Microbial reduction of nitrous oxide at low pH

by

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Abstract

Nitrous oxide (N_2O) is a potent greenhouse gas and an ozone-depleting substance. Its main emission comes from soil microorganisms in agricultural sectors. Recently, the diversity of the *nosZ* gene encoding for nitrous oxide reductase (NosZ) catalyzing the conversion of N_2O to N_2 was found to be much broader than expected. The microorganisms that possess *nosZ* split into two clades that follow denitrifying (clade I) and non-denitrifying (clade II) bacteria. With the unexpected diversity of bacteria possessing *nosZ*, environments that are not ideal for denitrifiers may be habitable for other microorganisms. Low pH environments are known to inhibit denitrification, especially the conversion of N_2O to N_2 starting at pH 6.8, resulting in N_2O accumulation. Impacts for these microorganisms include silage which has recently been shown to emit the third highest quantity of N_2O in the agricultural sector. The fermentation process within silage reduces the pH to 4-5 inhibiting the conversion of N_2O to N_2. The goal of this research is to develop an inoculum that can be used in silage to mitigate N_2O emissions at low pH. It is essential to learn how pH affects *nosZ* and how the bacteria may be able to protect against acidity. Samples from agricultural fields and activated sludge were used to gradually enrich the microorganisms to lower pH values at 0.5-unit increments, starting from pH 7 to pH 5. Results showed that the enrichment cultures reduced N_2O at pH as low as 5.5, but not at pH 5. At the end of each enrichment period at different pH values, the Michaelis-Menten analysis and the metagenomic analysis were performed. Clade II bacteria were more dominant than clade I bacteria at all conditions, however, clade I microorganisms increased in relative abundance over the decrease in pH. Michaelis-Menten showed that the affinity of bacteria at pH 7 is greater than that of bacteria at pH 5.5, which can be confirmed with the observed reduction rates of N_2O. Due to the wide taxa range of clade II *nosZ*, kinetics and metagenomic data cannot be conclusive

since only clade II A and clade II B primers were analyzed. Further experiments are needed to understand the kinetics of the microorganisms at differing pH values and the impacts on *nosZ*.

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Chapter 1 - Introduction

Nitrous oxide (N_2O) is a greenhouse gas that is 300 times more potent than carbon dioxide on a 100-year scale.[1] Additionally, N_2O is the most impactful ozone depleting compound in the stratosphere.[2] N_2O is converted to nitric oxide in the stratosphere by ultraviolet light.[3,4] Nitric oxide then reacts with ozone to produce nitrite and oxygen further reacting with free oxygen molecules to form nitric oxide to start the process over again.[2]

Equation 1: Ozone depletion by N_2O.

$$
\begin{aligned}
N_2O &\xrightarrow{UV\ Light} NO \\
NO + O_3 &\rightarrow NO_2 + O_2 \\
O + NO_2 &\rightarrow NO + O_2 \\
net: O + O_3 &\rightarrow 2O_2
\end{aligned}
$$

N_2O is naturally produced from oceans, forests, and savannas; however, anthropogenic sources are from agriculture (294 million metric tons of CO_2 equivalent (MMT CO_2 eq)), power plant combustion (22.1 MMT CO_2 eq), wastewater treatment plants (20.9 MMT CO_2 eq), manure management (17.4 MMT CO_2 eq), and mobile combustion (16.2 MMT CO_2 eq).[5–7] These anthropogenic sources constitute 40% of the total N_2O emissions while the remaining 60% are from natural sources. Microorganisms in the soil account for roughly two-thirds of anthropogenic N_2O emissions as a result of fertilizer application.[8,9] The United States' N_2O emissions in 2021 accounted for 6.2% of the total greenhouse gas emissions but is correspondent to 405.1 MMT CO_2 eq.[7] Globally, the mean soil N_2O emission rate was 1111.8 µg N m^{-2} d^{-1} as calculated from 6016 field observations.[10] N_2O emission rates are positively correlated with the mean air annual temperature, soil pH, cation exchange capacity, soil moisture, soil organic carbon, total soil nitrogen, dissolved organic nitrogen, ammonium, nitrate, available phosphorus concentrations, microbial biomass carbon, and microbial biomass nitrogen.[10] N_2O is a critical component of the nitrogen cycle producing dinitrogen gas (N_2) (Figure 1).

N_2O within the Nitrogen Cycle

Inside of the nitrogen cycle, both the nitrification and the denitrification pathways have the potential to emit N_2O into the atmosphere.[9] N_2O is formed within nitrification from the oxidation of hydroxylamine (NH_2OH). Additionally, dissimilatory nitrate reduction to ammonium (DNRA) also has the potential to simultaneously emit N_2O when reducing NO_2^-, though this amount is on a smaller scale compared to the largest emission pathway of denitrification.[5,11] Denitrification was shown to be a larger N_2O pathway than nitrification by Lv et al.[11] The researchers took sludge aggregates in wastewater and measured N_2O production on the outside layer (<1000 μm) where nitrification occurred and the inside layer (>1000 μm) where denitrification occurred. The inside layers produced more N_2O indicating denitrification plays a larger role than nitrification. Additionally, when the pH decreased, denitrification was more apparent to have a larger N_2O pathway compared to nitrification.[11]

The quantity of N_2O emissions from DNRA have not been tested in comparison to denitrification and nitrification.[12,13] The majority of denitrifying bacteria are facultative anaerobes meaning that the bacteria can choose to use oxygen as an electron acceptor if it is present or use another electron acceptor if oxygen is not present. Consequently, oxygen will inhibit denitrification as microorganisms will use O_2 as an electron acceptor instead of using nitrate.[14] The only known biological sink of N_2O is through nitrous oxide reductase (NosZ) which catalyzes N_2O to N_2.[15] The conversion of N_2O to N_2 must occur under anoxic conditions.[16] However, an experiment quantified O_2 intrusion at 4 μM and still N_2O reduction was completed.[17] It was believed that the microorganisms were simultaneously utilizing both O_2 and

N_2O as electron acceptors. Further tests are needed to understand how microoxic environmental conditions allowed for N_2O degradation.

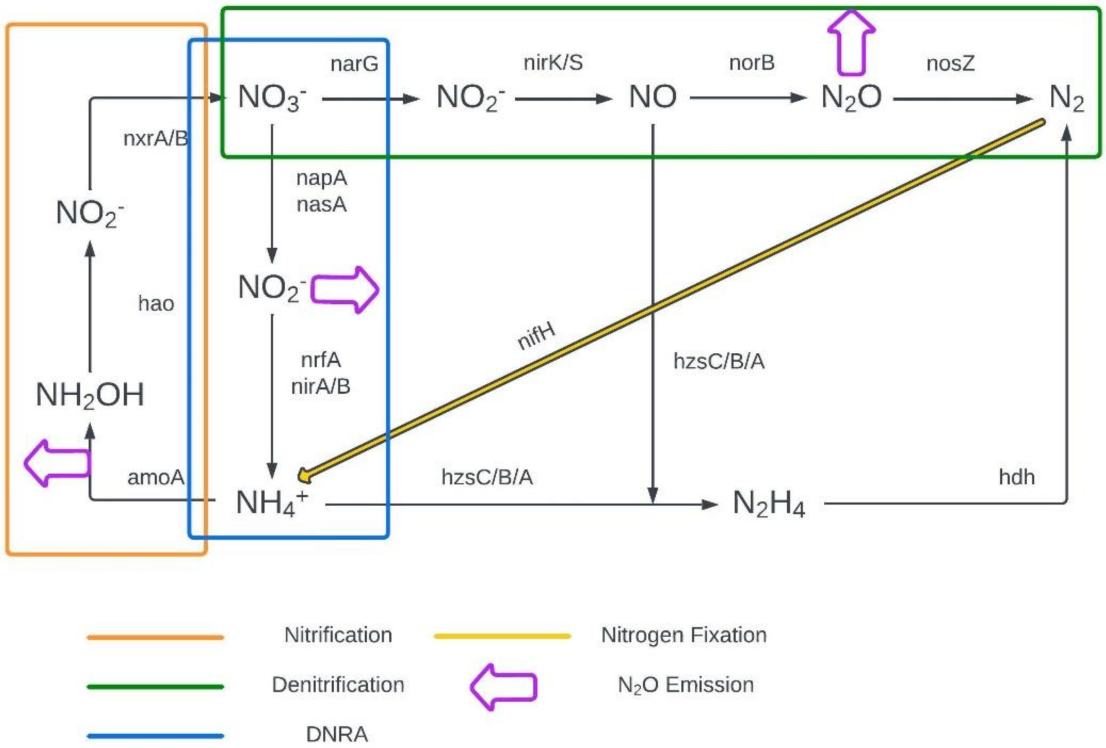

Figure 1: Sources of N_2O within the nitrogen cycle. Adapted from[5,18].

Denitrification shown by the green pathway in the figure above is the conversion of NO_3^- to N_2. The enzymes that catalyze the intermittent conversions are NarG, NirK, NirS, NorB, and NosZ. The major end products of denitrification are both N_2O and N_2, however, only one-third of studies between 1975 and 2015 reported both production fluxes.[19] Soil denitrification accounts for an average of 0.25 kg of nitrogen per hectare per day.[20] Greenhouse vegetable production systems in China over the growing season ranged from 76 to 422 kg of nitrogen per hectare in the top soil layer (0 - 40 cm).[21] When integrating the previous data with nitrogen leaching, plant nitrogen uptake, N_2O emissions, and NH_3 volatilization, the researchers calculated that on average 50% of added nitrogen from fertilizers are lost due to denitrification.[21] Nitrogen is needed by all living organisms and for crop production, fertilizers are used containing ammonium or nitrate as the nitrogen source (Figure 2).

The Haber-Bosch process is the most common technology for producing NH_3. Roughly 50% of the ammonium from this process goes to the production of rice, wheat, and corn.[22] This process, unfortunately, requires a high temperature (400-600°C) and a high pressure (up to 30 MPa) resulting in a large ecological footprint.[23] Applying fertilizer requires an understanding of the nitrogen use efficiency which is how much of the nitrogen goes into the plant. Nitrogen utilization rate is generally lowered by N_2O emission and NO_3^- loss.[22] Nitrogen enrichment increases the amount of N_2O emissions as well as the emissions of methane (CH_4).[24] The enrichment inhibits CH_4 oxidation leading to less CH_4 converting to CO_2. On the other hand, all three pathways of N_2O emissions (i.e., nitrification, DNRA, and denitrification) are enhanced and produce more N_2O.[24] Using ammonium-based fertilizers over nitrate-based fertilizers, biochar amendments, straw addition, and biological nitrogen inhibiters are effective ways to reduce soil denitrification.[20,21] To improve the scientific communities understanding of soil denitrification, it is recommended broaden access to technologies measuring soil N_2 production rates.[19] These technologies can then be applied to tropical soil, subsoils, and unmanipulated soils where very few studies have been performed.

