to look upon, and well proportioned; others were less white like mulattoes; others again were as black as Ethiops[4], and so ugly both in features and in body, as almost to appear (to those who saw them) the images of a lower hemisphere. But what heart could be so hard as not to be pierced with piteous feeling to see that company?"[84]

This report was widely circulated throughout Europe, significantly influencing European perceptions of Africans as innately predisposed to servitude[85]. Consequently, it laid the foundation for the burgeoning significance attributed to Africans within the framework of slave labor in the nascent system of mercantilism.

The Portuguese pioneered the model of the plantation system of the Americas, laying its foundations in their Atlantic possessions in the Azores, Cape Verde, and Madeira[59]. Madeira was possibly the first of such examples wherein the landscape was entirely repurposed for sugar production with a diverse mix of sub-Saharan African, North African, Mediterranean, and Levantine slaves for labor. São Tomé, however, emerged as the archetypal exemplar of plantation agriculture characterized by racialized slave labor, notably with sugar production bolstered by copious imports of slaves from the Congo[86]. Due to navigational prowess the Iberian peninsula emerged as the preeminent power in the Age of Discovery as Horne explains "From the early 1440s to 1521 an estimated 156,000 Africans arrived in Spain, Portugal, and the Atlantic islands, mostly from today's Guinea-Bissau, Guinea-Conarky, Senegal, the Gambia, and parts of Mali and Burkina Faso."[59] As a result, upon Columbus' "discovery" of the New World in 1492, the groundwork had been laid to translate the experiment of cash crop agriculture in the

[4] Again there is the reference to Africans as black as Ethiops, indicating a widespread association with Ethiopians with blackness.

Atlantic islands to an industrial scale bringing along with it the decimation of Indigenous peoples and the mass forced migration of Africans. These developments were paralleled by escalating debates over the ethical grounds of Indigenous versus African enslavement epitomized by the seminal debate between de las Casas and Sepulveda that ultimately determined the Indigenous having never encountered Christianity had "souls" and were thus eligible for conversion, whereas Africans having been exposed and proving recalcitrant to conversion relegating them to non-Christians and thereby justifying their enslavement[87]. These debates alongside the perception of the Indigenous as inherently feeble and unfit for physical labor gave the proverbial "green light" to early Portuguese and Spanish colonial authorities to pursue African enslavement with the utmost vigor.

The culmination of these historical developments resulted in the congealing of the Human as the protagonist in the narrative of discovery, Christianization, and civilization and the Inhuman as its necessary negation forming a dialectic prompting blackness to become coterminous with slaveness. However, it is a fallacy and act of historical obscurantism to assert that racialized theories of inferiority drove the enslavement of Africans, the reality is quite the converse, the economical and political imperatives of African slavery demanded an ideology that rationalized and encouraged African enslavement based on humanist perceptions of personhood centered in Western ontologies of categorization and extraction. In doing so the Slave is reduced to "nonagentic matter" through "thingification" paralleling geological designations of mineral matter as Yusoff explains in engagement with Hartman and Spiller:

"The slave in this formulation is rendered as matter, recognized through an inhuman property relation—what Saidiya Hartman calls fungibility—as a commodity with properties, but without subjective will or agency (or "flesh", as Hortense Spiller has it). Rendering subjects as inhuman matter, not as persons, thereby facilitated and incorporated the historical fact of extraction of personhood as a quality of geology at its inception."[69]

This process was essential for the transfiguration of New World peoples, landscapes, and cosmologies into the constitutive inputs for mercantile production as Wynter describes in her seminal work *Black Metamorphosis* "the reduction of Man to Labour and of Nature to Land under the impulsion of the market economy."[88] The emerging system of production that completely reoriented social relations, land management, and existing practices of commerce was entirely contingent on the relegation of the black to the Inhuman, an entity entirely devoid of personhood or autonomy for the purposes of exclusive control and extraction of surplus labor. The overt valorization of the black to its labor power was done for the sole purpose of fungibility to be transported, utilized, and disposed as a modular input for the capitalist system of production as Wynter explains:

"The not-I of Western idealist philosophy, that humanism which later spawned the concept of the rights of man, was the non-white sub-human assimilated to Nature and the ultimate non-white was black. The systematic devaluation of the black as human went hand in hand with the systematic exploitation of his labor power."

Hence, it would be a tautology to make the assertion that the Slave is utilized for its labor power or that the Inhuman lacks agency or will as compared to the Human because the Slave is inherently a manifestation of Colonial Man's desire for fungible labor and the Inhuman is intrinsically an object of extraction to materialize the Human's property rights. The fact that these designations are inextricably imbricated with blackness carries the implications of Black people by virtue of existing in the world as presently constituted are merely infinite reservoirs to deposit the material, spiritual, and epistemological violence that structures the world. The natural question one would ask is how does this at all relate to nitrogen as a pollutant in the current world order? The simple answer to this question is because gaseous nitrogen as etymologically indicated is mephitic, phlogisticated, lifeless, and inert but at the same time vital to the Human conceptual understanding of life and sentience. Nitrogen, abstracted from its relational paradigms as an agent of both life and death, is ontologically rendered to a state of unbeing, as an immaterial object, which is ripe for separation, quantification, commodification, and extraction. The current world order that is structured around state monopolized violence for the extraction of labor power for commodity production in market-oriented economies is critically dependent on the black being the epicenter of subjugation and barbaric violence just as it is dependent on nitrogen as a lifeless entity that can be extricated and commercialized. Both of these paradigms endeavor to transmute the Slave and Nitrogen as an element into inert nonagentic matter that can be morphed into modular inputs of production as both labor and capital. In the eyes of the Human, the Inhuman and Nitrogen are constituted of the same lifeless matter that both justifies and encourages its exploitation and expropriation. With this understanding, we can now proceed to examine the history of these extractive relationships and how these relationships have been shaped by various political, economic, and historical developments.

1.2.3 Nitrogen in Agriculture from pre-history to the Mercantile Period

Early humans adopted a variety of peripatetic hunting and foraging techniques prior to the Holocene in order to maintain sufficient intake of dietary nitrogen[89]. Populations were managed effectively in order to ensure finite resources would not be overextended, thereby mitigating anthropogenic perturbations on the nitrogen cycle to a negligible extent[90]. Evidence of diverse iterations of sedentary agricultural practices emerges sporadically throughout prehistoric epochs, spanning from the cultivation of yam fields in the Borneo highlands approximately 30,000 years ago to the inception of bread-making using wild cereals in the Levant around 11,000 years ago[91]. However, it is generally accepted that by about 10,000-8,000 years ago, a significant transition from hunter-gatherers to sedentary agricultural settlements had been made in various places across the globe[89]. This progression likely transpired in a non-linear fashion, with hunter-gatherer communities and agricultural societies cohabiting for a certain duration[92]. Scholarly discourse persists regarding the motivating factors underlying the transition from hunter-gatherer lifestyles to agriculture, encompassing hypotheses ranging from climatic stability during the Holocene's onset to the decline of large game populations[935]. The shift from nomadic patterns of

[5] Transitions to sedentary agricultural settlements was also a self-reinforcing cycle, for example the domestication of animals would restrict mobility, construction of semi-permanent and permanent dwelling and storage would disincentivize migratory patterns. See *The Emergence of Agriculture* (Smith, 1995)

habitation and resource utilization to enduring settlement precipitated significant alterations to the nitrogen cycle, concurrently establishing fundamental frameworks conducive to the emergence of capitalist systems.

While it is highly probable that ancient societies lacked a comprehensive understanding of the mechanisms dictating soil fertility, one might reasonably infer that repeated cultivation on the same land over successive years would compel early agriculturalists to recognize declines in soil fertility through empirical observation. Through adaptive selection and experimental adjustments many societies likely learned to develop soil fertility management practices or depend on natural resources to provide fertilization. One of the earliest soil management practices was likely the intentional practice to let fields remain fallow wherein farmers would observe the gradual recovery of soil fertility over time[46]. This practice was likely complemented by a proto-sedentary cyclical slash and burn where forested areas were systematically cleared and burned, releasing accumulated nutrients[6] and fertilizing the soil prompting a perpetual pattern of migration to fresh territories[46]. Nevertheless, this practice was constrained to regions devoid of acute land scarcity or those situated in tropical areas where fallow fields could regenerate into forest cover within a decade or two[94]. Farmers also learned to leverage natural resources; many of the prominent Ancient civilizations including Egypt, Mesopotamia, Indus Valley, and Han China, emerged in river valleys or floodplains that flooded annually and provided rich alluvial deposits that would provide an influx of nitrogen to the soil[46]. Organic recycling of crop residues probably developed concomitantly with the emergence of sedentary agriculture[3]. Other civilizations facing land scarcity such as Han China developed an awareness of animal[7] and human waste as natural fertilizers deploying them extensively to compensate for the challenges posed by a high population-to-arable-land ratio[958] and this knowledge had almost certainly been popularized in Europe by the apex of Greek and Roman civilization[96–989].

The awareness of integrating leguminous crops (beans, lentils, peas, clover, alfalfa) developed independently throughout many regions with historical indications of lentils and pea rotations with wheat and barley in the Middle East by 7000-6000 B.C.[99] and documents of beans, lentils, and chickpeas in Old Kingdom Egypt[3] serving as some of the oldest examples. However, intercropping and crop rotations of legumes with different root crops and cereals was common in Europe, sub-Saharan Africa, Asia, and the Americas[3]. The utilization of legumes in crop rotations facilitated the evolution of knowledge regarding their efficacy as green manures with some of the earliest historical recordings of these practices dating back to fifth century B.C. Han

[6] Most nitrogen was probably lost through NO_x combustion in the fires and only a small proportion returned to the soil through deposition but this was likely enough to yield a few good harvests-see General Energetics (Smil 1991)
[7] The use of animal waste as a fertilizer was in most cases contingent on the use of draft animals to plow fields, and this was constrained to the "Old World" as the use of draft animals in the Americas did not seem to be widely prevalent.
[8] Nitrogen available to crops through traditional manuring practices was actually quite limited due to volatilization and nitrate leaching
[9] Many reconstructions of Greek and Roman agriculture come from the works of philosopher Theophrastus (371-287 BC) and Roman soldier, senator, and historian Marcus Porcius Cato (234-149 BC) and Pliny the Younger (born 61 AD)

China records[100] as well as Ancient Greek and Roman text[97,101]. By integrating organic recycling, manuring techniques, crop rotations, and the cultivation of leguminous crops, the foundational principles of pre-Industrial agriculture were solidified which according to Smil may have provided on average 100-150 kg of N/ha annually or enough to feed roughly 5 people/ha[310]. Innovations in farming such as Chinese double cropping with soybeans and rice extended to the upper limit of this range and may have been enough to feed roughly 7 people/ha[11] on largely vegetarian diets[3]. Furthermore, the transition from two-crop[12] to three-crop rotations in Medieval Europe, eventually leading to the English Norfolk rotation, may have provided similar nitrogen inputs which could sustain 3-4 people/ha on diets rich in dairy and meat[3].

The development of agricultural societies instigated alterations to the nitrogen cycle initially centered around the widespread cultivation of wetlands eliminating their function as a sink for fixed nitrogen and the redistribution of alluvial nitrogen through irrigation projects[46]. Agrarian food production also heralded the inception of surpluses that could be stored or traded, incentivizing the establishment of markets and trade relationships[13] with neighboring settlements prompting transformative changes in the social structures of human interaction. This development precipitated the establishment of a centralized mode of governance that would be able to manage surpluses, land and irrigation projects, regulate trade, and construct a legal framework to mediate conflict[46]. In most pre-industrial societies, the predominant portion of labor was dedicated to growing sufficient food to sustain the population as scholar Hugh Gorman explains "No ancient society could maintain a population of soldiers, miners, artisans, judges, accountants, and administrators unless peasants were able to produce enough food to feed them. And generating that surplus presented a significant challenge. A rough rule of thumb is that, in a traditional agricultural society, about 90 percent of the population was needed to produce, process, and transport enough food to feed the entire society."[46] Put differently, for an empire to ascend, surplus value must be extracted from the labor of the peasantry. These societal changes heralded the advent of the first codified inscriptions of exploitation in the form of taxes, tributes, slavery[14], or a combination of all three[46]. Surplus facilitated the emergence of non-agrarian populations, laying the groundwork for the first towns and cities, and the establishment of centralized systems of trade and commerce, thereby exacerbating existing disparities in societal wealth distribution. The other transformative social change that agrarian societies engendered was the transition to patriarchy. In hunter-gatherer societies matriarchally centered social

[10] These estimates are based on an assumption of a minimum nitrogen efficiency (the amount of nitrogen taken up by the crops relative to nitrogen applied) somewhere in the range of 20-30% and an average protein intake of ~16 kg protein/person. See Enriching the Earth (Smil, 2004)

[11] These estimates are based on historical reconstructions of crop rotations estimating a maximum nitrogen use efficiency of around 50-60% that would theoretically be able to feed 12-15 people/ha but must be adjusted for nonfood crops and limits on multicropping and water supply at different scales

[12] Two crop rotations had been popular in Europe since the Romans

[13] Markets and trade relationships are known to exists prior to sedentary agriculture, hunter-gatherers likely traded obsidian and ocher. See "Long-Distance Stone Transport and Pigment Use in the Earliest Middle Stone Age" (Brooks et al., 2018)

[14] Slavery is theorized to have existed in hunter-gatherer societies prior to sedentary agriculture. See The Evolution of Lethal Intergroup Violence (Kelly, 2005)

networks were common given that procurement of food, aside from hunting, and other crafts were overseen by women, rendering paternal lineage relatively inconsequential[102]. Following the transition to sedentary lifestyles, starting with pastoralism, the capacity to generate large surpluses (like herds of animals) prompted a societal reconfiguration that underscored the importance of paternal lineage in delineating inheritance patterns[102]. This restructuring marginalized women from the central roles in production within hunter gatherer societies to mere instruments for producing heirs and undertaking domestic labor[15]. The extraction of surplus value from labor, facilitating the accumulation of initial capital and the transition to patriarchy constitute some of the most radical shifts in social relations that establish the prevailing global socioeconomic structure.

1.2.4 Colonial Man and the Roots of Nitrogen Asymmetries

Before the advent of the mercantile era, characterized by the urbanization of European nation-states[103] and the expansion of global commerce, nitrogen cycling was highly localized promoting the cyclical exchange of nitrogen between the biosphere, lithosphere, and hydrosphere at local and regional scales[46]. The cascade of historical developments precipitating colossal redistributions of nitrogen across extensive latitudinal gradients is rooted squarely in the system of market-oriented agriculture or *cash cropping*. Cash crops, although not the genesis of capitalist systems of production, have wielded significant influence in expediting the penetration of this mode of production into emerging markets. Producing crops for a market or their "exchange value" has entirely different implications than producing food crops for subsistence or their "use value" on the labor and natural resources used to produce them. The system of commodity agriculture serves as the impetus for the proliferation of plantation farming and mercantile trade relations, intrinsically linked to Indigenous genocide and African enslavement, and constitutes the foundational underpinning for the prevailing syncopation of global nitrogen asymmetries.

Growing crops for a market is hardly a mercantile inception, the Ancient Greek economy was almost entirely dependent on cash crops fueled by slave labor and the Roman latifundium emulated their model to propel extensive trade networks throughout their empire anteceding their descendants in plantations of the Americas by almost two millennia[82]. While the European conquest of the Americas is often synonymous with the pursuit of gold and spices, the desire for sugar held comparable significance. The cultivation of sugar in Portuguese colonies in the Atlantic, dating back to the 1440s, finds its origins in the impetus provided by their Genoese financiers and colonial predecessors.[82]. The Italian city states had exploited islands in the Aegean for centuries during the Middle Ages and dominated the highly remunerative sugar trade that flourished in Cyprus following its establishment by returning Crusaders in the late 13th century[104]. The Medieval Genoese and Venetians established a commercial hegemony in the Mediterranean following a model of slavery and colonization that facilitated the emergence of

[15] This is not the case in all post-agricultural societies. Matriarchal social and family networks persisted in societies in the pre-Columbian Americas, sub-Saharan Africa, and South Asia and there's actually quite a bit of debate against this view of archaic matriarchal societies. See *The Myth of Matriarchy: Why Men Rule in Primitive* Society (Bamberger, 1974)

financial innovations such as permanent trading firms, double-entry booking, debt instruments, and banking[82]. Following climactic changes attributable to the Little Ice Age, compounded by the ravages of the Black Death and consequent demographic downturn, Cyprus collapsed as the nucleus of sugar production in Europe creating an enormous hole to fill voracious European sugar demand[82]. The Portuguese, seeking to leverage this opportunity, invested heavily in the sugar trade, outfitting Maderia, the Azores, and Sao Tome as sugar factories[105]. However, rapid soil degradation, limited land supply, and incessant slave revolts tapered their projections and presaged what was to become of European possessions in the Americas[86].

Following the "discovery" of the Americas and subsequent colonization, burgeoning European nation states sought to consolidate hegemony into a nascent political economy through mercantile economic policies such as market monopolization, high tariffs on manufactured goods, and the accrual of monetary specie that was highly contingent on slavery and Indigenous dispossession in what scholar Sven Beckert calls "war capitalism"[106]. War capitalism as Beckert describes was "Slavery, the expropriation of indigenous peoples, imperial expansion, armed trade, and the assertion of sovereignty over people and land by entrepreneurs"[106]. This epoch signifies what Marx would categorize as "primitive accumulation"[107] — the foundational accumulation of wealth requisite for the emergence of industrial capitalism during the eighteenth and nineteenth centuries, a process facilitated through coercive acts of dispossession and expropriation. While cotton eventually emerged as the quintessential colonial commodity and catalyst for the transition from war capitalism to industrial capitalism, sugar was the original proverbial apple of Europe's eye at the dawn of the mercantile period and this apple sprouted rapidly in the West Indies. Slave imports to Jamaica alone are estimated to be 610,000 from 1700 to 1786[108], 353,000 to Barbados between 1651 and 1807[109], and 800,000 to Saint Domingue between 1697 and 1789[11016]. Exports of sugar from the British colonies alone went from a negligible amount in the 1640s to nearly 26,000 tons/year in 1700 to nearly 100,000 tons/year in the 1770s[111].

Sugarcane, the lifeblood of the West Indies in the early colonial period, held a value nearly equivalent to that of gold, aptly earning the moniker "white gold" among planters and merchants of the time. The commerce of sugar was so important to the British economy, Scottish economists Adam Smith commented in his seminal treatise "The profits of a sugar plantation in any of our West Indian colonies are generally much greater than those of any other cultivation that is known either in Europe or America."[112] Sugar along with other colonial commodities constituted the basis of the renowned Triangular Trade, a system characterized by the exportation of raw materials from the colonies to European metropoles, the exportation of manufactured goods[10817] to acquire slaves in West Africa, and the transportation of slaves to the colonies in exchange for raw materials, thus culminating the cycle. This pattern of commerce epitomizes mercantile trade relations between colony and motherland, effectively monopolizing trade, and asserting hegemonic control that isolates colonial markets, even among colonies under the same

[16] Spain should not be excluded from this consideration, however sugar was not a serious enterprise in Spanish Caribbean colonies until the nineteenth century.

[17] The most popular manufactured goods exchanged for slaves were guns, cotton cloth, and rum. See *Capitalism and Slavery* (Williams, 1944)

crown. Furthermore, the production of commodity crops within the plantation system foreshadows and exemplifies industrial capitalism in a distinct manner as Beck describes "the true importance of the Caribbean planters was not the cotton that was shipped, though that remained essential, but the institutional innovation that the Caribbean experiment produced: the re-creation of the countryside through bodily coercion, something only possible under war capitalism." Meaning that systematic hegemony imposed by industrialization derived its rhythms directly from the genocide, dispossession, and enslavement of war capitalism. This is manifested in the recognition that the plantation served as a true precursor to the factory, given the systematic, centralized, and highly efficient extraction of labor it employed as Wynter describes "the plantation and its mass labor force prefigured important aspects of the organization of modern industrial/technological society. And, as both the earlier and later theorists insist, the "slave functioned as an early form of the assembly line modern proletariat". The seasoning process which the slave underwent was not only a process of adaptation to this new environment. It was also a breaking in of a peasant accustomed to a different rhythm of group labor into a gang system of intensive carrying out carefully allocated tasks."[88] However, the draconian violence and regulation of slave labor in the West Indies also contributed to shaping working-class consciousness in Europe, particularly through a racial paradigm as Wynter explains "By being allowed to terrorize the freed slave, the poor whites are induced to accept the relatively milder forms and modalities of social repression exercised by the bourgeois[ie] against them. The slave too used his vicarious identification with the rich masters to look down on the poor whites. As Fanon says, the Negro *too* wants to be master. The proletariat wants to be the bourgeoisie, as the middle class black wants to be the white master"[88]. In this context, Wynter contributes to the transition of capitalism from a framework focusing solely on internal and external systems of production and exploitation to a broader understanding of a world system structured by the dominance of frontier markets[113].

An often overlooked aspect of the Triangular Trade paradigm is the ecological overdraft and underlying environmental imperialism intrinsic to these trade relations. Cash crop agriculture is fundamentally predicated on the cultivation of crops intended for commercial markets, thereby adhering to a prevailing practice of monocropping, primarily focusing on the cultivation of a single crop variety. Such agricultural practices deplete the soil of its essential nutrients, hastening the depletion of fertility and exacerbating erosion processes. The deleterious effects of monocropping on West Indian soil has a long and well documented history. Colonial reports in Barbados in 1661[114] cite decreasing soil fertility throughout the Caribbean instilling the fear of entire landscapes being washed away like a 1668 downpour that excavated hundreds of coffins from a churchyard and subsequently carried them out to sea[115]. By 1784 Antiguan planter Samuel Martin exclaimed the soil was completely lost of nutrients and called for a complete overhaul of soil management practices[116]. The demand for virgin soils precipitated the decimation of forest cover in most of the Caribbean islands which may have been semi-sustainable for larger islands such as Jamaica but virtually impossible for smaller islands in the Windwards and Leewards who had to turn to aggressive manuring and organic recycling to

maintain productivity[115,11718]. Nevertheless, these incentives were insufficient to prompt a substantial consideration of established and efficacious soil management techniques, such as crop rotations. This stems from the fact that sugarcane, along with the entirety of the commodity agriculture system, effectively externalized the adverse environmental impacts associated with its cultivation. The ecological degradation wrought by sugarcane cultivation, as perceived by Colonial Man, is not viewed as an insurmountable barrier constraining profit generation, but rather as a threshold to be extended in pursuit of greater economic gains as scholars Brett Clark and Richard York explain "rather than acknowledging metabolic rifts, natural limits, and/or ecological contradictions, capital seeks to play a shell game with the environmental problems it generates, moving them around rather than addressing the root causes."[65] Moreover, the preoccupation with maximizing profits in the sugar colonies resulted in a scarcity of land earmarked for the sustenance of the requisite slave population essential for cultivating sugarcane. This dilemma found resolution through British mercantilist strategies, whereby the vast majority of the food and fiber and even much of the lumber needed to feed, clothe and house the slaves in sugar colonies came from the British North American colonies as the eminent scholar Eric Williams writes in his classic *Capitalism and Slavery*:

"This was a deliberate policy on the part of the statesmen in England and the planters in the colonies. Many of the articles exported by New England to the islands could have been produced in the islands themselves. But, as a Jamaican planter asked, "If this island were able to maintain itself with diet and other necessaries what would become of the New England trade?" The answer is that without the sugar islands the mainland colonies would have received a serious setback. They became "the key to the Indies," without which the islands would have been unable to feed themselves except by a diversion of profitable sugar land to food crops, to the detriment not only of New England farmers but also British shipping, British sugar refining, and the customs revenue, glory, and grandeur of England."[108]

Put differently, the ability of the British sugar colonies to yield substantial quantities of sugarcane for export relied entirely on the North American colonies to furnish them with virtually all other essential provisions. In exchange, the colonies received West Indian sugar, rum, and molasses that was used to develop their own manufacturing capabilities[108]. Hence, when the obstreperous colonists' fear of the motherland turning them into slaves pushed them to revolution, it portended an ominous future for the British West Indies. One crucial aspect that warrants attention is the mercantile system honed by the British, which strategically harnessed the biogeochemical redistribution of vast quantities of nitrogen, manifested in food, fiber, and timber, facilitating subsequent nitrogen displacements from the Antilles to the European continent. This occurrence represents what could arguably be considered the first globally orchestrated multidirectional diversion of nitrogen flows to sustain the circulation of capital. The tricontinental trade regulating flows of raw material, manufactured goods, and human chattel, was inherently predicated upon a bidirectional exchange of nitrogen in various manifestations. British aspirations to monopolize the sugar trade and later cotton underscore a precipitous

[18] Mill trash (cane tops) were common organic fertilizers in sugar growing colonies. See *Sea and Land: An Environmental History of the Caribbean* (Morgan et al.,

disruption to localized nitrogen cycling across the globe, presaging the transformative trajectory aligned with the emergence of industrial capitalism.

1.2.5 The Saltpeter Trade, Early Formation of Nation-States, and the Crystallization of Whiteness

Up to this point, we have examined the role of nitrogen in agriculture and the roots of its appropriation for the incipience of capitalist production, the other complementary and reciprocally linked story is nitrogen as a direct instrument of war and conquest. Saltpeter, or "niter", crystallized potassium nitrate formed primarily from decaying organic matter, assumed a corporeal manifestation of nitrogen before its users could even grasp the concept of atomic forms of matter or nitrogen as an element. What they did understand through pure empiricism was that a combination of primarily saltpeter mixed with brimstone (sulfur) and coal produced gunpowder, a concoction that could concentrate the power to eviscerate flesh and stone alike within an individual's hand[46]. The relative abundance of brimstone and coal accentuated saltpeter's role as the limiting factor in the state's capacity to conduct warfare and execute mass genocide and enslavement in the colonies. This instigated aggressive strategies to control domestic saltpeter sources and consolidate foreign and domestic saltpeter markets which ultimately led to further centralization of power in nascent nation-states, extension of political economy beyond state borders, and the codification of whiteness in the colonies through the monopolization of arms.

The chemical mixture to produce gunpowder had been known to Chinese alchemists looking for an elixir for eternal life as early as the 9th century, standardized formulas were popularized by the 11th century, and gunpowder based projectiles and hand cannons followed in the 12th and 13th centuries[118]. This knowledge was disseminated throughout the Middle East, India, and Central Asia over the course of hundreds of years eventually reaching Europe[19] as evidenced by the earliest documented instance of gunpowder in Europe from the Franciscan Roger Bacon[119] in 1267[20]. The demand for saltpeter in Europe escalated dramatically, coinciding with the widespread adoption of cannons and firearms within military structures by the late 14th century[11821], cannon mounted naval vessels standard by the fifteenth century[120], and the conquest of the Americas all requiring copious amounts of gunpowder collectively transforming European nations into what scholar David Cressy calls "gunpowder states"[119]. By the denouement of the feudal epoch and the advent of the mercantile age, saltpeter had entrenched itself deeply within alchemical tradition becoming widely acknowledged as an effective fertilizer[119]. The chemical dualism inherent in saltpeter, serving both as a formidable explosive and a vital fertilizer, posed a conundrum to early scientists, suggesting a semblance of divine or mythological significance as Elizabethan theorists Joseph Duchesne described "a notable mystery the which, albeit it be taken from out of the earth, yet it may lift up our eyes to heaven"[121], or a tellurian reverence "a

[19] Likely via the Silk Road

[20] Saltpeter often had to be extracted from a mixture of salts containing potassium nitrate and calcium nitrate which could easily react with water giving undesirable properties. Thus many alchemist endeavored to come up with processes to refine pure salt mixtures to obtain saltpeter.

[21] Earliest evidence of widespread use of cannons in military formations is at the Battle of Crecy between England and France in 1346.

quintessence of qualities…convertible to all the elements" as described by gunner Robert Norton[122]. The confluence of saltpeter's status as a product of death and yet an enabler of life contributed to pervasive mythologization surrounding its material characteristics and its elusive nature as the primary constituent of gunpowder. A comprehensive understanding of the chemical properties of saltpeter would elude gun manufacturers until scientific advancements of the nineteenth century.

Saltpeter was known to naturally accumulate in dry places harboring decaying organic matter such as dung or corpses[119]. Owing to the imperative for saltpeter in gunpowder production[22], domestic reservoirs underwent rigorous regulation by state intervention, exemplified by the "saltpeter men" of Elizabethan era England[119]. Their comprehension of its formation mechanisms remained elusive, yet they possessed a pragmatic understanding of its whereabouts: sites where human or animal waste accumulated within domiciles or where cadavers were interred. Saltpeter men had sovereign authority to search wherever they deemed likely to contain saltpeter as Cardwell describes:

"soldiers who had the universal right to invade any property, home, or even church (though this caused some controversy) to dig up dried-up old urine, bones, and dung. Historical records show that for approximately two hundred years between the fifteenth and seventeenth centuries, English people were plagued in their homes by salpetre gathering, and this "grief and discontentment" and "great grievance and disturbance" was a significant problem for those in power, who needed "to provide for her majesty's stores by all means possible" regardless of "damage, intrusion, discommodity, and distress"[40]

Hence, the state's efforts to procure saltpeter from diverse sources underscored a profound convergence of its biopolitical and necropolitical imperatives. In this manner, nitrogen emerged as the fulcrum facilitating the state's exercise of sovereignty over the lives of its populace as Cardwell further elucidates "Not only did the state have "sovereign right" over saltpetre, this power extended to the right to procure it anywhere, regardless of the privacy or personal boundaries of the lower classes; thus the oppressive intrusions of the saltpetre men. Foucault's sovereign right over death, then, had a material as well as a judicial aspect, which focused on gunpowder."[40] This sovereignty was extended when the collection of saltpeter transitioned into the deliberate cultivation of saltpeter in nitre beds, where the incorporation of urine and dung into heaps of decomposing organic material yielded salts amenable to subsequent refinement and processing.[46]. Instead of invading peoples homes to scrape saltpeter off of the walls they hauled away entire pieces of the home to toss into nitre beds.

The technological advancements in weaponry facilitated through saltpeter precipitated what some historians dubbed a "military revolution". Until the sixteenth century, numerous armies dispatched to engage in warfare across Europe were assembled as needed and subsequently disbanded, or alternatively, sustained through the enlistment of mercenaries. The pervasive integration of portable firearms within armed forces precipitated a requisite enhancement in

[22] Saltpeter was also used in other industries like dyeing but the vast majority went to gunpowder production. See *Saltpeter: The Mother of Gunpowder* (Cressy, 2012)

training standards and the establishment of permanent military contingents, culminating in the expansion and heightened expenses of armies, thereby mandating centralized state authority to manage their fiscal and administrative exigencies as scholar Michael Roberts argues "the modern art of war made possible —and necessary—the creation of the modern state"[123]. This implies that the modern conceptualization of the state manifests its contemporary structure through the political hegemony of military might intertwined with the biopolitical hegemony exerted over its populace. Nitrogen as the '*miraculum mundi*' or '*materia universalis*'[121] which had attained the status of a mythical substance that "hath the sovereignty and quality of every element"[124] was not simply the catalyst for the consolidation of state power but also its fuel and calcifying agent. Following the consolidation of military administrative authority under the auspices of the state, the imperative to procure ample quantities of saltpeter materialized through acts of colonial and imperial violence. Emulating their Dutch predecessors[23] the British East India Company, the architectonic epitome of war capitalism, assumed the mantle of satisfying Britain's demand for saltpeter. Benefiting from climatic conditions favorable to saltpeter formation, India emerged as a significant hub for the production of this efflorescent salt, prompting the East India Company to eagerly capitalize on this remunerative trade opportunity.[119]. Saltpeter exports from India through the British East India Company went from tens of tons per year in the 1620s to an average of 500 tons annually in the 1660s to 1000 tons per year by the 1740s to nearly 30,000 tons per year in the nineteenth century[125]. Hence, the global restructuring of nitrogen cycles by European powers manifested not only through commodity agriculture but also through the instrumentalities requisite for conducting warfare. The dialectical interplay between capital and state militarism engendered a symbiotic relationship that contributed to the organization of the movement of populations, commodities, and ideologies, all mediated by the circulation of nitrogen.

Fueling the growth of mercantilism, the consolidation of state administrative power, and the biopolitical extension of state sovereignty into everyday life was the ravenous appetite for genocide, dispossession, and enslavement in the Americas. These complementary phenomenon were wholeheartedly facilitated and ossified by the use and monopolization of firearms. Firearms were so important to the identity of Colonial Man in the Americas there were laws erected in many colonies that compelled male colonists to carry arms at all times including in church such as a 1619 Virgina statue that mandated "all men that are fittinge to beare arms, shall bring their pieces to the church"[126] and "all suche as beare armes shall bring their pieces, swords, pouder, and shotte" or face a three shilling fine. This extended to the New England colonies as well "Sabath-day procession up the hill to worship, every man armed and marching three abreast"[127]. These regulations were born out of a pervasive apprehension regarding Indigenous incursions, perpetually placing colonists in a state of heightened vigilance. Legislation mandating the constant bearing of arms by white colonists coexisted with statutes prohibiting the dissemination of weapons to Indigenous populations. A 1619 Virginia statue proclaimed "That no man do sell or give any Indians any piece, shot, or powder, or any other arms offensive or defensive, upon pain of being held a traitor to the colony and of being hanged as soon as the fact

[23] The Portuguese were the first to get into the saltpeter trade in India, the Dutch and British East India Companies did not arrive until the 1600s.

is proved, without all redemption."[126] This prohibition was soon extended to African slaves, Virginia passed its first law against black slaves possessing arms in 1639 with this extended both enslaved and free blacks in 1680[128], Massachusetts passed a law in 1656 prohibiting both blacks and Natives from owning firearms[129]. Other colonies emulated this model, New York passed a law prohibiting the transfer of guns to Natives in 1664[130] and South Carolina passed a law precluding blacks from gun ownership in 1740[131]. The prohibition of firearm possession among Indigenous peoples and enslaved Africans functioned as a mechanism to operationalize the concept of whiteness as an arbiter of violence. Colonial Man was deeply vested in safeguarding both his property and his prerogative to make property of others by maintaining control over firearm possession. This phenomenon represents an extension of whiteness as a mechanism of exclusion aimed at demarcating the propertied from property. It additionally fortified the subordination of both Indigenous peoples and enslaved individuals by consolidating hegemonic control over the arms trade, thereby preempting either group from acquiring the means to mount rebellions or engage in warfare. In this way colonial law sought to coalesce gun ownership and whiteness, making them both coterminous and exclusive to Colonial Man. It is evident from this analysis that saltpeter, as the pivotal ingredient of firearms and, by extension, nitrogen, played a role in delineating the contours of whiteness. Hence, Colonial Man's abstraction of nitrogen from its relational contexts, despite an incomplete understanding of its nature, resulted in the monopolization of violence that underpinned racial ontologies critical to shaping contemporary legal frameworks. The implications of this phenomenon will be further elucidated in due course.

1.2.6 The Scientific Revolution and Evolving Understandings of Nitrogen

Up until the advent of the Scientific Revolution, Western knowledge production and dissemination was dominated by brute trial and error to confirm or expand on theories posited in Classical Antiquity[46]. Much of this can be attributed to the Church's hegemony of learning and the study of classical works that occluded contemporary philosophers from accessing and engaging with the insights of their predecessors[57]. Consequently, the pace of technological innovation lagged considerably behind the progress achieved by civilizations in China, South Asia, and the Islamic world, which demonstrated a greater receptivity to, and elaboration upon, the teachings of earlier scientific endeavors. Advancements in comprehending the nuances of nitrogen, primarily within the confines of agricultural discourse, progressed at a notably sluggish pace, mirroring the gradual trajectory of agricultural innovation. Famines prior to the Industrial Revolution were frequent occurrences. Consequently, any unsuccessful agricultural endeavor posed an immediate threat of widespread starvation, distinguishing it from more localized inquiries such as those within the realms of chemistry and medicine[94]. Nevertheless, the Scientific Revolution, catalyzed by the revelatory insights of Nicolas Copernicus, gradually undermined the hegemonic influence of the Church, thereby facilitating a paradigm shift towards scientific experimentation rooted in theoretical frameworks and subsequently mathematics, markedly accelerating the rate of scientific advancement[46]. However, this occurrence cannot be assessed in isolation from the concurrent efforts to colonize the Americas, which fostered a symbiotic nexus between science and colonialism. This nexus served as a means to codify Western ontologies to segregate and categorize elements of nature from the colonies for the purposes of extraction and later to rationalize racial ideology in scientific discourse[132]. Both of

these phenomena are grounded in the escalation of Western universalism as a means to perpetuate the marginalization and epistemicide of non-Western cosmologies[57]. At the heart of this sequence of events lies the evolving comprehension of nitrogen, reaching its zenith with its isolation and extraction, thereby laying the groundwork for its subsequent commodification within capitalist agricultural practices.

Prior to the scientific revolution, the practical knowledge of chemistry and medicine was manifested through alchemy. Alchemists, however, derived the majority of their knowledge through sheer trial and error and lacked a cohesive theoretical framework to systematically guide their endeavors[46]. However, notwithstanding their primary pursuit of transmuting base metals into gold or concocting an elixir for eternal life, numerous alchemists exhibited considerable expertise in synthesizing practical chemicals as Gorman describes "Most knew, for example, how to make acidum salis (hydro-chloric acid) and aqua fortis (nitric acid) as well as how to combine the two in a special mixture, aqua regia (nitrohydrochloric acid), capable of dissolving gold."[46] Hence, alchemists were quite familiar with saltpeter, how to refine it, process it, and produce nitric acid from it. However, alchemy was also predicated on secrecy[24] which hampered the dissemination of knowledge and perpetuated a methodology reliant on brute force trial and error to replicate experiments conducted by alchemists in preceding generations[133]. They also adhered to the Aristotelian paradigm positing that all matter was composed of four elements (air, earth, water, and fire)[133] which severely limited their capacity to make connections between their observations and to use it to develop predictive theories[134]. Consequently, they remained as distant from comprehending the link between the combustion of saltpeter within gunpowder and its potential utility as a fertilizer as they were from transmuting base metals into gold.

A pivotal juncture in the evolution of nitrogen science is delineated within the literary corpus of the Swiss physician and alchemist, Paracelsus (1493-1541), during the early sixteenth century. Paracelsus, characterized by a robust skepticism toward classical philosophical constructs of matter, adopted an experimental methodology reminiscent of contemporary scientific paradigms and underscored the inseparable interconnection between religion and science[135]. Paracelsus postulated a relationship between the air what he called "heavenly firmaments" and solid objects or "internal firmaments". In this regard, he proposed a reciprocal interplay between gaseous and solid constituents, postulating their interaction through what he termed "astral emanations"[94]. Employing this rationale, he forged a correlation between the combustion of saltpeter and its capacity to enrich soils, thereby advancing the notion of "aerial nitre"[94]. The concepts introduced by Paracelsus were profoundly thought-provoking, catalyzing a renewed discourse on the intricate interplay between solid and gaseous states of matter[25]. His propositions gained further clarity through the work of his ideological sympathizers including Robert Fludd's famous

[24] Most alchemists operated in secrecy to avoid being accused of practicing black magic or to be coerced into the service of the elite or the state. See *The Story of N* (Gorman, 2013)

[25] The limitation of Paracelsus' views on aerial nitre is that it was evaluated primarily from a medical lens. Paracelsus as a physician was very interested in developing systematic treatments for illnesses based on the knowledge of chemistry, thus most of his theories were developed with the intention of application to medicine and physiology. See *The World's Greatest Fix* (Leigh, 2004)

candle experiment[26] and John Mayow's treatises on aerial nitre that helped to consolidate the notion of "fixed salts"[27], elucidating the process by which aerial nitre was fixed from the air into salts suitable for agricultural fertilization[94]. By the end of the seventeenth century, the concept of aerial nitre was very popular and circulated commonly in scientific and agricultural discourse, with the idea of nitre integration into plant life beginning to germinate.

In the late seventeenth century, a departure from classical philosophy and alchemy gained momentum, marked by an increasing number of scientists embracing an approach akin to the modern scientific method, characterized by theory-driven experimentation[46]. Robert Boyle's investigations into the combustion-related behaviors of gases in sealed vessels, expounded in *The Sceptical Chymist*, appeared to signal a paradigm shift away from the Aristotelian conception of matter[136]. Subsequent to Boyle's critique of the Aristotelian framework, chemists propelled by the pursuit of predictive theory endeavored to address the vacuum left by the abandonment of a framework that had endured for close to two millennia. This vacuum was filled by the phlogiston theory of combustion proposed by German physician and alchemist Johann Joachim Becher in the 1660s[46]. The phlogiston theory posited that all matter contained an invisible substance called phlogiston that was released upon combustion[137]. Although not entirely precise, this theory emerged through numerous experiments observing changes in mass from burned substances indicating a significant shift from antiquated theories towards frameworks backed by experimentation and empirical evidence. The phlogiston theory swiftly garnered favor among chemists, thereby prompting an evaluation of every experiment aimed at elucidating the behavior of various materials through the prism of combustion and respiration to harmonize with phlogiston. The theory itself persisted for over a hundred years and helped to lay the foundations for the discovery of nitrogen in 1772.

The phlogiston theory satisfied early chemists primarily due to its qualitative explanatory power, chemists at the time knew that materials were heavier after combustion which seemingly contradicted the notion of phlogiston release, Boyle sought to reconcile this by suggesting that phlogiston had a negative mass[94]. Nevertheless, following the phlogiston theory other chemists aimed to elucidate the true nature of air which gained specific interest after the concept of gases[28] had been further developed. The notion of air being a combination of different gases began to emerge with the work of Scottish chemist Joseph Black whose distillations of vegetable matter allowed him to isolate "fixed air" or carbon dioxide. Following these advancements, English

[26] Robert Fludd (1547-1637) was an English philosopher and ardent follower of Paracelsus. He conducted experiments with candles in which he burned a candle enclosed in a glass bulb until the residual air could no longer support the flame. He simultaneously observed an accumulation of water at the base of the candle and a change in the volume of gas in the bulb. However, he was not able to make the connection that the candle material was consumed. See *The World's Greatest Fix* (Leigh, 2004)

[27] John Mayow (1640-1679) was an English physician and chemist who wrote a series of treatises on the nature of aerial nitrogen. He wrote of the idea of the earth drawing sal nitrum from the air that produced "fixed salts". See *The World's Greatest Fix* (Leigh, 2004)

[28] The concept of a gas started to be taken seriously after the work of J.B. van Helmont (1579-1644) with his famous tree experiment. See *The World's Greatest Fix* (Leigh, 2004)

chemist, Joseph Priestly isolated oxygen in 1774[29] by heating a sample of mercuric oxide claiming "I have discovered an air five or six times as good as common air" which he termed "dephlogisticated air" due to its enhanced ability to combust[94]. Concurrently, Daniel Rutherford, often credited as the foremost to "discover" nitrogen, a student of Black, isolated nitrogen by replicating his vegetable distillation experiments and employing alkali to remove the "fixed air" resulting in a mixture predominantly composed of nitrogen which they termed "mephitic air" or "phlogisticated air"[94]. Operating within the confines of the phlogiston theory, they remained unaware of the distinction between oxygen and nitrogen as separate chemical elements. It was not until their contemporary, French chemist Antoine Lavoisier, whose work on combustion fully developed a conservation of mass theory that provided the requisite framework to elucidate the intricate processes through which various elements amalgamated to yield novel compounds[46]. Lavoisier was aware of Priestly's work and ascertained that the substance Priestly had isolated from air was a novel element he named "oxygen" or acid-generating substance and his quantitative methods determined that oxygen was added to any material that was combusted, thus ensuring the principle of conservation of mass, whereby the mass of the resultant products equaled that of the initial reactants. In this endeavor, Lavoisier ushered in an era of stoichiometry and quantitative chemical analysis, heralding a departure from the constraints of the phlogiston theory and laying the groundwork for the evolution of modern chemistry.

The culminating strand interwoven into the narrative of nitrogen's progression, spanning from the era of saltpeter to the recognition of nitrogen as an elemental constituent, pertains to nitrogen fixation and its profound significance in the realm of plant biochemistry. With the understanding that air could be separated into different gases and that nitric acid, which was derived from saltpeter, contained oxygen, the English chemist Henry Cavendish[30] demonstrated in 1785 that nitrogen "phlogisticated air" and oxygen "dephlogisticated air" reacted together through an electric spark that produced a mixture containing nitric acid[138]. At this juncture, the conceptualization of "fixing" atmospheric nitrogen reached fruition, thereby establishing the pivotal linkage between "aerial nitre" and saltpeter. Following this revelation, the two entities were linguistically and scientifically interlinked, as "phlogisticated air" was designated as nitrogen, stemming from the root nitre, by Jean-Antoine Chaptal in 1790[94]. These strides were swiftly complemented by the comprehensive evolution of atomic theory, a framework pioneered by the English chemist John Dalton, who in 1805 elucidated that elements combined in fixed ratios as whole numbers, propounded through his seminal theory of multiple proportions[46]. Dalton's theoretical framework, undoubtedly influenced by the contributions of luminaries such as Lavoisier and his peers, provided the bedrock for contemporary chemistry by establishing rigorous quantitative methodologies. Hence, early chemists could finally conceptualize various

[29] Priestly's discovery comes around the same time that Swedish apothecary, Karl Wilhelm Scheele claims to have made a similar observation. See *The World's Greatest Fix* (Leigh, 2004)

[30] Cavendish was a contemporary of Priestly and Lavoisier and was also working on the isolation of oxygen or as he knew it "dephlogisticated air". Many of his experiments isolating hydrogen and combining it with oxygen were reproduced by Lavoisier to confirm his theory of combustion and acidity observations. He also worked extensively on characterizing the properties of hydrogen and estimating the density of gases. See *Henry Cavendish (1731–1810): hydrogen, carbon dioxide, water, and weighing the world* (West, 2014)

nitrogenous compounds as compositions comprising nitrogen, hydrogen, and oxygen. For instance, the nitrogen dioxide resulting from Cavendish's electric spark experiment arose from the amalgamation of one nitrogen atom with two oxygen atoms.

Following these developments the pace at which scientific advancements in nitrogen cycling and agriculture accelerated rapidly. Humphrey Davy, an English chemist, through a series of public lectures demonstrated the properties of various nitrogenous compounds including nitrous oxide known for its euphoric effects as "laughing-gas" and nitric oxide which he synthesized through a refined electric-arc process with nitrogen and oxygen[46]. His seminal contributions to agriscience include his estimations of the elemental composition of plants, wherein he identified the primary constituents of plant biomass as carbon, oxygen, hydrogen, nitrogen, phosphorus, and sulfur, albeit in lesser proportions[94]. Davy was one of the first proponents of albumen, which today would be interpreted as protein, positing its significance as the principal nitrogen-containing component within plant biomass[94]. However, he postulated that plants primarily acquired nitrogen through the absorption of ammonia[31], which by that time had been shown to be a combination of nitrogen and hydrogen, rather than through the capacity to fix atmospheric nitrogen. Heavily influenced by the prevailing sentiment of the era regarding the soil fertility crisis, he conducted extensive studies on manures, recognizing them as a viable nitrogen source. With the groundwork laid, the eminent German agricultural scientists and "father of modern agriculture" Justus von Leibig wrote in his seminal work published in 1840, *Organic Chemistry in Its Applications to Agriculture and Physiology* "By the deficiency or absence of one necessary constituent, all the others being present, the soil is rendered barren for all those crops to the life of which that one constituent is indispensable."[139] Von Leibig, through a series of meticulous analyses established the connection between nitrogen and plant growth by promulgating the concept of the growth limiting nutrient or the idea that plants were limited in growth by the nutrient that is the least available[3]. Following the work of Davy he also made the connection between conventional fertilizers like manure and plant growth asserting that "every part of the organism of a plant contains azotized matter in varying proportions" and linking this to manure "A manure containing several ingredients acts in this wise. The effect of all of them in the soil accommodates itself to that one among them which, in comparison to the wants of the plant, is present in the smallest quantity"[139]. Through these understandings von Leibig established the crucial linkage between plant growth, nitrogen, and fertilizer, excising nitrogen from nature, identifying its role in commodity production, and rationalizing its use as a modular input for production. An inherent gap in von Leibig's agricultural nitrogen theories lies in his oversight of nitrogen fixation. He operated under the presumption that plants received the majority of their nitrogen from "humus" or decomposing organic material in the soil[3], a theory that would not be successfully challenged and replaced until the work of Boussingault[94].

The evolution of nitrogen from a combustible salt to an element that can be conceptualized in modalities amenable for extraction and production forms a consilience with the trajectory of

[31] The compound of ammonia had been known since antiquity but its true nature as a combination of nitrogen and hydrogen was not fully elucidated in Western science until the work of C.L. Berthollet who in 1789 showed the ammonia could be decomposed into his constituents nitrogen and hydrogen though an electric ark. See *The World's Greatest Fix* (Leigh, 2004)

modern scientific development that aims to reconfigure the world along the axioms of whiteness and property. Nitrogen as “mephitic” or “phlogisticated air” is an artefact of epistemes with the ambition to extricate, classify, and compartmentalize complex natural systems for the purposes of conquest. The praxis molding the formation of modern sciences such as biology, chemistry, geology, and physics form a dialectical relationship with the epistemological frameworks distinguishing the Human from the Other. As early proponents of science and the forefathers of technocracy asserted, science could be used to conquer nature as philosopher Francis Bacon notes “For man being the minister and interpreter of nature, acts and understands so far as he has observed of the order, the works and mind of nature, and can proceed no further; for no power is able to loose or break the chain of causes, nor is nature to be conquered but by submission: whence those twin intentions, human knowledge and human power, are really coincident; and the greatest hindrance to works is the ignorance of causes.”[140] This praxis was reinforced and extended by rational humanism that emerged in the Enlightenment shifting the epistemic center of knowledge to the Human that can be used to appropriate the Earth as Descartes explicates “the invention of an infinity of artifices that would enable us to enjoy, without any pain, the fruits of the earth and all the goods to be found there.”[141] Thus, science as a praxis rooted in rational humanism is the epistemic operationalization of ontologies of separation and reduction to facilitate the expropriation of nature as an object of the Human’s wills and desires. This praxis was manifested through the violence of colonialism: taxonomic classifications of flora-the roots of modern biology-stems from efforts to characterize new plants for commodity agriculture[132], early studies of motion and kinematics were inspired by efforts to understand the Earth’s rotation to plot optimized navigation routes to colonies[132], and chemistry derived from Medieval alchemy was studied with the intention to identify and extract precious metals[69]. In this pursuit, scientific endeavors conformed to paradigms of property rights, seeking to delineate and differentiate immaterial entities ripe for extraction. These paradigms followed inherently racialized logics exemplified by Yusoff’s explanation of the inception of geology “The birth of racial subject is tied to colonialism and the conquest of space and the codification of geology as property and properties. Thereby geologic resources and bodily resources (or racialized slavery) share a natal moment”[69]. From these reflections, it becomes evident that the frameworks governing geology and chemistry are oriented towards delineating inert materials amenable to extraction. Similarly, these material relations determine what holds value and what does not as Yusoff further explains “they establish unfolding *geologics*, for particular bodies and subject positions, as disposable in the shadow economy of extraction.”[69] This serves to contextualize the predominant inclination towards examining nitrogen through its material applications. Early alchemists and chemists scrutinized nitre as saltpeter primarily for its combustive properties, while subsequent scientists investigated it in relation to plant fertility. The penultimate stages of nitrogen science unfolded in parallel with escalating concerns regarding soil fertility and the widespread adoption of fertilizers sourced from pre-existing reserves. Hence, the conceptual framework surrounding nitrogen, its abstraction from the atmospheric matrix of "aire," and its presence within plant matter all converge as endeavors to compartmentalize it, attributing to it the status of a material amenable to extraction and commercialization. In this manner, nitrogen, stripped of its relational context, is reduced purely to fertilizer that can be used as input for production much in the same way that the African slave is atomized to the Inhuman for the commoditization of labor power.

The same ontological framework that renders natural elements and human beings alike as immaterial objects pushes the notion of "universalism" which necessitates the deliberate marginalization of different systems of knowledge and scholarship[57]. European science and rationalism emerged as formidable tools within the arsenal of colonial authorities to disrupt Indigenous social and land management practices in settler colonies while simultaneously undermining precolonial scientific traditions in Asia and Africa[132]. Within the framework of early chemistry, particularly in its examination of nitrogen, European universalism was intrinsically associated with a perception of epistemic supremacy, which marginalized alternative chemical knowledge systems as mere "parachemistries" as Mukharji describes them as forms of knowledge that through colonial globalization, came to be forced into a subordinated dialog with 'modern chemistry' whilst remaining at least partially assimilated into 'modern chemistry'. From their subaltern positions they continued to contest the right of 'modern chemistry' alone to define and regulate chemical operations"[142]. This phenomenon is notably discernible throughout the annals of scientific progress, particularly concerning nitrogen, wherein non-European advancements in its study have often been relegated to the periphery or condensed into a mere footnote, contributing to the overarching European narrative of discovery. The Chinese were aware of the presence of nitrogen in the air by the eighth century A.D. nearly a millennium before Europeans came to the same understanding[143]. Islamic text describe the formation of nitric acid from saltpeter in the eighth century A.D., centuries before this knowledge was circulated in Europe[94]. The universalism that fuels Western science relegates the knowledge of nitrogen accrued through non-Western scientific traditions as "unserious" or simply primitive antecedents to European scientific advancements. It is imperative to thoroughly recognize and contextualize these trends within the framework of European conceptions regarding the "discovery" of nitrogen to comprehensively elucidate the theoretical and practical evolution of knowledge surrounding nitrogen, serving as antecedents for the phenomenon of ecological imperialism. Understanding the history of nitrogen science through this lens is paramount to grounding the role of nitrogen in conceptual frameworks charting the relationship between capitalist systems of production and the environment.

1.2.7 The Convergence of War Capitalism, Industrial Capitalism, and the Roots of the Nitrogen Crisis

Commodity agriculture, colonialism, genocide, land dispossession, the trans-Atlantic slave trade, mercantilism all amalgamating into war capitalism are the social, political, and economic pillars upon which the edifice of the modern world rests. All of these historical developments are prerequisites for the emergence of industrial capitalism in late eighteenth, early nineteenth century Britain that embodies the prevailing contemporary global socio-economic world order. Both of these modes of production, rooted in the epistemological frameworks of science, rational humanism, and racial alchemy, are the engines for the perpetuation of colonial land relations that are oriented towards the unidirectional extraction, processing, commerce, and disposal of natural amenities from the land. The convergence of war capitalism and industrial capitalism, though inherently reciprocal and mutually reinforcing, paradoxically signaled the decline of war capitalism as a state-sanctioned endeavor.[106]. At this convergence point the ideology of liberal political economy with a seemingly infinite reverence and confidence in markets arrived to

hegemonize the political terrain of nation states beginning to assume their modern form. This paradigm shift in political thought spelled a bevy of social and economic implications for the masses congealing historical materialism into legal frameworks that fully delineated the dichotomy between proletariat and the bourgeoisie, as conceptualized in contemporary discourse. Nestled within the interstices of these political and economic developments lies the escalation of nitrogen appropriation, a phenomenon unfolding in tandem with the ontological transition of nitrogen as an abstraction to nitrogen as a tangible material product. This consilience stands as a pivotal element directly implicated in the historical developments that have delineated the current trajectory of the world.

The manufacturing sector and the exportation of finished goods constituted pivotal components within mercantilist policies, underscored by aggressive endeavors aimed at dominating foreign markets. The principal contradiction to this commercial framework in eighteenth century Britian was the Indian domination of finished cotton products[32] that were inexpensive, high quality, and in high demand all over Europe[106]. In order to fortify its mercantile prowess, Britain instituted a prohibition on the importation of Indian cotton goods via the Calico Acts of 1700 and 1721. This policy shift redirected commercial networks towards fostering the growth of domestic cotton manufacturing[144]. Hence, in the wake of Lancashire and Cheshire's rich textile manufacturing heritage, urban centers dedicated to cotton production began to burgeon.

Concurrent with these geopolitical and economic complexities, there unfolded a rapid surge of engineering innovation, spurred by the ideological transformations stemming from the Enlightenment and the Scientific Revolution. These innovations included: the flying shuttle designed by John Kay in 1734; the water frame designed by Richard Arkwright in 1764; the spinning jenny designed by James Hargreaves in 1770; the spinning mule designed by Samuel Crompton in 1779; the power loom designed by Edmund Cartwright in 1785; and James Watt's steam engine in 1784[33]. All of these served as the technological catalysts for industrialization, dramatically escalating the speed and efficiency of cotton manufacturing[106]. Nevertheless, the pivotal element for the advent of industrial capitalism, namely capital in capitalism, largely originates from the proceeds garnered through the British slave trade and sugar commerce[108]. Samuel Greg, the principal investor of Quarry Bank Mill, the preeminent cotton factory of the Lancashire and Chesire area, derived much of his wealth from family inheritances in the British West Indies[106]. James Watt and his partner Matthew Boulton who patented the steam engine

[32] Britain had always been a center for textile production primarily in wool and flax, this was facilitated primarily through the putting out system where merchants would advance capital and raw materials to domestic producers in the countryside. However, the scale of this trade was miniscule in comparison to the Indian trade in cotton products and later the domestic production of cotton products during the Industrial Revolution. See *Empire of Cotton* (Beckert, 2014)

[33] The steam engine was not a new invention when Watt and Boulton patented it in 1784. Steam engines had been known and in use since the first were popularized by Thomas Newcomen in 1712. Watt did make significant modifications by adding a separate cooling condenser which allowed the power cylinder to maintain continuously without the need to be cooled.

depended on family estates derived from the triangular trade and the iron industry[34] and were financed from the West Indian trade[108]. The esteemed scholar Eric Williams, posits that as much as one third of Lancashire's exports were allocated to procure slaves in West Africa, with one half directed to the plantations of the British West Indies.[108]. Hence, the historical continuum from plantation slavery to industrial capitalism, evident in both operational methodologies and financial underpinnings, renders palpable the enduring impact on contemporary legal and economic structures, particularly regarding racial and class marginalization. The other important development from British industrialization is the imbrication of commodity production with globalized environmental catastrophe through the use of fossil fuels. Advocates of a technocratic perspective on human progress often romanticize the adoption of Watt's steam engine as a pivotal moment showcasing humanity's technical ingenuity. However, a nuanced examination of the circumstances surrounding the transition from water power to steam power reveals a more complex narrative. The technological sophistication of steam engines notably trailed behind that of water-powered systems, albeit possessing the distinct advantage of spatial adaptability. Consequently, the primary impetus driving cotton capitalists to adopt steam engines in their factories stemmed not from heightened efficiency, but rather from their inherent flexibility and versatility, facilitating the exploitation of labor power as Malm describes "steam did not offer any *absolute* emancipation in space, whatever that would have looked like, but a *relative* one, real and precious. Given the convergence between supplies of labour power and agglomeration economies on the one hand and supplies of coal and waterways on the other, the spatial liberty afforded by steam was all capital could wish for at this stage in history."[36] This spatially unrestricted ability to exploit labor power was coupled to what Malm calls "necromancy" or the use of dead organic matter in coal to produce a motive force for commodity production[36]. The geological redirection of carbon reservoirs underscores profound biogeochemical implications, extending spatially and temporally, mirroring the concurrent emergence of the nitrogen crisis. The system of capital, honed through British industrialization, conceptualized elemental reservoirs as interchangeable modalities to be strategically relocated and traded akin to pieces in a game of Tetris. The ramifications for the carbon cycle, exacerbated in the current era by climatic shifts, are overshadowed on a magnitude scale by the disruptions to the nitrogen cycle. Nevertheless, Britain embraced this economic opportunity wholeheartedly going on to dominate the global cotton goods market, exploding by a factor of sixteen from a total export value of £355,060 in 1780 to £5,854,057 in 1800[106].

As the nineteenth century dawned, indications of the waning influence of war capitalism became apparent. Colonies initiated movements for independence from their European metropoles, while the specter of slave uprisings materialized vividly in events such as the Haitian Revolution. Concurrently, mercantilist strategies incurred disapproval from the emerging industrial capitalist class.[108]. The one exception to this trend was the cotton empire of the U.S. antebellum South. Following the denouement of the Revolution, the institution of slavery, deeply entrenched within the legal and political fabric of the United States, was anticipated to undergo a gradual demise in

[34] The British iron industry made a large share of its profits from the manufacture of iron shackles for slaves, anchors for slave ships and other iron products associated with the triangular trade. See *Capitalism and* Slavery (Williams, 1944)

the Southern states. This expectation stemmed from the precipitous downturn of the tobacco trade in the late eighteenth century[82]. This paradigm shifted dramatically with the advent of the cotton gin in 1793, a pivotal innovation that rendered cotton cultivation exceedingly remunerative. Following the introduction of the cotton gin the slave populations of the South and cotton exports exhibited an isomorphic exponential trend, the population of black slaves went from approximately 650,000 in 1790 to 1.5 million by 1820 to nearly 4 million by 1860[35] and cotton exports went from 1.5 million pounds in 1800 to 167.5 million pounds in 1820 to over 2 billion pounds in 1860[106]. The United States went from supplying under 1% of the global raw cotton product to the market in 1790 to almost 50% by 1831[106]. This phenomenon was significantly facilitated by the systematic eradication and displacement of Indigenous populations, alongside the institutionalization and financialization of the slave system, deeply entrenched within the political framework of the nation, eventually reaching a scale akin to industrial operations. as Beck describes "What distinguished the United States from virtually every other cotton-growing area in the world was planters' command of nearly unlimited supplies of land, labor, and capital, and their unparalleled political power"[106]. This trend aligned with the overarching pattern of capitalism, characterized by overproduction[65], a phenomenon that became increasingly pervasive. This led to a notable decline in the price of raw cotton throughout the initial decades of the nineteenth century[106]. The decline in the price of cotton served as a potent incentive for Britain, the nucleus of cotton manufacturing, to make unbounded investments in the American cotton venture resulting in a dramatic shift, with U.S. cotton accounting for less than 1% of British cotton imports in 1790, soaring to over 80% by the onset of the Civil War. Yet, the expansion of the cotton empire and the sustained growth in cotton exports hinged entirely upon the Western expansion of cotton-growing regions and territories reliant on slave labor. Cotton monocropping rapidly depleted virgin soils, necessitating continual land acquisition by cotton planters to sustain profitability[145]. Financially, this strategy appealed to planters who adhered to the belief that employing slaves to clear new land was more cost-effective than investing in manure recycling or, later in the 1840s and 1850s, purchasing imported fertilizers.[145]. During the nascent stages of the cotton empire, this predicament was not evident, as the United States actively facilitated Indigenous land dispossession, forcibly relocating millions from their ancestral territories in Georgia, Alabama, Tennessee, and Mississippi, thus making these fertile lands available for cotton cultivation[106]. Emerging states with pristine soil swiftly garnered the lion's share of cotton exports, eclipsing older slave states. This shift is epitomized by the Mississippi delta region, which, by the onset of the Civil War, accounted for nearly a quarter of U.S. cotton exports[106]. This phenomenon can be interpreted as a substantial depletion of nitrogen reserves in the soil, as planters exhaust the land's fertility akin to the manner in which oil capitalists traverse regions in search of the next viable well to exploit. Concurrently, the Northern states reaped substantial benefits from the cotton empire, witnessing the emergence of textile manufacturing in New England, alongside the rise of financiers involved

[35] This was facilitated primarily through the domestic slave trade. Even before the ban of the slave trade in 1808, a large portion of slaves in the Piedmont areas of North Carolina, South Carolina, Georgia and eventually the newer slave states in Alabama and Mississippi received the majority of their slaves from the domestic trade sourced in Virginia and Maryland. By the apex of the Antebellum, Virginia derived most of its income from the domestic slave trade.

in the slave trade and cotton commerce in New York. These entities played a pivotal role in furnishing essential capital to Southern planters[106]. Ultimately, the eruption of the Civil War stemmed from the conflict between the industrial capitalists of the North, who advocated for the expansion of state authority to drive industrialization and restrict the spread of slavery, and the planter class of the South, staunchly opposed to state intervention and adamant about preserving political autonomy, particularly concerning slavery[106].

At this juncture, the convergence of war capitalism and industrial capitalism ignited the fuse of the Civil War, fundamentally altering global cotton markets and disrupting the trajectory of industrial growth in European metropoles. Of greater significance to the course of historical development, particularly concerning the evolution of nitrogen cycling, is the defeat and marginalization of the final remnants of war capitalism and the complete adoption of industrial capitalism. British industrialists had been decrying mercantile policies since well before the American Revolution chiefly centered in the West Indian monopoly of sugar production[108] that began to prove unprofitable with the escalation of Fresh competition[36]. Following the Revolution and the dissemination of laissez-faire economic principles attributed to the seminal work of Adam Smith, the allure of free markets permeated the aspirations of industrial capitalists, evoking a metaphorical fragrance of liberation and economic autonomy[108].
The burgeoning advocacy for liberalized markets and the concurrent marginalization of the West Indies are intricately interwoven with the remnants of war capitalism and the prevailing cash crop system, which systematically depleted the soil's fertility and compromised its ecological integrity as Williams explains "Between 1813 and 1833, Jamaica's production declined by nearly one-sixth; the exports of Antigua, Nevis and Tobago by more than one-quarter, St. Kitts by nearly one-half, St. Lucia's by two-thirds, St. Vincent's by one-sixth, Grenada's by almost one-eighth"[108].
Hence, a recognition of war capitalism's role in driving colonies to their ecological thresholds coincided with fervent appeals to embrace the ideology of industrial capitalism in its entirety. This nascent form of capitalism though was inextricably tied to a reimagination of state power as Beckert describes "Paradoxically, industrial capitalism made state power less visible as it amplified it. No longer did the personal authority of the king, the lord, or the master, or age-old custom, regulate the market; instead the market was made by explicit rules relentless enforced by contracts, laws, and regulations"[106]. In alternative terms, the viability of industrial capitalism rested entirely upon the valorization of property rights, safeguarding domestic markets, and the relentless expansion into international frontiers, each necessitating regulatory and political constructs manifested through state authority. The authority and range of state power also marginalized the importance of war capitalism's ability to hegemonize land, labor, and markets[106]. Henceforth, capitalism became intertwined with the state's capacity to consolidate institutions of authority, essentially engendering a symbiotic nexus between capital and the state. However, this symbiosis was challenged by war capitalism's insistence on the unfettered prerogative of Colonial Man to dictate terms concerning land, labor, and markets.[106].

[36] British sugar manufacturers constantly decried the monopoly that they thought was financially inflexible whereas as sugar from the French Caribbean islands such as Sainte Domingue was substantially cheaper. See *Capitalism and Slavery* (Williams, 1944)

The demise of the Southern states in the Civil War marked the conclusive denouement of an ideology steeped in war capitalism, which had been ailing irreparably for several decades prior.

With the transition to industrial capitalism underway in Europe, the mobilization of masses of landless proletariats to industrial metropoles began to escalate. In Britain alone roughly 28% of the population resided in cities in 1800[46] and that increased to 77% by 1900[37]. This engendered a range of social, political, and environmental changes in the network of spatial material flows or a disruption to what Marx calls the “metabolism between man and the earth”[107]. The industrial proletariats laboring in the cotton factories of British manufacturing centers, often crowded into slums with insalubriously vile living conditions, required food and fiber to feed and clothe themselves and their families. This phenomenon precipitated a significant redistribution of nutrients from rural locales to urban hubs, where, via human excretion and material refuse, they ultimately contributed to environmental pollution[65]. The disturbance of conventional production methods, which historically facilitated the reintegration of extracted nutrients back into the soil, engendered what Marx delineated as the "metabolic rift"[107]. Marx, who found resonance in the ideas of von Leibig, perceived capitalism as a framework that relegated nature to the periphery of its production apparatus, thereby stripping it of inherent value[65]. In this way, capitalism as a mode of production not only exploits labor power but also expropriates nature. As nature occupies a peripheral position within the system, the expenditure associated with waste disposal is not accounted for in the production cost, consequently leading to its perpetual externalization. This phenomenon is unmistakably apparent in the widespread environmental deterioration witnessed in British industrial hubs, characterized by a notable decline in air quality attributed to the pervasive emissions from industrial smokestacks, alongside the transformation of water bodies into repositories for toxic waste disposal[36]. By the 1850s, the Thames River in London had become inundated with a confluence of human and industrial effluents to such an extent that the entire city of London found itself enveloped in an olfactory miasma so noxious that it earned the appellation "The Great Stink."[146]. The onslaught of waterborne cholera epidemics claimed the lives of thousands, exacerbated by prevailing theories attributing disease transmission to "miasma," which engendered institutional inertia towards enacting infrastructural or policy measures to alleviate the crisis. The pivotal shift came with the research conducted by Dr. John Snow, whose findings elucidated the waterborne nature of cholera, attributed the outbreak to water contamination by fecal matter. This revelation prompted London city authorities to enact more stringent regulations governing waste disposal into the Thames and implement elevated standards for water treatment[147]. As a result, this development laid the groundwork for the emergence of contemporary wastewater treatment systems, which intersected with the imperative to reintegrate nutrients from human waste back into agricultural ecosystems.

As Gorman elucidates, across the annals of human history, the demographic composition of a society uninvolved in agrarian pursuits hinges entirely upon the capacity to generate ample food

[37] British proletarianization follows a distinct trend of land enclosures beginning in the fifteenth century that is mirrored throughout other European countries. The system of land enclosure is thought date back to the twelfth century but especially catalyzed by the Black Death and the English breakaway from the Catholic church that facilitated the seizure and sale of thousands of acres of Church lands.

resources, thereby facilitating the detachment of individuals from agricultural labor. Thus, agricultural innovations to increase productivity was a prerequisite for the massive population redistribution to fuel industrialization[46]. These innovations, albeit still under scholarly scrutiny, unfolded throughout the span of the eighteenth and early nineteenth centuries, yielding what certain academics have characterized as an "agricultural revolution"[14838]. Better crop rotations, improved use of technology, and increases in livestock breeding efficiency increased agricultural productivity over the course of the eighteenth century[148]. Land enclosures undoubtedly constituted a pivotal aspect of the augmentation in agricultural productivity, given that private land ownership fostered incentives for enhancing land output. The British Agricultural Revolution is epitomized by the Norfolk Four Course system, which was likely developed in the sixteenth century but popularized by British agriculturalist Charles Townshend in the late eighteenth century[149]. The Norfolk rotation utilizes a four year cycle including wheat, turnips, barley, and clover where the turnips and clover are used as fodder crop for livestock[149].
The widespread adoption of the Norfolk rotation method facilitated a departure from the customary practice of fallowing land. Instead, it introduced the cultivation of clover, a nitrogen-fixing crop known for its soil fertility restoration properties, alongside turnips and clover utilized as fodder to sustain livestock during the winter months. By this point, the utilization of leguminous cover crops to rejuvenate soil fertility had become a well-established practice, seamlessly integrated into a systematic crop rotation framework that facilitated the intensive cultivation of arable land. These innovations were amenable to a system of commodity agriculture that began to take root in England as early as the seventeenth century but was calcified by the advent of the Industrial Revolution. This was bolstered by mercantilist policies exemplified in the Corn Laws enacted by Parliament in 1815, which imposed restrictions on the importation of foreign cereals, thereby fortifying the profitability of domestic cereal cultivation and correspondingly escalating food prices[36]. For this reason, the Corn Laws were widely unpopular, especially amongst cotton capitalist who had to set their wages commensurate to the fluctuating price of bread, and after pedantically litigious discourse over several years and the devastation of the Irish Potato Famine, were finally repealed in 1846[36]. This marked a decisive shift in Britain away from mercantile policies towards the laissez faire economic ideology inherent in industrial capitalism. Following the repeal of the Corn Laws, a surge of inexpensive imported grain inundated British markets, thereby enabling the newly innovated agricultural system to transition fully into the framework of commodity agriculture, heralding the advent of modern industrial agricultural practices. This transition, empowered by the compartmentalization of nitrogen into fertilizer for agricultural growth, engendered the incipience of the world's first commercialized nitrogen market that precipitated continental translocation of nitrogen to support the flows of capital.

1.2.8 Knocking on the Doorsteps of Ecological Limits

[38] There's still a significant amount of debate over whether or not there was actually a "revolution" that occurred in the eighteenth century. Some scholars argue the changes observed in the eighteenth century is just an amalgamation of incremental changes that happened since the fourteenth century while some argue that the metrics are reflective of increased land privatization. See *Re-Establishing the Agricultural Revolution* (Overton, 1996)

Mercantilism defeated, industrial capitalism ascendant, liberal political economy exalted, the foundations of the modern state and its symbiotic interplay with capitalism began to calcify. Nevertheless, lingering within the collective consciousness of industrial capitalists and economists were the admonitions of imminent scarcity, ecological depletion, and demographic decline, initially murmured but progressively amplifying in resonance with each successive milestone of capitalist development. The subdued utterance emanated from the Reverend Thomas Malthus, an English cleric and economist, whose seminal treatise, *An Essay on the Principle of Population* issued in 1798, prophesied the inexorable encroachment of an exponentially expanding populace upon finite natural resources[150]. Malthus emerged in the wake of Adam Smith, whose oeuvre espoused the doctrine of free markets and diminished economic constraints, thereby fostering a trajectory within capitalism toward a confluence with perpetual expansion as its inherent outcome. Grow or die was the name of the game. To maintain viability, capital necessitated a perpetual quest for novel markets in which to extend its reach and proliferate. Malthus, undoubtedly influenced by the burgeoning discourse of racial eugenics, posited that the indispensability of food for human survival coupled with the intrinsic nature of the "passion between the sexes" inevitably leads to the assertion that "The power of population vastly exceeds the capacity of the earth to provide sustenance for humanity, necessitating some form of premature mortality within the human species"[150]. According to Malthus, it was inevitable that the Earth's resources would be depleted by a growing population. This sentiment forms a distinct contrast from the thought of contemporary political economist David Ricardo whose theories on rents, wages, and labor based on agricultural output posited "that the "power of the soil" on any given plot of land was "indestructible" and hence "inexhaustible""[65]. Both of these divergent perspectives exhibit inherent flaws as they presuppose colonial land relations that dissociate the Human from nature, consequently marginalizing ecological methodologies that have historically sustained Indigenous communities worldwide over millennia. Neo-Malthusian theories would subsequently serve as a catalyst for the eugenicist discourse, which in turn furnished the theoretical underpinning for egregious atrocities against humanity, most notably exemplified by the Holocaust[151]. During the nineteenth century, these theories precipitated a profound apprehension regarding food insufficiency, instigating a fervent pursuit to ascertain the ecological limitations of agricultural output and surpass them.

The contributions of von Liebig were integral to the discourse surrounding the inherent constraints of agricultural productivity and their ramifications for demographic expansion. His elucidation of the "law of the minimum" posited that plant growth was constrained by the scarcest nutrient available, consequently asserting that agricultural practices progressively depleted soil nutrients[39], leading to the declaration that "the majority of our cultivated fields are depleted"[65]. At the same time, the great powers of Europe, emulating England's model were making significant transitions from an agrarian society to an industrial one. Between 1800 and 1890 the share of the population living in towns with at least 10,000 people increased from 18.9% to 34.5% in Belgium, 28.8% to 33.4% in the Netherlands, 5.5% to 28.2% in Germany, 8.8% to 25.9% in France, and 3.7% to 16% in Switzerland[152]. With the advent of

[39] Von Leibig was under the impression that phosphorus limitation was the most significant for plant growth and largely advocated for phosphorus fertilizers. See *The Story of N* (Gorman, 2013)

industrialization in Europe, the once predominantly localized food networks and markets of the Medieval era commenced a process of globalization, expanding to encompass a significantly broader geographical scope. Food imports from the colonies of the Global South began to contribute a much larger piece of the market share and radically restructured colonies traditional modes of production for export in what scholar Eric Holt-Giménez calls a "colonial food regime"[153]. Via the scholarly endeavors of von Liebig and other agricultural scientists, the constraints inherent in conventional soil fertility and land management methodologies were increasingly accentuated within agrarian discourse as scientists began to associate the nitrogen content with the quality of a diet or what scholar Arnaud Page calls "nitrogenometrics"[154]. The nascent centrality of nitrogen to dietary requirements also began to assume a racialized undertone through the backdrop of imperial ambitions in the European metropoles. These sentiments fused with the mist of racial eugenics to rationalize notions of racial inferiority as Page describes "what nitrogenometrics offered was a scientific rationale to the alleged superiority of the high protein diet of the wheat-meat eating English as opposed to the potato-eating Irish or the rice-eating Indian"[154]. In other words the nitrogen quality of the diet was the principle demarcation between being a civilized Human and being a savage as Cardwell explicates "Lots of nitrogen in your diet and body made you savage, wild, and turbulent; a lack of it led to mild, tractable, and lethargic temperament"[40]. Imperial ambitions to monopolize raw materials and forcefully convert imperial populaces into new markets was functionalized through the confluence of nitrogen and protein intake with racial delineations. Thus, the Malthusian fear of scarcity fused with racialized underpinnings of dietary quality that caused a flurry of efforts to secure more nitrogen. To remedy this issue, the great powers of Europe turned to imported fertilizers starting first with Peruvian Guano.

