COUPLED IRON REDUCTION-AMMONIUM OXIDATION (FEAMMOX) IN ALKALINE SOILS POLLUTED WITH NITROGEN

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ABSTRACT

Although nitrogen fertilizers help stimulate plant and microbial growth in N-limited soils, the use of excess N fertilizers to improve agricultural yield in Canada can cause adverse side effects of groundwater and surface water pollution, greenhouse gas production, soil acidification, and human health issues. Two N-removal pathways currently used to treat N-polluted wastewater and groundwater include denitrification and anaerobic ammonium oxidation (anammox). This study explored a novel anaerobic N-removal pathway that converts ammonium (NH_4^+) to inert nitrogen gas (N_2) or nitrite (NO_2^-) while reducing Fe(III) to Fe(II), a.k.a. iron ammonium oxidation (Feammox) via a 118-day anaerobic incubation which included four sequential biostimulation experiments. The goal of the incubation was to identify Feammox in neutral-alkaline soil samples from a N-polluted site in Alberta by stimulating the bioremediation of NH_4^+. This was done amending the soils with vitamins and sources of NH_4^+ and Fe(III). The treatments for the anaerobic controls and soil slurries included one or more of the following: ammonium chloride (A), 2-line ferrihydrite (FH), and ferric citrate (FC). Amendments were added to these treatments in four sequential Feammox biostimulation experiments: 1) FC, FH, and A, 2) FC and FH, 3) FC and A, and 4) vitamin and molybdate solutions. The soil slurry with ferric citrate and NH_4Cl amendments (S-FCA) had the most notable dissolved NH_4^+-N loss during the 118-day incubation, particularly when FC and A were added concurrently, i.e. a decrease of 14 ± 1.7 mg L^{-1} dissolved NH_4^+-N in the first experiment and a decrease of 13 ± 6.5 mg L^{-1} dissolved NH_4^+-N in the third experiment. S-FCA also exhibited signs of Fe(III) reduction throughout the incubation. In the incubation all samples generated minimal dissolved NO_2^- (0-2 mg L^{-1}). Following the 118-day incubation the S-FCA treatment was subcultured to reproduce results; however, the subcultures did not show notable NH_4^+ loss, possibly due to dilution or N mineralization. Overall, this study showed a correlation between concurrent ferric citrate and NH_4Cl amendments and dissolved NH_4^+-N loss in near-neutral anaerobic conditions; however, it did not provide clear evidence of Feammox. Additional experiments are necessary to isolate Feammox.

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LIST OF ABBREVIATIONS

A	Ammonium
A6	*Acidimicrobiaceae* sp. A6
Anammox	Anaerobic ammonium oxidation
AOA	Ammonia-oxidizing archaea
AOB	Ammonia-oxidizing bacteria
AQDS	9,10-anthraquinone-2,6-disulfonate
ATP	Adenosine Triphosphate
DIRB	Dissimilatory Iron reducing bacteria
DNRA	Dissimilatory nitrate reduction to ammonium
E_{env}	Environmentally relevant midpoint potential
E_h	Reduction potential or redox potential
E_m	Midpoint potential
ESA	Environmental site assessment
FC	Ferric citrate
Feammox	Iron ammonium oxidation
FH	Ferrihydrite
GHG	Greenhouse gas
IRB	Iron reducing bacteria
Kp	*Klebsiella pneumoniae*
LAB	Lactic acid bacteria
Mnammox	Manganese ammonium oxidation
NADH	Nicotinamide adenine dinucleotide + hydrogen
NDFO	Nitrate/Nitrite dependent Fe(II) oxidation
NPK	Nitrogen Phosphorous Potassium
NS	No soil
OC	Organic carbon
OM	Organic matter
OTU	Operational taxonomic unit
PCE	Tetrachloroethylene

PFAS	Per- and polyfluoroalkyl substances
RSN	Residual soil nitrogen
S	Soil
SEM	Scanning electron microscope
SND	Simultaneous Nitrification-Denitrification
SOM	Soil organic matter
TCE	Trichloroethylene
UV/Vis	Ultraviolet-visible spectroscopy
WWTP	Wastewater treatment plant
XRD	X-ray diffraction

1. General Introduction

Agricultural production is a complex issue because feeding the global population of humans and livestock creates numerous environmental and health concerns. Some major concerns are rising water consumption, greenhouse gas (GHG) emissions, soil degradation and loss, and pollution in surface water and groundwater. Nitrogen fertilizers introduce nitrite (NO_2^-), nitrate (NO_3^-), and ammonium (NH_4^+) to soil that contribute to the latter three issues. Plants and soil microbiota require N to form essential molecules; therefore, adding N to soil typically increases yield and enriches soil organic matter in N-limited environments. However, aggressive N fertilization and loss, or leaching of soil N interferes with potential benefits. Remaining, or residual soil N, can leach into groundwater as NO_2^- and NO_3^-, erode into water bodies via runoff, or be transformed into harmful GHGs like nitrous oxide (N_2O). Furthermore, NH_4^+ threatens freshwater life due to its equilibrium with toxic ammonia gas (NH_3). Excess NO_2^- and NO_3^- in drinking water are linked to several serious human health issues.

Between 1981 and 2011, average N inputs into Canadian soils have nearly doubled while average N outputs from soil have increased by 1.6x (Agriculture and Agri-food Canada, 2020b). The N emissions from fertilized land have doubled, and 35% of NH_3 emissions are attributed to fertilizers (Agriculture and Agri-food Canada, 2020a). Therefore, the residual soil N in Canadian soils has increased by approximately 2.5x in 30 years. As of 2011, the Canadian prairie provinces of Saskatchewan and Alberta were responsible for 85% of fertilized land in Canada; therefore, the concerns associated with N pollution are relevant in these areas.

In 2016, a N fertilizer storage and distribution site in Lomond, Alberta was assessed for groundwater and soil pollution that may pose an environmental risk. The assessment concluded that levels of groundwater ammonia-N, NO_2^-, and NO_3^- exceeded Alberta Tier 1 Guidelines for Commercial, Residential and Agricultural Land Use using fine-grained criteria (Nichols Environmental, 2018; Government of Alberta, 2017). It is important to remedy this N pollution issue at the site. Standard treatment methods for excessive soil N include digging and dumping

polluted soil in a landfill, and standard treatments for groundwater N include installing a permeable reactive barrier that both intercepts and treats groundwater or treating wastewater in a wastewater treatment plant. A popular bioremediation technique used in these technologies is denitrification, which is a heterotrophic pathway that converts NO_2^- and/or NO_3^- into inert nitrogen gas (N_2) that comprises 78% of the atmosphere, but can produce GHG byproducts like N_2O. Therefore, it is worthwhile to explore alternate bioremediation pathways that generate N_2 without generating GHGs.