The denitrifying genes *nirS* and *nirK* are significantly increased in abundance due to the application of fertilizers.[24] Additionally, organic fertilizer treatment activated higher copy numbers of *nirS*, *nirK*, and *nosZ* compared to mineral fertilizer treatment.[24] Soil content plays an important role in the selection of which genes will be amplified. In a study examining N_2O emissions from fertilized sites, 332 experiments found increases in N_2O emissions while only 6 found the reverse.[24]

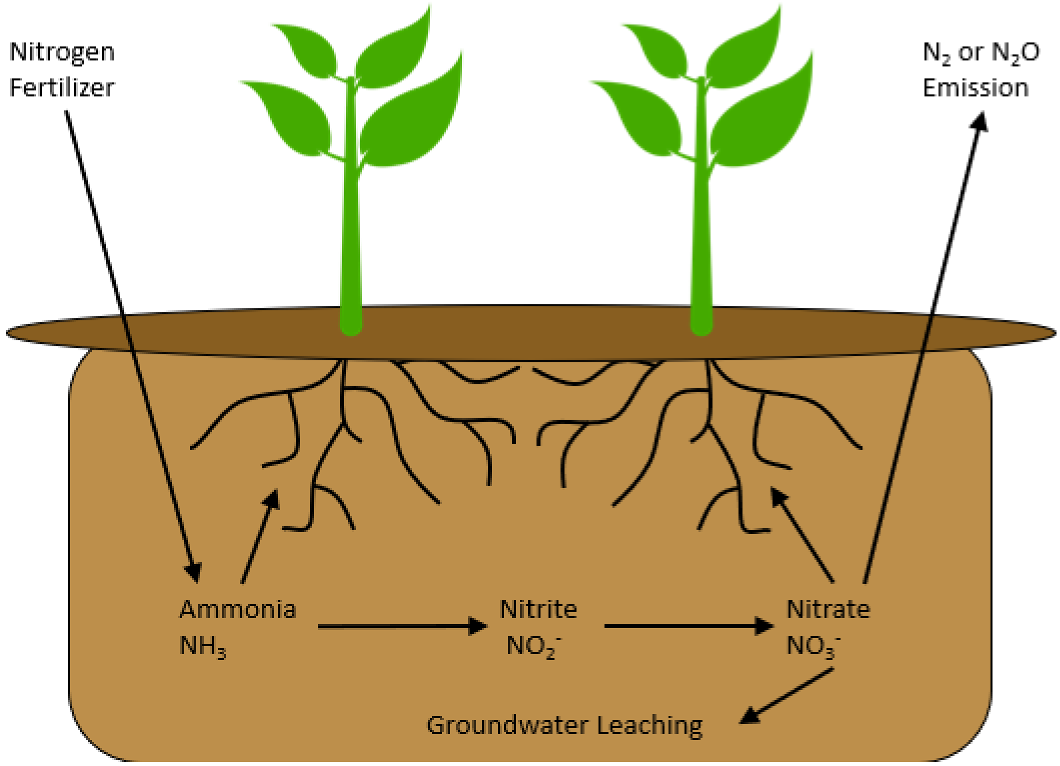

Figure 2: Fertilizer application and denitrification.

Anaerobic ammonium-oxidizing (ANAMMOX) bacteria are gaining a considerable amount of attention within the wastewater treatment sector. ANAMMOX have the capability to remove nitrogen through a shortcut directly transforming ammonium and nitrite to nitrogen gas.[25] Although evidence linking ANAMMOX bacteria to N_2O production is not clear, bacteria coexisting in the ANAMMOX system may produce a significant amount of N_2O.[26] The majority of the produced N_2O came from nitrifier denitrification.[26–28] N_2O emissions were quantified based on influent nitrogen loading to be 1.1-6.6% in a partial nitrification-ANAMMOX system and 0.58-2.4% in traditional nitrogen removal system.[25,29–33] The use of aeration increases the emission of N_2O not only due to gas stripping, but also larger formation rates of the gas.[31] Further experimentation is needed to understand the role ANAMMOX may play in N_2O formation and how that will impact the wastewater sector.

The role of salinity in soils on N_2O and CH_4 emissions have been summarized in a review paper by Fagodiya et al..[34] Saline soils affect 0.831 billion ha of agricultural land worldwide out of 1.87 billion ha (44%).[34,35] Salinity adversely affects soil microbial diversity and enzyme activity. Low N_2O emissions correspond with high salinity concentrations due to lower nitrification resulting in lower substrate for denitrification.[34] Additionally, CH_4 emissions follow the same trend because salinity suppresses both methanogens (CH_4 producing bacteria) and methanotrophs (CH_4 consuming bacteria), with the prior having greater inhibitory effects.[34]

In this same review, soil amendments (e.g., biochar, organic manure, gypsum, etc.) were analyzed to understand which amendments may be the best for soil reclamation. The process of reclamation will encourage higher emissions of both N_2O and CH_4 and therefore the main focus was on the mitigation of these gases. Gypsum proved to be the most effective amendment mitigating CH_4 by introducing sulfate reducing bacteria as competition, however, N_2O emissions went unchecked.[34] Biochar amendments helped mitigate both N_2O and CH_4 and became more apparent when aged biochar was used.[34] Investigations are still needed to fully understand and quantify the effects of these amendments on N_2O and CH_4 emissions.

Other interesting factors affecting the nitrogen cycle include cover crops and microplastics. Cover crops have also been used to reduce emissions as well as other benefits for the land. Cover crops improve soil structure, promote nitrogen cycling, improve soil fertility and microbial activity, control soil erosion, inhibit weeds, retain soil moisture content, and absorb excess mineral nutrients which leads to less N_2O and CH_4 emissions.[36] Microplastics (< 5 mm) are gaining attention as a new pollutant in soil, oceans, freshwater, and the atmosphere. Within the soil, microplastics can have adverse effects on the carbon, nitrogen, and phosphorus cycles.[37] In the nitrogen cycle, microplastics can affect nitrogen sources, nitrogen fixation, ammonification, nitrification and denitrification processes by changing gene abundance, enzyme activity, microbial community composition.[37] Microplastic pollution is at a quantity that cannot be ignored in future research and its implications must be recognized.

Biological nitrogen inhibitors (BNIs) are gaining attention for increasing the available nitrogen content for plants by inhibiting bacterial nitrification.[22,38] BNI can significantly improve nitrogen use efficiency in crops while simultaneously reducing N_2O emissions.[22] BNIs are low cost and environmentally friendly as they reduce N_2O and CH_4 emissions.[38] For example, the BNI from Signalgrass (*Brachiaria humidicola*) accounted for 60-90% of the total inhibition of nitrification.[22] Coupling BNIs and bacteria containing *nosZ* may be a promising future of completely reducing N_2O emissions in agricultural soils. Experiments are needed to understand if BNI and *nosZ* can work together.

Effect of pH on NosZ

Oxygen is not the only inhibitor of N_2O reduction. Multiple experiments have investigated N_2O emission under low pH because most soil ecosystems have a pH value between 5 and 8.[9,39,40] The ratio of $N_2O/(N_2 + N_2O)$ increased as soil pH decreased from 8 to 4; due to changes in the denitrifying community.[39] N_2O consumption decreased as the pH decreased even though N_2O production was maintained at the same rate. The low pH inhibited *nosZ* from converting N_2O to N_2, but the other denitrifiers were not inhibited. N_2O is the only intermittent denitrifier gas that is correlated with pH.[41] Another potential source of N_2O is from microorganisms that have truncated denitrification.[39,41,42] Truncated denitrification is achieved by denitrifying bacteria that do not possess *nosZ* therefore they emit N_2O as an end product.[39]

Researchers explored the effects of biochar or lime on N_2O emissions by increasing the soil pH.[40,43] Biochar was tested at a 2% application rate to understand how N_2O emissions would change compared to the control soil sample. The use of biochar increased the soil pH from 5.48 to 6.11, decreased N_2O emissions by 3.3 times and augmented the abundance of *nosZ* and *nirK* genes.[40] Gene copy numbers of *nosZ* in 2% biochar soil increased 1.8 per gram of dry soil more than the control over 35 days.[40] Accumulation of N_2O did not occur in the experiment due to the increase in *nosZ* catalyzing N_2O to N_2. On the other hand, the lime amendment increased soil pH from 6.1 to 7.0 and increased N_2O accumulation.[43] N_2O accumulation was observed because the lime increased the abundance of denitrifying genes increasing the production of N_2O whilst *nosZ* could not consume N_2O as rapidly as it was produced. N_2O production rates typically outweigh N_2O consumption rates at pH 7-7.5.[44]

Wastewater treatment plants produce N_2O via denitrifying microorganisms in the pursuit of nitrogen removal in wastewater.[44,45] The dissolved portion of N_2O could be relatively high with a dimensionless Henry's constant of 0.595.[46] A low solid retention time (< 10 days) and C:N ratio (< 2.5 COD:NO_3^-—N) and a pH less than or equal to 6.5 were conditions identified as favorable for N_2O production.[30] An additional study on wastewater treatment experimentally determined the maximum amount of N_2O gas was produced at a pH between 5 and 6, while no N_2O was emitted above a pH of 6.8.[44,45,47,48] The effect of pH on denitrification was evaluated at lower pH ranges in other studies. Denitrifying microorganisms from an acidic fern at an optimal pH of 5 were tested for their N_2O emission. The results showed that N_2O was 40% of the total nitrogen gases at pH 5 and increased to 100% at pH 3 or less.[49] Thus, exposure to low pH may cause the growth of acid-tolerant or acidophilic *nosZ*-containing denitrifiers.[48,50] Acidophilic bacteria were more phylogenetically clustered as the soil pH decreased from neutral indicating the pH is a selective filter on community membership.[51]

However, *nosZ* is not the only gene in the context of denitrification that is affected by pH.[50,52] Low pH lowers all denitrification conversion rates.[48] The genes that catalyze the conversion of nitrite to nitric oxide and then nitric oxide to nitrous oxide are *nirS*/*nirK* and *norB*, respectively. *nirS* denitrifiers favor a pH greater than 5.7, while *nirK* denitrifiers are less affected by acidic pH values.[50] Suboptimal pH values below 7 severely inhibit *nosZ*, whereas *nirK* and *norB* were not severely inhibited leading to N_2O accumulation.[47] N_2O emissions were noted to be closely related to *nirS*, *napA*, and *narG* copy numbers; however, the understanding of this is unknown.[39] *nosZ* gene abundance is lower compared to gene abundance of other denitrifying genes (i.e., *napA*, *narG*, *nirK*, *nirS*, and *norB*).[51,53] Denitrification response of N_2O production based on pH depends upon the entire denitrifier community composition.[52,54] A study observed transcription rates of *nosZ* versus *nirS* and *norB* were not influenced by pH in the model organism *Paracoccus denitrificans*.[47] This indicates that the inhibition of *nosZ* occurred during protein assembly and not transcriptionally. The leading hypothesis for inhibition is unsuccessful post-transcriptional assemblage/folding of the protein in the periplasm due to low pH and partial inhibition of the copper center of NosZ.[5,42,47,55]

The periplasm is the region between the cytoplasmic membrane and the outer cell membrane in gram-negative bacteria. NosZ, NapA, NirS, and NirK are located in the periplasm, while NorB is in the cell membrane, and NarG is in the cytoplasm.[44,56] Gram-negative bacteria cannot regulate periplasmic pH because it is outside of the cytoplasmic membrane. However, recent work has studied the use of chaperones in protecting the proteins within the periplasm.[55,57] Molecular chaperones are an array of proteins that maintain the activity of proteins during cell growth and under stress conditions.[58] Gut bacteria (e.g., *Escherichia coli*) are exposed to extremely acidic pH levels and use molecular chaperones to survive in this environments.[59]

Small periplasmic chaperones named HdeA and HdeB are critical for preventing irreversible aggregation of periplasmic proteins.[59–62] Kern et al.[61] isolated both HdeA and HdeB from *Escherichia coli* (strain MG1655). At pH 2, HdeA and HdeB working together can prevent the aggregation of periplasmic proteins. At pH 3, HdeB alone is more effective at protecting the proteins compared to HdeA alone.[61] Further research is needed on the use of HdeA and HdeB for protecting periplasmic proteins. Research questions examining how to increase the abundance of these chaperones to protect periplasmic proteins at low pH will be profitable.