Peruvian Guano remained largely on the fringes of European awareness since the writings of the Spaniard Garcilaso de la Vega, whose seminal volumes on the Spanish conquest of Peru, *Comentarios Reales* first published in 1609, elucidated the Inca civilization's utilization of guano sourced from coastal islands as fertilizers[94]. The revelation of guano's high nitrogen content to Europeans occurred when German explorer and naturalist, Alexander von Humboldt, dispatched a specimen for analysis to French chemist Antoine-Francois de Fourcroy[40] in 1804[3]. The recognition of guano's elevated nitrogen content coincided with the comprehensive comprehension of nitrogen as an elemental entity, paralleled by the emergence of novel methodologies facilitating the elucidation of the elemental composition across various substances. Peruvian Guano was found to contain around 15% and sometimes as high as 20% nitrogen by mass nearly an order of magnitude more than conventional organic fertilizers such as manures (2-3%) and plant litter (1-2%)[3]. This phenomenon primarily stems from the geological characteristics of Guano deposits, typically situated in arid coastal islands where seabirds, sustained by nitrogen-rich sardines, deposit their excrement that would accrue due to minimal precipitation[94]. This resulted in the accumulation of substantial mounds of guano, amassed over millennia to heights of several meters, often enveloping entire islands in a literal sense. Following the disintegration of the Spanish empire and the subsequent independence of its

[40] Fourcroy (1755-1809) was a contemporary of Lavoisier and co-authored *Méthode de Nomenclature Chimique*, a landmark text and one of the first taxonomic systems to classify chemical substances.

former colonies in the 1820s, which ensued after years of tumultuous upheaval instigated by various colonial powers, Peru entered a phase of nascent stability. However, it found itself burdened with substantial indebtedness to Britain, primarily attributable to wartime expenditures[155]. Britain capitalized on this dependency to assert its dominance in the Guano trade, initially importing limited quantities in the 1820s and 1830s, affording farmers the opportunity to evaluate its efficacy as a fertilizer. Subsequently, from 1841 onwards, shipments expanded to full vessel loads, with only a select few firms like Antony Gibbs & Son exercising virtual monopolistic control over the trade[155].

British imports of Guano went from 95,000 tons per year in 1850 to 200,000 tons the following year to over 300,000 tons by 1858[94]. The second highest importer was the United States which imported 175,000 tons in 1858[94]. The fervor surrounding Guano, under the sway of a quasi-oligopolistic structure, engendered enduring apprehensions regarding scarcity and produced what scholars Brett Clark and John Bellamy Foster describe as "ecological imperialism"[155]. The commerce surrounding Peruvian Guano precipitated an intensified quest for alternative Guano reservoirs, thereby initiating a continual exploration for islands potentially harboring such deposits and inundating the market with Guano of inferior quality [41]. This culminated in the enactment of the Guano Islands Act by Congress in 1856 "Whenever any citizen of the United States discovers a deposit of guano on any island, rock, or key, not within the lawful jurisdiction of any other Government, and not occupied by the citizens of any other Government, and takes peaceable possession thereof, and occupies the same, such island, rock, or key may, at the discretion of the president, be considered as appertaining to the United States"[156]. This provision effectively granted individuals the authority to claim ownership of an island based on the belief that it contains Guano, while also authorizing the use of military force at the discretion of the executive branch, to safeguard these claimed islands. This lead to the seizure of 94 islands, rocks, and keys in various places around the world between 1856 and 1903, with 66 ultimately being recognized as U.S. territories[157]. The Guano Islands Acts represent early manifestations of United States aspirations for global imperial influence, exemplified by the extension of its military prowess beyond the confines of its continental territories. The burgeoning demand for Guano in the United States predominantly revolved around the escalating concerns regarding soil depletion, particularly prominent in the cotton-producing states of the South[158]. Driven by apprehensions regarding obstacles to Western expansion, Southern plantation owners began adopting Guano as a soil amendment during the 1840s and 1850s, particularly in established slave-holding states grappling with soil exhaustion[145]. The importation of Guano was seen as an alternative to driving slave labor to clear more land as agricultural newspaper editor Daniel Lee explains "farmers should use more guano---…instead of investing their money in more Negroes the sooner to impoverish the best lands of the South."[158] Although demand was there, direct importation into the South and widespread commercialization did not start to escalate until after the Civil War.

[41] The problem of low-quality Guano became so pervasive, Guano quality standardization became the norm in places such as Britain where farmers came to rely only on Guano that had been tested and certified to be the same quality as Peruvian Guano. See *Ecological Imperialism and the Global Metabolic Rift* (Clark & Foster, 2009)

The labor employed in harvesting Guano from the islands was predominantly sourced from Chinese indentured laborers, commonly referred to as "coolies" who were procured at a rate of "30 pesos per head"[155]. The Chinese laborers endured such severe working conditions that numerous commentators asserted their plight exceeded that of enslaved individuals, with notable figures such as Marx and Engels characterizing it as a form of "disguised slavery"[159]. Chinese coolies toiled incessantly, with daily quotas averaging around five tons of Guano, amidst meager provisions and the perpetual specter of severe reprisal.[160]. The fifteen year mortality rate was 25-30%[155]. Between 1849 and 1874, Peru imported more than 90,000 Chinese coolies, whose exploitation fueled the profitability of the Guano trade. Paradoxically, the proceeds derived from their labor contributed to Peru's decision to abolish slavery in the 1850s[155]. The Guano trade was so profitable to Peru it went from making up five percent of state revenue in 1846-7 to 80 percent by 1869[161], radically transforming the political terrain of the country but also subjecting it to enduring dependency on the British, who wielded monopolistic control over the trade. The wealth generated from the Guano trade accrued mostly into the pockets of the wealthy elite and failed to stimulate the domestic economy. The country was still widely dependent on imports to meet the demand for general commodities and the trade agreements with Britain left the country in a perpetual state of debt[155]. The Guano deposits, basically exhausted by 1870[42] left Peru in a state of massive debt with little natural resources to help ameliorate its position[94].

The Guano trade, incited by Malthusian fears of soil exhaustion, scarcity, and impending famine, delivered Guano primarily to Britain, facilitating its transition towards "high farming" or proto-industrial commodity agriculture[155]. With the repeal of the Corn Laws in 1846 and the inundation of cheap grain into British markets, the price of domestic cereals began to fall. This impetus led to British agricultural consolidation, marked by the amalgamation of farms into fewer holdings, which in turn embraced the novel agricultural methodologies introduced during the Agricultural Revolution. These practices were oriented towards the production of marketable food commodities, notably meat and dairy products[153]. Henceforth, the Norfolk Rotation system underwent adaptation, integrating substantial quantities of imported Guano to bolster livestock development. This evolutionary shift lays the groundwork for the contemporary capitalist agricultural production paradigm, characterized by the consolidation of vast land holdings under the control of a minority, uniform land utilization conducive to economies of scale, and the excessive application of fertilizers to generate fodder for livestock destined for market trade[162]. This system relied on the intercontinental transfer of nutrients, the "metabolic rift" proclaimed by Marx escalated to a planetary scale, all tied to the accumulation of capital and the hegemony of the state. Britain was so invested in procuring nutrients for its high farming it harvested bones from other countries to use as phosphate fertilizers amounting to a "robbery of nature" as von Leibig claims Britain "deprives all countries of the conditions of their fertility. It has raked up the battle-fields of Leipsic, Waterloo, and the Crimea; it has consumed the bones of many generations accumulated in the catacombs of Sicily…Like a vampire it hangs on the breast of

[42] Guano exports continued into the 1880s and 1890s but by the mid 1880s imports were half the peak of 1870. See *Ecological Imperialism and the Global Metabolic Rift* (Clark & Foster, 2009)

Europe, and even the world, sucking its lifeblood without any real necessity or permanent gain for itself"[163]. The configuration of capitalist agricultural practices intertwined with ecological imperialism echoes Marx's concept of "primitive accumulation" a notion further elaborated by scholar David Harvey as "accumulation through dispossession"[164]. This forms the central tenet of Marx's examination of capitalist systems concerning nature, positing that capital exhibits a relentless pursuit to transcend ecological constraints, often achieved by incorporating peripheral economies into the orbit of capitalist production systems through processes of dispossession. The confluence of mercantilism, colonialism, and industrial capitalism converges upon the acknowledgment of nitrogen as a pivotal determinant for growth, with strategies of dispossession and exploitation emerging as mechanisms to circumvent this threshold.

As the decline of Guano became apparent, imperial focus shifted towards the exploitation of Chilean nitrates. Nitrate deposits had been known to Europeans since they were discovered by Spanish-Peruvian naturalist and chemist, Mariano Eduardo de Rivero in the coastal province of Tarapacá in 1821[3], and in 1853 deposits were found in the neighboring province of Antofagasta[155]. The nitrate deposits were composed primarily of a crude mineral called *caliche*, an amalgamation of different oxidized salts, magnesium, calcium, potassium, and sodium nitrates, sulfates, borates and iodates that rests 1-3 meters under thin layers of *chuca* (sand, silt, and clay) and *costra* (pebbles, cobbles, and rock fragments)[43]. The sodium nitrate proportion of caliche ranged from 40-50% and sodium nitrate itself has a nitrogen content of 16% roughly on the same order of magnitude as Peruvian Guano[3]. These deposits were accumulated over thousands of years from nitrogen rich sea-sprays in the Atacama Desert and its preservation was facilitated by the dry conditions of the desert which mitigated their erosion. Exports of nitrate soon took off, exporting around 8,300 tons in 1830 to 30,000 tons by 1850 to over 300,000 tons by 1870[3]. The discovery of the nitrates almost immediately precipitated geopolitical tension between the newly formed countries of Peru, Bolivia, and Chile[155]. Tarapacá initially constituted a Peruvian province, while Antofagasta was originally part of Bolivia; nonetheless, during the 1830s and 1840s, the predominant demographic in these regions comprised Chilean inhabitants[94]. Chile asserted control over a significant portion of Antofagasta in 1842 and subsequently negotiated a treaty with Bolivia in 1866, delineating the division of the territory between the two nations and establishing a framework for the shared exploitation of profits derived from the nitrate trade[94]. Recognizing the depletion of its Guano reserves, Peru sought to consolidate authority over its nitrate reserves in Tarapacá. In pursuit of this objective, Peru entered into a nonaggression and mutual support treaty with Bolivia in 1873, subsequently implementing a state monopoly on nitrate production in 1875[155]. Following Bolivia's imposition of heightened taxes on nitrates exported from Antofagasta, Chile dispatched troops to the titular port, precipitating the outbreak of the War of the Pacific fought between Chile against Peru and Bolivia, spanning from 1879 to 1883. The conflict was arguably catalyzed by Britain, driven by aspirations to broaden its dominion over fertilizers to encompass the lucrative nitrate deposits as well[165]. Supported by British investors and bolstered by a navy constructed in Britain and an

[43] Caliche was normally extracted from underneath the chuca and costra either manually or through blasting and required subsequent processing to remove the nitrates from the raw minerals. See *Enriching the Earth* (Smil, 2004).

army trained by France, Chile launched military operations into Tarapacá and Antofagasta and by 1881, Chile had effectively gained extensive control over the nitrate reserves in the region[155]. The conflict represented yet another instance of European imperialism, characterized by the strategic manipulation of nascent states' aspirations for economic progress to incite rivalries among them, ultimately serving the interests of the European powers involved. Amidst the conflict, British speculators opportunistically acquired Peruvian government certificates, leveraging them as instruments of influence following the war to compel the Chilean government into conceding exclusive ownership of nitrate enterprises based solely on possession of said certificates[166]. British ownership of nitrate deposits in Tarapacá went from 13 percent before the war to 34 percent immediately after the war and 70 percent by 1890[167]. Under the leadership of President José Manuel Balmaceda, Chile endeavored to nationalize its nitrate reserves in 1890 and impede the sale of state-owned nitrate fields to British interests. In response, Britain precipitated a civil conflict by supporting Balmaceda's adversaries with financial backing and weaponry, subsequently imposing a naval blockade along the Chilean coast to obstruct exports[155]. Following Balmaceda's demise and the ascension of a pro-British administration, the control over Chilean nitrate resources became largely concentrated in British hands, with nearly three-quarters of its exports directed to Britain by the early 1890s[155].

Although the primary impetus behind the trade resided in the demand for Chilean nitrates as fertilizers, a notable secondary market emerged in the explosives industry[44], quickly becoming its second most significant consumer[46]. Following the introduction of trinitrotoluene (TNT) as an explosive in 1891, armed forces swiftly adopted it as an adjunct for shell casings, thereby catalyzing a surge in demand for nitrates.[46]. These developments are concurrent with the escalating industrialization observed within armed forces during the latter portion of the nineteenth century. The full embrace of industrial capitalism led to an interlocking of industry and warfare which was accompanied by advances in military technology, strategy, and logistics. The state embraced a paradigmatic shift away from conventional military strategy to one embodied by the leveraging of a large industrial base to mobilize massive armies supported by robust transportation and communication networks. Science constituted an indispensable component of this emergent strategy aimed at harnessing technological advancements capable of yielding tangible advantages on the battlefield. With this paradigmatic shift the consumption of nitrates in the production of explosives skyrocketed, in 1890 45,000 tons of nitrogen-based explosives were produced and in 1900 that figure increased to 115,000 tons[46]. The United States in 1900 utilized almost half of its nitrate imports for making explosives[3]. The state's reliance on nitrogen for military endeavors has been evident since the widespread utilization of gunpowder in cannons. However, with the exploitation of Chilean nitrates, which accounted for approximately 80% of the world's nitrogen supply by the conclusion of the late nineteenth century[168], the state's ability to mobilize armies became intricately linked to the export capabilities of Valparaiso and Antofagasta. Exclusion from this market could precipitate dire consequences for an industry. This juncture signified a pivotal shift in the trajectory of nitrogen imperialism, which had played a significant role in shaping the evolution of both the state and capitalist structures. Moreover, it delineates the outer boundaries of capitalism's ecological

[44] Other industries needed nitrates as well, dyes, photograph films, and metals. See *The Story of N* (Gorman, 2013)

constraints. As previously underscored, capitalism operates without inherent limitations, extending to the realm of nitrogen and beyond. Thus, the imminent surpassing of thresholds delineates the onset of the contemporary biogeochemical epoch.

1.2.9 Breaking Through Ecological Limits

By the dawn of the twentieth century Malthusian anxiety over nitrogen scarcity had crept into political, economic, and social discourse throughout Europe. This is best exemplified by the British chemist and physicist, William Crooke's address to the British Association for the Advancement of Science in 1898 "The fixation of nitrogen is vital to the progress of civilized humanity, and unless we can class it among the certainties to come, the great Caucasian race will cease to be foremost in the world, and will be squeezed out of existence by races to whom wheaten bread is not the staff of life."[169] Nitrogen at this point in time was the key to civilization or more specifically White civilization. The perception of access to reactive nitrogen within the purview of Imperial Europe delineated a stark dichotomy between sustenance and mortality, epitomizing the enduring legacy of Empire, and serving as the catalyst for technological modernization and industrial advancement. It was also the corporeal and metaphysical boundary between racial supremacy and racial downfall. Amidst the dominance of Chilean nitrates as the principal source meeting global nitrogen requisites, the specter of depletion akin to the historical case of Peruvian Guano, coupled with the potential imposition of military blockades, instilled a burgeoning apprehension reflective of the pervasive imperialistic fervor characterizing Europe during that era, thereby nearly fomenting widespread hysteria. Capitalism thrives on the fear of scarcity, if the world demand of Chilean nitrates at the start of the First World War persisted indefinitely the deposits were projected to last for an additional 340 years[3]. However, the ghost of Malthus ensured the perpetual fear of scarcity and impending doom for the "great Caucasian race", exacerbated by rapidly increasing population[45] and threats of land scarcity[46], and thus the impetus to find an alternative and renewable source of reactive nitrogen amongst the great powers of Europe had reached a tipping point in the opening of the twentieth century. By this time the work of Boussingault in 1858[47] had shown that leguminous crops added nitrogen to the soil and the work of Hermann Hellriegel and Hermann Wilfarth in 1885[48] demonstrated nitrogen

[45] World population took millennia to reach 500 million by 1500 then increased to roughly 1 billion by 1800 and had increased to roughly two billion by the 1920s.

[46] There was a growing fear of scarcity of arable land, as the majority of the most fertile lands for grain and cereal production in North America and Europe had already been cultivated. See *Enriching the Earth* (Smil, 2004)

[47] Jean Baptiste Boussingault (1802-1887) was a French chemist and agro-scientists who conducted extensive work on the nitrogen composition of plants and the determinants of biological nitrogen fixation. He conducted extensive field experiments in which he demonstrated that leguminous crops (peas, clover, beans, etc.) added nitrogen to the soil by fixing atmospheric nitrogen. His work motivated further field experiments that confirmed his observations including the famous Rothamsted field experiment, the longest running agricultural field experiment that showed the effect of different plants and crop rotation strategies on the nitrogen content of the soil. See *The World's Greatest Fix* (Leigh, 2004)

[48] Hermann Hellriegel (1831-1895) and Hermann Wilfarth (1853-1904) were Prussian chemist who were the first to discover biological nitrogen fixation through symbiotic diazotrophs through their famous root splitting experiment. They separated the roots of a legume into two different vials, one sterilized and one inoculated with soil and observed that only the roots inoculated with soil were able to grow the root nodules associated with nitrogen

was fixed through symbiotic diazotrophic bacteria. These two advancements ultimately refuted the humus theory advocated by Davy and von Leibig, thereby establishing a definitive biological constraint on the natural capacity for nitrogen fixation[94]. These prerequisites provide the contextual framework for the endeavors of Fritz Haber and Carl Bosch, whose objective was to circumvent this ecological constraint and secure unhindered access to reactive nitrogen. This marks the zenith in the narrative of nitrogen imperialism, representing the apex attained after centuries characterized by episodes of genocide, enslavement, dispossession, mercantilism, industrial capitalism, and state-sanctioned violence. This is the crystallization of anthropogenic nitrogen geoengineering into the historical substratum. This juncture represents the pivotal moment where racialized paradigms, deeply entrenched in extraction-based ontologies, are integrated into the intricate tapestry of geohydrological, biogeochemical, and sociopolitical economies, thus shaping the foundational structures of the contemporary global landscape. Nitrogen as an idea, to nitrogen as co-constitutive of the Inhuman, to nitrogen as commodity.

At the turning point to the nineteenth century the natural limits to agricultural productivity were becoming increasingly defined and accentuated. Smil estimated the global average for grain productivity was on the order of 800 kg/ha, which would yield approximately 120 kg protein per hectare or enough to support around 7 people per hectare[3]. Approximately 70% of the nitrogen supplied to produce that harvest originated from manures, organic recycling, crop rotations, and atmospheric deposition[3]. In response to burgeoning population growth and finite land resources, industrializing nations perceived augmenting land productivity as the sole viable recourse[46]. Following the endorsement of inorganic fertilizers by von Leibig, they swiftly evolved into coveted commercial commodities, sparking the emergence of markets for phosphorus and potassium fertilizers[49] However, nitrogen, owing to its biochemical constraints, remained the most elusive and sought-after fertilizer, garnering the highest demand. This engendered a substantial impetus to penetrate this novel domain, assert hegemonic influence, and consolidate control thereof. Hence, the intensifying technological pursuit aimed at discovering an industrialized method for nitrogen fertilizer synthesis commenced in the mid-nineteenth century and culminated during the transition from the eighteenth to the nineteenth century.

The three most viable industrial processes for nitrogen fixation in the nineteenth century include the production of ammonia by-product from coking, the cyanamide process, and the electric-arc process. Ammonia by-product collection from coking leverages the release of trace nitrogen (1% to 1.6%) from the combustion of coal[3]. Coal, when heated anaerobically to produce coke for iron smelting or coal gas releases approximately 12-17% of the nitrogen as ammonia, after washing and condensation the resulting ammonia liquor is combined with calcium hydroxide to

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fixation. This knowledge comes after the solidification of germ theory and bacteria through the work of Pasteur and Koch. See *The World's Greatest Fix* (Leigh, 2004)

[49] Phosphorus fertilizers had been available since the 1820s, mostly from the mineral coprolites which was found in a variety of places around England, apatites were found in Norway in 1851, and rock phosphates were found in North Carolina and Florida in the 1860s and 1870s. Given the demand for phosphate fertilizers in relation to nitrogen, the long term supply was relatively secure. Potassium fertilizers come from potash (potassium chloride) and those reserves were also considered safe for the long term. See *Enriching the Earth* (Smil, 2004).

yield a solution of ammonium sulfate[3]. However, this process is limited by the low yield of nitrogen (15-20% of the original content) resulting in only 2.75-3.25 kg ammonia per ton of coal, even if all of the 80 Mt of coal used for coking at the beginning of the 20th century was used for ammonia by-product collection the yield would have only been 200,000 t N[50]. In reality only a small fraction of this was being recovered which paled in comparison to the roughly 400,00 t N provided through Chilean nitrates[3]. The cyanamide process leverages the production of calcium cyanide ($CaCN_2$) through the reaction of calcium carbide (CaC_2) and dinitrogen at high temperatures (>1000°C)[51]. The commercial process pioneered in Germany through the work of Adolf Frank and Nikodemus Caro decomposed the calcium cyanamide into calcium carbonate and ammonia through a reaction with superheated steam[94]. A few commercial plants were built in Europe, Japan, and Canada but the process was incredibly energy intensive (130-200 GJ/t N) due to the need for electricity to produce calcium carbide and to produce coke to heat the main reaction[3]. The total output of cyanamide plants amounted to only 100,000 t N in 1913 and 325,000 t N by 1918[3]. The electric arc process leveraged the conversion of dinitrogen and oxygen into nitric oxide through electric fixation, a natural atmospheric process, that had been known since the work of Priestly and Cavendish and popularized by Davy[94]. The resulting nitric oxide can form nitric acid in the presence of oxygen and water. Because of the need for electricity[52] to carry out the reaction this process was limited to locations with an inexpensive sustained supply of electricity which occurred predominantly at hydropower stations[94]. Such stations were constructed at Niagara Falls and the most serious commercial endeavor was constructed in Norway through the innovations of Kristian Birkeland and Samuel Eyde that produced the Birkeland-Eyde furnace which could provide an alternating current arc. However, even with these innovations the energy requirements were still incredibly high (180-270 GJ/ t N) and the spatial restrictions to hydropower stations resulted in the production of only about 70,000 t of calcium nitrate by 1913[3]. All of these processes, based in chemistry that was well studied and characterized were limited by either spatial, material, or energetic constraints.

The moment of Haber's discovery and culmination of years of work forever cemented in his famous letter to BASF (Badische Anilin-und Sodafabrik) on July 3, 1909 "Yesterday we began operating the large ammonia apparatus with gas circulation in the presence of Dr. Mittasch and were able to keep its uninterrupted production for about five hours. During this whole time it

[50] This process was also limited to industrial locations with a large demand for coke needed for iron smelting or coal gas. It also had the material requirements of coal to burn. Thus, only a few industrial nations seriously pursued this option included the U.S., Britain, and Germany. See *Enriching the Earth* (Smil, 2004)

[51] The reaction of barium carbonate and nitrogen to produce barium cyanamide $Ba(CN)_2$ had been known since the work of F. Margueritte and A. de Sourdeval in 1860. Barium carbonate was replaced with calcium carbide, a cheaper material to produce, after the work of Adolf Frank (1834-1916) and Nikodemus Caro (1871-1935). Frank and Caro helped to build several plants in Germany mostly powered with hydroelectricity. See *Enriching the Earth* (Smil, 2004)

[52] This reaction could only proceed at temperatures above 3000°C which could only be provided through electric sparks. The process was patented in Britain in 1859 by Madame L.J.P.B Lefebure, possibly one of the first female chemical engineers. The only way the reaction could really be feasible was through continuous discharge of electric sparks and this was realized through the work of Norwegian physicist, Kristian Birkeland (1867-1917) and English engineer Samuel Eyde (1866-1940) who built the Birkeland-Eyde furnace that could do this with an input of just 2.2 kW. See *The World's Greatest Fix* (Leigh, 2004)

had functioned correctly and it produced continuously liquid ammonia"[3] did not occur in a political or scientific vacuum. Germany, the burgeoning nation-state forged through the amalgamation of diverse German-speaking territories, heralded its inception following the denouement of the Franco-Prussian conflict in 1870. Its nascent national identity coalesced around principles of imperialism, industrial advancement, and the establishment of a formidable array of state-sponsored scientific institutions[170]. As a nascent state endeavoring to vie with longstanding empires in Western Europe, Germany expanded its territorial acquisitions into West, East, and South Africa, as well as East Asia, with the primary aim of procuring raw materials to fuel its swiftly advancing industrialization efforts, albeit at the cost of perpetrating acts of genocide in the process[171]. The escalation of Anglo-German tensions in the prelude to the First World War, notably exacerbated by the outbreak of the Boer Wars[172], ignited a heightened sense of apprehension regarding the availability of nitrogen supplies. This concern reached a climax amidst fears that a British blockade of Chilean nitrates could sever vital access routes, precipitating a dramatic surge in geopolitical anxiety. Consequently, Germany established a robust state apparatus that actively fostered industrial collaborations with scientific institutions, a strategic endeavor aimed to develop alternatives to reliance on Chilean nitrates, thereby mitigating vulnerabilities to potential British blockades and safeguarding vital national interests[3]. Thus, German chemical enterprises had been actively engaged in the exploration of diverse industrial nitrogen fixation methodologies throughout the latter half of the nineteenth and early twentieth centuries. This is exemplified by their interest in the cyanamide process, the collection of ammonia byproducts from coking, and collaborative ventures with Norwegian counterparts aimed at scaling up electric furnaces for the electric-arc process[3]. German chemists had long been engaged in the groundwork that underpinned the development of the Haber process, predating the involvement of Fritz Haber himself. This is evidenced most explicitly by the work of Wilhelm Ostwald, who reported to BASF in 1900 that he had succeeded at producing ammonia from hydrogen and nitrogen in the presence of an iron catalyst[53]. Even before Ostwald, chemists had been trying to realize the insights of Berthollet, Lavoisier, de Morveau, and de Fourcroy that ammonia could be produced from the synthesis of nitrogen and hydrogen. Johann Wolfgang reported ammonia synthesis from catalysts such as platinum in 1823, Otto Erdmann and Richard Marchand reported trace amounts of ammonia from passing nitrogen and hydrogen over red hot coals in the 1840s, Henri Saint-Claire Deville reported the persistence of ammonia with prolonged sparking in 1865, and William Ramsay and Sydney Young reported on the decomposition of ammonia at temperatures above 800°C in 1884[3]. Le Chatelier calculated the required temperature, pressure, and amount of iron catalyst to produce ammonia in 1901 but dropped his experiments after his apparatus blew up[94]. Consequently, upon commencing his research endeavors in 1903, Haber entered into a scientific milieu where chemists were already familiar with the synthesis of ammonia via a catalyzed, high-temperature, high-pressure process.

[53] Ostwald reported to BASF that he had developed a process for ammonia synthesis with an iron catalyst. BASF deployed Bosch to confirm his claims and Bosch reported that the ammonia Ostwald detected in his study was actually coming from the hydrogenolysis of iron nitride formed on the iron catalyst during its treatment. Bosch also reported he was unable to detect any catalysis for the same reaction under pressures of 0.5 MPa. See *Enriching the Earth* (Smil, 2004)

Haber's pivotal contribution to this continuum of inquiry lay in refining an optimized synthesis method achievable under practical operating conditions.

Haber started his work on ammonia synthesis at the behest of the Österreichische Chemische Werke, a chemical company in Vienna in 1903. During his tenure as a professor at the Technische Hochshule in Karlsruhe, Haber embarked upon his initial investigations concerning the equilibrium dynamics governing the interaction between ammonia and its precursor elements, nitrogen and hydrogen[54]. Building upon the research conducted by Ramsay and Young, Haber elucidated the modest ammonia yields achievable under equilibrium conditions at 1000°C. However, he inferred that the establishment of an economically viable system predicated on this reaction necessitated the utilization of a catalyst operating at atmospheric pressure and temperatures not exceeding 300°C[55]. In light of these findings, Haber terminated his engagement with the aforementioned project and redirected his focus primarily towards research endeavors within the domain of electrochemistry. In the wake of a public scholarly dispute with Walter Nernst, wherein accusations were made regarding the accuracy of Haber's findings[56], Haber resumed his engagement with the project with the aim of reinstating his academic credibility. He refined his experiments in 1907, further elucidating the equilibrium dynamics governing ammonia synthesis, and in the process ascertained that optimal ammonia yields were theoretically possible at lower temperatures (200-300°C) and elevated pressures (>3 MPa). At this juncture, Haber's parallel investigations into nitric oxide production via the electric arc method[57] had garnered the interest of BASF, still aspiring for commercialization of the arc process. BASF initiated discussions with him regarding a collaborative effort, subsequently, two contracts were negotiated: one pertaining to the advancement of the arc process, and the other encompassing his broader research endeavors in nitrogen fixation Through this partnership, Haber continued his work on ammonia synthesis ultimately building an experimental apparatus (75 cm tall) that was able to realize appreciable ammonia synthesis (80 g NH_3/hour) with an osmium catalyst at temperatures below 600°C and pressures in the range of

[54] Haber's initial experiments conducted in 1903 were predominantly fundamental to try to understand the equilibrium conditions of the reaction between hydrogen and nitrogen to produce ammonia. With his assistant Gabriel van Oordt, Haber found that the yield of ammonia under equilibrium conditions at 1000°C was somewhere between 0.0005 and 0.0125%. See *Enriching the Earth* (Smil, 2004)

[55] Haber did do some theoretical calculations which predicted that a higher yield cold be produced at lower temperatures. See *Enriching the Earth* (Smil, 2004)

[56] Walter Nernst (1865-1941) most famously known for the Nernst equation that makes up the Third Law of Thermodynamics did a lot of work on the equilibrium conditions of various reactions and according to his theorem the yield of ammonium should have been much lower than what Haber had observed. Haber corrected the results and presented them at a meeting of the Bunsen Society in 1907 but Nernst gave him a strong critique that suggested his work was not just incorrect but strongly inaccurate. This prompted Haber to pick his work on ammonia synthesis back up. *Enriching the Earth* (Smil, 2004)

[57] Haber's primary expertise was in electrochemistry and thus he had conducted an extensive amount of research into the electric-arc process. BASF was heavily invested in the arc process and was still attempting to commercialize it but ultimately chose not to pursue it further after 1908. *Enriching the Earth* (Smil, 2004)

17.7-20.2 MPa[58]. It was this pioneering experimental apparatus that solidified Haber's enduring legacy as the progenitor of the ammonia synthesis process, a seminal achievement that merited him the Nobel Prize in 1918. Nevertheless, it was Carl Bosch, an engineer with a longstanding tenure at BASF since 1899, who undertook the pivotal measures necessary to transition this experimental apparatus into a commercially viable endeavor. Drawing upon his extensive expertise in metallurgy and mechanical engineering, Bosch was granted full autonomy to undertake the task of upscaling Haber's experimental system[59]. The principal obstacles he confronted included ensuring a steady provision of feedstock gases—nitrogen and hydrogen—identifying a catalyst that was both stable and economically viable, and fabricating a reaction vessel capable of withstanding the elevated pressures requisite for the reaction.

With remarkable ingenuity, Bosch devised a double-tube apparatus featuring a steel jacket, ingeniously designed to prevent corrosion of the converter wall and thus capable of withstanding the requisite high pressures[60]. Through collaborations with colleague Alwin Mittasch, they identified an inexpensive mixed-iron based catalyst that could operate at low temperatures and high pressures[61]. Finally he solved the feedstock problem by utilizing water gas produced by the reaction of coke and water vapor as a hydrogen source[62]. In an impressively swift endeavor akin to a sprint, Bosch managed to translate Haber's 75-centimeter experimental reaction vessel into an expansive 8-meter commercial-scale apparatus within the span of a year. The inaugural large-scale prototype system was deployed at BASF's principal compound in Ludwigshafen, marking a significant milestone in May of 1910.

The original prototype system was converted to a fully operational system that began operating in August of 1910 which produced about 10 kg NH_3 per day in 1910 and 18 kg NH_3 daily by the beginning of 1911 and 100 kg daily by July of 1911. Another converter was added in 1912 soon reaching 1000 kg NH_3 output daily[3]. A massive 500,000 m^2 plant was built and completed at Oppau in 1913 producing 10 t of NH_3 a day. By early 1914 the plant was fixing 20 t of NH_3 a day and by late 1914 40 t of NH_3 a day[3]. The ecological boundary, comprehensively delineated merely three decades earlier, was rapidly breached with astonishing speed. The historically elusive nitrogen, hitherto accessible primarily through imperialistic ventures and the exploitation of racialized populations in regions such as India and South America, could now be extracted

[58] This work was very heavily dependent on the contributions of Haber's assistant Robert Le Rossignol who had extensive experience in the manufacturing of the components required for Haber's experimental vessel. *Enriching the Earth* (Smil, 2004)

[59] Bosch was ultimately the voice of approval for Haber's experimental apparatus and advocated for the company to move forward with commercializing it. *Enriching the Earth* (Smil, 2004)

[60] The addition of the steel jacket became necessary after Bosch's initial experiments resulted in the collapse of the converter tubes due to the inner tube becoming brittle because of hydrogen leakage. See *The World's Greatest Fix (*Leigh, 2004)

[61] Alwin Mittasch (1869-1953) a chemist employed by BASF worked meticulously to test a variety of different catalyst ultimately testing 2500 different catalyst in 6500 separate runs. *The World's Greatest Fix (*Leigh, 2004)

[62] This reaction absorbs heat and produced carbon monoxide which required air to be recirculated through the coal beds to maintain the temperature and the deployment of the water gas shift reaction to convert carbon monoxide to carbon dioxide which was removed by passing it through water under pressure. *The World's Greatest Fix (*Leigh, 2004)

directly from the atmosphere. The significance of this monumental event, akin to a geological shift, finds its proper contextualization in the emergence of a modern military-industrial complex, epitomized by the transformative repercussions emanating from the Oppau industrial site. Germany's fervent strain of bellicose nationalism compelled its involvement in a conflict predominantly instigated by the imperialistic and nationalistic aspirations pervasive throughout Europe, which had already etched deep divisions into the fabric of the continent for numerous decades. The amalgamation of state-sponsored scientific innovation coalesced into a nexus where scientific fervor intersected with the nationalistic agenda of warfare[170]. Ludwig Fulda's manifesto *To the Civilized World* claiming "[w]ere it not for German militarism, German civilization would long since have been extirpated…The German army and the German people are one"[3] signed by 93 scientists including Haber epitomized the notion of mobilizing a country's entire industrial base to support the institution of war. Subsequent to the precipitous decline in the expectation of swift military victory following the First Battle of Marne, coupled with the projected blockade of German ports by the British navy, thereby severing access to Chilean nitrates, the German military authority sought direct assistance from BASF to bolster its wartime endeavors. The military necessitated nitric acid for the production of explosives crucial to wartime combat. Concurrently, BASF faced a crisis stemming from the wartime disruption of conventional markets and the impediment to importing pyrite required for ammonium sulfate production. Consequently, a strategic partnership was forged between the state and industrial capitalists, setting a precedent for the interplay between military and capitalist interests that would endure throughout the remainder of the century. With the support of the German government BASF entirely restructured the plant at Oppau to produce nitric acid and produce a new plant at Leuna in central German that could both produced roughly 31,700 t N per year[3]. These transitions were crucial to Germany's ability to produce munitions and prolong the war effort despite blockades. During the war, Haber went on to lead the German chemical weapons program that produced chlorine gas first deployed at the Battle of Ypres in 1915. The introduction of this novel weapon swiftly ingrained itself within the collective consciousness of unprepared combatants, evoking a level of brutality and sadism reminiscent of the practices observed within Medieval torture chambers. Haber himself rationalized the use of these weapons "The aversion that is feed by the strangeness of the weapon, is further enhanced by the imagination of extreme cruelty and the doubt if it isn't harming the fundaments of international law, that have to be kept sacrosanct in the interest of the whole humanity even in war.[…]Gas as a weapon is not in the least more cruel than flying pieces of metal"[173]. Haber's involvement in the utilization of chemical weaponry prompted deliberation within the Nobel Prize committee regarding the appropriateness of awarding him the accolade in 1918. Nonetheless, he steadfastly defended his participation in the development of such weapons as an expression of patriotism. These examples underscore the employment of nitrogen as a tool of warfare, which was propelled to an industrial magnitude through the collaboration of the military-industrial complex. The endeavor to assert state control over nitrogen production epitomizes a convergence between the state's necropolitical capability to monopolize violence and its capacity to dominate life.

In reviewing the political and economic context surrounding the endeavors of Haber and Bosch, it becomes abundantly clear that the narrative surrounding this seminal epoch in history is not characterized by altruistic motives or a commitment to addressing global hunger. The primary

impetus driving BASF's pursuit of the ammonia synthesis process was to assert dominance in the fertilizer market, augment profitability, and amass additional capital for the purpose of market expansion, thus perpetuating a cycle of continual growth and market penetration. This scenario unfolded within the framework of state-sponsored industrialization, intricately interwoven with imperialistic aspirations in Africa and Asia, aimed at securing essential raw materials to fuel subsequent phases of expansion. This phenomenon also mirrors the prevailing sentiment across Europe during this era, wherein access to nitrogen was regarded as pivotal for racial sustainability. There was a prevailing notion that the populace, the "meat-wheat eating" populaces of Europe, needed to uphold their nitrogenometric composition to safeguard their long-term endurance. Consider that at the height of the Chilean nitrate trade, over 90% of nitrates were being exported to Europe (~70%) and the United States (~20%)[3]. Concurrently, imperial territories underwent profound restructuring to meet the export requisites imposed by their imperial overseers, resulting in the disruption of traditional subsistence patterns in favor of commodity agriculture. This restructuring coincided with historic droughts, exacerbating famines across Africa, East Asia, India, and Indonesia, leading to an estimated death toll of approximately 20 million individuals in the late 1890s[174]. The endeavor to assert hegemony over reactive nitrogen was not propelled by a genuine concern for addressing the dietary requirements of the global populace. On the contrary, the prevailing Malthusian anxiety pervasive within European metropolises fostered an illusion of scarcity, thereby compelling a relentless pursuit to secure existing nitrogen reservoirs through any available means. This fundamental reality solidifies nitrogen, encapsulated in the steel jackets of Haber and Bosch's invention, into its present status as a commodity. By definition exclusive, isolated, unidirectional to be bought, sold, and trafficked on a market. The commodification of nitrogen, closely intertwined with the aspirations of the state and the agenda of industrial militarism, bestows upon nitrogen a biopolitical and necropolitical significance, delineating the parameters of its utilization in terms of location, method, and agent. In this manner, the state exercises authority over the allocation of life and death through the regulation of nitrogen distribution. This phenomenon is evident on a global scale, guided by racialized ideologies that enable the dominance of an entire biogeochemical cycle to serve the interests of a privileged minority. Nitrogen as an inert, nonagentic, immaterial object is essential to the global structure of power and commerce consubstantially mimetic of the anathema that is the Inhuman. Nitrogen delineates the contours of power systems much like the Inhuman defines the boundaries of what it is to be Human. It is a misconception and a form of historical oversimplification to associate Haber-Bosch with a benevolent endeavor aimed at alleviating the world from perpetual famine. Rather, Haber-Bosch represents an extension and ossification of the racialized colonial frameworks that facilitated mass genocide, land expropriation, and enslavement. It solidifies the metamorphosis of land into a foundational element for production and consolidates nitrogen as a commodity to enable enhanced extraction from the land. It perpetuates racialized eugenics by delineating access to nitrogen in the form of sustenance and dictating the manner in which it is acquired. The moment that Haber demonstrated to BASF his experimental apparatus to synthesize ammonia, the world had forever changed. For millennia, the Human, constrained by inherent biological limitations, was subject to the Earth's provision, with little agency in their "metabolism" with the planet. However, with the advent of Haber-Bosch, the Human gained the ability to extract nitrogen

directly from the air, thereby transcending these historical constraints, assuming dominion over nature, and anointing himself as God.

1.2.10 Preaching the Gospel of Industrial Agriculture and the Green Revolution

With the Haber-Bosch process appearing to be on the verge of surpassing the ecological limitations inherent in biological nitrogen fixation, the groundwork was laid for the emergence of the contemporary paradigm of nitrogen-intensive agriculture. Nevertheless, the dissemination of the technology and the escalation in scale took several decades to truly make an imprint on the geological record. Following its defeat in the First World War, Germany found itself compelled to relinquish its technological and scientific capabilities upon surrender. British and French engineers were dispatched to observe the operations at the Oppau and Leuna plants, ultimately resulting in the establishment of the first Haber-Bosch facilities outside of Germany in Versailles, France, and North Yorkshire, England, in 1919[94]. A small plant with a 5000 t N per year capacity soon followed in Alabama in 1921[3]. Amidst the gradual dissemination of the Haber-Bosch process, a significant innovation emerged in the 1920s, marked by the utilization of steam reforming to extract hydrogen from natural gas as a primary feedstock[94]. Subsequently, this advancement facilitated the establishment of the inaugural ammonia production plant employing natural gas as a feedstock in Pittsburgh, California in 1931. By this point the Haber-Bosch process was producing roughly 1 Mt N per year or approximately three quarters of all industrially fixed nitrogen[3]. Throughout the 1920s, various process innovations emerged, encompassing the adoption of rotary compressors in lieu of reciprocating compressors, continuous enhancements in catalyst development, and the continual enlargement of plant infrastructure[94]. Nonetheless, beyond these iterative modifications, the fundamental framework of the Haber-Bosch process has remained largely unchanged since the late 1920s.

Concurrent to these technological developments that increased the scale and efficiency of the Haber-Bosch process are the institutional changes to the food system that significantly increased the politicization and commercialization of the agricultural sector in the United States. In the aftermath of the First World War, European farmers reverted to pre-war levels of agricultural production, resulting in a global surplus of food commodities and subsequent precipitous decline in prices[153]. This phenomenon precipitated widespread bankruptcies among farmers during the zenith of the Roaring Twenties, delineating an "Agricultural Depression" antecedent to the Great Depression. In the wake of the Great Depression, farmers endeavored to alleviate their financial burdens by "farming their way out of debt" intensifying food production efforts. However, with a substantial portion of the population unemployed and thus unable to purchase the surplus produce, the cycle of oversupply persisted, further exacerbating the downward spiral of prices[153]. To mitigate this crisis, the federal administration under President Roosevelt's New Deal endeavored to stabilize the agricultural sector through the enactment of the Agricultural Adjustment Act (AAA). This legislation sought to regulate supply by incentivizing farmers to withdraw lands from production, imposing levies on processors and intermediaries, and instituting a nationwide food assistance program aimed at alleviating both overproduction and poverty[175]. With the commencement of the Second World War, a vast industrial mobilization aimed at bolstering the war effort precipitated a significant exodus of laborers from the

agricultural sector, leading to a labor shortage. This crisis prompted the United States to address the issue by importing inexpensive labor from Mexico through the Mexican Farm Labor Program Agreement of 1942, implementing subsidies to elevate land values, and instituting price-fixing measures to sustain productivity levels[153]. This development paralleled a surge in conservation strategies following the Dust Bowl crisis, alongside advancements in mechanization epitomized by the adoption of tractors in lieu of traditional horse-drawn ploughs[176]. Lastly, the surge in wartime demand for explosives prompted the United States to erect numerous large-scale ammonia synthesis facilities, culminating in the annual production of approximately 880,000 metric tons of NH_3 by 1945, distributed across approximately 10 plants[3]. The value of U.S. agricultural exports increased from $517 million in 1940 to $669 million in 1941 to $3.2 billion by 1946[177]. Following the conclusion of the war, the industrial infrastructure accrued to support war production was redirected into agriculture to maintain productivity. The transition of ammonia synthesis plants, formerly dedicated to the production of explosives during wartime, towards the manufacture of fertilizers became prevalent. This shift was facilitated by the availability of inexpensive credit, enabling farmers to procure affordable fertilizers and pesticides. Additionally, the accessibility of cheap petroleum facilitated heightened mechanization in agricultural practices, while an expansion in land acquisition augmented the scale of farm operations[3]. Henceforth, the modern iteration of capitalist agriculture, distinguished by its reliance on substantial chemical inputs, automated and mechanized harvesting systems, the utilization of cost-effective immigrant labor, and the concentration of expansive land holdings within the control of a select few, commenced its maturation process.

The surplus production in the United States led to a saturation of the fertilizer and pesticide market, prompting a shift towards expansion into the European market. This in turn engendered overproduction in Europe, precipitating efforts to penetrate new markets, particularly in the Global South, through the negotiation of trade agreements. These agreements, characterized by predatory practices, facilitated the inundation of grain markets with inexpensive produce, exacerbating the challenges faced by already vulnerable farmers in these regions[153]. By 1950, industrial ammonia production amounted to roughly 2 Mt N per year[3]. This set the stage for what is now known as the “Green Revolution” which spread the American model of capitalist agriculture to other developing countries in the world[153]. The Green Revolution finds its epitome in the contributions of Norman Borlaug, whose breeding methodologies for rice and maize yielded hybrid varieties responsive to fertilizer application and characterized by uniform growth patterns. Consequently, these strains became conducive to capitalist agricultural models prioritizing economies of scale. Borlaug’s model encompassed by the utilization of hybrid seeds, irrigation, fertilizers, and pesticides was disseminated across the globe at breakneck speed “saving a billion people from hunger”[153]. The implementation of capitalist agricultural methodologies in developing nations undoubtedly constituted a Cold War strategy, strategically employed to divert emerging countries from the influence of Communism[176]. In actuality, this paradigm shift incentivized an annual reliance on new hybrid seeds, designated as "genetic property," consequently precipitating the financial insolvency of small and medium-sized family farms due to the expansion of farm sizes. Moreover, it engendered a proliferation in both the land area utilized and chemical inputs within agricultural systems[153]. The principles underpinning the Green Revolution were additionally fortified by subsequent global economic upheavals, such as

the Vietnam War and the 1972 oil crisis. These events prompted Western banks, buoyed by the influx of newfound "petrodollars," to extend loans to nations in the Global South. These financial resources were subsequently utilized by these nations to acquire Western technology and expertise[153]. The developmental fervor witnessed during the 1970s and 1980s significantly dismantled the remaining barriers of isolated markets in the Global South, inundating them with inexpensive food and Western agricultural innovations. This influx further exacerbated the downward pressure on prices. Additionally, the proliferation of predatory trade accords throughout the 1980s and 1990s ensnared developing nations in a perpetual cycle of indebtedness, rendering their domestic markets entirely vulnerable to the whims of Western capitalist forces. This exacerbated the shift towards capitalist agricultural methodologies that prioritize the establishment of large-scale mechanized farms, characterized by the intensive utilization of chemical fertilizers, pesticides, herbicides, and fungicides. Concurrently, this trend has posed significant challenges to small and medium-sized farms, which persist in contributing to approximately 70% of the global food supply[153]. Notwithstanding, major corporations involved in processing, handling, and distribution, such as Walmart, Carrefour, and General Mills, wield significant influence over the logistical pathways facilitating the journey of food from cultivation to consumption. The onset of neoliberalism and deregulation of financial sectors starting in the 1980s continues to accentuate this trend. Meanwhile the production of nitrogen fertilizers from Haber-Bosch going lockstep with the advancement of the Green Revolution went from 4,780 kt N per year in 1950 to 31,600 kt N per year in 1970 to 77,100 kt N per year in 1990 to 85,700 kt N per year in 2000[3]. Simultaneously, a considerable proportion of farmers residing in impoverished and developing nations are unable to procure these inputs due to financial constraints.

Henceforth, the prevailing global nitrogen regime is principally shaped by the tenets of capitalism, which exert dominion over the intricate biogeochemical cycles regulating Earth's natural processes. The commoditization of land, food, human and nonhuman life serves as the material and ontological basis for the abstraction and compartmentalization of nitrogen. Nitrogen, is the manifestation of the state’s dual capacity to generate life while simultaneously exerting control over it, a paradigm intricately entwined with the racialized relegation of nitrogen to a lifeless, amorphous ether that derives meaning solely from the utilitarian function bestowed upon it by the state. Reflecting upon the contemporary landscape, the United Nations Food and Agriculture Organization (FAO) forecasts a compelling future scenario: by the year 2050, the necessity to generate 60% more sustenance emerges to accommodate an estimated population of 9.3 billion individuals[178]. This projection invariably portends a heightened demand for the production and utilization of nitrogen fertilizer. However, juxtaposed against this prognostication lies the striking reality that the world presently yields 1.5 times the required food supply to sustain its current populace, which approximates 8 billion individuals[153]. The nitrogen problem proselytized for the past two centuries is not a question of scarcity rather it is a question of the fundamental dichotomy between common goods and private use. As long as the concept of private property, attached to a biocentric actant known as the Human, ensured by the state and it’s biopolitical agenda, and racialized through its extraction point the Inhuman, persist nitrogen will always be used and abused for private gain. Simultaneously, the accumulation of waste stemming from this epistemological deviation will persist, exerting deleterious effects on both

the planet and the individuals deemed suitable recipients thereof. This is the thrown upon which the current Imperial regime rests, this is the metaphysical and corporeal embodiment of Azotic Empire. The outlook for the planet and its racialized peoples will be evaluated in due course.

1.3 The Future of Azotic Empire

1.3.1 Mephitic Air and the Expansion of Empire

The trajectory of historical events and the evolution of social institutions, which delineate the contours of the modern global landscape, are intricately interwoven with the historical narrative of nitrogen, encompassing both its evolving comprehension and exploitation throughout time. The empires of old and the empires of new built transcontinental dominions by exploiting nitrogen reserves to feed commodity agriculture, industrialization, and as a material basis for war. In pursuit of these objectives, they promulgated a worldview that positioned their conception of an autonomous, free-thinking entity endowed with the capacity to exert dominion over nature as central, relegating racialized others to the periphery of this paradigm. This phenomenon has engendered the sustained perpetration of violent genocide and epistemicide against Indigenous populations on a global scale, accompanied by the transformation of land into a locus of production. Additionally, it has facilitated the dehumanization, dehistoricization, and marginalization of Black peoples, serving the expedient purpose of facilitating widespread enslavement. The "world" as we know it to exists today is a transcultural vacuum oriented around the ontological underpinnings of categorization as a means of commodification, structured by vicious dispossession and state monopolized violence. The axiological animus of this structure is the subjugation of the black. What are the implications of this sociopolitical configuration for the planet and the marginalized "other" going into the future of this world regime?

To answer this question, we must first acknowledge that the empire is alive and well. This empire thrives on the persistence of settler colonialism which serves as a material basis for the perpetuation of colonial land relations and the marginalization of Indigenous knowledge. Colonialism transcends its historical manifestations; rather, it persists as an enduring framework employed to dispossess Indigenous communities of their territories, thereby severing their ancestral ties to the land and disrupting the cosmological and epistemological knowledge rooted in relationality that has guided them for countless generations[179]. Settler colonialism undergoes a transformation of the land, configuring it into a composite of various exploitable resources, thereby precluding the establishment of reciprocal or symbiotic interactions with the land[63]. This process relies on categorical frameworks to overlook the inherent relationality intrinsic to each natural object. Such classification systems form the ontological foundation for the abstraction of nitrogen, perpetuating its exploitation and misallocation. Colonial legacies serve as the impetus behind the appropriation of land, thereby facilitating the utilization of nitrogen as a tool to optimize land productivity. The outcomes of this utilization are not intended for communal benefit; rather, they contribute to a system of commodity exchange driven solely by profit motives. Profit drives the overapplication of nitrogen, profit drives the fungibility of nitrogen to be trafficked, profit drives the waste of nitrogen as an expansion of colonial property rights

manifested as a right to pollute. In this way, nitrogen persist as a tool and a paradigm to drive the distinction between the Human and the Inhuman.

Smil estimates that roughly 50% of the global population is dependent on synthetic fertilizers produced by Haber-Bosch to meet their dietary nitrogen requirements[3]. This figure simultaneously underestimates and overestimates the impact of Haber-Bosch on the daily existence of the average individual, with nuanced implications contingent upon the geographical location of the individual. In Western nations such as those in Europe and the United States, a significant portion of nitrogen fertilizer application on agricultural lands is allocated to grain production intended for livestock feed. Smil's estimation indicates that approximately two-thirds of the grains produced in these regions are ultimately utilized for livestock consumption[3]. The protein conversion efficiencies of meat and dairy products derived from these agricultural processes vary, with values ranging from approximately 30% in milk and eggs to as low as 5% for beef. This suggests that only a minute fraction of the nitrogen initially applied to fertilizer for grain cultivation, aimed at sustaining livestock, ultimately contributes to the nitrogen content of the final product[3]. The bulk of these agricultural products are allocated for either export or domestic markets, resulting in daily per capita protein intakes averaging approximately 100 g in developed nations, with 55 g derived from animal products. In contrast, low-income countries in Latin America and Africa exhibit a lower daily per capita protein intake of 66 g, with merely 18 g sourced from animal products[3]. Reflect upon the fact that nitrogen fertilizer utilization in low-income nations surpassed that of high-income nations merely three decades ago. Consequently, of the aggregate nitrogen fertilizer consumed globally since 1961, more than half has been consumed by the Global North, which during that period comprised approximately one-third of the global population. Conversely, the remaining portion has been utilized by the Global South (excluding China), encompassing roughly two-thirds of the global populace[180]. While these figures are approximations, they underscore the extent of nitrogen disparity, wherein nearly half of the nitrogen utilized is dedicated to generating food items for export, while the remaining portion is employed in regions where it is indispensable for generating the dietary nitrogen essential to support the populace. Nations grappling with pronounced arable land scarcity and elevated population densities, exemplified by China, Bangladesh, India, and Indonesia, are fundamentally compelled to rely on nitrogen fertilizer to avert widespread famine. In contrast, affluent nations tend to excessively utilize nitrogen to exploit food commodity markets[3]. Fig 2. demonstrates the spatial changes in nitrogen fertilizer application globally from 1961 to 2013 and this highlights the growing dependence on high population density countries on nitrogen fertilizer applications[181]. Conversely, to exemplify the dichotomy between common goods and private utilization, Smil conjectures that the United States, notwithstanding a halt in food exports and a substantial alteration in the typical dietary patterns, could feasibly generate adequate sustenance for its populace sans the reliance on industrially synthesized nitrogen fertilizer[3]. This disparity is further compounded by the observation that more than 40% of food undergoes wastage at the consumer level within developed nations, while approximately 60% is lost within developing countries, primarily during processing and distribution phases[153]. As population growth is projected to persist until 2050, an additional 1.5 billion people are anticipated to inhabit the planet[182], primarily concentrated in developing nations with constrained capacity to

expand arable land cultivation. Consequently, this trend is poised to augment the reliance on synthetic nitrogen fertilizers.

While nitrogen endures as a racialized bio-constitutive metric indicative of civility and sophistication, wielded as a state apparatus to delineate the parameters of human existence, it is imperative to acknowledge the disparities in nitrogen pollution risks and their deleterious impacts across the globe. Oita et al. (2016) elucidate that the nitrogen footprint attributed to individual countries, delineated by their import and consumption patterns, diverges notably from the tangible nitrogen pollution experienced within those nations[183]. The nitrogen footprint of a nation delineates its consumer behaviors, encompassing elements such as transportation habits, dietary preferences, and consumption of commercial goods[183]. The proliferation of these markets has fostered the global diffusion of capital, enabling the relocation of agricultural and industrial manufacturing processes to developing nations. This practice effectively shifts the adverse impacts associated with reactive nitrogen utilization to these regions, thereby obscuring the accountability for its accumulation. This phenomenon finds its epitome in China's exportation of NO_x, estimated at approximately 1000 Gg annually[183]. This exportation is rooted in China's role as the "chimney of the world"[36] where it serves as the primary manufacturer of a considerable portion of global consumer goods. This production relies on abundant reservoirs of inexpensive labor and electricity derived from inefficient combustion of brown coal, resulting in substantial NO_x emissions. This effectively parallels the same phenomenon responsible for China being the leading CO_2 emissions producer as Malm describes "China had, relatively speaking, low wages and high carbon intensity, certain other countries had high wages and low carbon intensity, and *capital flowed from the latter to the former*"[36]. The globalization of capital has engendered its heightened spatial fluidity, traversing nations in pursuit of cost-effective labor and operational expenditures akin to a parasite. This process obfuscates the underlying reality, wherein nations importing goods manufactured through the exploitation of inexpensive labor in developing regions are the principal consumers, thereby perpetuating the cycle of pollution. Oita et al. elucidates this opacity by delineating that the principal consumers of nitrogen pollution are predominantly affluent Western nations, affording them the ability to evade culpability and mitigate the adverse consequences of pollution on their populations[183]. This phenomenon resonates with the consumer tendencies dictating import allocations, exemplified by Japan's heavy reliance on imported food items, notably meat which carries a substantial nitrogen footprint[184]. Meanwhile studies demonstrate that nitrogen air pollution continues to be disproportionately shared with racialized minorities and those of lower socioeconomic statuses both in countries of the Global South and minoritized communities in the Global North[185,186].
Anthropogenic nitrous oxide emissions, predominantly from agriculture, are expected to double from roughly 5.3 Tg N per year in 2005-2010 to roughly 9.7 Tg N per year in 2050[187].
With a greenhouse gas potential approximately 300 times greater than that of carbon dioxide, this will exert a disproportionate influence on warming potential, incrementally propelling the Earth toward the critical 2°C threshold, indicative of climatic shifts of cataclysmic proportions.
Once more, individuals residing in the Global South are poised to bear the brunt of climate change repercussions, encompassing escalating sea levels, alterations in temperature and precipitation regimes amplifying risks to food and water availability, and precipitating monumental climatic catastrophes with heightened frequency. In contrast, the Global North is

poised to possess the means to mitigate the most severe repercussions of such phenomena, thus creating a socio-economic disparity in vulnerability and resilience[188]. This serves as yet another exemplification of capital's systemic capacity to harness state-endorsed mobility to exploit labor across diverse geographies, consolidate the gains within a privileged minority, and shift the burdens onto individuals considered dispensable. In this context, nitrogen is once more wielded as a tool of capital to extract surplus value from peripheral economies, delineating those capable of conducting extraction from those subject to extraction

With the share of urban populations expected to increase from its current level of 55% to 68% by 2050[182] alongside the concomitant rise in the scale of reactive nitrogen emissions into the environment, the translocation of nitrogen and heightened perturbation of the nitrogen cycle are poised to intensify. Nitrogen discharges from wastewater treatment effluent to surface waters is expected to increase from 13.5 Tg N in 2010 to 17.9 Tg N in 2050[189]. It is noteworthy that as of 2020, the collective worldwide production of crop residues is purportedly around 20.9 Tg N annually[190] meaning that the nitrogen content discharged from wastewater treatment facilities theoretically harbors sufficient nitrogen to sustain the cultivation of half of the world's crops. While posing economic and political impediments, the technology necessary to make the paradigm shift to redivert the nutrients in wastewater and "close the loop" is well documented and theoretically viable. However, the system of capitalism does not bend to what is environmentally or societally beneficial. Capital is subject only to the pursuit of profit. Despite the widespread exposure of the global populace to the adverse ramifications of this system, the allocation of these detrimental byproducts is rationalized through an embedded racial animus intrinsic to the capitalist system. Thus the solutions needed to rectify the technical challenges of this crisis have little effect on a problem that is inherently political and by extension economic. Hence, it is necessary to clarify and explain that science and technology will not save us from this crisis.

1.3.2 Science will not save us

In the era of technical modernity, science is preached as a universal panacea to solve all of society's problems. In the epoch of technological modernity, science is extolled as a universal panacea for remedying all societal maladies. The pervasive faith in science as a remedy for historical injustices, a solution to contemporary dilemmas, and a bulwark against future anxieties approaches a near-deific ubiquity. Science is demanded to render the human body impervious to disease and aging, address perceived material scarcity through a transformative process reminiscent of alchemy, and remediate environmental damages on a global scale.
The rationale positing science as a universal panacea, devoid of contextual nuances or relational considerations, mirrors the logic underpinning knowledge generation, portraying the breadth of the Human's rationalism as analogous to a divine omniscience. Science is the operationalization of the Human's will to subjugate nature and appropriate it for his benefit. Furthermore, the Human being a willing biopolitical subject of the state links science to the materialization of nationalism. Hence, the mythos surrounding science, and its extension into universalism, seeks not solely to confer epistemic superiority upon the state but also to de-politicize inherently political social matters by framing them within the domain of science. Therefore, scientific

solutions are oriented not toward grappling with the core social or economic issues intrinsic to the problem, but rather toward fortifying the ideological underpinnings of the system that initially engendered the problem. Thus, in order to affect meaningful change, a critique of the modern form of science linked to the system of capitalism, the military-industrial complex, and the marginalization of Indigenous and alternative epistemes is warranted.

Since the genesis of the Scientific Revolution in Europe, science has been instrumentalized as a mechanism to bolster European agendas pertaining to the dispossession of Indigenous lands and the propagation of settler colonialism. This initially manifested as a deliberate marginalization of Indigenous land stewardship, social structures, and spiritual practices, portraying them as intrinsically inferior to European equivalents and thereby serving as a justification for land expropriation as Liboiron describes "Western science has long been identified as a practice that assumes mastery over Nature, reproduces the doctrine of discovery, revels in expropriation and appropriation of Indigenous Land, and is invested in a rigorous self-portraiture in which valid scientific knowledge is created only by proper European subjects"[63]. This "self-portraiture" served to extend universalism in which all scientific knowledge generated by Europeans was valid everywhere, all the time independent of context or circumstance[63]. As the ideas of liberal political economy and industrial capitalism aligned with the interest of the state began to take hold a new brand of "scientific nationalism" began to take hold in Europe. These ideas of human centric rationalism began to be increasingly pervasive in Europe especially after the onset of the French revolution as Somsen describes "patriotic values began to be associated with the pursuit of knowledge. Whereas the fruits of science used to be reaped by all of humanity, during the Napoleonic consulate and empire, its accomplishments were increasingly presented in terms of national prestige"[170]. The pursuit of scientific excellence became intertwined with national identity, forging a dialectical relationship with cultural uniformity and, by extension, notions of racial superiority. This accelerated with the unification of the German states in the late nineteenth century as Somsen explicates "By the late nineteenth century, *Wissenschaft* was still seen as a crucial component of German culture, and advancements made by German scientists were regarded as reflecting the greatness of the nation-just like economic and military achievements did"[170]. The phenomenon of scientific nationalism underwent a heightened escalation and confluence with the ideological precepts of political economy during the Cold War era. Scientific rivalry emerged as an additional arena of contention in the Cold War, as the United States and the Soviet Union vied ceaselessly to surpass each other in the pursuit of global hegemony[170]. Thus, science as an institution from its incipience has been an integral part of the state's strategy to exert its influence both politically and ideologically. Capitalism, entwined in a symbiotic relationship with the state, thrives on the perpetual innovation fostered by scientific advancements, relying on them to transcend limitations in labor and production efficiency, as well as to explore novel markets. Consequently, science, functioning as a tool of both the state and capital, is inherently aligned with the agenda of state interests and remains invariably subservient to its dictates.

Central to the discourse on the utilization of science for depoliticization is the pivotal role played by Western academic institutions in perpetuating settler colonial ideology. Moreover, these institutions serve as incubators for the state-sponsored agenda of militarized surveillance and the

development of imperial technologies. This is epitomized through Land-Grant institutions, conceived out of the Morrill Act of 1862, that granted land to public institutions primarily for the advancement of agricultural research[191]. The project of land-grant institutions is inherently contingent on the presupposition of access to Indigenous land and forms a material manifestation of the state's will to transform land into capital and advance the scientific interest of the state as la paperson explains "Land as *capital* and not as *campuses* is an innovation of the land-grant university. That is, states are able to trade, develop, and sell land to fund the construction of public universities"[192]. The land-grant university or what la paperson calls the "first-university" is an extension of the state's prerogative to dispossession and a confluence of scientific nationalism with settler colonial ideology[192]. Moreover, the Western university serves as the epicenter of technological innovation, particularly in the development of imperial instruments of war and military surveillance technology. These advancements are often deployed for the purpose of policing and exerting control over racialized communities, perpetuating systems of oppression and social inequality. The alignment of interest between military, industrial, and academic institutions was realized during the height of the Cold War where the Department of Defense partnering with weapons companies such as Raytheon, Northrup Gruman, and Lockheed Martin began to develop strategic partnerships with academic institutions coalescing into what scholar Stuart Leslie terms the "military-industrial-academic complex"[193].
The outcomes of these collaborative endeavors are subsequently employed by settler-colonial apartheid states, exemplified by Israel's utilization of such technologies to enforce the colonization of Palestinian territories. This dynamic is further reinforced by academic investments in Israel and its affiliated corporations, forging increasingly intricate connections among the state, corporate entities, and academic institutions. Western academic institutions are also implicated in the development of military surveillance technology such as remote sensing satellites and facial recognition software that are utilized by police departments and state militaries and militia alike to terrorize domestic populaces and enact genocide[194,195]. Lastly, the Western academic institution is a breeding ground for the ideology that subtends white supremacy and settler colonialism. Racial eugenics was an academic discipline that attained high levels of credibility at prominent academic institutions including Harvard, Columbia, Cornell, Brown, the University of Wisconsin, and Northwestern[196]. The discourse of these disciplines was used to rationalize forced sterilizations, institutionalizations, and social stigmatization. Thus, the Western academic institution is directly implicated in the goal of the state to perpetuate racial subjugation as a means of dispossession and extraction. They are deeply entrenched both materially and ideologically in upholding the structures of capitalism, state authority, and settler colonialism, thereby precluding any inclination toward fostering critical discourse that challenges these foundational ideologies. Even the institutions harboring critiques of capitalism the "second-university" as la paperson suggest, are at odds with decolonial agendas as their material ethos is reflective of the first university as la paperson explains:

"One of the tautological traps of the second world university is mistaking its personalized pedagogy of self-actualization for decolonial transformation. When people say "another university is possible," they are precisely saying that "a second university is possible," and they are often imaging second world utopias, where the professor ceases to profess, where hierarchies

disappear, where all personal knowledges are special, and, in other words, none are. Their assumption is that people will "naturally" produce freedom, and freedom's doppelganger is critical consciousness. They are rarely talking about a university that rematriates land, that disciplines scholar-warriors than "liberating" its students, that repurposes the industrial machinery, that supports insurrectionary nationalisms as problematic antidotes to imperialist nationalism, that acts upon financial systems rather than just critiquing them, that helps in the accumulation of third world power rather than simply disavowing first world power, that is a school-to-community pipeline, not a community-to-school pipeline."[192]

In essence, institutions purportedly aligned with a legacy of liberalism, social justice, and political activism often fail to tangibly promote decolonial initiatives. Instead, they represent a spatially delineated accumulation of intellectual elitism, masquerading as anti-colonialism while remaining deeply entrenched in upholding the material and ideological frameworks of settler colonialism.. Therefore, the academic institution will not save us, they cannot even save themselves from the paradoxically parasitic and symbiotic relationship with capital and the state.

Scientific nationalism and the military-industrial-academic complex are the institutions that form the structural basis of scientific depoliticization of societal issues. However, a principal practice in this depoliticization is the confabulation of the myth of "we". When atmospheric chemist Paul Crutzen declared in 2000 that humanity had embarked into a new geological era known as the "Anthropocene", it quickly assumed a scholarly connotation coterminous to the collective influence of human activities on nature: "Human activities have become so pervasive and profound that they rival the great forces of Nature and are pushing the Earth into planetary *terra incognita"*[197]. In doing so, Crutzen and all of his ideological sympathizers began a project of homogenization that attempted to find a consilience between the pervasive effects of climate change and the collective actions of all humans. However, here the distinction must be made that the human Crutzen refers to is in actuality the Human that by definition has entitlement to dominion over the Earth. Scholars raced to modify this new geological designation with its proper historical context coining the "Capitolacene"[36], the "Plantationocene"[198], the "Cthulucene"[199] and the "Negrocene"[200]. What is most dangerous about the geological demarcation of the Anthropocene is the historical obscurantism that functionalizes the myth of "we". Asserting that "we" as in all human life forms share equal responsibility for the degradation of the Earth, "we" as in all human activities contribute to the Earth's exploitation, "we" as in all human's just by virtue of existing are deleterious to the Earth's wellbeing. "We" attempts to co-opt a myth of collective responsibility that sanitizes the oversized impact of an infinitesimally small number of people to produce a narrative of all peoples by virtue of living and breathing on the planet as contributing to its downfall. "We" disperses the legacy of a small group of British capitalist in nineteenth century Lancashire to the commercial choices and consumer patterns of individuals. "We" both dehistoricizes and depoliticizes the crises of biogeochemical disruption to absolve the systems that both cause and perpetuate the ongoing crises: racial capitalism, settler colonialism, and their complementary tools of epistemic superiority and imperial militarism. "We" is a product of the Anthropocene not as a scientific endeavor but as a political endeavor as Yusoff writes:

"If the imagination of planetary peril coerces an ideal of "we," it only does so when the entrappings of late liberalism become threatened. This "we" negates all responsibility for how the wealth of that geology was built off the subtending strata of indigenous genocide and erasure, slavery and carceral labor, and evades what that accumulation of wealth still makes possible in the present-lest "we" forget that the economies of geology still largely regulate geopolitics and modes of naturalizing, formalizing, and operationalizing dispossession and ongoing settler colonialism"[69]

"We" is a distraction. "We" is a historical and political fallacy. "We" only serves to impart a obfuscation of scale that is necessary to impose feelings of helplessness and apathy to the state of the world and its current direction. This is the praxis that is crucial to the depoliticization of science and technology. If "we" as individual consumers make better choices and subscribe to the implications of technological modernity than the crisis will solve itself. It inherently constructs a narrative of universalism that perpetuates science as a ubiquitous tool to save humanity. In order to break the fog, properly contextualize the problem, hold responsible parties accountable, and formulate strategies that redress societal harms and ensure sustainable and equitable solutions for the planet and marginalized peoples we must break through the paradigm of "We".

To put it simply, nitrogen as a commodity, nitrogen as a biogeochemical cycle, nitrogen as an arbiter life of death is inherently a political and societal issue, not a technical one. The technology exists to ensure more efficient uptake of fertilizer nitrogen in plants, limiting overapplication cropland leakage, volatilization, and nitrous oxide emissions. It's not widely used because it conflicts with the capitalist practice of uniform application amenable to mechanized harvesting for larger economies of scale. The technology exists to dramatically scale up renewable energy and electric vehicles which would reduce NO_x generated through the combustion of coal and natural gas for electricity generation and gasoline for combustion engines. The barriers that prevent its replacement of conventional energy and transportation structures are predominantly economic. The technology exists to capture nitrogen released in wastewater treatment effluent and return it to sites of agricultural production. It's not pursued as a viable option for fertilizers because of the logistical challenges hampering its scale and profitability. If all these challenges were purely technical, they would have been solved decades ago. Moreover, in numerous instances, technical solutions have the potential to deepen the material enactment of settler colonial land dynamics. Heightening efficiency via technology within capitalist frameworks typically results not in diminished output but rather augmented productivity, thereby yielding increased profits[65]. Consequently, technologies ostensibly capable of curbing nitrogen emissions may not translate into reduced emissions; rather, they may incentivize heightened emissions by enabling greater nitrogen removal capacity. The veracity remains that these challenges are not isolated within a political or economic vacuum; rather, they are shaped by the material ramifications of a system wholly geared towards profit acquisition through any available means. Profit over nature. Profit over people. That is the mantra. That is the reality that structures every political, economic, and social decision that governs our lives. Profit is contingent on property, it's dependent on scarcity, and thrives on exploitation. Nitrogen as it exists in its current paradigm as a lifeless commodity to be exploited is necessary to the

perpetuation of private benefit that creates profit. This commoditization is entirely dependent on a regime of violence that determines who is and who is not deserving of life, will, or agency. Nitrogen is an instrument of the state that reinforces the contours of the Inhuman, the immaterial object that is necessary to conceptualization of living free under the authority of the state. Both being consubstantial, they are rendered lifeless as a means of fungibility for the purposes of extraction and accumulation. The alteration of this paradigm hinges upon a reintegration of nitrogen into its contextual relational framework, recognizing its nuanced dynamics across various contexts. Until nitrogen is disentangled from its commoditized status and its role as a mere production input, there will be no change. There will be no progress. Nitrogen will continue to be emitted to the atmosphere, sinking deeper and deeper into subsurface aquifers, exported and disseminated to the deepest gorges of the ocean, and accumulate in the biosphere imparting untold consequences for nonhuman and human life alike. No technical solution will change this paradigm until we do the work to change it ourselves. Nevertheless, "solutions" abound out of the specter of technical modernity. Attempts to ameliorate the effects of the issue persist in scientific and engineering discourse. One of these solutions and the subject of the remainder of this dissertation will be expounded upon in due course.

1.4 Strategies to Mitigate Nitrogen Pollution in Wastewater

1.4.1 Nitrogen Pathways

Through the ambitions of scientific hegemony, engineers have endeavored to manipulate natural systems to ameliorate the issues caused by anthropogenic nitrogen pollution. These solutions leverage the capabilities of microbial life that has evolved over eons to utilize nitrogen in varying capacities. The biogeochemical cycling and transformation of reactive nitrogen in the environment is mediated by a phylogenetically and metabolically diverse groups of bacteria[201]. Ammonia, the standard equivalency of biological nitrogen, is used for the production of amino acids which accounts for the vast majority of cellular nitrogen[202,203]. As a result of this the ability to reduce organic nitrogen and other forms of reactive nitrogen to ammonia is ubiquitous throughout most major groups of microorganisms[204]. However, the ability to oxidize and reduce different nitrogen species for energy generation is much more specific to different groups of bacteria and archaea. Some of these pathways are strongly linked to phylogeny (e.g. anammox) while others are widely distributed throughout various bacterial and archaeal domains. Nevertheless, microorganisms performing different nitrogen transformations are often classified based on the metabolic pathways that they perform as shown in Figure 1.32.

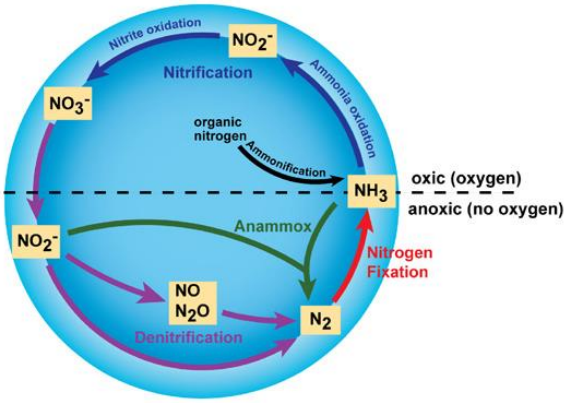


Figure 1.2: Nitrogen cycle pathway including nitrogen fixation, nitrification, denitrification, and anammox. Acquired from Bernhard (2010)

Nitrogen fixation is performed by symbiotic diazotrophic microorganisms to produce ammonia. Ammonia is oxidized aerobically to nitrite and nitrate, denitrification anaerobically reduces nitrate and nitrite to dinitrogen gas. Ammonium reacts with nitrite through anaerobic ammonium oxidation (anammox) to produce dinitrogen gas and nitrite is reduced to ammonia through dissimilatory nitrate reduction (DNRA). The design of wastewater treatment systems for biological nitrogen removal leverages the physiological attributes and metabolic capabilities of these different groups of bacteria to accomplish the desired treatment goal. Different nitrogen removal communities are enriched from diverse inocula based on a strong selection pressure determined by reactor conditions.