Iron ammonium oxidation (Feammox) is an anaerobic bioremediation pathway where bacteria utilize Fe(III), a ubiquitous and abundant terminal electron acceptor in anaerobic conditions, to oxidize NH_4^+, thus generating NO_2^- and ideally N_2. Feammox research implies that the pathway is most effective in acidic soils containing sufficient inorganic carbon (C), ferrihydrite as an Fe(III) source, and NH_4^+. In addition, organic C may enhance Feammox efficiency but is not a requirement. The soils and groundwater at Lomond are Fe- and NH_4^+-rich, so Feammox may already occur on site. However, the alkalinity (pH 7-9) of the site's soil and groundwater impose thermodynamic constraints related to Fe solubility and reduction potential, indicating that Feammox may be less efficient in site soils than in acidic soils. Stimulating Feammox could nevertheless enhance N_2 outputs to mitigate the N pollution risk at Lomond. Due to potential constraints imposed by the alkalinity, it is worthwhile to treat the soil with different Fe(III) sources and NH_4^+ to assess Feammox potential in neutral to alkaline conditions.

Soils were selected from the Lomond fertilizer site and incubated anaerobically for 118 days to determine which amendments stimulate Feammox, including ammonium chloride, 2-line ferrihydrite, and ferric citrate. Previous work showed successful Feammox with NH_4Cl and 6-line ferrihydrite amendments, and only Fe reduction when ferric citrate and NH_4Cl were amended to anaerobic soil slurries. Therefore, the hypothesis for the anaerobic 118-day incubation is that ferrihydrite will stimulate Feammox, and not ferric citrate. The Feammox rate, if discovered, was expected to be lower than previously seen in acidic soils, given the Fe(III) solubility and thermodynamic constraints in alkaline conditions. If Feammox is isolated in the Lomond soils, or if NH_4^+ oxidation can otherwise be stimulated on Lomond soils, this may influence future bioremediation *in situ*, and the most successful treatment could be applied to other N-polluted alkaline soils globally.

2. LITERATURE REVIEW

2.1 Nitrogen Fertilizers and Management

2.1.1 Nitrogen fertilizer use and associated risk in Canadian soils

In cropland, N often originates from commercial fertilizers and manure. Nitrogen-Phosphorous-Potassium (NPK) fertilizers are applied to improve plant growth and soil organic matter (SOM) inputs (Poffenbarger et al., 2018). Nitrogen fertilizer contents vary, but typically are a direct source of ammonium (NH_4^+), ammonia (NH_3), nitrate (NO_3^-), and indirect source of nitrite (NO_2^-). Manure, urea, and uric acid excreted by livestock and poultry are also sources of NH_4^+ and NH_3 (Agriculture and Agri-Food Canada, 2020a). Globally, N fertilizers are the largest source of N input in cropland, with the goal of improving crop yields in N-limited environments (Poffenbarger et al., 2018). Abundant N fertilization relates to economic necessity, food production in N-limited areas, and the low efficiency of N uptake globally.

The mechanisms by which N fertilization affects yield varies with soil type, vegetation type, and fertilizer type and rate. There is a limit to the positive effects of N fertilizers on yield (Sainju et al., 2019). Plants have an N uptake limit and residual fertilizer N can be detrimental to soil and plant health. The average N fertilizer uptake by plants is 50% (Zhaohui et al., 2012), and 33% for cereal grains (Delgado et al., 2002). This relationship is also known as N-use efficiency, which is the crop yield, or N uptake, per unit of applied N fertilizer (Sainju et al., 2019). The leftover, or residual N typically sorbs to the soil or is lost from the rooting zone via water or to the atmosphere (Poffenbarger et al., 2018) For example, NH_4^+ can sorb to organic matter (OM) and mineral surfaces, or volatilize into gaseous NH_3 which influences aquatic health and atmosphere conditions, while NO_2^- and NO_3^- leach into groundwater, and nitrogen gas (N_2) and nitrous oxide (N_2O) can enter the atmosphere.

In Canada, the N fertilizer usage has increased in modern history. In Canada, N losses and residual soil N are quantified on a local scale with empirical methods and estimated on a

national scale. Residual Soil N (RSN) is a method of estimating how efficiently soil N is used (Agriculture and Agri-Food Canada, 2020b). The RSN amount is calculated as the difference between N inputs into agricultural soils (fertilizers, N fixation by legumes, and atmospheric deposition) and the N outputs from agricultural soils (crop harvests, gaseous N loss) (Agriculture and Agri-Food Canada, 2020b). Between 1981 and 2011, average N inputs nearly doubled, from 44 kg/ha to 81 kg/ha while average N outputs increased from 35 kg/ha to 57 kg/ha (Agriculture and Agri-Food Canada, 2020b). Therefore, RSN values increased from 9 kg/ha in 1981 to 24 kg/ha in 2011 (Agriculture and Agri-Food Canada, 2020b). Although the RSN may support the following year's crop, or be lost from the soil via water or the atmosphere (Agriculture and Agri-Food Canada, 2020b). As of 2011, the RSN N performance index in Canadian soils was at a moderate risk level, indicating that accumulated N is a growing concern (Agriculture and Agri-Food Canada, 2020b).

In 2011, 25 million hectares in Canada were fertilized with commercial fertilizers (Dorff and Beaulieu, 2014). As of 2010, Alberta and Saskatchewan were responsible for 85% of fertilized areas in Canada (Dorff and Beaulieu, 2014). Although the national risk of N contamination in Canada is low, due to the drier climate and low drainage in these Canadian prairies, as of 2011 some areas of Canada are considered to have high to very high risk of N contamination in water (Agriculture and Agri-Food Canada, 2020b). In particular, there is contamination concern in fertilized areas of the Central and Atlantic regions which experience high precipitation (Agriculture and Agri-Food Canada, 2020b).

Nitrogen emissions from fertilized land in Canada have increased from 63,000 to 130,000 kilotons N between 1981 and 2011 (Agriculture and Agri-Food Canada, 2020a). Agricultural NH_3 emissions are measured and/or estimated as one method of indicating negative environmental and human health impacts of excess N (Agriculture and Agri-Food Canada, 2020a). Between 1981 and 2011, NH_3 emissions increased in the Canadian prairies due to increased fertilization and expansion of cropland relative to livestock grazing areas. National NH_3 emissions have increased from 333,000 to 370,000 tons between 1981 and 2011, and 35% of the 2011 NH_3 emissions were attributed to fertilizers (Agriculture and Agri-Food Canada, 2020a).

Excessive N from annual soil fertilization can acidify and damage soil. The breakdown of N fertilizers releases protons via nitrification and ammonium hydrolysis (Barak et al., 1997). In

addition, nitric acid (HNO_3) can accumulate in aerobic soils (Barak et al., 1997). Left untreated, continued fertilizer use can result in loss of base cations, enhanced mineral weathering, and loss of cation exchange capacity (Barak et al., 1997). These changes reduce soil nutrient retention and plant access to nutrients, as well as render toxic nutrients bioavailable (Barak et al., 1997). Therefore, excess N fertilization can decrease soil quality.