Partial inhibition of NosZ may also be due to the copper centers of the enzyme. NosZ has two copper centers, CuA and CuZ (Figure 3), that transfer electrons from CuA close to the enzyme surface to the active site CuZ.[44,56] N_2O reduction is limited by this transfer of electrons. CuA has two copper molecules while CuZ has one sulfur molecule surrounded by four copper molecules. Conformational changes to CuA occur in low pH causing a disruption of the electron transfer.[44,63] Shen et al.[64] experimentally determined that the addition of Cu ions significantly increased the consumption of N_2O by promoting *nosZ* expression. Further studies are needed to understand how *nosZ* is affected by Cu on a molecular level as well understanding the best application rate to avoid Cu contamination.[64]

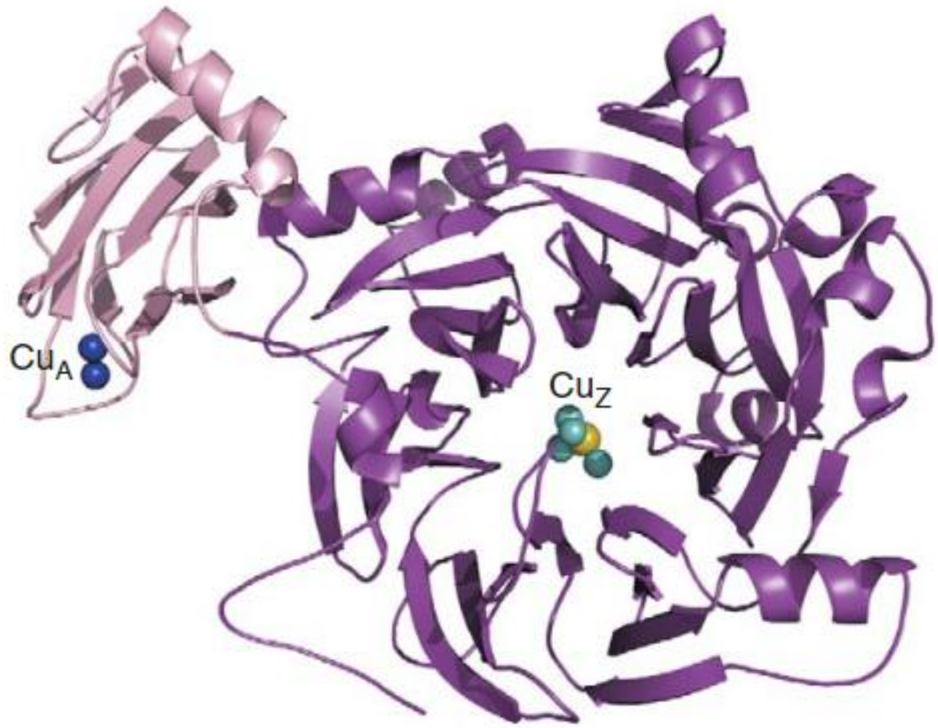

Figure 3: Ribbon diagram of NosZ with CuA and CuZ centers. Adapted from[65].

If *nosZ* is able to reduce N_2O at low pH, the bacteria can mitigate N_2O emissions within wastewater treatment or agricultural processes (e.g., silage, manure management). Microorganisms with *nosZ* were classified into clade I *nosZ* and clade II *nosZ* as recently as 2012.[66,67] The unexpected discovery of the latter indicates that previous studies have underestimated the capability of microorganisms to reduce N_2O.[66,67] The differences between the two clades of *nosZ* may be able to help N_2O degradation in environments that were previously overlooked before the discovery of clade II *nosZ*. The hypothesis of this paper is that microorganisms possessing clade II *nosZ* will be more abundant than clade I *nosZ* at low pH and may be able to continue N_2O degradation.

NosZ and its Clades

Phylogenetic differences between the clades are shown in their transmembrane pathway where clade I bacteria encode for Tat (twin-arginine translocation pathway) and clade II bacteria encode for Sec (secretion pathway) (Figure 4).[66,68] Microorganisms containing clade II *nosZ* are found in almost every ecosystem with varying conditions (e.g., anoxic, microaerophilic, oxic, thermophilic, psychrophilic, piezophilic, and halophilic).[67,69,70] Traditional denitrifiers possess clade I *nosZ* and bacteria that possess clade II *nosZ* are non-denitrifying. Bacteria with clade II *nosZ* have the potential to reduce N_2O emissions without contributing to the production of N_2O. Short-read metagenomic shotgun sequencing of sandy and silty loam agricultural soils revealed that 70% of microorganisms were clade II *nosZ* and 15% were related to the genus *Anaeromyxobacter*.[69] Clade II *nosZ* outnumbered clade I *nosZ* in almost all publicly available records of soil metagenomics.[69] Masudo et al.[71] identified that roughly 80% of the bacteria containing *nosZ* in waterlogged paddy soils were of the genus *Anaeromyxobacter*. Deep-sea hydrothermal vent clade II microorganisms were discovered to reduce N_2O, demonstrating that the capabilities are not limited to traditional denitrifying organisms and traditional environments.[72] Bacteria were taken from a hydrothermal vent in the Southern Mariana Trough and grown in a synthetic sea water media using N_2O and H_2 as the electron acceptor and the electron donor, respectively. 90% of the original N_2O gas in the headspace was consumed after 42 hours of inoculation.[72] The availability of N_2O is not a driver for either clade to become more dominant.[73]

Clade II organisms most likely acquired *nosZ* from horizonal gene transfer due to the fact that N_2O is a favorable electron acceptor.[16] The Gibb's free energy of the conversion from N_2O to N_2 is between -314 and -339.5 kJ/mol.[18,56] N_2O accumulation is toxic to cells as low as 0.1

mM, but researchers observed that *nosZ* helped resist this toxicity and may be a reason as to why non-denitrifiers carry the gene.[15,74] Known features of clade II *nosZ* include high phylogenetic diversity and involvement in a broad range of taxa.[75,76] Conventional polymerase chain reaction (PCR) primers do not include markers identifying clade II *nosZ* and offer a very narrow range of clade I *nosZ*.[77,78] Because of the broad diversity of clade II *nosZ*, a universal primer does not work to accurately quantify the role of microorganisms possessing clade II *nosZ*. Therefore, primer sets were developed to target subclades of clade II microorganisms.[78,79]

There are a few experiments that looked at the effect of nitrate (NO_3^-) on *nosZ* and N_2O production. *Thauera linaloolentis* (strain 47Lol^T) was cultivated in the presence of N_2O and in the presence of N_2O and NO_3^-. *Thauera linaloolentis* was chosen as the bacteria is one of three types that contains both clade I *nosZ* and clade II *nosZ*.[80] Observations showed that NO_3^- possibly inhibited clade II *nosZ* expression but not clade I *nosZ* expression.[80] The mitigation of N_2O emissions will change depending on the habitat of the bacteria. Accumulation of NO_2^- or incomplete removal of NO_3^- always accompanied N_2O formation in wastewater treatment.[30] An experiment comparing N_2O versus NO_3^- as electron acceptors with the same wastewater inoculum discovered that clade II *nosZ* became dominate in the N_2O reactor while clade I *nosZ* became dominate in the NO_3^- reactor.[81] The isolates from the N_2O reactor demonstrated higher N_2O consumption rates and lower half saturation constants than the isolates from the NO_3^- reactor. Furthermore, higher specific growth rates were observed in clade II *nosZ* bacteria that used N_2O as the sole electron acceptor compared to using NO_3^-.[81]

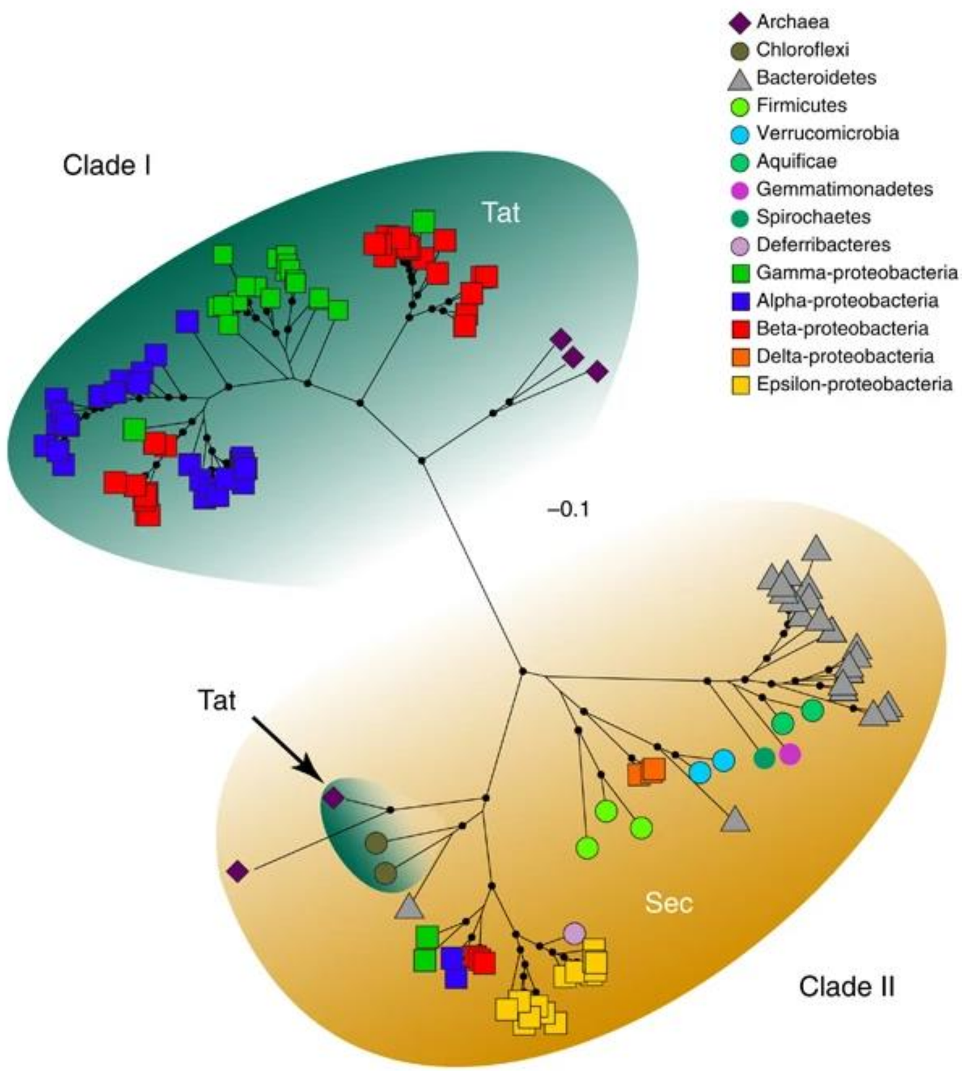

Figure 4: Unrooted maximum likelihood phylogenetic tree of major taxonomic groups. Adapted from[66].