Nitrification is an aerobic process facilitated by chemoautotrophic bacteria in which ammonia is utilized as an electron donor and oxygen is utilized as an electron acceptor[205]. Nitrifiers form two distinct groups: ammonia oxidizers which oxidize ammonia to nitrite via hydroxylamine through ammonia monooxygenase and hydroxylamine oxidoreductase enzymes, and nitrite oxidizers which oxidize nitrite to nitrate using nitrite oxidoreductase enzymes[205]. A third group of nitrifiers constitutes comammox bacteria, which can perform complete nitrification of ammonia directly to nitrate[206]. These reactions can be characterized using equations 1.1 and 1.2

$$\textbf{(1.1)}\ NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O$$

$$\textbf{(1.2)}\ NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

Bacteria performing the reaction in equation 1.1 are generally classified using the prefix "Nitroso-" with lineages in Betaproteobacteria, Gammaproteobacteria, and Alphaproteobacteria. Bacteria performing the reaction in equation 1.2 are classified using the prefix "Nitro-" and have lineages in Alphaproteobacteria as well as the monophyletic clade Nitrospira[205,207]. Wastewater treatment plants often apply nitrification either through nitrifying activated sludge or through a separate nitrification process. Both options rely on the optimization of reactor conditions to balance the distinct kinetics and physiology of ammonia and nitrite oxidizers.

Nitrification is often combined with denitrification, where first influent ammonia is oxidized to nitrate and then anaerobically reduced to dinitrogen gas using organic carbon as an electron

donor. Denitrification proceeds in four separate steps, nitrate reduction to nitrite via nitrate reductases (narG/H or napA/P), nitrite reduction to nitric oxide via nitrite reductases (nirK or nirS), nitric oxide reduction to nitrous oxide via nitric oxide reductases (norB/C), and nitrous oxide reduction to dinitrogen via nitrous oxide reductases (nosZ)[208]. Some bacteria contain all of the genes for complete denitrification of nitrate to dinitrogen and some only contain some of the genes making them capable of only partial denitrification. Heterotrophic denitrification is associated with a broad range of taxonomies including *Pseudomonas* and *Bacillus*[209] but can span a diverse array of phyla including Proteobacteria, Chloroflexi, and Bacteroidetes[210], while autotrophic denitrification is more exclusively associated with *Thiobacillus* and *Parococcus*[211]. Denitrifiers can utilize either nitrate produced by complete nitrification or nitrite from ammonia oxidation or nitrate reduction. These reactions can be characterized by equation 1.3 and 1.4.

(1.3) $4NO_2^- + 3CH_2O + 4H^+ \rightarrow 2N_2 + 3CO_2 + 5H_2O$

(1.4) $4NO_3^- + 5CH_2O + 4H^+ \rightarrow 2N_2 + 5CO_2 + 5H_2O$

Denitrification can be leveraged to remove nitrate from wastewater through a variety of means including a two stage sequential nitrification-denitrification reactor system, an anoxic pretreatment utilizing activated sludge recycle, or a one-stage nitrification-denitrification reactor[212].

DNRA bacteria catalyze the eight electron reduction of nitrate to ammonium with nitrite as an intermediate. This metabolic pathway is widely ubiquitous across different bacterial domains and includes genera such as *Klebsiella*, *Citrobacter*, *Escherichia*, and *Enterobacter* in *Proteobacteria*, *Ignavibacterium*, and *Draconibacterium*[209]. DNRA can proceed through two pathways, the first involving organic matter mineralization and the second involving sulfur oxidation[213,214], characterized by equations 1.5 and 1.6.

(1.5) $2NO_3^- + 2CH_2O + 2H^+ \rightarrow NH_4^+ + 2CO_2 + H_2O$

(1.6) $HS^- + NO_3^- + H^+ \rightarrow SO_4^- + NH_4^+$

The first step in DNRA involves the reduction of nitrate to nitrite via the nitrate reductase enzyme and the second step is the reduction of nitrite to ammonium via the nitrite reductase enzyme. DNRA are often considered undesirable in wastewater treatment systems because they retain nitrogen through conversion of nitrite to ammonia. However, some treatment systems have endeavored to incorporate DNRA with other nitrogen pathways including anammox and denitrification to remove nitrogen from wastewater[215].

Anammox: The anammox bacteria are phylogenetically classified into the phylum *Planctomycetes* and to date six "Candidatus" genera and 16 species have been identified[216,217]. Their overall metabolism can be described by Equation 1[218].

(1) $NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15}$

Equation 1 is the net sum of two reactions, ammonium oxidation coupled to nitrite oxidation for energy generation and the oxidation of nitrite coupled to the reduction of bicarbonate for biomass synthesis. The anammox are autotrophic and are capable of using carbon dioxide/bicarbonate as

their sole carbon source[219]. Some strains of anammox bacteria have also been suggested to utilize short fatty acids and formate as a carbon source. However, they primarily use the Wood-Ljungdhal pathway in order to catalyze the conversion of carbon dioxide to organic carbon via acetyl-coA[220–222]. Nitrite is both simultaneously reduced for the production of energy and oxidized to produce reducing equivalents for biosynthesis generating nitrate[223].

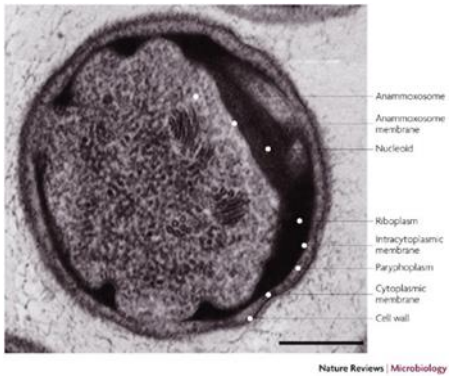


Figure 1.3: Cross section of anammox bacteria. Acquired from Kuenen (2008)

The conversion of ammonium and nitrite to nitrogen gas is executed in three consecutive redox reactions within the specialized subcellular organelle specific to anammox bacteria, the anammoxosome[224]. During this process two intermediates are generated, nitric oxide and the highly toxic hydrazine[223,225]. The four electron oxidation of hydrazine to nitrogen gas is facilitated using the hydrazine dehydrogenase enzyme which generates the necessary electrons for hydrazine production facilitated by hydrazine synthase through the condensation of ammonium and nitric oxide and reduction of nitrite using nitric oxide reductase[225]. Both the hydrazine dehydrogenase and hydrazine synthase genes are used to indicate the presence of anammox since both are specific to the anammox metabolism.

$$\textbf{(2)}\ N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$$

$$\textbf{(3)}\ NH_4^+ + NO + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O$$

$$\textbf{(4)}\ NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$$

Thus anammox enrichments often contain bacteria from three different nitrogen cycling pathways, anammox, denitrifiers, and bacteria capable of performing dissimilatory nitrate reduction to ammonium (DNRA)[226]. All three of these groups actively compete for nitrite for energy production.

1.4.2 Anammox for Wastewater Treatment

Anammox Reactors: The physiological capabilities of anammox bacteria make them an attractive option for the removal of nitrogen from concentrated side streams in municipal wastewater treatment plants. These side streams, which can contain 15-20% of total plant influent nitrogen, are an aggregation of supernatant liquid from anaerobic digesters treating sludge from primary and secondary treatment processes as well as centrate/filtrate return from anaerobically-digested sludge dewatering processes[227]. Full scale installations of anammox reactors have been demonstrated to remove up to 9.5 kg NH_4^+-N/L·d making them more than capable of removing ammonium in side streams that can have concentrations of up to 2500 mg NH_4^+-N/L[228,229].

In most applications of anammox for side stream nitrogen removal, it is coupled with partial nitritation facilitated through ammonia oxidizing bacteria (AOB)[229–231]. Partial nitritation provides the nitrite that is required by the anammox to oxidize ammonium. AOBs are aerobic chemolithoautotrophs that oxidize ammonium to nitrite using oxygen as an electron acceptor. They are carefully selected over aerobic nitrite oxidizing bacteria (NOBs) by optimized control of temperature, pH, dissolved oxygen concentrations, as well as solid and hydraulic retention times[230,232]. Application of partial nitritation-anammox systems (PN/A) can be implemented sequentially where the two processes are placed in separate reactors[229] or in a single stage reactor in which a mixed community of AOB and anammox coexists[233–235]. Single stage reactors carry several benefits over two stage primarily through the reduction of costs due to less equipment as well as environmental footprint. However, these systems can be difficult to maintain because of the physiological differences between AOBs and anammox. AOBs typically grow significantly faster than anammox which can lead to nitrite accumulation causing reactor instability due to nitrite's inhibitory effects on anammox in high concentrations (> 100 mg NO_2^-N/L)[238]. Oxygen supply to support AOB growth can also have deleterious effects on anammox performance due to their oxygen sensitivity as well as inadvertent growth of NOBs contributing nitrate to reactor effluent[239–241].

Many PN/A reactors try to leverage the propensity of anammox bacteria to form biofilms and aggregates to increase the solids retention time of the reactor to compensate for their slow growth[239–241]. This is done by utilizing reactor configurations including moving bed biofilm reactors (MBBRs), upflow anaerobic sludge blanket reactors (UASBs), and membrane bioreactors (MBRs) all of which stimulate the formation of biofilms and granules to retain biomass. These configurations also create a large pool of organic carbon that supports the growth of heterotrophic bacteria within the reactor.

Microbial Communities in Anammox Reactors:

Anammox reactors create conditions that select for a unique microbial community. Many of the bacteria within anammox enrichments utilize alternative nitrogen metabolisms such as denitrification and DNRA in order to gain energy. Both denitrifiers and DNRA bacteria utilize nitrate produced as a byproduct of the anammox reaction, but can also directly utilize nitrite which can help to reduce nitrite toxicity but also leads to competition with the reactor. However, the diversity of bacteria in anammox reactors is not limited to denitrifying and DNRA bacteria. Genomic studies have identified a core community or a group of bacteria that are consistently

present within anammox reactors. This implies that they play an essential ecophysiological role that supports the function and activity of anammox bacteria[242–244]. The phyla that co-exists most often with anammox include *Proteobacteria*, an incredibly diverse group of bacteria that spans a range of metabolic and physiological types, *Ignavibacteria*, which includes the order of obligately anaerobic non-photosynthetic bacteria *Ignavibacteriales*, *Chloroflexi*, a diverse group containing anoxygenic phototrophs, aerobic thermophiles, and anaerobic halorespirers, and *Bacteriodetes*, non spore-forming aerobic and anaerobic bacteria often found in gut microbiomes. All of these bacteria form a variety of mutualistic, competitive, and symbiotic relationships with the anammox and other core community members[246,249,250].

Metabolic Interactions and Carbon Cycling

Heterotrophs form a multitude of complex metabolic exchanges that can be beneficial for anammox bacteria but simultaneously sustains subpopulations that can opportunistically destabilize reactor performance[249,251]. These relationships are constrained and enhanced by the availability of labile organic carbon[252–254]. In anammox-based treatment systems, the presence of labile organic carbon is often limited, making carbon the growth limiting factor for bacteria inside of the reactor. Due to this fact, the anammox often serve as the primary source of organic carbon for heterotrophic bacteria within the system. Thus, the majority of the carbon utilized by heterotrophic bacteria would either consist of extracellular polymeric substances produced by the anammox, soluble microbial products excreted through anabolic processes, and decaying biomass from cell death. This would suggest that the bacteria within anammox systems have substantial metabolic diversity for carbon acquisition strategies. While the anammox bacteria supply carbon to other heterotrophic bacteria, they are also likely dependent on others for the biosynthesis of certain vitamins, co-factors, and secondary metabolites such as Molybdopterin Cofactor (MOCO) and folate (vitamin B12) both of which most anammox strains lack the biosynthetic pathways for. Given the various metabolic interdependencies, anammox growth as well as the overall community stability is heavily dependent on a delicate network of materials exchanges and metabolic handoffs. However, the strategies that bacteria deploy to acquire, utilize, and cycle carbon and how they interplay with one another is at the center of this network.

Extracellular polymeric substances secreted by anammox bacteria are used to create an adhesive mechanism to induce the growth of aggregates and granules[252–254]. EPS is a complex matrix comprised of different macromolecules which can vary widely in composition depending on the bacterial species, growth strategy, and reactor type[259,260]. In anammox reactors, the EPS consists largely of proteins, polysaccharides, humic substances, DNA, and lipids. The EPS within anammox granules has been found to contain a large amount of hydrophobic side chains which are thought to form hydrophobic interactions with surface functional groups on cell membranes to promote aggregation[259,260]. The EPS also serves as a large reservoir of organic carbon for heterotrophic bacteria within the reactor. Genomic studies have identified bacteria in anammox reactors that have a multitude of extracellular peptidases that could be used to cleave long chain peptides[262]. Bacteria with hydrolytic enzymes could liberate macromolecules from the bulk matrix making smaller oligopeptides and oligosaccharides for other community members. Thus, the balance of activity between the anammox and heterotrophs is important because controlled

stimulation of certain bacteria through EPS secretion could support robust community function while overgrowth of bacteria degrading EPS could lead to destabilization of the matrix and disaggregation.

Motivation

Anammox reactors have displayed immense potential to remove nitrogen from wastewater side-streams with a lower carbon and energy footprint than other biological nitrogen removal systems. However, they are plagued by long start up periods, reactor instability, and performance crashes that can take months for recovery. All of these disadvantages are compounded by gaps in the understanding of anammox physiology, biochemistry, and the microbial ecology of anammox reactor systems especially as it pertains to heterotrophic bacteria. The metabolic interdependencies that support anammox growth and reactor stability have yet to be fully elucidated and have only been partially inferred from genomic information. Developing a robust understanding of the microbial networks that regulate community diversity, function, and stability is critical to designing, building, and operating more robust treatment systems.

In order to understand the metabolic networks that promote increased anammox activity and the long-term stability of the reactor, the microbial interactions of the anammox community must be ascertained, especially as they relate to carbon dynamics. A robust understanding of the carbon cycling and materials exchanges within the reactor will be able to contextualize information derived from genomic studies and to inform strategies that encourage the growth of bacteria advantageous for reactor performance and resiliency. Furthermore, it is necessary to condense high dimensional genomic data into modalities that can be applied to construct comprehensive frameworks that can accurately conceptualize the system. This is a critical step in being able to model the behavior of anammox systems and to predict how the microbial community responds to specific conditions. Robust predictive modeling capabilities would further our ability to maintain anammox reactors and to predict microbially provoked instabilities before they actually occur.

Dissertation overview

This dissertation seeks to characterize microbial interactions in anammox reactor systems. The goal of this work is to develop a comprehensive understanding of the various interactions that occur between the anammox and other heterotrophic bacteria as well as the dynamics between different groups of heterotrophs. The culmination of this work is the development of models that can accurately represent the microbial dynamics of the system and provide insights for predictive models. The remainder of this dissertation is organized into four chapters, three of which detailing experimental investigations into anammox treatment systems and the fourth examines modeling approaches using genomic data, followed by a conclusion chapter summarizing the current work and illuminating future directions.

Chapter 2 examines the response of a lab scale anammox membrane bioreactor to changes in the influent nitrogen loading ratio. A reactor time series experiment was conducted using shotgun metagenomic and 16S rRNA data that tracks changes in the microbial community composition

over time. Trends in the metabolic and phylogenetic composition of the community are investigated and analysis into the factors driving such trends is pursued.

Chapter 3 investigates the response of a lab scale anammox membrane bioreactor to an induced performance crash through adjustment of the solids retention time (SRT). The performance of the bioreactor is monitored and shotgun metagenomic sequencing and 16S rRNA sequencing is conducted to elucidate changes in the microbial community. Analysis into the drivers of community change and its implications on microbial ecology is explored.

Chapter 4 seeks to elucidate the microbial degradation of EPS produced by anammox bacteria. Through interpretation of EPS chemical characterization data and heterotrophic batch enrichment experiments using EPS as a carbon source, the capability of heterotrophic bacteria within anammox reactors to degrade anammox EPS is investigated. The implications of this analysis on the microbial ecology of anammox systems is then expounded upon.

Chapter 5 attempts to develop a comprehensive reaction network to represent the microbial ecology of anammox systems. Trait-based modeling using genomic information and reactive transport modeling is utilized to model and predict microbial behavior. The inferences drawn from these models is then used to outline a generalizable framework to describe anammox reactors.

Chapter 2: Synergistic Interactions between Anammox and dissimilatory nitrate reducing bacteria sustains reactor performance across variable nitrogen loading ratios

This chapter is largely adapted from the manuscript, White et al., "Synergistic Interactions between Anammox and dissimilatory nitrate reducing bacteria sustains reactor performance across variable nitrogen loading ratios." *Frontiers in Microbiology*, 2023, 1243410. DOI: https://doi.org/10.3389/fmicb.2023.1243410[11,227,263].

2.1 Introduction

Nitrogen removal from wastewater is paramount to the remediation of anthropogenic nutrient pollution and the protection of sensitive aquatic environments[264,265]. One strategy to remove nitrogen from wastewater is anaerobic ammonium oxidation (anammox), in which ammonium (NH_4^+) is anaerobically oxidized using nitrite (NO_2^-) as an electron acceptor[239,264,265]. Anammox is a biogeochemical process facilitated by chemolithoautotrophic bacteria in the Phylum Planctomycetes[266,267] and is estimated to account for up to 70% of fixed nitrogen removal in marine environments[268,269]. The anammox process has shown great potential for robust side-stream nitrogen removal, with full scale installations demonstrating nitrogen removal efficiencies up to 90% and ammonium removal rates up to 9.5 kg NH_4^+-N/ L·d[270–272] while using 60% less energy and producing 90% less sludge than conventional nitrification-denitrification systems[270,273,274]. Despite these benefits, anammox-based water treatment faces multiple challenges including long start up periods (6 months-2 years)[275–277] and process instability due to inhibitory compounds[278,279], operational fluctuations, and microbially-provoked destabilizations[280]. Thus, prior anammox research has sought to better-understand microbial community dynamics in order to design, maintain, and operate more resilient reactors. Anammox reactors harbor phylogenetically and functionally diverse bacteria that engage in a variety of synergistic, competitive, and mutualistic interactions[261,281–283]. The presence of a core microbial community identified alongside anammox bacteria—which have never been isolated in pure culture—suggests an ecological niche specific to the conditions found inside of the reactor. Previous research consistently identifies bacteria belonging to the phyla Proteobacteria, Chloroflexi, Ignavibacteria, and Bacteroidetes as part of this core community[243,249,251,281,282]. Many of the interactions occurring among different anammox community members are predicated on an exchange of organic carbon substrates, secondary metabolites, and various nitrogen species[243,249,251,284,285]. These complex relationships are key to improving nitrogen removal efficiency and sustaining microbial growth. For example, heterotrophic bacteria performing nitrate reduction through *nar* or *nap* nitrate reductases can reduce nitrate (NO_3^-) to nitrite, providing a substrate for anammox; anammox bacteria then produce organic carbon that feeds heterotrophic partners, forming a nitrite loop. However, other nitrogen metabolisms can disrupt this loop. Bacteria performing dissimilatory nitrate reduction to

ammonium (DNRA) with *nrfA* or *nrfH* cytochrome nitrite reductases convert nitrite back to ammonium, forming antagonistic relationships with anammox bacteria that can disrupt reactor performance[286–288]. Heterotrophs performing DNRA actively compete with denitrifiers for organic carbon substrates and nitrite, a competition that has been shown to depend on C/N ratio, carbon source availability, and hydraulic retention time[289–291].

The competition between bacteria performing DNRA and denitrification can also be altered by influent resource concentrations[292]. In full scale systems, anammox reactors are coupled with a partial nitritation reactor that provides a nitrite source, generally resulting in a nitrogen influent stream of approximately 50% ammonium (NH_4^+) and 50% nitrite (NO_2^-), or a NH_4^+:NO_2^- ratio of 1:1[290]. However, the theoretical stoichiometric ratio of ammonium to nitrite in anammox systems is 1:1.146 [293]. Because of the competition between denitrifying bacteria and DNRA for nitrite, the NH_4^+:NO_2^- ratio needed within an anammox reactor tends to be higher. Highly enriched anammox cultures remove ammonium and nitrite at a ratio between 1:1.2 and 1:1.32[294]. Any divergence from this ratio can lead to poor reactor performance, or in extreme cases, reactor crashes. While changes to reactor performance during ratio changes has been well documented, the underlying changes to the microbial community that drive performance changes are poorly understood.

Given that full-scale anammox reactors are susceptible to performance destabilization due to variable nitrogen loading[295], it is important to evaluate the effects of fluctuating nitrogen species ratios on the complex network of metabolic interdependencies between anammox, DNRA, and denitrification bacteria. Here we constrain the microbial community response to perturbations induced by influent nitrogen loading ratio changes in a lab scale anammox reactor. We evaluate the effects of variable nitrogen loading ratios on reactor performance, anammox activity, and microbial community dynamics; we also assess the changes in microbial interactions as a result of changing influent conditions using 16s rRNA amplicon sequencing and shotgun metagenomic sequencing analysis. The results provide insight into the competitive and synergistic relationships between bacteria employing different nitrogen metabolisms, and how these complex relationships affect reactor performance, stability, and resiliency.

2.2 Materials and Methods:

2.2.1 Bioreactor Operation

A 1 L anaerobic membrane bioreactor (MBR) was operated for one year prior to the experiment. The reactor was enriched for anammox bacteria from anaerobic digester solids. The specific details of initial inoculation and operation can be found in[293]. A polyvinylidene fluoride membrane with a pore size of 0.22 µm was mounted to the inside of the reactor and a gas mix (Argon:CO_2=95:5; 50 ml/min) was continuously supplied to purge the system of oxygen and maintain circumneutral pH (6.9-7.2) (Figure 3, Appendix 2). Temperature was maintained at 37 °C using a heating jacket (Eppendorf, Hauppauge, NY) and mixing was provided through an impeller at a rate of 200 rpm. A synthetic media containing ammonium, nitrite, bicarbonate, and trace nutrients prepared anaerobically under nitrogen was continuously fed to the reactor; the exact composition can be found in Table 1, Appendix 1. Influent and effluent samples were collected every other day to monitor concentrations of ammonium, nitrite, and nitrate using HACH test kits (HACH, Loveland, CO), as described in the manufacturer's methods 10031, 10019, and 10020, respectively. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured according to standard methods[296].

The experiment took place over 252 days during which the hydraulic retention time (HRT) of the reactor was maintained at 12 hours (via an effluent pump) and the solids retention time (SRT) was maintained at 50 days (via biomass wasting). For the first 88 days of the experiment, $NH_4^+$and NO_2^-were loaded at the conventional 1:1.32 ratio at concentrations of 500 mg-N/L and 660 mg-N/L respectively. On day 89, the influent ammonium concentration was raised to 600 mg-N/L, decreasing the NH_4^+:NO_2^-ratio to 1:1.1. On day 169, the influent nitrite concentration was raised to 680 mg-N/L, increasing the NH_4^+:NO_2^- ratio to 1:1.13. From day 200 to 252, the total nitrogen loading was slowly increased while also increasing the NH_4^+:NO_2 ratio from 1:1.13 to 1:1.2 as shown in Table 2.1. This range of NH_4^+:NO_2^- ratios was selected for the experiment in order to maintain stable operation of the reactor, as lower ratios (< 1:1) have been demonstrated to lead to the accumulation of free ammonia (FA) [297] and higher ratios (>1:1.3) have been shown to lead to nitrite inhibition [298]. The dominant anammox strain in our MBR (*Brocadia sinica*) has also been shown to have higher sensitivity to nitrite inhibition than other strains such as *Kuenenia* [299]. Thus the influent nitrogen ratios tested in this experiment were selected to avoid these issues.

Day	Influent Ammonium (mg-N/L)	Influent Nitrite (mg-N/L)	NH_4^+:NO_2^- Ratio	Nitrogen Loading Rate (g-N/L/d)
0	500	660	1.32	2.32
89	600	660	1.1	2.52
169	600	680	1.13	2.52
200	600	700	1.17	2.6
217	600	710	1.18	2.62
218	600	720	1.2	2.64
235	640	768	1.2	2.82
243	660	792	1.2	2.9
250	680	816	1.2	2.99

Table 2.1: Influent nitrogen loading and NH_4^+:NO_2^- ratio data

2.2.2 DNA Extraction

Biomass samples were collected every two to ten days via syringe through an extraction port, flash frozen in liquid nitrogen, and stored at -80 °C until further use. Genomic DNA was extracted from the samples using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA) as described in the manufacturer's protocol. DNA quality was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). DNA was quantified using a Qubit fluorometer (Thermofisher Scientific, Walthan, MA), diluted to 10 ng/ µL with nuclease free water (Thermo Scientific, Waltham, MA), and stored at -20 °C until further use. Shotgun metagenomic sequencing samples were sent to the Joint Genome Institute (JGI) in Walnut Creek, CA. There, DNA was sequenced (150 bp paired-end) on an Illumina HiSeq 2500 1T sequencer (Ilumina, San Diego, CA). 16S rRNA sequencing for samples collected from day 1 to 45 were sequenced at the Institute for Environmental Genomics at the University of Oklahoma and the remaining samples were sequenced at JGI on an Illumina MiSeq sequencer (Illumina, San Diego, CA).

2.2.3 16s rRNA gene analysis

The microbial community composition was evaluated by 16s ribosomal RNA sequencing of 28 DNA samples collected throughout the experiment. The V4 region was amplified using primers 515F (5'-GTGCCAGCMGCCGCGG-3′) and 806R (3′-TAATCTWTGGVHCATCAG-5′), with barcodes attached to the reverse primer. Amplicons were pooled at equal molarity and purified with the QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD). Paired-end sequencing (250 bp paired-end) was then performed on the Illumina MiSeq sequencer (Illumina, San Diego, CA). The full protocol is provided by[300]. Sequence processing and data analysis was conducted using MOTHUR v.1.39.5, following the MiSeq Standard Operating Procedure (SOP)[301], and OTUs were assigned based on a 97% sequence similarity threshold.

2.2.4 Metagenomic sequencing, assembly, and binning

Three DNA samples were used for metagenomic sequencing, two from single timepoints on day 37 and 140 and one bulked from samples taken on days 232, 235, and 237. Resulting sequences from each time point were processed separately according to the procedure previously reported in (Keren et al. 2020). KEGG Automated Annotation Service (KAAS) was used to annotate predicted gene sequences using Hidden Markov Models (HMMs). Single time point genome abundances were calculated using reads per kilobase per million (RPKM). The log ratio change for each genome was calculated based on the procedure previously reported in (Keren et al. 2020). Briefly, three genomes with stable coverage across the three time points were selected as reference frame genomes. The coverage of each genome was then divided by the coverage of the three reference frame genomes. These ratios were then used to calculate the log ratio changes between samples yielding three values, one for each reference frame genome. This was done in order to account/adjust for differences in sequencing depth between samples, which can otherwise lead to biased results. Relative replication rates were calculated using iRep[302] Briefly, the replication rates of bacteria can be estimated by calculating the coverage ratio between the origin of replication and the terminus of replication. In a population that is not actively replicating, the coverage at the ratio and terminus will be the same, and the ratio will be one. For populations that are actively replicating the coverage will be greater around the origin of replication because of replication forks that have not finished replicating. Thus, the higher this ratio the higher the proportion of the population that is actively replicating.

2.2.5 Statistical Analysis

Principal coordinate analysis (PCA) was applied to evaluate the correlation between taxa abundance for different pathways (anammox, denitrification, and DNRA) and reactor performance parameters. In order to assign pathways to specific taxa, 16s rRNA sequences obtained from metagenomes with genes encoding for each pathway were aligned to representative sequences from amplicon sequencing following the procedure described in (Keren et al. 2020). For MAGs not containing 16s rRNA sequences but classified down to the species level, sequences were obtained from NCBI. PCA analysis was conducted using R (https://www.r-project.org/) with the "factoextra" package in RStudio (https://www.rstudio.com/).

2.3 Results

2.3.1 Bioreactor Performance

Under the initial NH4+:NO2- ratio of 1:1.32, the average ammonium and nitrite removal efficiencies were 99.77±0.04 % and 96.85±2.31% respectively (Table 2.2), and the average NRR was 1.98±0.03 g·N L-1d-1. This resulted in a nitrate production rate of 0.075±0.002 g·N L-1d-1 and a NO3-:NH4+ ratio of 0.298±0.0007:1, which is slightly above the conventional ratio of 0.26:1300. Following the influent ratio shift on day 89, the average ammonium concentration in the effluent increased from 1.14±0.22 mg-N/L to 55.19±1.68 mg-N/L, which was expected due to the increased NH4+ in the influent. Of the 100 mg-N/L NH4+ added to the influent, about half was emitted in the effluent, while the remaining ammonium was likely removed through the anammox reaction or was assimilated for biomass synthesis.

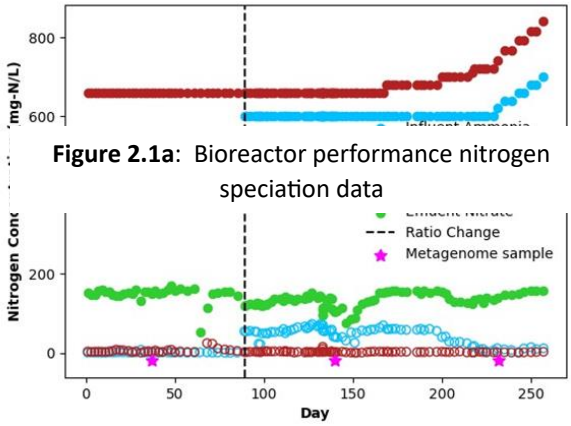


Figure 2.1a: Bioreactor performance nitrogen speciation data

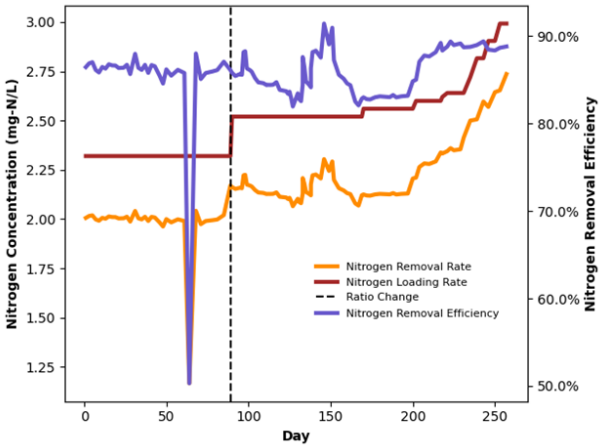
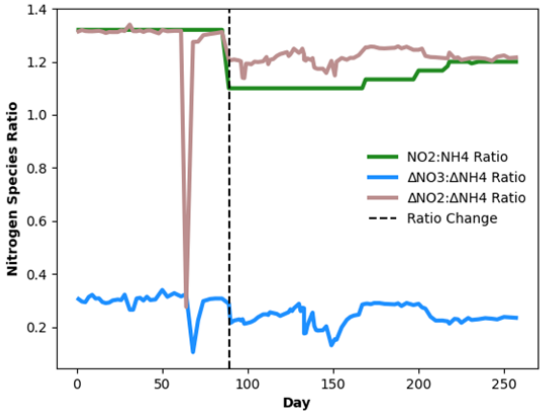


Figure 2.1b: Bioreactor performance during the experiment in terms of NRR, NRR, NRE



After increasing the NO2- influent concentration, the NLR was steadily increased from 2.60 gN/L/d on day 200 to 2.99 gN/L/d on day 250 (Table 2.1). From day 89 to day 202 the average ammonium removal efficiency fell to 90.74±0.25 % and the nitrite removal efficiency increased to 99.57±0.04 %; the NRR increased to 2.16±0.01 g·N L-1d-1. This resulted in a nitrate production rate of 0.066±0.002 g·N L-1d-1 and a NO3-:NH4+ ratio of 0.24±0.005:1. From day

202 to day 253, ammonium removal efficiency increased back to 97.28±0.45 %, and the NO3-:NH4+ ratio decreased again to 0.237±0.005:1.

Day	NH_4^+ Removal Efficiency	NO_2^- Removal efficiency	Nitrogen removal efficiency	Nitrogen removal rate (g N/L-d)	Nitrate production rate (g N/L-d)	ΔNO3-:ΔNH4+
0-89	99.77±0.04%	96.85±2.34%	85.26±0.01%	1.98±0.03	0.08±0.01	0.30±0.01
89-169	90.85±0.29%	99.56±0.05%	85.49±0.01%	2.15±0.01	0.06±0.01	0.23±0.01
169-200	90.14±0.09%	99.65±0.02%	83.04±0.01%	2.13±0.01	0.08±0.01	0.29±0.01
200-252	97.28±0.45%	99.76±0.03%	88.21±0.01%	2.37±0.03	0.07±0.01	0.24±0.05

Table 2.2: MBR performance and effluent data

2.3.2 Microbial Community in MBR through 16s Amplicon Sequencing

16s rRNA amplicon sequencing was conducted to ascertain changes in abundance of taxonomic groups throughout the reactor lifecycle. Figure 2.2 shows the relative abundance of 16s rRNA genes at the phylum level. At the beginning of the experiment, the dominant phylum in the reactor was Planctomycetes, accounting for 77.19% of total reads. Other significant phyla included Chloroflexi, Ignavibacteria, and Proteobacteria accounting for 7.73%, 4.57%, and 8.67% of total reads respectively, which is consistent with previously reported results (Pereira et al. 2017). Of the reads belonging to Chloroflexi, over 99% belonged to the class Anaerolineae and of the reads belonging to Proteobacteria, 50.4% belonged to the class Alphaproteobacteria. Over the course of the experiment, the relative abundance of Planctomycetes decreased from 77.19% to 12.24%. Meanwhile, the relative abundance of Chloroflexi and Ignavibacteria increased from 7.73% and 4.57% to 23.36% and 38.22%, respectively. The relative abundance of the phylum *Bacteroidetes* also increased from <0.05% at the beginning of the experiment to 7.30% by the end. The Shannon, Simpson, and Chao indices were calculated using relative abundance data from sequenced amplicons, which indicated that the microbial diversity of the community increased over the course of the experiment. Further information on diversity calculations and results can be found in Table 3, Appendix 2.

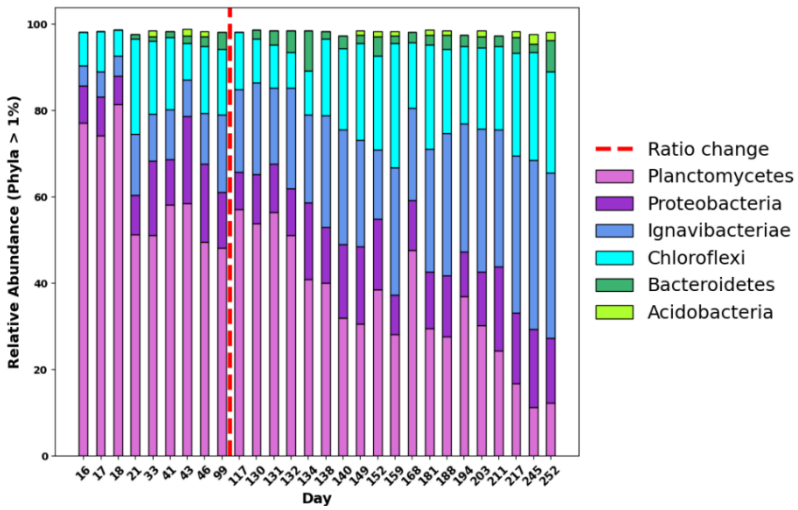


Figure 2.2: Changes in relative abundance at the phylum level of 16s rRNA amplicon sequences over time from day 16 of the experiment to day 252.

The abundance of taxonomic groups was also aggregated at the genus level (Figure 2.3). These results are consistent with relative abundances at the phylum level, demonstrating high abundance genera in Chloroflexi, Ignavibacteria, Proteobacteria, and Bacteroidetes phyla. Several genera associated with the families Anaerolineaceae, Rhodocylacea, Burkholderiaceae and the order Ignavibacteriales were consistently abundant throughout the experiment which was consistent with previously reported results[303–306]. Several of the genera from Anaerolineaceae and Ignavibacteriales increased by at least one order of magnitude post-ratio change. Multiple genera from Rhodospiralles, Flavobacteriales, Sphingobacteriales, and Chitinophagales that were previously undetected or low abundance increased by at least two orders of magnitude post-ratio change.

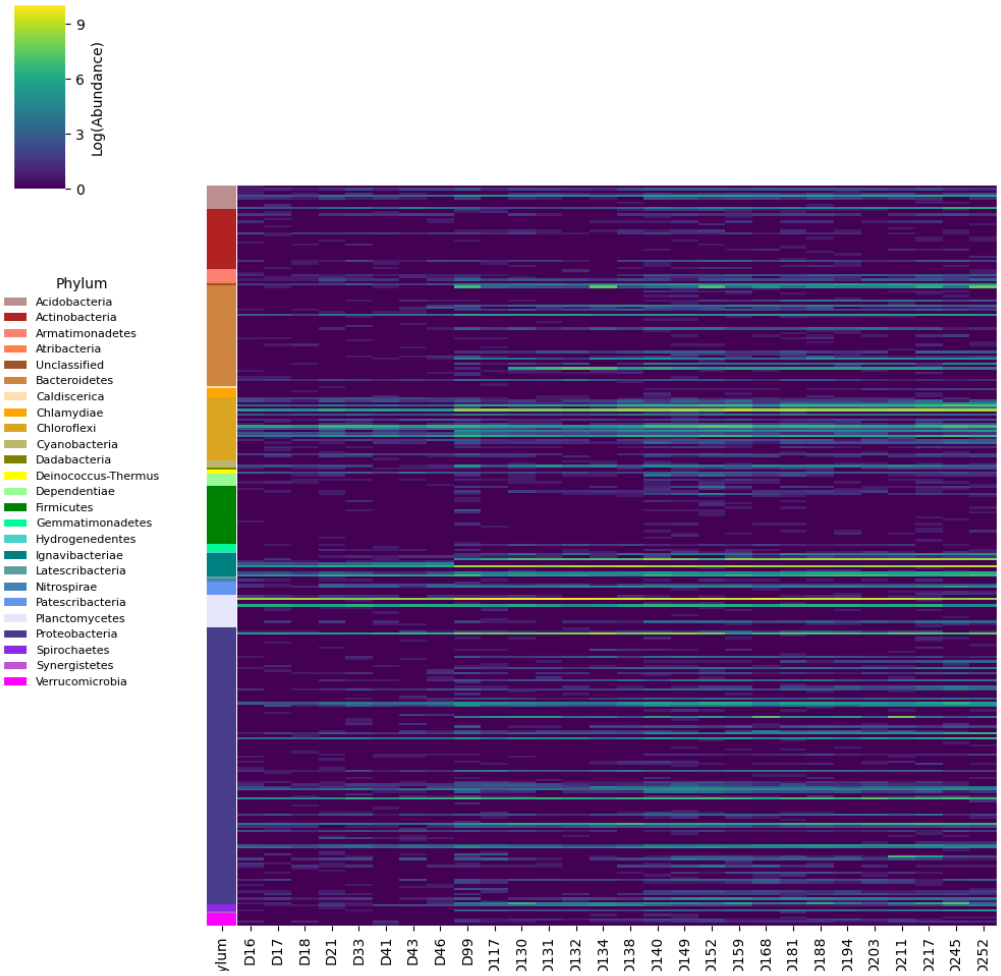


Figure 2.3: Changes in logarithmic abundances of 16s rRNA amplicon reads aggregated at the genus level over time from day 16 of the experiment to day 252.

2.3.3 Metagenome Sequencing Results

After read quality control 342,892,526 reads were obtained from all three metagenome samples. Reads were assembled into Metagenome Assembled Genomes (MAG), resulting in 27,836 contigs with a median N50 of 2138 bp. Contigs were binned to draft genomes resulting in 129 draft genomes accounting for 74.62% of quality filtered reads on average across all three samples (Table 4, Appendix 2). These MAGs represented twenty bacterial phyla and one archaeal phylum, as shown in Figure 2.4. The most abundant genomes based on coverage were affiliated with Planctomycetes, Proteobacteria, Ignavibacteriae, Chloroflexi, and the super-phylum Candidate Phyla Radiation (CPR). A full list of genomes is available in Table 5, Appendix 2. AMX1 (*Brocadia sinica*), the only anammox MAG recovered, accounted for 44.97% of all reads on average across all three metagenome samples. AMX1 had an average GC content of 42.29%, a genome size of 3.1 Mbp, and 2859 open reading frames (ORFs) which was consistent with other previously reported anammox genomes for this strain[307,308].

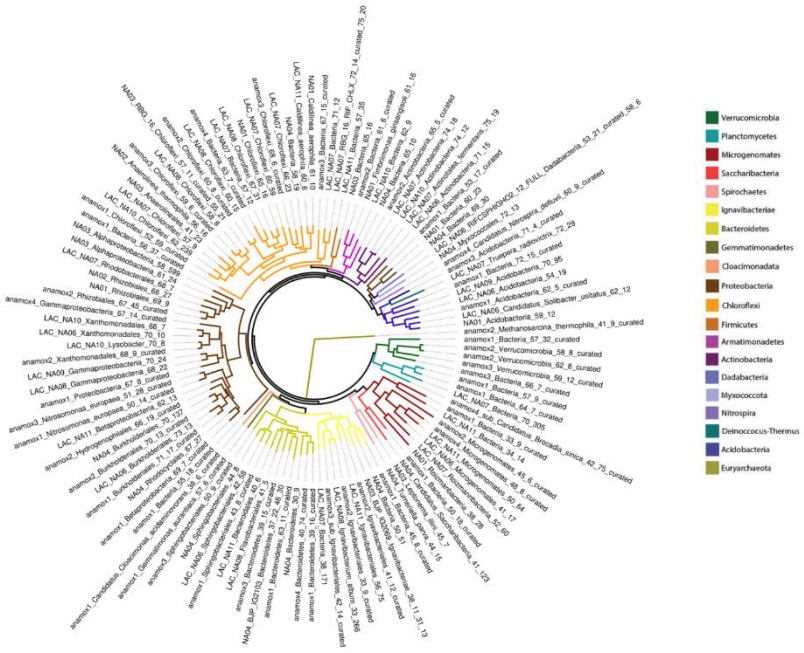


Figure 2.4: Maximum likelihood phylogenetic tree from concatenated ribosomal proteins of three metagenomic samples taken during the experiment

2.3.4 Nitrogen Cycle Gene Abundances

To assess the changes in abundance of nitrogen metabolic genes, the relative abundance of these genes was calculated for each metagenomic sampling timepoint (Figure 2.5). The metabolic genes with the highest abundance throughout the duration of the experiment included hydrazine dehydrogenase (*hdh*), hydrazine synthase (*hzsA/B/C*), hydroxylamine oxidoreductase (*hao*), respiratory nitrate reductase (*narGH*), nitrous oxide reductase (*nosZ*), and cytochrome c membrane associated nitrite reductases (*nrfAH*) with relative abundances ranging from 4.3% to 32.3%. For the purposes of this study, we define DNRA as the one-step reaction of nitrite to ammonium facilitated by *nrfAH* genes, and denitrification as the conversion of nitrite to nitrogen gas via nitric oxide and nitrous oxide facilitated by nitrite reductases *nirKS*. The abundance of functional analogs *nirK* and *nirS*, both used for nitrite reduction to nitric oxide, was only 0.9%

and 0.1% respectively; however, the relative abundance of respiratory nitrate reductases *narGH* was 30.99% on average, indicating the wide prevalence of nitrate reduction capacity. The nitrogen gene relative abundances for samples from day 37 and 140 were nearly identical; by day 232, the relative abundance of *nrfAH* nitrite reductases and *narGH* nitrate reductases increased slightly and the relative abundance of anammox associated hydrazine synthase and hydrazine dehydrogenase decreased slightly (Figure 2.5). The taxonomic variability and relatively stable metabolic composition suggest high levels of functional redundancy amongst bacteria in the reactor.

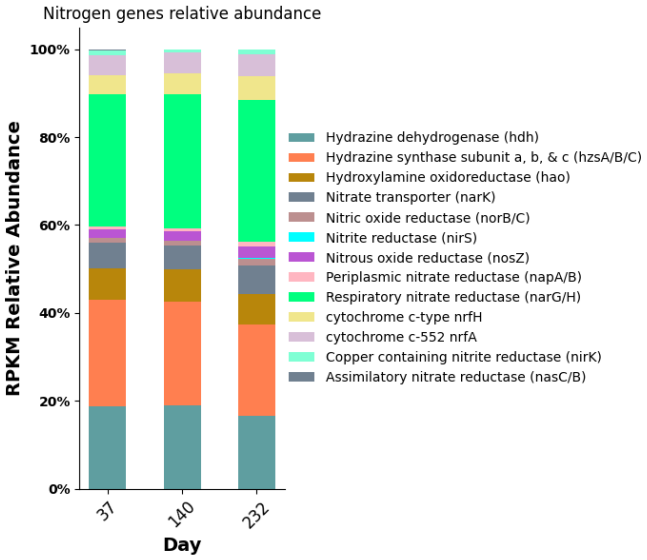


Figure 2.5: Nitrogen gene relative abundances from metagenomic sequencing from three time points during the experiment

To understand how different nitrogen metabolisms influenced microbial competition throughout the duration of the experiment, the abundance of key individual MAGs was calculated for each timepoint as shown in Figure 2.6. *narGH* nitrate reductases were the most abundant of any nitrogen metabolic gene and widely distributed amongst MAGs. The next most abundant nitrogen metabolism gene identified in key MAGs was *nrfAH*. The top 5 most abundant MAGs including AMX01 (*Brocadia*), IGN01 (*Ignavibacterium*), CFLX01 (Chloroflexi), PROT01 (Burkholderiales), and BAC01 all contained *narGH.* AMX01, CFLX01, and IGN01 also contained *nrfAH*. The abundance of AMX01 decreased from 9.67 RPKM on day 37 to 7.81 RPKM on day 232, while the abundances of CFLX01 and IGN01 increased from 14.11 and 9.18 RPKM on day 37 to 23.02 and 15.98 RPKM on day 232. Many of the MAGs that encoded for *narGH* also lacked the remaining genes necessary for the full denitrification pathway, which could result in the accumulation of nitrite. This is consistent with previously reported results[287] supporting the presence of a nitrite loop, in which nitrate is recycled back to anammox bacteria via partial denitrification of nitrate to nitrite[306]. Many genomes encoding for DNRA also encode genes for partial denitrification but for the purposes of our analysis, we assume these bacteria will carry out DNRA as the most energetically favorable pathway.

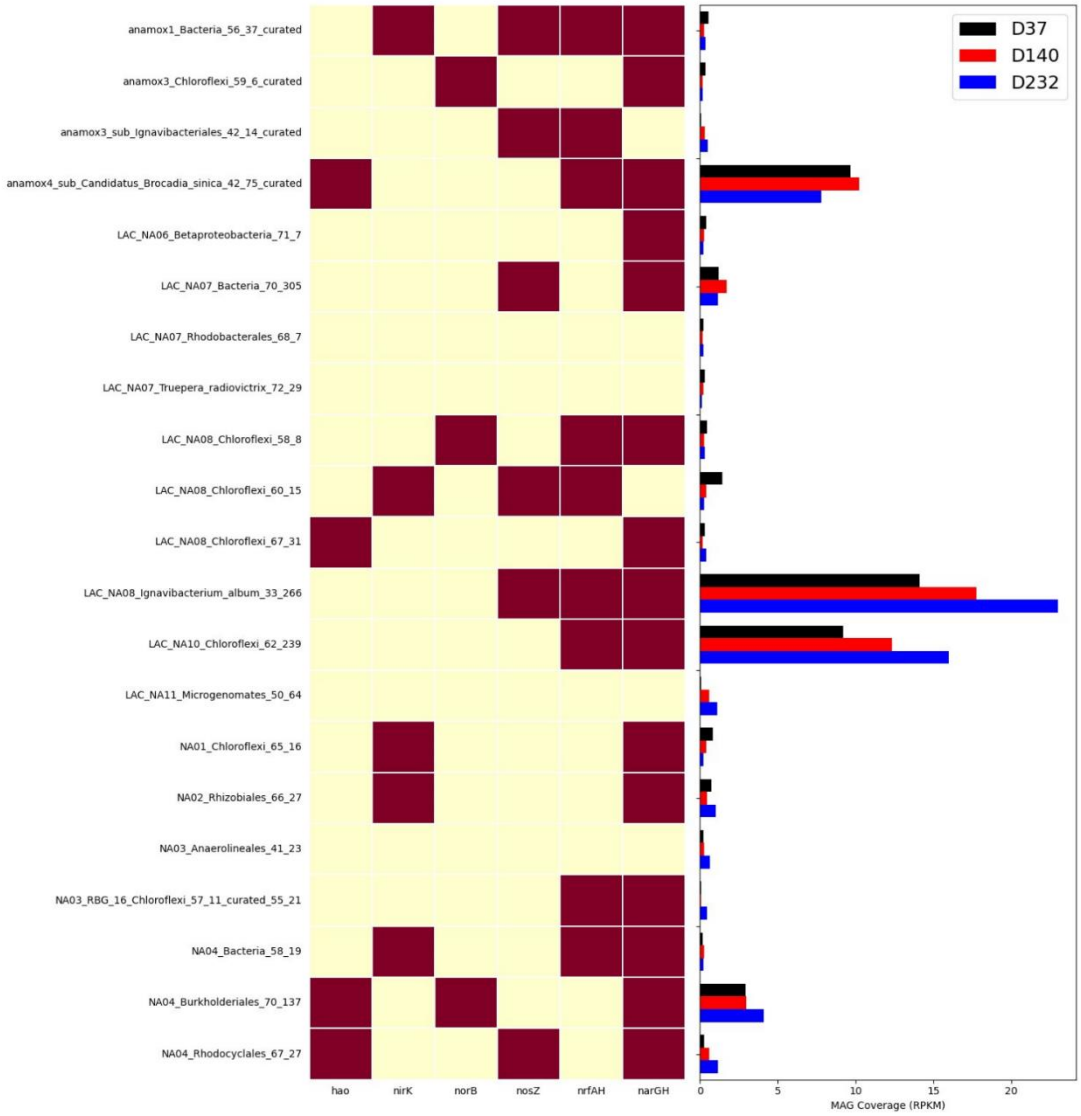


Figure 2.6: Heatmap showing presence of nitrogen genes in MAGs and abundance of MAGs from three timepoints with metagenome samples measured as RPKM

Nitrogen transport and sensing genes, which can be used to infer substrate affinity, were identified in the top 14 most abundant MAGs by coverage (Figure 2.7), and all MAGs with genome coverage above 40% (Figure 5, Appendix 2). This analysis was performed to assess how effectively different organisms compete for various nitrogen substrates. Nitrate, a byproduct of anammox biosynthesis, is frequently abundant in anammox reactors, while nitrite is kept limiting to prevent nitrite toxicity. Therefore, the bacteria with the greatest affinity for nitrite will have a competitive advantage. Most genomes contained transporters belonging to the Nitrate/Nitrite Porter (NNP) family, including *narK*, *nrtP*, and *nasA* for nitrate and nitrite membrane transport[309]. Consistent with previously reported anammox metagenomes[310,311], AMX01 contained Nitrate Transporter superfamily (NRT) nitrate/nitrite transporters, the high

affinity nitrate/nitrite transporter *nrtB*, and a polytopic membrane transporter specifically for nitrite, *nirC*. Several genomes, excluding those of anammox bacteria, also encoded genes for putative formate-nitrite transporters from the formate-nitrate transporter (FNT) superfamily, including formate channel *focA* and formate permease *fdhC*. Previous research has shown that some denitrifying bacteria have a higher affinity for nitrite than nitrate[312]. This was supported by the presence of FNT transporters in a higher proportion of MAGs without *nrfAH* than those with these nitrate-metabolizing genes. The majority of genomes also encoded nitrate/nitrite sensor protein complexes *narX/L* and *nar Q/P*. These genes, which are often colocated on the *nar* operon, have been demonstrated to regulate expression of nitrate/nitrite reductases as well as other respiratory proteins[313].

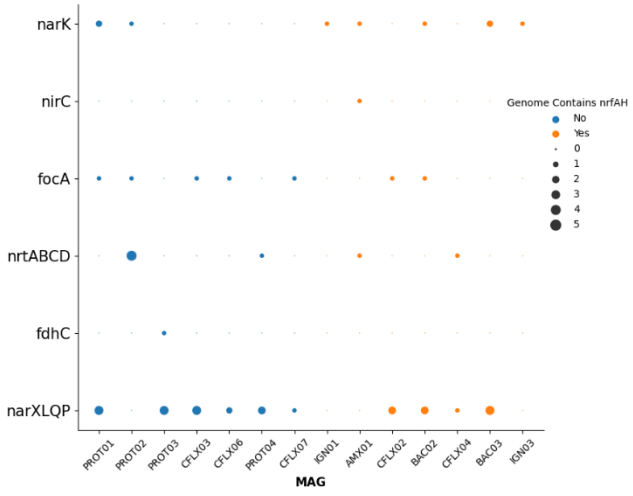


Figure 2.7: Putative nitrate/nitrite transporters and sensors encoded in MAGs

2.3.5 Microbial Community Dynamics

Log-ratio (LR) changes between day 232 and day 37 were calculated using each MAG's coverage normalized to three reference genomes (Figure 2.8a). Of the 50 MAGs that experienced LR changes above the upper 95% confidence interval (CI) (Table 7, Appendix 2), 25 had RPKM abundances lower than the 95% CI (Table 9, Appendix 2) and 32 contained *narGH*, 15 contained *nirKS*, and 17 contained *nrfAH*. Of the 57 MAGs that experienced LR changes below the lower 95% CI, 36 had RPKM abundances lower than the lower 95% CI, and 35 contained *narGH*, 21 contained *nirKS*, and 13 contained *nrfAH*. Significant log-ratio changes in coverage did not have a strong correlation with taxonomy (Table 6, Appendix 2).

iRep values, which estimate each MAG's replication rate, ranged between 1.12 and 2.29 (Figure 2.8b). As expected, *Brocadia* had the lowest replication rates during the loading ratio experiment at all three time points with an average of 1.14. CFLX13 (Chloroflexi) had the highest replication rates with an average of 2.15. PROT03 (Rhodocyclales) experienced the most substantial decrease in replication rates during the course of the experiment, falling from 1.85 at day 37 to 1.43 at day 232 (a 23% decrease); CPR01 experienced the most significant

increase, from 1.39 on day 37 to 2.15 on day 232 (a 55% increase). With the exception of CPR01, all of the MAGs that demonstrated increased replication rates between time points had negative log-ratio changes between day 37 and 232. This observation could be an indication of higher mortality rates suggestive of r-type strategists, or organisms well adapted to optimize growth during unstable environmental conditions. These types of organisms have shorter life cycles but very high reproduction rates associated with low efficiency substrate utilization[314–316]..

2.3.6 Taxon Abundance and Reactor Operational Parameters

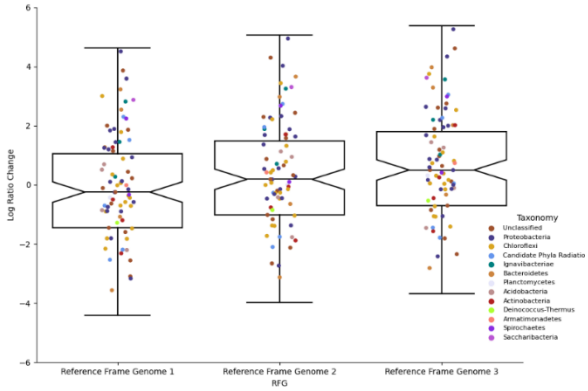


Figure 2.8a: Log ratio changes between D37 and D232 using different genomes as reference frames

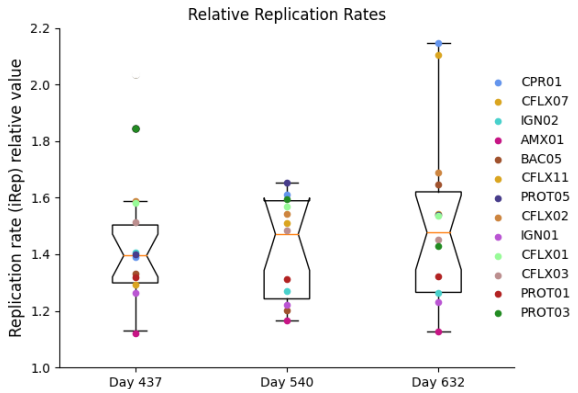


Figure 2.8b: Replication rate values on D37, D140, and D232

To evaluate the effects of different nitrogen pathways on reactor performance, PCA analysis was conducted for the abundance of anammox, denitrifying, and DNRA associated taxa (Figure 2.9). The abundance of anammox bacteria is positively correlated with ammonium removal efficiency and the nitrate production rate, indicating the anammox bacteria are primarily responsible for ammonium removal and nitrate production. The abundance of DNRA and denitrifying bacteria is positively correlated with nitrogen removal efficiency, nitrite removal efficiency, and the nitrogen removal rate, and negatively correlated with the ammonium removal efficiency, nitrate production rate, and effluent nitrite concentrations. However, the abundance of DNRA bacteria is more strongly associated with nitrite removal efficiency than denitrification. These results are consistent with the performance of the MBR throughout the course of the experiment, as the abundance of anammox bacteria decreased after the ratio change on day 89 the ammonium removal efficiency decreased as well. Nevertheless, the overall nitrogen removal rate and nitrogen removal efficiency increased along with the increased growth of DNRA bacteria.

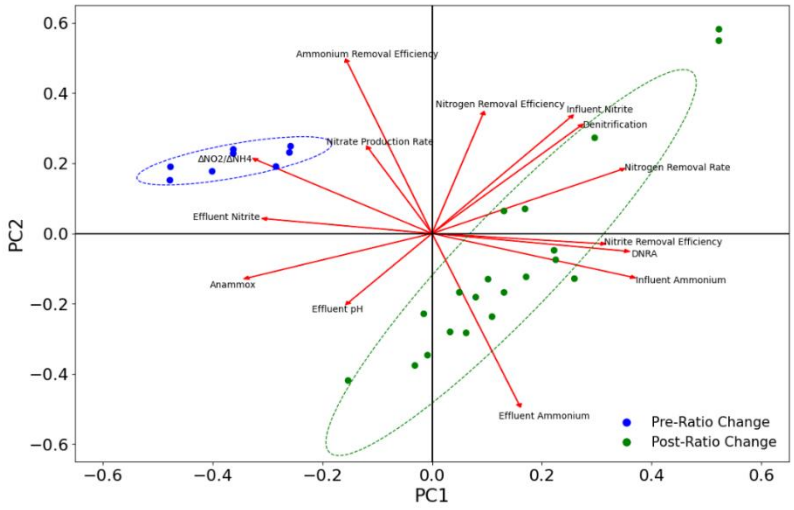


Figure 2.9: PCA plot of changes in taxa abundance for anammox, denitrification, and DNRA with reactor operational parameters. Ellipses represent 95% confidence intervals.

2.4 Discussion

2.4.1 Microbial Community Shifts in Reactor

While previous studies have examined the performance of anammox reactors under variable influent loading ratios, analyses of the effects on the microbial community are limited. After the introduction of decreased NH_4^+:NO_2^- ratios, we observed an increase in the microbial diversity of the system. Of the 317 genera identified through amplicon sequencing, 36% increased by at least one order of magnitude after the ratio change, and the alpha diversity also increased steadily. This increased diversity may correspond to increased availability of ammonium as a growth-limiting nitrogen source. Of the 129 MAGs identified, only 25 contained canonical assimilatory nitrate/nitrite reductases to utilize nitrate or nitrite as nitrogen sources; this suggests that most of the microbial community may require ammonium as a fixed nitrogen source for cell growth. During the initial nitrogen loading ratio, effluent ammonium concentrations remained low, within the range of 0-0.143 mM. Anammox bacteria are known to have high affinities for ammonium, as evidenced by the presence of multiple ammonium transporters in their genomes[236,317,318]. This gives anammox microbes a competitive advantage for ammonium uptake, even at low concentrations. When the ratio of nitrogen substrates changed, the low fixed nitrite concentration would have limited ammonium oxidation, leading to an increased concentration of ammonium in the reactor, in excess of what anammox bacteria could use. This resulted in increased reactor ammonium concentrations (Figure 2.1), which may have supported the growth of other organisms with lower affinities for ammonium uptake to support biosynthesis.

Increased microbial diversity could also be explained by the interaction of diversifying stochastic drift constrained by deterministic homogenous selection. Based on theory supporting these processes, a microbial community will trend towards functional homogeneity during static environmental conditions (homogenous selection), but will also undergo variable changes in composition due to weak selection pressures (drift)[319,320]. In anammox reactors, these processes

would result in the selection of bacteria well-adapted to the conditions created by reactor operational parameters and the dominance of anammox bacteria, independent of taxonomy, as shown through the log-ratio changes (Fig. 2.8a). However, when the reactor maintains these environmental conditions for extended periods of time, weak selection can promote fluctuations in the microbial community composition. Throughout the duration of the experiment, the reactor maintained relatively stable performance, except for effluent ammonium concentrations rising post-ratio change. Despite this consistency, populations of bacterial taxa from Ignavibacteriae, Chloroflexi, and Bacteroidetes still fluctuated. This is similar to results presented by Ya et al. (2023) where increased abundance of Chloroflexi and Proteobacteria was observed despite stable reactor operation. Functional redundancy amongst these bacterial groups could contribute to their increased abundance, despite the lack of any operational or natural perturbations. This hypothesis, combined with the sustained high activity of anammox bacteria despite low abundance, could provide an explanation for the maintenance of efficient bioreactor performance (>85% nitrogen removal) despite significant changes to the microbial community.

2.4.2 Synergistic Interactions between DNRA and Anammox

After the influent NH_4^+:NO_2^- ratio change, we observed increased abundance of bacteria capable of performing DNRA. Amplicon sequencing and shotgun metagenomic sequencing revealed increased abundance of bacteria belonging to Ignavibacteriae, Chloroflexi, and Bacteroidetes, phyla previously associated with DNRA in anammox reactors[247,286,321]. While there is not sufficient evidence to conclude that the ratio change directly led to increased abundance of DNRA bacteria in this reactor system, the co-occurrence of this trend with stable reactor operation is an intriguing observation worth consideration. Previous studies have reported the presence and increased replication of DNRA bacteria to coincide with decreased reactor performance[321,322]. However, DNRA bacteria have also been shown to form symbiotic interactions with anammox bacteria, promoting robust reactor performance[322,323]. Many anammox species can also perform DNRA using simple organic acids as substrates[324], further complicating the characterization of the interplay between these metabolisms. Zhou et al., (2023) reported very similar results to those observed in this work, demonstrating increased abundance of DNRA bacteria with improved nitrogen removal efficiency (NRE) at lower NH_4^+:NO_2^- ratios, but deleterious effects on NRE at higher NH_4^+:NO_2^-[325]. The results from that study illustrate the delicate balance between anammox and DNRA, and how the complexity of these interactions is impacted by influent loading rates, organic carbon concentrations, and anammox species niche differentiation.

The observation of increased abundance of bacteria utilizing DNRA post-ratio change could be explained using a few key considerations. Previous research on the competitive balance between DNRA and denitrification suggests that DNRA is generally favored over denitrification at higher C/N ratios[290]. This observation has also been purported to apply for both nitrate and nitrite and under dual limitation conditions when both the supply of electron donor and electron acceptor are limited in the environment[323]. Organic carbon concentrations, estimated from the biodegradable fraction of biomass measured through MLVSS (Figure 4, Appendix 2) remained substantially higher than effluent nitrate and nitrite concentrations throughout the duration of the experiment indicating conditions favorable for DNRA. From day 0 up until day 89 when the influent NH_4^+:NO_2^- ratio was 1:1.32, anammox bacteria, DNRA bacteria, and denitrifiers actively competed for nitrite and anammox bacteria were able to sustain relative dominance likely due to a higher affinity for nitrite as evidenced by multiple nitrite transporters (Fig.

2.7). However, on day 89 when the NH_4^+:NO_2^- ratio was shifted to 1:1.1, the influent ammonium concentration was increased, and this would have required more nitrite to undergo the anammox reaction to completely oxidize ammonium to dinitrogen gas. Lowering the NH_4^+:NO_2^- ratio could have intensified the competition for nitrite, and this increased competition could select for bacteria that are capable of utilizing already limited substrates more efficiently. From a thermodynamic perspective, the theoretical amount of energy produced per mole of nitrite for DNRA and anammox is comparable[324]. However, the yield of biomass produced per mole of nitrite through DNRA is effectively higher than through anammox, and bacterial growth rates are also significantly higher under DNRA (Fig. 2.8b). Thus, when the ratio shift occurred and nitrite limitation was intensified, DNRA bacteria could have been poised to proliferate because of their ability to grow and biosynthesize more efficiently. It is important to consider that anammox bacteria also convert nitrite to nitrate to generate reducing equivalents for carbon fixation and biomass production[323]. Additional competition for nitrite could lead to anammox bacteria diverting more nitrite towards energy generation than carbon fixation. This could result in decreased biomass production, which could offer a possible explanation for the decrease in cell abundance observed throughout the duration of the experiment. This explanation neglects the capability of anammox bacteria to utilize partial DNRA to convert nitrate back to nitrite to use for the anammox reaction. However, this reaction has only explicitly been identified to support the oxidation of volatile fatty acids (VFAs)[324] and is uncharacterized for alternative carbon substrates.

Reactor configuration and biomass growth type adds another dimension of complexity to the dynamics between anammox, DNRA, and denitrifying bacteria. Bryson et al. (2022) reported higher abundance of Ignavibacteriae and Phycisphaerae with *nrfAH* genes used for DNRA in two stage anammox configurations as compared with one stage configurations, citing a negative selection pressure on facultatively aerobic denitrifiers in a strictly anaerobic environment[321]. Our findings are consistent with those observations given that the strict anaerobic conditions provide a niche for fermentative bacteria[326]. These observations could provide insight into the conditions that favor DNRA in anammox systems. Many of the bacteria with *nrfAH* could be coupling DNRA to the fermentation of extracellular amino acids and exogenous carbon substrates. This reaction is more bioenergetically favorable than pure fermentation[327–329], and could be even more competitive than alternative nitrogen pathways when the carbon substrate is more reduced. IGN01 and IGN03 both contain genes encoding for acetate kinase, and CFLX01 encodes genes for acetate ligase, suggesting that these strains have the ability to ferment acetate or propionate. This would be especially advantageous under the conditions found in the MBR where the concentrations of fermentable substrates, such as extracellular amino acids and polysaccharides, are likely much higher than non-fermentable substrates such as acetate, ethanol, or methanol. Fermentable sugars such as D-Arabinose, D-Ribose, and D-Mannose and sialic acids such as neuraminic acid, commonly found in anammox extracellular polysaccharides[330], have also been shown to selectively enrich DNRA bacteria over denitrifiers[322,330]. When the ratio shift occurred, and the competition for nitrite intensified, the ability to couple nitrite reduction to fermentation of highly reduced carbon substrates could have also contributed to the enrichment of DNRA. Thus, the type of carbon source and the oxidative conditions in anammox systems also has a substantial impact on the competitive balance between anammox, denitrifiers, and DNRA bacteria.

The balance between DNRA and anammox is predicated on an exchange of nitrate, ammonium, and organic carbon substrates as shown in Figure 2.10. DNRA bacteria are thought to utilize substrates derived from extracellular polymeric substances (EPS) produced by anammox bacteria[323,331], which would necessitate a symbiotic relationship between these two pathways. DNRA can provide anammox bacteria with nitrite through the reduction of nitrate via *nar* and *nap* nitrate reductases, or ammonium through the reduction of nitrite with *nrfAH* nitrite reductases. This steady flow of material exchanges can result in enhanced nitrogen removal in a variety of ecosystems[323,331]. Despite this, DNRA can still actively compete with anammox for nitrite, and even potentially destabilize aggregates through overconsumption of EPS, leading to anammox cell death[332,333]. This tipping point is a pivotal junction to distinguish in order to optimize reactor efficiency and resiliency. It is also important to evaluate how this relationship changes at more extreme NH_4^+:NO_2^- ratios (<1:1.1 and >1:1.3). Thus, further research is needed to identify and parameterize the equilibrium of this dynamic.

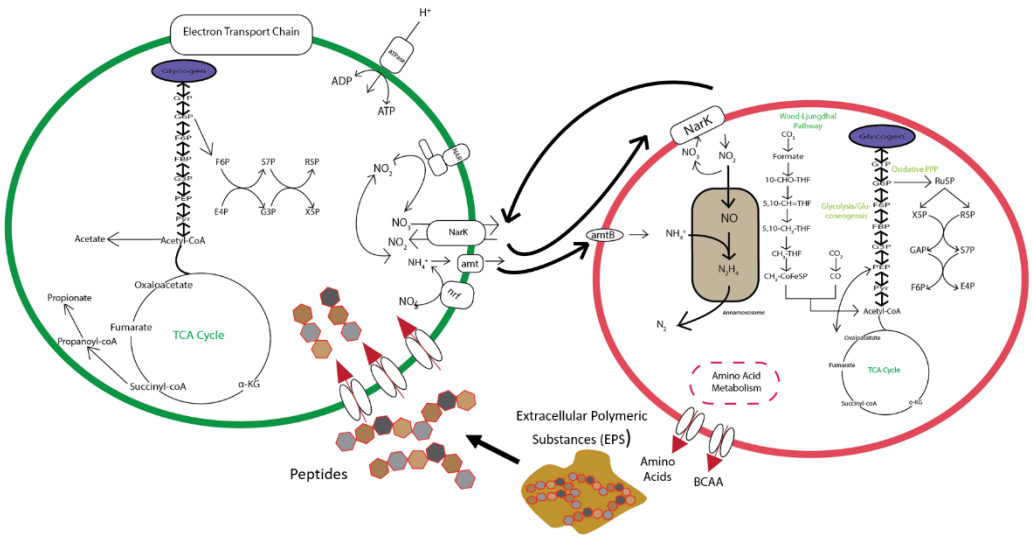


Figure 2.10: Conceptual diagram of metabolic exchanges occurring between anammox and DNRA bacteria within the tested bioreactor

2.4.3 Reactor Performance Implications

Anammox reactors are known to be sensitive to operational conditions and are susceptible to destabilizations after perturbation. These systems are particularly sensitive to nitrite fluctuations[325,334]. The formation of synergistic relationships between anammox and DNRA can help to alleviate the deleterious effects of nitrite inhibition by keeping nitrite concentrations low. These interactions have been demonstrated to assist in the recovery of anammox reactor performance following nitrite inhibition[325,334], and to help stimulate the recovery of anammox bacteria from dormancy[335]. Promoting synergy between anammox and DNRA bacteria could be even more important at higher nitrogen loading rates, which cause the reactor to be more susceptible to nitrite inhibition[249,251,337]. Throughout the duration of this study, nitrite concentrations rarely rose above detectable levels in the effluent, which can be attributed to the abundance of excess ammonium for the anammox reaction (1 mole of NH_4^+ requires 1.32 moles

of NO_2^- to be fully oxidized) but also to the reduction of nitrite to ammonium by DNRA bacteria and, to a lesser extent, nitrite reduction to dinitrogen gas by denitrifying bacteria. The abundance of DNRA bacteria was also positively correlated with the nitrite removal efficiency, nitrogen removal efficiency, and nitrogen removal rate (Figure 2.9). These results provide evidence for the positive contributions of DNRA towards robust reactor performance. The interactions between anammox and DNRA bacteria can be enhanced by organic carbon amendments to encourage the growth of DNRA microbes, which has been previously reported to have beneficial effects on reactor performance[268,269,337]. Taking advantage of the synergy between anammox and DNRA could be especially advantageous during reactor startup, where anammox activity is vulnerable to performance disruptions due to nitrite inhibition. These interactions could also be leveraged during periods of elevated nitrite concentrations by dosing with organic carbon to stimulate the growth of DNRA bacteria.

2.5 Conclusions

In a single-stage anammox MBR, adjusting influent NH_4^+:NO_2^- from the conventional ratio of 1:1.32 to 1:1.1 led to a significant change in the microbial community. Despite relatively minor changes in total nitrogen removal efficiency (85.26±0.01% pre-ratio change vs 85.833±0.002% post-ratio change), the relative abundance of anammox bacteria in the system decreased from 77.19% to 12.24% by 16s rRNA amplicon sequencing. This coincided with the growth of bacteria capable of performing DNRA; the phyla Ignavibacteriae and Chloroflexi increased from 7.73% and 4.57%, respectively, to 23.36% and 38.22%. These results demonstrate the positive effects of a stable dynamic between anammox and DNRA, which can result in robust reactor performance and enhanced nitrogen removal.

Chapter 3: Growth strategies determined response to SRT driven crash in anammox reactor

This chapter is largely adapted from the manuscript, White et al., "Life-history strategies determine response to SRT driven crash in anammox reactor" submitted to Water Research and currently under review at the time of this dissertation writing.

3.1 Introduction:

Anaerobic ammonium oxidation (anammox) is a biological process performed by a group of bacteria in the phylum Planctomycetes[268,269,338]. Anammox is estimated to account for 30-70% of fixed nitrogen removal in marine environments[276,339] and is commonly applied to sidestream nitrogen removal from anaerobic digester effluents in wastewater treatment plants[276,339]. Anammox bacteria obtain energy by coupling ammonium oxidation to nitrite reduction[239,264,265]. In practice, anammox is combined with partial-nitritation (PN) either simultaneously in a one-stage or separately in a two-stage configuration. In both cases, approximately half of the ammonium is first aerobically oxidized to nitrite via nitritation to provide the appropriate stoichiometric ratio of ammonium and nitrite required for the anammox reaction[243,262,282,321]. Treatment technologies utilizing the anammox process have demonstrated exceptional potential to remove nitrogen from highly concentrated waste streams while using 60% less energy and producing 90% less sludge than conventional nitrification-denitrification systems[243,262,285,324]. Despite these advantages, anammox-based treatment systems often experience process instability and performance crashes due to slow bacterial growth rates and environmental instability[243,262,285,325]. These issues are exacerbated by an insufficient understanding of the community interactions between the autotrophic anammox bacteria and co-existing heterotrophic microbes.

Anammox bioreactors are known to harbor a taxonomically and functionally diverse array of organisms that form various mutualistic and competitive interactions with anammox bacteria[261,281–283]. Previous genomic studies have identified similar uncultured phyla coexisting with anammox bacteria, including Proteobacteria, Chloroflexi, Bacteroidetes, and Ignavibacteriae. These same groups are found across numerous reactor configurations, suggesting that the unique conditions within anammox bioreactors consistently select for this core community[247,249,308]. Many of these coexisting bacteria employ denitrification or dissimilatory nitrate reduction to ammonium (DNRA)[243,262,285,325] as an energy strategy, and utilize microbial decay products or amino acids derived from extracellular polymeric substances (EPS) as a carbon source[340,341], which can recycle biomass and stimulate growth of symbionts. Some of these bacteria also play an important role in the formation of cell aggregates[243] and the circulation of vitamins, co-factors, and secondary metabolites, for which some anammox bacteria lack biosynthetic pathways[247,249,308]. Despite metabolic inferences drawn from large amounts of genomic data on these mutualistic organisms, the precise ecological niche of these organisms and their interactions with anammox bacteria in reactor systems remains unclear.

The low growth rate of anammox bacteria is a key factor affecting the microbial ecology of anammox-based treatment systems. With minimum doubling times ranging from 5-11 days

depending on the growth strategy (e.g., suspended, granular, biofilms) and reactor conditions[343] , the solids retention time (SRT) must be sufficiently long to enable the growth and enrichment of anammox bacteria[344]. Systems employing longer SRTs (i.e., > 45 days) can contain higher biomass concentrations[345,346], which are generally conducive to stable reactor operation and higher nitrogen removal efficiencies. However, excessively long SRTs, which are often coupled to longer hydraulic retention times (HRT), are impractical from an operations standpoint and can result in the accumulation of dead biomass that supports the growth of undesirable heterotrophic bacteria. Conversely, shorter SRTs (i.e., < 20 days) can lead to insufficient biomass retention and washout[347,348], which have deleterious effects on reactor stability and nitrogen removal performance[349]. Thus, optimizing the SRT to strike a balance between stable community composition and treatment viability and effectiveness is important for the effective application of anammox for wastewater treatment. While the effects of SRT on reactor performance has been previously studied, SRT effects on the microbial communities within anammox reactors remains understudied.