The concern associated with NH_4^+ and NH_3 in soil, surface water, and groundwater is due to the equilibrium between these N-species. The equilibrium primarily depends on changes in pH and temperature, where in alkaline conditions and higher temperatures NH_4^+ can readily volatilize to NH_3 gas (CCME, 2010; Government of Alberta, 2019) and at low pH and low temperature, NH_3 is protonated, generating NH_4^+. Although neutral NH_3 is useful for plants and microbes because it is readily assimilated (Burkovski, 2003), it can be problematic because it is highly soluble in water and very toxic to aquatic life (Government of Alberta, 2019) because it easily diffuses through biological membranes (CCME, 2010). Conversely, ionic NH_4^+ is not toxic to aquatic organisms (CCME, 2010); however, it poses further risk due to this equilibrium and because it can sorb to clay surfaces (Sieczka and Koda, 2016), active fractions of OM (Wen-Zhao et al., 2013) and other cation exchange sites in soil, as well as forming complexes with metal ions and sorbing to suspended sediment in aquatic environments (CCME, 2010). Due to the sensitivity to volatilization into NH_3, NH_4^+ continuously poses a threat to aquatic life.

To account for the NH_3-NH_4^+ equilibrium, environmental assessments typically include guidelines for "total ammonia" or "total ammonia-N" as a sum of both compounds (NH_3 + NH_4^+) (CCME, 2010). Canadian water quality guidelines for total ammonia-N are stricter in high pH and higher temperature waters as seen in the Canadian Council of Ministers of the Environment (CCME) (2010) guidelines (Table 2.1). With every unit pH increase, the NH_3 concentration can increase by tenfold, and each 5°C increase can result in a 40-50% increase in NH_3 (CCME, 2010). Two equations are used by the CCME to assess the equilibrium in aquatic environments where T=temperature in K, f = fraction of total un-ionized ammonia, and pKa is the dissociation constant (Eq. 1-2).

(1) $pKa = 0.0901821 + 2729.92/T$
(2) $f = 1/[10^{(pKa-pH)} + 1]$

Table 2.1. Water quality guidelines for total ammonia-N ($NH_3 + NH_4^+$) (mg L^{-1}) for the protection of aquatic life in freshwater at a given temperature and pH in Canada. Guidelines are stricter at higher pH and temperature as the NH_3-NH_4^+ equilibrium favors NH_3 (Source: CCME, 2010)

Temp (°C)	**pH**							
	6.0	*6.5*	*7.0*	*7.5*	*8.0*	*8.5*	*9.0*	*10*
0	231†	73	23.1	7.32	2.33	0.759	0.25	0.042
5	153	48.3	15.3	4.84	1.54	0.502	0.172	0.034
10	102	32.4	10.3	3.26	1.04	0.343	0.121	0.029
15	69.7	22.0	6.98	2.22	0.715	0.239	0.089	0.026
20	48.0	15.2	4.82	1.54	0.499	0.171	0.067	0.024
25	33.5	10.6	3.37	1.08	0.354	0.125	0.053	0.022
30	23.7	7.50	2.39	0.767	0.256	0.094	0.043	0.021

† Values are in mg L^{-1} NH_3 and can be converted to total ammonia-N (mg L^{-1}) by multiplying by 0.8224.

Nitrogen fertilizer runoff and infiltration introduces NO_3^- and NO_2^- into surface water and groundwater reservoirs. Nitrate salts, HNO_3, and NO_3^- are highly mobile and soluble, exacerbating this issue (CCME, 2012). Surface water bodies are often N-limited, so leached N can stimulate excessive algal growth, leading to death and hypoxia in a process known as eutrophication. Although NO_3^- is less toxic than NH_3 or NO_2^- (CCME, 2012), excessive NO_3^- poses a risk to aquatic organisms, particularly if converted to NO_2^- or NH_3 (Government of Alberta, 2019). Overall, excess N decreases regional water quality (Poffenbarger et al., 2018).

Excessive N fertilization also affects climate regulation of gases and associated human health. For example, there is risk associated with volatilized NH_3 in the atmosphere. The NH_3 can react with acidic gases in the atmosphere, generating NH_4^+ compounds classified as fine particulate matter which contributes to smog formation and is a harmful pollutant when ingested by humans (Agriculture and Agri-Food Canada, 2020a). Furthermore, smog contributes to the greenhouse gas effect. Another risk includes denitrification pathways which can produce NO and N_2O (Poffenbarger et al., 2018), with the former influencing acid precipitation, ozone formation, and ozone destruction and the latter a GHG that contributes to climate change (Oertel et al., 2016). As soil temperature increases up to 37°C, so do N_2O emissions, thus exacerbating the climate change effect (Oertel et al., 2016). Globally, soils are responsible for the following proportions of annual GHG emissions: 35% CO_2, 47% CH_4, and 53% N_2O (Oertel et al., 2016). Overall, excess N is associated with decreased air quality.

Nitrogen pollution also threatens human life because excess NO_2^- and NO_3^- are considered pollutants in drinking water. Furthermore, oxidation-reduction pathways that convert NO_3^- to NO_2^- or NO_2^- to NO_3^- increases the risk associated with both ions. The NO_2^- molecule is highly reactive and its ingestion has been associated with cancer, birth defects, and immune system changes (Fewtrell, 2004). Ingesting excess NO_2^- can cause methemoglobinemia, a condition where NO_2^- reduces blood's O_2 carrying capacity (CCME, 2012). This limited O_2 transport to body tissues can result in blue skin and even death, especially in infants. Elevated NO_3^- in drinking water has been linked to cancer, reproductive issues, diabetes, and thyroid conditions (Ward et al., 2005) and elevated nitrate salts can interfere with osmoregulation in aquatic animals (CCME, 2012). In remediation efforts, NO_3^- and NO_2^- are assessed as soil and water pollutants to determine guidelines for environmental and human health. Most toxicity assessments involve aquatic organisms with varying sensitivities to NO_3^- and NO_2^- (CCME, 2012). Guidelines for environmental and human health are expressed in terms of nitrate-N, nitrite-N, or nitrate+nitrite-N in mg L^{-1} (Government of Alberta, 2019). Overall, NH_3 and NH_4^+ are a concern in aquatic environments and in the atmosphere. Furthermore, their transformation into other N-species via the N cycle influences their mobility and risk level, e.g. NO_2^- and NO_3^- as water pollutants, N_2 as a safe and inert gas, and N_2O as a GHG.

2.1.2 Nitrogen, carbon, and microbe metabolism

The connections between the C and N cycles and microbial metabolism are important for understanding the N cycle and N bioremediation. Nitrogen is essential for protein development and other cellular processes in microbes, while C is an important source of energy and/or growth (Jurtshuk Jr., 1996). Metabolism is a term used to describe all biochemical reactions within a cell, such as assimilation reactions, energy-yielding, and energy-requiring reactions (Jurtshuk Jr., 1996). The main metabolic strategies differ based on the energy source (heterotrophic versus autotrophic), O_2 presence, and what is used as a terminal electron acceptor. In soil, OM is the primary electron source (DeLaune and Reddy, 2005), and energy source for microbial activity. Heterotrophic metabolism occurs in microbes that cannot perform C fixation, and therefore organic C (OC) from other life is their main source of nutrients, energy, and growth, i.e. they cannot produce organic compounds from inorganic compounds but can consume reduced C

(Jurtshuk Jr., 1996). Three heterotrophic pathways in soils are aerobic respiration, anaerobic respiration, and fermentation (Jurtshuk Jr., 1996).