Differences between the two clades include enzyme kinetics relating to N_2O reduction. Most experiments indicate clade II *nosZ* has a lower affinity than clade I *nosZ*, but growth yields varied by experiment.[15,73,81,82] Through the enrichment of pure cultures, clade II *nosZ* growth yields exceeded clade I *nosZ* by 1.5- to 1.8-fold based on optical density.[82] Conversely, media inoculated with activated sludge observed that clade I *nosZ* had higher growth yields than clade II *nosZ*.[15] The physiology of the two *nosZ* clades may not be distinguishable by kinetics alone as taxa differences may be more important than clade differences.[73] The principle roles of soil microorganisms in the production and consumption of N_2O must be examined to mitigate future N_2O emissions. Exploration of functional genes and enzymes and their regulatory mechanisms are crucial to controlling N_2O emissions.[6]

Chapter 2 - Methods and Materials

Media and experimental preparation

N_2O reducing bacteria were gradually enriched to lower pH values at 0.5-unit increments, starting from pH 7 to pH 5 under anoxic conditions. Microorganisms from wastewater activated sludge in the aeration basin (Manhattan, KS, USA) and agricultural soil (lat: 39.206003; long: -96.594166) were tested in triplicates, as well as one negative control duplicate. Standard deviation was calculated for each condition and represented as error bars on the figures. The media was composed of 45.3 mM sodium acetate anhydrous, 13.3 mM ammonium chloride, 7.4 mM monopotassium phosphate, 2.1 mM magnesium sulfate heptahydrate, 0.5 mM sodium hydroxide, 2 mg/L yeast extract, and 2.5 mL/L trace element solution.[15] Trace element solution per liter contained HCl (25% solution w/w), 10 mL; $FeCl_2$ x $4H_2O$, 1.5 g; $CoCl_2$ x $6H_2O$, 0.19 g; $MnCl_2$ x $4H_2O$, 0.1 g; $ZnCl_2$, 70 mg; H_3BO_3, 6 mg; Na_2MoO_4 x H_2O, 36 mg; $NiCl_2$ x $6H_2O$, 24 mg; $CuCl_2$ x $2H_2O$, 2mg. The pH of the media was adjusted using HCl or NaOH as needed to reach the target pH value. At pH values below 6, 7.4 mM MES (2-ethanesulfonic acid) was used in place of monopotassium phosphate as the media buffer. Acetate is used as the electron donor in this experiment due to the novelty of the gradual enrichment to low pH. However, volatile fatty acids could be substituted for more realistic applications in the future.

The media was prepared using a 3-necked flask with glass stoppers and a condensing tube flushed with ultra-pure N_2 gas (Matheson, Manhattan, KS). The media was boiled over a Bunsen burner and then cooled to room temperature before adjusting the pH of the media. Each 1 L bottle (Fisherbrand GL45, Hampton, NH) was prepared under anoxic conditions and filled with 300 mL of media, resulting in a liquid to headspace ratio of roughly 1:3. The initial N_2O (Aldrich, St. Louis, MO) injection and each subsequent spike of N_2O was 150 mL of the gas.

N_2O was used as the sole electron acceptor in the media. Concentrations of N_2O were monitored roughly every 2-3 days until the concentration neared 0 mM in a gas chromatography (GC) machine. Henry's constant of N_2O (dimensionless 0.595) was used to calculate the concentration within the aqueous phase and the entire bottle.[46] An electron capture detector (ECD) was used for this analysis. N_2O standard curves were prepared once a month to correlate peak area from GC to concentration of N_2O. After N_2O had successfully degraded 3-4 times, new media was prepared at a pH value 0.5 units lower and 5 mL of the media was transferred to continue the experiment. 50 mL of the media from each bottle were retained for microbial analysis.

DNA extraction and analysis

From the retained samples, DNA was extracted using DNeasy UltraClean Microbial Kit (Qiagen, Hilden, Germany). PCR was used to analyze the DNA using primers targeting clade I and clade II *nosZ*. The instrument used for PCR was a MiniAmp Thermal Cycler (Applied Biosystems, Waltham, MA). PCR protocol combined 1 µL for each forward and reverse primer (at 10 µM concentrations), 2 µL template DNA, 10 µL Taq 5x master mix (New England Biolabs, Ipswich, MA), and 36 µL nuclease-free water for each 50 µL reaction. For gel electrophoresis, E-Gel™ Power Snap Electrophoresis Device and Camera (Invitrogen, Watham, MA) were used in conjunction with E-Gel™ EX 1% Agarose gel plates. A FX Opus 96 real-time PCR System (Bio-Rad Laboratories, Hercules, CA) was used to quantify gene copy numbers. The primers and standards were obtained from Genewiz (South Plainfield, NJ). A total of 9 primers were used targeting clade I broad and clade II subclades A, B, C_1, C_2, D, F, G, and H (Table 1). The primers targeted small amplicon product sizes ranging from 203 to 230 base pairs ideal for PCR.[79] The PCR method involved the touchdown thermal cycle as follows: 94°C for 5 minutes, 10 cycles of [95°C for 30 seconds, 65°C for 30 seconds, and 72°C for 30 seconds;

decreasing the annealing temperature by 0.5°C per cycle], 25 cycles of [95°C for 30 seconds, 55°C for 90 seconds, and 72°C for 2 minutes], and 72°C for 10 minutes.[79] Quantitative PCR (qPCR) data were normalized by 16S results from the respective samples. qPCR protocol combined 0.6 µL for each forward and reverse primer (at 10 µM concentrations), 2 µL template DNA, 10 µL SYBR® Green Supermix (Bio-Rad Laboratories, Hercules, CA), and 6.8 µL nuclease-free water for each 20 µL reaction. Samples were loaded into a 96-well plate and spun down before the analysis.

DNA samples from the initial wastewater, pH 7, pH 6, pH 5.5 WW 1, and pH 5.5 WW 3 were analyzed by metagenomic sequencing (MR DNA, Shallowater, TX). Reads were taken by IluminaSeq 6000 at 2x150 base pairs for a total of 30 million paired ends per sample. MobaXTerm interface was used to complete the metagenomic analysis coding. Samples were then trimmed using Trimmomatic version 0.39.[83] After trimming the sequences, the paired forward and reverse sequences were merged using paired-end read merger (PEAR).[84] Reference libraries were prepared from FunGene Pipeline and the National Center for Biotechnology Information for denitrifying genes.[85] A supercomputer was used to BLAST (Basic Local Alignment Search Tool) the reference library and the unknown DNA samples.[86] The samples were normalized by the 16S copy number acquired from qPCR with the respective DNA sample. Since two samples were analyzed from pH 5.5, the qPCR and metagenomic sample from WW 1 were the same, but for WW 3 a factor of 0.35 was applied to account for the change in community composition.

Table 1: Primers targeting clade I and clade II nosZ. Adapted from[79].

***nosZ* Target Group**	**Primer Name**	**Sequence 5'→3'**	**Amplicon Size (bp)**
Clade II (A)	NosZ 1374F-A	CCBYTBCAYACSCARTTYG	203
	NosZ 1577R-A	TGSGASAGCTTGTTSAGSS	
Clade II (B)	NosZ 1374F-B	CIYTSCACACSGARTTCGA	207
	NosZ 1577R-B	GRTCCTTCGTGATCTTGTTSA	
Clade II (C_1)	NosZ 1374F-C_1	CVYTICAYACCGARTTYGA	208
	NosZ 1577R-C_1	CKRTCTTTBGTRATYTTRTT	
Clade II (C_2)	NosZ 1374F-C_2	CCIYTICAYACWGARTTTGA	209
	NosZ 1577R-C_2	CKRTCTTTBGTRATYTTRTT	
Clade II (D)	NosZ 1374F-D	CCIYTICAYACSCARTWYGA	206
	NosZ 1577R-D	ATSGMSAGCTTGTTSADSS	
Clade II (E)	NosZ 1374F-E	CCIYTICAYACSCARTWYGA	230
	NosZ 1577R-E	GASAKCTTGTTSATSGCIAC	
Clade II (G)	NosZ 1374F-G	CGCTCCACASSCARTTCGA	211
	NosZ 1577R-G	CGGTCCTTSGASAGCTTGT	
Clade II (H)	NosZ 1374F-H	CCDYTKCAYACICARTTYGA	206
	NosZ 1577R-H	TCYTTVGCHAKYTTRTTYARIG	
Clade I Broad	NosZ1mod-F	WCSYTSTTCMTSGAYAGCCAG	248-251
	NosZ1mod-R	ATRTCGATSARCTGVKCRTTYTC	

Michaelis-Menten kinetics

To analyze the Michaelis-Menten parameters of V_{max} and k_m, an experimental protocol was followed of that below. 160 mL serum bottles were flushed with N_2 gas and sealed with rubber stoppers. 48 mL of the media sample were added to each of the prepared bottles (48 mL allows the same ratio of liquid to headspace as the experimental bottles). The bottles were then injected with small concentrations of N_2O gas ranging from 0.1 mL to 0.3 mL. After injection, 10-60 minutes equilibration time was allowed before GC measurements were taken every 10-60 minutes. The time interval was split, and the degradation rate measured over each short time frame. The analysis was completed within a few hours so that the assumption of no biomass formation remained true. The Michaelis-Menten parameters were calculated after graphing a Lineweaver-Burke plot. Standard error of the regression was calculated and shown in the error bars.

Chapter 3 - Results & Discussion

Gas Chromatography

The soil bacteria easily degraded N_2O at pH 7, completing all four degradations within 10 days (Figure 5). Nevertheless, after one complete degradation at pH 6.5, the soil bacteria stopped degrading N_2O. It is not understood why the reduction of N_2O stopped, especially considering the first degradation was successful. Experiments in the past have explored using acidic soil as an inoculant.[40,49,87] The use of acidic soil compared to neutral pH soil may be advantageous for attaining microorganisms that are already thriving in a low pH environment. These microorganisms may already have adapted a way to protect *nosZ* from low pH. After monitoring the soil bottles for 60 days without any significant degradation, the bottles were abandoned.