Previous research on microbial community dynamics in activated sludge reactors suggests that longer SRTs result in higher levels of functional and taxonomic diversity[345] leading to the emergence of rare taxa and metabolic functions such as nitrification and trace organic contaminant removal[346]. This increase in diversity is owed in part to the selection of organisms based on growth strategies[296]. Longer SRTs enable the growth of K-strategists, which make significant metabolic investments in resource acquisition at low concentrations, while shorter SRTs favor r-strategists, which are specialized for rapid resource utilization[300]. Manipulating the SRT in a controlled environment such as a bioreactor has been shown to illuminate the growth strategies and ecological niches of uncultured bacteria within activated sludge systems[301]. This same strategy could be applied in anammox bioreactors.

Here, we observe the response and recovery of the microbial community within a lab scale anammox membrane bioreactor following a controlled SRT-driven crash. We evaluate the effects of this crash on reactor performance, anammox activity, and microbial community interactions through 16S rRNA amplicon and shotgun metagenomic sequencing analyses. The results help to illuminate microbial interactions and metabolic interdependencies that contribute to reactor stability, function, and resilience.

3.2 Materials and Methods:

3.2.1. Bioreactor operation

A 1 L anaerobic membrane bioreactor was enriched for anammox bacteria from anaerobic digester solids and operated for 1 year prior to the experiment. The specific details of initial inoculation and operation can be found in Keren et al, 2020[347]. A polyvinylidene fluoride membrane with a pore size of 0.22 μm was mounted to the inside of the reactor and a gas mix (Argon:CO2 = 95:5; 50 mL/min) was continuously supplied to purge the system of oxygen and maintain circumneutral pH (6.9–7.2) (Table 2, Appendix 2). Temperature was maintained at 37°C using a heating jacket (Eppendorf, Hauppauge, NY) and mixing was provided through an impeller at a rate of 200 rpm. A synthetic medium (Table 1, Appendix 2) containing ammonium, nitrite, bicarbonate, and trace nutrients prepared anaerobically under nitrogen was continuously

fed to the reactor. Influent and effluent samples were collected every other day to monitor concentrations of ammonium, nitrite, and nitrate using HACH test kits (HACH, Loveland, CO), as described in the manufacturer's methods 10031, 10019, and 10020, respectively. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were measured according to standard methods[349].

The experiment took place over 405 days, during which the hydraulic retention time (HRT) of the reactor was maintained at 12 h via an effluent pump and the solids retention time (SRT) was varied between 50 and 28.5 days via biomass wasting (Table 3.1). For the first 210 days of the experiment, the SRT was maintained at 50 days. On day 210 the SRT was adjusted to 40 days, to 33.3 days on day 224, and to 28.5 days on day 245. The influent ammonium and nitrite concentrations were increased stepwise from 600 mg-N/L and 660 mg-N/L on day 0 to 818 mg-N/L and 982 mg-N/L on day 210, respectively. Following the performance crash, the influent ammonium and nitrite concentrations were decreased to 201 mg-N/L and 249 mg-N/L on day 296 and the SRT was adjusted back to 50 days on day 297. During the recovery period, the influent ammonium and nitrite concentrations were increased stepwise to a final concentration of 909 mg-N/L and 1091 mg-N/L on day 364. Experimental parameters specifically for SRT changes are listed in Table 3.1 and a full list is available in Table 1, Appendix 3.

Day	Influent ammonium (mg-N/L)	Influent nitrite (mg-N/L)	Nitrogen loading rate (g N/L-d)	SRT
0	600	660	2.52	50
210	818	982	3.6	40
224	818	982	3.6	33.3
245	818	982	3.6	28.5
297	201	249	0.9	50

Table 3.1: Solids retention time regimes over the course of the experiment with complementary influent nitrogen loading. For full list of nitrogen loading amendments reference Table 1, Appendix 3

3.2.2. DNA extraction

Biomass samples were collected every 2–10 days via syringe through an extraction port, flash frozen in liquid nitrogen, and stored at −80°C until further use. Genomic DNA was extracted from the samples using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA) as described in the manufacturer's protocol. DNA quality was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). DNA was quantified using a Qubit fluorometer (Thermofisher Scientific, Waltham, MA), diluted to 10 ng/µl with nuclease free water (Thermo Scientific, Waltham, MA), and stored at −20°C until further use. Shotgun metagenomic samples were sent to the Joint Genome Institute (JGI) in Walnut Creek, CA for sequencing (150 bp paired-end) on an Illumina HiSeq 2500 1 T sequencer (Illumina, San Diego, CA).

3.2.3. 16S rRNA gene analysis

The microbial community composition was evaluated by 16S ribosomal RNA sequencing of 54 DNA samples collected throughout the experiment. The V4 region was amplified using primers 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (3′-TAATCTWTGGVHCATCAG-5′), with barcodes attached to the reverse primer. Amplicons were pooled at equal molarity and purified with the QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD). Paired-end sequencing (250 bp paired-end) was then performed on the Illumina MiSeq sequencer (Illumina, San Diego, CA). The full protocol for paired-end amplicon sequencing is provided by Wu et al. (2015)[300]. Sequence processing and data analysis was conducted using MOTHUR v.1.39.5, following the MiSeq Standard Operating Procedure (SOP)[350], and OTUs were assigned based on a 97% sequence similarity threshold. Merging of 16S rRNA sequences and 16S sequences obtained from metagenomes was conducted according to the procedure described in Keren et al. (2020)[351]. 16S rRNA copy numbers for the top 130 most abundant taxa were predicted using rrndb[352] with classifications down to the genus level at 70% confidence.

3.2.4. Metagenomic sequencing, assembly, and binning

Eight DNA samples were used for metagenomic sequencing: Four from single time points on day 41, 248, 293, and 378; and four samples bulked from multiple days as described in Table 2, Appendix 3. Resulting sequences from each time point were processed separately and assembled into metagenome assembled genomes (MAGs) according to the procedure previously reported in Keren et al. (2020)[353]. KEGG Automated Annotation Service (KAAS) was used to annotate predicted gene sequences using Hidden Markov Models (HMMs), and single time point MAG abundances were calculated using reads per kilobase per million (RPKM). Phylogenetic analysis was conducted using a set of 15 ribosomal proteins (L2-L6, L8, L10, L12, L14, L16, L18, L22, L24, S3, S8, S11, S17, S19)[345]; each protein was aligned independently using MAFFT with the default auto-parameterization settings[352], and alignments were then manually concatenated prior to maximum-likelihood tree construction in IQ-TREE using the MFP substitution model[311]. Enzyme commission (EC) analysis was conducted according to the procedure reported in Mansfeldt et al. (2019)[314]. The log ratio change for each MAG was calculated based on the procedure previously reported in Keren et al. (2020)[315]. Briefly, three MAGs with stable coverage across the three time points were selected as reference frame MAGs. The coverage of each MAG in the study was divided by the coverage of each of the three reference frame MAGs, and the resulting ratios were used to calculate the log ratio changes between samples. This process allows for accounting or adjusting for differences in sequencing depth between samples, which can otherwise bias results.

3.2.5. Statistical analysis

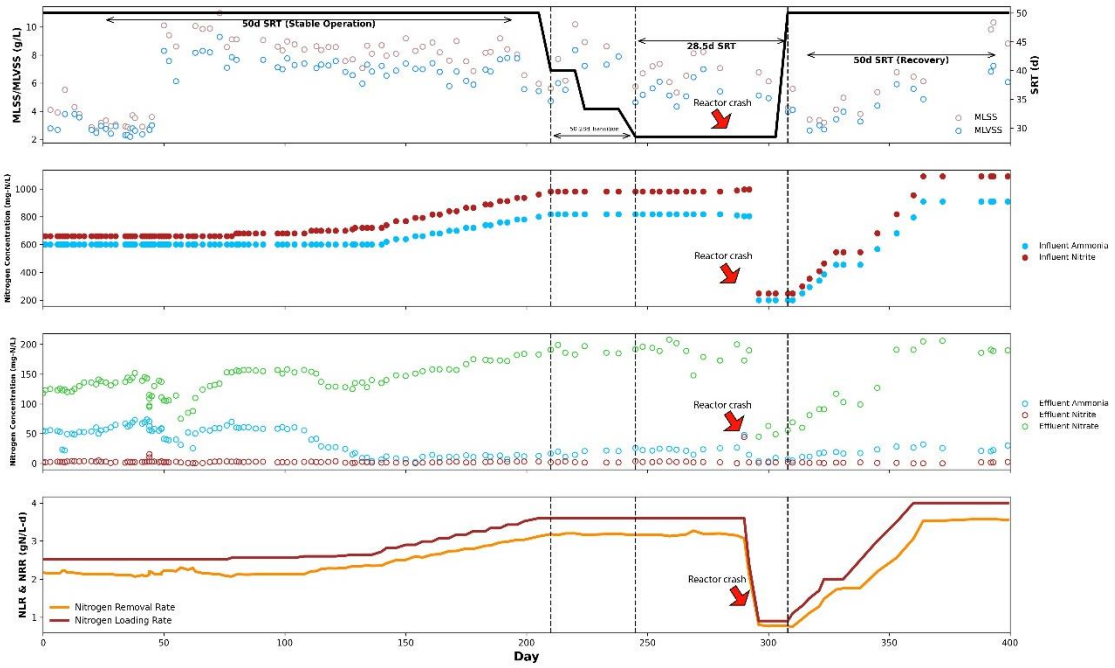
Temporal association among MAGs was tested according to the procedure reported in Keren et al. (2020)[353]. From pairwise relative abundance values across all metagenomes and time points, Spearman rank correlation coefficients were calculated. A distance matrix (Euclidean) was then generated and clustered using the ward.D method with a distance cutoff of 15 used to define clusters. Associations among MAGs with specific time points were assessed using nMDS analysis (non-parametric MultiDimensional Scaling) with MAGs and time points.

Redundancy analysis (RDA) was applied to evaluate the correlation between taxa and reactor performance parameters. Statistical significance of RDA results was determined using a one-way ANOVA test. A two-sample t-test and Kruskal-wallis test was used to evaluate statistically significant differences between datasets using $p < 0.05$ as the threshold of significance.

3.3 Results and Discussion:

3.3.1 Bioreactor Performance

The MBR experienced incremental improvements in reactor performance over the initial 210 days of the experiment, demonstrating increases in the MLVSS concentrations from 2.8 g/L on day 3 to 7.8 g/L on day 196 (Figure 3.1a) and modest increases in nitrogen removal rate up to 3.2 g-N/L-d by day 210 (Figure 3.1d). Over this time period, the ammonium removal efficiency, nitrite removal efficiency, and total nitrogen removal efficiency were 93.0±0.39% and 99.6±0.28%, and 86.1±0.25% respectively. In the transition period between day 210 and day 245, the SRT steadily decreased from 50 days to 28.5 days. Following the reduction of the SRT to 28.5 days, the MBR maintained stable operation until a performance crash occurred on day 290. During this period the MLVSS concentration decreased from 7.90 g/L on day 238 to 4.0 g/L on day 308 . The change was accompanied by visual indication of performance failure including discoloration/graying of biomass and disintegration of biomass granules. On days 296 and 297, the nitrogen loading rate (NLR) was decreased from 2.4 g-N/L-d to 0.9 g-N/L-d, and the SRT was adjusted back to 50 days, respectively, resulting in a rapid period of performance recovery. The nitrogen removal rate (NRR) rapidly increased from 0.8 g-N/L-d on day 296 to 3.6 g-N/L-d on day 364, and the MLVSS concentration increased from a low of 2.6 g/L on day 317 to 8.8 g/L on day 399.



3.3.2 Microbial Community Shifts

3.3.2.1 Metagenomic Results Overview

After read quality control, 1,055,259,456 reads were obtained from all eight metagenome samples. Reads were assembled into metagenome assembled genomes (MAGs), resulting in 27,836 contigs with a median N50 of 2,138 bp. Contigs were binned into 129 draft genomes accounting for 77.99% of quality filtered reads on average across all eight samples (Table 3, Appendix 3). The number of MAGs present at each time point (using a Coverage > 1 and Breadth > 0.5 as a threshold) ranged from 50 (Day 388) to 70 (~Day 143). The most abundant MAGs based on coverage were affiliated with Planctomycetes, Proteobacteria, Ignavibacteriae, Chloroflexi, Bacteroidetes, and the super-phylum Candidate Phyla Radiation (CPR). A full list of MAGs is available in Table 4, Appendix 3.

The phylogeny of the recovered MAGs showed similar topology to that displayed in previously-reported anammox metagenomic studies[354], and demonstrates a close phylogenetic relationship among a core group of anammox bioreactor bacteria (Figure 3.2). In addition to the anammox MAGs (Planctomycetes), various closely related MAGs from Bacteroidetes, Ignavibacteriae, Chloroflexi, Proteobacteria and CPR were highly abundant in all three studies. The tree demonstrates dozens of instances in which identical or nearly-identical MAGs were recovered from different bioreactors, indicating key taxonomies that recur in anammox systems. These close phylogenetic relationships persist in spite of different reactor configurations and growth strategies indicating unique metabolic interactions that occur between anammox and supporting community members.

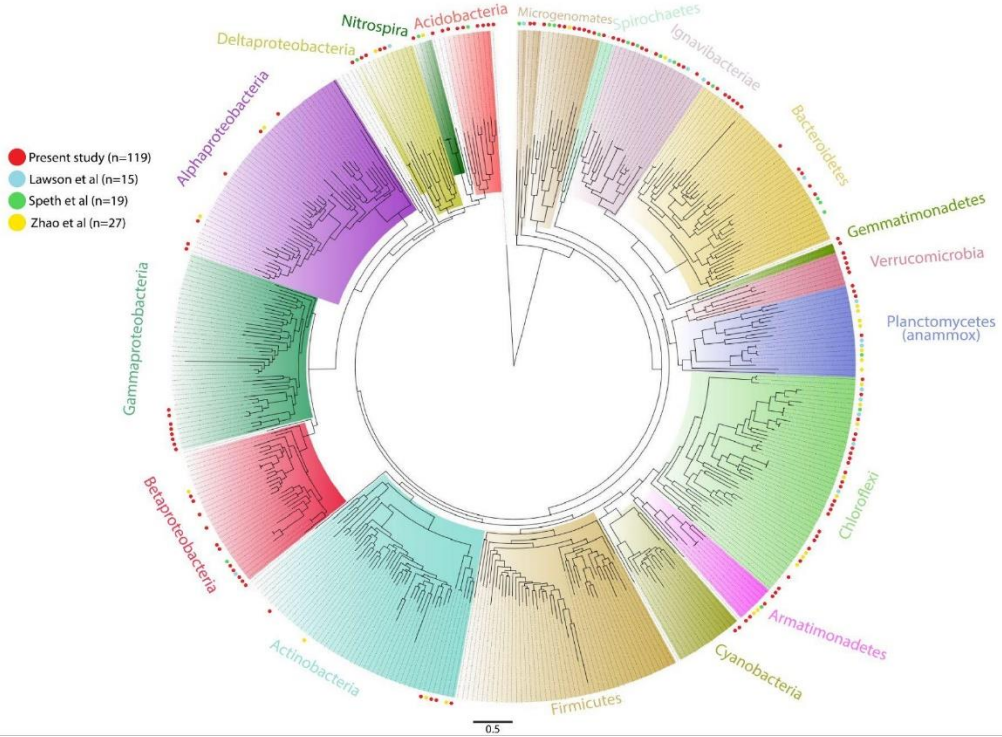


Figure 3.2: Maximum likelihood tree of concatenated ribosomal proteins with genomes from previous studies and present study

3.3.2.2 Temporal Community Dynamics

Based on the physiological and biomass performance changes that occurred in response to SRT changes, we hypothesized that strong selection pressures would cause the emergence of sub-communities throughout the reactor's lifespan. In order to evaluate this hypothesis, we looked to identify groups of MAGs that were closely associated with each other based on abundance. We conducted pairwise cross-correlation analysis of MAG coverage (Figure 3.3a) which yielded five distinct clusters (Groups 1-5). Group 3 was the largest group with 32 MAGs, Group 1 and 5 both contained 28 MAGs, Group 2 contained 24 MAGs, and Group 4 was the smallest with 17 MAGs (Table 4, Appendix 3).

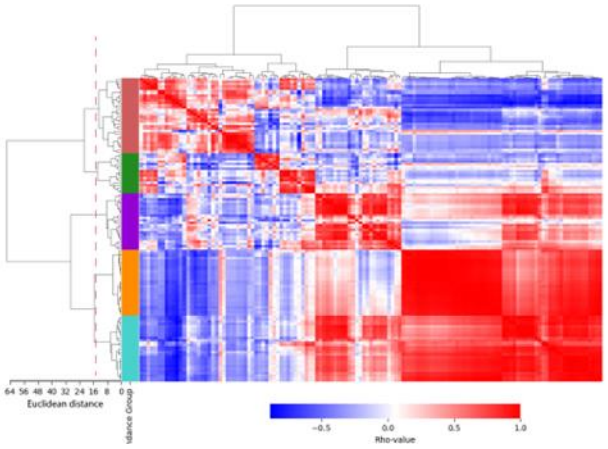


Figure 3.3a: Hierarchical clustering of genome correlation

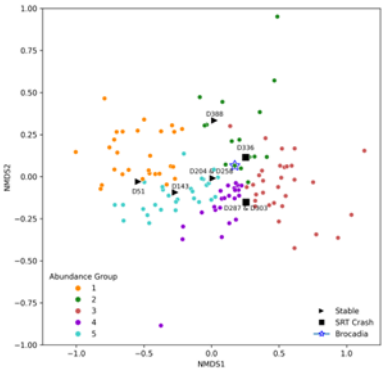


Figure 3.3b: nMDS analysis of genome relative abundance and sampling timepoints

In order to understand which clusters were most associated with specific time points, we performed a non-metric multidimensional scaling (nMDS) analysis using MAG relative abundance (Figure 3.3b). This analysis revealed distinctive patterns between the abundance clusters and specific time points. Groups 1 and 5 are more closely associated with earlier time points prior to the SRT changes, with Group 1 more affiliated with Days 0-51 and Group 5 with Days 51-258. Groups 2 and 3 were more associated with the early stages of the crash (Days 287-303) and Group 4 was more related to the post-crash recovery period (Day 388). AMX01, the only anammox MAG recovered from this study, was part of Group 4, but was skewed closer to Group 5 because it was highly abundant during the early period of the experiment. Based on these relationships the crash seems to have prompted a strong temporal shift in community composition where sub-communities were differentially abundant at various stages of the reactor's lifespan. This indicates disparate community taxonomic compositions before, during, and after the crash.

To examine the relative abundances of Groups 1-5 at finer temporal and taxonomic scales, 16S rRNA sequences obtained from metagenomes were merged with direct 16S rRNA sequencing data into operational taxonomic units (OTUs). Of the 129 recovered MAGs, 20 contained 16S sequences that matched to a sequence obtained from amplicon sequencing. These sequences

comprised on average 71.7% of the total 16S rRNA reads. These reads were summed for each abundance group to calculate the relative abundances (Figure 3.4a) throughout the duration of the experiment. Two groups exhibited a decrease and rebound in abundance: the anammox bacteria (43.8% on day 253, 1.9% on day 294, and 46.9% on day 371) and Group 2 (38.4% on day 253, 8.8% on day 294, and 31.6% on day 364). Group 3 showed the opposite trend (15.8% on day 253, 89.1% on day 294, and 16.5% on day 364) and Group 4 started with low abundance but increased over the latter stages of reactor operation.(0.2% on day 294 and 36.9% on day 401). Group 1 and 5 had low relative abundances throughout most of the experiment. These results are consistent with the trends identified through MAG abundance analysis and demonstrate a strong temporal partitioning of sub-community abundance.

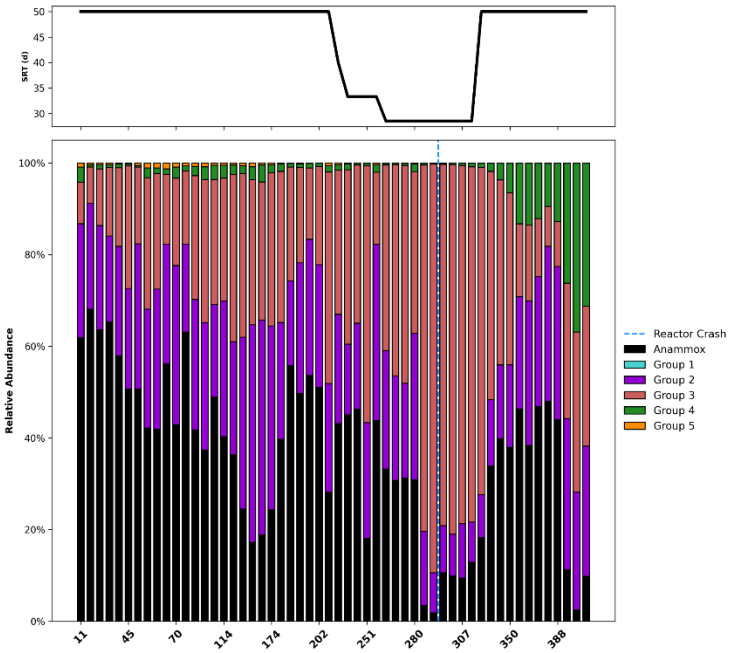


Figure 3.4a: Relative abundance of 16S sequences matched to genomes

In order to assess the accuracy of the relative abundances calculated from matching amplicon and metagenome 16S sequences, the relative abundances of Groups 1-5 were calculated directly from metagenome coverage (Figure 3.4b). These results were slightly different in magnitude but reflect the same patterns shown in the relative abundances calculated from matching sequences. The 16S data tended to overestimate the abundance of Group 4 and underestimate the abundance of Groups 1, 2, and 5 relative to the shotgun sequencing data. These results, being consistent with the abundances derived from matching 16S sequences, suggest the SRT driven crash imparted a strong selective pressure on bacteria within the bioreactor. This selective pressure prompted a transient destabilization in community composition during the crash followed by a reconfiguration of the community during its recovery.

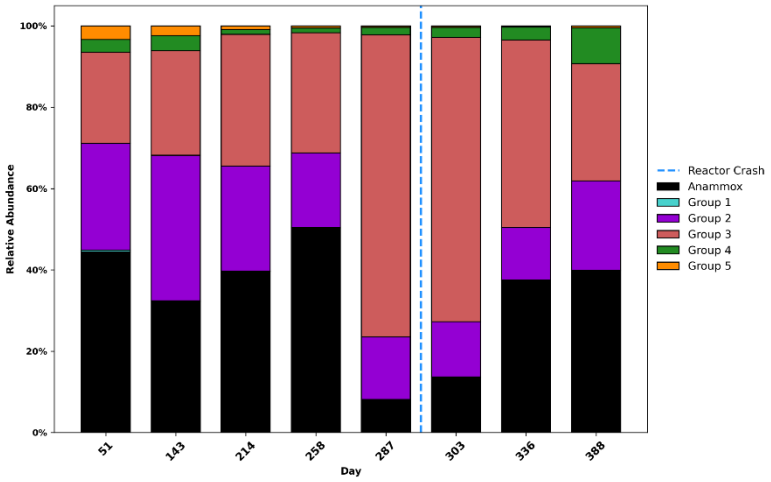


Figure 3.4b: Relative abundance of genome abundance groups from metagenomic data

3.3.2.3 Taxonomic Succession at the Order level

Relative abundance data from 16S rRNA reads at the Order level were also evaluated to illuminate microbial succession patterns at lower taxonomic levels (Figure 3.5a). These results demonstrate a strong alignment of specific taxonomic groups to time periods before, during, and after the crash. Sphingobacteriales and Rhodospirillales showed the highest increase in relative abundance in response to lowered SRTs, increasing from 1.0% and 11.9% on day 223 to 31.9% and 18.1% on day 294, respectively. Brocadia (of the Planctomycetes) and Anaerolineales (which on average represented 83.8% of reads classified as Chloroflexi) experienced the most significant negative response, decreasing from 17.3% and 28.3% to 7.3% and 1.4% over the same time period, respectively. During the reactor recovery period, taxonomic group SJA-28[354], a lower taxonomic level of Ignavibacteriae, increased in relative abundance from 0.6% on day 315 to 26.7% on day 401. These taxonomic trends align with the abundance clusters identified through MAG analysis. Two MAGs from Group 3 classified as Bacteroidetes and Alphaproteobacteria contained 16S sequences that matched to OTUs classified as Sphingobacteriales and Rhodospirillales and one MAG from Group 4 matched to an OTU classified as SJA-28. An RDA of reactor operational parameter effects on taxonomic order abundance revealed that Anaerolineales was positively correlated with the SRT, MLSS, and MLVSS concentrations (Figure 3.5b). Conversely, Sphingobacteriales and Rhodospirillales were negatively correlated with the SRT ($p < 0.001$). Alpha diversity metrics were calculated (Table 5, Appendix 3) and the species richness significantly increased during lowered SRTs (days 218-315) compared with higher SRT (days 0-218) ($p = 0.02$, Kruskal-wallis). The increase in richness can be explained by the decrease in abundance of the anammox bacteria that coincided with higher abundances of bacteria affiliated with Group 3. Overall, these results indicate that changes in the SRT are strongly associated with significant shifts in dominance of certain taxonomic groups.

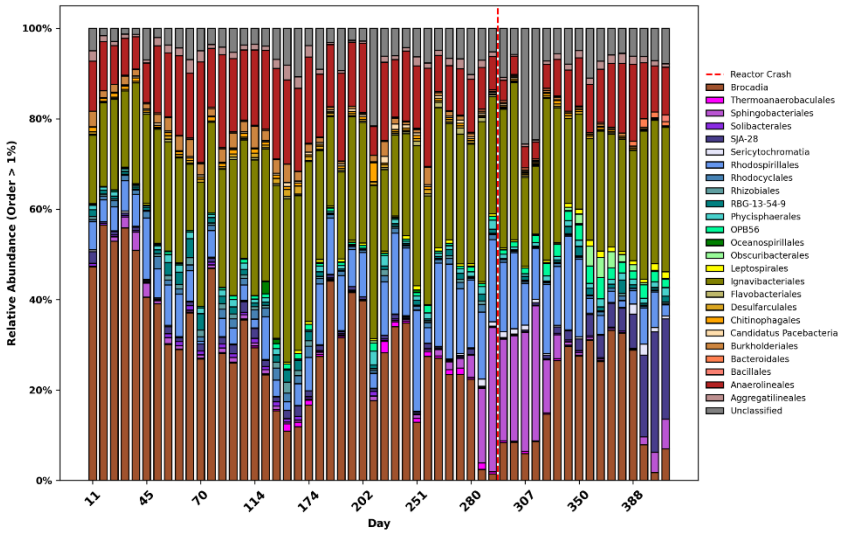


Figure 3.5a: Relative abundance of 16S sequences at the order level

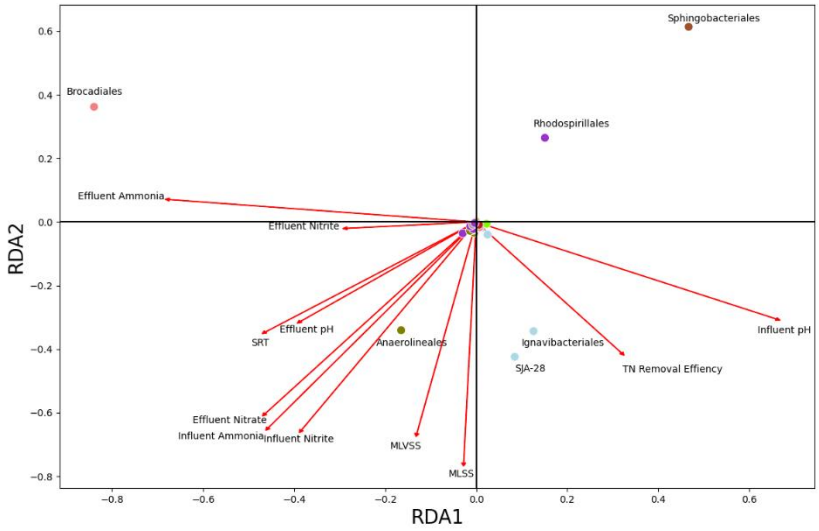


Figure 3.5b: Redundancy analysis (RDA) of 16S rRNA gene abundances aggregated at order level colored by phyla and reactor operational parameters as covariates as red arrows

3.3.2.4 Growth strategies of temporal sub-communities

The decline in *Brocadia* abundance, higher F/M ratios, and change in biomass morphology coincided with the proliferation of Sphingobacteriales and Rhodosprillales. The magnitude and rate of increasing abundance at lower SRTs and higher F/M ratios is consistent with previous literature purporting these organisms' r-strategist growth traits[355]. R-strategists are generally considered to be organisms that can rapidly replicate when substrate conditions are favorable, in contrast to K-strategists, which maintain slow, steady growth even under oligotrophic conditions[356,357]. This dichotomy is often used synonymously to describe the copiotrophy-

oligotrophy spectrum which is based on the physiological traits that regulate an organism's growth (Michaelis-Menten constants, K_s and maximum growth rates, μ)[355]. Indeed, Sphingobacteriales can be enriched in activated sludge systems operating under lower SRTs (<5 days)[355,359], and genomic analyses have identified terrestrially sourced Rhodospirillales genomes enriched in traits associated with copiotrophy[356]. In contrast, Anaerolineales, which decreased in abundance after the SRT was decreased, has been reported to exhibit K-strategist behavior, being enriched at lower F/M ratios and higher SRTs[357,358]. These trends indicate that the SRT induced crash selected for bacteria based on their growth strategies, selecting for faster growing r-strategists in favor of slower growing K-strategists.

To determine whether this trend applied more broadly throughout the microbial community we examined traits associated with growth strategies using high 16S copy numbers as an indicator of copiotrophic bacteria[359]. When considering the 130 most abundant taxa, those which increased in relative abundance in response to the crash had significantly higher copy numbers than those that decreased (p=0.02, Kruskall-wallis). Other traits often associated with growth strategies (i.e., minimum generation time and genome size) did not associate with 16S copy number. These results support the idea that a binary model for copiotrophy and oligotrophy may not be an adequate framework to explain microbial growth strategies[356,360]. Furthermore, different traits including stress tolerance, nutrient acquisition, and carbon use efficiency contribute towards the determination of ecological niches[356].

3.3.3 Microbial Metabolic Network

3.3.3.1 Metabolic Clustering

To understand how metabolic differences could have impacted SRT-induced selection, we examined the presence of genes belonging to Clusters of Orthologous Groups of proteins (COGs). Using this information, we constructed a matrix of COG categories and clustered the MAGs based on Bray-Curtis distance using the complete method (Figure S4). This clustering resulted in four distinct groups (A-D), the metabolic and taxonomic composition of which closely resembled the metabolic groups identified in our previous study[362]. Group A comprised MAGs classified as Ignavibacteriae and Bacteroidetes, which had similar metabolic capabilities to Group B and C but had multiple auxotrophies including several amino acids. Group B comprised mostly Chloroflexi MAGs as well as smaller numbers of Verrucomicrobia, Acidobacteria, Armatimonadetes, and Actinobacteria MAGs, characterized by hydrogen oxidation, oxidative phosphorylation with cytochrome BD complex and a wide array of transporters. Group C was predominantly Proteobacteria with other MAGs including AMX01, that had diverse metabolism including oxygen respiration and denitrification, the ability to biosynthesize certain vitamins, cofactors, and several amino acids (tyrosine, phenylalanine, proline). Group D was composed exclusively of CPR bacteria, which are known to have reduced genomes and metabolisms[361]. Of the 32 MAGs that became most dominant during the crash (i.e., Group 3), 10 belonged to Group A, 12 belonged to Group B, 6 belonged to Group C, and 4 belonged to Group D, suggesting that the taxonomy of the crash-associated bacteria were metabolically diverse.

In order to directly examine the effects of the crash on different metabolic groups, we calculated the log ratio (LR) change in MAG coverage between Day 258 and Day 303, directly before and after the crash (Keren et al., 2020) (Figure 6). Log ratio changes above or below the confidence intervals (CIs) are considered to be significantly increased or decreased in abundance. A full list of LR changes and CIs is available in Table 6 and 7, Appendix 3.

Metabolically similar clusters were affected differently by the crash. Groups B and C had roughly equal numbers of MAGs with LR changes above and below the upper and lower CIs, indicating that the metabolisms defining these clusters were less important to the crash. Group D, composed exclusively of CPR bacteria had only 2 MAGs above the upper CI, which suggests that CPR bacteria were negatively affected by the decrease in SRT and resulting crash, which makes sense given their limited metabolic abilities. Group A, composed of Ignavibacteriae and Bacteroidetes, contained 17 MAGs above and 6 MAGs below the upper and lower CIs respectively. These results would suggest the crash positively impacted bacteria with metabolisms similar to Ignavibacteriae and Bacteroidetes.

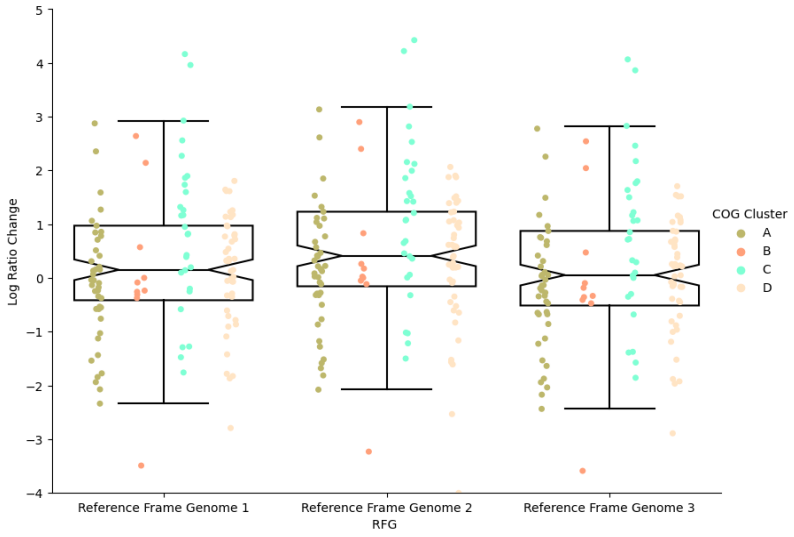


Figure 3.6: Log ratio changes of genomes by COG group, COG groups were determined by hierarchical clustering of COG gene abundance (Figure 3, Appendix 3), and are representative of metabolic similarities between MAGs within the group

The ecophysiology of CPR bacteria remains elusive given their small genomes and lack of central carbon pathways, thus their exact role in anammox bioreactors remains unclear. However, previous research suggests they may subsist off of chitin related substrates derived from anammox biomass and produce fermentation by-products for other heterotrophic bacteria[362]. Based on these observations, the decrease in CPR bacteria in this study could be tied to a gradual decrease in the anammox bacteria, depriving them of their primary carbon source. Ignavibacteriae and Bacteroidetes are known to metabolize high molecular weight organic compounds and reduce nitrite both through denitrification and DNRA. Thus, we hypothesized the capability to utilize high molecular weight compounds may have imparted a selective advantage to certain bacteria in response to the overall community crash.

To determine if specific metabolic functions provided a selective advantage in response to the crash, we evaluated the KEGG module completeness of modules related to synthesis and nutrient acquisition of each abundance group. This analysis was restricted to near complete MAGs (>70% completeness). We checked 27 KEGG modules for amino acid synthesis, 14 modules for lipid synthesis, 36 modules for vitamin and cofactor synthesis, and 39 modules for transporters. We also analyzed the ratio of abundance and absolute abundance (Figure S5) of extracellular and outer membrane carbohydrate active enzymes (CAZymes) and peptidase (MEROPS) superfamilies.

The mean module completeness and extracellular enzyme abundance was compared between MAGs belonging to Group 3 (crash-associated) and the remaining MAGs across all time points. For datasets that followed a normal distribution (Anderson-Darling test $p < 0.05$), a two-sample t-test was performed. For MAG groups not following a normal distribution, the ratio of mean abundance or completeness of Group 3 MAGs to remaining MAGs were calculated and a 90% confidence interval was calculated based on those ratios. Ratios above or below the confidence intervals were considered significantly different than the mean[366,367].

Vitamin and cofactor synthesis were the only modules that followed a normal distribution ($p = 0.17$) and a t-test revealed no statistically significant difference in module completeness between MAGs in Group 3 and the remaining combined groups. Similarly, an equal number of amino acid synthesis and lipid synthesis modules were above and below the CIs. Modules encoding for biosynthesis of aromatic (phenylalanine, tyrosine) and nonpolar aliphatic (valine, leucine, isoleucine) amino acids, which tend to be more biosynthetically costly[363], were lower in completeness while modules for Kdo2-Lipid A, a critical lipopolysaccharide for Gram(-) bacteria, was higher for crash-associated MAGs. Transport modules for metal ions and amino acid precursors were also lower for crash-associated MAGs. Extracellular CAZymes and MEROPS both had ratio CIs above 1; 44 and 25 superfamilies had ratios above the CIs for crash-associated MAGs respectively. Enzymes belonging to serine peptidase superfamilies showed the greatest increase in abundance in crash-associated MAGs being on average 2.4 times more abundant than non-crash MAGs. Based on these results, the SRT crash appears to have selected for bacteria with a higher abundance of extracellular and outer membrane enzymes but a lower capacity to produce biosynthetically intensive amino acids. This indicates community changes that maximize the benefits of obtaining complex biomolecules through extracellular means over the ability to biosynthesize them intracellularly. One explanation for this could be the increase in decaying biomass from anammox cell death which could promote the growth of saprotrophic bacteria like Bacteroidetes and Ignavibacteriae.

To elucidate metabolic disparities that could have aided the formulation of the post-crash community, we also examined the differences in the same set of modules and superfamilies between Group 4 (associated with post-crash recovery) and pre-recovery MAGs (Groups 1,2,3, & 5). Metabolic analysis of modules related to vitamin and cofactor biosynthesis revealed a significant difference in the module completeness for post-crash recovery MAGs (Supplementary Data File 2), compared with all other MAGs ($p < 0.005$, two-sample t-test). This suggests that Group 4 MAGs may have a higher capacity to biosynthesize certain vitamins

and cofactors which could have aided in the recovery of the anammox bacteria to initial levels of abundance and activity. Thus, the rapid recovery of anammox bacterial abundance was likely supported by symbiotic microbial interactions with other community members which could have aided in the rapid nitrogen removal recovery despite the modified community composition. However, because it is not possible to directly infer metabolic activity from metagenomic data, further experimental work would be required to confirm these conclusions.

3.3.3.2 Polysaccharide Cross-feeding

The observed disintegration of flocs and granules and the loss of biomass indicates a decrease in the ability of bacteria inside the bioreactor to aggregate. This aggregation is often facilitated by the synthesis of EPS which requires the polymerization of nucleotide-sugar and amino-sugar precursors into chains of irregular repeating units[364–366] (Fig. 7A). Thus, we hypothesized that the crash may have induced a disruption of the production of these precursors and elongation into heteropolysaccharides.

To evaluate this hypothesis we examined the relative abundance of the top 30 most abundant genes related to amino-sugar and nucleotide-sugar metabolism for all metagenome sampling time points (Fig. 7B). Genes decreasing in abundance during the crash included *glgC*, *GPI*, and *glmM*, and *murQ*, the latter two being important for the formation of UDP-GlcNAcA and UDP-MurNAc, precursors for exopolysaccharide production. UGP2, a gene catalyzing the conversion of Glc-1P to UDP-Glc was already low abundance prior to the crash, likely limiting the formation of UDP-Glc, UDP-Gal, and UDP-GlcA other key nucleotide-sugar monomers. The synthesis of polysaccharides is also constrained by the activity of glycosyltranserases that polymerize amino-sugar and nucleotide-sugar monomers[243]. Many of these genes are phylogenetically concentrated within Chloroflexi which decreased in abundance in response to the crash. Thus, with decreasing production of UDP-GlcNAcA and UDP-MurNAc and a decrease in glycosyltransferase producing Chloroflexi bacteria, the biosynthesis of exopolysaccharides could have been severely hampered.

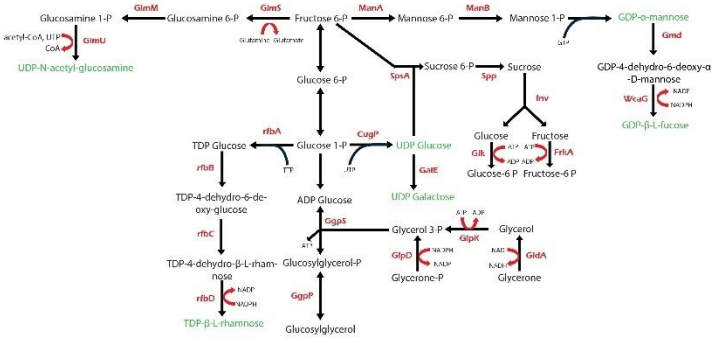


Figure 3.7a: Amino-sugar and nucleotide-sugar biosynthesis pathways

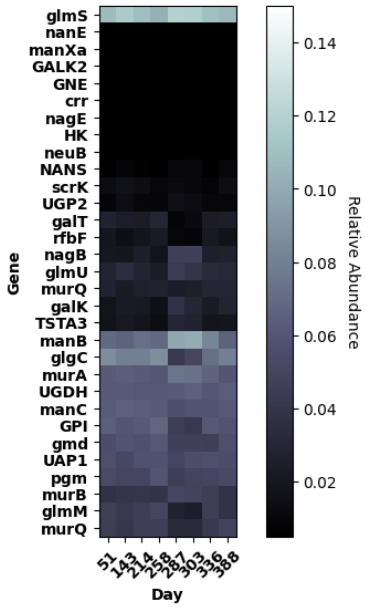


Figure 3.7b: Amino-sugar and nucleotide-sugar biosynthesis gene abundances at each metagenomic sampling time point

Previous research has reported on the unique ecological niche that supports the growth of Chloroflexi bacteria in anammox reactors[367,368]. Many Chloroflexi in the order Anaerolineales have filamentous morphologies[369,370] and the ability to polymerize sugars to produce extracellular polysaccharides that promote cell aggregation[243,371]. Specifically, *Ppgk* and *gtrB*, essential genes for exopolysaccharide production, as well as *cpaf* genes involved in surface adherence are mostly reserved to Chloroflexi MAGs (Figure 5, Appendix 3). Both of these traits are important for the formation and structural integrity of flocs and granules[372,373]. Anammox bacteria are often found within the interior of biomass aggregates, which provides insulation from oxygen and environmental perturbations[374,375]. These structures similarly create substrate gradients, which are conducive to the selection of K-type strategists[378] such as Chloroflexi. This may explain the observed decrease in abundance for both Anaerolinales and Aggregatlineales, both Orders within Chloroflexi, as a result of lowered SRTs. Only 3 of 32 MAGs in Abundance Group 3 (crash-associated) were classified as Chloroflexi. Washout of Chloroflexi, with subsequent deterioration of flocs and granules, could have resulted in the observed changes in biomass morphology. The interior of flocs and granules also provide an environment where nitrite concentrations are limiting due to substrate gradients[376,377]. After the disintegration of aggregates, *Brocadia* could have been left exposed to higher levels of nitrite, which can lead to severe and irreversible nitrite inhibition[332,378]. Compounded with the inability to retain biomass, this toxicity could have resulted in unfavorable conditions for K-type strategists like *Brocadia,* resulting in lower abundance and decreased bioreactor performance.

3.3.3.3 Nitrogen Pathway Dynamics

Given the decline in the nitrogen removal efficiency of the bioreactor during the crash, we sought to evaluate changes in the nitrogen metabolism within the microbial community. The relative abundance of nitrogen pathway genes was calculated for each metagenomic sampling timepoint (Figure 3.8). The most dominant pathways based on gene presence in the bioreactor were anammox (performed by Brocadia, AMX01), nitrate reduction (performed by 82 MAGs), denitrification (performed by 44 MAGs), and DNRA (performed by 34 MAGs). Anammox pathway genes quantified included hydrazine synthase (subunits alpha, beta, and gamma), *hzs*, and hydrazine dehydrogenase, *hdh.* Nitrate reduction was quantified using the abundance of respiratory (*narG*) and periplasmic (*napA*) nitrate reductases. Denitrification genes were independently evaluated for NO_2^- reduction (*nirK*, *nirS*), NO reduction (*norB*), and N_2O reduction (*nosZ*). DNRA pathway abundance was quantified based on the presence of *nrfAH* cytochrome c nitrite reductase, which catalyzes the reduction of nitrite to ammonium[379].

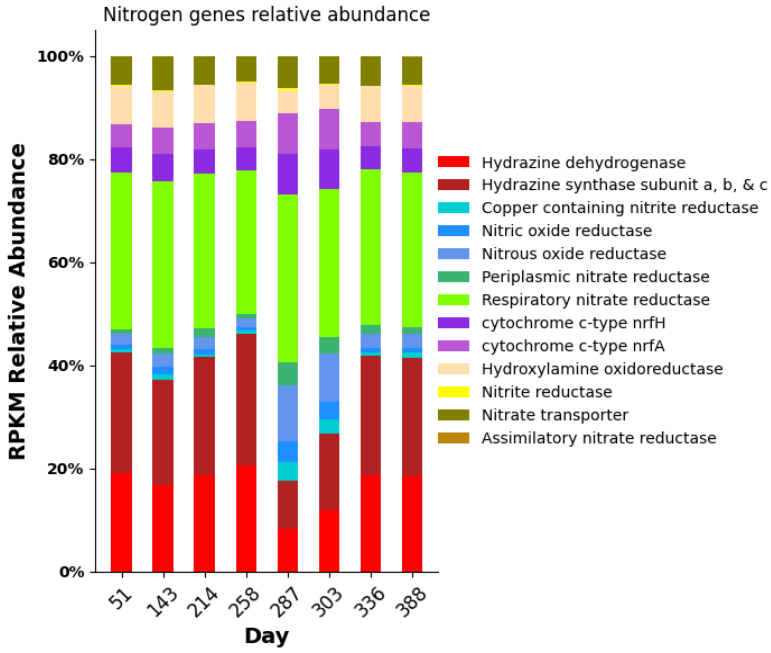


Figure 3.8: Nitrogen pathway genes RPKM relative abundance

Prior to the crash, anammox pathway genes accounted for an average of 41.9% of nitrogen pathway reads. Nitrate reduction genes accounted for 31.3% of nitrogen pathway reads on average. During the crash, anammox pathway genes decreased to an average of 22.2% while nitrate reductases increased slightly to an average of 34.5%. Over this same time period following the crash, genes associated with both DNRA and the individual steps of denitrification increased in relative abundance, with nitrous oxide reduction undergoing the highest fold increase going from 2.1% on average before the crash to 10.1% during the crash (5-fold increase). Of the 46 MAGs that experienced positive significant LR changes, 18 (39% of MAGs) contained genes encoding for *nirK* used for nitrite reduction; 17 (37% of MAGs) contained genes encoding for *norB* used for nitric oxide reduction; 9 (20% of MAGs) contained genes encoding for *nosZ* used for nitrous oxide reduction; and 13 (28% of MAGs) contained genes for *nrfAH* for nitrite reduction to ammonium. Of the 13 genomes capable of performing DNRA, 8 were also capable of performing partial denitrification. Of the 56 MAGs that decreased in coverage below the lower CI, 14 (25% of MAGs) contained genes for *nirK*, 14 (25% of MAGs) contained genes for *norB*, 26 (46% of MAGs) contained genes encoding *nosZ*, and 11 (20% of MAGs) contained genes for *nrfAH*. This distribution is inconsistent with crash-driven selection of either pathway over the other.

The overall effects of SRT on nitrogen removal in anammox systems remains unclear. SRTs below the doubling time of anammox bacteria have a clear negative effect on anammox pathway gene abundance, but they also affect the relative abundance of nitrate reducers, which recycle nitrite back to anammox bacteria; and partial denitrifiers, which remove nitrogen from the system through the complete reduction of nitrite to dinitrogen gas via *norB* and *nosZ*. Lower SRTs can enrich partial denitrifiers over bacteria capable of full denitrification due to kinetic

limitations of some denitrification steps[379]. In this study, the relative abundance of the individual steps in the denitrification pathway, as well as DNRA, increased at lower SRTs, but the relative abundance of nitrate reduction genes remained the same. This could point to a selective enrichment of bacteria capable of performing nitrite, nitric oxic oxide, and nitrous oxide reduction over nitrate reduction. As a result, a bottleneck could form for the conversion of nitrate to nitrite, which would diminish the symbiotic nitrite loop between anammox and heterotrophic nitrate reducers. However, these conclusions are not fully supported strictly from metagenomic data, so future research on the expression of these genes would be needed to provide a complete understanding of the observations.

3.3.3.4 Functional Redundancy and Reactor Performance

Changes in the microbial residence time of a system are known to influence the functional diversity of microbial communities, with previous studies demonstrating a positive correlation between SRT and functional richness and evenness[381–383]. To determine if this trend applied to our system, we examined changes in functional diversity by calculating the relative abundance, richness, and Shannon's diversity index for enzymes classified by the Enzyme Commission (EC) system. The taxonomic richness and evenness was also calculated based on the genus-level organism origin annotation that each protein was assigned to (Table 8, Appendix 3).

The functional richness and diversity in enzyme classes showed modest decreases in response to the SRT decreases (Table 8, Appendix 3) and the relative abundance of EC subclasses was stable, which is consistent with previously reported results[384]. Taxonomic richness and diversity trended in opposite directions over the course of the study (Figure S7). Evenness, measured as Shannon's diversity index, increased on average by 16.9% in highly abundant EC subclasses (>1% relative abundance) and by 10.6% in rare EC subclasses (<0.05% relative abundance). Taxonomic richness decreased on average by 13.4% for highly abundant enzyme subclasses and by 9.6% in rare enzyme subclasses. According to this trend, a small subset of organisms dominate specific functions at higher SRTs but this dominance is conceded at lower SRTs, allowing the enzyme function to be more equally distributed. This points to more organisms contributing to various ecological functions, thus increasing interspecies competition which could explain increases in diversity previously noted.

The observed changes in functional diversity and richness are likely associated with the selection of organisms based on growth strategies, with higher functional diversity and novel functions tied to slower-growing K-strategists[380] This suggests a deterministic community assembly mechanism characteristic of press disturbances, where the system undergoes sustained perturbation. This is in contrast to stochastic processes which predominate at intermediate disturbance levels characteristic of pulse disturbances[381]. This observation is especially salient in comparison to other studies examining anammox reactor pulse disturbances which exhibit much more variable microbial community responses dependent on the intensity and intermittency of the perturbation[382–384].

Despite the deterministic nature of these assembly processes, they still produce divergent trends for functional and taxonomic diversity. Species richness increased, but functional richness

decreased, resulting in a loss of ecosystem function. This conflicts with the expectation that increased biodiversity results in higher functional diversity[385], but is consistent with the hypothesis that lower SRTs result in lower levels of observed functional richness[386]. This could also be partially explained by dynamic reactor conditions temporarily increasing the range of ecological niches in response to the absence of the anammox bacteria as the primary ecosystem regulators. This hypothesis is supported by the observed increase of taxonomic diversity in EC subclasses in response to the crash, which indicates greater functional evenness.

Despite the significant taxonomic changes and loss of ecosystem function, reactor performance recovered quickly to a pre-crash NRR and NRE within 50 days of the initial decline. Restoration of original ecosystem function was not accompanied by the recovery of the microbial community to its original state (Fig. 3.1c and Fig. 3.4a). These results support the functional redundancy hypothesis, which states that environmental conditions are the primary driver for the selection of ecological functions[386]. Under the assumptions of this hypothesis, the community reassembled itself following the reactor crash to maintain the same ecosystem functions. This idea is supported by the analysis of EC subclasses, which revealed that the relative abundances of both common and rare functions remained relatively unchanged between the first and last metagenomic sampling points.

3.3.4 Implications for Reactor Performance

The SRT in a bioreactor is an important governing constraint that determines the kinetic limitations for bacterial growth within the reactor[357,387]. This study sought to illuminate how these limitations impact microorganisms employing different growth strategies by artificially inducing an SRT-driven reactor perturbation. In response to this perturbation, the relative abundance of anammox bacteria decreased dramatically, from 39.8% on day 200 to 1.4% on day 294, according to 16S rRNA analysis. Although *Brocadia* has high doubling times ranging from 5-11 days and would thus be disadvantaged by lower SRTs, these growth rates are still lower than the 28.5 day SRT that resulted in the crash. Thus, the decreased abundance of *Brocadia* alone is not sufficient to explain the crash and loss of nitrogen removal efficiency. A more likely scenario involves a complete consideration of the microbial ecology and interactions that sustain reactor performance. Lower SRTs impart a negative selection pressure on slower growing organisms, which in this study led to the decreased abundance of Chloroflexi. With the loss of Chloroflexi, the anammox lost a key partner in the production of sticky macromolecular exopolysaccharides that aid in cell aggregation. A decrease in aggregation led to the disintegration of flocs and granules, disrupting substrate gradients and exposing *Brocadia* to unfavorable environmental conditions such as high nitrite concentrations. More stringent kinetic limitations resulted in the transient growth of r-strategists such as Rhodospirillales and Sphingobacteriales. Once the longer SRT was reestablished and the microbial community stabilized, functional redundancy and increased circulation of vitamins and co-factors aided in the rapid recovery of reactor performance and anammox abundance.

This hypothesis aims to account for the range of metabolic interactions that are likely disrupted from a sustained press disturbance. Anammox bioreactors are dependent on a delicate network of metabolic interdependencies that determine resilience in response to perturbation[243,248,388].

Anammox bacteria are the primary source of organic carbon in these reactors[268,269,335] and produce a range of metabolites that can be used to support heterotrophic growth[388]. However, anammox bacteria are also reliant on other organisms to provide certain vitamins, co-factors, secondary metabolites, and EPS macromolecules for biomass aggregation[243,247]. This relationship in many ways is analogous to the trophic interactions between autotrophic phytoplankton and heterotrophic bacteria that exchange organic carbon for inorganic nutrients and secondary metabolites[389,390]. Thus, any disturbance that negatively affects organisms performing key ecological functions carries the risk of destabilizing the entire community.

3.4 Conclusions:

In a single-stage anammox MBR, adjusting the SRT progressively from 50 days to 28.5 days led to the destabilization of reactor performance and significantly changed the microbial community. The relative abundance of anammox bacteria decreased from 39.8% on day 192 to 1.4% on day 294 at the peak of the crash. The SRT-driven crash prompted the loss of ecosystem function and the enrichment of copiotrophic Sphingobacteriales and Rhodospirillales. Despite these changes, the reactor underwent a rapid period of recovery, regaining initial levels of NRR and NRE within 52 days of the performance crash. Recovery of ecosystem function was accompanied by the reassembly of the microbial community based not on its original taxonomic composition, but on essential traits for ecosystem function. Growth strategies along with functional diversity and redundancy provide potential explanations for the observed decrease in reactor performance, transient microbial community changes, and post-crash recovery community assemblage. These results demonstrate the response of a stable anammox bioreactor to an SRT induced crash, which can be used to more efficiently monitor and maintain anammox treatment systems for robust nitrogen removal.

Chapter 4: Heterotrophic degradation of anammox extracellular polymeric substances

4.1 Introduction:

Point source anthropogenic nitrogen pollution, such as wastewater treatment effluent discharges, increases reactive nitrogen loading into waterways and disrupts biogeochemical fluxes between atmospheric and terrestrial nitrogen reservoirs[274–276]. One strategy to remove reactive nitrogen from wastewater is anaerobic ammonium oxidation (anammox)[387] which is a biogeochemical process that is estimated to account for up to 70% of fixed nitrogen removal in marine environments[389]. Anammox bacteria, classified into the phylum Planctomycetes[390], deploy a unique metabolism utilizing nitrite as an electron acceptor to oxidize ammonium to dinitrogen gas[239,264,265]. Full scale anammox-based treatment systems have been demonstrated to remove up to 90% of influent ammonium at rates up to 9.5 kg NH_4^+-N/L·d with a lower carbon and energy footprint than conventional sequential nitrification-denitrification processes[393–395]. However, anammox has not been widely deployed in wastewater treatment plants due to a lack of process stability and slow recovery from reactor performance crashes (up to 6 months)[394–396]. These issues are associated with the slow growth rates of anammox bacteria[391] that prevent the accumulation and retention of biomass necessary to facilitate the reaction at the rates needed for viable treatment[391]. This problem is exacerbated by a lack of understanding behind the mechanisms of anammox cell aggregation which is critical for biomass retention and more specifically the bacterial interactions that promote or disrupt this aggregation.

Previous studies have demonstrated that anammox cell aggregation is enabled by cell membrane surface adhesiveness and extracellular polymeric substances (EPS)[392–394]. EPS secreted by anammox bacteria are used to create an adhesive mechanism to induce the growth of aggregates and granules[252,259]. EPS are a complex matrix comprised of different macromolecules which can vary widely in composition depending on the bacterial species, growth strategies, and reactor configurations[260,398]. In anammox reactors, the EPS consists largely of proteins, polysaccharides, humic substances, DNA, and lipids[260,398]. EPS within anammox granules have been found to contain a large amount of hydrophobic side chains as well as alpha-helix secondary structures which are thought to form hydrophobic interactions with surface functional groups on cell membranes to promote aggregation[400]. EPS extracted from anammox granules has also been shown to have high concentrations of long chain polysaccharides, sialic acids, and sulfated glycosaminoglycans which are all negatively charged molecules that can stimulate surface adhesion[400,401]. EPS have been demonstrated to assist in the biosorption of ions and other substrates as shown in Figure 4.1 (Li et al. 2016). Higher concentrations of EPS in reactor biomass have been shown to positively correlate with higher biomass concentrations, settleability, and nitrogen removal rates[400,401]. However, EPS also serves as a significant reservoir of organic carbon for heterotrophic bacteria within the reactor[403].

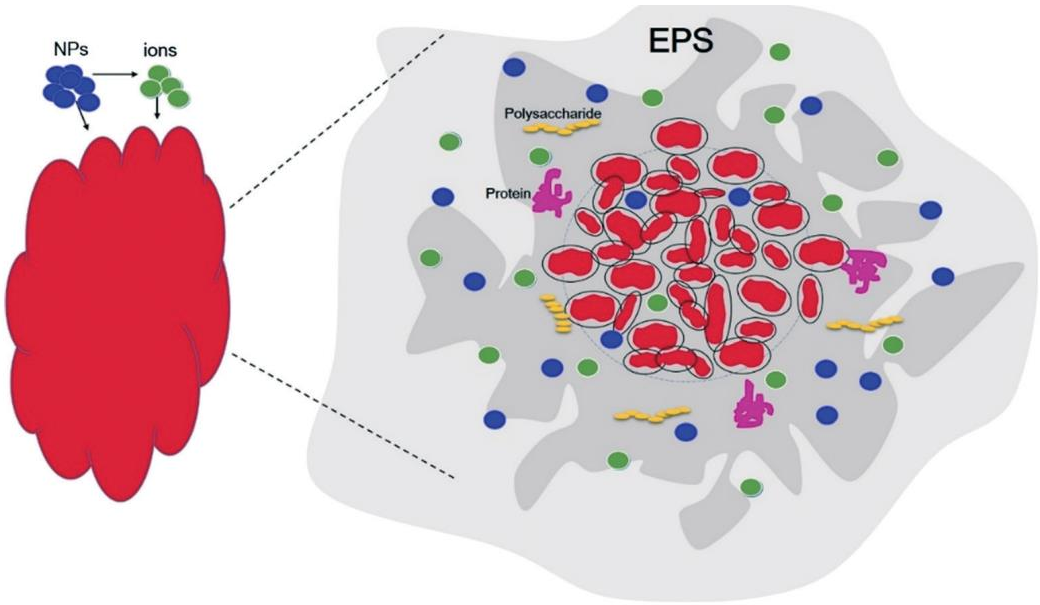


Figure 4.1: Anammox EPS composition, polysaccharides are displayed in yellow, proteins are displayed in red, nanoparticles (NPs) are in blue, ions are in green. Acquired from Li et al. (2019).

Genomic studies have identified bacteria in anammox reactors that have a multitude of extracellular peptidases and glycoside hydrolases that could be used to cleave long chain peptides and polysaccharides[310,321,402]. Some of these bacteria include uncultured members of the phylum Ignavibacteriae as well as Chloroflexi which are both highly abundant in anammox reactor systems[282]. Other less abundant bacteria with high levels of hydrolytic capacity include Bacteroidetes, Acidobacteria, and Actinobacteria which fluctuate in abundance depending on the levels of organic carbon in the system[282,365,403,404]. Bacteria with hydrolytic enzymes could liberate macromolecules from the bulk matrix making smaller oligopeptides and oligosaccharides available to other community members. Thus, the balance of activity between anammox and heterotrophs is important because controlled stimulation of certain bacteria through EPS secretion could support robust community function while overgrowth of bacteria degrading EPS could lead to destabilization of the matrix and disaggregation. This phenomenon has been reported in previous studies where the growth of Ignavibacteriae and Chloroflexi bacteria with multiple extracellular hydrolytic enzymes occurred simultaneously with the loss of anammox cell aggregation and reactor destabilization[403].

In this study, we seek to elucidate the role of EPS as a carbon source for heterotrophic bacteria and the differential contributions of various anammox community members to EPS degradation. This was accomplished by observing the bacterial responses to different EPS fractions extracted from anammox biomass. We evaluate the effects of EPS enrichment on community composition and function through 16S rRNA amplicon and shotgun metagenomic sequencing analysis. The results of this research will help to illuminate microbial interactions between anammox bacteria and heterotrophs that can support or undermine cell aggregation for reactor stability and sustained nitrogen removal.

4.2 Materials and Methods:

4.2.1 EPS Extraction and Characterization

EPS was extracted from a suspended anammox culture cultivated in a 1 L anaerobic membrane bioreactor (MBR) operating under stable conditions (> 3 years without significant disruption). The specific details of initial inoculation and operation can be found in (Keren et al., 2020)[404]. The MBR was supplied with a gas mix (Argon:CO2 = 95:5; 50 mL/min) was continuously supplied to purge the system of oxygen and to maintain circumneutral pH (6.9–7.2) (SI). Temperature was maintained at 37°C using a heating jacket (Eppendorf, Hauppauge, NY) and mixing was provided by an impeller at a rate of 200 rpm. A synthetic media containing ammonium, nitrite, bicarbonate, and trace nutrients prepared anaerobically under nitrogen was continuously fed to the reactor; the exact composition can be found in the appendix.

EPS was extracted from MBR biomass using a modified heat extraction method[405] and an alkaline extraction method[406] designed to extract different fractions of EPS. Briefly, 15 ml of biomass was pelleted by centrifugation at 10,000 *g* for 10 minutes at room temperature (RT) and resuspended in a solution of 5% NaCl (v/v). This solution was then incubated at 70°C and 300 rpm for 30 minutes using a magnetic stir plate and then centrifuged at 5,000 *g* for 20 minutes at 4°C. The resulting supernatant was then filtered through a 0.22 µm polyvinyl filter and then dialyzed using a cellulose dialysis membrane in MilliQ water at 4°C for 24 hours. The resulting solution after dialysis was taken as the heat-extracted EPS fraction (H-EPS). For the alkaline extractions, 15 ml of biomass was pelleted by centrifugation at 10,000 *g* for 10 minutes at room temperature (RT) and resuspended in a solution of 0.6% formamide (v/v) and incubated at 4°C and 300 rpm for 1 hour. Then the pH was adjusted to 13 by adding 15 ml of a 1 M NaOH solution and then incubated again 4°C and 300 rpm for 3 hours. Following the incubation the solution was centrifuged at 10,000 *g* for 15 minutes at 4°C, filtered through a 0.22 µm polyvinyl filter and the resulting supernatant was dialyzed using a cellulose dialysis membrane in MilliQ water at 4°C for 24 hours. The resulting solution after dialysis was taken as the alkaline extracted fraction of EPS (A-EPS).

The chemical composition of both EPS solutions was then assessed using the following analytical methods. The total organic carbon content was measured using a Shimadzu TOC Analyzer. The protein content was measured using the Lowry Method[407], the humic acids measured using the Modified Lowry Method[408], and the polysaccharide content measured using the Phenol-Sulfuric Acid Method[409]. The specific UV absorbance at 254 nm (SUVA254) was calculated by measuring the absorbance of both EPS solutions at 254 nm and then dividing the absorbance by the total organic carbon content. The ratios of absorbance at 254 nm and 203 nm and ratios of absorbance at 445 nm and 665 nm were calculated to assess changes in spectroscopic properties of organic EPS fractions. To evaluate the presence of different functional groups in both EPS solutions, Fourier Transform Infrared Spectroscopy (FTIR) was used. 3D Excitation-Emission Matrix analysis was used to determine changes in the fluorescent properties of organic fractions[410].

4.2.2 Batch Enrichments

Batch cultures were used for EPS enrichment experiments using five treatments: a control inoculated with anammox MBR biomass and fed with regular synthetic media, two negative controls with regular synthetic media not inoculated but amended with H-EPS or A-EPS, and two experimental treatments with anammox synthetic media with nitrite but no ammonium inoculated with anammox biomass and amended with either H-EPS or A-EPS. All conditions were established in triplicate. 50 ml of synthetic anammox media was prepared anaerobically in 150 ml serum bottles based on a recipe modified from Van de Graaf et al. (1996)[409] with an initial nitrogen concentration of 15 mg-N/L and then autoclaved. Control and experimental treatments were inoculated with biomass by extracting 15 ml of biomass from the MBR, washing 3 times with a phosphate buffer solution (PBS), and then axenically inserted into serum bottles with flame sterilized syringes. Following inoculation the headspace of the serum bottles was flushed with a gas mix (N_2:CO_2 = 90:10; 50 mL/min) for 10 minutes to ensure anaerobic conditions. EPS was amended to batch cultures by inserting 5 ml of EPS solution into the serum bottles using a flame sterilized syringe. Batch cultures were then incubated in a shake incubator at 37°C and 150 rpm for 14 days. 2 ml of aqueous solution was extracted from batch cultures and filtered through polyvinyl filters every 2 days for chemical analysis and 1 ml of synthetic media was amended to batch cultures to maintain sufficient levels of electron acceptor. DNA was extracted from biomass destructively sampled from batch cultures at the end of the incubation period.

4.2.3 DNA extraction

Biomass samples were collected every 2–10 days via syringe through an extraction port, flash frozen in liquid nitrogen, and stored at −80°C until further use. Genomic DNA was extracted from the samples using the DNeasy PowerSoil Kit (Qiagen, Carlsbad, CA) as described in the manufacturer's protocol. DNA quality was assessed using a NanoDrop Spectrophotometer (Thermo Scientific, Waltham, MA) and Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). DNA was quantified using a Qubit fluorometer (Thermofisher Scientific, Walthan, MA), diluted to 10 ng/µl with nuclease free water (Thermo Scientific, Waltham, MA), and stored at −20°C until further use. Shotgun metagenomic sequencing samples were sent to the Novogene Sequencing Facility (Tianjin,China). There, DNA was sequenced (150 bp paired-end) on an Illumina HiSeq 2500 sequencer (Ilumina, San Diego, CA).

4.2.4. 16S rRNA gene analysis

The microbial community composition was evaluated by 16S ribosomal RNA sequencing of 28 DNA samples collected throughout the experiment. The V4 region was amplified using primers 515F (5′-GTGCCAGCMGCCGCGG-3′) and 806R (3′-TAATCTWTGGVHCATCAG-5′), with barcodes attached to the reverse primer. Amplicons were pooled at equal molarity and purified with the QIAquick Gel Extraction Kit (QIAGEN Sciences, Germantown, MD). Paired-end sequencing (250 bp paired-end) was then performed on the Illumina MiSeq sequencer (Illumina, San Diego, CA). The full protocol is provided by Wu et al. (2015)[300]. Sequence processing and data analysis was conducted using MOTHUR v.1.47.0, following the MiSeq Standard Operating Procedure (SOP)[408], and OTUs were assigned based on a 97% sequence similarity threshold.

4.2.5. Metagenomic sequencing, assembly, and binning

Metagenomes from metagenome assembled genome (MAG) datasets for the MBR previously reported in Keren et al. (2020)[409] and White et al. (2023)[409] were used in addition to MAGs obtained in this study. Four DNA samples were used for metagenomic sequencing, one from the MBR source inoculum, one bulked from the control batch enrichment triplicates, one bulked from the A-EPS batch enrichment triplicates, and one bulked from the H-EPS batch enrichment triplicates. Resulting sequences from each time point were processed separately based on an adapted procedure reported in Keren et al. (2020)[410]. To briefly summarize, raw sequencing reads were quality filtered using sickle v-1.33 with a quality threshold of 25 and a length threshold of 125 and then assembled into contigs using MegaHit v-1.2.9[411]. Then contigs were binned using MaxBin v-2.2.7[412] and the quality of binning was assessed using CheckM v-1.1.0[413] both with the default settings. Following binning, bins were automatically curated using a script available through ggkbase and then manually curated to remove erroneous bins. Preliminary phylogenetic classification was conducted using PhyloSift v-1.0.1[414] with the default settings. Prodigal v-2.6.3[415] was used to call open reading frames and KEGG Automated Annotation Service (KAAS) was used to annotate predicted gene sequences using Hidden Markov Models (HMM). Diamond v-2.1.8 blastx[416] was used to identify carbohydrate active enzymes from the CAZymes database, peptidases from the MEROPS database, and transporter from the Transporter Classification Database (TCDB) with a E-value threshold of 1e-30. Transmembrane secretion factors prediction used to indicate extracellular enzymes was conducted using SignalP 5.0[417] with the default settings. Coverage was calculated by mapping of raw sequences to obtained MAGs using bbmap v-39.5[418] with ambiguous bases called at random and the minimum identification threshold set to 95%. Relative abundance of MAGs was calculated according to the procedure described in Zhao et al. (2018)[243] and log ratio (LR) coverage calculations were done according to the procedure described in Keren et al. (2020)[404,417].

4.2.6 Statistical Analysis

Confidence intervals (CI) at 90% for LR changes in A-EPS and H-EPS samples were calculated and MAGs with LR changes higher or lower than the CI were considered different from the mean. 16S rRNA gene abundance differential abundance between control and experimental treatments was determined using ALDEx2 v-1.4.0 using a t-test and $p<0.05$ was taken to be significantly different. Kruskal-Wallis test were used to assess statistical significance of CAZymes, MEROPS, and TCDB genes with $p<0.05$ taken to be statistically significant.

4.3 Results and Discussion:

4.3.1 EPS Chemical Characterization

The concentrations of proteins, polysaccharides, humic acids, and total organic carbon content are normalized by the grams of volatile suspended solids (VSS) of the original biomass (Fig. 4.12). Overall, both EPS solutions are largely proteinaceous which is consistent with the findings from previous studies on anammox EPS[258,259,392]. The H-EPS solution had a much higher organic carbon content than the A-EPS (1022 mg/g-VSS vs 9.59±0.20 mg/g-VSS) but the A-EPS was significantly more proteinaceous and had a higher level of aromaticity as indicated by the

SUVA254 (41.40 ± 0.90 L/m-mg vs 7.95 ± 0.18 L/m-mg). The protein to polysaccharide ratio is also higher for the A-EPS (3.22±0.41 vs 2.15±0.60). DNA was not detected in either EPS solution, this suggest low intracellular contamination from the extraction procedures[406,419]. The FTIR spectra (Figure 1, Appendix 4) indicates two major peaks, corresponding to the vibrational frequencies of different bonds, one between 3400-3300 and one between 1700-1600. The peak around 3400 corresponds to O-H and N-H stretching from hydrogen bonds and the peak around 1700 corresponds to C=C and C=O double bonds from peptides[420,421]. The peak around 3400 is more prominent for the H-EPS solution which indicates a great number of hydrogen bonds. This could suggest a different predominant protein secondary or tertiary structure as compared to the A-EPS[421]. 3D-EEM spectra reveal a dominant peak occurring around Em 275-400 and Ex 300-380 for both A-EPS and H-EPS (Fig. 4.23). This emission spectra corresponds to tyrosine-like proteins and the peak intensity corresponds to the concentration of the fluorescent species[410,422]. These results are consistent with the results of other anammox EPS studies utilizing 3D-EEM matrix analysis[258,392,398].

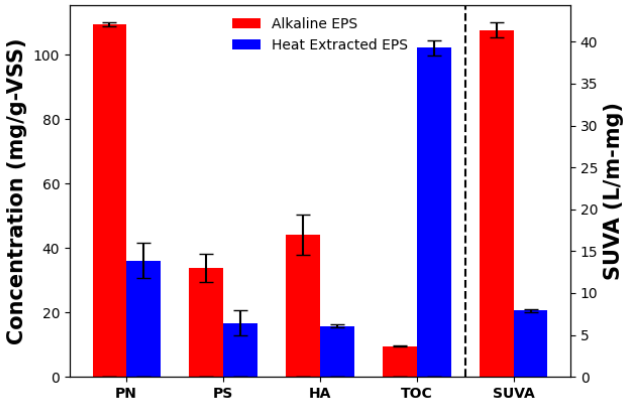


Figure 4.2: EPS Chemical composition for alkaline and heat extracted EPS. Proteins (PN), polysaccharides (PS), Humic Acids (HA), Total Organic Carbon (TOC) and Specific UV Absorbance at 254 nm (SUVA).

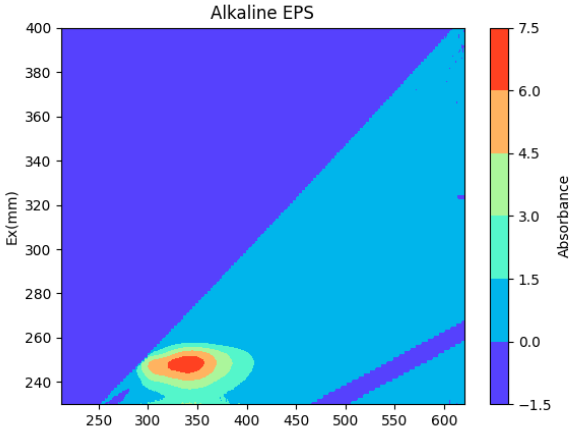


Figure 4.3a: 3D Excitation Emission Spectra for Alkaline EPS

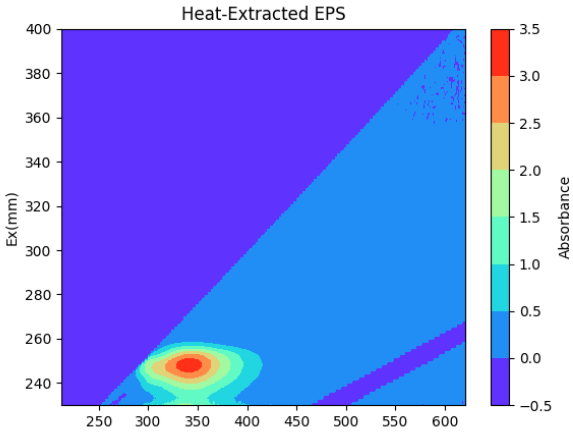


Figure 4.3b: 3D Excitation Emission Spectra for Heat Extracted EPS

4.3.2 Batch Enrichment Results

TOC measurements display a steady decrease in EPS concentrations over the course of the experiment (Fig. 4.4). H-EPS decreased from 42.07±1.31 mg TOC/L on day 0 to 18.00±1.85 mg TOC/L on day 14 while A-EPS decreased from 8.98±0.75 mg TOC/L to 6.43±0.05 mg TOC/L over the same time span representing a 57.1% and 28.4% decrease respectively. This is in comparison to abiotic controls which experienced 16.6% decrease and 16.9% increase respectively. The ratio of UV absorbance at 254 nm and 203 nm (A254/A203), an indicator of increasing aromaticity over aliphatic compounds (proteins and peptides)[423], increased from 0.29±0.01 on day 0 to 0.74±0.02 on day 14 for H-EPS and 0.39±0.01 to 0.72±0.03 over the same time span for A-EPS (SI Fig. 2a & 2b)., Appendix 4). The full UV absorption spectrum (Fig 3a-3e, Appendix 4) also demonstrates a clear shift in the peak from 205 nm (the wavelength peptide bonds absorb at) to 240 nm. The TOC and A254/A203 data may point to a higher degree of biodegradation in H-EPS than A-EPS which would be expected given the higher level of aromaticity detected in A-EPS. Park and Novak (2006)[424] also reported minimal degradation of alkaline extracted EPS from activated sludge flocs under anaerobic conditions which points to a difference in biodegradability due to extraction associated differences in composition.