Aerobic respiration is a form of heterotrophic metabolism where an organic compound/substrate, such as glucose, is oxidized, generating energy for the microbe, H_2O, and reduced C such as CO_2 (Jurtshuk Jr., 1996). For example, heterotrophic denitrifying bacteria can perform aerobic and anaerobic respiration with organic compounds (Jetten, 2008; Yang et al., 2012; Kamp et al., 2019; Yang et al., 2020) to generate energy in the form of adenosine triphosphate (ATP) as well as simple organic compounds for assimilation and/or biosynthesis (Jurtshuk Jr., 1996). To access N heterotrophs can break down proteins and/or amino acids in the OM and generate NH_3 via ammonification (Jurtshuk Jr., 1996). Ammonification is one aspect of mineralization, a process where OM decomposes into inorganic material (Jurtshuk Jr., 1996). Immobilization is the inverse of mineralization, and is the act of incorporating, or assimilating N into biomass (Barret and Burke, 2000). Autotrophic metabolism (a.k.a. chemoautotrophy, chemolithotrophy, and chemotrophy) allows bacteria to grow via the use of inorganic compounds such as CO_2, and therefore involves CO_2 fixation and (Jurtshuk Jr., 1996). Here, inorganic compounds are oxidized, yielding energy needed for assimilating CO_2 to generate glucose for biosynthesis (Jurtshuk Jr., 1996). Autotrophs gain N from inorganic NH_3, NO_3^-, and N_2 (Jurtshuk Jr., 1996).

Anaerobic respiration and fermentation allow microbes to decompose OM in anaerobic conditions. Aerobic respiration uses O_2 as the electron acceptor, which is the most favorable electron acceptor for OM oxidation (DeLaune and Reddy, 2005). Conversely, in anaerobic conditions less thermodynamically favorable electron acceptors are available for OM oxidation via anaerobic respiration, and are expressed as a hierarchy of least reduced to most reduced (electron acceptor → product): NO_3^- → N_2 followed by Mn(IV) → Mn^{2+}, Fe(III) → Fe^{2+}, sulfate (SO_4^{2-}) → hydrogen sulfide (H_2S), and methanogens CO_2 → methane (CH_4) (Beck and Mann, 2010; DeLaune and Reddy, 2005). In anaerobic conditions thermodynamic favorability often dictates OM decomposition rate because the energy required to perform decomposition may outweigh the energy gained by the microbe (DeLaune and Reddy, 2005).

Fermentation, anaerobic respiration, and aerobic respiration all begin with a step where a carbohydrate is broken down, but differ in subsequent steps (Jurtshuk Jr., 1996). What differentiates aerobic from anaerobic respiration is whether O_2 (aerobic) or another terminal

electron acceptor (e.g. NO_3^-, SO_4^{2-}, and CO_2) is used (anaerobic). Fermentation involves the oxidation of organic molecules and what differentiates it is the generation of waste products like acid or alcohol (Jurtshuk Jr., 1996). Fermenters can be subdivided into homofermenters and heterofermenters, with the former producing lactic acid from glucose fermentation, while the latter can produce multiple end products like acetic acid, ethanol, H_2, CO_2, and formic acid from glucose (Jurtshuk Jr., 1996). Typically, different fermenter species generate different end products and fermentation generates less ATP than respiration (Jurtshuk Jr., 1996).

As mentioned, N and C assimilation reactions are an important component of metabolism, and the C and N released from substrate decomposition (mineralization) is important for heterotrophic growth (Jurtshuk Jr., 1996). All biota must maintain an essential C:N ratio. Therefore, the C:N ratio of degradable biomass is crucial to decomposition rates, C:N balance in soils and microbes, and other microbial activity (Kuzyakov et al., 2000; Barrett and Burke, 2000). A material with a higher C:N ratio (more C than N) will take longer to decompose because the energy demand to decompose the material outweighs the N gained by the microbe (Barrett and Burke, 2000). Conversely, adding OM with a low C:N ratio (easily decomposable organic substrate) can rapidly satisfy microbial N needs (via immobilization) and stimulate population growth, thus increasing C and N needs, which stimulates microbial decomposition of other material with a low C:N ratio (e.g. SOM), in a process known as priming (Kuzyakov et al., 2000). Priming can degrade long-term soil N and C stores and may enhance leaching of mobile components (e.g. NO_3^-) (Kuzyakov et al., 2000). Soil fertilization also influences mineralization, immobilization, and priming (Kuzyakov et al., 2000). Adding commercial N fertilizers (typically a very low C:N ratio) stimulates N immobilization via plants and microbes, which can enhance SOM mineralization to satisfy C and N needs (Kuzyakov et al., 2000).

2.1.3 Microbial nitrogen cycling

To be used by most biota atmospheric N_2 must be converted to NH_3 via natural processes or the Haber-Bosch process (Poffenbarger et al., 2018). Natural fixation is carried out by N-fixing rhizobia (Denison and Kiers, 2004), N-fixing lichens (Marks et al., 2015), and N-fixing cyanobacteria (Issa et al., 2014; Marks et al., 2015). Rhizobium bacteria fix N in root nodules and exchange it for the plant's fixed C in a symbiotic relationship (Denison and Kiers, 2004). Rhizobia can also live independently in the soil and fix N nonsymbiotically, but this is less

common (Denison and Kiers, 2004). N-fixing lichens (cyanolichens) are important in wet forest environments and arctic environments and can participate in bipartite symbiosis with cyanobacteria and fungus, as well as tripartite symbiosis (Marks et al., 2015). Certain cyanobacteria can fix N in aerobic or anaerobic aquatic environments (Issa et al., 2014).

Bacteria that cannot fix N can assimilate biologically fixed N and/or fertilizer N. The Haber Bosch process synthesizes inorganic NH_3 via a reaction between H_2 and N_2 that is sped up with a catalyst (CCME, 2010) (Eq. 3) in a pressurized system (Mitsushima et al., 2018) at high temperatures (~600°C) (CCME, 2010). The NH_3 is primarily used in fertilizer manufacturing, e.g. in ammonium nitrate, ammonium phosphate, urea, and ammonium sulphate fertilizers (CCME, 2010). The fertilizer anhydrous ammonia is sold as a pressurized liquid that is injected directly into soils and quickly volatilizes into NH_3 gas (CCME, 2010), rendering it accessible to plant roots and the soil microbial community.

(3) $3H_2\,(g) + N_2\,(g) = 2NH_3\,(g)$

The process of assimilation converts inorganic N (e.g. NH_3, NO_3^-, and NO_2^-) into organic N that microbes can incorporate (Herrero et al., 2018) into cellular components like proteins, nucleic acids, and cell wall components (Burkovski, 2003). This may occur via assimilatory reduction, dissimilatory reduction (Herrero et al., 2018), or dissimilatory nitrate reduction to ammonium (Sparacino-Watkins, 2014). Due to its neutral nature, bacteria prefer to assimilate NH_3 which easily diffuses into the cytoplasm (Burkovski, 2003). When NH_3 is limited, bacteria use other forms of inorganic N (e.g. NO_3^-, NO_2^-) or organic N sources that may require more energy to assimilate (Herrero et al., 2018). The NO_3^- is first reduced to NO_2^- and then NH_3 (Herrero et al., 2018). Organic N is rendered accessible via mineralization, which is when organic material (e.g. plant material, microbes) decomposes, causing the conversion of organic N (e.g. amines, amides) into available inorganic N (e.g. NO_3^- and NH_4^+ (via ammonification)) (Barak et al., 1997).