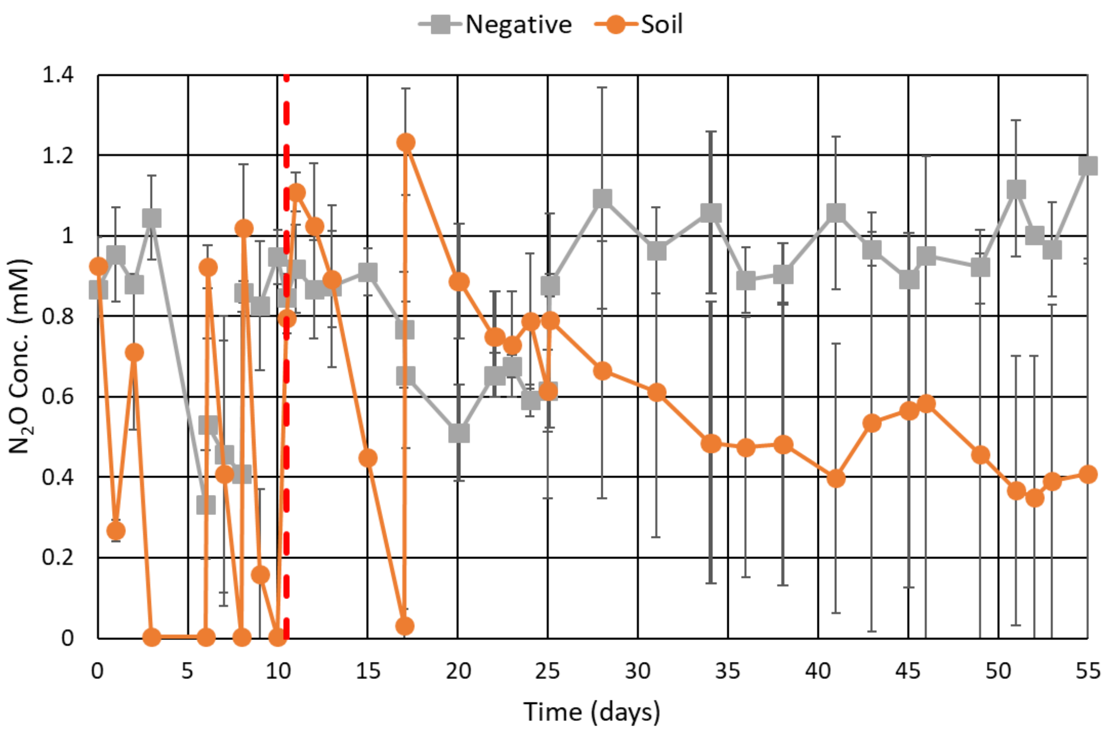

Figure 5: Soil bottles at pH 7-6.5. Red dashed line indicated transfer to lower pH.

On the other hand, the wastewater bottles rapidly degraded N_2O in pH conditions 7 and 6.5 (Figure 6). Four degradations of N_2O at pH 7 were completed within 10 days. Four degradations of N_2O at pH 6.5 were completed within 15 days. At pH 6, four degradations of N_2O slowed down to 30 days. The results are supported by an experiment with pH 6 and pH 7 inoculated with *Paracoccus denitrificans.*[47] The bottle at pH 7 degraded N_2O, but the bottle at pH 6 did not until researchers took sample from pH 7 and added it to pH 6. Then N_2O was able to be reduced at a significantly slower rate analogous to what was observed in this experiment.

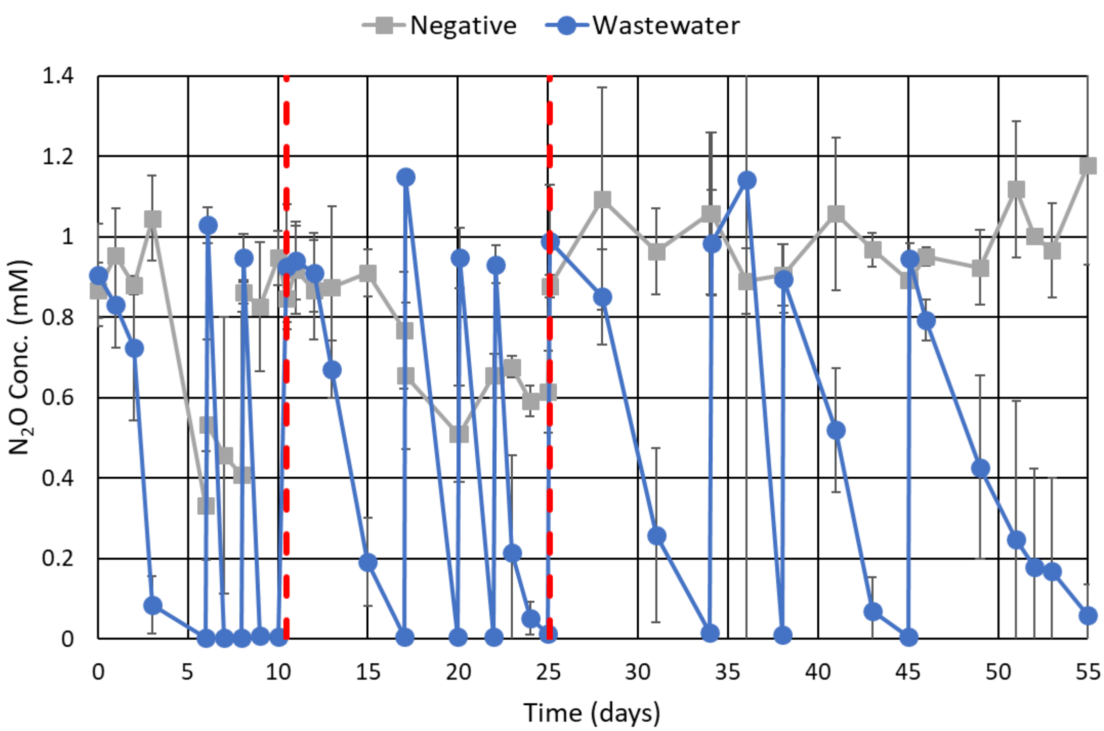

Figure 6: Wastewater bottles at pH 7-6. Red dashed line indicated transfer to lower pH.

The bottles at pH 5.5 were monitored individually because each bottle had different rates of N_2O reduction (Figure 7). Bottle 3 (WW 3) remained at the same concentration while both bottle 1 (WW 1) and bottle 2 (WW 2) reduced N_2O within 30 days. Successful N_2O degradation is presented here at pH 5.5 even at a diminished rate. In wastewater treatment, the maximum amount of N_2O gas was produced at a pH between 5 and 6, while no N_2O was emitted above a pH of 6.8.[30,45,47,48] The pH of the bottles was measured at day 83 and found that WW 3 was still at the desired pH 5.5 while WW 1 and WW 2 had increased in pH to 6. WW 3 was then inoculated by 5 mL of WW 2 on day 83 as a move to kick start the degradation of N_2O in WW 3. This lead to WW 3 degrading N_2O over a 30 day time span after which pH was measured in the bottle to be 6.2. A similar result occurred when researchers inoculated soil samples at pH 5.4 and it drifted up to pH 6.6.[48] Denitrifying bacteria were able to grow in the liquid media but the converions of N_2O to N_2 did not occur until the pH approached neutral.[48] The proposed reasoning behind the pH shift was that growing denitrifiers consumed 6 moles of H+ per reduced mol of NO_3^-. The process for the pH to increase took only a few days, whereas in this experiment the pH shift took multiple weeks due to the different starting nitrogen compound. This is a feasable explanation of why a pH shift occured. When WW 3 was ready to be spiked, a new bottle was prepared (WW 3.1) to confirm kinetic data. WW 3.1 worked quicker than the other bottles at pH 5.5 and degraded all the N_2O within 15 days of being inoculated.

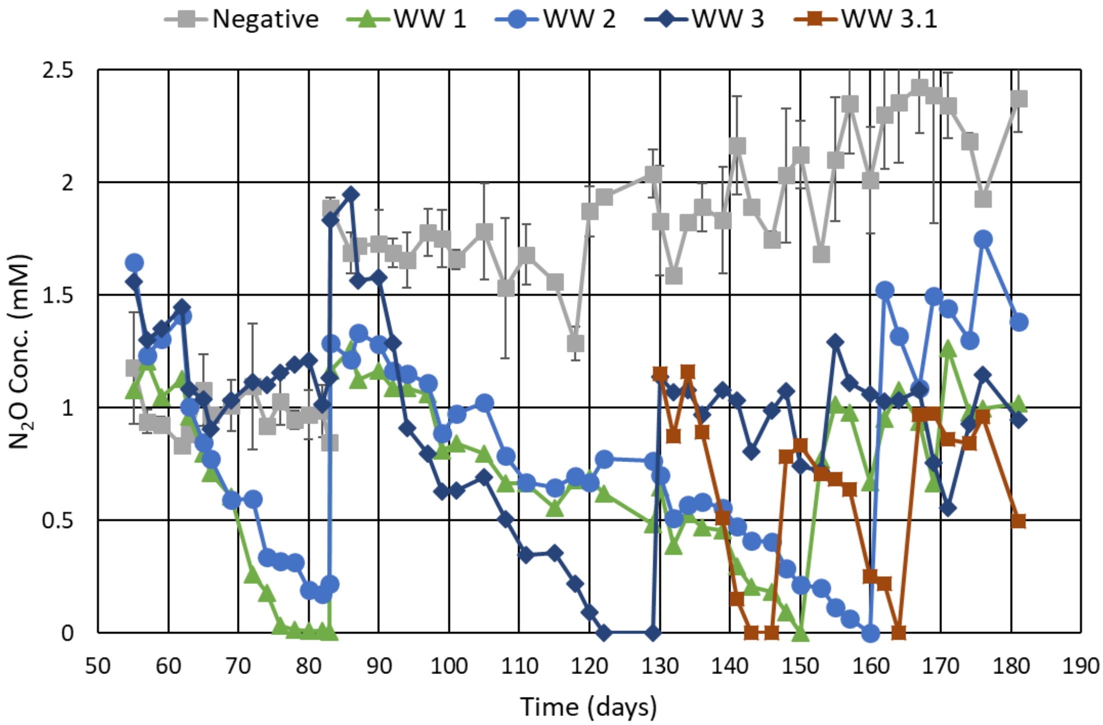

Figure 7: Wastewater bottles at pH 5.5. Bottles were monitored individually due to varying degradation rates.

Bottles at pH 5 were prepared and inoculated with sample from WW 3.1 (Figure 8). After 20 days, no N_2O reduction was observed; a second inoculation with 5 mL from WW 3.1 was performed. New media was prepared and inoculated on day 160, and no N_2O degradation was observed. Based on optical density, a little to no bacteria were able to grow at pH 5 from the transfer. Indicating that from this method of experimentation, microorganisms can degrade N_2O down through pH 5.5 but not including pH 5.