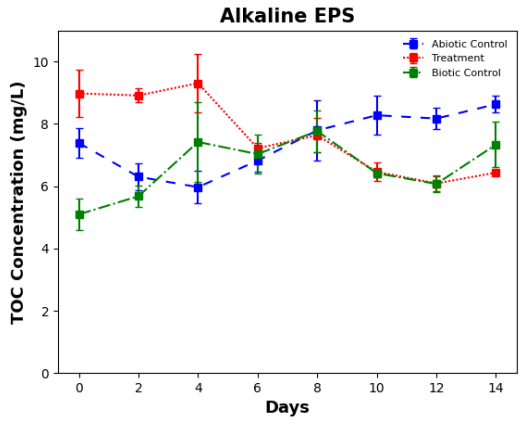


Figure 4.4a: TOC removal for alkaline EPS enrichments

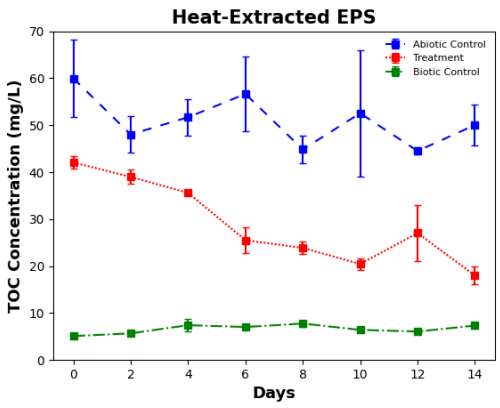


Figure 4.4b: TOC removal for heat extracted EPS enrichments

The higher levels of organic carbon in H-EPS than A-EPS also likely affects the kinetics of the reaction resulting in faster rates of biodegradation in H-EPS. Nitrite concentration data demonstrates a near complete removal of nitrite amended every two days, indicating EPS degradation was likely linked to nitrite reduction (Figure 4, Appendix 4). The differences in degradation of the two EPS solutions parallel the results reported in Zhang and Bishop for biofilms[425] where easily biodegradable EPS is consumed rapidly and more recalcitrant EPS is degraded more slowly. It is not known what fraction of anammox EPS are biodegradable and non-biodegradable but studies on aerobic sludge have estimated that at least 50% of EPS in aerobic granules is biodegradable[426]. This would indicate a significant portion of EPS remains intractable to biodegradation and this fraction could vary significantly depending on the

extraction method. Nevertheless, these results point to a clear decrease in EPS content in batch cultures as a result of microbial activity.

4.3.3 Community Dynamics

Metagenomic analysis revealed a diverse microbial community with the most abundant MAGs classified as Proteobacteria, Ignavibacteriae, Chloroflexi, and Planctomycetes (Fig. 3.4.5). The number of MAGs present at each time point (using Coverage >1 and Breadth>0.5 as a threshold) ranged from 41 in A-EPS and H-EPS samples to 54 in the control sample. A-EPS samples demonstrated modest changes in MAG abundance. The most abundant MAG by coverage, AMX1, the only anammox MAG present, accounted for 24.1% of reads per kilobase per million (RPKM) abundance in the control samples but decreasing to 21.5% in the A-EPS samples. IGN1 and IGN3 increased in relative abundance from 9.78% and 1.57% in the control sample to 13.09% and 2.19% in the A-EPS sample respectively. Strong phylogenetic patterns in coverage changes emerged for MAGs classified as Burkholderiales (5 out of 7 MAGs increased in coverage) and Actinobacteria (4 out of 7 MAGs increased in coverage) based on LR coverage calculations (Table 2, Appendix 4). This is consistent with previously reported studies that have observed increased abundances of these lineages with the availability of large macromolecular compounds[427–430].

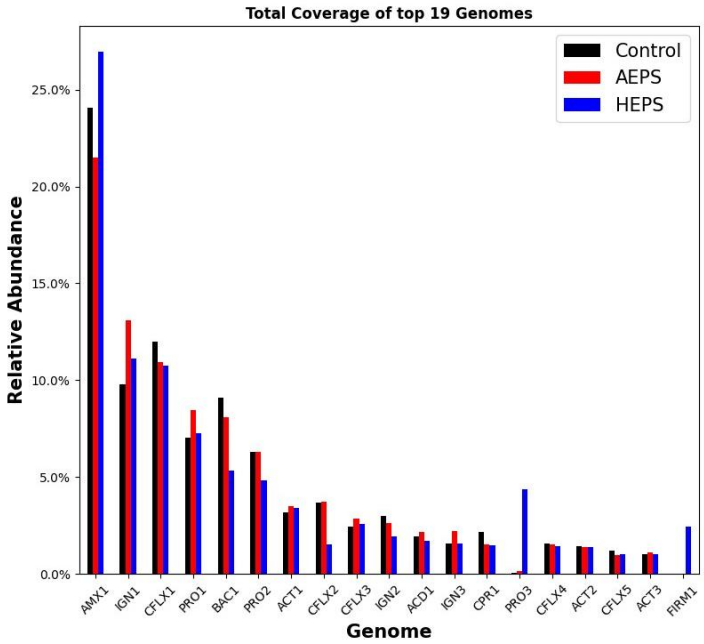


Figure 4.5: Relative abundance of MAG coverage calculated as reads per kilobase per million (RPKM) for the control, alkaline, and heat-extracted EPS enrichments for the top 19 most abundant MAGs

The H-EPS sample demonstrated a distinct difference in microbial community responses as compared to A-EPS. PRO3 and FIRM1 increased in coverage by several orders of magnitude, increasing from 0.07% and 0.01% in the control sample to 4.36% and 2.46% in the H-EPS sample. BAC1 and CFLX2 decreased from 9.08% and 3.69% in the control sample to 5.33%

and 1.52% in the H-EPS sample. More MAGs decreased in coverage (54) than increased (40) based on LR coverage calculations (Table 4, Appendix 4) and Burkholderiales MAGs displayed a congruent pattern in coverage changes with the A-EPS sample. These results indicate that the different EPS solutions differentially enriched various subsets of the microbial community. However, there is overlap with the lineages enriched between the two samples. This may indicate metabolic commonalities in phylogenetic lineages that could impart a selective advantage for metabolizing EPS constituents.

4.3.4 Taxonomic Patterns at the Order Level

Differential abundance analysis using 16S rRNA genes at the order level was conducted to illuminate taxonomic shifts that occurred in response to EPS enrichment. This analysis revealed that distinct taxonomic shifts occurred in both the A-EPS and H-EPS enrichments (Fig. 4.56). Comamonadaceae, Ruminococcaceae, and Acidimicrobiales incertae-sedis all exhibited positive differential abundances in both samples with Ruminococcaceae experiencing the highest relative abundance changes increasing 6.1 and 9.2 fold in A-EPS and H-EPS samples respectively. This is consistent with the results of the metagenome analysis that demonstrated a collective increase in coverage for MAGs in the Burkholderiales, Actinobacteria, and Firmicutes lineages.

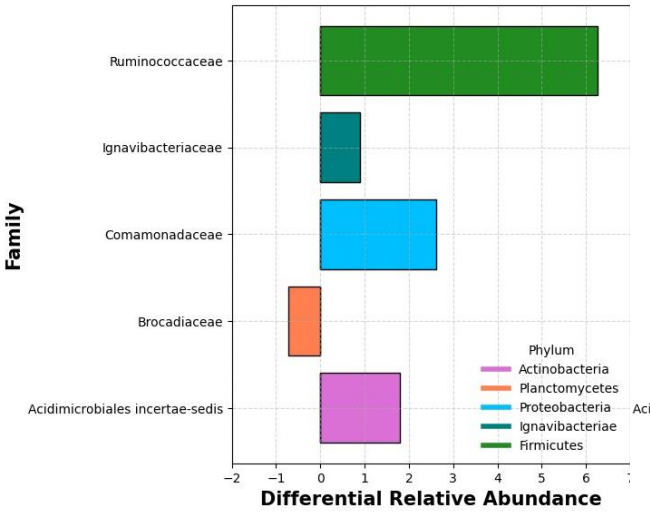


Figure 4.6a: Differential relative abundance of 16S rRNA genes aggregated at the Order level in alkaline EPS enrichments. Only Orders that displayed significant ($p<0.05$) increases or decreases are displayed. Bars are colored by phylum.

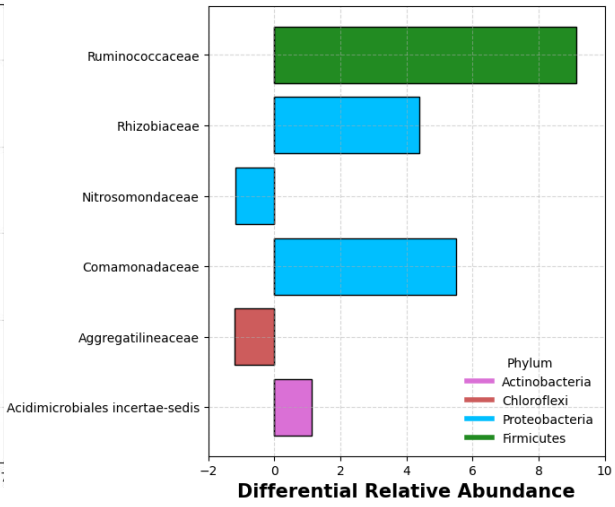


Figure 4.6b: Differential relative abundance of 16S rRNA genes aggregated at the Order level in heat extracted EPS enrichments. Only Orders that displayed significant ($p<0.05$) increases or decreases are displayed. Bars are colored by phylum.

These results also agree with other studies observing the bacterial degradation of EPS in both cyanobacterial and activated sludge microbial communities[431,432]. These observations may be explained by the ability of these lineages to degrade complex organic macromolecules and aromatic compounds. Ignavibacteriaceae increased in relative abundance in A-EPS samples and

Rhizobiaceae increased in H-EPS samples. Ye et al. (2020)[431] also reported increased abundance for Rhizobiales in enrichments with cyanobacterial tightly-bound (TB) EPS. Increased abundance of Rhizobiales in H-EPS may correspond with the lower aromaticity of H-EPS relative to A-EPS which could indicate higher levels of bioavailability. Brocadiaceae decreased in relative abundance in A-EPS samples and Nitrosomonadaceae and Aggregatilineaceae decreased in relative abundance in H-EPS samples. The latter observation is consistent with results reported in Xu et al. (2022)[432] that demonstrated a significant decrease in Chloroflexi abundance during both anoxic and aerobic EPS biotransformation.
These observations differ from those reported in studies on bacterial degradation of diatom produced EPS. Taylor et al. (2013)[433] and Haynes et al. (2007)[434] reported the enrichment of Alphaproteobacteria and Gammaproteobacteria from diatom associated EPS, that consists largely of hexose heteropolymers. The results presented here displayed a diverging trend, of the 7 MAGs classified as Gammaproteobacteria only 3 were present (Coverage>1 & Breadth>0.5) in the A-EPS enrichments and only 1 increased in coverage while 4 were present but none increased in coverage for H-EPS enrichments. This could be explained by the differences in composition between EPS extracted from anammox biomass and EPS extracted from diatoms. Moreover, this supports the conclusion that EPS composition is the primary determinant in the selective enrichment of organisms from the initial inoculum.

4.3.5 Metabolic Cohesiveness in EPS Degraders

Given that the principal components of EPS are large macromolecules with intricate complexes of proteins, glycoproteins, humic substances, and polysaccharides, it would be expected that a distinct assemblage of enzymatic machinery would be needed to metabolize EPS as a carbon source. Since these macromolecules generally exceed the molecular size of compounds that can be transported through cell membranes at viable rates, extracellular enzymes are critical to the capacity of a microbe to access substrates in EPS[435]. To determine if this was the case in the anammox bioreactor, extracellular hydrolytic enzymes including carbohydrate active enzymes and peptidases in each MAG were assessed (Fig. 4.7a).

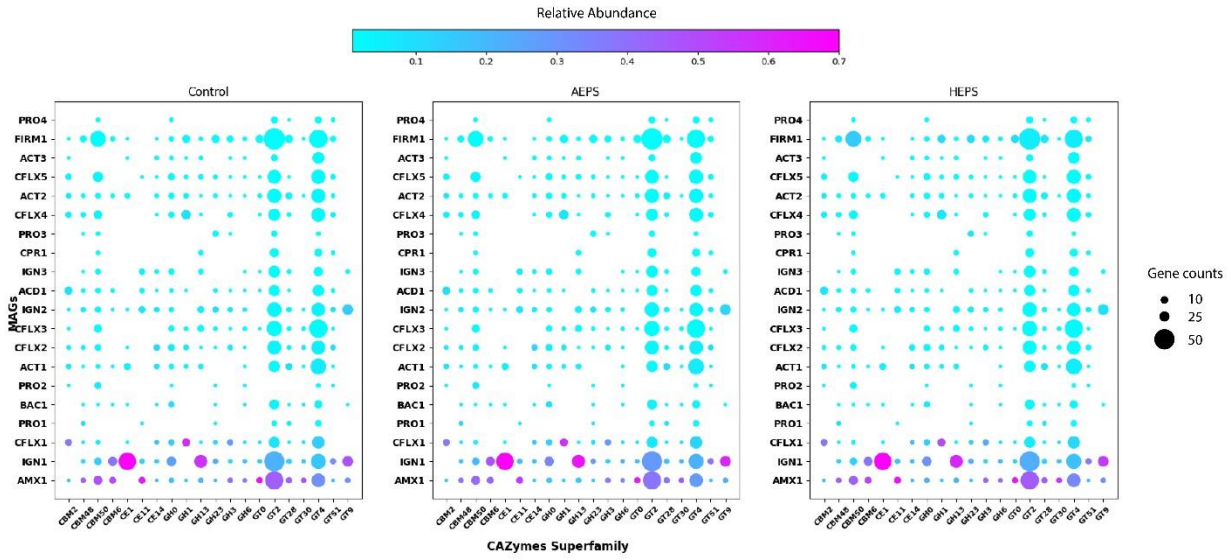


Figure 4.7a: Extracellular and outer membrane CAZymes in top 19 most abundant MAGs. Color indicates relative abundance, size indicates gene counts in each MAG

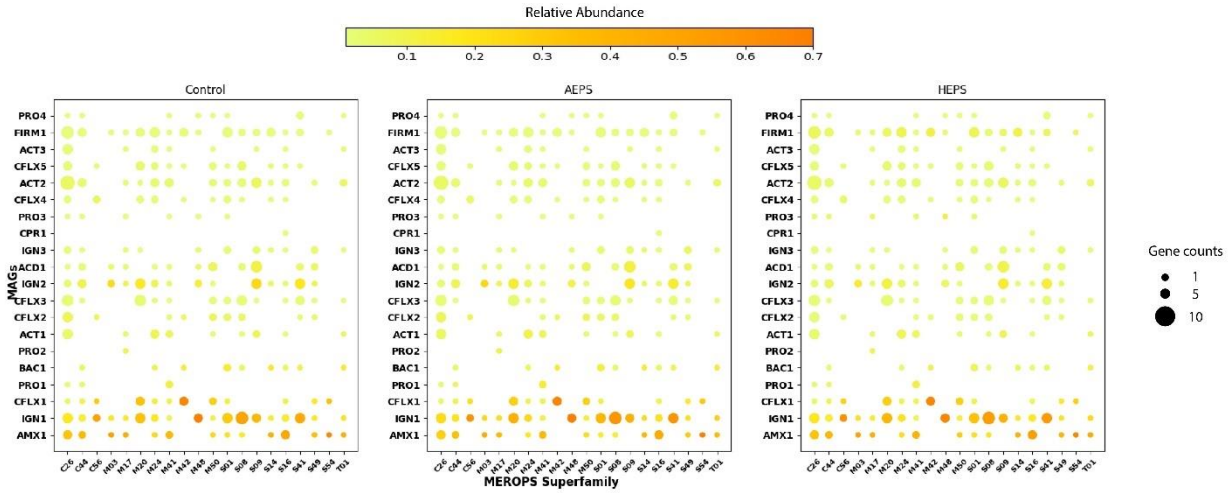


Figure 4.7b: Extracellular and outer membrane MEROPs peptidases in top 19 most abundant MAGs. Color indicates relative abundance, size indicates gene counts in each MAG

The results of this analysis suggest that extracellular enzymes are widely distributed across MAGs of diverse phylogenetic lineages with enzymes belonging to glycosyl transferase (GT) families GT4 and GT8 being the most prominent. These families contain amino-sugar and nucleotide sugar GTs indicating that these enzymes may be widely utilized in the bioreactor. The abundances for different classes of CAZymes and peptidases between MAGs that experienced LR changes above or below the upper and lower CIs were directly compared to identify enzyme classes that were enriched in different samples (Table 6, Appendix 4). The results of this analysis show that glycoside hydrolases (GH) were significantly enriched in both the A-EPS (Kruskal-

Wallis, $p<0.001$) and H-EPS (Kruskal-Wallis, $p<0.05$) samples. Peptidase classes including asparagine, cysteine, serine, threonine, and metallo-peptidases were not significantly enriched in either the A-EPS or H-EPS sample ($p>0.05$). These results suggest that the presence of extracellular glycoside hydrolases imparts a stronger advantage than other CAZymes or peptidases for microbes in the presence of both types of EPS. This would explain the increases in abundance for IGN1 which had multiple extracellular enzymes belonging to GH0 and GH13 families which includes pullulanases, α-amylases, and various monosaccharide transferases. The enrichment of glycoside hydrolases over peptidases in MAGs that increased in coverage despite proteins being the primary constituent of EPS may suggest the significance of glycoproteins. Glycoproteins, proteins carrying glycosidically linked carbohydrate chains, have been demonstrated to comprise a significant fraction of proteins embedded in granular anammox EPS[436]. They have been suggested to make significant contributions to the structural integrity of EPS that is critical to the maintenance of aggregation mechanisms[392,437]. Branched polysaccharides linked to protein chains with various protein secondary structures that promote protein seclusion through hydrophobic mechanisms[259,394] could preclude the accessibility of proteins to bacteria with hydrolytic capacities. Furthermore, previous research has suggested that polysaccharides in EPS contribute more to the molecular mechanisms of aggregation than proteins in anammox biomass[436], calling into question the dominant narrative of adhesion being facilitated by proteins. Given this information, it is possible that the structural reformation of EPS, which is likely a prerequisite for increased bioavailability, may be more dependent on the molecular configuration of polysaccharides. Thus, the ability to cleave carbohydrates glycosidically linked to protein chains may be a more significant resource acquisition strategy in the context of this system. However, further experimentation with the use of proteomics or transcriptomics and analytical assessment of polysaccharide molecular structures would be needed to confirm these conclusions.

4.3.6 Amino Acid & Monosaccharide Uptake Support Growth

Equally paramount to the utilization of EPS as a carbon source is the capacity to transport and uptake exogenously hydrolyzed monomers. To determine if this was a contributing factor to the microbial dynamics of the system, the abundance of Transporter Classified DataBase (TCDB) superfamilies was compared between MAGs that experience LR coverage changes above or below the upper CI (Figure 5, Appendix 4). This analysis revealed that transporters belonging to the General Secretory, Heterodisulfide Oxidoreductase, Fatty Acid Translocation, ATP Binding Cassette (ABC), and Major Facilitator (MFS) superfamilies were all significantly enriched (Kruskal-Wallis, $p<0.05$) in both A-EPS and H-EPS samples. The latter two of which encompass transporter families for monosaccharides, carbohydrates, various types of amino acids, and peptides. This would suggest an enhanced capacity to uptake sugars and amino acids provides a competitive advantage to bacteria in the presence of EPS. Thus, the capability to cleave peptide and glycosidic bonds of EPS macromolecules to generate smaller oligomers alone may not be sufficient to effectively utilize it as a carbon source. The combined ability to hydrolyze large macromolecules and uptake the resulting oligomers and monomers would afford a stronger advantage. Furthermore, the ability to utilize the amino acids liberated from EPS as either an energy source or carbon skeleton for anabolic reactions may also contribute to the fitness of an

organism in the system. Previous research has demonstrated the importance of amino acid auxotrophy to community assembly and function in anammox bioreactors[437]. Thus, the metabolic versatility of an organism to catabolize different amino acids could have a large effect overall on its competitive fitness. This was evident in the pathway completion for different amino acid degradation pathways (Fig. 4.78). IGN1 and IGN3 both increased in coverage in A-EPS enrichments and had higher pathway completions for asparagine and hydrophobic amino acids such as tryptophan, phenylalanine, and tyrosine. FIRM1, which increased in coverage in H-EPS enrichments, had higher pathway completions for polar amino acids such as serine, threonine, and glutamine. PRO3 increased in coverage in H-EPS enrichments, and it also possesses the genes to degrade methionine as do both IGN1 and FIRM1. These results indicate that increased metabolic versatility toward amino acids likely also contributed to increases in abundance for specific bacteria. They also provide evidence for the use of EPS not only as a carbon source but also as an amino acid source for bacteria with amino acid auxotrophies.

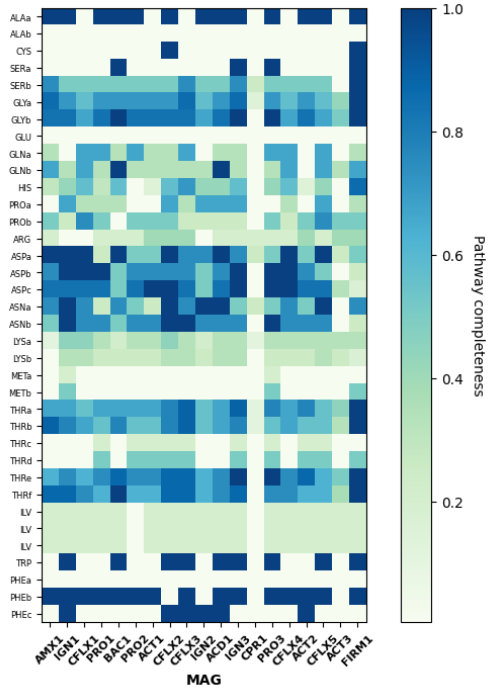


Figure 4.8: Amino acid degradation pathway completion for the top 19 most abundant MAGs. Different degradation pathways are labeled for specific amino acids (a,b,c) and colored by completion.

4.3.7 Study Limitation and Reactor Implications

The results provided in this study illuminate potential microbial interactions occurring between anammox and other heterotrophic bacteria. However, the conclusions of this study are limited by experimental constraints. Most extraction methods in some way alter the chemical composition and structure of the EPS, generating a proxy that at best approximates the intrinsic characteristics of EPS[438]. The EPS supplied to enrichment cultures in these experiments were

also in excess of the EPS intrinsically present in anammox biomass under normal conditions. This would be expected to change the kinetics of the system and favor the growth of organisms that are better adapted to growth at higher substrate concentrations, obscuring the actual dynamics of the system. Furthermore, a positive response by an organism in enrichment cultures does not unequivocally equate to their ability to degrade EPS. Bacteria exhibiting positive responses could be scavengers utilizing hydrolytic byproducts produced by organisms directly hydrolyzing EPS or they could be utilizing microbial necromass generated by an increase in decay following rapid growth. Future studies could aim to circumvent these limitations by leveraging transcriptomics to measure gene expression or isotope tracer experiments to directly track the incorporation of EPS molecules into biomass.

The results presented in this study suggest the ability to degrade anammox EPS may be distributed across a broad range of diverse phylogenetic lineages including Ignavibacteriae, Firmicutes, Actinobacteria, Alphaproteobacteria, and Betaproteobacteria. These organisms occupy different ecological niches within the system both from a resource use and growth strategy perspective. Several Alphaproteobacteria and Betaproteobacteria lineages, especially Comamonadaceae and Rhizobiales, are known to perform denitrification in anammox systems[242,282,439]. Ignavibacteriae have been shown to utilize DNRA[321,325,440] while Firmicutes are thought to rely more on fermentation than respiration[441]. Ignavibacteriae, are thought to exhibit oligotrophic behavior[442], slow growth and efficient resource utilization, whereas Firmicutes, Actinobacteria and certain lineages within Alphaproteobacteria and Betaproteobacteria are more associated with copiotrophy, fast growth and high resource utilization rates[442]. These metabolic and biophysical differences point to niche partitioning which occurs when organisms competing over a similar resource inhabit different ecological niches, lowering competition and ensuring ecological stability[443]. This has many implications on the diversity, function, and succession of anammox microbial communities. Intense resource competition over EPS could lead to competitive exclusion resulting in the extinction of specific groups of organisms which could disrupt the delicate network of metabolic interdependencies needed for sustained ecosystem function. Phylogenetically and metabolically diverse organisms utilizing EPS ensures that community diversity and stability are maintained.

EPS production is a critical trait for the aggregation of cells necessary for the retention of biomass in a bioreactor and the spatial localization of anammox in granule and biofilm interiors that helps to insulate them from adverse environmental conditions. Thus, EPS destabilization could lead not only to biomass washout but also to deleterious exposure to hostile conditions for anammox. The growth of EPS degrading bacteria could have a number of different implications on the stability and function of anammox microbial communities and reactor performance. Previous research demonstrates the importance of metabolic interdependencies between anammox and heterotrophic bacteria predicated on an exchange of vitamins, cofactors, and secondary-metabolites[243,247,261], many of which the anammox lack the biosynthetic pathways to perform. Growth supported by EPS could promote these interactions and ensure the adequate circulation of these metabolites. However, overgrowth of these organisms could result in the deterioration of the extracellular matrix and disintegration of granules and biofilms which could lead to biomass washout. This phenomena has been suggested to be the cause of reported

anammox bioreactor crashes[443]. Thus, finding a balance between stimulation and overgrowth for these organisms and the tipping point between symbiotic and antagonistic interactions with the anammox bacteria is critical to sustained operation of bioreactors. Further studies may endeavor to understand this tipping point by identifying the conditions that enrich these organisms in anammox systems.

4.4 Conclusions

Bacteria present in a single-stage anammox MBR exhibited increases in abundance in response to enrichment on EPS extracted from anammox biomass. Predominantly proteinaceous EPS solutions with protein to polysaccharide ratios of 3.22±0.41 and 2.15±0.60 for A-EPS and H-EPS respectively enriched specific fractions of the microbial community. Shotgun metagenomic and 16S rRNA gene analysis revealed distinct microbial community dynamics under different experimental conditions. Bacteria classified as Firmicutes, Actinobacteria, and Betaproteobacteria were shown to increase in abundance in response to enrichment with A-EPS and H-EPS. Bacteria classified as Ignavibacteriae increased in abundance in A-EPS enrichments and Alphaproteobacteria increased in H-EPS enrichments. MAGs experiencing increases in coverage were also were found to be enriched in extracellular glycoside hydrolase enzymes which may be linked to the cleavage of carbohydrates from glycoproteins. Membrane transport proteins for monosaccharides, carbohydrates, amino acids, and peptides were also significantly higher in abundance for MAGs increasing in coverage. These results demonstrate the growth of heterotrophic microorganisms in the presence of EPS extracted from anammox biomass, which can be used to cultivate a better understanding of the formation, breakdown and life-cycle of EPS. This information contributes towards a more robust understanding of anammox microbial community dynamics and can lead to better operational strategies for resilient reactor performance for nitrogen removal.

Chapter 5: Ecological niche partitioning of metabolically diverse bacteria in anammox bioreactors through trait-based modeling

5.1 Introduction:

Anaerobic ammonium oxidation (anammox) is a microbial process that is estimated to account for up to 50% of fixed nitrogen removal in marine environments[268,269] and is commonly applied for side-stream nitrogen removal in wastewater treatment plants[276,339]. Anammox based wastewater treatment systems have immense potential to reduce nutrient loadings in wastewater sidestreams with lower carbon and energy footprints than other biological nutrient removal technologies such as sequential nitrification-denitrification[444,445]. The system conditions inside anammox reactors characterized by mesophilic temperatures (30-40°C), high total nitrogen (TN) concentrations (>1000 mg-N/L), and low biodegradable carbon (BOD/TN<0.5)[270], creates a unique environment that selects for a distinct microbial community. The bacteria often found inside anammox reactors form a variety of symbiotic and antagonistic relationships with anammox bacteria predicated on an exchange of organic carbon and secondary metabolites as well as competition over nitrogen substrates[243,261]. These metabolic interdependencies constitute an extensive network of material exchange that influence the overall function of the reactor ecosystem. Previous research, primarily based on genomic analyses, have illuminated the various interactions occurring between community members[247,248,281] but has not yet provided a comprehensive assessment to enable process-based modeling of these systems. Despite the strong selective pressure in anammox systems, and anammox bacteria often being the dominant taxa, a phylogenetically and metabolically diverse array of microorganisms are present[282] with functional redundancy within and between reactors.

In these reactors, most of the bacteria that often co-exist with anammox are heterotrophs that deploy a wide range of resource acquisition and energy generating strategies including denitrification[283] and dissimilatory nitrate reduction to ammonium (DNRA)[323,325]. Based on genomic studies, it is suspected that some heterotrophs in anammox enrichments subsist off the degradation of microbial decay products and extracellular polymeric substances (EPS) while others are reliant on anammox for vitamins and biosynthetic cofactors[261,284]. These genomic inferences provide us with an understanding of what bacteria are present and their metabolic capabilities but fail to illustrate the overall microbial ecology of anammox systems. A critical step in understanding the microbial ecology of anammox reactors is discerning, quantifying, and modeling material exchange between bacteria within the community. An analysis of the major metabolite fluxes would allow us to contextualize the functional predictions from genomic studies and identify the molecular interactions between anammox and heterotrophs that are beneficial for reactor performance. Modeling complex systems such as these requires a formal approach to simplifying the complexity of microbiomes, while simultaneously retaining the key mechanisms and their microbial catalysts. Furthermore, anammox bacteria have never been isolated in pure culture and have only been studied in enrichments, necessitating the utilization of population genomics represented through metagenome associated genomes which can produce highly dimensional datasets. One strategy to address the challenges of using metagenomic

datasets is the use of trait-based modeling frameworks to reduce dimensionality, while representing the ecologically relevant traits that determine the fitness of organisms and their contribution to the cycling of carbon and nutrients[446].

Trait-based approaches have been suggested as an alternative to phylogeny-based classifications of microbial communities to highlight the functional traits that contribute to organism fitness and ecosystem function[447,448]. Such approaches can be used to classify anammox associated microorganisms into functional guilds, or groups of organisms that have similar trait profiles[449]. Classifying organisms into functional guilds enables the selection of representative organisms that capture the functional diversity within the system. In doing so, the complexity of microbial communities often represented by metagenomes, which are themselves consolidations of strain level metabolic and genetic diversity, is reduced allowing for the identification of physiological traits that impact microbial diversity and function[356]. This approach can be effective in reducing the dimensionality of complex systems, facilitating analysis of organismal interactions through trait-based process modeling. Trait based analyses have been performed on seaweed[450], marine[451], gut[452], and sponge[453] associated microbiomes, demonstrating the capacity of these analyses to represent functional diversity in low dimensional spaces. Identifying these traits and their relationship to anammox reactor performance is critical to an ecological understanding of microbial community composition differences in anammox reactor systems. Previous research has demonstrated the differences in microbial diversity for anammox reactors with differing biomass types[454,455], suggesting biofilms and granules promote the growth of strictly anaerobic bacteria as opposed to suspended growth which promotes the growth of facultative aerobes[281]. Other studies have examined the functional and taxonomic differences between freshwater and marine anammox systems indicating that marine anammox systems promote the growth of bacteria with higher ammonium affinities and cold stress response mechanisms[456]. Understanding the differences in traits between systems allows for the selection of microbial communities to achieve specific treatment goals (i.e. side-stream treatment, mainstream treatment, industrial wastewater treatment).

In the present study, we curate an extensive database of anammox bioreactor associated metagenomes and seek to characterize the functional trait space of these organisms using *microTrait*, a framework and associated suite of computational tools to identify ecologically relevant microbial traits. In doing so we investigate the drivers of functional diversity in different anammox reactors and elucidate the presence of functional guilds that mediate exchanges of metabolites at the system level. We look to evaluate whether trait based modeling frameworks can adequately capture the metabolic diversity that is relevant to anammox bioreactors. Developing this understanding will allow us to generate modeling frameworks that can accurately represent the bioenergetics of the system, model resource partitioning, and predict the influences of microbial diversity on reactor performance.

5.2 Methods:

5.2.1 Metagenome Assembled Genomes Database Curation

A metagenomic database was assembled and curated from publicly available metagenome assembled genomes (MAGs) uploaded to the National Centre for Biotechnology Information

(NCBI) GenBank database. Searches were narrowed to metagenomic datasets from anammox bioreactor sludge metagenomes. A full list of MAGs and their corresponding BioSample and BioProject accession numbers is available in Table 1, Appendix 5. Initial MAG canvassing yielded a dataset of 1215 MAGs from 17 different reactor systems of varying configurations, biomass growth strategies, and dominant anammox strains. MAGs were curated based on completeness and contamination estimated by CheckM[413]; only MAGS with completeness greater than 70% and containing contamination less than 10% were retained for further analysis. Initial curation yielded 992 MAGs of sufficient quality for analysis.

5.2.2 Trait-Based Analyses with microTrait

Trait based modeling was conducted on the MAG dataset using microTrait[457]. The details of microTrait's conceptual framework and functionality has been previously reported. Briefly, microTrait uses a set of specified Hidden Markov Models (HMM)[458] to identify genes corresponding to ecologically relevant traits. Using a modular rules matrix, microTrait then condenses the HMM matrix into a trait matrix representing nearly 200 traits corresponding to different resource acquisition, resource use, and stress tolerance strategies that are measured either gene counts or binary traits per MAG. Count traits were normalized by MAG size (bp). microTrait also estimates biophysical life history traits for each MAG including the optimum growth temperature and minimum generation time based on genomic imprints across the whole genome. Based on the trait matrix, microTrait then represents MAGs as functional guilds based on a trait covariance threshold determined by the user. For this dataset, microTrait was used with the default settings and a variance threshold of 70% was used to distinguish functional guilds. The only exceptions to the default settings was the use of a lower HMM detection threshold (i.e. trusted cutoff TC) to identify anammox associated genes including hydrazine synthase subunit alpha, beta, and gamma (hzsABC) as well as hydrazine dehydrogenase (hdh) and NADP+ dependent formate dehydrogenase genes (fdh). This was done due to the limited reference database sizes used to generate HMMs for hzsABC, hdh, and fdh which results in the specificity threshold being too stringent to identify potential hits. After preliminary data processing another round of curation was conducted to remove outliers potentially skewing results which reduced the final dataset to 966 MAGs.

5.2.3 Phylogenetic Analyses

Phylogenetic relationships between MAGs were inferred based on a multitude of single copy phylogenetic markers using the genome taxonomy database toolkit (GTDB-Tk) v2.3.2[459]. Taxonomies were assigned to MAGs based on classifications from the genome taxonomy database. A phylogenetic tree was constructed in FastTree v2.1.0[460] using concatenated ribosomal protein alignments obtained from the GTDB-Tk align function. The resulting tree was visualized and annotated in iTOL[461]. Within guild phylogenetic diversity was estimated using the mean nearest taxon distance (MNTD) which is a measure of the average phylogenetic distance of each taxon to its nearest neighbors. The net relatedness index (NRI) and the nearest taxon index (NTI) was calculated by comparing the calculated MPD and MNTD values to that of a null distribution. Bootstrapped phylogenetic trees were generated for each guild using the S3

ribosomal protein using RAxML-NG v1.2.2[462] and the MPD and NRI was calculated for each tree in using the mntd function of the picante package in R[463].

5.2.4 Statistical Analyses

Data ordination to represent the functional diversity of different guilds was conducted using non-metric multidimensional scaling (NMDS) analysis and clustering was conducted using k-means with the vegan and stats packages in R respectively. Clustering quality was assessed using within sum of squares (wss) analysis (Figure 5, Appendix 5)

Multivariate generalized linear modeling was applied to examine functional differences associated with the reactor configuration (i.e. Partial-Nitritation/Anammox (PN/A) vs. Anammox), biomass growth type (i.e. Granular, Suspended, Biofilm), and the dominant anammox strain (Brocadia, Jettenia, Kuenenia, Scalindua). Tests were performed using the "manyglm" function in the R package mvabund v4.1.3[464]. To account for the effects of abundance, count traits were scaled by the relative abundance of the MAG determined by mapping raw reads to metagenomic contigs. A negative binomial distribution was utilized to model continuous count traits and a binomial distribution was utilized to model binary traits. Significant effects for each model were determined using likelihood ratio tests (LRTs) to compare fitted models to null models. Multivariate analysis of variance (MANOVA) and Chi-Squared testing was conducted on individual count and binary traits to determine significant levels of enrichment or depletion in different reactor configurations, biomass types, and dominant strains.

5.3 Results:

5.3.1 Phylogenetic analysis

Following initial filtering of MAGs, 992 met the designated threshold for contamination and completeness (>70% and <10%). Of the 992 MAGs selected after initial curation, 67.4% had an estimated completeness of greater than 90%, and 94.9% had an estimated contamination level of less than 5%, while 64.2% had completeness over 90% and contamination less than 5% indicating high quality population genomes recovered from metagenomic data. Taxonomic analysis of the MAGs utilized in the trait based analysis revealed the occurrence of 34 bacterial phyla and Euryarchaeota as the single archaeal phylum (Fig 5.1). The bacterial phyla represent all of the supergroups including Candidate Phyla Radiation (CPR), Sphingobacteria, Proteobacteria, Planctobacteria, and Terrabacteria. The largest phyla represented in this study include Proteobacteria (230 MAGs), Bacteroidetes (207 MAGs), Chloroflexi (127 MAGs), Planctomycetes (113 MAGs), and Ignavibacteria (46 MAGs) comprising 74.8% of the 966 MAGs included in the phylogenetic analysis. MAGs recovered from reactors dominated by the Brocadia strain of anammox overwhelmingly represented analyzed MAGs (80.9% of the 966 included). Biomass growth type was split with the majority being biofilm associated (59.1%), followed by suspended (27.5%), and granular (13.5%). Reactor configuration was primarily dominated by PN/A reactors (64.7%) in comparison to anammox reactors (35.3%). Interestingly, 56% of MAGs classified as Chloroflexi were recovered from anammox reactors, and 86.5% of MAGs classified as Bacteroidetes were recovered from PN/A reactors, the highest

respective phyla percentages for either reactor configuration. The tree reveals over 80 instances of identical or nearly identical MAGs based on estimated branch length being recovered from different studies with 18 of these instances involving five or more MAGs from different studies.

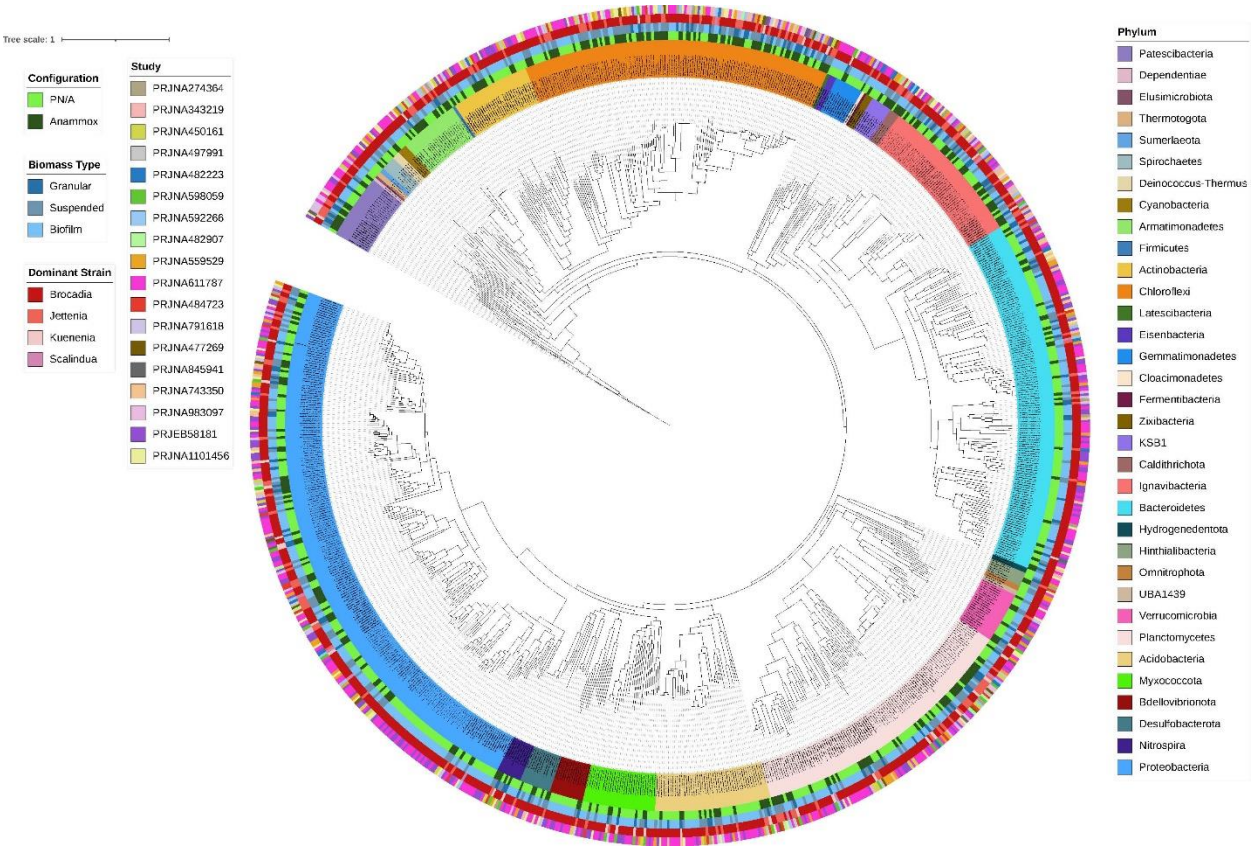


Figure 5.1: Maximum likelihood tree constructed from concatenated ribosomal proteins, inner circle colors indicate phylum (classified from GTDB-Tk), second inner ring indicates reactor configuration, third inner circle indicates biomass type, outer circle indicates dominant strain

5.3.2 Genome-inferred traits associated with anammox bioreactors

Of the 188 functional traits tested in microTrait, 160 were detected with protein degradation and heat shock proteins having the highest prevalence (100%) and hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis, and anoxygenic photosynthesis having the lowest prevalence, only being detected in 2 MAGs. Biophysical life history traits estimated in *microTrait* revealed average minimum generation times and optimum growth temperatures of 6.43 days and 31.57 °C. 241 MAGs had minimum generation times below the 25th percentile (5.47 days) and 223 MAGs had minimum generation times above the 75th percentile (7.59 days) while 241 MAGs had optimum growth temperatures above the 75th percentile (34.06 °C) and 241 MAGs were below the 25th percentile (28.53°C).

To determine if the set of traits identified through *microTrait* could accurately represent the functional differences in the underlying trait space of the analyzed MAGs, a correlation analysis

was conducted to determine independence. Pairwise pearson correlation analysis of trait occurrence in MAGs was calculated revealing the predominant independence of traits from one from another ($|\rho| < 0.2$) (Figure 1 and 2, Appendix 5). A few exceptions to this trend were Resource Use and Acquisition traits including anoxygenic photosynthesis, biopolymer transport, lipid transport, and ammonia assimilation that had moderately negative correlations ($-0.44 < \rho < -0.2$) with various traits. The photosynthetic traits are likely not relevant to the functioning of these systems, although the negatively correlated traits would be indicative of strategic tradeoffs.

After trait correlation testing, a trait matrix was generated demonstrating the clustering of MAGs based on trait similarities (Fig 5.2). Distance metrics were calculated utilizing the Wishart distance metric which accounts for both binary and count traits. Resource Acquisition traits including protein degradation, amino acid transport, and metal ion transport were widely distributed (>90% prevalence). Stress Tolerance traits degradation of misfolded and denatured proteins, and heat shock proteins were also widely distributed while photosynthetic traits, and energy generating traits using arsenic, selenium, and various sulfur compounds were completely absent. Interestingly, Resource Use traits for complete sulfate reduction ($SO_4^{2-} \rightarrow S^{2-}$) was completely absent within individual MAGs, despite numerous MAGs having the capability to reduce sulfate to sulfite or sulfite to sulfide. Iron reduction ($Fe^{3+} \rightarrow Fe^{2+}$) was also rare (<1 % prevalence). Carbon fixation was moderately prevalent, being detected in 133 MAGs with the Calvin cycle and dicarboxylate-hydroxybutyrate pathways accounting for 74.5% of detected carbon fixation pathways. Stress tolerance traits appeared to be the most common amongst MAGs, thus hierarchical clustering placed MAGs together predominantly based on differences in Resource Acquisition and Resource Use traits. Some clusters showed a higher prevalence of Resource Acquisition traits related to ion transport and Resource Use traits related to partial denitrification, while other clusters have a higher prevalence of oligosaccharide transporters and carotenoid synthesis related traits.

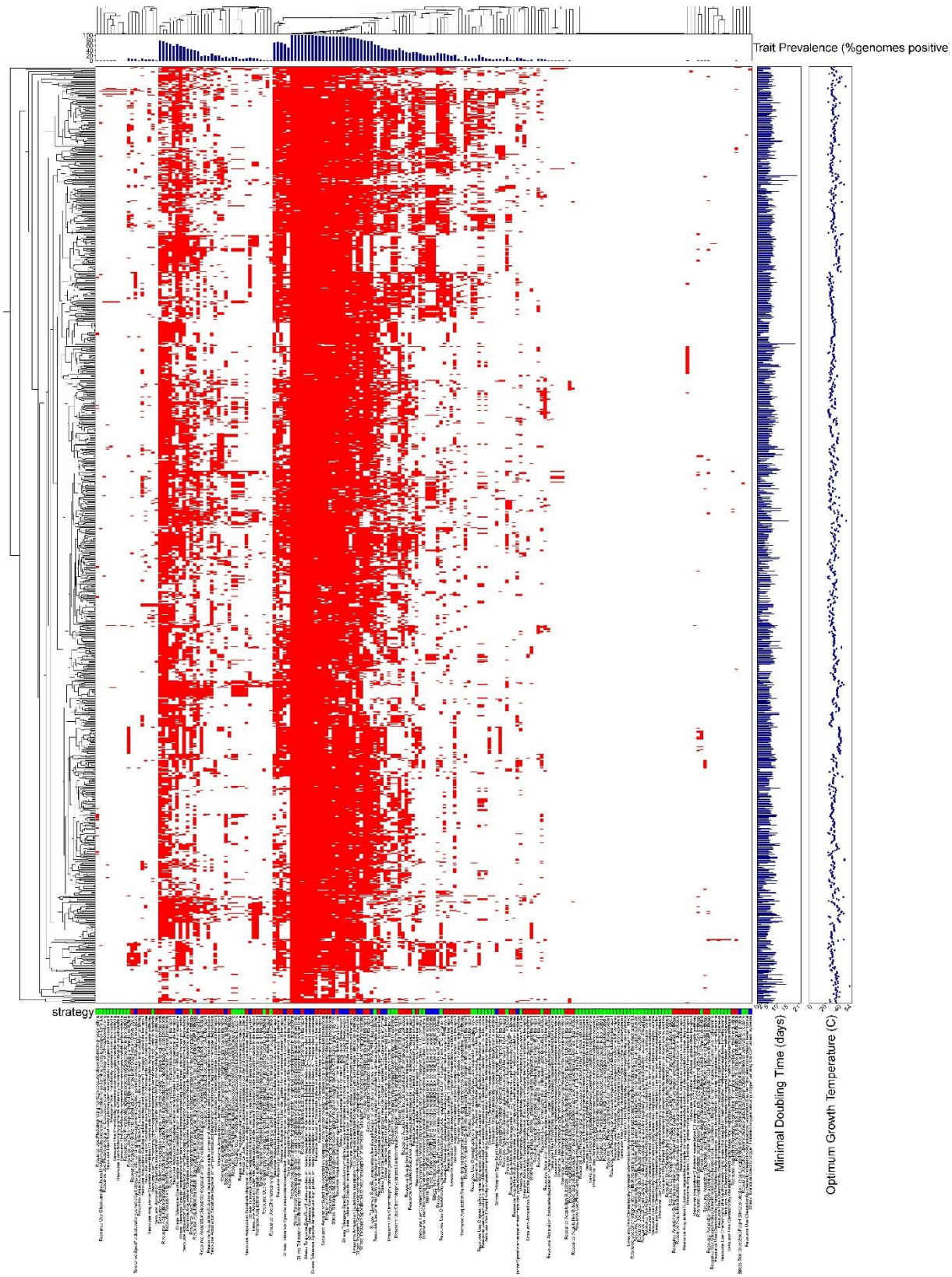

Trait Prevalence (%genomes positive
strategy
Minimal Doubling Time (days)
Optimum Growth Temperature (C)

Figure 5.2: Trait matrix based on presence/absence of traits identified through microTrait for each MAG. Trait prevalence is annotated at the top (scale of 0-100%), minimum doubling time (days) and optimum growth temperature (C) are annotated on the right. Trait type (strategy) is indicated by color on the bottom axis, stress tolerance (blue), resource use (green), and resource acquisition (red)

Based on trait cooccurrence, MAGs were grouped into functional guilds that represent similar ecological functions. This was conducted by calculating the inter-guild variance as a function of the number of guilds. (Figure 3, Appendix 5). In the trait matrix, 142 guilds captured 70% of the inter-guild variance, with the vast majority of guilds containing only one or two MAGs (50% of all guilds) and only 9 guilds containing more than 25 MAGs (Figure 4, Appendix 5) stressing the orthogonal functional diversity represented in the genomes from bioreactors. MAGs belonging to the 9 largest guilds accounted for 42.4% of all MAGs in the study. Average trait occurrences for each guild were calculated to determine functional differences between each guild and the statistical significance of the differences across all guilds was determined based on the null hypothesis that all the trait averages were the same across guilds (Fig. 5.3). Guild 7, guild 6, and guild 21, with 101, 29, and 27 MAGs respectively had similar trait profiles characterized by enrichment in a diverse array of transporters for inorganic ions as well as redox related stress tolerance traits ($p < 0.001$). Guild 8 and guild 15 with 104 and 27 MAGs respectively had similar trait profiles being enriched in nucleoside and peptide transporters ($p<0.001$) but deficient in various sugar degradation pathways ($p<0.001$). Guild 20 is enriched in various traits for inorganic nitrogen and nucleoside transport, pH stress tolerance, and carotenoid biosynthesis. Guild 9 is representative of the anammox bacteria, containing traits for anaerobic ammonium oxidation and carbon fixation through the reductive acetyl CoA pathway. Guild 22 is deficient in several traits related to inorganic ion transport, and nitrogen, sulfur, and phosphorus assimilation but is enriched in hydrogenotrophy and oxidative stress tolerance.

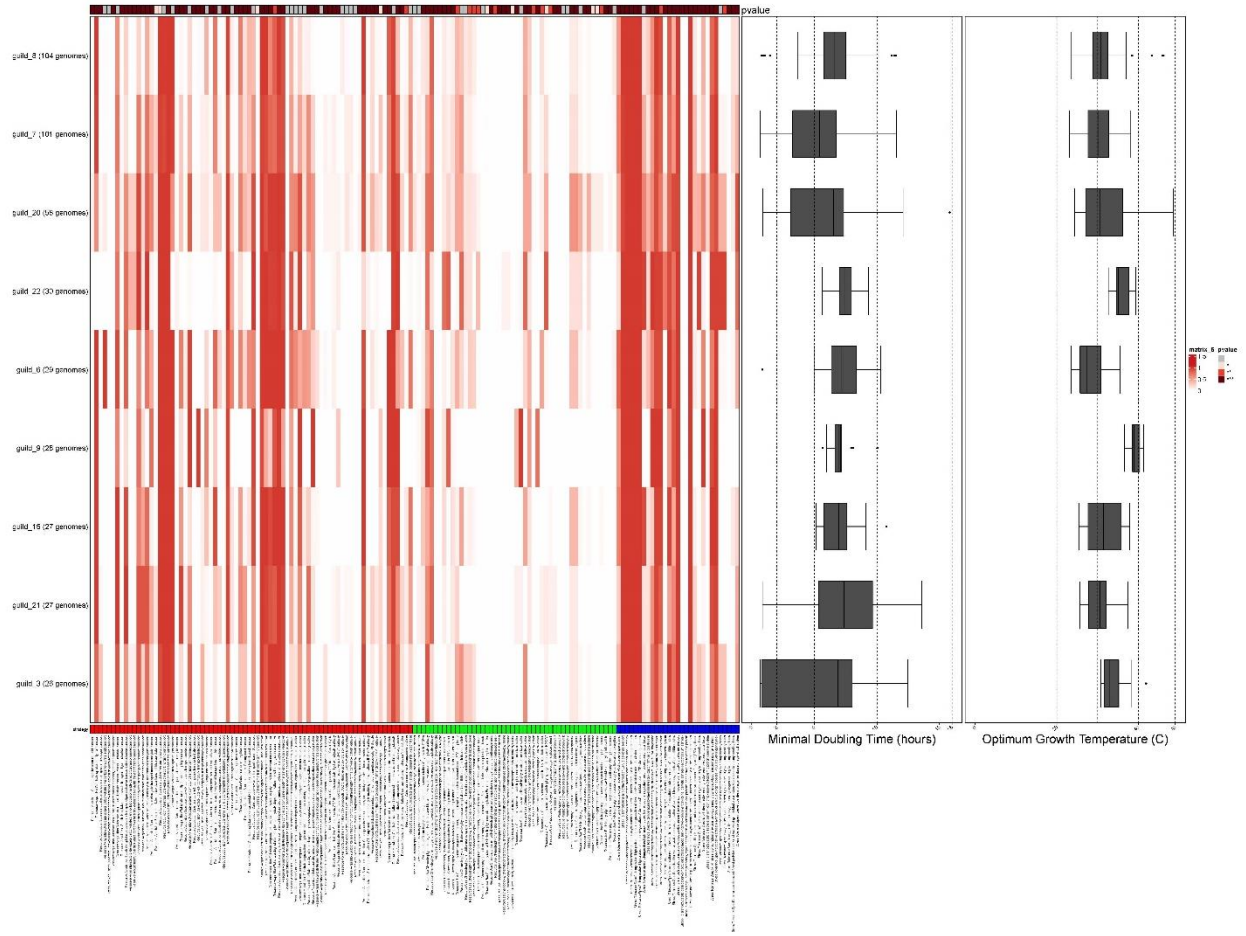


Figure 5.3: Guild trait profiles, colored cells are trait presence averages for each guild, statistical significance (Kruskal-Wallis) is annotated at the top, life history traits mini. Trait type is indicated by color, stress tolerance (blue), resource use (green), and resource acquisition (red)

Biophysical life history traits demonstrated varying levels of convergence for different guilds. Guild 22, guild 21, and guild 6 had the highest average minimum generation times of 7.52, 7.31, and 7.19 days, however MAGs in guild 22 converged tightly around the average (σ=0.86 days) while guild 21 and guild 6 had wider distributions of generation times (σ=3.39 d and σ=1.85 d). On the other hand, guild 7 and guild 20 had the lowest average minimum generation times of 5.03 and 5.65 days but the second and fourth widest distributions of times (σ=3.13 d and σ=2.55 d). Optimum growth temperatures displayed slightly more convergence than minimum growth temperatures ($CV_{avg} = 0.14$ for optimum growth temperature and $CV_{avg} = 0.41$ for minimum generation time). Most average optimum growth temperatures were mesophilic with five of the most abundant guilds averaged between 28.5 and 32.0 °C while guild 9, guild 22, and guild 3 had average optimum growth temperatures of 39.3, 35.8, and 33.8 °C respectively.

To determine the functional similarities of all guilds in relation to the most abundant guilds, average trait occurrences for guilds with three or more MAGs were analyzed using non metric multidimensional scaling (nMDS) producing a two dimensional ordination of trait profiles (Fig. 5.4). Guilds were aggregated into five superclusters using k-means clustering (Figure 5, Appendix 5.) bounded by 95% confidence ellipses.

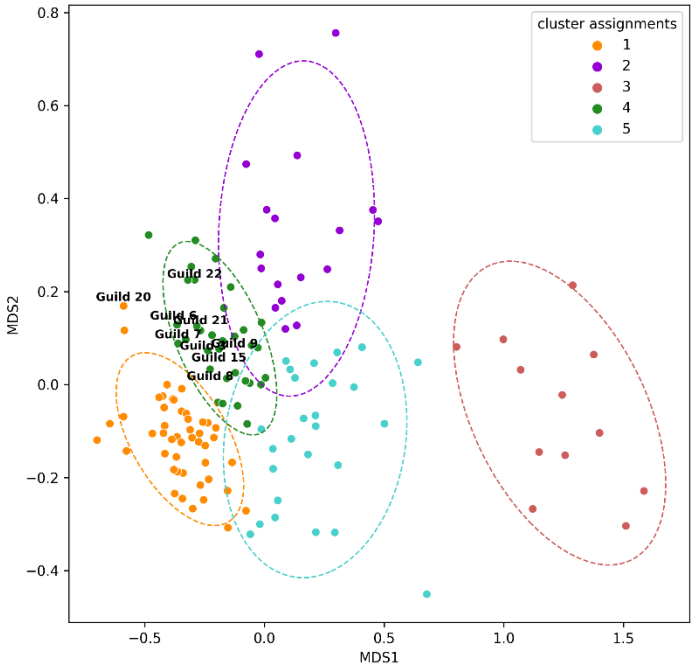


Figure 5.4: Nonmetric multidimensional scaling (nMDS) for guild trait profiles (guild size >3). Data points are colored by supercluster determined from k-means clustering. Ellipses represent 95% confidence intervals

Supercluster 1, which contains guild 20, were enriched in traits for the transport of ammonium, nitrite, vitamin B, and lipids as well as carboxylates, alcohols, ammonia, sulfur, and phosphate assimilation and energy generating traits utilizing the glyoxylate cycle and TCA cycle, electron transport chain, and denitrification. These guilds were also particularly enriched in traits related to PHB cycling as well as stress tolerance traits related to cold stress and oxidative stress but were deficient in complex carbon breakdown pathways. Supercluster 5 containing 3 of the largest 4 guilds is deficient in aerobic respiration traits but is enriched in traits for polysaccharide and oligosaccharide transport, complex carbon breakdown, the EMP pathway, and hydrogenotrophy. Supercluster 3, the most divergent from the rest of the guilds, was predominantly composed of CPR bacteria, known to have small genomes that lack many of the primary catabolic and anabolic pathways normally found in bacteria. Supercluster 4 is deficient in traits for inorganic ion transport, aerobic respiration, and pH and oxidative stress tolerance but is enriched in traits for complex carbon breakdown, peptide uptake, EPS biosynthesis, and mannose degradation. Supercluster 4 contains eight of the nine most abundant guilds and with the exception of guild 22 which skews towards supercluster 2 and guild 8 which skews towards supercluster 5 are more similar in trait composition to each other than the rest of the guilds. This supercluster also includes guild 9 which contains the majority of the anammox MAGs. Overall this supercluster is deficient in several traits related to pH and osmotic stress but is enriched in traits related to the EMP pathway as well as different sugar degradation pathways. Interestingly, the vast majority of MAGs capable of carbon fixation are contained in guilds clustering in Supercluster 1 and 4 indicating a distinctive impact of autotrophy on guild similarity.

5.3.3 Phylogenetic Relatedness of Guilds

To elucidate the degree to which phylogeny underlies guild assignment for different guilds, we have calculated net relatedness index (NRI) and nearest taxon index (NTI) to quantify phylogenetic relatedness among genomes within each guild (Table 2, Appendix 5). To assess the effects of deep branching clades on the phylogenetic structure of guilds bootstrapped mean nearest taxon distance (MNTD) for each guild were calculated (Fig. 5.5). Guild 3 and guild 21 have the highest average MNTD values ($MNTD_{avg}$ = 1.47 and $MNTD_{avg}$ = 0.78) but divergent NRI (-3.60 vs 2.24) and NTI (-0.05 vs 2.02) values. Guild 3 is comprised of MAGs classified as Phycisphaerae, a class of Planctomycetes, as well as Hinthialibacteria, Desulfobacteria, and Armatimonadetes, while guild 21 is predominantly comprised of Phycisphaerae. Guild 6, guild 8, and guild 15 are comprised predominantly of Ignavibacteriae and Bacteroidetes, both phyla of the Sphingobacteria supergroup, however they displayed a range of NRI and NTI values with guild 6 having the lowest values (NRI=3.85 and NTI=3.52) and guild 8 having the highest values (NRI=8.86 and NTI=5.27). Guild 7, the second largest guild, had the lowest NRI value amongst guilds (NRI=0.14) and the second lowest NTI value (NTI=1.82) and was comprised predominantly of Phycisphaerae, Acidobacteria, Myxococcota as well as smaller numbers of Gammaproteobacteria, Chloroflexi, and Bacteroidetes. Guild 20 which contained a mixture of MAGs classified as Actinobacteria, Chloroflexi, Myxococcota, and Pirellulaes, an order of Planctomycetes, had the third highest average MNTD values ($MNTD_{avg}$=0.64) but the third lowest NRI and NTI values (NRI=1.40 and NTI=2.15). Guild 9 and guild 22, both comprised almost entirely of MAGs classified as Planctomycetes and Chloroflexi respectively had the lowest average MNTD values ($MNTD_{avg}$ = 0.02 and $MNTD_{avg}$ = 0.15).

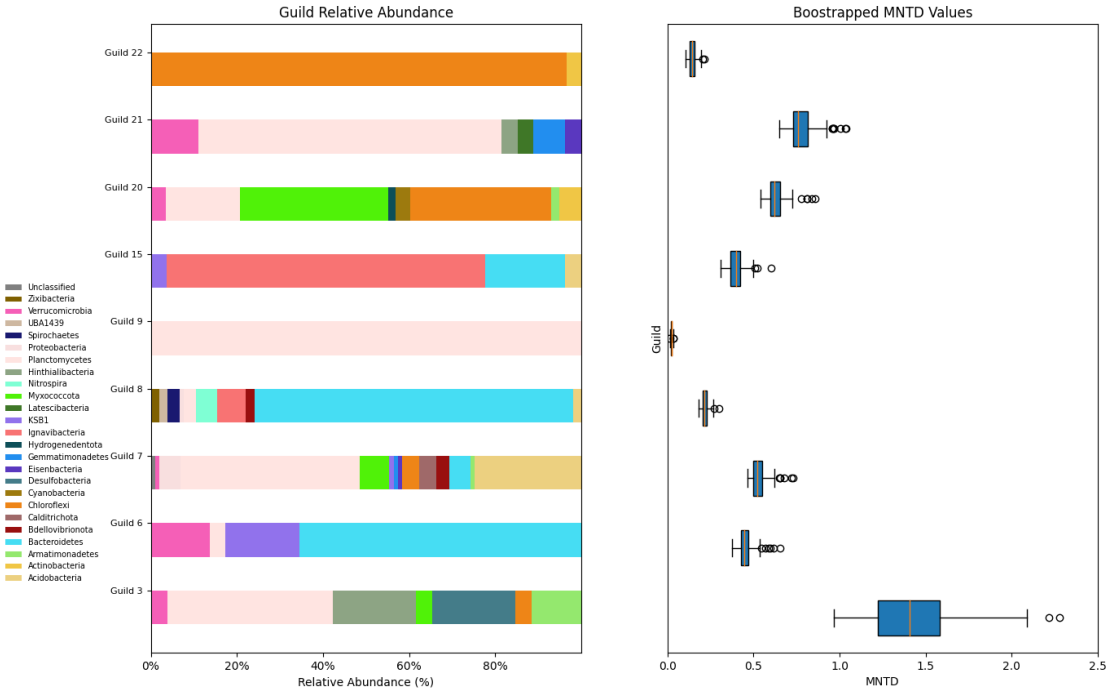


Figure 5.5: Phylogenetic composition of guilds (a) taxonomic composition at the phylum level (classifications from GTDB-Tk) (b) bootstrapped mean nearest taxon distance (MNTD) (n=100)

5.3.4 Trait Differences between reactor types

Multivariate analyses and non-parametric testing were performed to elucidate trait differences occurring between different reactor types. These tests were performed separately on count traits and binary traits producing likelihood ratio test metrics (Table 5.1). Multivariate modeling for count traits demonstrated the significant effects ($p<0.001$) of reactor configuration (PN/A vs. Anammox), biomass type (Granular, Suspended, and Biofilm), and the dominant anammox strain (*Brocadia, Jettenia, Kuenenia, and Scalindua*). MANOVA testing confirmed trait counts were significantly different ($p<0.05$) for 27 and 25 traits between different reactor configurations and biomass types but only 14 for dominant strain. Significantly different traits in different biomass types included Resource Acquisition traits for inorganic ion transport, oligosaccharide transport, and stress tolerance traits for temperature, reactive oxygen, pH, and osmotic stress. MAGs associated with suspended biomass demonstrated higher instances of stress tolerance traits for temperature as well as phage resistance which could be indicative of abiotic stresses present in suspended cultures. Traits significantly different in different reactor configurations included Resource Acquisition traits for oligosaccharide transport, secondary metabolite transport, and complex carbohydrate depolymerization as well as stress tolerance traits for temperature, pH, and dessication (Table 2-4, Appendix 5) possibly indicating a different set of abiotic stresses. Multivariate modeling for binary traits indicated the strain, reactor configuration, and biomass type had a significant effect ($p<0.05$) on trait presence and absence. Chi-squared testing indicated the largest differences ($|\chi^2|>1$) occurred in Resource Use traits for anaerobic and aerobic ammonia oxidation, with MAGs in Jettenia dominated reactors being particularly deficient but being enriched in Resource Acquisition traits for PHB cycling (Fig. 6a-6c, Appendix 5).

	Count Traits		Binary Traits	
Predictor	LRT	P-value	LRT	P-value
Configuration	22.93	0.001	8.97	0.092
Biomass Type	15.97	0.001	7.49	0.064
Dominant Strain	17.19	0.001	6.87	0.031

Table 5.1: Multivariate analysis of system factors influencing trait prevalence. Likelihood ratio tests (LRT) and p-values are reported for each category.

5.4 Discussion

5.4.1 Ecological Strategies in Anammox Reactors

Anammox reactors are a unique environment that select metabolically and phylogenetically distinct assemblages of bacterial and archaeal organisms. These organisms harbor traits amenable to surviving inside of anammox reactors in anaerobic, carbon-limited, and mesophilic conditions. Based on ecological theory it is expected that these organisms would develop different niches that would allow them to co-exist in highly competitive, high stress, resource poor environments[465]. The trait-based analysis conducted here supports and expands this idea by positing the significance of functional guilds in resource acquisition and utilization. This is most

apparent in the diversity of carbon utilization strategies that is evident in the functional guilds identified through *microTrait*. In anammox reactors, organic carbon is generated almost exclusively through chemoautotrophic carbon fixation by anammox bacteria and AOB[220,222], thus carbon dynamics between autotrophic and heterotrophic bacteria contribute substantially to ecological dynamics within the reactor. The ability to fix carbon amongst MAGs marks a clear distinction in guild clustering that drives ecological niche partitioning. A substantial proportion of carbon fixed by the anammox especially goes towards the production of extracellular polymeric substances (EPS)[252,466] which has been reported to be a complex mixture of predominantly proteins and polymerized carbohydrates as well as smaller amounts of lipids, DNA, and aliphatic and aromatic humic compounds[259,260,436]. Thus, there are many bacteria that have traits specifically adapted to hydrolyze polymerized molecules and uptake the resulting monomers and then ferment through the EMP pathway to various end products[261,402]. Guilds in supercluster 2 and 4 appear to fit this description with both being enriched in traits for glucan, heteromannan, and xyloglucan breakdown as well as the EMP pathway. However, they follow different strategies that would seem to indicate the utilization of different resources. Supercluster 5 is more enriched in peptide transport, whereas supercluster 2 is more enriched in oligosaccharide and polysaccharide uptake as well as pathways for fructose, maltose, mannose, and trehalose degradation. This could indicate guilds in supercluster 4 being more adapted to the degradation of EPS which previous research has shown to be comprised predominantly of proteins[392,393,399]. Supercluster 2 may be more adapted to breaking down cellular decay products from dead cells such as cellulose and amino sugar polymers which is evidenced by their enrichment in various oligosaccharide and polysaccharide transporters and sugar degradation pathways. Supercluster 1 appeared to be more invested in the utilization of simple carbon compounds coupled to aerobic respiration or denitrification, being enriched in traits for the transport of aromatic amino acids, carboxylates (mono-, di-, and tri-), sugar alcohols as well as the Enter-Doudnoff pathway (trades-off for lower yield with lower protein cost) for simple carbon utilization, but are deficient in various complex carbohydrate depolymerization traits. The utilization of the ED pathway has been suggested to be more common in facultative anaerobes and aerobes to limit unnecessary protein biosynthesis to maximize growth, which would suggest lower minimum generation times[467]. The guilds of supercluster 1 are likely adapted to metabolic versatility being able to switch between aerobic respiration and anaerobic denitrification, being enriched in traits for the glyoxylate cycle, TCA cycle, electron transport chain as well as traits for partial denitrification to nitrite, nitric oxide, nitrous oxide, and dinitrogen.

The ecological niches inferred from the metabolic differences between guild superclusters closely aligns with reaction networks hypothesized from metagenomic studies of anammox microbial communities. Zhao et al. (2019) posited a reaction network premised around the production of organic carbon by the anammox bacteria in the form of EPS and cellular detritus that was then fermented by anaerobic bacteria to fermentation byproducts that were utilized by denitrifiers[468]. This reaction network is informed by the work of Chu et al. (2015)[469] and Lawson et al. (2017)[261] which both highlight the contributions of anaerobic bacteria capable of utilizing EPS and other cellular decay products produced by the anammox as both energy sources and carbon skeletons for anabolic processes. Xiao et al. (2022) also demonstrated the activity and

carbon assimilation from soluble microbial products (SMP) by heterotrophic fermenters[388]. This reaction network is also consistent with experimental studies suggesting the role of readily biodegradable fermentation byproducts in the growth of heterotrophic denitrifiers[470,471]. Also posited within this reaction network is hydrogenotrophic methanogenesis supported by the production of hydrogen from complex carbon fermentation which could then support methanotrophic denitrification. However, in this study, traits related to methanogenesis were only detected in 2 archaeal MAGs (*Methanomicrobia* and *Methanosarcina*), which suggests that methane metabolism may not be a key determinant in the microbial ecology of the system

5.4.2 Phylogenetic Conservation of Traits

Analysis of within guild phylogenetic diversity indicated varying levels of metabolic coherence amongst different taxonomic groups. This is indicative of the degree to which various traits are phylogenetically conserved and if taxonomy has strong effects on trait profiles. Many traits that were low prevalence (<2% prevalent) had strong phylogenetic convergences. Traits such as anammox and aerobic ammonia oxidation have high levels of phylogenetic conservation which is evidenced by the lower average MNTD and higher NRI and NTI values for guild 9 which is comprised exclusively of anammox bacteria, while guild 12 was comprised exclusively of MAGs classified as Nitrosomonas. Traits for the utilization of dimethyl sulfoxide (DMSO) as an electron acceptor and tetrathionate utilization were strongly phylogenetically conserved to guilds 22 (NRI=11.0 and NTI=4.55) and 106 predominantly comprised of Chloroflexi and guild 106 comprised exclusively of Burkholderiales. Other traits also had strong phylogenetic conservation but were not determinants of guild classification, sulfur oxidation traits were observed predominantly in MAGs classified into the order Burkholderiales, ferric iron respiration was restricted to Proteobacteria, galacturonate degradation traits were found almost exclusively in Chitinophagales, and carotenoid related traits were limited primarily to Chloroflexi. However, these MAGs were split across several guilds indicating that these traits did not have the strongest impact on guild classification.

Varying levels of phylogenetic conservation for traits indicate differences in niche spaces occupied by various guilds[472]. The system conditions of anammox reactors select for a distinct assemblage of traits that are highly prevalent across MAGs of varying taxonomies including stress tolerance traits particularly for biofilm formation and osmotic stress as well as complex carbon depolymerization for cellulose, chitin, and heteroxylan. This is consistent with previous studies that have observed this functional redundancy in anammox reactor systems[243,248,473]. However, according to ecological theory resource competition constrains functional redundancy, therefore metabolic diversity must be present in order to define different niche spaces[443]. Rare and highly conserved traits help to differentiate organisms in their resource acquisition, use, and stress tolerance strategies[474]. In anammox reactors the most desirable resources that maximize energetic tradeoffs of acquisition and utilization are simple carbon compounds and various nitrogen species like nitrate and nitrite, making the competition for these resources high in relation to other nutrients. Thus, organisms that are able to utilize different resources such as sulfur, iron, or methane have the competitive advantage of metabolic specialization that allows

them to operate independently from highly competitive resources. This is especially pertinent considering the carbon limited conditions often found inside of anammox reactors.

Guilds in supercluster 5 and guild 8 were predominantly comprised of MAGs classified as Ignavibacteriae and Bacteroidetes[261,475]. The traits associated with these guilds including peptide uptake and fermentation are consistent with the observations of previous traits that align these organisms with the hydrolyzation and fermentation of proteinaceous substrates for energy generation[354,476]. Guilds in supercluster 1 are predominantly composed of Proteobacteria which contains a substantial amount of both phylogenetic and metabolic diversity that precludes a strong functional convergence amongst MAGs. Guilds from supercluster 3 including guilds 9, 12, and 48 strongly converge around traits that have high levels of phylogenetic conservation including carbon fixation and aerobic and anaerobic ammonia oxidation. Guilds from supercluster 2 are the most diverse containing mixtures of MAGs classified as Chloroflexi, Actinobacteria, Desulfobacteria, and Verrucomicrobia and are enriched in traits related to complex carbon depolymerization and sugar degradation. This could be related to the scavenging of necromass which recycles carbon in anammox bioreactors and is performed by a variety of metabolically and phylogenetically diverse bacteria[477].

5.4.3 Reactor conditions contribute to the divergence of trait prevalence

While taxonomy may be a significant contributor to trait differences between guilds, selection under distinct reactor conditions would drive significant trait variations. Reactor configuration and biomass growth type are the most significant factors explaining differences in count traits but also likely contribute to differences in binary traits. The trait differences that emerge between PN/A and anammox reactors are based on the environmental conditions that change between an aerobic and anaerobic system[321]. Complex carbon depolymerization and oligosaccharide and polysaccharide uptake are significantly enriched in anammox associated MAGs relative to PN/A while PN/A MAGs appear to be slightly more enriched in aerobic respiration traits. This would indicate that the degradation of complex carbon is more ecologically relevant in anammox reactors as opposed to PN/A systems where aerobic conditions may favor the utilization of simple organic compounds. The degradation of complex carbon compounds is likely related to anaerobic metabolisms such as fermentation that would be more selected for in anammox reactors. Aerobic respiration being enriched in PN/A reactors is consistent with previously reported studies highlighting the metabolic versatility of organisms aerobic systems like PN/A[287,478].

MAGs associated with biofilm and granular anammox systems demonstrated significant enrichment in traits related to complex carbon depolymerization, peptide uptake as well as various nitrogen and sulfur transporters. This aligns with the selection of organisms capable of using complex macromolecular compounds present in EPS that would be in higher abundance in biofilm and granular systems in relation to suspended growth systems. This was also the case for MAGs in reactors dominated by Brocadia as opposed to other anammox strains which could be related to the tendency of Brocadia to form flocs and granules as opposed to Jettenia and Kuenenia which are often found in suspended growth systems[479,480]. Differences in transporter abundance may be related to the substrate diffusion limitations inside of biofilms in granules as

opposed to suspended growth[481]. MAGs associated with suspended growth systems were significantly enriched in stress tolerance traits related to degradation of misfolded proteins and high temperature stress but were deficient in traits related to reactive oxygen scavenging, desiccation, and pH stress which suggests different environmental stresses in anammox reactors of different biomass configurations.