Within the N cycle, NH_4^+ can undergo a 2-step process of nitrification: 1) aerobic ammonium oxidation followed by 2) aerobic nitrite oxidation. In aerobic ammonium oxidation, NH_4^+ is oxidized by ammonia-oxidizing bacteria (AOB) (typically *Nitrosomonas*) (Cabello et al., 2009) and/or ammonia-oxidizing archaea (AOA) (Gonzalez-Martinez et al., 2018). The O_2-

dependent hydroxylation of NH_3 produces hydroxylamine (NH_2OH) which is oxidized to nitric oxide (NO) and then NO_2^- (Caranto and Lancaster, 2017). During aerobic nitrite oxidation the NO_2^- is oxidized to NO_3^- (Jetten, 2008), which is catalyzed by nitrite oxidizing bacteria (NOB) (Daims et al., 2016). *Nitrobacter* species are well known NOB that oxidize NO_2^- for energy and use NO_2^- as an electron source in CO_2 fixation for growth (Cabello et al., 2009).

The process of NH_4^+ oxidation also occurs in anaerobic conditions. Some autotrophic bacteria perform anaerobic ammonium oxidation (anammox) and are known as anammox bacteria (Huang et al., 2014; Gonzalez-Martinez et al., 2018). In the natural environment anammox may be responsible for 30-50% of N removal in marine sediments (Tomaszewski et al., 2017) and 67% of N_2 production in marine and freshwater ecosystems (Yang et al., 2012). Anammox occurs inside their membrane-bound anammoxosome compartment where enzymes catalyze coupled NH_4^+ oxidation-NO_2^- reduction to yield N_2, with NO, hydrazine (N_2H_4), and NH_2OH as intermediates (Gonzalez-Martinez et al., 2018). Although N_2H_4 accumulation is toxic to anammox bacteria, the N_2H_4 is confined within the anammoxosome membrane, preserving the anammox reaction (Gonzalez-Martinez et al., 2018). Anammox bacteria perform CO_2 fixation as a central component of their growth, and the proton consumption causes a local pH increase (Gonzalez-Martinez et al., 2018). The anammox reactions are available in Eqs. 4-6, where NO_2^- is reduced to NO via nitrite reductase (Eq. 4), the NO combines with NH_4^+ into N_2H_4 via hydrazine hydrolase (Eq. 5), and the N_2H_4 is oxidized to N_2 via hydrazine/hydroxylamine oxidoreductase (Eq. 6) (Gonzalez-Martinez et al., 2018). In environments where anammox occurs, anaerobic NO_2^- oxidation can generate some NO_3^- (Gonzalez-Martinez et al., 2018).

(4) $NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O$
(5) $NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O$
(6) $N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$

Another pathway in the N cycle is anaerobic respiration by heterotrophic bacteria where NO_3^- is reduced to NH_4^+, a.k.a. anaerobic dissimilatory nitrate reduction to ammonium (DNRA) (Pilegaard, 2013). Since NO_3^- is the most oxidized form of N with an oxidation state of +5 (CCME, 2012), it is an important energy source for microbes. First NO_3^- is reduced to NO_2^- via nitrate reductase, then NO_2^- is reduced to NH_4^+ via nitrite reductase while oxidizing OM (Bu et al., 2017), which produces energy. DNRA typically dominates in high temperature, electron

donor-rich sediments (Bu et al., 2017). Excess NO_2^- is toxic to the cell, and DNRA helps with detoxification (Sparacino-Watkins, 2014) by minimizing NO_2^- stored in the cell (Bu et al., 2017).

Denitrification is a heterotrophic process which can occur in mixed aerobic and anaerobic conditions and can produce atmospheric N_2 (Kamp et al., 2019). Denitrification involves the reduction of NO_3^- and/or NO_2^- to NO, N_2O, and/or N_2 (Shapleigh, 2009). In aerobic conditions NO_3^- and O_2 are available electron acceptors, with O_2 being the preferred electron acceptor for aerobic respiration. In aerobic conditions aerobic denitrifiers simultaneously use NO_3^- and O_2 as electron acceptors during co-respiration (Yang et al., 2020). Anaerobic denitrification involves sequential reduction of N-species, first NO_3^- is reduced to NO_2^- via nitrate reductase, then nitric oxide NO via nitrite reductase, NO into N_2O via nitric oxide reductase (Yang et al., 2020), and finally N_2O to N_2 via nitrous oxide reductase (Jetten, 2008; Yang et al., 2012; Yang et al., 2020). In anaerobic conditions, bacteria that perform DNRA and denitrifying bacteria compete for NO_3^- (Bu et al., 2017). Two examples of denitrification are represented, the sequential reduction series (Eq. 7) and a complete denitrification redox reaction (Eq. 8) (Bernhard, 2010).

(7) $NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$
(8) $2NO_3^- + 10e^- + 12H^+ \rightarrow N_2 + H_2O$

Although biotic denitrification is often addressed, abiotic chemodenitrification, or chemical denitrification by ferrous iron (Fe(II)) is a lesser known pathway in the N cycle (Jamieson et al., 2018). Here, chemical NO_2^- reduction is coupled to Fe(II) oxidation in anaerobic conditions to produce ferric (oxyhydr)oxide minerals along with NO and N_2O (Eq. 9). This pathway could be responsible for substantial Fe(II) oxidation and NO_2^- consumption in anoxic and sterile conditions, or can occur as an extracellular process (Jamieson et al., 2018).

(9) $4Fe(II) + 2NO_2^- + 5H_2O \rightarrow 4FeO(OH) + N_2O + 6H^+$

2.2 Wastewater Treatment of Nitrogen Pollution

Denitrification is the most common bioremediation method for N in wastewater treatment plants (WWTPs). In WWTPs the N treatment occurs in a 2-step nitrification-denitrification process that involves multiple treatment tanks within a reactor. In the initial aerobic nitrification

step, wastewater is pumped into a tank and the NH_4^+/NH_3 is oxidized to NO_2^- via AOB and then NO_3^- via NOB (Kamp et al., 2019). Aerobic nitrification may also generate N_2O (Oertel et al., 2016). The resultant wastewater is transferred to a secondary tank where anaerobic denitrification generates N_2 (Kamp et al., 2019) or N_2O (Tomaszewski et al., 2017) in the case of incomplete denitrification. In the initial nitrification step, the aeration (Kamp et al., 2019) and energy use can be expensive (Ruiz-Urigüen et al., 2019). Furthermore, if the wastewater has a low C:N ratio (Yang et al., 2018), the denitrification step may require sludge or methanol as a biodegradable inorganic C source, which increases cost (Kamp et al., 2019). Another problem is the potential generation of N_2O, a GHG.