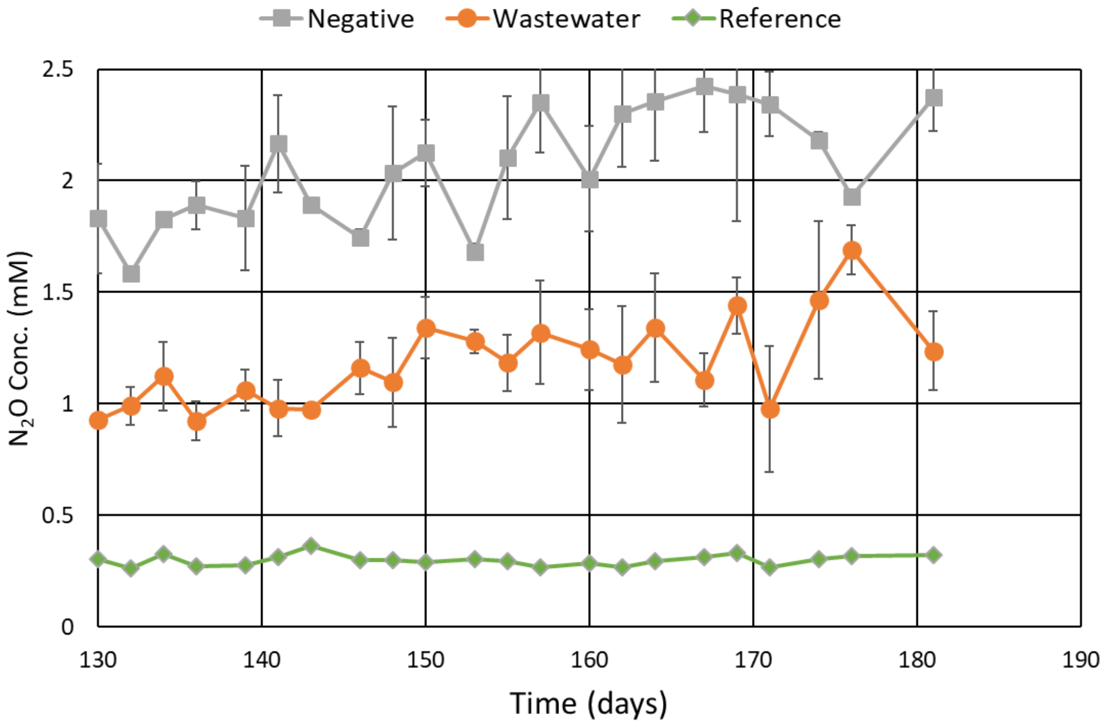

Figure 8: Wastewater bottles at pH 5. A reference of 5% N_2O is shown by the green line.

Kinetics

The Michaelis-Menten parameters obtained from the Lineweaver-Burke plots are shown in Table 2 and Figure 9. The results from this experiment indicate that K_m increased in value from pH 7 to pH 5.5. This insinuates that the affinity of the bacteria at higher pH is greater than the affinity of bacteria at lower pH. Distinctions between the kinetics of two clades of nosZ are evaluated in the metagenomic analysis. V_{max} decreased from pH 7 to pH 6, but dramatically increased from pH 6 to pH 5.5. An increase in V_{max} through pH drop does not relate to the observed effect on N_2O reduction rates. Palmer et al.[49] noted that V_{max} decreased with decreasing pH for both NO_3^- and N_2O amended soil. The metagenomic analysis may shed some light onto why K_m increased with the decrease in pH. It was found that clade I *nosZ* increased in relative abundance as the pH dropped. Literature surrounding kinetics between the two clades of *nosZ* agree than clade II has a lower affinity than clade I.[15,73,81,82] The kinetics experiment for pH 5.5

has a significantly higher starting N_2O concentration compared to pH 7 and pH 6 due to the very low reduction rate.

Cary 60 UV-VIS Spectrophotometer (Agilent Technologies, Santa Clara, CA) was used to measure samples before and after the pH 5.5 experiment to validate that biomass growth was not a factor. The absorbances at 600 nanometer wavelengths were 0.0762 and 0.0794, before and after respectively. This demonstrates that there is no significant biomass growth, and the kinetics experimental data is reliable. The analysis performed for pH 6, however, is not ideal as the linear range of the degradation curve seems to occur right before sampling begins. Kinetics data cannot be concluded due to the limited data noted in this experiment and the broad diversity of clade II microorganisms. The high sensitivity of the ECD in the GC can make it difficult to monitor the degradation of low N_2O concentrations. For pH 5.5, increasing the initial N2O concentration and allowing a longer degradation time allowed for a more efficient analysis and should be considered for future work. The kinetics of different environmental samples should be compared in future analyses.

Table 2: Michaelis-Menten Parameters.

Sample	**V_{max} (nM/min/16S)**	**K_m (μM)**
pH 7	0.92 (± 0.38)	2.759 (±11.311)
pH 6	0.33 (± 0.00)	7.892 (± 1.058)
pH 5.5	1.34 (± 0.00)	19.702 (± 0.077)

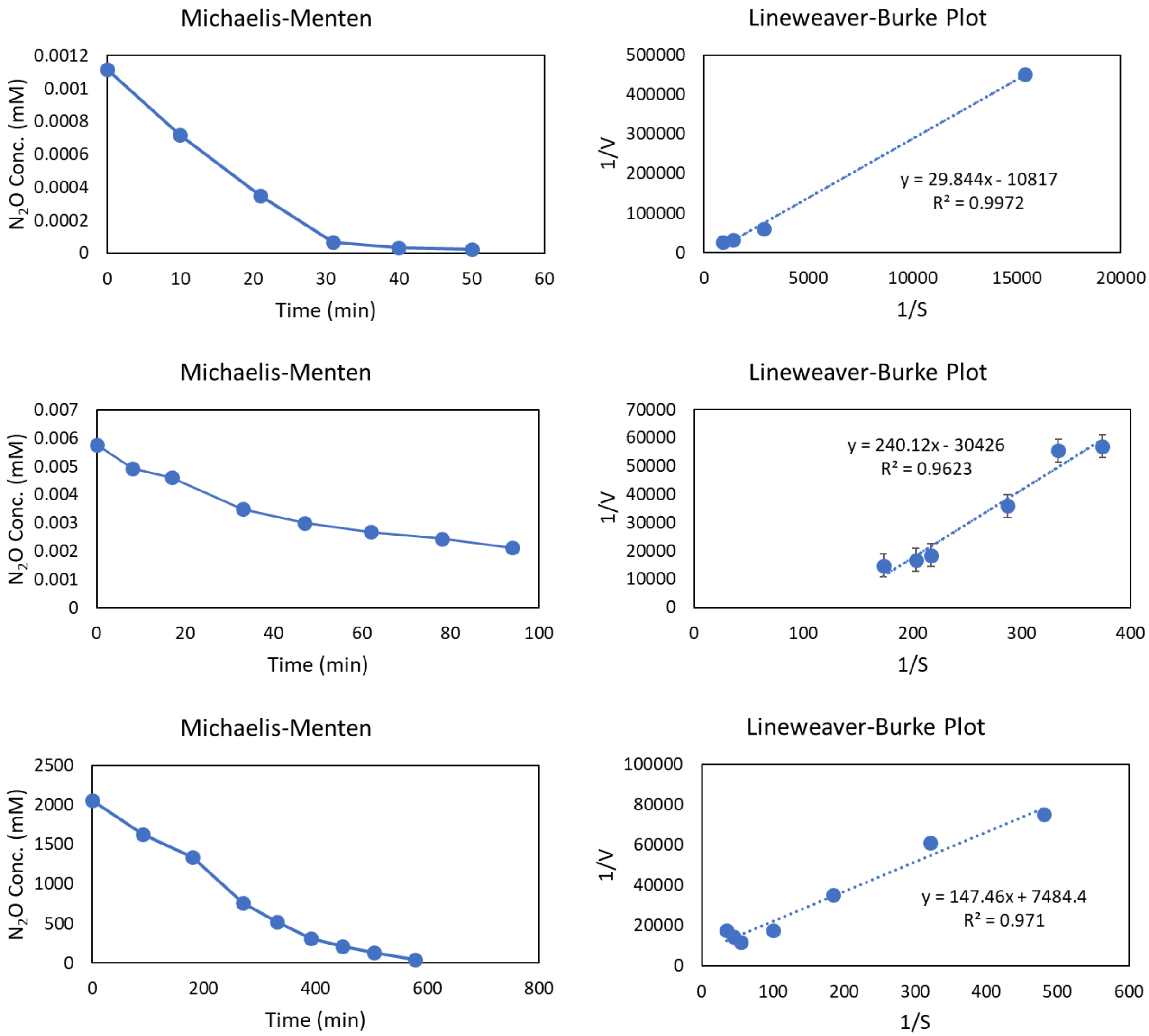

Figure 9: Michaelis-Menten and Lineweaver-Burke plots for kinetics analysis. The top row is pH 7, the middle row is pH 6, and the bottom row is pH 5.5.

Microbial Analysis

Clade II subclades A and B were the only two primers/standards able to quantify DNA in the samples. Subclades D, G, and H were not viable because they did not give positive results on gel electrophoresis indicating problems with either the primer or standard. Subclades C_1, C_2, and E did show positive results on gel electrophoresis, but qPCR standard curve quality was not reliable and the sample data could not be determined acurrately. qPCR was preformed twice for these subclade primers with the same results. Standards should be reordered from a different manufacturer to confirm these results. Bacterial 16S copy numbers were quantified using primers starting at 1055 to 1392. pH 7 contained the most copy numbers of 16S with pH 6, pH 5.5, and Initial WW following behind (Figure 10). Optical density seemed to confirm those same results when compared to pH 6 or pH 5.5. The initial WW sample contained the least amount of 16S copy numbers suggesting a diluted sample. The remaining primers are shown per 16S copy number to normalize the data.

Clade II A microorganisms were the most dominant of the 3 clades/subclades tested. pH 7 had 286% more clade II A bacteria than the next highest condition. Clade II B microorganisms were detected in each sample but at minute levels comparatively. The initial WW sample was composed of the most clade II B bacteria, however, it was only 1/1600 of clade I bacteria at that condition. Surprisingly, clade I bacteria were detected at a maximum at the pH 5.5 condition. The hypothesis would have believed that clade I bacteria would minimize in quantity as the pH decreased due to unfavorable environmental conditions. The ratio of clade II (subclades A and B only) to clade I at pH 7 was 7.98 while at pH 5.5 it was 0.48 suggesting that clade II bacteria decrease and clade I bacteria increase from pH 7 to pH 5.5. Nevertheless, clade II is too broad in taxa to conclude those results, particularly without the other clade II subclade primers functioning.