Energy availability associated with different redox conditions is a significant driver of functional diversity in microbial systems and results in selection of different microbial growth strategies[482] . In anammox reactor systems, the redox conditions are likely a primary driver of metabolic diversity[321]. Anammox reactors as well as granular and biofilm reactors promote the growth of organisms that are well adapted to anaerobic lifestyles whereas PN/A and suspended growth systems likely encourage the enrichment of organisms with metabolic versatility to switch between aerobic and anaerobic growth[321]. However, different biomass growth conditions also likely impart different stresses and can even modulate stress responses amongst different organisms. Biofilm systems have been demonstrated to be more resilient to temperature stress and pH stress perturbations than suspended growth systems[382,483]. This is due to the conditions of the biofilm creating a barrier that can insulate organisms in the interior of the biofilm to adverse environmental conditions in the bulk solution[391]. This may promote the depletion of different stress tolerance related traits in biofilm and granular systems due to the growth strategy inherently provided a stress tolerance mechanism. However, the effects of different stresses also influence the dominant anammox strain. Different strains have been demonstrated to be more resilient to environmental stresses such as pH and osmotic stress[274]. Scalindua specifically is more adapted to survive in high osmotic stress conditions, dominating anammox activity in marine systems[484,485]. Strain differences also likely influence resource acquisition traits, as different strains have the capability to synthesize different metabolites and form various metabolic interactions with other heterotrophic bacteria[486,487]. This is indicative of strain level variation that occurs for highly conserved traits[488], a trend observed for different anammox strains for kinetic, stress tolerance, and resource utilization traits[485,489]. Thus, reactor configuration and biomass growth type are likely larger drivers of stress tolerance and resource utilization traits, while strain differences are likely more significant for resource acquisition traits. However, more research with a larger diversity of anammox strains would be needed to confirm this hypothesis.

5.4.4 Growth Strategies in Anammox Reactors

The conventional paradigm of oligotrophy and copiotrophy, describing organisms as being adapted to either resource poor conditions and slow growth or high resource availability and fast growth[315], to describe the spectrum of life history strategies in microbial systems has inherent limitations being predominantly focused on substrate preferences and growth rates. Other attempts have been made to adapt the C-S-R life history strategies to describe plant growth to microbes[316], but this framework also has limitations in that it is largely driven by stress and disturbance gradients whereas resource acquisition is a significant determinant of microbial fitness. New frameworks have been developed that seek to capture the nuanced differences in microbial growth strategies that adequately represent the strategic tradeoffs and bioenergetic

investments in growth over stress tolerance and resource acquisition. One of these frameworks is the Y-A-S model[490] which describes microorganisms as following one of three life history strategies: (Y) or high yield characterized by investments in biomass synthesis and high carbon use efficiency, (A) or resource acquisition characterized by investments in cellular machinery, such as extracellular enzymes, to capture resources, or (S) stress tolerance which follows the stress tolerance niche from the traditional C-S-R framework closely. These strategies operate on a two-dimensional spectrum of resource availability and abiotic stress with different strategies being favored under different conditions.

In applying this framework to the guild superclusters identified through trait identification, several patterns become evident. Guilds from supercluster 4 especially guilds 9 and 12, which include the anammox and AOB can be considered to fall within the A strategy centered around resource acquisition. This is driven primarily by their ability to fix inorganic carbon which gives them an advantage in carbon limited environments like anammox reactors. Guilds from supercluster 5 which include guilds predominantly comprised of MAGs classified as Bacteroidetes and Ignavibacteriae also fall squarely within the domain of A-strategist as they are enriched in extracellular enzymes to degrade heteromannan, glucan, and xyloglucan as well as transporters for peptide uptake, however, they are severely deficient in stress tolerance traits. The MAGs within this supercluster make the investments to acquire resources through complex carbon depolymerization which is especially critical inside anammox reactors where simple carbon compounds are limited. These two superclusters clearly follow the delineations of the A-strategists and do not appear to make other investments in high yield or stress tolerance based on the trait analysis[490].

Superclusters 1 and 5, however, do not adhere to such clear demarcations. Guilds from supercluster 5 are also enriched in extracellular enzymes, transporters, and several stress tolerance traits including osmotic stress and desiccation, phage resistance, and low and high temperature stress (OGT_{avg} = 35.54℃) but are deficient in several pH stress tolerance traits. However, these MAGs also appear to be enriched in carotenoid biosynthesis which is a pigment used in photosynthetic organisms but has been shown to be related to cold stress and oxidative stress in heterotrophic bacteria[491] indicating even more enzymatic investment in stress tolerance. The ability to acquire resources through extracellular means as well as a diverse array of stress tolerance traits places the guilds of this supercluster somewhere between the A-strategists and the S-strategists. Lastly, guilds from supercluster 1 are enriched in traits related to different central carbon pathways including the glyoxylate cycle, TCA cycle, and ED pathway as well as ammonia and sulfur assimilation but are also enriched in stress tolerance traits for osmotic stress and desiccation, temperature stress, redox stress, and pH stress. The presence of different central carbon pathways would indicate an investment in biosynthesis that would result in higher yields[490], although this could not be confirmed without experimental data to estimate carbon turnover. However, guilds of this supercluster are also enriched in aerobic respiration which would suggest that they may have an energetic advantage that would result in higher investments in anabolic processes than catabolic processes. Nevertheless, this supercluster appears to fall somewhere in between Y-strategist and S-strategist which may fall closer to the traditional

ruderal categorization characterized by high growth rates that can quickly proliferate after high intensity perturbations.

The application of the Y-A-S framework to functional guilds from the anammox system can be useful in constructing trait-based ecosystem models to predict carbon fluxes, substrate utilization, and microbial succession patterns. For anammox reactors, it is critical to be able to model system perturbations that would disrupt reactor performance and resultingly the microbial ecology of the system, thus incorporating stress tolerance strategies into such a model would greatly increase the accuracy and predictive capabilities. Furthermore, carbon fluxes within the system are mediated by the primary production of the anammox and other autotrophs within the system, therefore the ability to accurately model the degradation and utilization of complex carbon substrates from EPS and cellular decay products and couple it to the growth patterns of the anammox would also be a significant advancement. Trait based analyses such as the one described in this study would be able to significantly reduce the dimensionality of microbial traits by identifying representative guilds and organisms that can appropriately represent the functional traits and kinetic growth parameters (maximum substrate uptake rate and maximum growth rate) of organisms with similar trait profiles[492]. This would lead to the development of more robust and accurate models that would be able to predict resource allocation patterns that impact the ecosystem function of the microbial community and ultimately reactor performance.

5.5 Conclusion

Anammox reactors select for a distinct microbial community that possess an array of specific traits and growth strategies that enhance organism fitness under the unique environmental conditions of the reactor. Here we apply a trait based framework study to understand the diversity of microbial growth strategies in the microorganisms inhabiting anammox reactors. This analysis identified 190 microbial traits across 966 MAGs describing various resource acquisition, resource utilization, and stress tolerance strategies, classifying them into 136 functional guilds. Analysis of resulting trait profiles demonstrated clear distinctions in taxonomy and metabolic traits amongst guilds with varying degrees of trait prevalence and phylogenetic conservation. The life history strategies of these guilds generally follows conceptual frameworks that classify organisms based on investments in yield, resource acquisition, or stress tolerance. Ultimately, we found that the life history strategies of these guilds places organisms on a spectrum of resource acquisition and stress tolerance resulting in enrichment of various extracellular enzymes or pH and oxidative stress tolerance traits. The results of this study have substantial implications for the development of trait based models that can accurately represent the diversity of growth strategies and functional traits of microorganisms in anammox reactors. The development of such models will enable the prediction of microbial succession patterns that can better assist with reactor design, operation, and stress response.

Chapter 6: Conclusions and Future Work

The specter of Haber and Bosch looms large over the biogeochemical substratum of technological modernity. In the quest for dominion over the natural world including nonhuman, and racialized human life, humans have endeavored to obliterate the ecological thresholds placed before them. Pursued through a biopolitical and necropolitical modality of subjugation, the "civilized races of the world" have transfigured nitrogen from an alchemical enigma into a compartmentalized projection of relational matter for the aim of ceaseless extraction, commerce, and accumulation. However, the conflation of all human life with the arbiters of a capitalist fueled environmental apocalypse is not only a historical fallacy but a political reorientation of the nature of environmental harm. The aim of this dissertation was to expose this erroneous myth and recast the full scope of nitrogen transmutation into the broader spectrum of ecological imperialism and the manipulation of the Earth's natural amenities to support the hegemony of the self-proclaimed, deified, obstruse actant that is the "Human". It is this political and epistemic entity that has constructed modern modes of production and commerce from the relics of feudal peasant relations, which utilized race as a tool to subjugate ethnic minorities as a means of labor extraction, and desecrated Indigenous cosmologies and land relations to commodify the land as a basis for production. It is this same entity that endeavors to deploy technological and scientific interventions to address sociopolitical problems, which perpetuates the narrative of technological modernity as a solution to environmental catastrophe, and seeks to marginalize restorative frameworks that aim to center the healing of Indigenous and African diaspora communities as a means to redress environmental harm. It is this entity that will advocate for utilizing the anammox process as a panacea to anthropogenic nitrogen pollution without considering the historical, political, or even biogeochemical implications of such an endeavor. The hope for this dissertation was to demonstrate that the efforts to resolve centuries long issues of environmental harm intricately woven with the corporeal realities of genocide, enslavement, and land dispossession with technological solutions is not only a distraction but moreover an effort to avoid the actual problem. The anammox bacteria are slow growing, they are sensitive to a wide variety of inhibitors including their own substrate nitrite, they recover slowly from toxic shocks, and can even be cannibalized by their heterotrophic partners for whom they depend on essential metabolites and nutrients. This makes a scientifically intriguing engineering process difficult to align with the standards of a capitalist system that emphasizes efficiency, maximum resource utilization, and incessant productivity. Despite this systematic misalignment, the anammox bacteria are not the problem. It is not the fault of the anammox bacteria that a small group of humans have aimed to perpetuate the extraction of natural resources by manipulating natural flows of nitrogen. It is not the fault of the anammox bacteria that the manipulation of this cycle has precipitated unprecedented levels of reactive nitrogen pollution into the environment causing incalculable ecological and human harm. It is not the fault of the anammox bacteria that instead of attempting to regulate the inputs of reactive nitrogen in accordance with natural cycles that have self-regulated over billions of years, the political systems of the Earth have done nothing but incentivized more nitrogen use resulting in more nitrogen pollution resulting in more environmental harm. While the nuances of reactive nitrogen utilization are multidimensional, with many around the globe being critically dependent on inputs of fixed nitrogen to maintain

their elemental body composition, the primary actuator of nitrogen's systematic exploitation is the desire to produce agricultural commodities, fossil energy, and weapons for a system of nation states aiming to hegemonize the world's resources at the expense of those deemed disposable. The primary aim of this dissertation was to display the full breadth of the ecological networks that support the anammox bacteria, which continue to defy scientific efforts to classify, taxonomize, and compartmentalize them. This is a testament to nature's unequivocal impetus to exist independently of human efforts to place them within an ontology amenable to extraction and commodification. The anammox bacteria are not the problem, nor are they alone the solution to nitrogen pollution, however, they form the basis for an incredible ecosystem that harbors immense phylogenetic and metabolic diversity that is worth appreciating independent of their capacity to support capitalist growth.

Chapter 2 of this dissertation was structured in a way to fully appreciate the nuance of metabolic interactions in anammox systems. The influent nitrogen loading ratio of anammox bioreactors, generally adhering to a guideline of an NH_4^+:NO_2^- =1:1.32 carries large implications for the substrate utilization patterns of microorganisms with diverse nitrogen pathways. Changing this ratio to 1:1.1 led to a surprising result in that bioreactor performance was maintained (NRE>90%), but the relative abundance of the anammox bacteria decreased to less than 12%. While this change occurred, the abundance of DNRA associated bacteria including Ignavibacteriae and Chloroflexi increased. This would suggest a potentially symbiotic relationship between anammox bacteria and DNRA bacteria which contrast with the results of previous work which suggest that increased replication of DNRA bacteria has the potential to result in a reactor performance crash. The co-existence of these observations would suggest that the interactions between anammox and DNRA bacteria are much more complex than originally suggested and can take different forms falling on a spectrum of mutualism to parasitism depending on the conditions of the reactor and taxonomic and metabolic composition of the microbial community. However, this work would be significantly improved upon by testing nitrogen loading ratios at extreme ends of the spectrum including higher ratios (NH_4^+:NO_2^- < 1:1) and lower ratios (NH_4^+:NO_2^- > 1:1.4). This work would also benefit from metatranscriptomic analysis that could elucidate the activity of various organisms over metagenomics that can only provide information on their abundance, as well as nitrogen isotopic studies that could track the partitioning of nitrogen through various pathways (anammox, denitrification, DNRA).

Chapter 3 of this dissertation aimed to assess the microbial response to the solids retention time of anammox bioreactor systems which imparts a kinetic shift influencing the growth range of organisms within the reactor. The solids retention time was adjusted stepwise from 50 days to 28.5 days until the reactor experienced a performance crash as indicated by nitrogen removal performance and biomass degradation. Shotgun metagenomic and 16S rRNA analysis demonstrated that the relative abundance of the anammox bacteria as well as Chloroflexi decreased in response to decreased SRTs, while the abundance of Sphingobacteriales and Rhodospirillales increased. These results suggest that an SRT driven crash, characteristic of a press disturbance to a stable system, selected for organisms primarily based on their life historygrowth strategies with faster growing organisms being selected over slower growing

organisms. However, this alone could not explain the decreased abundance of the anammox bacteria and further examination of the metabolic pathways for exopolysaccharide production suggests that decreased abundance of sticky exopolysaccharide bacteria may have precipitated a decreased capacity for biomass aggregation resulting in further biomass washout. Taxonomic analysis also suggest the reassembly of the microbial community following the crash based on functional redundancy and not the original composition of the reactor. This work could also benefit immensely from metatranscriptomic analysis that elucidate the activity of different organisms in response to the crash as well as during the recovery period. Chemical analysis of the EPS and biomass aggregation would also support the conclusions of this work and provide evidence for decreased biomass aggregation. Furthermore, testing longer SRTs (>50 days) to investigate the effects of biomass decay and the accumulation of anammox necromass on the microbial community would provide interesting complementary results for the present study.

Chapter 4 of this dissertation seeks to investigate the role of EPS as a carbon source for heterotrophic bacteria within anammox reactors. EPS was extracted using an alkaline and heat extraction method and then used as a carbon source for batch enrichments using anammox biomass as inoculum. Chemical analysis confirmed the degradation of EPS over the course of the enrichment and sequencing analysis demonstrated differential enrichment of various subsets of the community with different EPS solutions. Metagenomic analysis suggest that extracellular glycoside hydrolases could be more important for EPS degradation and utilization than extracellular peptidases despite the EPS being predominantly composed of proteins. These results suggest that multiple organisms across varying ecological niches within the reactor have the ability to degrade EPS suggesting functional redundancy in complex carbon depolymerization. This study could be greatly enhanced by the utilization of FTIR and 3D-EEM to track the conversions of different carbon compounds throughout the experiment. Metatranscriptomic analysis would also be useful to determine the activity of different organisms independent of abundance and the expression of specific enzymes. Further studies may also endeavor to make stronger links of EPS degradation and carbon assimilation using stable isotope probing with ^{13}C labeled EPS. The degradation of different sub-fractions of EPS including tightly bound EPS and loosely bound EPS as well as the microbial community response would also be an interesting addition to this work.

Chapter 5 provides a culmination of this work by examining the functional diversity of organisms across different reactor systems to assess patterns in the metabolic capabilities of anammox bioreactor associated microbiomes. Trait- based modeling was conducted using microTrait to assess the presence of various traits related to different microbial strategies including resource acquisition, resource use, stress tolerance, and biophysical life history traits. The results of this analysis identified functional guilds of organisms that share similar trait profiles which aligned with reaction networks previously predicted by metagenomic studies. Phylogenetic analysis of identified guilds suggested varying levels of metabolic coherence amongst different taxonomic groups with Proteobacteria being some of the most metabolically diverse and Ignavibacteriae and Bacteroidetes being some of the most metabolically convergent. Analysis of all functional guilds with respect to the Y-A-S life history framework determined that functional guilds align to a spectrum of different ecological strategies including stress

tolerators and resource acquisition. Further studies may endeavor to utilize the functional guilds identified in this study as representative organisms to construct kinetic models that can simulate substrate utilization, resource partitioning and growth of different organisms under varying conditions. This can be done by utilizing the biophysical life history traits to predict the kinetic parameters of microbial growth including the maximum substrate uptake and maximum growth rate. Studies such as these will also benefit from benchmarking the predictions of such models against experimental data such as the utilization of various substrates by isolates as well as biomass anabolism from isotope tracer studies.

Excess reactive nitrogen in the environment is not a historical or geochemical anomaly, rather it is a manifestation of structures of power that accumulated over thousands of years of human interaction with the environment. Developing an understanding of the current state of the nitrogen cycle with respect to the historical processes that have helped to mold it is critical to elucidating the true nature of its disruption and current trajectory. Reactive nitrogen is vital to the perpetuation of both nonhuman and human life on the planet. Humans cannot dispense with it just as they cannot dispense with water or air. Yet, the manipulations of nitrogen to actuate the expropriation of land is not representative of the intrinsic nature of humans, it is a materialization of the voracious appetite of the capitalist system which will literally cannibalize itself from the inside out to perpetuate the accumulation of profit. Science and technology are merely tools in this process and by themselves cannot be mistaken for a robust solution to the current state of nitrogen pollution. Wholistic redress for the environmental harm caused by nitrogen pollution cannot be done without a critical re-examination of the systems that continue to proliferate reactive nitrogen pollution: the global agricultural commodity chain, the fossil fuel industry, and the military-industrial complex. Without holding these systems accountable for their appropriation of nitrogen as well as restorative justice for the billions of racialized others who have experienced centuries of genocide and epistemicide, and re-centering the land and nonhuman life, there can be no substantive change. The anammox process is no more a solution to the nitrogen problem than other technologies that have been attempted and deemed insufficient to meet the standards of the capitalist system. Independent of its capacity to fit within the mold carved out for it by capitalism, the anammox bacteria carry an immense reservoir of unprecedented biochemical novelty and ecological distinctiveness. Studying the anammox as well as their heterotrophic partners for their biological novelty is not a project of grand interest for technological modernity. Yet, it is an endeavor that attempts to truly appreciate the scientific superfecundity of a process that carries immense implications for the biogeochemical cycling of nitrogen around the globe. The work done in this dissertation is meant to highlight this phenomenon and appreciate the anammox bacteria not as a tool to incentivize more nitrogen pollution but as a gift from nature to be properly respected and appreciated.

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Appendix 1: Supplementary Information for Chapter 1-Synergistic Interactions between Anammox and dissimilatory nitrate reducing bacteria sustains reactor performance across variable nitrogen loading ratios

Constituent	Concentration	Unit
NH_4HCO_3	0-2000	mg-N/L
$NaNO_2$	0-2000	mg-N/L
NaCl	1000	mg/L
$MgCl_2{\cdot}6H_2O$	500	mg/L
KH_2PO_4	27.2	mg/L
KCl	10	mg/L
$CaCl_2{\cdot}2H_2O$	180	mg/L
$NaHCO_3$	500	mg/L
$FeCl_2{\cdot}4H_2O$	17.89	mg/L
$CoCl_2{\cdot}6H_2O$	0.24	mg/L
$MnCl_2{\cdot}4H_2O$	0.99	mg/L
$ZnCl_2$	0.2	mg/L
H_3BO_3	0.014	mg/L
$Na_2MoO_4{\cdot}2H_2O$	0.22	mg/L
$NiCl_2{\cdot}6H_2O$	0.19	mg/L
$CuCl_2{\cdot}2H_2O$	0.17	mg/L
$Na_2SeO_3{\cdot}5H_2O$	0.16	mg/L
pH	6.8-7.0	

Table 1: MBR Media Composition

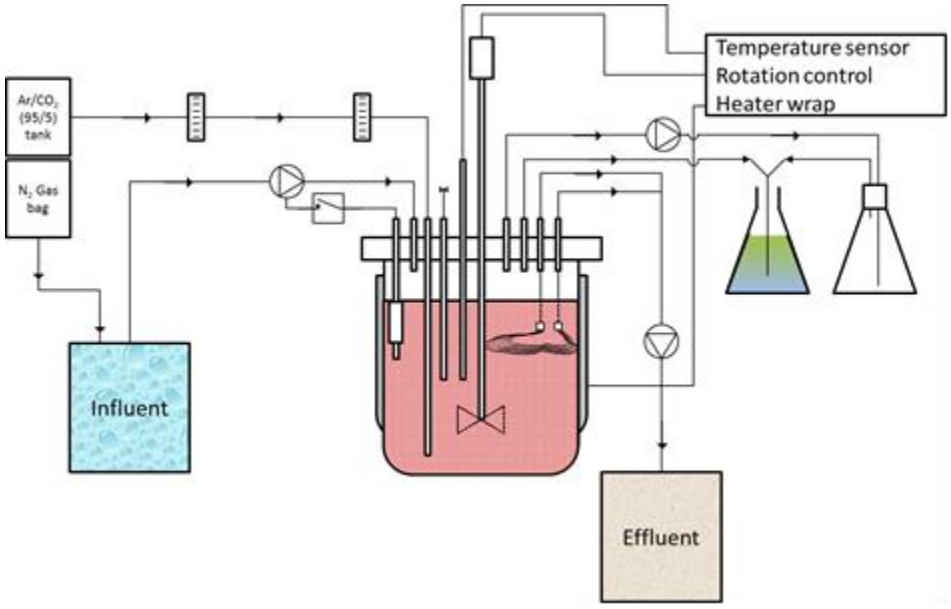

Figure 1: Mechanical sketch of MBR

Operational Parameter	Setting	Unit
Gas flow rate (Ar/CO_2:95/5)	0.2	LPM
Temperature	37	℃
Mixing	200	RPM
HRT	18-24	hr
SRT	50-60	days
Volume	1	L

Table 2: MBR Operational parameters

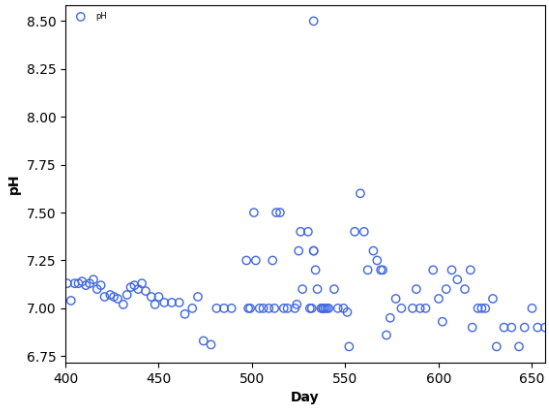

Figure 2: MBR effluent pH

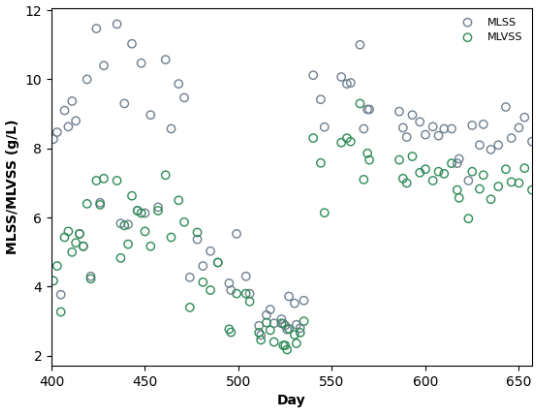

Figure 3: MBR MLSS/MLVSS Measurements

Day	Chao	Shannon	Simpson
16	363.5769	1.522672	0.496445
17	500	1.623997	0.548803
18	276	1.211802	0.402098
21	671.3125	2.368713	0.767801
33	573.6471	2.053709	0.689854
41	466.3684	2.089925	0.709396
43	269.7143	2.368648	0.781668
46	448.9545	2.387704	0.780954
99	718.1429	2.223641	0.743966
117	577.1714	1.714986	0.640657
130	581.9024	1.948363	0.689643
131	518	1.856925	0.662794
132	553.8571	1.873487	0.6956
134	642.025	2.170183	0.780811
138	724	2.178913	0.778882
140	778.35	2.727573	0.858208
149	942.2885	2.655922	0.852828
152	869.1111	2.701284	0.825935
159	811.2128	2.653657	0.852464
168	558.9268	2.201301	0.744807
181	955.5714	2.651988	0.854377
188	1025.325	2.749838	0.866492
194	819.15	2.502905	0.82332
203	787.0857	2.578343	0.848444
211	772.0612	2.693486	0.873011
217	991.626	2.93486	0.891122
245	973.9608	3.012237	0.899178
252	1167.917	2.97098	0.897576

Table 3: 16s rRNA Alpha Diversity Indices

Sample	Total Reads	Mapped Reads	Mapped Percentage	Total Bases	Mapped Bases	Mismatches	Error rate
LAC_NA07 (D37)	119945232	93129120	77.64303628	16160242400	12597551874	75765735	6.01E-03
LAC_NA08 (D140)	95301016	72438066	76.00975209	1321888537	10079415025	57323417	5.69E-03
LAC_NA01 (D232, D235, D237)	127646278	89632124	70.21914419	17694180953	12442322754	61468369	4.94E-03

Table 4: Metagenome alignment statistics

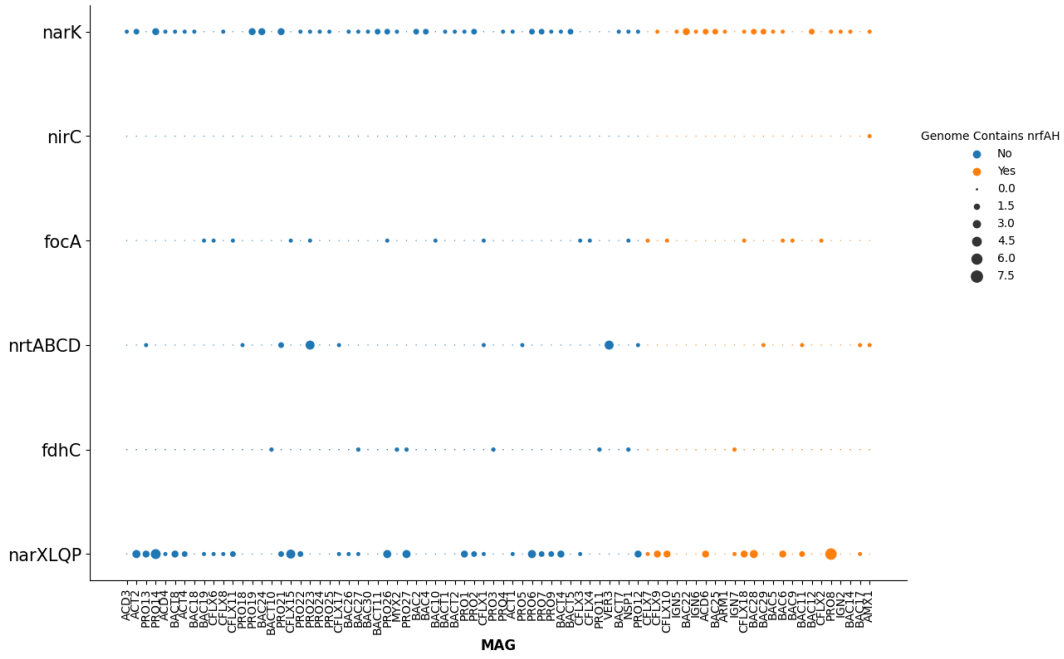

Figure 5: Nitrogen transport and sensing genes for MAGs with at least 40% genome coverage

Table 5: Metagenome assembled genome (MAG) statistics

Genome	Genome code (ggkbase)	Phyla	Size	GC%	Contigs	ORFS	Completeness (%)
ACD01	LAC_NA09_Acidobacteria_70_95	Acidobacteria	3,730,000	69.99	45	3263	92.74%
ACD02	NA01_Acidobacteria_59_12	Acidobacteria	5,590,000	59.15	159	4789	92.98%
ACD03	LAC_NA06_Candidatus_Solibacter_usitatus_62_12	Acidobacteria	4,520,000	62.17	166	3970	84.35%
ACD04	anamox3_Acidobacteria_71_4_curated	Acidobacteria	1,330,000	69.62	772	1759	35.00%
ACD05	anamox1_Acidobacteria_62_5_curated	Acidobacteria	3,110,000	61.59	565	3251	69.92%
ACD06	LAC_NA06_Acidobacteria_54_19	Acidobacteria	2,710,000	54.24	26	2505	94.02%
ACT01	LAC_NA10_Actinobacteria_74_12	Actinobacteria	2,540,000	73.88	170	2460	96.24%
ACT02	LAC_NA07_Actinobacteria_74_18	Actinobacteria	1,210,000	73.80	141	1247	58.54%
ACT03	anamox2_Actinobacteria_65_5_curated	Actinobacteria	2,800,000	63.61	662	3147	66.92%
ACT04	LAC_NA06_Actinobacteria_71_15	Actinobacteria	3,180,000	70.59	57	3196	95.83%
ACT05	LAC_NA07_Actinotalea_fermentans_75_19	Actinobacteria	2,910,000	75.44	31	2757	95.95%
AMX01	anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	Planctomycetes	3,110,000	42.29	64	2859	100%
ARC01	anamox2_Methanosarcina_thermophila_41_9_curated	Euryarchaeota	2,960,000	41.19	82	2703	97.85%
ARM01	NA01_Fimbriimonas_ginsengisoli_61_16	Armatimonadetes	2,770,000	60.97	47	2570	93.98%
BAC01	LAC_NA07_Bacteria_70_305	Bacteria	2,750,000	70.12	54	2386	90.91%
BAC02	anamox1_Bacteria_56_37_curated	Bacteria	4,970,000	55.88	43	4404	91.52%
BAC03	NA04_Bacteria_58_19	Bacteria	7,560,000	57.56	112	6122	97.27%
BAC04	LAC_NA07_Bacteria_38_171	Bacteria	2,390,000	37.56	23	2128	95.08%
BAC05	LAC_NA07_Bacteria_57_12	Bacteria	5,860,000	57.26	271	5345	92.73%
BAC06	anamox2_Bacteria_68_6_curated	Bacteria	2,290,000	67.96	460	2621	60.64%

BAC07	anamox4_Bacteria_63_7_curated	Bacteria	3,450,000	62.64 %	351	3276	87.27%
BAC08	NA04_Bacteria_65_10	Bacteria	4,100,000	64.60 %	238	3590	91.67%
BAC09	LAC_NA07_Bacteria_71_12	Bacteria	2,760,000	71.10 %	206	2842	84.60%
BAC10	NA04_Bacteria_69_30	Bacteria	4,020,000	69.42 %	30	3566	93.33%
BAC11	NA01_Bacteria_60_23	Bacteria	3,780,000	60.01 %	48	3266	94.95%
BAC12	LAC_NA10_Bacteria_62_9	Bacteria	3,000,000	62.13 %	264	2819	87.04%
BAC13	LAC_NA11_Bacteria_34_14	Bacteria	863,630	34.10 %	17	886	79.15%
BAC14	NA04_Bacteria_50_51	Bacteria	2,400,000	49.62 %	31	1990	94.81%
BAC15	anamox3_Bacteria_67_15_curated	Bacteria	2,770,000	66.48 %	144	2980	90.76%
BAC16	anamox1__Bacteria_33_9_curated	Bacteria	775,490	32.38 %	45	793	62.09%
BAC18	anamox3_Bacteria_66_7_curated	Bacteria	2,270,000	65.35 %	286	2150	81.93%
BAC19	anamox1_Bacteria_64_7_curated	Bacteria	3,990,000	63.42 %	306	3679	89.37%
BAC20	anamox1_Bacteria_65_5_curated	Bacteria	2,090,000	64.54 %	434	2237	52.01%
BAC21	anamox2_Bacteria_61_6_curated	Bacteria	2,420,000	60.87 %	385	2646	65.68%
BAC22	LAC_NA11_Bacteria_57_35	Bacteria	6,060,000	56.96 %	90	5339	95.73%
BAC23	anamox1_Bacteria_45_8_curated	Bacteria	2,580,000	45.00 %	166	2221	89.32%
BAC24	anamox1_Bacteria_57_9_curated	Bacteria	3,470,000	57.28 %	74	3012	91.27%
BAC24	NA03_Bacteria_65_16	Bacteria	3,910,000	65.04 %	63	3719	91.45%
BAC25	anamox1_Bacteria_50_18_curated	Bacteria	820,570	49.55 %	22	874	63.77%
BAC26	anamox1_Bacteria_72_15_curated	Bacteria	2,940,000	72.45 %	24	2507	93.75%
BAC27	anamox1_Bacteria_53_17_curated	Bacteria	3,470,000	53.24 %	12	2895	95.91%
BAC28	anamox1_Bacteria_57_32_curated	Bacteria	2,940,000	56.91 %	76	2571	92.02%

BAC29	anamox3_Bacteria_67_13_curated	Bacteria	1,640,000	66.50	47	1442	92.02%
BACG11	anamox1_Sphingobacteriales_43_8_curated	Bacteroidetes	3,160,000	42.95	130	2826	95.13%
BACT01	LAC_NA08_Flavobacteriales_41_5	Bacteroidetes	1,240,000	41.09	665	1648	50.87%
BACT02	LAC_NA11_Bacteroidales_40_6	Bacteroidetes	2,280,000	40.14	900	2540	70.85%
BACT03	NA04_Bacteroidetes_30_9	Bacteroidetes	2,580,000	30.05	248	2356	95.63%
BACT04	LAC_NA06_Sphingobacteriales_42_58	Bacteroidetes	3,450,000	42.01	34	2968	97.04%
BACT05	NA04_Sphingobacteriales_44_8	Bacteroidetes	4,070,000	43.73	474	3624	86.45%
BACT06	NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	Bacteroidetes	2,930,000	45.76	35	2441	96.77%
BACT07	anamox1_Bacteroidetes_63_11_curated	Bacteroidetes	3,360,000	63.21	73	2915	99.46%
BACT08	anamox2_Sphingobacteriales_41_11_curated	Bacteroidetes	2,770,000	41.16	129	2490	85.67%
BACT09	anamox4_Bacteroidetes_40_74_curated	Bacteroidetes	2,630,000	39.91	15	2219	99.52%
BACT10	anamox3_Sphingobacteriales_50_9_curated	Bacteroidetes	4,310,000	49.73	216	2999	95.45%
BACT12	anamox3_Bacteroidetes_39_15_curated	Bacteroidetes	2,530,000	39.26	23	2188	98.57%
BACT13	anamox1_Bacteroidetes_39_16_curated	Bacteroidetes	2,900,000	38.84	40	2472	98.73%
BAC17	anamox1_Bacteria_55_18_curated	Bacteria	4,100,000	54.56	216	3365	99.46%
CFLX01	LAC_NA10_Chloroflexi_62_239	Chloroflexi	1,430,000	61.97	417	1859	49.63%
CFLX02	LAC_NA08_Chloroflexi_60_15	Chloroflexi	3,260,000	59.74	310	3038	86.36%
CFLX03	NA01_Chloroflexi_65_16	Chloroflexi	3,700,000	65.08	207	3252	93.64%
CFLX04	NA03_Anaerolineales_41_23	Chloroflexi	2,360,000	41.48	215	2322	85.45%
CFLX05	LAC_NA08_Chloroflexi_58_8	Chloroflexi	2,650,000	57.66	432	2733	74.18%
CFLX06	LAC_NA08_Chloroflexi_67_31	Chloroflexi	3,550,000	66.65	70	2905	90.91%

CFLX07	anamox3_Chloroflexi_59_6_curated	Chloroflexi	1,410,000	53.68 %	351	1507	32.37%
CFLX08	NA03_RBG_16_Chloroflexi_57_11_curated_55_21	Chloroflexi	3,440,000	55.06 %	367	3313	91.2%
CFLX09	LAC_NA07_Chloroflexi_57_9	Chloroflexi	2,090,000	56.86 %	325	2202	70.11%
CFLX10	LAC_NA11_Caldilinea_aerophila_60_6	Chloroflexi	3,520,000	59.88 %	1263	3974	70.72%
CFLX11	LAC_NA07_Chloroflexi_66_23	Chloroflexi	4,470,000	66.09 %	141	3687	94.55%
CFLX12	NA01_Caldilinea_aerophila_61_10	Chloroflexi	3,730,000	60.76 %	159	2098	83.64%
CFLX13	NA02_Anaerolinea_thermophila_56_16	Chloroflexi	2,140,000	56.04 %	540	2398	78.33%
CFLX14	anamox3_Chloroflexi_68_6_curated	Chloroflexi	2,290,000	67.35 %	479	2330	56.04%
CFLX15	LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	Chloroflexi	2,490,000	74.73 %	63	2327	91.20%
CFLX16	LAC_NA07_Chloroflexi_60_59	Chloroflexi	3,860,000	60.11 %	13	3453	89.55%
CFLX17	anamox1_Chloroflexi_52_59_curated	Chloroflexi	2,860,000	52.47 %	96	2759	92.73%
CFLX18	anamox2_Chloroflexi_60_8_curated	Chloroflexi	2,020,000	60.06 %	111	1885	44.42%
CLA01	anamox1_Candidatus_Cloacimonas_acidaminovorans_38_6_curated	Candidatus Cloacimonas	1,190,000	35.28 %	246	1132	53.43%
CPR01	LAC_NA11_Microgenomates_50_64	Microgenomates	869,170	49.81 %	63	986	55.88%
CPR02	anamox2_Microgenomates_45_6_curated	Microgenomates	740,220	44.28 %	99	904	59.54%
CPR03	NA01_Roizmanbacteria_38_28	Microgenomates	897,990	38.12 %	17	921	68.93%
CPR04	NA04_Candidatus_Saccharibacteria_41_123	Microgenomates	820,330	40.66 %	14	857	66.82%
CPR05	anamox4_Microgenomates_48_8_curated	Microgenomates	999,880	47.64 %	49	1055	63.30%
CPR06	LAC_NA07_Roizmannbacteria_52_60	Microgenomates	885,170	52.20 %	1	945	73.98%
CPR07	LAC_NA06_Microgenomates_41_17	Microgenomates	1,100,000	41.16 %	6	1203	67.89%
DADA01	LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	Dadabacteria	918,620	57.55 %	539	1314	43.08%
DEN01	LAC_NA07_Truepera_radiovictrix_72_29	Deinococcus-Thermus	1,320,000	72.29 %	165	1356	73.23%
GEM01	anamox1_Gemmatimonas_aurantiaca_57_6_curated	Gemmatimonadota	2,630,000	56.94 %	255	2549	90.66%
IGN01	LAC_NA08_Ignavibacterium_album_33_266	Ignavibacteriae	2,140,000	33.41 %	731	2621	67.42%

IGN02	anamox3_sub_Ignavibacteriales_42_14_curated	Ignavibacteriae	3,160,000	42.21	22	2498	95.63%
IGN03	anamox2_Ignavibacteriales_33_9_curated	Ignavibacteriae	2,940,000	33.09	166	2753	76.8%
IGN04	LAC_NA11_Ignavibacteriales_56_75	Ignavibacteriae	2,990,000	56.40	21	2685	96.72%
IGN05	NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	Ignavibacteriae	2,740,000	30.53	53	2344	96.89%
IGN06	anamox2_Ignavibacteriales_41_12_curated	Ignavibacteriae	3,160,000	41.32	42	2805	95.63%
IGN07	anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	Ignavibacteriae	1,160,000	34.03	347	1374	51.34%
MYX01	NA04_Myxococcales_72_13	Myxococcota	4,300,000	71.89	121	3695	89.03#
MYX02	anamox2_Myxococcales_71_5_curated	Myxococcota	1,430,000	70.21	495	1687	24.99%
NSP01	anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	Nitrospirota	3,090,000	60.30	94	3084	95.85%
PROT01	NA04_Burkholderiales_70_137	Proteobacteria	2,510,000	69.69	131	2498	90.41%
PROT02	NA02_Rhizobiales_66_27	Proteobacteria	2,950,000	66.38	163	3000	95.3%
PROT03	NA04_Rhodocyclales_67_27	Proteobacteria	2,480,000	66.98	295	2718	85.24%
PROT04	LAC_NA06_Betaproteobacteria_71_7	Proteobacteria	4,100,000	70.65	1841	5661	97.98%
PROT05	LAC_NA07_Rhodobacterales_68_7	Proteobacteria	1,330,000	68.41	473	1674	58.41%
PROT06	NA01_Rhizobiales_69_9	Proteobacteria	3,340,000	69.28	447	3602	79.77%
PROT07	LAC_NA10_Xanthomonadales_68_7	Proteobacteria	2,460,000	68.28	555	2584	68.74%
PROT08	LAC_NA10_Lysobacter_70_8	Proteobacteria	1,030,000	70.00	294	1180	37.95%
PROT09	LAC_NA06_Xanthomonadales_70_10	Proteobacteria	1,920,000	69.75	377	2039	69.12%
PROT10	anamox1_Betaproteobacteria_69_7_curated	Proteobacteria	2,110,000	66.39	531	2636	52.65%
PROT11	anamox2_Burkholderiales_67_5_curated	Proteobacteria	1,250,000	66.28	298	1434	38.05%
PROT12	LAC_NA08_Gammaproteobacteria_68_22	Proteobacteria	2,800,000	68.43	13	2594	89.07%

PROT13	NA03_Alphaproteobacteria_58_599	Proteobacteria	2,550,000	57.83	1	2485	95.81%
PROT14	LAC_NA09_Gammaproteobacteria_70_24	Proteobacteria	2,570,000	69.91	23	2420	88.23%
PROT15	LAC_NA11_Betaproteobacteria_62_13	Proteobacteria	2,820,000	62.38	76	2811	97.47%
PROT16	anamox1_Burkholderiales_71_17_curated	Proteobacteria	3,640,000	70.81	114	3448	61.64%
PROT17	LAC_NA06_Burkholderiales_73_13	Proteobacteria	3,680,000	72.87	335	3453	89.44%
PROT18	anamox4_Gammaproteobacteria_67_14_curated	Proteobacteria	3,270,000	66.91	73	3049	92.53%
PROT19	anamox2_Burkholderiales_68_9_curated	Proteobacteria	3,530,000	68.25	181	3452	95.74%
PROT20	anamox3_Nitrosomonas_europaea_51_28_curated	Proteobacteria	2,370,000	50.54	93	2302	95.55%
PROT21	NA03_Alphaproteobacteria_61_24	Proteobacteria	3,390,000	61.02	12	3189	99.50%
PROT23	anamox2_Burkholderiales_70_13_curated	Proteobacteria	3,070,000	70.33	39	2841	87.2%
PROT24	anamox1_Proteobacteria_67_8_curated	Proteobacteria	2,910,000	66.93	203	2910	83.43%
PROT25	anamox2_Xanthomonadales_68_9_curated	Proteobacteria	1,240,000	67.63	21	1168	55.44%
PROT26	anamox2_Rhizobiales_67_45_curated	Proteobacteria	4,840,000	66.56	24	4668	98.36%
PROT27	anamox1_Nitrosomonas_europaea_50_14_curated	Proteobacteria	2,130,000	50.43	37	2006	99.74%
PROT28	anamox2_Hydrogenophilales_66_19_curated	Proteobacteria	2,260,000	65.94	122	2331	87.57%
SPR01	NA03_Leptonema_illini_45_7	Spirochaetes	2,310,000	45.31	383	2382	77.25%
SPR02	NA04_Turneriella_parva_44_15	Spirochaetes	2,930,000	44.39	65	2790	93.19%
VER01	anamox2_Verrucomicrobia_62_8_curated	Verrucomicrobia	3,690,000	61.78	161	3134	95.27%
VER02	anamox3_Pedosphaera_parvula_66_5_curated	Verrucomicrobia	1,230,000	65.98	543	1384	52.71%
VER03	anamox2_Verrucomicrobia_58_8_curated	Verrucomicrobia	3,590,000	57.68	250	3293	89.46%
VER04	anamox3_Verrucomicrobia_59_12_curated	Verrucomicrobia	2,710,000	59.27	50	2592	96.62%

MAG	RFG1	RFG2	RFG3
anamox1_Acidobacteria_curated	-1.77982	-1.3459	-1.03193
anamox1__Bacteria_33_9_curated	0	0.433921	0.747891
anamox1_Bacteria_45_8_curated	1.264691	1.698612	2.012582
anamox1_Bacteria_50_18_curated	0.064567	0.498488	0.812458
anamox1_Bacteria_53_17_curated	-2.27411	-1.84019	-1.52622
anamox1_Bacteria_55_18_curated	-2.58838	-2.15446	-1.84049
anamox1_Bacteria_56_37_curated	-0.63401	-0.20009	0.113882
anamox1_Bacteria_57_32_curated	-3.22817	-2.79425	-2.48028
anamox1_Bacteria_57_9_curated	-1.2124	-0.77848	-0.46451
anamox1_Bacteria_64_7_curated	-1.40103	-0.96711	-0.65314
anamox1_Bacteria_65_5_curated	-0.03475	0.399174	0.713143
anamox1_Bacteria_72_15_curated	-2.77627	-2.34235	-2.02838
anamox1_Bacteroidetes_39_16_curated	-3.10719	-2.67327	-2.3593
anamox1_Bacteroidetes_63_11_curated	1.197916	1.631837	1.945806
anamox1_Betaproteobacteria_69_7_curated	-1.80718	-1.37325	-1.05928
anamox1_Burkholderiales_71_17_curated	-0.58284	-0.14892	0.165054
anamox1_Candidatus_Cloacimonas_acidaminovorans_38_6_curated	1.042035	1.475956	1.789925
anamox1_Chloroflexi_52_59_curated	-0.43994	-0.00602	0.307946
anamox1_Gemmatimonas_aurantiaca_57_6_curated	-0.13016	0.303763	0.617732
anamox1_Nitrosomonas_europaea_50_14_curated	-1.77595	-1.34203	-1.02806
anamox1_Proteobacteria_67_8_curated	-1.87713	-1.44321	-1.12924
anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	0.187597	0.621518	0.935488
anamox1_Sphingobacteriales_43_8_curated	-2.65684	-2.22292	-1.90895
anamox2_Actinobacteria_65_5_curated	-0.83256	-0.39864	-0.08467
anamox2_Bacteria_61_6_curated	1.299484	1.733405	2.047375
anamox2_Bacteria_68_6_curated	0.221993	0.655914	0.969884
anamox2_Burkholderiales_67_5_curated	0.930401	1.364322	1.678291
anamox2_Burkholderiales_68_9_curated	-0.73692	-0.303	0.010969
anamox2_Burkholderiales_70_13_curated	0.501921	0.935842	1.249812
anamox2_Chloroflexi_60_8_curated	-1.48376	-1.04984	-0.73587
anamox2_Hydrogenophilales_66_19_curated	1.051323	1.485244	1.799213
anamox2_Ignavibacteriales_33_9_curated	0.405233	0.839154	1.153123
anamox2_Ignavibacteriales_41_12_curated	0.216878	0.650799	0.964768
anamox2_Methanosarcina_thermophila_41_9_curated	-2.18282	-1.7489	-1.43493
anamox2_Microgenomates_45_6_curated	-0.70318	-0.26926	0.044711
anamox2_Myxococcales_71_5_curated	0.623163	1.057084	1.371054
anamox2_Rhizobiales_67_45_curated	0.911623	1.345544	1.659514
anamox2_Sphingobacteriales_41_11_curated	0.042353	0.476274	0.790243
anamox2_Verrucomicrobia_58_8_curated	-0.26585	0.168076	0.482045
anamox2_Verrucomicrobia_62_8_curated	-0.43392	0	0.313969

anamox2_Xanthomonadales_68_9_curated	-0.19986	0.234057	0.548027
anamox3_Acidobacteria_71_4_curated	-1.46911	-1.03519	-0.72122
anamox3_Bacteria_66_7_curated	-0.10137	0.332552	0.646521
anamox3_Bacteria_67_13_curated	-0.90291	-0.46899	-0.15502
anamox3_Bacteria_67_15_curated	-1.4235	-0.98958	-0.67561
anamox3_Bacteroidetes_39_15_curated	0.403564	0.837485	1.151454
anamox3_Chloroflexi_59_6_curated	-0.90286	-0.46894	-0.15497
anamox3_Chloroflexi_68_6_curated	-0.71449	-0.28057	0.033398
anamox3_Nitrosomonas_europaea_51_28_curated	-2.81727	-2.38335	-2.06938
anamox3_Pedosphaera_parvula_66_5_curated	-1.46866	-1.03474	-0.72077
anamox3_Sphingobacteriales_50_9_curated	-2.78219	-2.34827	-2.0343
anamox3_sub_Ignavibacteriales_42_14_curated	1.450668	1.884589	2.198558
anamox3_Verrucomicrobia_59_12_curated	-4.41896	-3.98504	-3.67107
anamox4_Bacteria_63_7_curated	-3.09306	-2.65913	-2.34516
anamox4_Bacteroidetes_40_74_curated	-3.56254	-3.12861	-2.81465
anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	-1.34792	-0.914	-0.60003
anamox4_Gammaproteobacteria_67_14_curated	-3.16664	-2.73271	-2.41874
anamox4_Microgenomates_48_8_curated	-2.90478	-2.47086	-2.15689
anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	-0.43375	0.000174	0.314144
LAC_NA06_Acidobacteria_54_19	-2.20477	-1.77085	-1.45688
LAC_NA06_Actinobacteria_71_15	-0.4978	-0.06388	0.250086
LAC_NA06_Betaproteobacteria_71_7	-0.90021	-0.46629	-0.15232
LAC_NA06_Burkholderiales_73_13	-1.58571	-1.15179	-0.83782
LAC_NA06_Candidatus_Solibacter_usitatus_62_12	-0.8555	-0.42158	-0.10761
LAC_NA06_Microgenomates_41_17	-2.53461	-2.10069	-1.78672
LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	-1.45	-1.01608	-0.70211
LAC_NA06_Sphingobacteriales_42_58	-1.08037	-0.64644	-0.33247
LAC_NA06_Xanthomonadales_70_10	-0.24986	0.184064	0.498034
LAC_NA07_Actinobacteria_74_18	-1.19828	-0.76436	-0.45039
LAC_NA07_Actinotalea_fermentans_75_19	-2.31665	-1.88273	-1.56876
LAC_NA07_Bacteria_38_171	-1.59721	-1.16329	-0.84932
LAC_NA07_Bacteria_57_12	-1.45178	-1.01786	-0.70389
LAC_NA07_Bacteria_70_305	-0.24661	0.187308	0.501277
LAC_NA07_Bacteria_71_12	-2.55738	-2.12345	-1.80948
LAC_NA07_Chloroflexi_57_9	-0.01693	0.416995	0.730965
LAC_NA07_Chloroflexi_60_59	-0.70041	-0.26649	0.047483
LAC_NA07_Chloroflexi_66_23	-1.81055	-1.37663	-1.06266
LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	-2.16044	-1.72652	-1.41255
LAC_NA07_Rhodobacterales_68_7	-0.1535	0.280423	0.594393
LAC_NA07_Roizmannbacteria_52_60	-2.1934	-1.75948	-1.44551
LAC_NA07_Truepera_radiovictrix_72_29	-1.28346	-0.84954	-0.53557
LAC_NA08_Chloroflexi_58_8	-0.58222	-0.1483	0.16567
LAC_NA08_Chloroflexi_60_15	-1.8236	-1.38968	-1.07571

LAC_NA08_Chloroflexi_67_31	0.060378	0.494299	0.808268
LAC_NA08_Flavobacteriales_41_5	1.271384	1.705305	2.019275
LAC_NA08_Gammaproteobacteria_68_22	-0.74789	-0.31397	0
LAC_NA08_Ignavibacterium_album_33_266	0.273581	0.707502	1.021472
LAC_NA09_Acidobacteria_70_95	0.51073	0.944651	1.25862
LAC_NA09_Gammaproteobacteria_70_24	4.50999	4.943911	5.257881
LAC_NA10_Actinobacteria_74_12	1.269679	1.7036	2.01757
LAC_NA10_Bacteria_62_9	3.396389	3.83031	4.14428
LAC_NA10_Chloroflexi_62_239	0.348056	0.781977	1.095946
LAC_NA10_Lysobacter_70_8	1.889056	2.322977	2.636946
LAC_NA10_Xanthomonadales_68_7	1.836755	2.270676	2.584646
LAC_NA11_Bacteria_34_14	3.864204	4.298125	4.612094
LAC_NA11_Bacteria_57_35	1.070316	1.504237	1.818206
LAC_NA11_Bacteroidales_40_6	1.204191	1.638112	1.952081
LAC_NA11_Betaproteobacteria_62_13	4.629397	5.063318	5.377288
LAC_NA11_Caldilinea_aerophila_60_6	1.559157	1.993078	2.307048
LAC_NA11_Ignavibacteriales_56_75	2.816129	3.25005	3.56402
LAC_NA11_Microgenomates_50_64	2.29927	2.733191	3.04716
NA01_Acidobacteria_59_12	0.693025	1.126946	1.440915
NA01_Bacteria_60_23	1.999411	2.433332	2.747302
NA01_Caldilinea_aerophila_61_10	-0.36419	0.069729	0.383698
NA01_Chloroflexi_65_16	-1.47319	-1.03927	-0.7253
NA01_Fimbriimonas_ginsengisoli_61_16	-0.01282	0.421102	0.735072
NA01_Rhizobiales_69_9	1.44392	1.877841	2.19181
NA01_Roizmanbacteria_38_28	1.515339	1.94926	2.26323
NA02_Anaerolinea_thermophila_56_16	3.002677	3.436598	3.750568
NA02_Rhizobiales_66_27	0.100987	0.534908	0.848877
NA03_Alphaproteobacteria_58_599	-0.88757	-0.45364	-0.13967
NA03_Alphaproteobacteria_61_24	3.589824	4.023746	4.337715
NA03_Anaerolineales_41_23	0.88398	1.317901	1.631871
NA03_Bacteria_65_16	0.833982	1.267903	1.581873
NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	-1.2438	-0.80988	-0.49591
NA03_Leptonema_illini_45_7	-0.33367	0.100248	0.414218
NA03_RBG_16_Chloroflexi_57_11_curated_55_21	1.781768	2.215689	2.529658
NA04_Bacteria_50_51	1.860578	2.294499	2.608469
NA04_Bacteria_58_19	-0.11038	0.323543	0.637513
NA04_Bacteria_65_10	1.142646	1.576567	1.890536
NA04_Bacteria_69_30	0.440185	0.874106	1.188076
NA04_Bacteroidetes_30_9	2.540491	2.974412	3.288382
NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	3.224898	3.658819	3.972789
NA04_Burkholderiales_70_137	0.124463	0.558384	0.872353
NA04_Candidatus_Saccharibacteria_41_123	2.871607	3.305528	3.619498
NA04_Myxococcales_72_13	3.460994	3.894915	4.208885

NA04_Rhodocyclales_67_27	1.250777	1.684698	1.998667
NA04_Sphingobacteriales_44_8	2.136662	2.570583	2.884552
NA04_Turneriella_parva_44_15	2.238999	2.67292	2.98689

Table 6: MAG D37/D232 LR Changes

	Upper Limit	Lower Limit
RFG1 (anamox1_Bacteria_33_9_curated)	0.143726	-0.47735
RFG2 (anamox2_Verrucomicrobia_62_8_curated)	0.577647	-0.04343
RFG3 (LAC_NA08_Gammaproteobacteria_68_22)	0.891617	0.270544

Table 7: LR Change Confidence Intervals

MAG	D37	D140	D232	totals
anamox1_Acidobacteria_62_5_curated	0.006211	0.002572	0.001277	0.01006
anamox1_Bacteria_33_9_curated	0.003004	0.003106	0.00369	0.0098
anamox1_Bacteria_45_8_curated	9.90E-05	0.000176	0.00045	0.000725
anamox1_Bacteria_50_18_curated	0.000115	3.91E-05	0.000151	0.000306
anamox1_Bacteria_53_17_curated	8.81E-05	5.15E-05	1.15E-05	0.000151
anamox1_Bacteria_55_18_curated	0.001926	0.001072	0.000186	0.003184
anamox1_Bacteria_56_37_curated	0.528339	0.288761	0.34461	1.16171
anamox1_Bacteria_57_32_curated	2.08E-05	1.03E-05	2.93E-06	3.40E-05
anamox1_Bacteria_57_9_curated	0.000327	0.00027	0.00012	0.000717
anamox1_Bacteria_64_7_curated	0.001251	0.000816	0.000401	0.002469
anamox1_Bacteria_65_5_curated	0.000789	0.000762	0.000878	0.002429
anamox1_Bacteria_72_15_curated	0.000124	2.36E-05	1.33E-05	0.000161
anamox1_Bacteroidetes_39_16_curated	8.44E-06	2.51E-06	8.57E-07	1.18E-05
anamox1_Bacteroidetes_63_11_curated	0.002264	0.008341	0.009036	0.019642

anamox1_Betaproteobacteria_69_7_curated	0.111081	0.026609	0.022672	0.160362
anamox1_Burkholderiales_71_17_curated	0.007112	0.014539	0.004898	0.02655
anamox1_Candidatus_Cloacimonas_acidaminovorans_38_6_curated	2.38E-06	3.05E-06	6.20E-06	1.16E-05
anamox1_Chloroflexi_52_59_curated	0.030156	0.025691	0.024239	0.080086
anamox1_Gemmatimonas_aurantiaca_57_6_curated	0.003277	0.015437	0.003565	0.02228
anamox1_Nitrosomonas_europaea_50_14_curated	0.000246	0.000106	5.48E-05	0.000407
anamox1_Proteobacteria_67_8_curated	0.001968	0.000821	0.00038	0.003169
anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	0.000651	0.000523	0.000942	0.002116
anamox1_Sphingobacteriales_43_8_curated	0.001037	0.001041	9.11E-05	0.002169
anamox2_Actinobacteria_65_5_curated	0.07197	0.052252	0.038705	0.162927
anamox2_Bacteria_61_6_curated	0.000155	0.000238	0.000674	0.001067
anamox2_Bacteria_68_6_curated	0.045385	0.204178	0.069537	0.3191
anamox2_Burkholderiales_67_5_curated	0.013811	0.018652	0.042328	0.074791
anamox2_Burkholderiales_68_9_curated	0.005229	0.011966	0.003099	0.020294
anamox2_Burkholderiales_70_13_curated	0.000834	0.00113	0.001606	0.00357
anamox2_Chloroflexi_60_8_curated	0.003463	0.001735	0.000965	0.006163
anamox2_Hydrogenophilales_66_19_curated	0.000727	0.001098	0.002463	0.004288
anamox2_Ignavibacteriales_33_9_curated	0.108968	0.150627	0.202631	0.462227
anamox2_Ignavibacteriales_41_12_curated	0.000603	0.001402	0.000918	0.002922
anamox2_Methanosarcina_thermophila_41_9_curated	8.54E-05	4.04E-05	1.38E-05	0.00014
anamox2_Microgenomates_45_6_curated	0.163884	0.078205	0.100563	0.342652
anamox2_Myxococcales_71_5_curated	0.000936	0.002248	0.00198	0.005164
anamox2_Rhizobiales_67_45_curated	0.000114	0.000105	0.000326	0.000545

anamox2_Sphingobacteriales_41_11_curated	0.002197	0.002492	0.002818	0.007507
anamox2_Verrucomicrobia_58_8_curated	0.000181	0.000218	0.000165	0.000564
anamox2_Verrucomicrobia_62_8_curated	0.007203	0.006882	0.005759	0.019844
anamox2_Xanthomonadales_68_9_curated	0.00024	0.000912	0.000247	0.001398
anamox3_Acidobacteria_71_4_curated	0.023815	0.012341	0.006766	0.042921
anamox3_Bacteria_66_7_curated	0.000631	0.001628	0.000695	0.002954
anamox3_Bacteria_67_13_curated	3.35E-06	3.14E-06	2.36E-06	8.85E-06
anamox3_Bacteria_67_15_curated	0.00892	0.002728	0.002638	0.014286
anamox3_Bacteroidetes_39_15_curated	2.22E-05	2.86E-05	3.50E-05	8.58E-05
anamox3_Chloroflexi_59_6_curated	0.351935	0.190165	0.175534	0.717635
anamox3_Chloroflexi_68_6_curated	0.061493	0.034993	0.037099	0.133585
anamox3_Nitrosomonas_europaea_51_28_curated	0.006325	0.00274	0.000472	0.009537
anamox3_Pedosphaera_parvula_66_5_curated	0.001103	0.000457	0.000332	0.001892
anamox3_Sphingobacteriales_50_9_curated	0.001807	0.001235	0.000151	0.003194
anamox3_sub_Ignavibacteriales_42_14_curated	0.092131	0.326489	0.491841	0.91046
anamox3_Verrucomicrobia_59_12_curated	2.75E-05	4.70E-06	2.02E-06	3.42E-05
anamox4_Bacteria_63_7_curated	0.233667	0.047579	0.012995	0.294241
anamox4_Bacteroidetes_40_74_curated	0.002813	0.000743	9.98E-05	0.003655
anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	0.001149	0.00072	0.000367	0.002236
anamox4_Gammaproteobacteria_67_14_curated	0.017333	0.003895	0.0009	0.022128
anamox4_Microgenomates_48_8_curated	0.004511	0.000962	0.000319	0.005793
anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	9.672443	10.25377	7.805721	27.73194
LAC_NA06_Acidobacteria_54_19	0.003664	0.001198	0.000497	0.005358

LAC_NA06_Actinobacteria_71_15	0.059425	0.027907	0.044429	0.131762
LAC_NA06_Betaproteobacteria_71_7	0.423245	0.279806	0.212422	0.915474
LAC_NA06_Burkholderiales_73_13	0.015869	0.005565	0.00419	0.025624
LAC_NA06_Candidatus_Solibacter_usitatus_62_12	0.099705	0.07025	0.051995	0.221949
LAC_NA06_Microgenomates_41_17	0.001591	0.001032	0.000158	0.00278
LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	0.039493	0.009404	0.011482	0.060379
LAC_NA06_Sphingobacteriales_42_58	0.037026	0.013769	0.015586	0.066381
LAC_NA06_Xanthomonadales_70_10	0.040064	0.11222	0.038219	0.190503
LAC_NA07_Actinobacteria_74_18	0.162363	0.058828	0.061087	0.282277
LAC_NA07_Actinotalea_fermentans_75_19	0.041313	0.017182	0.005042	0.063537
LAC_NA07_Bacteria_38_171	0.287491	0.067699	0.072364	0.427554
LAC_NA07_Bacteria_57_12	0.221275	0.106413	0.063713	0.391402
LAC_NA07_Bacteria_70_305	1.186839	1.698255	1.139331	4.024425
LAC_NA07_Bacteria_71_12	0.167489	0.035013	0.015919	0.218421
LAC_NA07_Chloroflexi_57_9	0.20102	0.154175	0.243924	0.599118
LAC_NA07_Chloroflexi_60_59	0.056022	0.00375	0.034162	0.093933
LAC_NA07_Chloroflexi_66_23	0.227458	0.069382	0.045569	0.342409
LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	0.086919	0.019389	0.012342	0.118651
LAC_NA07_Rhodobacterales_68_7	0.216367	0.194236	0.229316	0.639918
LAC_NA07_Roizmannbacteria_52_60	0.004357	0.000658	0.0006	0.005614
LAC_NA07_Truepera_radiovictrix_72_29	0.32921	0.212072	0.11242	0.653702
LAC_NA08_Chloroflexi_58_8	0.441165	0.292891	0.302911	1.036966
LAC_NA08_Chloroflexi_60_15	1.440842	0.427863	0.28559	2.154295

LAC_NA08_Chloroflexi_67_31	0.315572	0.199258	0.411202	0.926032
LAC_NA08_Flavobacteriales_41_5	0.030048	0.256915	0.131187	0.418151
LAC_NA08_Gammaproteobacteria_68_22	0.030248	0.025794	0.01755	0.073592
LAC_NA08_Ignavibacterium_album_33_266	14.10886	17.76094	23.01864	54.88844
LAC_NA09_Acidobacteria_70_95	0.121235	0.043512	0.248251	0.412998
LAC_NA09_Gammaproteobacteria_70_24	0.000345	0.001912	0.037637	0.039895
LAC_NA10_Actinobacteria_74_12	0.069063	0.106258	0.30697	0.482291
LAC_NA10_Bacteria_62_9	0.001946	0.002261	0.069944	0.074151
LAC_NA10_Chloroflexi_62_239	9.181714	12.35382	15.9766	37.51214
LAC_NA10_Lysobacter_70_8	0.014105	0.071352	0.113695	0.199152
LAC_NA10_Xanthomonadales_68_7	0.01465	0.128817	0.112009	0.255475
LAC_NA11_Bacteria_34_14	0.001217	0.000799	0.071575	0.073591
LAC_NA11_Bacteria_57_35	0.00012	0.000471	0.000397	0.000989
LAC_NA11_Bacteroidales_40_6	0.012362	0.099346	0.05127	0.162977
LAC_NA11_Betaproteobacteria_62_13	0.000246	0.000402	0.028601	0.029249
LAC_NA11_Caldilinea_aerophila_60_6	0.052892	0.07633	0.306149	0.435372
LAC_NA11_Ignavibacteriales_56_75	0.001156	0.004679	0.023713	0.029547
LAC_NA11_Microgenomates_50_64	0.091262	0.614933	1.127105	1.8333
NA01_Acidobacteria_59_12	0.059256	0.094173	0.145765	0.299194
NA01_Bacteria_60_23	0.009344	0.027154	0.084536	0.121033
NA01_Caldilinea_aerophila_61_10	0.138865	0.055083	0.118356	0.312303
NA01_Chloroflexi_65_16	0.853347	0.402605	0.240791	1.496744
NA01_Fimbriimonas_ginsengisoli_61_16	0.046217	0.026844	0.056038	0.129099

NA01_Rhizobiales_69_9	0.055615	0.200284	0.288574	0.544473
NA01_Roizmanbacteria_38_28	0.006454	0.005005	0.036326	0.047784
NA02_Anaerolinea_thermophila_56_16	0.00723	0.014861	0.177577	0.199668
NA02_Rhizobiales_66_27	0.742663	0.448988	1.010378	2.202028
NA03_Alphaproteobacteria_58_599	0.021953	0.018951	0.01113	0.052034
NA03_Alphaproteobacteria_61_24	7.94E-05	0.001738	0.003421	0.005238
NA03_Anaerolineales_41_23	0.218985	0.266058	0.657177	1.14222
NA03_Bacteria_65_16	0.000105	0.000119	0.000285	0.000508
NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	0.002173	0.00495	0.00077	0.007893
NA03_Leptonema_illini_45_7	0.029639	0.039308	0.026414	0.095362
NA03_RBG_16_Chloroflexi_57_11_curated_55_21	0.062375	0.083842	0.456644	0.602861
NA04_Bacteria_50_51	0.004698	0.01877	0.037412	0.06088
NA04_Bacteria_58_19	0.182447	0.288477	0.201094	0.672019
NA04_Bacteria_65_10	0.03248	0.068478	0.124895	0.225853
NA04_Bacteria_69_30	0.054346	0.044211	0.104293	0.202849
NA04_Bacteroidetes_30_9	0.005584	0.071644	0.084641	0.161868
NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	0.000465	0.038325	0.013532	0.052323
NA04_Burkholderiales_70_137	2.925124	2.992179	4.087406	10.00471
NA04_Candidatus_Saccharibacteria_41_123	0.00104	0.000679	0.023445	0.025164
NA04_Myxococcales_72_13	0.000137	0.002139	0.003983	0.006259
NA04_Rhodocyclales_67_27	0.268306	0.581367	1.155097	2.00477
NA04_Sphingobacteriales_44_8	0.003063	0.027064	0.031978	0.062106
NA04_Turneriella_parva_44_15	0.000869	0.004679	0.010115	0.015663

Table 8: MAG RPKM Values

	Upper Limit	Lower Limit
Day 37	0.935288	0.051942
Day 140	0.660955	0.07046
Day 232	0.775536	0.050206

Table 9: MAG RPKM Confidence Intervals

Appendix 2: Supplementary Information for Chapter 3-Growth strategies determined response to SRT driven crash in anammox bioreactor

Day	Influent ammonium (mg-N/L)	Influent nitrite (mg-N/L)	Nitrogen loading rate (g N/L-d)	SRT
0	600	660	2.52	50
80	600	680	2.52	50
111	600	700	2.6	50
128	600	710	2.62	50
129	600	720	2.64	50
142	620	740	2.72	50
150	640	768	2.82	50
157	660	792	2.9	50
161	680	816	2.99	50
168	700	840	3.08	50
175	720	864	3.17	50
183	740	888	3.26	50
189	760	912	3.34	50
196	780	936	3.43	50
205	800	960	3.52	50
210	818	982	3.6	40
224	818	982	3.6	33.3
245	818	982	3.6	28.5
287	811	989	3.6	28.5
290	804	996	3.6	28.5
296	201	249	0.9	28.5
297	201	249	0.9	50
314	250	300	1.1	50
317	295	355	1.3	50
321	341	409	1.5	50
323	386	464	1.7	50
328	455	545	2	50
345	568	682	2.5	50
353	682	818	3	50
360	795	955	3.5	50
364	909	1091	4	50

Table 1: Experimental MBR influent media and SRT data

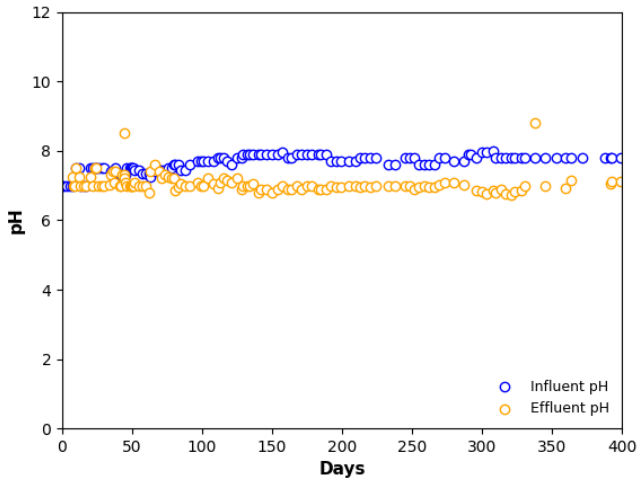

Figure 1: MBR Influent and Effluent pH

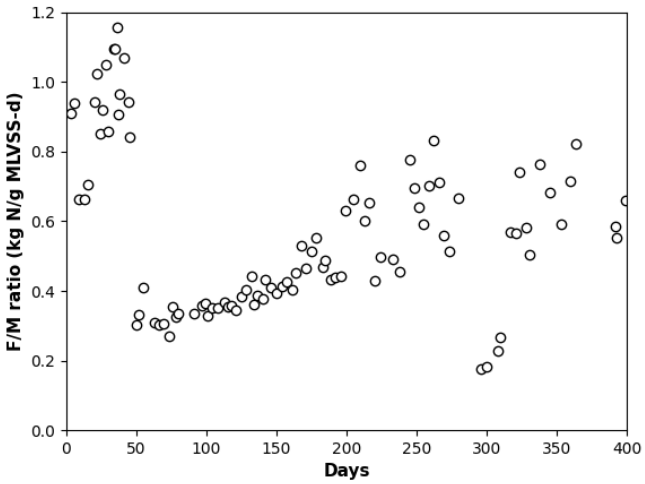

Figure 2: MBR Food to Microbe (F/M) ratios

Sample	Days collected	Total Reads
LAC_NA08	41	95301016
LAC_NA01	126, 133, 140	127646278
LAC_NA02	199, 206, 208	132292488
LAC_NA03	270, 277, 284	128552526
LAC_NA04	324, 326, 328	152499468
LAC_NA09	248	93267710
LAC_NA10	293	112149384
LAC_NA11	378	93605354

Table 2: Metagenome sampling data

Sample	Total Reads	Mapped Reads	Mapped Percentage	Total Bases	Mapped Bases	Mismatches	error rate
LAC_NA08	95301016	72438066	76.00975209	1321888537	10079415025	57323417	5.69E-03
LAC_NA01	127646278	89632124	70.21914419	17694180953	12442322754	61468369	4.94E-03
LAC_NA02	132292488	101795619	76.94739175	18436840767	14200752323	60607324	4.27E-03
LAC_NA03	128552526	100131252	77.89131425	17962511815	14002242931	53289819	3.81E-03
LAC_NA04	152499468	127248106	83.44167207	21268429587	17770627268	63460045	3.57E-03
LAC_NA09	93267710	75538253	80.9907877	12752032352	10360571717	55471755	5.35E-03
LAC_NA10	112149384	90405586	80.61175441	15461035235	12484776448	60817391	4.87E-03
LAC_NA11	93605354	73186094	78.18579907	12635399009	9908581128	55726332	5.62E-03

Table 3: Metagenome mapping statistics

Genome	Genome code (ggkbase)	Phyla	Genome length (bp)	GC %	Contigs	ORFs	Completeness (%)	Abundance Cluster	Metabolic Cluster
ACD01	LAC_NA09_Acidobacteria_70_95	Acidobacteria	3,730,000	69.99%	45	3263	92.74%	3	C
ACD02	NA01_Acidobacteria_59_12	Acidobacteria	5,590,000	59.15%	159	4789	92.98%	2	A
ACD03	LAC_NA06_Candidatus_Solibacter_usitatus_62_12	Acidobacteria	4,520,000	62.17%	166	3970	84.35%	5	A
ACD04	anamox3_Acidobacteria_71_4_curated	Acidobacteria	1,330,000	69.62%	772	1759	35.00%	5	D
ACD05	anamox1_Acidobacteria_62_5_curated	Acidobacteria	3,110,000	61.59%	565	3251	69.92%	1	A
ACD06	LAC_NA06_Acidobacteria_54_19	Acidobacteria	2,710,000	54.24%	26	2505	94.02%	1	C
ACT01	LAC_NA10_Actinobacteria_74_12	Actinobacteria	2,540,000	73.88%	170	2460	96.24%	2	D
ACT02	LAC_NA07_Actinobacteria_74_18	Actinobacteria	1,210,000	73.80%	141	1247	58.54%	5	D
ACT03	anamox2_Actinobacteria_65_5_curated	Actinobacteria	2,800,000	63.61%	662	3147	66.92%	5	D
ACT04	LAC_NA06_Actinobacteria_71_15	Actinobacteria	3,180,000	70.59%	57	3196	95.83%	5	D
ACT05	LAC_NA07_Actinotalea_fermentans_75_19	Actinobacteria	2,910,000	75.44%	31	2757	95.95%	1	D
AMX01	anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	Planctomycetes	3,110,000	42.29%	64	2859	100%	4	A
ARC01	anamox2_Methanosarcina_thermophila_41_9_curated	Euryarchaeota	2,960,000	41.19%	82	2703	97.85%	1	A
ARM01	NA01_Fimbriimonas_ginsengisoli_61_16	Armatimonadetes	2,770,000	60.97%	47	2570	93.98%	3	D
BAC01	LAC_NA07_Bacteria_70_305	Bacteria	2,750,000	70.12%	54	2386	90.91%	3	D
BAC02	anamox1_Bacteria_56_37_curated	Bacteria	4,970,000	55.88%	43	4404	91.52%	4	A
BAC03	NA04_Bacteria_58_19	Bacteria	7,560,000	57.56%	112	6122	97.27%	2	A
BAC04	LAC_NA07_Bacteria_38_171	Bacteria	2,390,000	37.56%	23	2128	95.08%	4	C
BAC05	LAC_NA07_Bacteria_57_12	Bacteria	5,860,000	57.26%	271	5345	92.73%	5	A
BAC06	anamox2_Bacteria_68_6_curated	Bacteria	2,290,000	67.96%	460	2621	60.64%	3	D
BAC07	anamox4_Bacteria_63_7_curated	Bacteria	3,450,000	62.64%	351	3276	87.27%	1	A
BAC08	NA04_Bacteria_65_10	Bacteria	4,100,000	64.60%	238	3590	91.67%	4	D
BAC09	LAC_NA07_Bacteria_71_12	Bacteria	2,760,000	71.10%	206	2842	84.60%	1	D
BAC10	NA04_Bacteria_69_30	Bacteria	4,020,000	69.42%	30	3566	93.33%	2	A
BAC11	NA01_Bacteria_60_23	Bacteria	3,780,000	60.01%	48	3266	94.95%	2	A
BAC12	LAC_NA10_Bacteria_62_9	Bacteria	3,000,000	62.13%	264	2819	87.04%	3	D
BAC13	LAC_NA11_Bacteria_34_14	Bacteria	863,630	34.10%	17	886	79.15%	3	B