Anammox is a new, less common technology in WWTPs relative to denitrification. As of 2015, over 114 anammox WWTP full-scale reactors have been implemented (Kamp et al., 2019). Autotrophic anammox generates N_2 anaerobically, thus reducing aeration and methanol costs associated with denitrification in WWTPs (Kamp et al., 2019) and reducing N-removal costs by up to 60% (Tomaszewski et al., 2017). Anammox does not produce N_2O, reducing GHG emissions relative to denitrification (Tomaszewski et al., 2017). However, anammox is less efficient than traditional denitrification in WWTPs. The anammox bacteria colonies are slow growing, with replication times of 11-22 days and low biomass yields (Gonzalez-Martinez et al., 2018). Furthermore, anammox is sensitive to temperature, oxygen, substrate concentration, and pH, being most active at 25-40°C (Kamp et al., 2019) and pH 6.7-8.3 conditions (Tomaszeski et al., 2017), which adds to operating costs. Buildup of excess N_2H_4 (Kamp et al., 2019) and NO_2^- (Gonzalez-Martinez et al., 2018) also inhibits anammox activity. Due to these limitations, anammox reactors may not completely replace denitrification reactors in WWTPs.

2.3 Iron Chemistry and Microbial Iron Assimilation

Iron (Fe) is an essential micronutrient and is required in most enzymes (Schröder et al., 2003). Ferric iron (Fe(III)) is an important terminal electron acceptor (Bird et al., 2011) that is ubiquitous in soil and ferrous iron (Fe(II)) is an abundant electron donor in acidic and anaerobic conditions (Schröder et al., 2003; Bird et al., 2011). However, Fe chemistry is often limited by thermodynamics and/or kinetics (Bird et al., 2011), especially in heterogeneous soil environments. The likelihood of reduction indicated by the species' redox or reduction potential (E_h), which is the potential to gain electrons and is measured in volts, with a higher value

favoring reduction and lower value favoring oxidation (DeLaune and Reddy, 2005). For example, the E_h of CO_2 is significantly lower than Fe^{3+} in anaerobic soils (DeLaune and Reddy, 2005). The pH, moisture, microbial activity, and complexing agents like OM influence Fe availability and redox activity (Schröder et al., 2003). The Fe(III) mineral solubility also dictates E_h, with more soluble phases having higher reduction potential (Bird et al., 2011). At neutral to alkaline pH, soluble Fe^{2+} readily oxidizes to less soluble Fe(III) (Schröder et al., 2003; Bird et al., 2011). The midpoint potential (E_m) is the point between reduction and oxidation (DeLaune and Reddy, 2005), and the E_m for Fe^{3+}/Fe^{2+} at pH 2 is about +0.77 V assuming that precipitation (occurs at pH < 3) does not occur (Bird et al., 2011). Conversely, at pH > 3, Fe(III) precipitates, making Fe oxidation more favorable (lowering the Em) (Bird et al., 2011).

Fe(III) reduction and Fe(II) oxidation have an important role in heterotrophic and autotrophic metabolism in aerobic and anaerobic conditions (Bird et al., 2011). Autotrophic Fe(II) oxidizers perform CO_2 fixation (Bird et al., 2011). Anaerobic respiration with Fe(III) reduces Fe(III) while oxidizing OC (Straub, 2011; Bird et al., 2011) and since the Fe^{3+}/Fe^{2+} Em is +0.77V at low pH, this redox reaction is favored in acidic conditions (Bird et al., 2011). Heterotrophic Fe reduction can also occur in neutral conditions, but Fe(III) is often in the solid/insoluble state which can limit bioavailability and E_h. (Bird et al., 2011). However, there are two methods for electron transfer between Fe(III) minerals and the bacteria's outer membrane to aid in neutral-alkaline Fe(III) reduction – electron shuttles and organic chelators. For example, microbes like anaerobic Fe-reducer *Shewanella* bacteria can colonize Fe(III) minerals and secrete flavins that act as electron shuttles (Bird et al., 2011) to transfer the electrons (Schröder et al., 2003; Bird et al., 2011). Organic chelators like citrate can bind tightly Fe(III), lowering its reduction potential, while other chelators bind tightly to Fe(II) (Bird et al., 2011). Siderophore molecules can also bind and transport Fe (Schröder et al., 2003). Furthermore, SOM further influences redox activity. Humic substances within SOM can chelate Fe^{3+} and render it bioavailable, can act as electron shuttles for Fe^{3+} oxides (Schröder et al., 2003), and the oxidized components of humic substances (e.g. quinone moieties) and reduced phenol moieties can undergo reduction and oxidation, respectively, (LaCroix et al., 2020) with Fe. Therefore, although Fe chemistry is constrained at neutral to alkaline conditions relative to acidic conditions, there are numerous mechanisms for enhancing Fe availability.

Lithotrophic (a.k.a. chemolithotrophic) microbes can oxidize Fe(II) while reducing O_2 or NO_3^- (Bird et al., 2011). They are grouped into facultative lithotrophs and lithoautotophs (a.k.a. chemoautotrophs) (Keenleyside, 2019). Chemical/abiotic Fe(II) oxidation occurs rapidly in the presence of O_2 at neutral pH, and can generate Fe(III) minerals (Bird et al., 2011). Therefore, lithotrophs typically perform Fe(II) oxidation in microaerobic or anaerobic conditions, or in very acidic conditions that minimize abiotic oxidation (Schröder et al., 2003; Bird et al., 2011). These metabolic pathways that involve Fe are important soil processes and influence Fe availability for microbial assimilation.

In regards to microbial use, microbes can only incorporate Fe^{2+} into cellular components (Schröder et al., 2003). In acidic conditions Fe^{3+} is more bioavailable and can be directly transported into a cell and reduced by ferric reductase enzymes (Schröder et al., 2003). At neutral to alkaline pH this Fe uptake requires more energy and the use of assimilatory ferric reductases (Schröder et al., 2003). At near neutral to alkaline pH, microbes may secrete the metabolic intermediate citrate, or siderophores that chelate Fe^{3+} (Schröder et al., 2003). Then assimilatory ferric reductases help take in and reduce that citrate- and/or siderophore-chelated Fe^{3+}, and the Fe^{2+} is incorporated into Fe-containing proteins (Schröder et al., 2003). These enzymes are found in all known biota excluding some lactic acid bacteria (LAB) (Schröder et al., 2003). Another class of ferric reductases, dissimilatory ferric reductases, are active in dissimilatory iron reducing bacteria (DIRB) respiration, which yields energy that stimulates population growth (Schröder et al., 2003). Here, inorganic Fe^{3+} precipitates or complexed Fe^{3+} are reduced, generating bioavailable Fe^{2+} for microbes (Schröder et al., 2003). Dissimilatory reduction consumes substantially more Fe(III) than assimilatory reduction (Ehrlich et al., 2015).