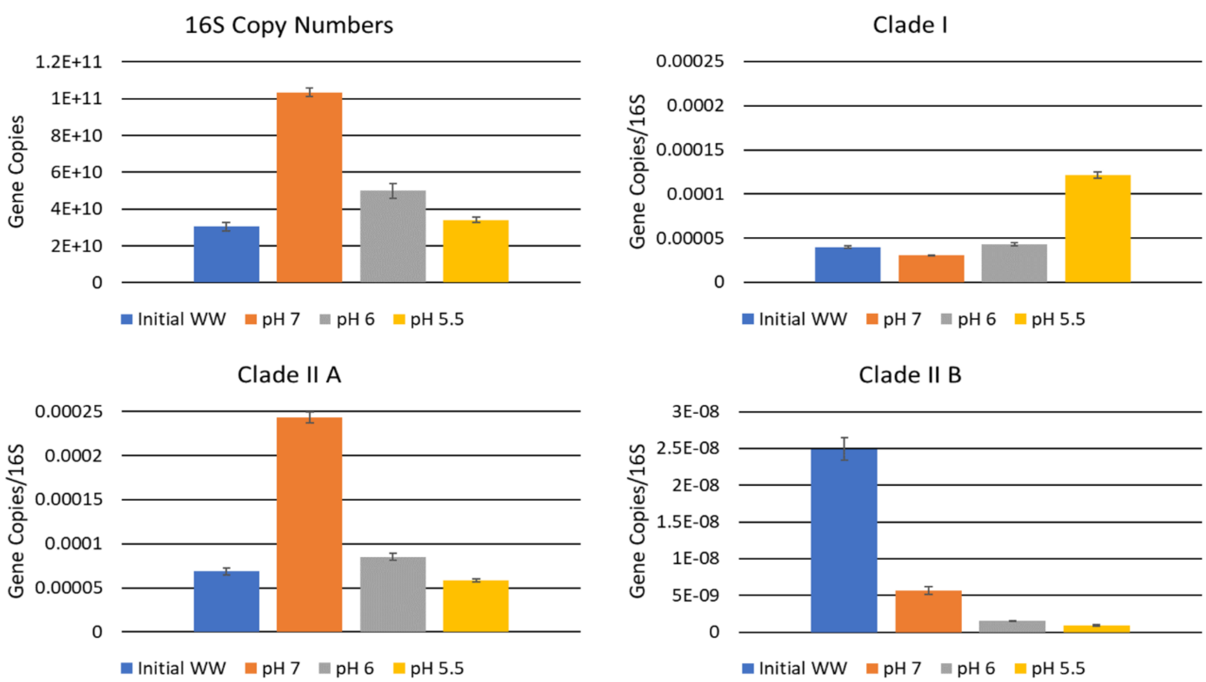

Figure 10: qPCR results for 16S, nosZ clade I, nosZ clade II A, and nosZ clade II B.

The metagenomic analysis relied heavily upon previously prepared tools such as Trimmomatic and PEAR. Table 3 shows the results from merging the sequences with PEAR. Figure 11 illustrates the changes in denitrifier gene abundance at each pH increment. Clade II *nosZ* was more abundant than clade I *nosZ* in every condition.[69] Detection of both clade I and clade II *nosZ* were highest in the pH 5.5 WW 1 sample. This metagenomic analysis confirms the qPCR results indicating that clade I *nosZ* are more prevalent at pH 5.5 compared to pH 7 or pH 6. This conflicts with what was thought in the hypothesis. While a decrease in the rate of N_2O reduction occurs with decreasing pH, the relative abundance of clade I *nosZ* implies that the enzyme is still capable of conversion down through pH 5.5. The relative abundance of bacteria possessing *nosZ* is a strong predictor of $N_2O/(N_2 + N_2O)$ ratio.[53] The metagenomic data does not, unfortunately, inform if clade I *nosZ* is expressed more than clade II *nosZ*. An RNA analysis would be needed for gene expression information, however, that is outside the scope of this paper. RNA samples were saved at each transfer and will be used in future work.

Table 3: PEAR percentages of assembled and unassembled reads.

Sample	Assembled Reads (%)	Unassembled Reads (%)
Initial-WW	49.6	50.4
pH-7	34.3	65.7
pH-6	37.6	62.4
pH-5-5-WW1	51.8	48.2
pH-5-5-WW3	38.2	61.8

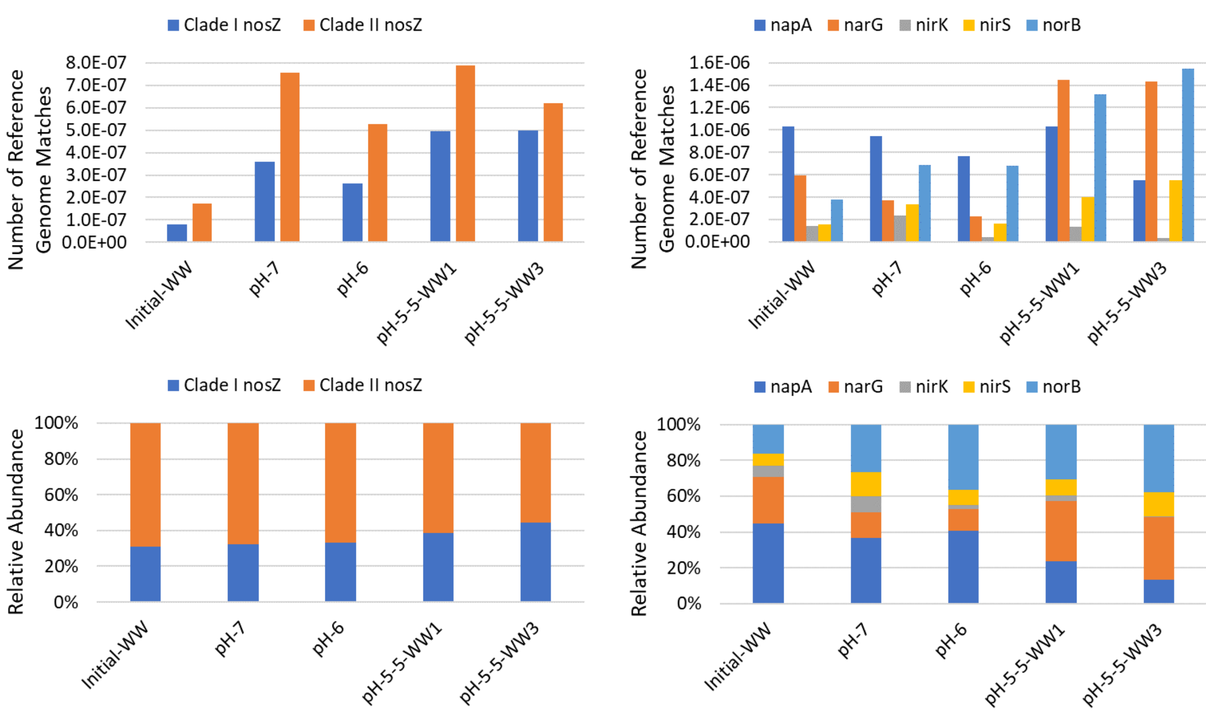

Figure 11: Metagenomic analysis for clade I and clade II nosZ and other denitrifying genes.

The denitrifying genes within the samples were analyzed in the metagenomic analysis to understand the whole denitrifying community. The denitrifying end product, either N_2 or N_2O, is dependent on the soil pH.[53] Low pH impeads all denitrifier conversion rates and can selectively shift microbial community composition.[41,48] As the pH decreased, the abundance of *narG*, *nirS* and *norB* increased, while the abundance of *napA and nirK* stayed the same. It is unexpected to see *nirS* increase due to the fact that *nirK* has a better adability to lower pH compared to *nirS*.[50] N_2O emissions were linked to *napA*, *nirS*, and *narG* two of which increased, but no significant relationship can be observed.[39] It is important to note that the enzymes NapA, NirS, and NirK are all located in the periplasm with NosZ, however, they are not affected by the low pH in the same manner as NosZ.[44,56]

After comparing with the reference library, clade I *nosZ* contained 1,073 sequences compared to clade II *nosZ* with 1,689 sequences. The relative taxa abundance for the top 20 taxa are shown in Figure 12. The bacteria that had the most similar sequences to the samples were from *Burkholderia pseudomallei*, *Brucella abortus*, *Brucella suis*, *Achromobacter xylosoxidans*, and *Stutzerimonas stutzeri*. 16.9% of clade I *nosZ* was comprised of *Brucella abortus*. Interestingly, 10.7% of the same bacteria comprised clade II *nosZ* showing that the bacteria may have some overlap between the two clades. Different species of Brucella have shown complete and partial denitrification based on the genes expressed.[88] This may be the same case for the bacteria in this study. 29.5% of clade II *nosZ* was comprised of the microorganism *Burkholderia pseudomallei*. This species of microorganism is commonly found in contaminated soils and waters and is responsible for the disease meliodosis. Since our inoculation was activated sludge, understandable, the bacteria was present. The other microorganism sources may be from cattle and swine products.

Figures 13 and 14 show the relative taxa abundance in the different pH conditions for clade I *nosZ* and clade II *nosZ*, respectively. For both clades, at low pH (< 6) the bacteria *Achromobacter xylosoxidans* was the most dominate. At pH 7, clade I *nosZ* was dominated by the microorganism *Brucella abortus* even though the initial wastewater and pH 6 samples have very minimal traces of the bacteria. It is unclear why this specific microorganism had such a large relative abundance at pH 7. Clade II *nosZ* in the initial wastewater and pH 7 was dominated by the microorganism *Burkholderia pseudomallei*, however, below pH 6 the bacteria is no longer present.

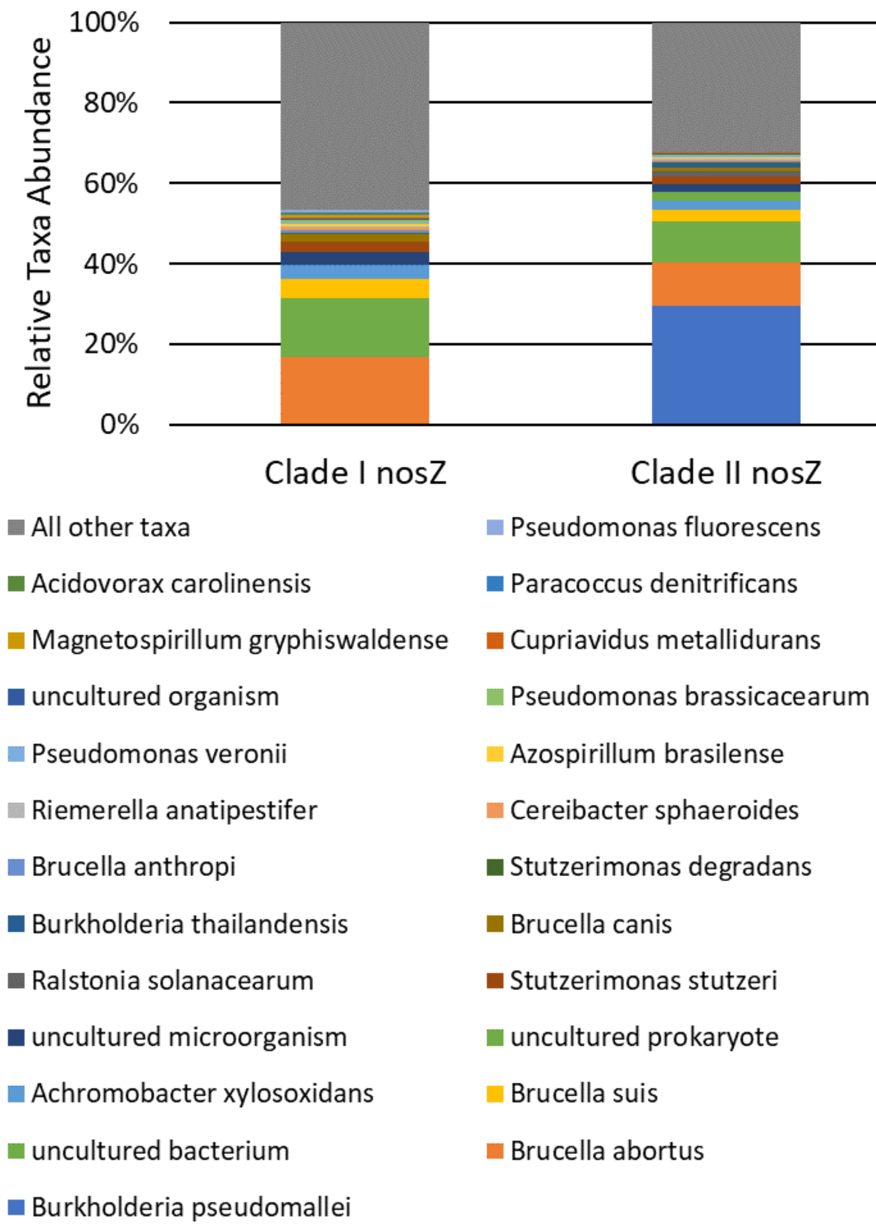

Figure 12: Relative taxa abundance from reference genome library.