BAC 14	NA04_Bacteria_50_51	Bacteria	2,400,000	49.62%	31	1990	94.81%	4	C
BAC 15	anamox3_Bacteria_67_15_curated	Bacteria	2,770,000	66.48%	144	2980	90.76%	5	D
BAC 16	anamox1__Bacteria_33_9_curated	Bacteria	775,490	32.38%	45	793	62.09%	2	A
BAC 18	anamox3_Bacteria_66_7_curated	Bacteria	2,270,000	65.35%	286	2150	81.93%	4	D
BAC 19	anamox1_Bacteria_64_7_curated	Bacteria	3,990,000	63.42%	306	3679	89.37%	1	D
BAC 20	anamox1_Bacteria_65_5_curated	Bacteria	2,090,000	64.54%	434	2237	52.01%	5	D
BAC 21	anamox2_Bacteria_61_6_curated	Bacteria	2,420,000	60.87%	385	2646	65.68%	4	D
BAC 22	LAC_NA11_Bacteria_57_35	Bacteria	6,060,000	56.96%	90	5339	95.73%	4	A
BAC 23	anamox1_Bacteria_45_8_curated	Bacteria	2,580,000	45.00%	166	2221	89.32%	3	C
BAC 24	anamox1_Bacteria_57_9_curated	Bacteria	3,470,000	57.28%	74	3012	91.27%	1	D
BAC 24	NA03_Bacteria_65_16	Bacteria	3,910,000	65.04%	63	3719	91.45%	3	D
BAC 25	anamox1_Bacteria_50_18_curated	Bacteria	820,570	49.55%	22	874	63.77%	3	B
BAC 26	anamox1_Bacteria_72_15_curated	Bacteria	2,940,000	72.45%	24	2507	93.75%	4	C
BAC 27	anamox1_Bacteria_53_17_curated	Bacteria	3,470,000	53.24%	12	2895	95.91%	1	A
BAC 28	anamox1_Bacteria_57_32_curated	Bacteria	2,940,000	56.91%	76	2571	92.02%	1	D
BAC 29	anamox3_Bacteria_67_13_curated	Bacteria	1,640,000	66.50%	47	1442	92.02%	4	D
BACG11	anamox1_Sphingobacteriales_43_8_curated	Bacteroidetes	3,160,000	42.95%	130	2826	95.13%	1	C
BACT01	LAC_NA08_Flavobacteriales_41_5	Bacteroidetes	1,240,000	41.09%	665	1648	50.87%	5	C
BACT02	LAC_NA11_Bacteroidales_40_6	Bacteroidetes	2,280,000	40.14%	900	2540	70.85%	3	C
BACT03	NA04_Bacteroidetes_30_9	Bacteroidetes	2,580,000	30.05%	248	2356	95.63%	4	C
BACT04	LAC_NA06_Sphingobacteriales_42_58	Bacteroidetes	3,450,000	42.01%	34	2968	97.04%	2	C
BACT05	NA04_Sphingobacteriales_44_8	Bacteroidetes	4,070,000	43.73%	474	3624	86.45%	3	A
BACT06	NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	Bacteroidetes	2,930,000	45.76%	35	2441	96.77%	3	C
BACT07	anamox1_Bacteroidetes_63_11_curated	Bacteroidetes	3,360,000	63.21%	73	2915	99.46%	3	C
BACT08	anamox2_Sphingobacteriales_41_11_curated	Bacteroidetes	2,770,000	41.16%	129	2490	85.67%	4	C
BACT09	anamox4_Bacteroidetes_40_74_curated	Bacteroidetes	2,630,000	39.91%	15	2219	99.52%	1	C
BACT10	anamox3_Sphingobacteriales_50_9_curated	Bacteroidetes	4,310,000	49.73%	216	2999	95.45%	1	C
BACT12	anamox3_Bacteroidetes_39_15_curated	Bacteroidetes	2,530,000	39.26%	23	2188	98.57%	3	C
BACT13	anamox1_Bacteroidetes_39_16_curated	Bacteroidetes	2,900,000	38.84%	40	2472	98.73%	1	C
BAC 17	anamox1_Bacteria_55_18_curated	Bacteria	4,100,000	54.56%	216	3365	99.46%	1	D
CFLX01	LAC_NA10_Chloroflexi_62_239	Chloroflexi	1,430,000	61.97%	417	1859	49.63%	2	D
CFLX02	LAC_NA08_Chloroflexi_60_15	Chloroflexi	3,260,000	59.74%	310	3038	86.36%	5	A

CFLX03	NA01_Chloroflexi_65_16	Chloroflexi	3,700,000	65.08%	207	3252	93.64%	5	D
CFLX04	NA03_Anaerolineales_41_23	Chloroflexi	2,360,000	41.48%	215	2322	85.45%	2	D
CFLX05	LAC_NA08_Chloroflexi_58_8	Chloroflexi	2,650,000	57.66^	432	2733	74.18%	5	D
CFLX06	LAC_NA08_Chloroflexi_67_31	Chloroflexi	3,550,000	66.65%	70	2905	90.91%	2	D
CFLX07	anamox3_Chloroflexi_59_6_curated	Chloroflexi	1,410,000	53.68%	351	1507	32.37%	5	D
CFLX08	NA03_RBG_16_Chloroflexi_57_11_curated_55_21	Chloroflexi	3,440,000	55.06%	367	3313	91.2%	3	D
CFLX09	LAC_NA07_Chloroflexi_57_9	Chloroflexi	2,090,000	56.86%	325	2202	70.11%	2	D
CFLX10	LAC_NA11_Caldilinea_aerophila_60_6	Chloroflexi	3,520,000	59.88%	1263	3974	70.72%	4	A
CFLX11	LAC_NA07_Chloroflexi_66_23	Chloroflexi	4,470,000	66.09%	141	3687	94.55%	5	A
CFLX12	NA01_Caldilinea_aerophila_61_10	Chloroflexi	3,730,000	60.76%	159	2098	83.64%	2	A
CFLX13	NA02_Anaerolinea_thermophila_56_16	Chloroflexi	2,140,000	56.04%	540	2398	78.33%	3	D
CFLX14	anamox3_Chloroflexi_68_6_curated	Chloroflexi	2,290,000	67.35%	479	2330	56.04%	5	D
CFLX15	LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	Chloroflexi	2,490,000	74.73%	63	2327	91.20%	5	D
CFLX16	LAC_NA07_Chloroflexi_60_59	Chloroflexi	3,860,000	60.11%	13	3453	89.55%	3	D
CFLX17	anamox1_Chloroflexi_52_59_curated	Chloroflexi	2,860,000	52.47%	96	2759	92.73%	5	D
CFLX18	anamox2_Chloroflexi_60_8_curated	Chloroflexi	2,020,000	60.06%	111	1885	44.42%	1	D
CLA01	anamox1_Candidatus_Cloacimonas_acidaminovorans_38_6_curated	Candidatus Cloacimonas	1,190,000	35.28%	246	1132	53.43%	2	C
CPR01	LAC_NA11_Microgenomates_50_64	Microgenomates	869,170	49.81%	63	986	55.88%	3	B
CPR02	anamox2_Microgenomates_45_6_curated	Microgenomates	740,220	44.28%	99	904	59.54%	2	B
CPR03	NA01_Roizmanbacteria_38_28	Microgenomates	897,990	38.12%	17	921	68.93%	2	B
CPR04	NA04_Candidatus_Saccharibacteria_41_123	Microgenomates	820,330	40.66%	14	857	66.82%	3	B
CPR05	anamox4_Microgenomates_48_8_curated	Microgenomates	999,880	47.64%	49	1055	63.30%	1	B
CPR06	LAC_NA07_Roizmannbacteria_52_60	Microgenomates	885,170	52.20%	1	945	73.98%	5	B
CPR07	LAC_NA06_Microgenomates_41_17	Microgenomates	1,100,000	41.16%	6	1203	67.89%	1	B
DADA01	LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	Dadabacteria	918,620	57.55%	539	1314	43.08%	5	C
DEN01	LAC_NA07_Truepera_radiovictrix_72_29	Deinococcus-Thermus	1,320,000	72.29%	165	1356	73.23%	5	D
GEM01	anamox1_Gemmatimonas_aurantiaca_57_6_curated	Gemmatimonadota	2,630,000	56.94%	255	2549	90.66%	1	C
IGN01	LAC_NA08_Ignavibacterium_album_33_266	Ignavibacteriae	2,140,000	33.41%	731	2621	67.42%	2	C
IGN02	anamox3_sub_Ignavibacteriales_42_14_curated	Ignavibacteriae	3,160,000	42.21%	22	2498	95.63%	3	C
IGN03	anamox2_Ignavibacteriales_33_9_curated	Ignavibacteriae	2,940,000	33.09%	166	2753	76.8%	3	C
IGN04	LAC_NA11_Ignavibacteriales_56_75	Ignavibacteriae	2,990,000	56.40%	21	2685	96.72%	3	C

IGN 05	NA03_BJP_IG2069_Ignavibacteriae_38 _11_31_13	Ignavibacter iae	2,740,000	30.5 3%	53	234 4	96.89%	3	C
IGN 06	anamox2_Ignavibacteriales_41_12_cur ated	Ignavibacter iae	3,160,000	41.3 2%	42	280 5	95.63%	5	C
IGN 07	anamox1_RBG_16_Ignavibacteria_36_ 9_curated_35_5_curated	Ignavibacter iae	1,160,000	34.0 3%	347	137 4	51.34%	4	C
MYX 01	NA04_Myxococcales_72_13	Myxococcot a	4,300,000	71.8 9%	121	369 5	89.03#	4	A
MYX 02	anamox2_Myxococcales_71_5_curated	Myxococcot a	1,430,000	70.2 1%	495	168 7	24.99%	4	C
NSP 01	anamox4_Candidatus_Nitrospira_deflu vii_60_9_curated	Nitrospirota	3,090,000	60.3 0%	94	308 4	95.85%	1	A
PRO T01	NA04_Burkholderiales_70_137	Proteobacte ria	2,510,000	69.6 9%	131	249 8	90.41%	2	A
PRO T02	NA02_Rhizobiales_66_27	Proteobacte ria	2,950,000	66.3 8%	163	300 0	95.3%	2	A
PRO T03	NA04_Rhodocyclales_67_27	Proteobacte ria	2,480,000	66.9 8%	295	271 8	85.24%	3	A
PRO T04	LAC_NA06_Betaproteobacteria_71_7	Proteobacte ria	4,100,000	70.6 5%	184 1	566 1	97.98%	5	A
PRO T05	LAC_NA07_Rhodobacterales_68_7	Proteobacte ria	1,330,000	68.4 1%	473	167 4	58.41%	2	D
PRO T06	NA01_Rhizobiales_69_9	Proteobacte ria	3,340,000	69.2 8%	447	360 2	79.77%	2	A
PRO T07	LAC_NA10_Xanthomonadales_68_7	Proteobacte ria	2,460,000	68.2 8%	555	258 4	68.74%	3	A
PRO T08	LAC_NA10_Lysobacter_70_8	Proteobacte ria	1,030,000	70.0 0%	294	118 0	37.95%	3	D
PRO T09	LAC_NA06_Xanthomonadales_70_10	Proteobacte ria	1,920,000	69.7 5%	377	203 9	69.12%	1	A
PRO T10	anamox1_Betaproteobacteria_69_7_c urated	Proteobacte ria	2,110,000	66.3 9%	531	263 6	52.65%	5	D
PRO T11	anamox2_Burkholderiales_67_5_curat ed	Proteobacte ria	1,250,000	66.2 8%	298	143 4	38.05%	2	D
PRO T12	LAC_NA08_Gammaproteobacteria_68_ 22	Proteobacte ria	2,800,000	68.4 3%	13	259 4	89.07%	5	A
PRO T13	NA03_Alphaproteobacteria_58_599	Proteobacte ria	2,550,000	57.8 3%	1	248 5	95.81%	3	D
PRO T14	LAC_NA09_Gammaproteobacteria_70_ 24	Proteobacte ria	2,570,000	69.9 1%	23	242 0	88.23%	3	A
PRO T15	LAC_NA11_Betaproteobacteria_62_13	Proteobacte ria	2,820,000	62.3 8%	76	281 1	97.47%	4	A
PRO T16	anamox1_Burkholderiales_71_17_cura ted	Proteobacte ria	3,640,000	70.8 1%	114	344 8	61.64%	1	A
PRO T17	LAC_NA06_Burkholderiales_73_13	Proteobacte ria	3,680,000	72.8 7%	335	345 3	89.44%	5	A
PRO T18	anamox4_Gammaproteobacteria_67_1 4_curated	Proteobacte ria	3,270,000	66.9 1%	73	304 9	92.53%	1	A
PRO T19	anamox2_Burkholderiales_68_9_curat ed	Proteobacte ria	3,530,000	68.2 5%	181	345 2	95.74%	3	A
PRO T20	anamox3_Nitrosomonas_europaea_51 _28_curated	Proteobacte ria	2,370,000	50.5 4%	93	230 2	95.55%	1	A
PRO T21	NA03_Alphaproteobacteria_61_24	Proteobacte ria	3,390,000	61.0 2%	12	318 9	99.50%	3	A
PRO T23	anamox2_Burkholderiales_70_13_cura ted	Proteobacte ria	3,070,000	70.3 3%	39	284 1	87.2%	2	A
PRO T24	anamox1_Proteobacteria_67_8_curate d	Proteobacte ria	2,910,000	66.9 3%	203	291 0	83.43%	1	A
PRO T25	anamox2_Xanthomonadales_68_9_cur ated	Proteobacte ria	1,240,000	67.6 3%	21	116 8	55.44%	1	D
PRO T26	anamox2_Rhizobiales_67_45_curated	Proteobacte ria	4,840,000	66.5 6%	24	466 8	98.36%	2	A
PRO T27	anamox1_Nitrosomonas_europaea_50 _14_curated	Proteobacte ria	2,130,000	50.4 3%	37	200 6	99.74%	1	A

PROT28	anamox2_Hydrogenophilales_66_19_curated	Proteobacteria	2,260,000	65.94%	122	2331	87.57%	2	A
SPR01	NA03_Leptonema_illini_45_7	Spirochaetes	2,310,000	45.31%	383	2382	77.25%	3	D
SPR02	NA04_Turneriella_parva_44_15	Spirochaetes	2,930,000	44.39%	65	2790	93.19%	3	D
VER01	anamox2_Verrucomicrobia_62_8_curated	Verrucomicrobia	3,690,000	61.78%	161	3134	95.27%	5	D
VER02	anamox3_Pedosphaera_parvula_66_5_curated	Verrucomicrobia	1,230,000	65.98%	543	1384	52.71%	5	C
VER03	anamox2_Verrucomicrobia_58_8_curated	Verrucomicrobia	3,590,000	57.68%	250	3293	89.46%	5	D
VER04	anamox3_Verrucomicrobia_59_12_curated	Verrucomicrobia	2,710,000	59.27%	50	2592	96.62%	1	D

Table 4: Metagenome assembly information with abundance and COG clustering information

Day	data_evenness	data_shannon	data_simpson	S.obs	S.chao1	se.chao1	S.ACE	se.ACE
10	0.348617	2.221255	0.742573	585	1454.833	156.5623	1260.677	21.18476
28	0.282484	1.73074	0.643117	458	1083.061	121.5023	1036.133	19.8132
41	0.308017	1.937341	0.68545	539	1329.932	139.3494	1345.518	23.52488
42	0.316896	1.854537	0.661534	348	626.7222	59.04967	715.834	17.07734
43	0.332669	1.879239	0.696332	284	550.0294	66.32321	555.914	13.89743
45	0.342146	2.181187	0.782487	587	1409.721	136.8809	1422.684	23.85096
49	0.346162	2.180456	0.778076	544	1618.49	195.9809	1592.558	25.75983
51	0.411967	2.737556	0.858452	769	1656.451	136.5401	1540.108	23.72138
60	0.434896	2.655922	0.852828	449	942.2885	96.89919	949.2377	18.99016
63	0.412214	2.725023	0.829191	743	1601.517	129.8875	1665.294	25.89406
70	0.406974	2.654321	0.851578	680	1515.775	136.5932	1388.201	21.79906
79	0.35373	2.212166	0.744988	520	1022.047	91.45341	1062.519	20.5008
92	0.398194	2.659775	0.854311	796	1963.471	171.0714	2011.269	28.83098
99	0.416401	2.765935	0.867723	767	2060.042	200.3389	1937.305	27.63469
105	0.387511	2.502067	0.821825	637	1418.667	128.2396	1413.275	23.623
114	0.402867	2.579787	0.848211	604	1073.447	78.47027	1155.481	21.68383
122	0.417331	2.699819	0.873344	645	1459.625	132.9095	1508.177	24.75376
128	0.444332	2.943282	0.891088	753	1709.413	139.7262	1874.155	27.46149
156	0.444783	3.031965	0.899905	913	2440.876	213.0371	2286.813	29.28382

163	0.453848	2.969941	0.896594	695	1582.451	136.54	1680.756	26.28191
174	0.423994	2.771515	0.877914	690	1433.821	116.2809	1409.567	22.86032
182	0.427662	2.71379	0.868078	570	1185.3	105.4275	1248.312	21.37644
188	0.422511	2.108511	0.759735	147	271.25	41.70782	290.1059	10.18611
195	0.39576	2.482553	0.837933	530	1421.596	168.6647	1182.575	20.77824
199	0.345899	2.123779	0.765354	464	1191.468	140.9182	1145.192	21.08792
202	0.345615	2.139467	0.779637	488	1268.122	147.5357	1236.524	22.74982
218	0.442022	2.971016	0.883845	830	1692.901	123.7115	1644.733	24.85292
223	0.44215	2.95394	0.881592	797	2075.762	186.1214	2020.473	28.59106
233	0.391893	2.564512	0.830307	695	1487.439	123.8319	1502.491	25.21003
245	0.380236	2.404065	0.824074	557	1370.333	144.6343	1302.33	22.71876
251	0.423924	2.761742	0.883412	675	1444.286	119.6848	1553.036	25.57911
253	0.411861	2.633268	0.845662	598	1184.197	98.28784	1198.236	21.21621
258	0.393397	2.492886	0.843787	565	1360.016	136.6338	1388.535	24.56031
266	0.420081	2.694861	0.866968	611	1538.188	155.3893	1522.479	25.38573
272	0.417706	2.810091	0.879398	835	2030.062	166.2628	2123.451	30.19767
280	0.431336	2.608449	0.867753	423	867.1875	91.1044	852.8515	17.42311
287	0.439109	2.842064	0.882143	647	1305.125	106.5446	1333.552	23.23659
294	0.381557	2.413788	0.826971	559	1225	115.2329	1261.353	22.87429
300	0.366041	2.562078	0.858715	1096	2813.65	199.1734	3124.766	37.39925
303	0.339246	2.372043	0.829325	1088	3415.684	281.4388	3521.442	37.92146

307	0.399087	2.649365	0.86797	764	1767.531	142.366	1939.635	29.20698
315	0.366788	2.477077	0.845242	857	2144.529	172.9152	2228.233	31.28517
323	0.388489	2.533191	0.84995	679	1624.288	150.5068	1527.937	24.61503
328	0.407857	2.603557	0.857332	592	1496.909	160.9987	1366.5	22.91631
345	0.418131	2.571756	0.848446	469	890.6667	79.31765	918.8055	18.65851
350	0.427418	2.677904	0.867032	526	1081.694	100.8477	1073.857	20.65114
359	0.437315	2.708909	0.862333	490	1237.024	150.4854	979.8421	19.15549
364	0.43259	2.720065	0.871204	538	1113.085	105.7046	1021.185	18.97716
371	0.405554	2.504647	0.834612	481	894.1429	75.24245	942.4781	18.60181
380	0.408251	2.575286	0.835574	549	1115.042	97.21673	1212.619	22.36214
388	0.405512	2.52813	0.847582	510	1021.479	89.63421	1100.465	20.77765
394	0.423546	2.685436	0.885184	567	1284.923	123.6316	1251.286	22.62025
401	0.439922	2.633575	0.872027	398	930.5238	111.8009	839.6231	17.62395
406	0.42154	2.711061	0.883439	621	1303.12	108.4414	1374.318	23.33734

Table 5: Alpha Diversity metrics

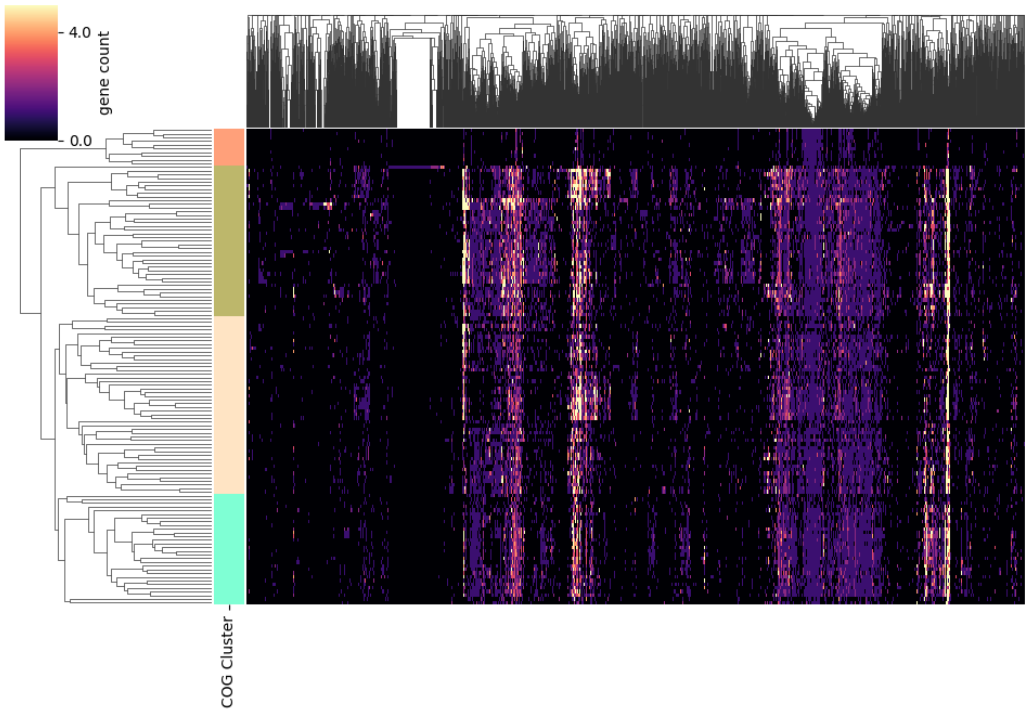

Figure 3: Hierarchical clustering of COG category gene counts

genome	RFG1	RFG2	RFG3
anamox1_Acidobacteria_62_5_curated	-1.77639	-1.51687	-1.87469
anamox1_Bacteria_33_9_curated	-0.25951	0	-0.35782
anamox1_Bacteria_45_8_curated	0.821175	1.080685	0.722867
anamox1_Bacteria_50_18_curated	2.13935	2.398861	2.041042
anamox1_Bacteria_53_17_curated	-2.33907	-2.07956	-2.43737
anamox1_Bacteria_55_18_curated	0.021023	0.280534	-0.07728
anamox1_Bacteria_56_37_curated	1.061516	1.321027	0.963208
anamox1_Bacteria_57_32_curated	0.769418	1.028929	0.671111

anamox1_Bacteria_57_9_curated	-0.32716	-0.06765	-0.42547
anamox1_Bacteria_64_7_curated	0.812357	1.071868	0.714049
anamox1_Bacteria_65_5_curated	0.542308	0.801819	0.444001
anamox1_Bacteria_72_15_curated	1.318906	1.578417	1.220598
anamox1_Bacteroidetes_39_16_curated	1.894582	2.154093	1.796275
anamox1_Bacteroidetes_63_11_curated	-1.27734	-1.01783	-1.37565
anamox1_Betaproteobacteria_69_7_curated	1.612745	1.872255	1.514437
anamox1_Burkholderiales_71_17_curated	0.844418	1.103929	0.74611
anamox1_Chloroflexi_52_59_curated	0.315067	0.574577	0.216759
anamox1_Gemmatimonas_aurantiaca_57_6_curated	2.924651	3.184161	2.826343
anamox1_Nitrosomonas_europaea_50_14_curated	0.512519	0.772029	0.414211
anamox1_Proteobacteria_67_8_curated	-0.58055	-0.32104	-0.67886
anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	-1.76016	-1.50065	-1.85847
anamox1_Sphingobacteriales_43_8_curated	2.556653	2.816163	2.458345
anamox2_Actinobacteria_65_5_curated	0.354284	0.613795	0.255977
anamox2_Bacteria_61_6_curated	1.615343	1.874854	1.517036
anamox2_Bacteria_68_6_curated	1.256266	1.515777	1.157958
anamox2_Burkholderiales_67_5_curated	-0.07038	0.189133	-0.16869
anamox2_Burkholderiales_68_9_curated	2.873994	3.133504	2.775686
anamox2_Burkholderiales_70_13_curated	-0.53614	-0.27663	-0.63445
anamox2_Chloroflexi_60_8_curated	-1.42347	-1.16396	-1.52178
anamox2_Hydrogenophilales_66_19_curated	-1.84443	-1.58492	-1.94274
anamox2_Ignavibacteriales_33_9_curated	0.098308	0.357818	0
anamox2_Ignavibacteriales_41_12_curated	-0.58308	-0.32357	-0.68139
anamox2_Methanosarcina_thermophila_41_9_curated	-0.55156	-0.29205	-0.64986
anamox2_Microgenomates_45_6_curated	0	0.259511	-0.09831
anamox2_Myxococcales_71_5_curated	0.809719	1.069229	0.711411
anamox2_Rhizobiales_67_45_curated	1.269691	1.529202	1.171384
anamox2_Sphingobacteriales_41_11_curated	0.195645	0.455156	0.097337
anamox2_Verrucomicrobia_58_8_curated	0.311249	0.570759	0.212941
anamox2_Verrucomicrobia_62_8_curated	0.354468	0.613979	0.25616
anamox2_Xanthomonadales_68_9_curated	0.789524	1.049035	0.691216
anamox3_Acidobacteria_71_4_curated	-0.01293	0.246576	-0.11124
anamox3_Bacteria_66_7_curated	1.640897	1.900408	1.54259
anamox3_Bacteria_67_13_curated	-4.26132	-4.00181	-4.35963
anamox3_Bacteria_67_15_curated	1.173827	1.433337	1.075519
anamox3_Bacteroidetes_39_15_curated	3.959191	4.218702	3.860883
anamox3_Chloroflexi_59_6_curated	-0.90666	-0.64715	-1.00496
anamox3_Chloroflexi_68_6_curated	-0.78473	-0.52521	-0.88303
anamox3_Nitrosomonas_europaea_51_28_curated	-0.23433	0.025177	-0.33264
anamox3_Pedosphaera_parvula_66_5_curated	1.596701	1.856212	1.498393
anamox3_Sphingobacteriales_50_9_curated	-1.4771	-1.21759	-1.57541
anamox3_sub_Ignavibacteriales_42_14_curated	1.732642	1.992153	1.634335

anamox3_Verrucomicrobia_59_12_curated	-0.7108	-0.45129	-0.80911
anamox4_Bacteria_63_7_curated	-0.76023	-0.50072	-0.85854
anamox4_Bacteroidetes_40_74_curated	-0.20212	0.057393	-0.30043
anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	-2.07424	-1.81473	-2.17255
anamox4_Gammaproteobacteria_67_14_curated	-0.34233	-0.08282	-0.44064
anamox4_Microgenomates_48_8_curated	-0.23718	0.022333	-0.33549
anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	-1.02979	-0.77028	-1.1281
LAC_NA06_Acidobacteria_54_19	0.948641	1.208151	0.850333
LAC_NA06_Actinobacteria_71_15	-1.09022	-0.83071	-1.18853
LAC_NA06_Betaproteobacteria_71_7	0.063922	0.323433	-0.03439
LAC_NA06_Burkholderiales_73_13	0.206002	0.465513	0.107694
LAC_NA06_Candidatus_Solibacter_usitatus_62_12	0.133422	0.392933	0.035114
LAC_NA06_Microgenomates_41_17	-3.4918	-3.23229	-3.59011
LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	0.141745	0.401255	0.043437
LAC_NA06_Sphingobacteriales_42_58	0.424599	0.684109	0.326291
LAC_NA06_Xanthomonadales_70_10	0.153778	0.413289	0.05547
LAC_NA07_Actinobacteria_74_18	-0.0376	0.221914	-0.1359
LAC_NA07_Actinotalea_fermentans_75_19	-1.78452	-1.52501	-1.88282
LAC_NA07_Bacteria_38_171	1.168288	1.427798	1.06998
LAC_NA07_Bacteria_57_12	-1.93971	-1.6802	-2.03802
LAC_NA07_Bacteria_70_305	1.22794	1.487451	1.129633
LAC_NA07_Bacteria_71_12	-1.82688	-1.56736	-1.92518
LAC_NA07_Chloroflexi_57_9	-0.86543	-0.60592	-0.96374
LAC_NA07_Chloroflexi_60_59	0.126561	0.386072	0.028253
LAC_NA07_Chloroflexi_66_23	-1.43713	-1.17762	-1.53544
LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	-0.29168	-0.03217	-0.38999
LAC_NA07_Rhodobacterales_68_7	0.673878	0.933389	0.575571
LAC_NA07_Roizmannbacteria_52_60	-0.37556	-0.11605	-0.47387
LAC_NA07_Truepera_radiovictrix_72_29	0.958993	1.218504	0.860686
LAC_NA08_Chloroflexi_58_8	-0.60735	-0.34784	-0.70566
LAC_NA08_Chloroflexi_60_15	-1.5399	-1.28039	-1.63821
LAC_NA08_Chloroflexi_67_31	-0.35363	-0.09412	-0.45194
LAC_NA08_Flavobacteriales_41_5	1.860568	2.120079	1.76226
LAC_NA08_Gammaproteobacteria_68_22	-0.09587	0.163638	-0.19418
LAC_NA08_Ignavibacterium_album_33_266	-0.2531	0.006409	-0.35141
LAC_NA09_Acidobacteria_70_95	0.389318	0.648828	0.29101
LAC_NA09_Gammaproteobacteria_70_24	0.97051	1.230021	0.872203
LAC_NA10_Actinobacteria_74_12	0.972848	1.232359	0.87454
LAC_NA10_Bacteria_62_9	0.48042	0.73993	0.382112
LAC_NA10_Chloroflexi_62_239	0.321847	0.581358	0.223539
LAC_NA10_Lysobacter_70_8	1.13843	1.397941	1.040122
LAC_NA10_Xanthomonadales_68_7	0.709071	0.968581	0.610763
LAC_NA11_Bacteria_34_14	-0.08617	0.173342	-0.18448

LAC_NA11_Bacteria_57_35	-1.12744	-0.86793	-1.22575
LAC_NA11_Bacteroidales_40_6	2.269814	2.529324	2.171506
LAC_NA11_Betaproteobacteria_62_13	-0.17621	0.083301	-0.27452
LAC_NA11_Caldilinea_aerophila_60_6	0.856495	1.116006	0.758188
LAC_NA11_Ignavibacteriales_56_75	1.158757	1.418268	1.060449
LAC_NA11_Microgenomates_50_64	0.57046	0.829971	0.472152
NA01_Acidobacteria_59_12	0.410649	0.67016	0.312341
NA01_Bacteria_60_23	0.778527	1.038038	0.680219
NA01_Caldilinea_aerophila_61_10	-0.03752	0.221988	-0.13583
NA01_Chloroflexi_65_16	-2.79338	-2.53386	-2.89168
NA01_Fimbriimonas_ginsengisoli_61_16	0.717657	0.977168	0.619349
NA01_Rhizobiales_69_9	1.588083	1.847593	1.489775
NA01_Roizmanbacteria_38_28	-0.31112	-0.05161	-0.40942
NA02_Anaerolinea_thermophila_56_16	-0.06744	0.192069	-0.16575
NA02_Rhizobiales_66_27	-0.04434	0.215169	-0.14265
NA03_Alphaproteobacteria_58_599	0.329605	0.589116	0.231297
NA03_Alphaproteobacteria_61_24	-0.37646	-0.11695	-0.47476
NA03_Anaerolineales_41_23	0.146212	0.405722	0.047904
NA03_Bacteria_65_16	10.80063	11.06014	10.70232
NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	7.882101	8.141612	7.783793
NA03_Leptonema_illini_45_7	-1.86979	-1.61027	-1.96809
NA03_RBG_16_Chloroflexi_57_11_curated_55_21	-0.0554	0.204114	-0.1537
NA04_Bacteria_50_51	1.261389	1.5209	1.163082
NA04_Bacteria_58_19	-0.57866	-0.31915	-0.67696
NA04_Bacteria_65_10	1.803626	2.063137	1.705319
NA04_Bacteria_69_30	0.154602	0.414113	0.056294
NA04_Bacteroidetes_30_9	-1.29078	-1.03127	-1.38909
NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	4.162608	4.422118	4.0643
NA04_Burkholderiales_70_137	-0.13546	0.124048	-0.23377
NA04_Candidatus_Saccharibacteria_41_123	2.638346	2.897857	2.540038
NA04_Myxococcales_72_13	2.353229	2.61274	2.254921
NA04_Rhodocyclales_67_27	-0.24619	0.013319	-0.3445
NA04_Sphingobacteriales_44_8	0.309014	0.568525	0.210706
NA04_Turneriella_parva_44_15	1.130256	1.389767	1.031948

Table 6: LR changes for reference frame genomes 1, 2, and 3

	Upper Limit	Lower Limit
RFG1 (anamox2_Microgenomates_45_6_curated)	0.01705	0.623911
RFG2 (anamox1_Bacteria_33_9_curated)	0.276561	0.883422
RFG3 (anamox2_Ignavibacteriales_33_9_curated)	-0.08126	0.525603

Table 7: LR change confidence intervals (CI)

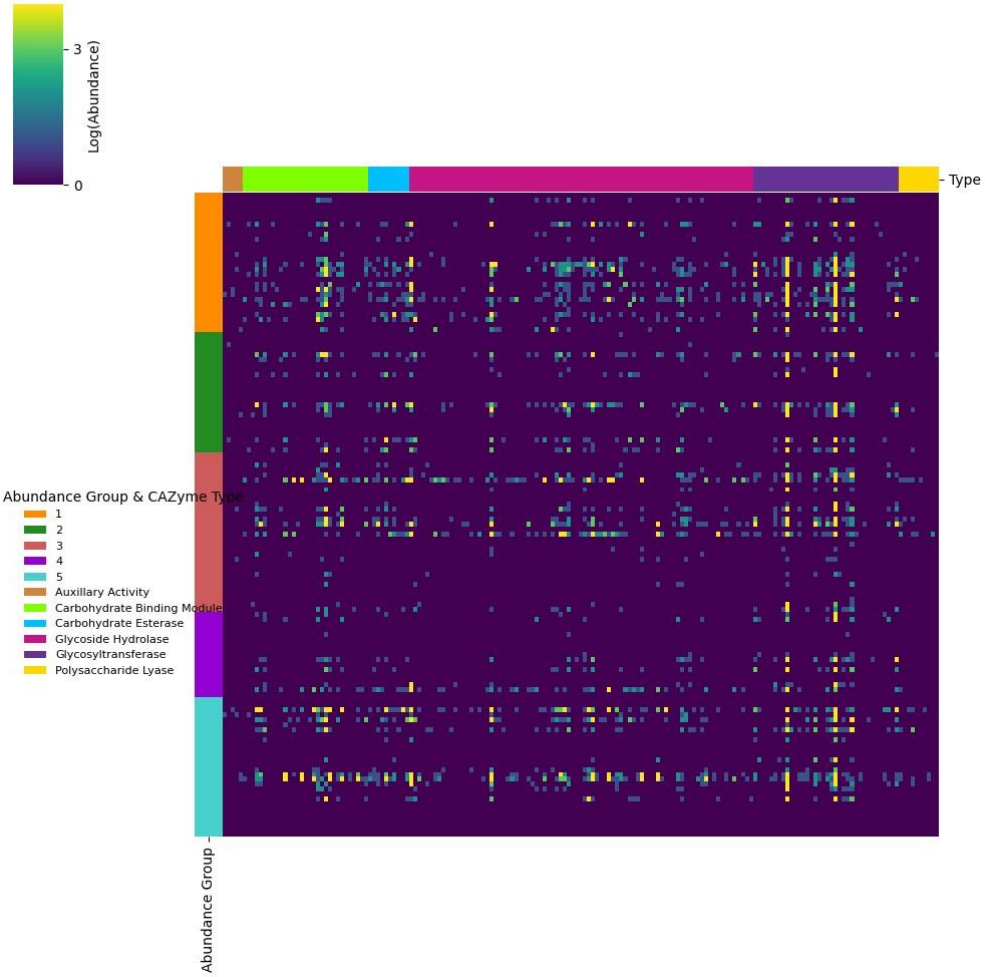

Figure 4: Extracellular and outer membrane CAZyme log abundances

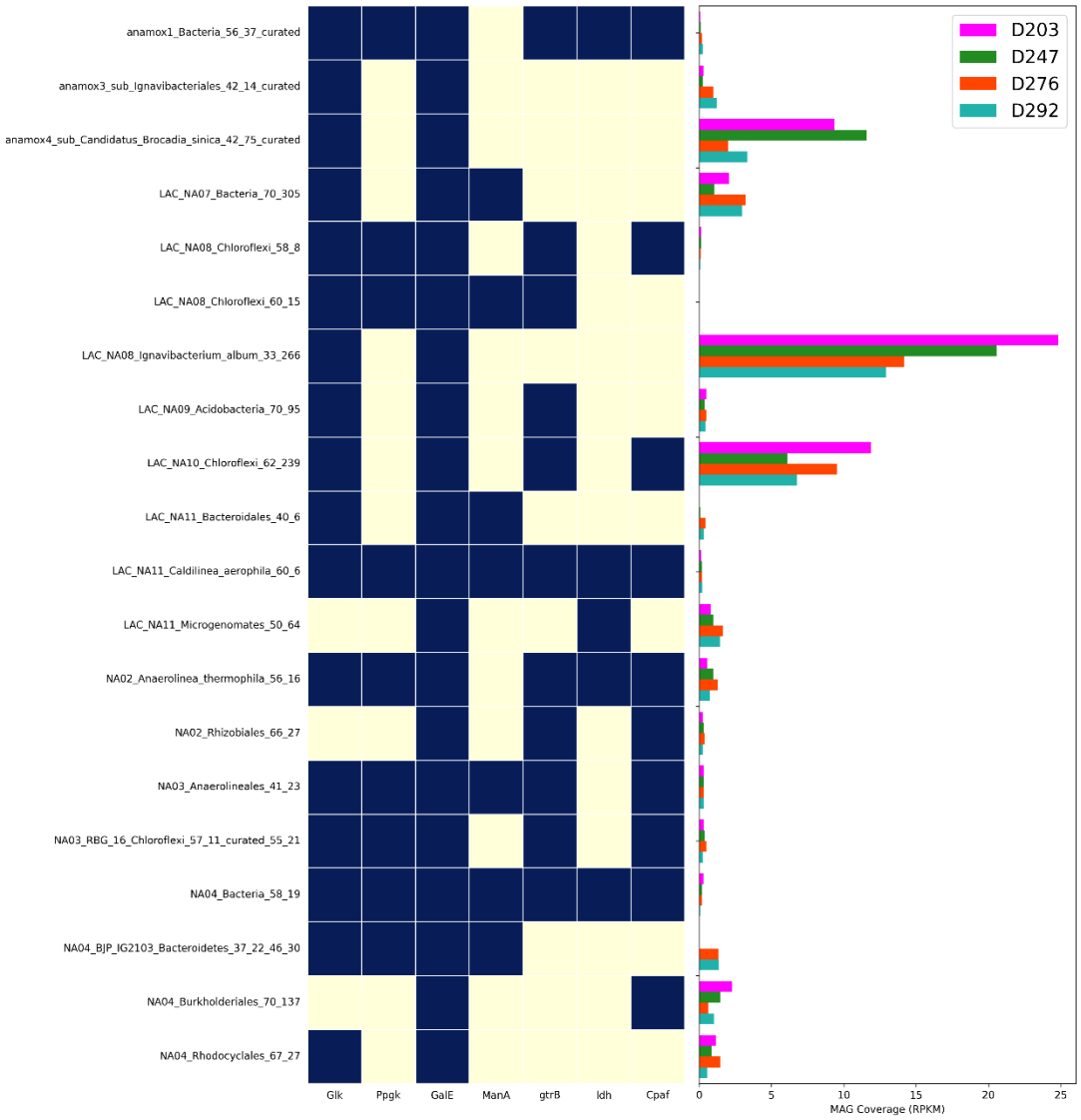

Figure 5: Amino-sugar and nucleotide sugar and cell adherence gene presence plotted against MAG abundance measured as RPKM

Day	51	143	214	258	287	303	336	388
EC number richness	1982	1983	1984	1982	1981	1980	1982	1980
EC subclass richness	218	218	217.8	218	218	218	218	218
EC number evenness	2.829164	2.863598	2.805686	2.819368	2.735922	2.740044	2.773432	2.77362
EC subclass evenness	2.233924	2.253599	2.21459	2.22719	2.151888	2.15578	2.186788	2.189239

Table 8: Enzyme commission (EC) number richness and evenness metrics and EC-subclass richness and evenness

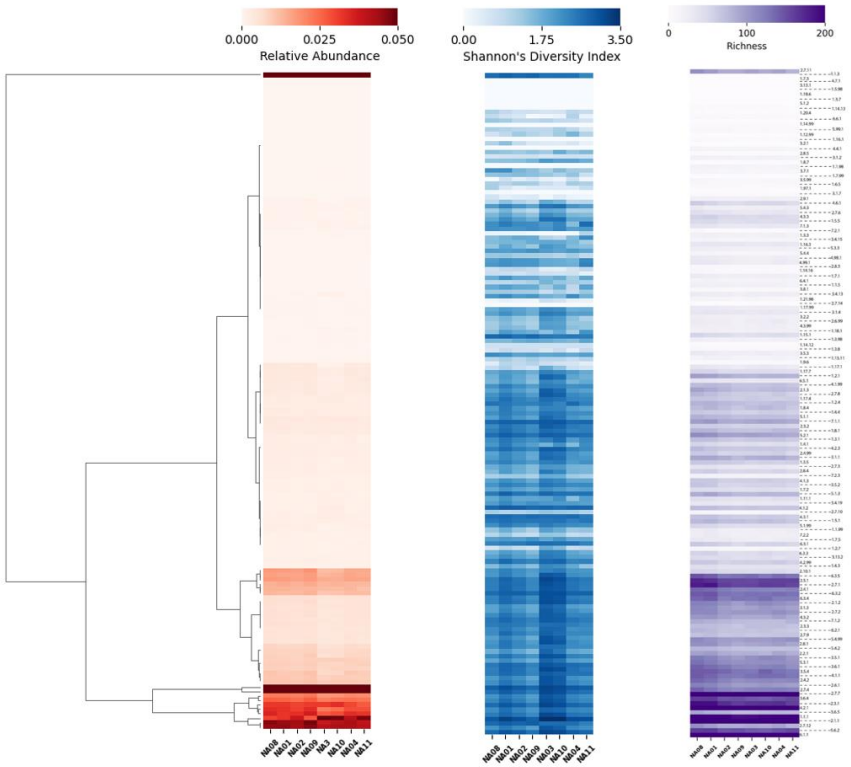

Figure 6: Enzyme Commission (EC) subclass relative abundance, taxonomic evenness (Shannon's Diversity Index), and taxonomic richness

Appendix 3: Supplementary Information for Chapter 4-Heterotrophic degradation of anammox extracellular polymeric substances

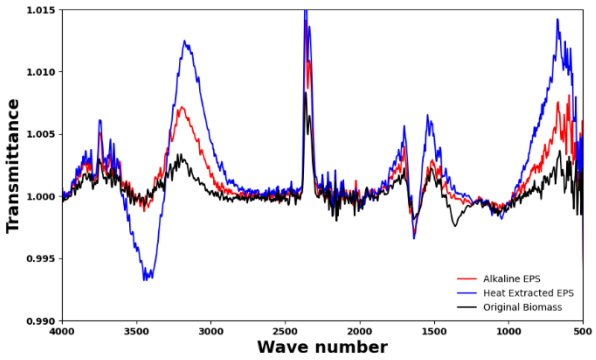

Figure 1: FTIR spectra of EPS solutions

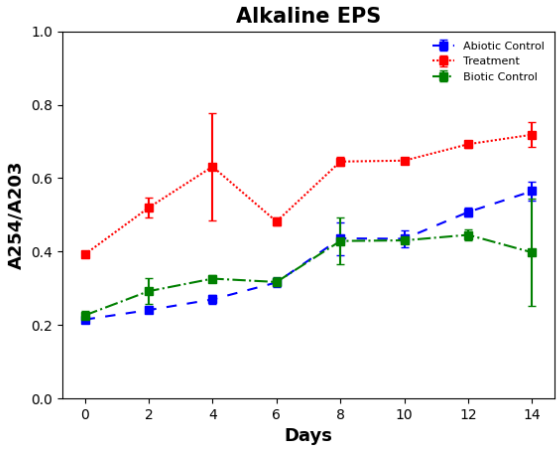

Figure 2a: A254/A203 UV absorbance data of alkaline-EPS enrichments

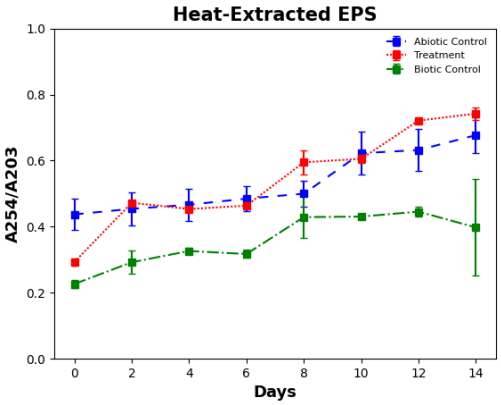

Figure 2b: A254/A203 UV absorbance data of heat-extracted-EPS enrichments

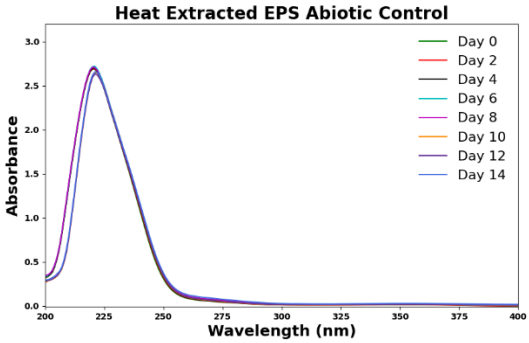

Figure 3a: UV-Vis Absorbance of samples from abiotic heat-extracted EPS controls

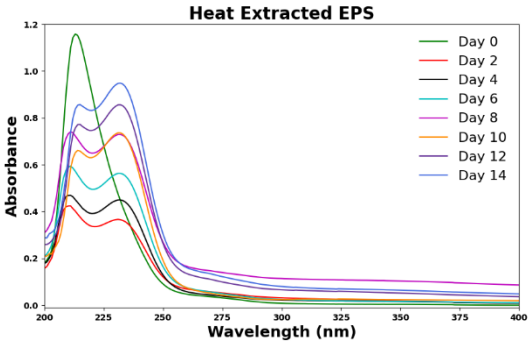

Figure 3b: UV-Vis Absorbance of samples from heat-extracted EPS treatments

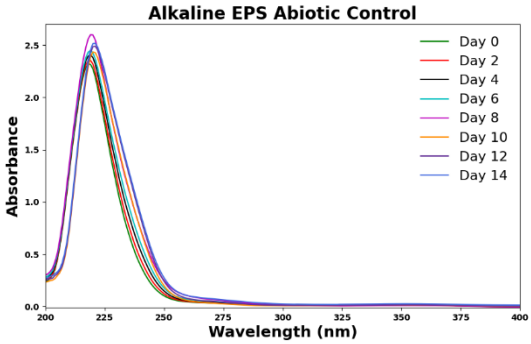

Figure 3c: UV-Vis Absorbance of samples from abiotic alkaline EPS controls

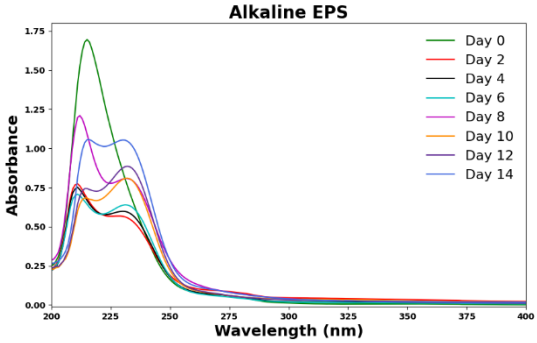

Figure 3d: UV-Vis Absorbance of samples from alkaline EPS treatments

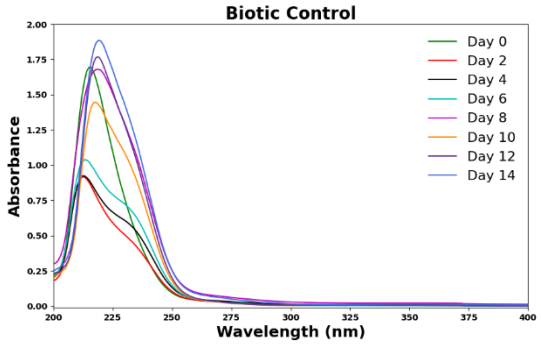

Figure 3e: UV-Vis Absorbance of samples from biotic controls

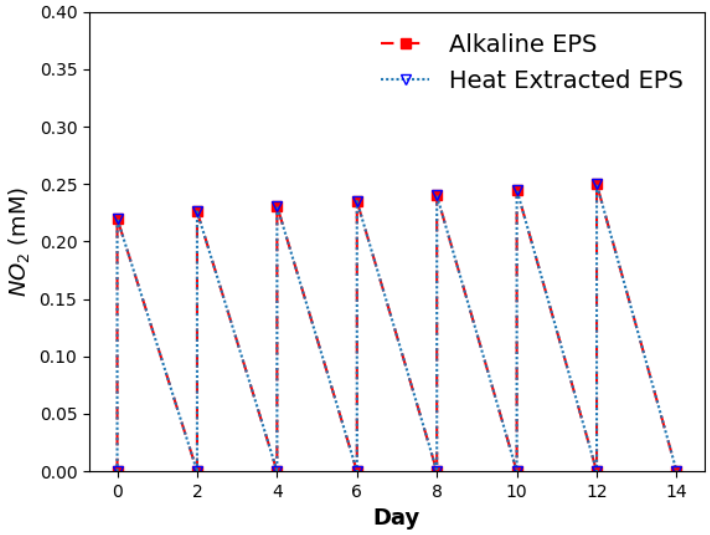

Figure 4: Nitrite concentration data of heat-extracted and alkaline EPS enrichments

Abrev.	MAG	Phyla	Genome length (bp)	GC	Contigs	OR	Completeness(%)
AMX1	anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	Planctomycetes	3110000	0.42	64	285	100%
IGN1	Control_bins.003	Ignavibacteriae	3700000	0.33	93	312	99.40%
CFLX1	HEPS_bins.003	Chloroflexi	4740000	0.64	298	414	94.50%
PRO1	NA04_Burkholderiales_70_137	Proteobacteria	2510000	0.69	131	249	90.41%
BAC1	LAC_NA10_Bacteria_62_9	Bacteria	3000000	0.62	264	281	87.04%
PRO2	NA04_Rhodocyclales_67_27	Proteobacteria	2480000	0.66	295	271	85.24%
ACT1	LAC_NA10_Actinobacteria_74_12	Actinobacteria	2540000	0.73	170	246	96.24%
CFLX2	AEPS_bins.007	Chloroflexi	6360000	0.61	245	555	
CFLX3	HEPS_bins.010	Chloroflexi	3140000	0.72	87	294	93.10%
IGN2	LAC_NA08_Ignavibacterium_album_33_266	Ignavibacteriae	2140000	0.33	731	262	67.42%
ACD1	LAC_NA09_Acidobacteria_70_95	Acidobacteria	3730000	0.69	45	326	92.74%
IGN3	LAC_NA11_Ignavibacteriales_56_75	Ignavibacteriae	2990000	0.56	21	268	96.72%
CPR1	anamox2_Microgenomates_45_6_curated	Microgenomates	740220	0.44	99	904	59.54%
PRO3	anamox2_Burkholderiales_68_9_curated	Proteobacteria	3530000	0.68	181	345	95.74%
CFLX4	Control_bins.014	Chloroflexi	5060000	0.61	351	455	
ACT2	R2_6_3_bins.015	Actinobacteria	4221632	0.65	167	399	98.30%
CFLX5	NA03_RBG_16_Chloroflexi_57_11_curated_55_21	Chloroflexi	3440000	0.55	367	331	91.2%
ACT3	LAC_NA07_Actinobacteria_74_18	Actinobacteria	1210000	0.73	141	124	58.54%
FIRM1	HEPS_bins.011	Firmicutes	7660000	0.41	210	691	98.00%
PRO4	NA02_Rhizobiales_66_27	Proteobacteria	2950000	0.66	163	300	95.3%

	anamox1_Bacteria_56_37_curated	Bacteria	4970000	0.55	43	440	91.52%
	NA04_Bacteria_69_30	Bacteria	4020000	0.69	30	356	93.33%
	AEPS_bins.021	Proteobacteria	3960000	0.66	541	397	
	HEPS_bins.016	Proteobacteria	5400000	0.61	119	516	
	AEPS_bins.024	Actinobacteria	2880000	0.69	577	331	
	LAC_NA11_Microgenomates_50_64	Microgenomates	869170	0.49	63	986	55.88%
	NA04_Bacteria_50_51	Bacteria	2400000	0.49	31	199	94.81%
	Control_bins.031	Chloroflexi	3690000	0.60	649	368	97.70%
	anamox2_Ignavibacteriales_41_12_curated	Ignavibacteriae	3160000	0.41	42	280	95.63%
	LAC_NA11_Bacteria_34_14	Bacteria	863630	0.34	17	886	79.15%
	LAC_NA10_Xanthomonadales_68_7	Proteobacteria	2460000	0.68	555	258	68.74%
	LAC_NA11_Caldilinea_aerophila_60_6	Chloroflexi	3520000	0.59	1263	397	70.72%
	LAC_NA10_Lysobacter_70_8	Proteobacteria	1030000	0.7	294	118	37.95%
	LAC_NA07_Bacteria_38_171	Bacteria	2390000	0.37	23	212	95.08%
	NA01_Rhizobiales_69_9	Proteobacteria	3340000	0.69	447	360	79.77%
	NA01_Acidobacteria_59_12	Acidobacteria	5590000	0.59	159	478	92.98%
	NA04_Bacteria_65_10	Bacteria	4100000	0.64	238	359	91.67%
	NA04_Candidatus_Saccharibacteria_41_123	Microgenomates	820330	0.40	14	857	66.82%
	LAC_NA06_Xanthomonadales_70_10	Proteobacteria	1920000	0.69	377	203	69.12%
	LAC_NA08_Gammaproteobacteria_68_22	Proteobacteria	2800000	0.68	13	259	89.07%
	LAC_NA11_Betaproteobacteria_62_13	Proteobacteria	2820000	0.62	76	281	97.47%
	LAC_NA09_Gammaproteobacteria_70_24	Proteobacteria	2570000	0.69	23	242	88.23%
	LAC_NA07_Bacteria_57_12	Bacteria	5860000	0.57	271	534	92.73%

	LAC_NA06_Candidatus_Solibacter_usitatus_62_12	Acidobacteria	4520000	0.62	166	397	84.35%
	anamox2_Ignavibacteriales_33_9_curated	Ignavibacteriae	2940000	0.33	166	275	76.8%
	anamox2_Burkholderiales_70_13_curated	Proteobacteria	3070000	0.70	39	284	87.2%
	anamox2_Hydrogenophilales_66_19_curated	Proteobacteria	2260000	0.65	122	233	87.57%
	NA01_Chloroflexi_65_16	Chloroflexi	3700000	0.65	207	325	93.64%
	LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	Chloroflexi	2490000	0.74	63	232	91.20%
	NA03_Alphaproteobacteria_58_599	Proteobacteria	2550000	0.57	1	248	95.81%
	NA04_Bacteroidetes_30_9	Bacteroidetes	2580000	0.30	248	235	95.63%
	NA01_Fimbriimonas_ginsengisoli_61_16	Armatimonadetes	2770000	0.60	47	257	93.98%
	NA03_Alphaproteobacteria_61_24	Proteobacteria	3390000	0.61	12	318	99.50%
	LAC_NA11_Bacteria_57_35	Bacteria	6060000	0.56	90	533	95.73%
	anamox2_Sphingobacteriales_41_11_curated	Bacteroidetes	2770000	0.41	129	249	85.67%
	anamox2_Burkholderiales_67_5_curated	Proteobacteria	1250000	0.66	298	143	38.05%
	anamox2_Rhizobiales_67_45_curated	Proteobacteria	4840000	0.66	24	466	98.36%
	NA01_Bacteria_60_23	Bacteria	3780000	0.60	48	326	94.95%
	LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	Dadabacteria	918620	0.57	539	131	43.08%
	NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	Ignavibacteriae	2740000	0.30	53	234	96.89%
	anamox3_sub_Ignavibacteriales_42_14_curated	Ignavibacteriae	3160000	0.42	22	249	95.63%
	NA02_Anaerolinea_thermophila_56_16	Chloroflexi	2140000	0.56	540	239	78.33%
	anamox2_Xanthomonadales_68_9_curated	Proteobacteria	1240000	0.67	21	116	55.44%
	anamox1_Betaproteobacteria_69_7_curated	Proteobacteria	2110000	0.66	531	263	52.65%

	LAC_NA06_Actinobacteria_71_15	Actinobacteria	3180000	0.70	57	319	95.83%
	LAC_NA07_Bacteria_71_12	Bacteria	2760000	0.71	206	284	84.60%
	LAC_NA06_Acidobacteria_54_19	Acidobacteria	2710000	0.54	26	250	94.02%
	LAC_NA07_Rhodobacterales_68_7	Proteobacteria	1330000	0.68	473	167	58.41%
	NA03_Bacteria_65_16	Bacteria	3910000	0.65	63	371	91.45%
	NA04_Myxococcales_72_13	Myxococcota	4300000	0.71	121	369	89.03%
	LAC_NA08_Chloroflexi_58_8	Chloroflexi	2650000	57.6	432	273	74.18%
	LAC_NA06_Sphingobacteriales_42_58	Bacteroidetes	3450000	0.42	34	296	97.04%
	NA01_Caldilinea_aerophila_61_10	Chloroflexi	3730000	0.60	159	209	83.64%
	NA04_Turneriella_parva_44_15	Spirochaetes	2930000	0.44	65	279	93.19%
	anamox2_Actinobacteria_65_5_curated	Actinobacteria	2800000	0.63	662	314	66.92%
	LAC_NA08_Chloroflexi_67_31	Chloroflexi	3550000	0.66	70	290	90.91%
	LAC_NA10_Chloroflexi_62_239	Chloroflexi	1430000	0.61	417	185	49.63%
	anamox3_Bacteria_67_15_curated	Bacteria	2770000	0.66	144	298	90.76%
	NA04_Sphingobacteriales_44_8	Bacteroidetes	4070000	0.43	474	362	86.45%
	anamox2_Bacteria_68_6_curated	Bacteria	2290000	0.67	460	262	60.64%
	anamox1_Chloroflexi_52_59_curated	Chloroflexi	2860000	0.52	96	275	92.73%
	anamox1_Bacteria_33_9_curated	Bacteria	775490	0.32	45	793	62.09%
	anamox2_Verrucomicrobia_62_8_curated	Verrucomicrobia	3690000	0.61	161	313	95.27%
	anamox3_Acidobacteria_71_4_curated	Acidobacteria	1330000	0.69	772	175	35.00%
	anamox3_Nitrosomonas_europaea_51_28_curated	Proteobacteria	2370000	0.50	93	230	95.55%
	anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	Ignavibacteriae	1160000	0.34	347	137	51.34%

	LAC_NA07_Chloroflexi_60_59	Chloroflexi	3860000	0.60	13	345	89.55%
	LAC_NA08_Chloroflexi_60_15	Chloroflexi	3260000	0.59	310	303	86.36%
	anamox1_Proteobacteria_67_8_curated	Proteobacteria	2910000	0.66	203	291	83.43%
	LAC_NA07_Actinotalea_fermentans_75_19	Actinobacteria	2910000	0.75	31	275	95.95%
	anamox2_Myxococcales_71_5_curated	Myxococcota	1430000	0.70	495	168	24.99%
	anamox1_Gemmatimonas_aurantiaca_57_6_curated	Gemmatimonadota	2630000	0.56	255	254	90.66%
	anamox1_Nitrosomonas_europaea_50_14_curated	Proteobacteria	2130000	0.50	37	200	99.74%
	NA04_Bacteria_58_19	Bacteria	7560000	0.57	112	612	97.27%
	anamox1_Sphingobacteriales_43_8_curated	Bacteroidetes	3160000	0.42	130	282	95.13%
	anamox4_Bacteria_63_7_curated	Bacteria	3450000	0.62	351	327	87.27%
	anamox4_Gammaproteobacteria_67_14_curated	Proteobacteria	3270000	0.66	73	304	92.53%
	anamox3_Bacteroidetes_39_15_curated	Bacteroidetes	2530000	0.39	23	218	98.57%
	LAC_NA07_Chloroflexi_66_23	Chloroflexi	4470000	0.66	141	368	94.55%
	LAC_NA08_Flavobacteriales_41_5	Bacteroidetes	1240000	0.41	665	164	50.87%
	NA03_Leptonema_illini_45_7	Spirochaetes	2310000	0.45	383	238	77.25%
	LAC_NA11_Bacteroidales_40_6	Bacteroidetes	2280000	0.40	900	254	70.85%
	LAC_NA07_Roizmannbacteria_52_60	Microgenomates	885170	0.52	1	945	73.98%
	anamox3_Bacteria_66_7_curated	Bacteria	2270000	0.65	286	215	81.93%
	anamox1_Bacteria_72_15_curated	Bacteria	2940000	0.72	24	250	93.75%
	anamox3_Chloroflexi_68_6_curated	Chloroflexi	2290000	0.67	479	233	56.04%
	anamox4_Bacteroidetes_40_74_curated	Bacteroidetes	2630000	0.39	15	221	99.52%
	anamox1_Bacteria_64_7_curated	Bacteria	3990000	0.63	306	367	89.37%

	anamox1_Acidobacteria_62_5_curated	Acidobacteria	3110000	0.61	565	325	69.92%
	NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	Bacteroidetes	2930000	0.45	35	244	96.77%
	anamox2_Chloroflexi_60_8_curated	Chloroflexi	2020000	0.60	111	188	44.42%
	anamox3_Chloroflexi_59_6_curated	Chloroflexi	1410000	0.53	351	150	32.37%
	anamox2_Bacteria_61_6_curated	Bacteria	2420000	0.60	385	264	65.68%
	NA03_Anaerolineales_41_23	Chloroflexi	2360000	0.41	215	232	85.45%
	anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	Nitrospirota	3090000	0.60	94	308	95.85%
	anamox1_Bacteria_45_8_curated	Bacteria	2580000	0.45	166	222	89.32%
	anamox3_Pedosphaera_parvula_66_5_curated	Verrucomicrobia	1230000	0.65	543	138	52.71%
	anamox1_Bacteroidetes_63_11_curated	Bacteroidetes	3360000	0.63	73	291	99.46%
	anamox2_Verrucomicrobia_58_8_curated	Verrucomicrobia	3590000	0.57	250	329	89.46%
	anamox1_Bacteria_55_18_curated	Bacteria	4100000	0.54	216	336	99.46%
	anamox4_Microgenomates_48_8_curated	Microgenomates	999880	0.47	49	105	63.30%
	anamox1_Bacteria_53_17_curated	Bacteria	3470000	0.53	12	289	95.91%
	anamox1_Bacteria_50_18_curated	Bacteria	820570	0.49	22	874	63.77%
	anamox1_Bacteria_57_9_curated	Bacteria	3470000	0.57	74	301	91.27%
	anamox3_Bacteria_67_13_curated	Bacteria	1640000	0.66	47	144	92.02%
	anamox1_Bacteria_65_5_curated	Bacteria	2090000	0.64	434	223	52.01%
	NA01_Roizmanbacteria_38_28	Microgenomates	897990	0.38	17	921	68.93%
	anamox1_Bacteroidetes_39_16_curated	Bacteroidetes	2900000	0.38	40	247	98.73%
	anamox1_Bacteria_57_32_curated	Bacteria	2940000	0.56	76	257	92.02%
	anamox3_Sphingobacteriales_50_9_curated	Bacteroidetes	4310000	0.49	216	299	95.45%

	anamox3_Verrucomicrobia_59_12_curated	Verrucomicrobia	2710000	0.59	50	259	96.62%
	anamox2_Methanosarcina_thermophila_41_9_curated	Euryarchaeota	2960000	0.41	82	270	97.85%
	anamox1_Candidatus_Cloacimonas_acidaminovorans_38_6_curated	Candidatus Cloacimonas	1190000	0.35	246	113	53.43%

Table 1: Metagenome assembled genomes (MAGs) statistics

Table 2: LR coverage changes for alkaline EPS enrichments

RFG	Upper CI	Lower CI
RFG1	-0.126820938	-0.290372549
RFG2	-0.158976708	-0.32252832
RFG3	-0.200135977	-0.363687589

MAG	RFG1	RFG2	RFG3
AEPS_bins.007	-0.04943	-0.08159	-0.12275
AEPS_bins.021	0.799413	0.767257	0.726098
AEPS_bins.024	0.048465	0.016309	-0.02485
anamox1_Acidobacteria_62_5_curated	-0.03026	-0.06242	-0.10358
anamox1_Bacteria_33_9_curated	-0.21375	-0.24591	-0.28707
anamox1_Bacteria_45_8_curated	-0.31692	-0.34907	-0.39023
anamox1_Bacteria_50_18_curated	0.508077	0.475921	0.434762
anamox1_Bacteria_53_17_curated	-0.99311	-1.02526	-1.06642
anamox1_Bacteria_55_18_curated	0.041402	0.009246	-0.03191
anamox1_Bacteria_56_37_curated	0.064918	0.032762	-0.0084
anamox1_Bacteria_57_32_curated	-0.87116	-0.90332	-0.94448
anamox1_Bacteria_57_9_curated	0.613611	0.581455	0.540296
anamox1_Bacteria_64_7_curated	-0.15145	-0.18361	-0.22477
anamox1_Bacteria_65_5_curated	-0.59752	-0.62967	-0.67083
anamox1_Bacteria_72_15_curated	0.028034	-0.00412	-0.04528
anamox1_Bacteroidetes_39_16_curated	-0.9432	-0.97536	-1.01652
anamox1_Bacteroidetes_63_11_curated	-0.41387	-0.44602	-0.48718
anamox1_Betaproteobacteria_69_7_curated	-0.0309	-0.06306	-0.10422
anamox1_Chloroflexi_52_59_curated	-0.15703	-0.18919	-0.23035
anamox1_Gemmatimonas_aurantiaca_57_6_curated	-0.71457	-0.74672	-0.78788
anamox1_Nitrosomonas_europaea_50_14_curated	0.08991	0.057754	0.016595
anamox1_Proteobacteria_67_8_curated	-0.07648	-0.10864	-0.1498
anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	-0.0649	-0.09705	-0.13821
anamox1_Sphingobacteriales_43_8_curated	-1.01258	-1.04474	-1.0859
anamox2_Actinobacteria_65_5_curated	0.023473	-0.00868	-0.04984
anamox2_Bacteria_61_6_curated	-0.19019	-0.22235	-0.26351
anamox2_Bacteria_68_6_curated	0.119794	0.087639	0.046479
anamox2_Burkholderiales_67_5_curated	0.295773	0.263617	0.222458
anamox2_Burkholderiales_68_9_curated	0.729783	0.697627	0.656468
anamox2_Burkholderiales_70_13_curated	0.352887	0.320731	0.279572
anamox2_Chloroflexi_60_8_curated	0.045499	0.013344	-0.02782
anamox2_Hydrogenophilales_66_19_curated	-0.88041	-0.91256	-0.95372
anamox2_Ignavibacteriales_33_9_curated	-0.10675	-0.13891	-0.18007
anamox2_Ignavibacteriales_41_12_curated	-0.36122	-0.39338	-0.43454
anamox2_Methanosarcina_thermophila_41_9_curated	NA	NA	NA
anamox2_Microgenomates_45_6_curated	-0.36319	-0.39534	-0.4365
anamox2_Myxococcales_71_5_curated	-0.84226	-0.87442	-0.91558
anamox2_Rhizobiales_67_45_curated	-0.23296	-0.26512	-0.30628
anamox2_Sphingobacteriales_41_11_curated	-0.04025	-0.0724	-0.11356
anamox2_Verrucomicrobia_58_8_curated	-0.23781	-0.26997	-0.31113

anamox2_Verrucomicrobia_62_8_curated	-0.11818	-0.15034	-0.1915
anamox2_Xanthomonadales_68_9_curated	0.410124	0.377969	0.336809
anamox3_Acidobacteria_71_4_curated	-0.22561	-0.25777	-0.29893
anamox3_Bacteria_66_7_curated	-1.24716	-1.27932	-1.32048
anamox3_Bacteria_67_13_curated	0.019457	-0.0127	-0.05386
anamox3_Bacteria_67_15_curated	-0.25119	-0.28334	-0.3245
anamox3_Bacteroidetes_39_15_curated	-0.30409	-0.33625	-0.37741
anamox3_Chloroflexi_59_6_curated	-0.17725	-0.20941	-0.25057
anamox3_Chloroflexi_68_6_curated	-0.04151	-0.07367	-0.11483
anamox3_Nitrosomonas_europaea_51_28_curated	-0.52325	-0.55541	-0.59657
anamox3_Pedosphaera_parvula_66_5_curated	-0.40209	-0.43425	-0.47541
anamox3_Sphingobacteriales_50_9_curated	-0.59948	-0.63164	-0.6728
anamox3_sub_Ignavibacteriales_42_14_curated	-0.04997	-0.08213	-0.12329
anamox3_Verrucomicrobia_59_12_curated	-0.83676	-0.86892	-0.91008
anamox4_Bacteria_63_7_curated	-0.03617	-0.06833	-0.10948
anamox4_Bacteroidetes_40_74_curated	-0.66585	-0.69801	-0.73917
anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	-0.48939	-0.52155	-0.56271
anamox4_Gammaproteobacteria_67_14_curated	0.069189	0.037033	-0.00413
anamox4_Microgenomates_48_8_curated	-0.59261	-0.62476	-0.66592
anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	-0.12616	-0.15831	-0.19947
Control_bins.003	0.283831	0.251675	0.210516
Control_bins.014	-0.0579	-0.09005	-0.13121
Control_bins.031	0	-0.03216	-0.07332
HEPS_bins.003	-0.09481	-0.12696	-0.16812
HEPS_bins.010	0.144377	0.112221	0.071062
HEPS_bins.011	0.013223	-0.01893	-0.06009
HEPS_bins.016	-0.01525	-0.0474	-0.08856
LAC_NA06_Acidobacteria_54_19	-0.33212	-0.36428	-0.40544
LAC_NA06_Actinobacteria_71_15	-0.01044	-0.0426	-0.08376
LAC_NA06_Candidatus_Solibacter_usitatus_62_12	0.064435	0.03228	-0.00888
LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	-0.19315	-0.2253	-0.26646
LAC_NA06_Sphingobacteriales_42_58	0.185584	0.153428	0.112269
LAC_NA06_Xanthomonadales_70_10	-0.70949	-0.74165	-0.78281
LAC_NA07_Actinobacteria_74_18	0.032156	0	-0.04116
LAC_NA07_Actinotalea_fermentans_75_19	0.157036	0.12488	0.083721
LAC_NA07_Bacteria_38_171	-0.66671	-0.69887	-0.74003
LAC_NA07_Bacteria_57_12	0.042567	0.010411	-0.03075
LAC_NA07_Bacteria_71_12	-0.02841	-0.06056	-0.10172
LAC_NA07_Chloroflexi_60_59	-1.95019	-1.98234	-2.0235
LAC_NA07_Chloroflexi_66_23	-0.12426	-0.15641	-0.19757
LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	-0.13189	-0.16405	-0.20521

LAC_NA07_Rhodobacterales_68_7	0.154396	0.12224	0.081081
LAC_NA07_Roizmannbacteria_52_60	-0.556	-0.58816	-0.62932
LAC_NA08_Chloroflexi_58_8	-0.07511	-0.10727	-0.14843
LAC_NA08_Chloroflexi_60_15	-0.08116	-0.11331	-0.15447
LAC_NA08_Chloroflexi_67_31	-0.11142	-0.14357	-0.18473
LAC_NA08_Flavobacteriales_41_5	-0.68819	-0.72035	-0.76151
LAC_NA08_Gammaproteobacteria_68_22	-0.38152	-0.41368	-0.45484
LAC_NA08_Ignavibacterium_album_33_266	-0.13703	-0.16919	-0.21035
LAC_NA09_Acidobacteria_70_95	0.100931	0.068775	0.027616
LAC_NA09_Gammaproteobacteria_70_24	0.370191	0.338035	0.296876
LAC_NA10_Actinobacteria_74_12	0.073315	0.041159	0
LAC_NA10_Bacteria_62_9	-0.12776	-0.15992	-0.20108
LAC_NA10_Chloroflexi_62_239	-0.47424	-0.5064	-0.54756
LAC_NA10_Lysobacter_70_8	0.540145	0.507989	0.46683
LAC_NA10_Xanthomonadales_68_7	-0.64963	-0.68178	-0.72294
LAC_NA11_Bacteria_34_14	-0.58196	-0.61411	-0.65527
LAC_NA11_Bacteria_57_35	-0.48479	-0.51695	-0.55811
LAC_NA11_Bacteroidales_40_6	-0.11117	-0.14332	-0.18448
LAC_NA11_Betaproteobacteria_62_13	-0.20484	-0.23699	-0.27815
LAC_NA11_Caldilinea_aerophila_60_6	0.35741	0.325254	0.284095
LAC_NA11_Ignavibacteriales_56_75	0.324416	0.29226	0.251101
LAC_NA11_Microgenomates_50_64	-0.51072	-0.54287	-0.58403
NA01_Acidobacteria_59_12	-0.2888	-0.32096	-0.36211
NA01_Bacteria_60_23	-0.18966	-0.22181	-0.26297
NA01_Caldilinea_aerophila_61_10	0.06201	0.029854	-0.01131
NA01_Chloroflexi_65_16	-0.12031	-0.15246	-0.19362
NA01_Fimbriimonas_ginsengisoli_61_16	-0.18555	-0.2177	-0.25886
NA01_Rhizobiales_69_9	-0.29926	-0.33142	-0.37257
NA01_Roizmanbacteria_38_28	-2.80997	-2.84213	-2.88329
NA02_Anaerolinea_thermophila_56_16	0.044469	0.012313	-0.02885
NA02_Rhizobiales_66_27	-0.36277	-0.39493	-0.43609
NA03_Alphaproteobacteria_58_599	-0.3297	-0.36185	-0.40301
NA03_Alphaproteobacteria_61_24	0.980325	0.948169	0.90701
NA03_Anaerolineales_41_23	0.2901	0.257945	0.216785
NA03_Bacteria_65_16	-0.28721	-0.31937	-0.36053
NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	-0.19538	-0.22753	-0.26869
NA03_Leptonema_illini_45_7	-0.37621	-0.40837	-0.44953
NA03_RBG_16_Chloroflexi_57_11_curated_55_21	-0.22175	-0.25391	-0.29507
NA04_Bacteria_50_51	-0.47218	-0.50434	-0.5455
NA04_Bacteria_58_19	-0.13364	-0.16579	-0.20695
NA04_Bacteria_65_10	0.056509	0.024353	-0.01681

NA04_Bacteria_69_30	-0.04895	-0.08111	-0.12227
NA04_Bacteroidetes_30_9	-1.11999	-1.15214	-1.1933
NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	0.381266	0.34911	0.307951
NA04_Burkholderiales_70_137	0.175713	0.143557	0.102398
NA04_Candidatus_Saccharibacteria_41_123	-0.64066	-0.67282	-0.71398
NA04_Myxococcales_72_13	-0.69821	-0.73036	-0.77152
NA04_Rhodocyclales_67_27	-0.00292	-0.03507	-0.07623
NA04_Sphingobacteriales_44_8	-0.27504	-0.3072	-0.34836
NA04_Turneriella_parva_44_15	-1.19104	-1.2232	-1.26436
R2_6_3_bins.015	-0.1021	-0.13426	-0.17542