The relationship between Fe and soil C can in some ways be compared to C:N ratios. Similar to how a soil amendment's C:N ratio influences and OM protection, adding a labile material with a high C:Fe ratio can trigger priming (Adhikari et al., 2017). To understand this relationship it is important to note that aerobic conditions can favor Fe(II) oxidation and respective OM stability. Fe(II) oxidation can generate minerals and Fe(III) precipitates, e.g. via chemical binding with OM to form mineral associated organic matter (MAOM) followed by the formation of Fe oxyhydroxides that can precipitate with dissolved OM (Chen et al., 2020). MAOM formation is an important OM protection mechanism and can improve long term soil C storage, with Fe playing a crucial role in binding SOM together in aggregates, sorbing SOM, and

influencing electron transfer (Chen et al., 2020). However, due to the reactivity of Fe, the level of aggregation and O_2 presence heavily influences SOM vulnerability, with anaerobic conditions favoring Fe(III) reduction-OM oxidation and OM exposure and solubilization (Chen et al., 2020).

2.3.1 Ferric citrate

Ferric citrate ($C_6H_5FeO_7$) is a soluble Fe(III)-C complex (Ruiz-Urigüen et al., 2019). Ferric citrate is a family of compounds with different structures. Ferric citrate describes several complexes of Fe^{3+} and conjugate bases derived from citric acid ($C_6H_8O_7$) (Abrahamson et al., 1994), such as citrate ($C_6H_5O_7^{3-}$), $HC_6H_5O_7^{2-}$, and $H_2C_6H_5O_7^{-}$. Citric acid is a tricarboxylic acid (three carboxyl groups (-COOH) as well as one hydroxyl group (-OH)), and citric acid is more prevalent at pH 2-4, while citrate (one hydroxyl group and three carboxylate groups ($-COO^-$)) is more prevalent at pH ~7 or higher (Martell and Motekaitis, 1992). Citric acid is naturally found in the soil, plant exudates, and is produced by many microorganisms (Francis and Dodge, 1993). Ferric citrate is an important transporter and source of Fe for microbes via assimilatory ferric iron reductases (Schröder et al., 2003). In alkaline conditions, which render Fe less bioavailable, soil microbes can release citrate to extract Fe, and can assimilate that ferric citrate (Pierre and Gautier-Luneau, 2000). There may be specific ferric citrate reductases for assimilating ferric citrate, but the existence of these specific enzymes is debated (Schröder et al., 2003). Ferric reductases that can reduce ferric citrate have been isolated in some bacteria species (e.g. *R. sphaeroides, S. oneidensis, P. aeruginosa, L. pneumophila, P. islandicum*) (Schröder et al., 2003). The E_{env} of Fe(III)-citrate/Fe(II)-citrate at pH 7 is +0.385 V, which is lower than the Eh of Fe^{3+}/Fe^{2+} at pH 2 (+0.77 V) but higher than the E_{env} of solid ferrihydrite/Fe^{2+} at pH 7 (+0.1 to -0.1 V) (Bird et al., 2011). When Fe(III) binds strongly to citrate, this may decrease the E_h (Bird et al., 2011), and therefore bioavailability (Lipson et al., 2010).

The degradation of ferric citrate releases Fe into solution and corresponds to increased bacterial growth, access to Fe(III), and citrate usage (Schröder et al., 2003). The biodegradation tendency depends on the particular structure, with the free hydroxyl group playing a large role in binding (Francis and Dodge, 1993). Mononuclear bidentate complexes are readily biodegraded, but the mononuclear tridentate, binuclear, and polynuclear complexes with the hydroxyl of

citrate are not biodegraded unless first degraded into a bidentate form (Francis and Dodge, 1993).

2.3.2 Ferrihydrite

Ferrihydrite is a solid, poorly crystalline, thermodynamically unstable (Tang et al., 2016) oxyhydroxide (Zhou et al., 2016) that is the most bioavailable mineral for Fe-related microbial respiration due to its surface structure and active relationship with OM (Tang et al., 2016). Like other Fe-minerals, the bioavailability and reactivity of ferrihydrite is related to the soil pH, i.e. the E_h, or environmentally relevant midpoint potential (E_{env}) of solid ferrihydrite/Fe^{2+} at pH 7 is +0.1 to -0.1 V (Bird et al., 2011), thus limiting its reduction in neutral conditions. Ferrihydrite's bioavailability and reactivity is relatively unique in respect to other Fe-minerals because these factors are highly influenced by its formation conditions with OM that influences its structure, crystal size, and surface area.

The structure of the ferrihydrite mineral is debated (Eusterhues et al., 2008), but typically it includes Fe in tetrahedral and octahedral coordination (Vodyanitzkii and Shoba, 2016). Organic matter strongly influences ferrihydrite formation and reactivity (Vodyanitzkii and Shoba, 2016). Ferrihydrite develops in OM-rich soils (Vodyanitzkii and Shoba, 2016), usually via co-precipitation with OM (Cooper et al., 2017). Once formed, ferrihydrite has other important interactions with SOM because it contains pH dependent functional hydroxyl groups at the surface, which can sorb organic compounds (Eusterhues et al., 2008). Co-precipitation can influence the fate of the OM, because it can sorb to the ferrihydrite surface and remain active, or become occluded (via sorption) in the interstices of ferrihydrite crystals (Cooper et al., 2017). The presence of OM influences ferrihydrite particle size (Eusterhues et al., 2008; Vodyanitzkii and Shoba, 2016) by either increasing particle repulsion (producing smaller particles) or acting as bridging material (producing larger particles) (Adhikari et al., 2017). In Vodyanitzkii and Shoba (2016), microbes excreted polysaccharides that aggregated ferrihydrite particles, decreasing the specific surface area from 300 m^2/g in pure ferrihydrite to 40 m^2/g in ferrihydrite with organic polymers within its structure (Vodyanitzkii and Shoba, 2016). Ferrihydrite forms in variable conditions, and therefore it is rarely chemically pure in soils, usually containing oxyanion or cation admixtures that affect its sorptive capabilities and reactivity (Vodyanitzkii and Shoba, 2016).

Ferrihydrite can transform into other minerals like goethite and hematite depending on temperature, pH, OM, presence of Fe(II), and other conditions. The presence of Fe(II) can abiotically stimulate ferrihydrite's transformation into goethite (Adhikari et al., 2017) because soluble Fe(II) can adsorb to ferrihydrite and act as a reductant, stimulating reductive dissolution—the first step of goethite formation (Yee et al., 2006). Goethite is generated by the dissolution of ferrihydrite followed by nucleation and precipitation/crystallization, usually around pH 4 and 11 (Kukkadapu et al., 2003), or around pH 2-5 and 10-14 and low temperatures (Cudennac and Lecerf, 2006). Typically pH of ~7 means H_3O^+ and OH^- concentrations are too weak to dissolve enough ferrihydrite to form goethite (Cudennac and Lecerf, 2006). Conversely, hematite formation is favored in Fe(II)-free environments (Adhikari et al., 2017), where ferrihydrite dehydration and internal atomic rearrangement produces hematite at a pH <8 (Kukkadapu et al., 2003), and high temperatures (Cudennac and Lecerf, 2006). Furthermore, goethite can form hematite via a dehydration reaction (Lefèvre et al., 2006). Since ferrihydrite is poorly crystalline compared to goethite or hematite, this makes it more thermodynamically favorable for redox activity in the right pH conditions (Kukkadapu et al., 2003). Due to ferrihydrite's high surface area it has a higher degree of biological reduction and dissolution (Vodyanitzkii and Shoba, 2016), and likelihood of binding with OC (Adhikari et al., 2017) than goethite or hematite particles (Vodyanitzkii and Shoba, 2016; Adhikari et al., 2017). Furthermore, abiotic ferrihydrite reduction and reduction via DIRB is usually faster, and this reduction may generate secondary minerals like magnetite or goethite (Adhikari et al., 2017). However, certain impurities can significantly slow down ferrihydrite dissolution (Vodyanitzkii and Shoba, 2016).