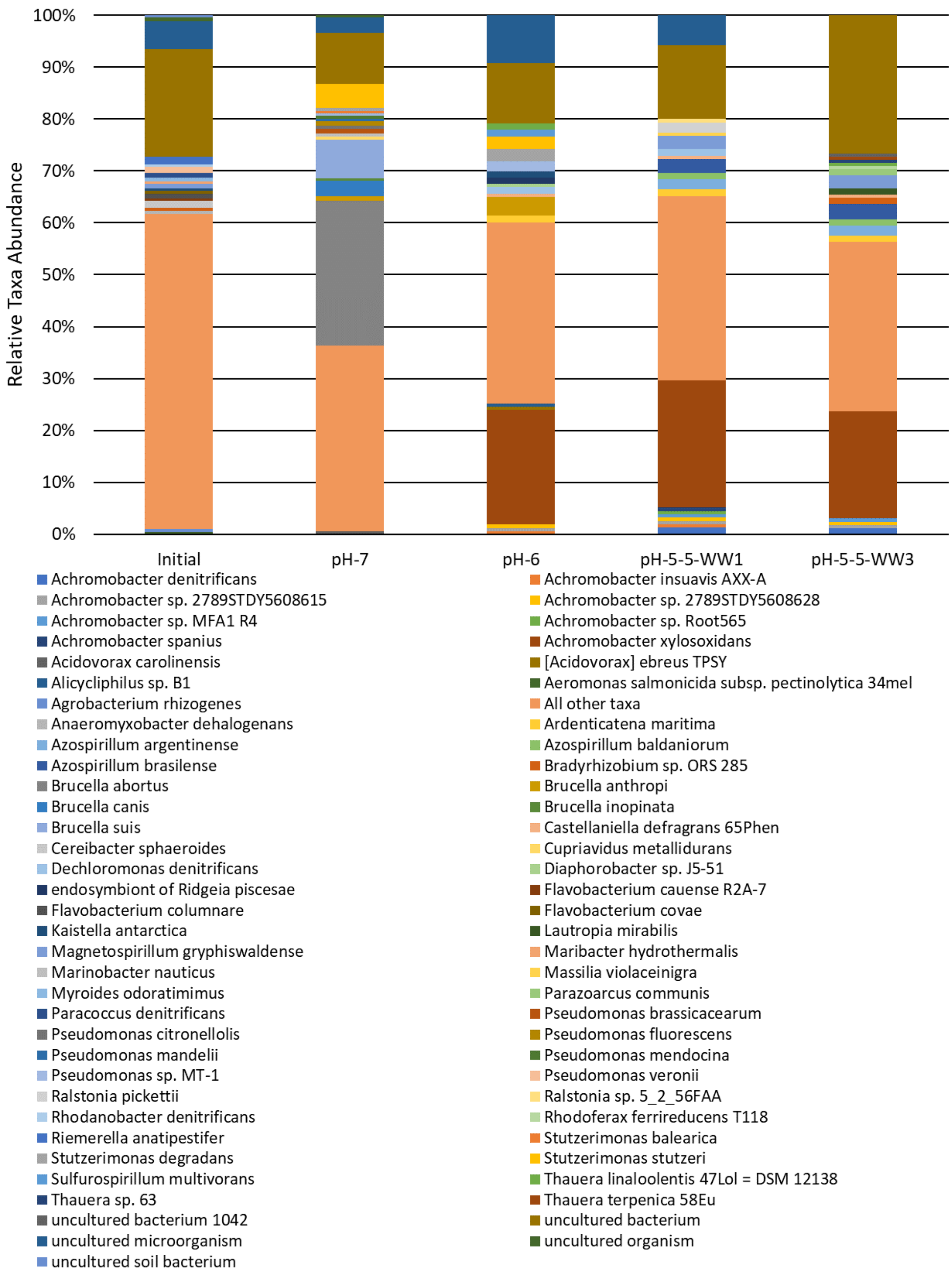

Figure 13: Clade I nosZ relative taxa abundance.

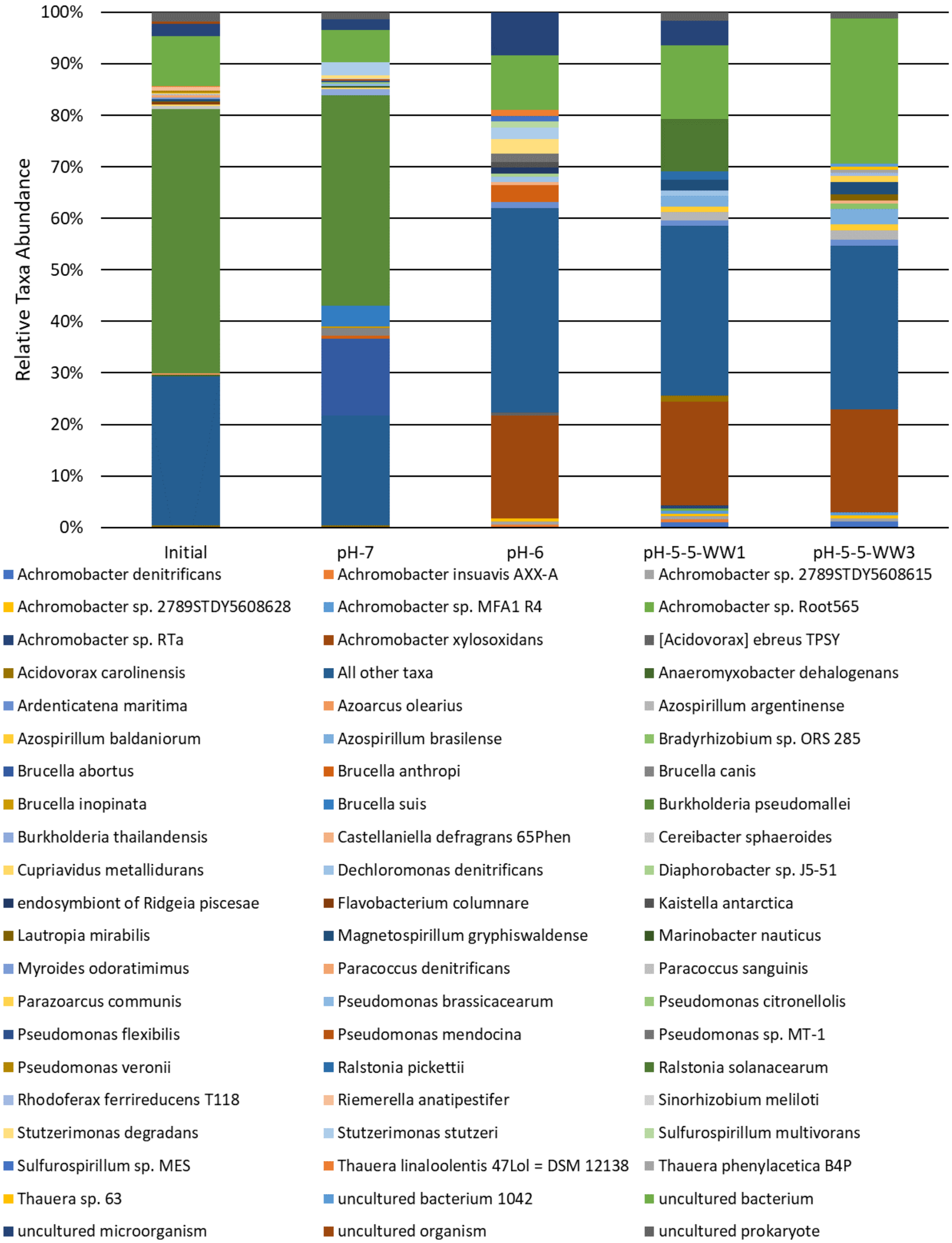

Figure 14: Clade II nosZ relative taxa abundance.

Chapter 4 - Conclusion

Clade I and clade II *nosZ* are not completely understood in various environments due to clade II's recent discovery and broad taxa range. In this experiment, gradual stepwise enrichment of bacteria containing *nosZ* were exposed to pH as low as 5. N_2O reduction occurred up through pH 5.5 but not at pH 5. The kinetics and gene abundance were analyzed throughout the experiment at pH changes. The kinetics revealed that at lower pH, K_m increases, however, nothing can be said of how pH affects V_{max} at this point. Clade II *nosZ* outnumbered clade I *nosZ* in all conditions. However, the relative abundance of clade I *nosZ* increased throughout the experiment while clade II *nosZ* decreased. Due to the wide taxa range of clade II *nosZ*, kinetics and metagenomic data cannot be conclusive since only clade II A and clade II B primers were analyzed. In future analyses, metagenomics should be compared to both the NCBI and the ENA (European Nucleotide Archive) as reference libraries to get a full range of bacteria that are present in the community.

Further experiments are needed to validate kinetic findings and understand the full effect of pH on *nosZ*. Additionally, understanding why the soil samples ceased degradation and experimenting with different soil communities will be beneficial future research. An RNA analysis will be helpful to determine which clade was more active at each condition. The wastewater sample used from this experiment came from the aeration basin, however, changes could be observed using bacteria from the anoxic basin where the denitrifying organisms are actively reducing nitrogen. Additionally, the soil sample was taken in October so soil samples taken after fertilization may contain different bacteria.

Previous laboratory experiments identified silage as a producer of N_2O and listed as the third highest emission source within the agricultural sector with 0.3 million metric tons CO_2

equivalent per year.[89] Within silage, anoxic conditions are satisfied, and fermentation occurs producing lactic acid to reduce the pH to 4-5.[89] The long-term goal of this experiment is to produce an inoculum that can be sprayed over silage to mitigate N_2O emissions. The inoculum would contain bacteria that are able to survive at low pH and possess *nosZ*. The temperature of silage falls between 20°C - 35°C so growing the bacteria at 30°C is applicable for this treatment option and should be conserved in future research. Wastewater treatment, manure management, and other low pH environments will likewise benefit from this research.

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