Table 3: LR coverage confidence intervals for alkaline EPS enrichments

MAG	**RFG1**	**RFG2**	**RFG3**
AEPS_bins.007	-0.1484	-0.24128	-0.31103
AEPS_bins.021	0.28907	0.196194	0.126437
AEPS_bins.024	-0.07356	-0.16644	-0.2362
anamox1_Acidobacteria_62_5_curated	-0.24524	-0.33812	-0.40787
anamox1_Bacteria_33_9_curated	-0.44769	-0.54057	-0.61032
anamox1_Bacteria_45_8_curated	-0.49685	-0.58972	-0.65948
anamox1_Bacteria_50_18_curated	1.181402	1.088526	1.018769
anamox1_Bacteria_53_17_curated	-0.04509	-0.13797	-0.20772
anamox1_Bacteria_55_18_curated	0.698163	0.605287	0.53553
anamox1_Bacteria_56_37_curated	0.055471	-0.0374	-0.10716
anamox1_Bacteria_57_32_curated	0.687526	0.59465	0.524893
anamox1_Bacteria_57_9_curated	0.549033	0.456157	0.3864
anamox1_Bacteria_64_7_curated	0.045058	-0.04782	-0.11758
anamox1_Bacteria_65_5_curated	-0.05592	-0.1488	-0.21855
anamox1_Bacteria_72_15_curated	0.582343	0.489467	0.41971

anamox1_Bacteroidetes_39_16_curated	-1.37574	-1.46862	-1.53838
anamox1_Bacteroidetes_63_11_curated	-0.13103	-0.22391	-0.29366
anamox1_Betaproteobacteria_69_7_curated	0.056582	-0.03629	-0.10605
anamox1_Chloroflexi_52_59_curated	-0.11107	-0.20394	-0.2737
anamox1_Gemmatimonas_aurantiaca_57_6_curated	-0.43738	-0.53026	-0.60001
anamox1_Nitrosomonas_europaea_50_14_curated	0.019667	-0.07321	-0.14297
anamox1_Proteobacteria_67_8_curated	-0.09107	-0.18395	-0.2537
anamox1_RBG_16_Ignavibacteria_36_9_curated_35_5_curated	-0.28933	-0.38221	-0.45197
anamox1_Sphingobacteriales_43_8_curated	-0.22991	-0.32278	-0.39254
anamox2_Actinobacteria_65_5_curated	-0.00971	-0.10259	-0.17235
anamox2_Bacteria_61_6_curated	-0.39556	-0.48844	-0.5582
anamox2_Bacteria_68_6_curated	0.153519	0.060643	-0.00911
anamox2_Burkholderiales_67_5_curated	0.30503	0.212154	0.142397
anamox2_Burkholderiales_68_9_curated	4.22947	4.136594	4.066837
anamox2_Burkholderiales_70_13_curated	0.171056	0.07818	0.008423
anamox2_Chloroflexi_60_8_curated	-0.17481	-0.26768	-0.33744
anamox2_Hydrogenophilales_66_19_curated	-0.80207	-0.89494	-0.9647
anamox2_Ignavibacteriales_33_9_curated	-0.3438	-0.43667	-0.50643
anamox2_Ignavibacteriales_41_12_curated	-0.03954	-0.13242	-0.20218
anamox2_Methanosarcina_thermophila_41_9_curated	NA	NA	NA
anamox2_Microgenomates_45_6_curated	-0.27547	-0.36835	-0.43811
anamox2_Myxococcales_71_5_curated	-0.55915	-0.65202	-0.72178
anamox2_Rhizobiales_67_45_curated	0.35419	0.261314	0.191557
anamox2_Sphingobacteriales_41_11_curated	-0.1873	-0.28018	-0.34994
anamox2_Verrucomicrobia_58_8_curated	0.041747	-0.05113	-0.12089
anamox2_Verrucomicrobia_62_8_curated	-0.32663	-0.41951	-0.48926
anamox2_Xanthomonadales_68_9_curated	-0.2391	-0.33198	-0.40173

anamox3_Acidobacteria_71_4_curated	-0.04428	-0.13715	-0.20691
anamox3_Bacteria_66_7_curated	-0.97918	-1.07206	-1.14181
anamox3_Bacteria_67_13_curated	-0.80294	-0.89582	-0.96557
anamox3_Bacteria_67_15_curated	-0.12322	-0.2161	-0.28586
anamox3_Bacteroidetes_39_15_curated	0.644376	0.5515	0.481743
anamox3_Chloroflexi_59_6_curated	-0.22983	-0.3227	-0.39246
anamox3_Chloroflexi_68_6_curated	0.099366	0.00649	-0.06327
anamox3_Nitrosomonas_europaea_51_28_curated	-0.02662	-0.1195	-0.18926
anamox3_Pedosphaera_parvula_66_5_curated	-0.78958	-0.88246	-0.95222
anamox3_Sphingobacteriales_50_9_curated	0.462095	0.369219	0.299462
anamox3_sub_Ignavibacteriales_42_14_curated	-0.56223	-0.65511	-0.72486
anamox3_Verrucomicrobia_59_12_curated	0.360712	0.267836	0.198079
anamox4_Bacteria_63_7_curated	-0.06382	-0.15669	-0.22645
anamox4_Bacteroidetes_40_74_curated	-0.5656	-0.65848	-0.72823
anamox4_Candidatus_Nitrospira_defluvii_60_9_curated	-0.64249	-0.73537	-0.80513
anamox4_Gammaproteobacteria_67_14_curated	-0.07208	-0.16495	-0.23471
anamox4_Microgenomates_48_8_curated	0.101243	0.008367	-0.06139
anamox4_sub_Candidatus_Brocadia_sinica_42_75_curated	0.209776	0.1169	0.047143
Control_bins.003	0.229821	0.136945	0.067188
Control_bins.014	-0.05135	-0.14423	-0.21398
Control_bins.031	0	-0.09288	-0.16263
HEPS_bins.003	-0.00108	-0.09396	-0.16372
HEPS_bins.010	0.151817	0.058941	-0.01082
HEPS_bins.011	2.423656	2.33078	2.261023
HEPS_bins.016	0.020841	-0.07203	-0.14179
LAC_NA06_Acidobacteria_54_19	-0.48649	-0.57936	-0.64912
LAC_NA06_Actinobacteria_71_15	0.0721	-0.02078	-0.09053

LAC_NA06_Candidatus_Solibacter_usitatus_62_12	-0.06164	-0.15452	-0.22427
LAC_NA06_RIFCSPHIGHO2_12_FULL_Dadabacteria_53_21_curated_58_6	-0.14838	-0.24125	-0.31101
LAC_NA06_Sphingobacteriales_42_58	0.102159	0.009283	-0.06047
LAC_NA06_Xanthomonadales_70_10	-0.20507	-0.29795	-0.36771
LAC_NA07_Actinobacteria_74_18	0.092876	0	-0.06976
LAC_NA07_Actinotalea_fermentans_75_19	-0.1759	-0.26877	-0.33853
LAC_NA07_Bacteria_38_171	-0.03632	-0.12919	-0.19895
LAC_NA07_Bacteria_57_12	0.119351	0.026475	-0.04328
LAC_NA07_Bacteria_71_12	0.024773	-0.0681	-0.13786
LAC_NA07_Chloroflexi_60_59	-0.14123	-0.23411	-0.30387
LAC_NA07_Chloroflexi_66_23	0.011056	-0.08182	-0.15158
LAC_NA07_RBG_16_RIF_CHLX_72_14_curated_75_20	-0.31358	-0.40646	-0.47622
LAC_NA07_Rhodobacterales_68_7	-0.19239	-0.28526	-0.35502
LAC_NA07_Roizmannbacteria_52_60	-0.37665	-0.46953	-0.53928
LAC_NA08_Chloroflexi_58_8	-0.15182	-0.24469	-0.31445
LAC_NA08_Chloroflexi_60_15	-0.03702	-0.12989	-0.19965
LAC_NA08_Chloroflexi_67_31	-0.08565	-0.17853	-0.24828
LAC_NA08_Flavobacteriales_41_5	-0.56122	-0.65409	-0.72385
LAC_NA08_Gammaproteobacteria_68_22	-0.23985	-0.33272	-0.40248
LAC_NA08_Ignavibacterium_album_33_266	-0.34744	-0.44031	-0.51007
LAC_NA09_Acidobacteria_70_95	-0.03228	-0.12515	-0.19491
LAC_NA09_Gammaproteobacteria_70_24	0.776341	0.683465	0.613708
LAC_NA10_Actinobacteria_74_12	0.162633	0.069757	0
LAC_NA10_Bacteria_62_9	-0.43516	-0.52803	-0.59779
LAC_NA10_Chloroflexi_62_239	-0.50347	-0.59635	-0.66611
LAC_NA10_Lysobacter_70_8	-0.21049	-0.30336	-0.37312
LAC_NA10_Xanthomonadales_68_7	-0.21483	-0.30771	-0.37747

LAC_NA11_Bacteria_34_14	-0.28694	-0.37981	-0.44957
LAC_NA11_Bacteria_57_35	-0.25169	-0.34457	-0.41432
LAC_NA11_Bacteroidales_40_6	0.321269	0.228393	0.158636
LAC_NA11_Betaproteobacteria_62_13	-0.21785	-0.31073	-0.38049
LAC_NA11_Caldilinea_aerophila_60_6	0.601346	0.50847	0.438713
LAC_NA11_Ignavibacteriales_56_75	0.078681	-0.01419	-0.08395
LAC_NA11_Microgenomates_50_64	-0.29088	-0.38375	-0.45351
NA01_Acidobacteria_59_12	-0.04705	-0.13992	-0.20968
NA01_Bacteria_60_23	-0.20117	-0.29404	-0.3638
NA01_Caldilinea_aerophila_61_10	0.069704	-0.02317	-0.09293
NA01_Chloroflexi_65_16	0.020006	-0.07287	-0.14263
NA01_Fimbriimonas_ginsengisoli_61_16	-0.20666	-0.29954	-0.36929
NA01_Rhizobiales_69_9	-0.1457	-0.23857	-0.30833
NA01_Roizmanbacteria_38_28	-1.61453	-1.70741	-1.77717
NA02_Anaerolinea_thermophila_56_16	0.188987	0.096111	0.026354
NA02_Rhizobiales_66_27	-0.38437	-0.47725	-0.547
NA03_Alphaproteobacteria_58_599	-0.23812	-0.331	-0.40076
NA03_Alphaproteobacteria_61_24	-0.45633	-0.54921	-0.61897
NA03_Anaerolineales_41_23	0.400861	0.307985	0.238228
NA03_Bacteria_65_16	0.075859	-0.01702	-0.08677
NA03_BJP_IG2069_Ignavibacteriae_38_11_31_13	-0.0648	-0.15768	-0.22744
NA03_Leptonema_illini_45_7	-0.14709	-0.23996	-0.30972
NA03_RBG_16_Chloroflexi_57_11_curated_55_21	-0.06716	-0.16004	-0.22979
NA04_Bacteria_50_51	-0.75405	-0.84693	-0.91668
NA04_Bacteria_58_19	-0.03308	-0.12596	-0.19571
NA04_Bacteria_65_10	-0.63509	-0.72796	-0.79772
NA04_Bacteria_69_30	-0.25828	-0.35115	-0.42091

NA04_Bacteroidetes_30_9	-1.25519	-1.34807	-1.41782
NA04_BJP_IG2103_Bacteroidetes_37_22_46_30	0.382276	0.2894	0.219643
NA04_Burkholderiales_70_137	0.141273	0.048398	-0.02136
NA04_Candidatus_Saccharibacteria_41_123	-0.63532	-0.7282	-0.79795
NA04_Myxococcales_72_13	-0.2084	-0.30127	-0.37103
NA04_Rhodocyclales_67_27	-0.16824	-0.26112	-0.33088
NA04_Sphingobacteriales_44_8	-0.84711	-0.93999	-1.00975
NA04_Turneriella_parva_44_15	-0.39072	-0.48359	-0.55335
R2_6_3_bins.015	0.011725	-0.08115	-0.15091

Table 4: LR coverage changes for heat-extracted-EPS enrichments

RFG	Upper CI	Lower CI
RFG1	0.028768617	-0.17338113
RFG2	-0.064107289	-0.266257037
RFG3	-0.13386436	-0.336014107

Table 5: LR coverage confidence intervals for heat-extracted EPS enrichments

Enzyme Family	AEPS Increased	AEPS Decreased	HEPS Increased	HEPS Decreased
A08	0.05	0.0465116	0.025	0.0185185
A22	0.025	0	0.025	0
A24	0.175	0.1162791	0.1	0.1111111
A25	0.025	0	0.025	0
A31	0	0	0	0
C01	0.075	0.0465116	0.15	0.037037

C14	0.025	0	0.05	0
C15	0.075	0	0.05	0.0185185
C25	0.1	0.0232558	0.1	0.037037
C26	2.175	1.8139535	2.05	1.7592593
C39	0	0	0	0
C40	0.05	0.0232558	0.025	0
C44	1.05	1.1395349	1.075	1.1296296
C56	0.225	0	0.15	0.0555556
C69	0.075	0.0232558	0.075	0.0185185
C82	0.075	0.0465116	0.05	0
M01	0.05	0.1162791	0.05	0.0555556
M02	0.05	0.0465116	0.025	0.0740741
M03	0.25	0.3255814	0.325	0.3518519
M04	0.125	0.0930233	0.15	0.0555556
M06	0.025	0	0.025	0
M09	0	0.0232558	0	0.0185185
M10	0.025	0	0	0
M103	0.075	0.1395349	0.025	0.1296296
M13	0.1	0.0930233	0.05	0.1296296
M14	0.15	0.0697674	0.15	0.1666667
M15	0	0.0232558	0	0.037037
M16	0.45	0.4418605	0.475	0.3703704
M17	0.475	0.2790698	0.375	0.2777778
M18	0.025	0.0232558	0	0.0185185
M19	0.15	0.1162791	0.15	0.0925926

M20	1.35	1.0697674	1.375	1
M23	0.25	0.255814	0.15	0.1666667
M24	0.975	0.9069767	0.975	0.8333333
M28	0.1	0.1162791	0.1	0.1851852
M29	0.05	0.0232558	0.075	0
M32	0	0.1395349	0.1	0.1296296
M36	0	0.0465116	0.025	0.0185185
M38	0.6	0.4883721	0.725	0.3518519
M41	0.85	0.5581395	0.8	0.5185185
M42	0.4	0.0930233	0.35	0.2037037
M43	0.025	0.1627907	0.05	0.1481481
M48	0.475	0.4418605	0.325	0.4259259
M49	0.05	0.1627907	0.1	0.1666667
M50	0.8	0.5581395	0.7	0.4074074
M54	0	0.0232558	0	0
M55	0.025	0	0.025	0
M61	0.025	0.2093023	0.05	0.1296296
M75	0	0	0	0
M82	0	0.0232558	0	0
M90	0	0.0465116	0	0.0185185
M93	0	0	0.025	0
S01	1.1	0.9302326	1.25	0.7962963
S08	0.875	0.8139535	1.325	0.5740741
S09	1.275	1.0697674	1.175	1.1296296
S11	0.325	0.2093023	0.3	0.2037037

S12	0.55	0.5116279	0.5	0.7037037
S13	0.1	0.1162791	0.125	0.1481481
S14	0.55	0.3488372	0.55	0.4814815
S16	0.875	1.0465116	1.125	0.7222222
S24	0.025	0.0697674	0.05	0.0555556
S26	0.3	0.1162791	0.2	0.1481481
S33	0.5	0.255814	0.375	0.2592593
S37	0	0.0232558	0.025	0
S41	0.75	0.9069767	0.8	0.8703704
S45	0.15	0.0465116	0.125	0.0555556
S46	0.125	0.3488372	0.175	0.3518519
S49	0.35	0.372093	0.45	0.2962963
S51	0.025	0.0930233	0	0.0740741
S54	0.05	0.1162791	0.075	0.0740741
S55	0.025	0	0.025	0
S66	0.05	0.0465116	0.05	0.0925926
S82	0.025	0	0.025	0.0555556
S85	0.025	0	0.025	0
T01	0.375	0.1627907	0.225	0.2037037
T02	0.15	0.255814	0.15	0.1851852
T03	0.375	0.255814	0.325	0.2407407
T05	0.175	0.0930233	0.2	0.0925926
AA1	0.125	0	0.125	0
AA10	0	0	0.025	0
AA12	0	0	0.025	0

AA15	0	0	0	0.0185185
AA3	0.05	0.0697674	0.05	0.0185185
AA3_2	0.025	0	0	0
AA4	0.075	0.0465116	0.05	0.0185185
AA5	0.025	0.0697674	0.025	0.0555556
AA6	0.1	0	0.125	0.037037
AA7	0.05	0	0.025	0
CBM0	0.075	0	0.1	0.0925926
CBM10	0.1	0	0.1	0.0185185
CBM12	0.15	0	0.15	0.0185185
CBM13	1.025	0.8837209	1	0.8148148
CBM16	0.05	0	0.05	0
CBM2	1.125	1.5581395	0.9	1.462963
CBM20	0.45	0.5813953	0.475	0.5555556
CBM22	0.125	0.0232558	0.1	0.037037
CBM23	0.4	0.5581395	0.4	0.2777778
CBM25	0.05	0.0232558	0.075	0
CBM26	0.05	0	0	0
CBM3	0.175	0.1162791	0.225	0.0185185
CBM32	0.775	0.2790698	0.95	0.5555556
CBM34	0.075	0	0.05	0.0185185
CBM35	0.225	0.0465116	0.275	0.2407407
CBM37	0	0.0697674	0.05	0.037037
CBM38	0.1	0.0465116	0.125	0.2592593
CBM4	0	0.0232558	0	0

CBM41	0.325	0.0930233	0.25	0.2037037
CBM47	0.25	0	0.075	0.2037037
CBM48	1.225	0.6511628	1.125	0.8333333
CBM5	0.725	0.8837209	0.875	0.5925926
CBM50	3.775	3.5116279	4.325	2.7407407
CBM51	0.575	0.6046512	0.45	0.8333333
CBM54	0.025	0	0.025	0
CBM56	0	0.0697674	0.025	0.0555556
CBM57	0.375	0.2790698	0.35	0.3148148
CBM6	0.75	0.8139535	0.925	0.9814815
CBM61	0.025	0.0232558	0.025	0.037037
CBM62	0	0.0232558	0.025	0.037037
CBM63	0	0.0232558	0	0.0185185
CBM66	0.075	0.0232558	0.1	0.037037
CBM67	0.1	0.0232558	0.125	0.2037037
CBM8	0.025	0.0232558	0.025	0.037037
CBM9	0.375	0.1860465	0.375	0.2777778
CE0	0.375	0.1860465	0.325	0.2407407
CE1	1.475	0.2325581	1.425	0.4259259
CE11	0.9	1.255814	1.025	1.2962963
CE12	0.525	0.1162791	0.425	0.1666667
CE14	1.1	0.7209302	1.15	0.6851852
CE15	0.075	0	0.125	0
CE16	0	0.0232558	0	0.0185185
CE2	0.05	0	0.05	0.0185185

CE3	0	0	0	0
CE4	0.775	0.4651163	0.8	0.5
CE6	0.175	0.0697674	0.2	0.1111111
CE7	0.175	0.0232558	0.175	0.0925926
CE8	0.025	0	0.05	0.0185185
CE9	0.6	0.3488372	0.725	0.5555556
GH0	2.675	1.7906977	2.8	2.1481481
GH1	1.6	0.6744186	1.425	0.7962963
GH10	0.175	0.0465116	0.125	0.0925926
GH102	0.075	0.0697674	0.1	0.0555556
GH103	0.15	0.1395349	0.075	0.1111111
GH104	0.05	0.1162791	0.075	0.0925926
GH105	0.175	0.0232558	0.375	0.1296296
GH106	0.025	0	0.05	0.0740741
GH108	0	0.0232558	0	0.0185185
GH109	0.2	0.0930233	0.325	0.1851852
GH11	0	0.0232558	0.05	0.0185185
GH110	0	0	0.025	0.0185185
GH113	0.05	0.0232558	0.05	0
GH114	0.15	0.1162791	0.15	0.0740741
GH116	0	0	0	0.0740741
GH117	0	0	0	0.0185185
GH119	0	0.0232558	0.025	0
GH12	0.025	0.0232558	0.05	0.037037
GH120	0.025	0	0	0.037037

GH121	0.025	0	0	0.0185185
GH123	0	0	0.075	0.0185185
GH125	0.05	0.0232558	0.05	0.0185185
GH126	0.025	0	0.025	0
GH127	0.125	0	0.05	0.0925926
GH129	0.025	0	0.025	0
GH13	2.625	1.6744186	3.15	1.7592593
GH13_11	0.075	0	0.05	0
GH13_16	0	0	0	0
GH13_18	0	0	0	0
GH13_20	0	0	0	0
GH13_23	0	0	0	0
GH13_3	0.025	0	0.025	0
GH13_30	0.075	0	0.05	0
GH130	0.475	0.3023256	0.575	0.2592593
GH133	0.125	0.0465116	0.2	0.0925926
GH135	0.175	0.1162791	0.175	0.0740741
GH136	0	0	0.025	0
GH138	0	0	0	0
GH139	0	0	0	0.0740741
GH140	0.075	0	0.125	0.037037
GH141	0.05	0	0.05	0.0925926
GH143	0	0	0	0.0185185
GH144	0.175	0.0930233	0.225	0.1481481
GH146	0.025	0	0.025	0

GH147	0.025	0	0	0.0185185
GH148	0.025	0	0	0.0185185
GH15	0.225	0.1627907	0.4	0.1296296
GH151	0.15	0	0.15	0.037037
GH16	0.725	0.255814	0.625	0.4444444
GH17	0.05	0.0465116	0.05	0.0185185
GH18	0.6	0.4418605	0.725	0.3518519
GH19	0.825	0.7674419	0.675	0.7962963
GH2	0.7	0.3488372	0.95	0.537037
GH20	0.6	0.2093023	0.775	0.4259259
GH23	1.25	1.1162791	1.3	1.1296296
GH24	0.05	0	0.05	0.0185185
GH25	0.1	0.0930233	0.125	0.0740741
GH26	0.025	0.0697674	0.025	0.0555556
GH27	0.05	0.0465116	0.1	0
GH28	0.325	0.4883721	0.4	0.462963
GH29	0.575	0.255814	0.65	0.4444444
GH3	1.375	1.3488372	1.675	1.2592593
GH30	0.1	0.0465116	0.225	0
GH30_5	0.025	0	0.025	0
GH31	0.6	0.3023256	0.5	0.3148148
GH32	0.25	0.0232558	0.3	0.0925926
GH33	0.525	0.1395349	0.625	0.4444444
GH35	0.325	0.1395349	0.6	0.4074074
GH36	0.475	0.1860465	0.65	0.1851852

GH37	0.475	0.7209302	0.625	0.4814815
GH38	0.45	0.0697674	0.475	0.1111111
GH39	0.5	0.2325581	0.475	0.1296296
GH4	0.525	0	0.55	0.0925926
GH42	0.175	0	0.175	0.037037
GH43	0.55	0.255814	0.6	0.4814815
GH43_18	0.025	0	0.025	0
GH43_2	0.025	0	0.025	0
GH43_22	0.025	0	0.025	0
GH43_26	0.025	0	0.025	0
GH43_29	0.05	0	0.05	0
GH43_3	0.025	0	0.025	0
GH44	0.025	0.0232558	0	0.0185185
GH46	0.025	0	0.025	0
GH47	0.05	0.0930233	0.075	0.037037
GH5	0.425	0.1627907	0.4	0.5
GH5_28	0	0	0	0
GH5_46	0.025	0	0.025	0
GH5_5	0	0	0	0
GH50	0.05	0	0.15	0.0185185
GH51	0.25	0	0.15	0.0185185
GH52	0.025	0	0.025	0
GH53	0.05	0.0232558	0.05	0.0740741
GH55	0.05	0.0232558	0.025	0.037037
GH57	0.275	0.3255814	0.425	0.1851852

GH59	0	0	0	0.0185185
GH6	0.75	0.9767442	0.825	0.9444444
GH63	0.075	0	0.05	0.0185185
GH65	0.475	0.2790698	0.525	0.2777778
GH66	0	0	0	0
GH67	0	0	0	0
GH71	0	0	0.025	0
GH72	0.425	0.5813953	0.475	0.4444444
GH73	0.3	0.0930233	0.3	0.0555556
GH74	0.025	0	0	0
GH75	0	0.0232558	0	0.0185185
GH76	0	0	0	0.037037
GH77	0.175	0.1162791	0.15	0.1296296
GH78	0.325	0.4651163	0.4	0.6111111
GH8	0.025	0	0.05	0
GH84	0.025	0	0.05	0.0185185
GH88	0.075	0.0465116	0.25	0.0925926
GH89	0.075	0.0232558	0.05	0.0555556
GH9	0.175	0.2093023	0.175	0.2777778
GH91	0	0	0.025	0
GH92	0.15	0.1395349	0.2	0.2592593
GH93	0.05	0	0.05	0
GH94	0.275	0.0465116	0.2	0.1296296
GH95	0.15	0.0465116	0.2	0.0925926
GH97	0.075	0.0232558	0.175	0.1111111

GH99	0.1	0.0232558	0.075	0
GT0	1.075	1.0930233	1.125	1.2407407
GT1	0.45	0.0930233	0.425	0.1666667
GT10	0	0.0465116	0	0.037037
GT102	0.025	0	0.025	0
GT104	0.05	0.0465116	0	0.037037
GT105	0	0.0930233	0.075	0.0185185
GT11	0.05	0.0232558	0.1	0.037037
GT12	0.025	0	0	0
GT13	0.45	0.5348837	0.45	0.462963
GT14	0.075	0	0.075	0
GT19	0.2	0.372093	0.35	0.3888889
GT2	14.35	13.55814	16.075	11.648148
GT20	0.35	0.2325581	0.3	0.2407407
GT21	0.075	0.0465116	0.05	0.1111111
GT22	0.2	0.1860465	0.2	0.2222222
GT24	0.025	0.0232558	0.05	0.037037
GT25	0.025	0	0.1	0.0185185
GT26	0.725	0.4883721	0.8	0.462963
GT27	0.025	0	0.025	0
GT28	1.625	1.8139535	1.625	1.5
GT29	0.275	0.3023256	0.25	0.3703704
GT3	0.025	0.0232558	0.075	0
GT30	0.85	0.8372093	0.9	1
GT32	0	0	0	0

GT35	0.275	0.3255814	0.525	0.3518519
GT39	0.05	0.0232558	0.05	0.0185185
GT4	14.525	12.674419	15.75	10.574074
GT41	0.025	0	0	0.1111111
GT47	0.675	0.6511628	0.675	0.6666667
GT48	0	0.0232558	0	0
GT49	0.05	0.0697674	0.075	0.0185185
GT5	0.375	0.5581395	0.525	0.6111111
GT51	1.275	1.9069767	1.225	1.5
GT55	0	0	0	0
GT56	0	0	0.025	0
GT66	0.1	0.1860465	0.125	0.1851852
GT68	0.35	0.1860465	0.375	0.1111111
GT7	0.025	0	0.025	0.037037
GT70	0	0.0232558	0	0
GT71	0	0.0232558	0	0.0185185
GT8	0.475	0.3255814	0.35	0.2407407
GT80	0.025	0.0697674	0	0.037037
GT81	0.175	0	0.125	0.0185185
GT83	0.175	0.3255814	0.15	0.3148148
GT84	0	0	0	0
GT87	0.075	0.0232558	0.075	0.0185185
GT89	0.05	0.0465116	0.05	0.0185185
GT9	0.925	1.4651163	1.15	1.7037037
GT95	0.1	0.0232558	0.1	0.037037

GT96	0.025	0	0.025	0

Table 6: CAZyme and MEROPS enzyme abundance averages for MAGs increasing or decreasing in coverage

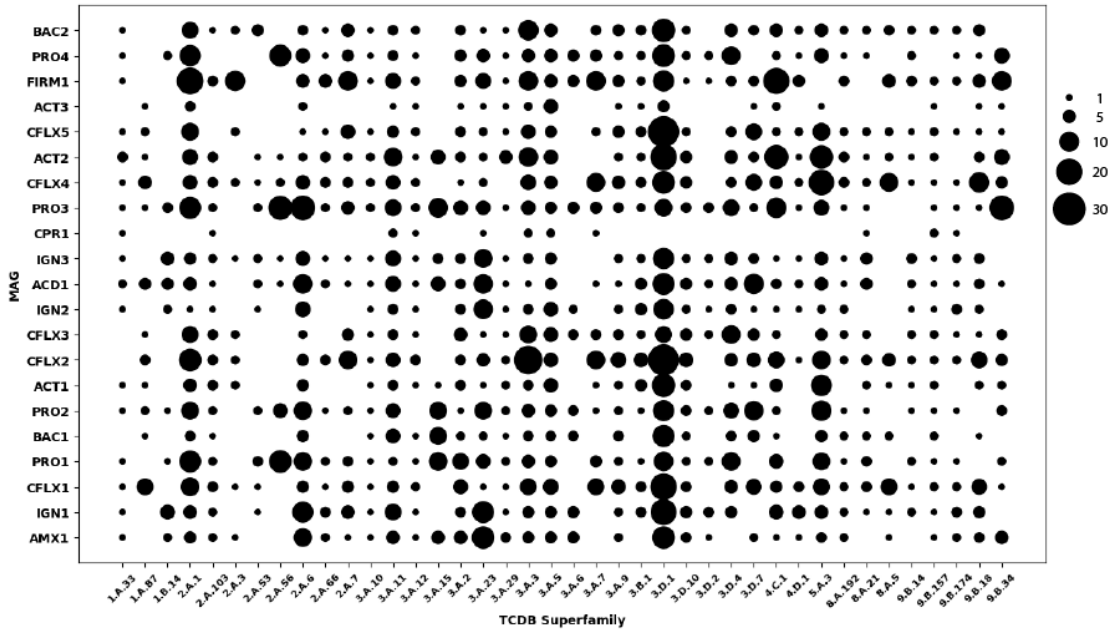

Figure 5: Membrane transport gene counts from Transporter Classification Database (TCDB) superfamilies

Appendix 4, Supplementary Information for Chapter 5-Ecological niche partitioning of metabolically diverse bacteria in anammox bioreactors through trait-based modeling

BioProject Accession	Institution	Reactor Configuration	Biomass Type	Dominant Strain	Publication
PRJNA482223	KAUST	Anammox	Biofilm	Jettenia	Application of an enrichment culture of the marine anammox bacterium "Ca. Scalindua sp. AMX11" for nitrogen removal under moderate salinity and in the presence of oganic carbon, Ali et al. (2020)
PRJNA592266	KAUST	Anammox	Biofilm	Scalinuda	Comparative Genome-Centric Analysis of Freshwater and Marine ANAMMOX Cultures Suggests Functional Redundancy in Nitrogen Removal Processes, Ali et al. (2020)
PRJNA559529	University of Wisconsin, Madison	PN/A	Suspended	Brocadia	Exploring the Meta-regulon of the CRP/FNR Family of Global Transcriptional Regulators in a Partial-Nitritation Anammox Microbiome, Beach et al. (2021)
PRJNA743350	University of Washington	Anammox/PN/A	Granular	Kuenenia/ Brocadia	Metagenomic Insights Into Competition Between Denitrification and Dissimilatory Nitrate Reduction to Ammonia Within One-Stage and Two-Stage Partial-Nitritation Anammox Bioreactor Configurations, Bryson et al. (2022)
PRJNA598059	East China University of Science and Technology	Anammox	Granular	Kuenenia	Flocs are the main source of nitrous oxide in a high-rate anammox granular sludge reactor: insights from metagenomics and fed-batch experiments, Zhuang et al. (2020)
PRJNA482907	Peking University	Anammox	Suspended	Jettenia	Deciphering bacterial social traits via diffusible signal factor (DSF) -mediated public goods in an anammox community, Guo et al. (2021)
PRJNA484723	Peking University	Anammox	Suspended	Jettenia	Metabolic acclimation of anammox consortia to decreased temperature, Huo et al. (2020)
PRJNA343219	University of Washington, Madison	Anammox	Granular	Brocadia	Metabolic network analysis reveals microbial community interactions in anammox granules, Lawson et al. (2017)
PRJNA58181	Lund University	PN/A	Biofilm	Brocadia	Biofilm colonization and succession in a full-scale partial nitritation-anammox moving bed biofilm reactor, Suarez et al. (2024)
PRJNA791618	Technical University of Denmark	Anammox	Suspended	Jettenia	Efficient management of the nitritation-anammox microbiome through intermittent aeration: absence of the NOB guild and expansion and diversity of the NOx reducing guild suggests a highly reticulated nitrogen cycle, Palomo et al. (2022)
PRJNA477269	Peking University	Anammox	Suspended	Jettenia	unpublished
PRJNA274364	Radboud University Nijmegen	PN/A	Granular	Brocadia	Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system, Speth et al. (2016)
PRJNA611787	University of Gothenburg	PN/A	Biofilm	Brocadia	Disturbance-based management of ecosystem services and disservices in partial nitritation-anammox biofilms, Suarez et al. (2022)
PRJNA983097	Chongqing University	Anammox	Granular	Jettenia	unpublished
PRJNA845941	Beijing University of Chemical Technology	Anammox	Granular	Kuenenia	Metagenomic insights into the symbiotic relationship in anammox consortia at reduced temperature, Ya et al. (2022)
PRJNA497991	Peking University	Anammox	Biofilm	Jettenia	Genome-centered omics insight into the competition and niche differentiation of Ca. Jettenia and Ca. Brocadia affiliated to anammox bacteria, Zhao et al. (2019)
PRJNA450161	Peking University	Anammox	Suspended	Brocadia	Genome-Centered Metagenomics Analysis Reveals the Symbiotic Organisms Possessing Ability to Cross-Feed with Anammox Bacteria in Anammox Consortia, Zhao et al. (2018)
PRJNA1101456	University of California, Berkeley	Anammox	Suspended	Brocadia	unpublished

Table 1: BioProject Accession numbers and associated metadata for genomes obtained through NCBI

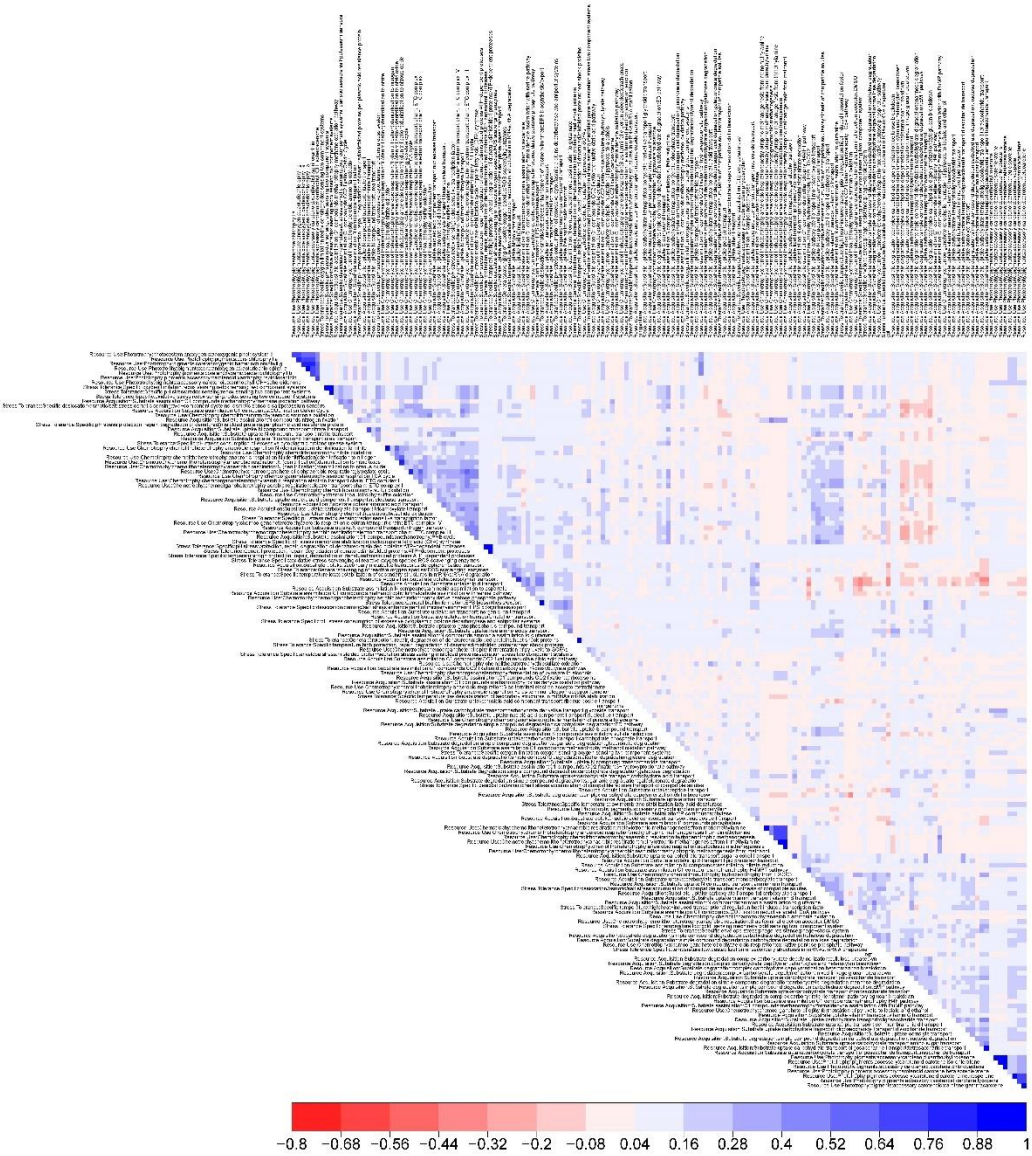

Figure 1: Trait correlation matrix, cells colored with Pearson correlation metrics (ρ)

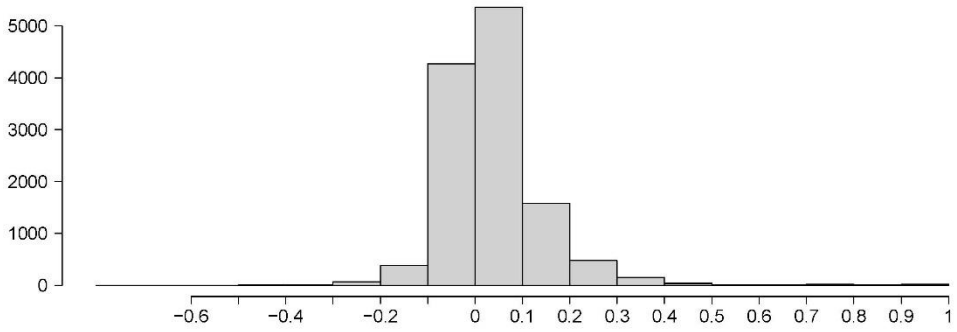

Figure 2: Trait correlations histogram, (ρ) on the x-axis, correlation counts on the y-axis

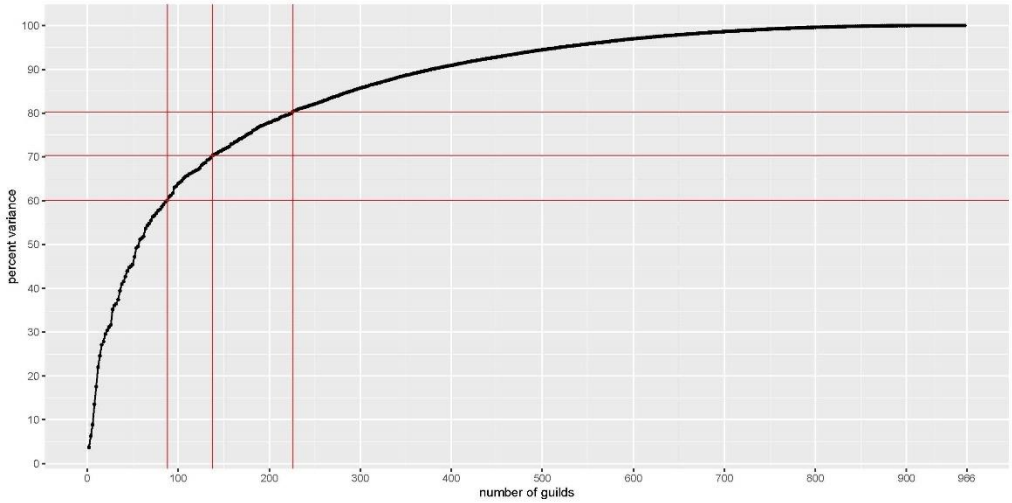

Figure 3: Percent variance explained as a function of the number of guilds

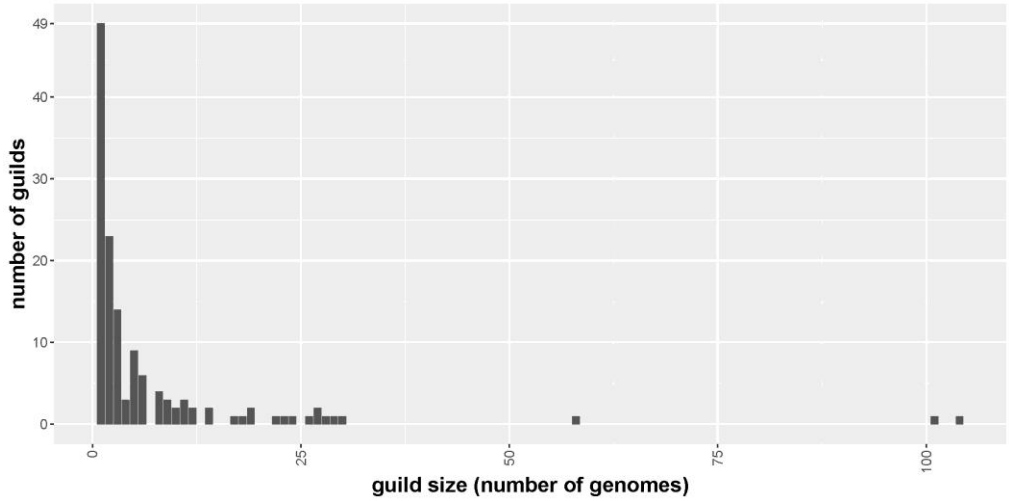

Figure 4: Guild size distribution

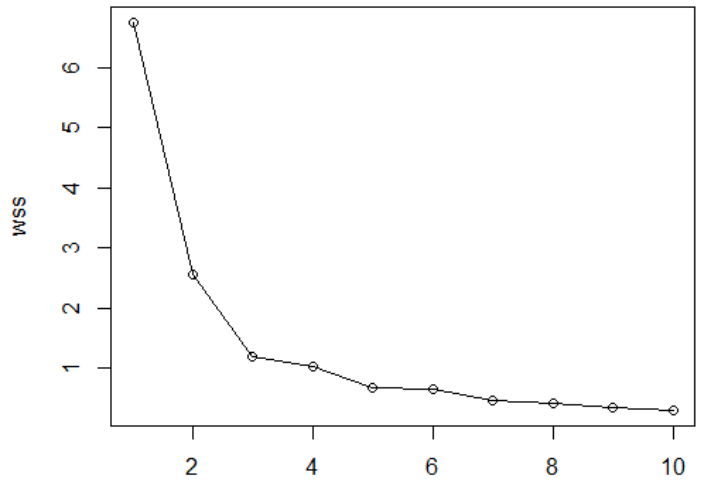

Figure 5: Within sum of squares (wss) for K-means clustering of nMDS guilds

Guild	Net Relatedness Index (NRI)	Nearest Taxon Index (NTI)
3	-3.6	-0.05
6	3.85	3.52
7	0.14	1.82
8	8.86	5.27
9	13.7	5.52
15	8.28	3.32
20	1.4	2.15
21	2.24	2.02
22	11	4.55

Figure 6: Guild nearest relatedness index (NRI) and nearest taxon index (NTI)

Trait	Anammox	PN/A	p-value (MANOVA)
Resource Acquisition:Substrate uptake:aromatic acid transport	0.064607	0.080328	0.37
Resource Acquisition:Substrate uptake:biopolymer transport	1.601124	2.018033	**8.268E-09**
Resource Acquisition:Substrate uptake:carbohydrate transport:amino sugar transport	0.044944	0.037705	0.5915
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate acid transport	0.061798	0.07541	0.4975
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate derivative transport:glycoside transport	0	0.001639	0.4452
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate phosphate transport	0.014045	0.016393	0.7761
Resource Acquisition:Substrate uptake:carbohydrate transport:monosaccharide transport	1.359551	1.404918	0.6544
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport	0.050562	0.029508	0.1054
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:disaccharide transport	0.845506	0.696721	**0.0394**
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:tetrasaccharide transport	0.129213	0.067213	**0.001146**
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:trisaccharide transport	0.129213	0.070492	**0.002609**
Resource Acquisition:Substrate uptake:carbohydrate transport:polysaccharide transport	1.162921	0.891803	**0.00008963**
Resource Acquisition:Substrate uptake:carbohydrate transport:sugar alcohol transport	0.174157	0.139344	0.1953
Resource Acquisition:Substrate uptake:carboxylate transport:dicarboxylate transport	1.429775	1.485246	0.5541
Resource Acquisition:Substrate uptake:carboxylate transport:monocarboxylate transport	0.55618	0.485246	0.2045

Resource Acquisition:Substrate uptake:carboxylate transport:tricarboxylate transport	0.117978	0.208197	**0.0005702**
Resource Acquisition:Substrate uptake:free amino acids transport	4.030899	3.827869	0.186
Resource Acquisition:Substrate uptake:ion transport:inorganic ion transport	4.25	4.086885	0.2598
Resource Acquisition:Substrate uptake:ion transport:metal ion transport	5.533708	5.396721	0.3665
Resource Acquisition:Substrate uptake:lipid transport	1.525281	1.6	0.3134
Resource Acquisition:Substrate uptake:lipid transport:cell membrane lipid transport	0.033708	0.034426	0.9567
Resource Acquisition:Substrate uptake:lipid transport:lipid precursor transport	0.171348	0.24918	**0.008135**
Resource Acquisition:Substrate uptake:N compound transport:amide transport	0.039326	0.085246	**0.008154**
Resource Acquisition:Substrate uptake:N compound transport:ammonium transport	0.463483	0.542623	**0.01758**
Resource Acquisition:Substrate uptake:N compound transport:nitrate transport	0.016854	0.045902	**0.01809**
Resource Acquisition:Substrate uptake:N compound transport:nitrite transport	0.078652	0.072131	0.7101
Resource Acquisition:Substrate uptake:N compound transport:nitrogen transport	0.196629	0.242623	0.09921
Resource Acquisition:Substrate uptake:N compound transport:urea transport	0.210674	0.237705	0.34
Resource Acquisition:Substrate uptake:N compound transport:uric acid transport	0	0	NaN
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleobase transport	0.036517	0.063934	0.1026
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleoside transport	0.331461	0.44918	**0.002774**
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleotide transport	0.095506	0.085246	0.5895
Resource Acquisition:Substrate uptake:nucleic acid component transport:ribonucleoside transport	0.025281	0.039344	0.2462
Resource Acquisition:Substrate uptake:organophosphorus compound transport	1.477528	1.519672	0.4652
Resource Acquisition:Substrate uptake:osmolyte transport	0.244382	0.270492	0.471
Resource Acquisition:Substrate uptake:other transport	0	0.003279	0.28
Resource Acquisition:Substrate uptake:peptide transport	0.435393	0.632787	**0.000001884**

Resource Acquisition:Substrate uptake:S compound transport	0.216292	0.218033	0.9519
Resource Acquisition:Substrate uptake:secondary metabolite transport:siderophore-based transport	0.269663	0.419672	**0.0001436**
Resource Acquisition:Substrate uptake:vitamin transport:vitamin B transport	1.620787	1.491803	0.1617
Resource Acquisition:Substrate uptake:vitamin transport:vitamin C transport	0.008427	0.004918	0.5036
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:cellulose breakdown	2.398876	2.409836	0.9002
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:chitin breakdown	2.213483	2.416393	**0.003449**
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:heteromannan breakdown	1.044944	0.922951	**0.02155**
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:mixed linkage glucan breakdown	1.351124	1.295082	0.4262
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xylan and heteroxylan breakdown	4.179775	4.039344	0.4423
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xyloglucan breakdown	1.901685	1.909836	0.9361
Resource Acquisition:Substrate degradation:simple compound degradation:protein degradation	23.61798	23.67869	0.8536
Stress Tolerance:General:scavenging of reactive oxygen species:ROS scavenging enzymes	0.477528	0.554098	**0.03709**
Stress Tolerance:General:biofilm formation:EPS biosynthesis/export	1.988764	2.165574	**0.002516**
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.682584	4.560656	**0.02235**
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.853933	1.831148	0.3703
Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.682584	4.560656	**0.02235**

Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.853933	1.831148	0.3703
Stress Tolerance:Specific:temperature:high:heat-induced transcriptional regulation:heat induced transcription factor	0.61236	0.54918	**0.05561**
Stress Tolerance:Specific:temperature:low:membrane stabilization:fatty acid desaturase	0.115169	0.195082	**0.001248**
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA chaperone	0.668539	0.640984	0.3865
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA stabilization	0.941011	0.936066	0.759
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:RNA degradation	0.924157	1.113115	**0.00005574**
Stress Tolerance:Specific:temperature:low:cold-sensing machinery:cold sensing two-component system	0.182584	0.088525	**0.00001661**
Stress Tolerance:Specific:desiccation/osmotic/salt stress:accumulation of compatible solutes:transport of compatible solutes	0.969101	1.045902	**0.0417**
Stress Tolerance:Specific:desiccation/osmotic/salt stress:accumulation of compatible solutes:synthesis of compatible solutes	0.823034	0.855738	0.5017
Stress Tolerance:Specific:desiccation/osmotic/salt stress:enhancement of microenvironment:EPS biosynthesis/export	1.988764	2.165574	**0.002516**
Stress Tolerance:Specific:desiccation/osmotic/salt stress:osmotic sensing:two-component systems, osmotic sensors salt/potassium sensors	0.05618	0.068852	0.4387
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:periplasmic acid resistance protein	0	0.001639	0.4452
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.853933	1.831148	0.3703
Stress Tolerance:Specific:pH stress:membrane stabilization:cyclopropane fatty acid (CFA) synthase	0.27809	0.327869	0.1066

Stress Tolerance:Specific:pH stress:redox sensing:redox sensing two component systems	0.202247	0.201639	0.9819
Stress Tolerance:Specific:pH stress:redox sensing:redox sensitive transcription factor	0.449438	0.488525	0.241
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:urease system	0.033708	0.088525	**0.001105**
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:decarboxylase and antiporter systems	1.148876	1.206557	0.2672
Stress Tolerance:Specific:oxidative stress:scavenging of reactive oxygen species:ROS scavenging enzymes	1.446629	1.554098	**0.02357**
Stress Tolerance:Specific:oxidative stress:redox sensing:redox sensing two component systems	0.202247	0.201639	0.9819
Stress Tolerance:Specific:oxygen limitation:redox sensing:redox sensing two component systems	0.202247	0.201639	0.9819
Stress Tolerance:Specific:oxygen limitation:oxygen sensing:oxygen sensing two component systems	0.002809	0.001639	0.7
Stress Tolerance:Specific:envelope stress:misfolded protein/secretion stress sensing:misfolded proteins/secretion stress two component systems	0.176966	0.139344	0.1958
Stress Tolerance:Specific:envelope stress:phage resistance:phage-shock system	0.429775	0.35082	**0.0147**

Table 3: MANOVA test results of effects of reactor configuration of count traits

Trait	**Biofilm**	**Granular**	**Suspended**	**p-value (MANOVA)**
Resource Acquisition:Substrate uptake:aromatic acid transport	0.077617	0.102362	0.056140351	0.2351
Resource Acquisition:Substrate uptake:biopolymer transport	2.009025	1.708661	1.652631579	**0.000009174**
Resource Acquisition:Substrate uptake:carbohydrate transport:amino sugar transport	0.036101	0.031496	0.052631579	0.4633
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate acid transport	0.081227	0.03937	0.063157895	0.3271
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate derivative transport:glycoside transport	0.001805	0	0	0.6899

Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate phosphate transport	0.021661	0.007874	0.007017544	0.2024
Resource Acquisition:Substrate uptake:carbohydrate transport:monosaccharide transport	1.391697	1.275591	1.431578947	0.6274
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport	0.030686	0.023622	0.056140351	0.1404
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:disaccharide transport	0.693141	0.724409	0.877192982	0.06301
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:tetrasaccharide transport	0.061372	0.062992	0.157894737	**0.00001064**
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:trisaccharide transport	0.064982	0.062992	0.157894737	**0.00003437**
Resource Acquisition:Substrate uptake:carbohydrate transport:polysaccharide transport	0.864621	1.007874	1.231578947	**0.000007301**
Resource Acquisition:Substrate uptake:carbohydrate transport:sugar alcohol transport	0.149819	0.141732	0.161403509	0.881
Resource Acquisition:Substrate uptake:carboxylate transport:dicarboxylate transport	1.5	1.401575	1.424561404	0.6578
Resource Acquisition:Substrate uptake:carboxylate transport:monocarboxylate transport	0.5	0.409449	0.578947368	0.147
Resource Acquisition:Substrate uptake:carboxylate transport:tricarboxylate transport	0.207581	0.141732	0.126315789	**0.01061**
Resource Acquisition:Substrate uptake:free amino acids transport	3.886282	4.149606	3.824561404	0.4031
Resource Acquisition:Substrate uptake:ion transport:inorganic ion transport	4.155235	4.031496	4.182456140	0.8012
Resource Acquisition:Substrate uptake:ion transport:metal ion transport	5.380866	5.527559	5.540350877	0.5748
Resource Acquisition:Substrate uptake:lipid transport	1.586643	1.614173	1.526315789	0.6838
Resource Acquisition:Substrate uptake:lipid transport:cell membrane lipid transport	0.036101	0.031496	0.031578947	0.9398

Resource Acquisition:Substrate uptake:lipid transport:lipid precursor transport	0.240072	0.173228	0.203508772	0.2269
Resource Acquisition:Substrate uptake:N compound transport:amide transport	0.090253	0.031496	0.042105263	**0.009203**
Resource Acquisition:Substrate uptake:N compound transport:ammonium transport	0.552347	0.362205	0.505263158	**0.0005177**
Resource Acquisition:Substrate uptake:N compound transport:nitrate transport	0.046931	0.007874	0.024561404	**0.05009**
Resource Acquisition:Substrate uptake:N compound transport:nitrite transport	0.070397	0.07874	0.080701754	0.8495
Resource Acquisition:Substrate uptake:N compound transport:nitrogen transport	0.243682	0.181102	0.210526316	0.2415
Resource Acquisition:Substrate uptake:N compound transport:urea transport	0.222022	0.251969	0.228070175	0.7736
Resource Acquisition:Substrate uptake:N compound transport:uric acid transport	0	0	0	NaN
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleobase transport	0.061372	0.055118	0.038596491	0.4628
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleoside transport	0.449458	0.370079	0.336842105	**0.02493**
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleotide transport	0.084838	0.070866	0.105263158	0.4586
Resource Acquisition:Substrate uptake:nucleic acid component transport:ribonucleoside transport	0.037906	0.047244	0.021052632	0.3051
Resource Acquisition:Substrate uptake:organophosphorus compound transport	1.505415	1.464567	1.519298246	0.8377
Resource Acquisition:Substrate uptake:osmolyte transport	0.281588	0.204724	0.245614035	0.3028
Resource Acquisition:Substrate uptake:other transport	0.00361	0	0	0.4755
Resource Acquisition:Substrate uptake:peptide transport	0.640794	0.488189	0.435087719	**0.00001243**
Resource Acquisition:Substrate uptake:S compound transport	0.212996	0.125984	0.266666667	**0.008806**
Resource Acquisition:Substrate uptake:secondary metabolite transport:siderophore-based transport	0.397112	0.307087	0.326315789	0.1325
Resource Acquisition:Substrate uptake:vitamin transport:vitamin B transport	1.51083	1.503937	1.610526316	0.5844
Resource Acquisition:Substrate uptake:vitamin transport:vitamin C transport	0.005415	0	0.010526316	0.426

Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:cellulose breakdown	2.373646	2.362205	2.487719298	0.452
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:chitin breakdown	2.377256	2.251969	2.312280702	0.404
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:heteromannan breakdown	0.931408	1.015748	1.01754386	0.2557
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:mixed linkage glucan breakdown	1.283394	1.354331	1.361403509	0.5428
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xylan and heteroxylan breakdown	4.01083	3.629921	4.452631579	**0.01074**
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xyloglucan breakdown	1.889892	1.850394	1.964912281	0.7202
Resource Acquisition:Substrate degradation:simple compound degradation:protein degradation	23.68051	23.06299	23.87368421	0.3005
Stress Tolerance:General:scavenging of reactive oxygen species:ROS scavenging enzymes	0.577617	0.440945	0.463157895	**0.002959**
Stress Tolerance:General:biofilm formation:EPS biosynthesis/export	2.146209	1.984252	2.063157895	0.1203
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.528881	4.732283	4.698245614	**0.002322**
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.810469	1.88189	1.877192982	**0.02249**
Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.528881	4.732283	4.698245614	**0.002322**
Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.810469	1.88189	1.877192982	**0.02249**
Stress Tolerance:Specific:temperature:high:heat-	0.543321	0.535433	0.645614035	**0.01181**

induced transcriptional regulation:heat induced transcription factor				
Stress Tolerance:Specific:temperature:low:membrane stabilization:fatty acid desaturase	0.196751	0.11811	0.126315789	**0.01025**
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA chaperone	0.640794	0.598425	0.694736842	0.1227
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA stabilization	0.935018	0.929134	0.947368421	0.7106
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:RNA degradation	1.122744	1.11811	0.856140351	**5.334E-07**
Stress Tolerance:Specific:temperature:low:cold-sensing machinery:cold sensing two-component system	0.099278	0.11811	0.171929825	**0.00982**
Stress Tolerance:Specific:desiccation/osmotic/salt stress:accumulation of compatible solutes:transport of compatible solutes	1.045126	0.976378	0.98245614	0.2138
Stress Tolerance:Specific:desiccation/osmotic/salt stress:accumulation of compatible solutes:synthesis of compatible solutes	0.844765	0.700787	0.905263158	**0.03147**
Stress Tolerance:Specific:desiccation/osmotic/salt stress:enhancement of microenvironment:EPS biosynthesis/export	2.146209	1.984252	2.063157895	0.1203
Stress Tolerance:Specific:desiccation/osmotic/salt stress:osmotic sensing:two-component systems, osmotic sensors salt/potassium sensors	0.066787	0.03937	0.070175439	0.4651
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:periplasmic acid resistance protein	0.001805	0	0	0.6899
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.810469	1.88189	1.877192982	**0.02249**

Stress Tolerance:Specific:pH stress:membrane stabilization:cyclopropane fatty acid (CFA) synthase	0.33935	0.212598	0.294736842	**0.01666**
Stress Tolerance:Specific:pH stress:redox sensing:redox sensing two component systems	0.200361	0.19685	0.207017544	0.9635
Stress Tolerance:Specific:pH stress:redox sensing:redox sensitive transcription factor	0.5	0.448819	0.435087719	0.1694
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:urease system	0.090253	0.03937	0.038596491	**0.007321**
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:decarboxylase and antiporter systems	1.18231	1.149606	1.207017544	0.7807
Stress Tolerance:Specific:oxidative stress:scavenging of reactive oxygen species:ROS scavenging enzymes	1.563177	1.472441	1.438596491	**0.04333**
Stress Tolerance:Specific:oxidative stress:redox sensing:redox sensing two component systems	0.200361	0.19685	0.207017544	0.9635
Stress Tolerance:Specific:oxygen limitation:redox sensing:redox sensing two component systems	0.200361	0.19685	0.207017544	0.9635
Stress Tolerance:Specific:oxygen limitation:oxygen sensing:oxygen sensing two component systems	0.001805	0	0.003508772	0.7535
Stress Tolerance:Specific:envelope stress:misfolded protein/secretion stress sensing:misfolded proteins/secretion stress two component systems	0.137184	0.165354	0.178947368	0.3987
Stress Tolerance:Specific:envelope stress:phage resistance:phage-shock system	0.359206	0.354331	0.431578947	0.1009

Table 4: MANOVA test results of effects of biomass type of count traits

Trait	**Brocadia**	**Jettenia**	**Kuenenia**	**Scalinuda**	**p-value (MANOVA)**

Resource Acquisition:Substrate uptake:aromatic acid transport	0.080052	0.03871	0.131579	0	0.1168
Resource Acquisition:Substrate uptake:biopolymer transport	1.912073	1.541935	2.131579	2.181818	**0.000389**
Resource Acquisition:Substrate uptake:carbohydrate transport:amino sugar transport	0.040682	0.045161	0.026316	0	0.8709
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate acid transport	0.066929	0.090323	0.078947	0	0.7023
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate derivative transport:glycoside transport	0.001312	0	0	0	0.9661
Resource Acquisition:Substrate uptake:carbohydrate transport:carbohydrate phosphate transport	0.015748	0.006452	0.026316	0.090909	0.157
Resource Acquisition:Substrate uptake:carbohydrate transport:monosaccharide transport	1.423885	1.232258	1.421053	1	0.4254
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport	0.03937	0.025806	0.052632	0	0.7385
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:disaccharide transport	0.751969	0.767742	0.710526	0.636364	0.9754
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:tetrasaccharide transport	0.085302	0.122581	0.078947	0	0.3407
Resource Acquisition:Substrate uptake:carbohydrate transport:oligosaccharide transport:trisaccharide transport	0.087927	0.122581	0.078947	0	0.3926
Resource Acquisition:Substrate uptake:carbohydrate transport:polysaccharide transport	0.965879	1.122581	1.052632	0.727273	0.2891
Resource Acquisition:Substrate uptake:carbohydrate transport:sugar alcohol transport	0.146982	0.148387	0.210526	0.363636	0.2652
Resource Acquisition:Substrate uptake:carboxylate transport:dicarboxylate transport	1.488189	1.283871	1.684211	1.636364	0.2757

Resource Acquisition:Substrate uptake:carboxylate transport:monocarboxylate transport	0.511811	0.477419	0.552632	0.818182	0.6108
Resource Acquisition:Substrate uptake:carboxylate transport:tricarboxylate transport	0.194226	0.070968	0.263158	0	**0.000763**
Resource Acquisition:Substrate uptake:free amino acids transport	3.850394	3.825806	5.184211	4.181818	**0.005676**
Resource Acquisition:Substrate uptake:ion transport:inorganic ion transport	4.087927	4.148387	5.078947	5	**0.02556**
Resource Acquisition:Substrate uptake:ion transport:metal ion transport	5.423885	5.483871	5.578947	6.090909	0.7703
Resource Acquisition:Substrate uptake:lipid transport	1.56168	1.503226	1.947368	2	0.08433
Resource Acquisition:Substrate uptake:lipid transport:cell membrane lipid transport	0.03937	0.012903	0.026316	0	0.4411
Resource Acquisition:Substrate uptake:lipid transport:lipid precursor transport	0.240157	0.122581	0.263158	0.090909	**0.01496**
Resource Acquisition:Substrate uptake:N compound transport:amide transport	0.07874	0.025806	0.052632	0	0.1003
Resource Acquisition:Substrate uptake:N compound transport:ammonium transport	0.526247	0.445161	0.578947	0.363636	0.1697
Resource Acquisition:Substrate uptake:N compound transport:nitrate transport	0.040682	0.006452	0.026316	0.090909	0.1365
Resource Acquisition:Substrate uptake:N compound transport:nitrite transport	0.066929	0.090323	0.157895	0.090909	0.1671
Resource Acquisition:Substrate uptake:N compound transport:nitrogen transport	0.234908	0.167742	0.236842	0.363636	0.2063
Resource Acquisition:Substrate uptake:N compound transport:urea transport	0.23622	0.219355	0.131579	0.090909	0.3267
Resource Acquisition:Substrate uptake:N compound transport:uric acid transport	0	0	0	0	NaN
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleobase transport	0.064304	0.019355	0	0	0.09098
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleoside transport	0.422572	0.309677	0.473684	0.363636	0.1528
Resource Acquisition:Substrate uptake:nucleic acid component transport:nucleotide transport	0.089239	0.083871	0.105263	0.090909	0.9816
Resource Acquisition:Substrate uptake:nucleic acid component transport:ribonucleoside transport	0.03937	0.019355	0	0	0.3362

Resource Acquisition:Substrate uptake:organophosphorus compound transport	1.498688	1.464516	1.710526	1.727273	0.3546
Resource Acquisition:Substrate uptake:osmolyte transport	0.278215	0.219355	0.131579	0.090909	0.1777
Resource Acquisition:Substrate uptake:other transport	0.002625	0	0	0	0.9112
Resource Acquisition:Substrate uptake:peptide transport	0.586614	0.419355	0.631579	0.454545	**0.01778**
Resource Acquisition:Substrate uptake:S compound transport	0.225722	0.187097	0.236842	0	0.2724
Resource Acquisition:Substrate uptake:secondary metabolite transport:siderophore-based transport	0.380577	0.296774	0.368421	0.181818	0.3054
Resource Acquisition:Substrate uptake:vitamin transport:vitamin B transport	1.526247	1.574194	1.631579	1.636364	0.9423
Resource Acquisition:Substrate uptake:vitamin transport:vitamin C transport	0.007874	0	0	0	0.6567
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:cellulose breakdown	2.410761	2.419355	2.315789	2.181818	0.9128
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:chitin breakdown	2.395013	2.141935	2.210526	1.909091	**0.01677**
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:heteromannan breakdown	0.939633	1.109677	1	0.818182	0.09671
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:mixed linkage glucan breakdown	1.30315	1.380645	1.394737	1	0.5957
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xylan and heteroxylan breakdown	4.141732	4.025806	3.631579	3.090909	0.4101
Resource Acquisition:Substrate degradation:complex carbohydrate depolymerization:xyloglucan breakdown	1.914698	1.896774	1.894737	1.545455	0.8855
Resource Acquisition:Substrate degradation:simple compound degradation:protein degradation	23.63517	23.59355	24.05263	24.63636	0.869

Stress Tolerance:General:scavenging of reactive oxygen species:ROS scavenging enzymes	0.519685	0.509677	0.631579	0.818182	0.193
Stress Tolerance:General:biofilm formation:EPS biosynthesis/export	2.119423	2.025806	2.026316	2.090909	0.6276
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.565617	4.729032	4.815789	4.909091	**0.02029**
Stress Tolerance:General:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.83727	1.83871	1.894737	1.818182	0.8356
Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:heat shock proteins	4.565617	4.729032	4.815789	4.909091	**0.02029**
Stress Tolerance:Specific:temperature:high:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.83727	1.83871	1.894737	1.818182	0.8356
Stress Tolerance:Specific:temperature:high:heat-induced transcriptional regulation:heat induced transcription factor	0.570866	0.593548	0.578947	0.363636	0.5221
Stress Tolerance:Specific:temperature:low:membrane stabilization:fatty acid desaturase	0.171916	0.122581	0.210526	0.181818	0.4128
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA chaperone	0.644357	0.677419	0.631579	0.818182	0.5651
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:mRNA stabilization	0.933071	0.974194	0.868421	1	0.05288
Stress Tolerance:Specific:temperature:low:destabilization of secondary structures in mRNAs:RNA degradation	1.045932	0.96129	1.184211	1.545455	**0.02655**
Stress Tolerance:Specific:temperature:low:cold-sensing machinery:cold sensing two-component system	0.104987	0.193548	0.184211	0.181818	**0.01107**
Stress Tolerance:Specific:desiccation/osmotic/sa	1.038058	0.941935	0.947368	0.909091	0.1901

lt stress:accumulation of compatible solutes:transport of compatible solutes					
Stress Tolerance:Specific:desiccation/osmotic/salt stress:accumulation of compatible solutes:synthesis of compatible solutes	0.867454	0.709677	0.921053	0.818182	0.09017
Stress Tolerance:Specific:desiccation/osmotic/salt stress:enhancement of microenvironment:EPS biosynthesis/export	2.119423	2.025806	2.026316	2.090909	0.6276
Stress Tolerance:Specific:desiccation/osmotic/salt stress:osmotic sensing:two-component systems, osmotic sensors salt/potassium sensors	0.059055	0.077419	0.105263	0.090909	0.5768
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:periplasmic acid resistance protein	0.001312	0	0	0	0.9661
Stress Tolerance:Specific:pH stress:protection, repair, degradation of denatured/misfolded proteins:ATP-dependent proteases	1.83727	1.83871	1.894737	1.818182	0.8356
Stress Tolerance:Specific:pH stress:membrane stabilization:cyclopropane fatty acid (CFA) synthase	0.324147	0.219355	0.421053	0.181818	**0.02107**
Stress Tolerance:Specific:pH stress:redox sensing:redox sensing two component systems	0.198163	0.219355	0.236842	0.090909	0.686
Stress Tolerance:Specific:pH stress:redox sensing:redox sensitive transcription factor	0.48294	0.393548	0.526316	0.818182	**0.0192**
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:urease system	0.076115	0.032258	0.078947	0	0.19
Stress Tolerance:Specific:pH stress:consumption of excessive cytoplasmic protons:decarboxylase and antiporter systems	1.183727	1.122581	1.473684	1.181818	0.1015
Stress Tolerance:Specific:oxidative stress:scavenging of reactive oxygen species:ROS scavenging enzymes	1.507874	1.496774	1.684211	1.636364	0.4506

Stress Tolerance:Specific:oxidative stress:redox sensing:redox sensing two component systems	0.198163	0.219355	0.236842	0.090909	0.686
Stress Tolerance:Specific:oxygen limitation:redox sensing:redox sensing two component systems	0.198163	0.219355	0.236842	0.090909	0.686
Stress Tolerance:Specific:oxygen limitation:oxygen sensing:oxygen sensing two component systems	0.002625	0	0	0	0.9112
Stress Tolerance:Specific:envelope stress:misfolded protein/secretion stress sensing:misfolded proteins/secretion stress two component systems	0.145669	0.167742	0.210526	0.272727	0.5973
Stress Tolerance:Specific:envelope stress:phage resistance:phage-shock system	0.35958	0.445161	0.447368	0.636364	**0.04712**

Table 5: MANOVA test results of effects of dominant strains of count traits

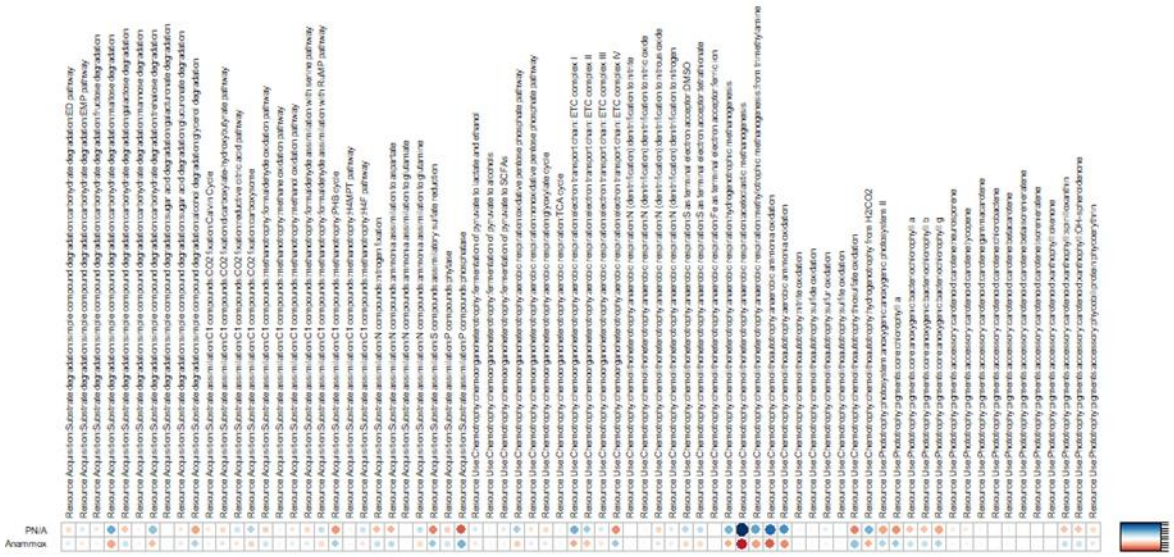

Figure 6a: Chi-Squared test results for binary trait comparisons of reactor configuration

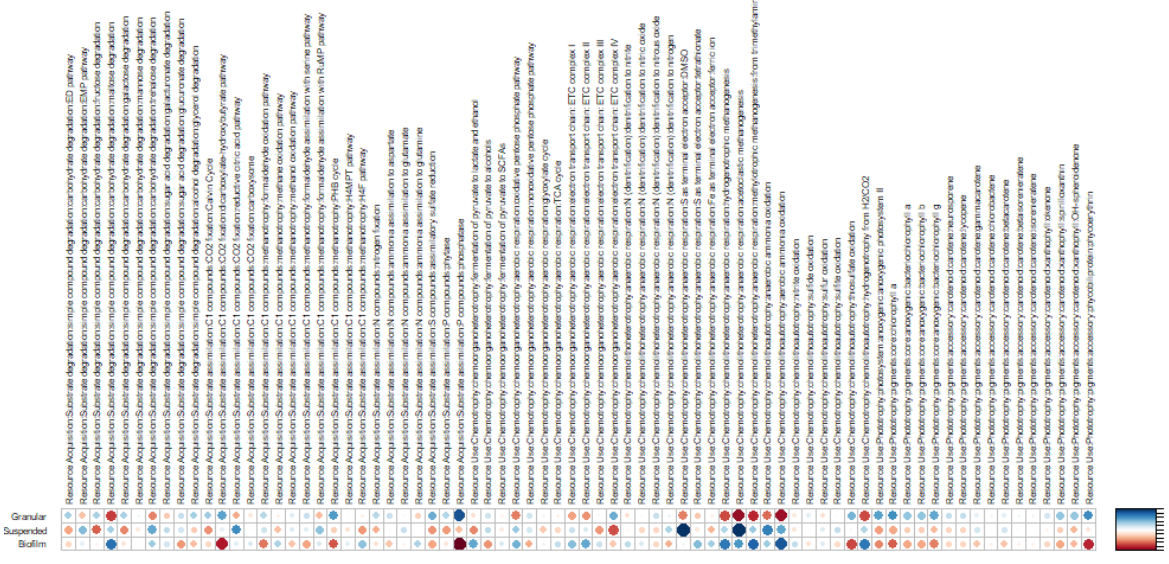

Figure 6b: Chi-Squared test results for binary trait comparisons of biomass type

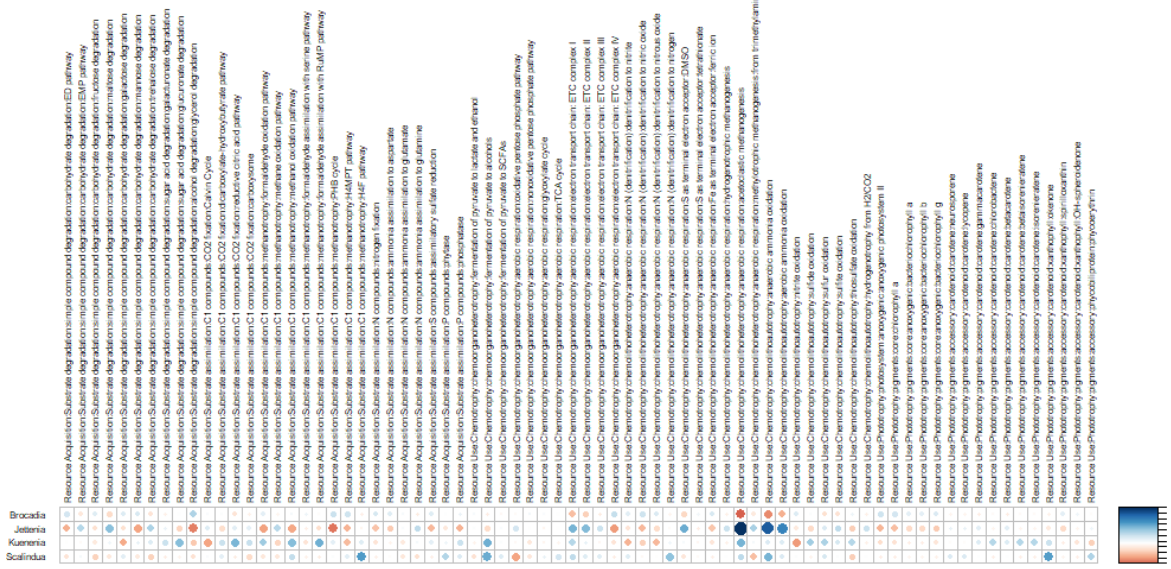

Figure 6c: Chi-Squared test results for binary trait comparisons of dominant strain