Organic matter can interact with ferrihydrite to enhance or inhibit Fe(III) reduction and crystallization (Eusterhues et al., 2008; Cooper et al., 2017). OC can complex with ferrihydrite to limit Fe(III) reduction and it can chelate Fe(II) which inhibits microbial ferrihydrite-Fe(III) reduction, Fe oxidation, and ferrihydrite dissolution and recrystallization (Adhikari et al., 2017). Conversely, ferrihydrite may stabilize and protect the OM from biodegradation (Eusterhues et al., 2008). Cooper et al. (2017) noticed enhanced dissolution in co-precipitated ferrihydrite-OM compared to ferrihydrite sorbed on OM, likely because the smaller crystal size and impurities in the former enhanced dissolution. Ferrihydrite forms in OM-rich environments that contain humic substances that may act as electron shuttles that enhance microbial Fe(III) reduction (Cooper et

al., 2017). Adhikari et al. (2017) found that the presence of material with high C:Fe ratios in soil enhanced Fe(III) reduction and OC release, which likely relates to the protective relationship between Fe and OC and associated reduction and mineralization activity (Chen et al., 2017). Therefore, priming may explain why this high C:Fe addition enhanced DIRB activity and OC release in Adhikari et al. (2017). Furthermore, the presence of OC inhibited ferrihydrite's transformation into more crystalline minerals, possibly because the added OC stimulated Fe(III) reduction (Chen et al., 2009) and ferrihydrite dissolution in anaerobic conditions, or because the OC prevented Fe(II) from interacting (e.g. via chelation) with ferrihydrite to form secondary minerals like goethite (Adhikari et al., 2017).

In terms of microbial accessibility, and to compare ferric citrate with ferrihydrite, Fe(III) oxides like ferrihydrite and OM-mineral complexes cannot pass through the outer membrane of Fe(III)-reducing microorganisms (Cooper et al., 2017). Therefore, there are two characterized methods for electron transfer between the cell and ferrihydrite: 1) direct transfer of electrons via enzymes anchored to the outer cell membrane, 2) mediated electron transfer via exogenous electron-shuttling compounds found in OM, endogenous electron-shuttling compounds, or via Fe(III)-chelating compounds like siderophores (Cooper et al., 2017).

2.4 Iron Ammonium Oxidation (Feammox)

Although denitrification and anammox are commonly addressed as the primary anaerobic N_2 generating pathways, a lesser-known N-removal pathway is Fe ammonium oxidation (Feammox). Feammox is an autotrophic (Yang et al., 2018) dissimilatory Fe(III) reduction pathway coupled with NH_4^+ oxidation (Yang et al., 2019), which helps obtain energy and improves cell growth (Schröder et al., 2003) for Feammox bacteria. The Feammox bacteria can reduce Fe(III) in clay minerals, oxides, and hydroxides into Fe^{2+} (Yang et al., 2019) while oxidizing NH_4^+ into NO_2^-, NO_3^-, and/or N_2 (Clément et al., 2005; Shrestha et al., 2009; Yang et al., 2012; Huang and Jaffé, 2015). In Feammox, N_2 formation is more thermodynamically favorable (-245 kJ mol^{-1}) than NO_2^- (-164 kJ mol^{-1}), or NO_3^- (-207 kJ mol^{-1}) formation (Luther et al., 1997; Clément et al., 2005; Shrestha et al., 2009; Kuypers et al., 2018). Feammox can produce N_2 directly, or via coupled Feammox-denitrification or Feammox-anammox (Huang and Jaffé, 2015). Following the initial Feammox or coupled reactions, freed Fe^{2+} can crystallize, or

re-oxidize into minerals or other forms of solid Fe(III) (Latta et al., 2012) and potentially refuel Feammox.

2.4.1 Feammox discovery and initial characterization

The first consideration of a Feammox-like pathway was in Luther et al. (1997) which defined the manganese (Mn) ammonium oxidation (Mnammox) reaction. Mnammox occurs in aerobic conditions when MnO_2 (Mn^{4+}) oxidizes NH_3 and/or NH_4^+ into N_2. In soils with built up Mn^{2+} or MnO_2, Mnammox transforms NO_3^-, organic-N, and NH_4^+ (Luther et al., 1997). This reaction influenced the discovery of Feammox by indicating that Fe^{2+} may undergo similar reactions as Mn^{2+}, i.e. react with NO_3^- to form N_2 in pH 1-14 conditions (Luther et al., 1997). The authors also note that Fe^{3+} catalyzed N_2 formation and could be regenerated at pH < 6.8 (Eqs. 11-14) (Luther et al., 1997).

(10) $FeOOH + NH_3 \rightarrow N_2 + Fe^{2+}$
(11) $FeOOH + NH_3 \rightarrow NO_3^- + Fe^{2+}$
(12) $Fe^{2+} + NO_3^- \rightarrow N_2 + FeOOH$
(13) $Fe^{2+} + O_2 \rightarrow H_2O + FeOOH$

Clément et al. (2005) hypothesized an anaerobic process where Fe(III) reduction is coupled with NH_4^+ oxidation to produce NO_2^-, i.e. Feammox. Clément et al. (2005) did not imply that Feammox could form N_2, which was corrected in other work, e.g. Yang et al. (2012). However, the experimental results in Clément et al., 2005 did not fully support NH_4^+ oxidation to NO_2^-, or Feammox. In anaerobic conditions, fresh acidic wetland soils were treated with urea at different rates, incubated, and the NO_3^- and NO_2^- was tracked. The Fe(II) produced in the incubation likely originated from Fe(III) hydroxides (e.g. goethite) in the soil. The Fe(II) and NO_2^- production increased with higher urea concentration, up to 500 mmol N g^{-1}, which was an effect NH_3 volatilization alone could not explain. The increase in NO_2^- and loss of total dissolved N coincided with a pH increase. Since NO_3^- was not detected, this was thought to be explained by anaerobic denitrification of NO_3^- (Clément et al., 2005). However, this 20-day incubation does not account for long-term Feammox activity. Clément et al. (2005) proposed several mechanisms and Gibb's free energies for their results. These reactions were thought to be thermodynamically feasible at pH 7 and include the theoretical Fe(III) reduction-NH_4^+ oxidation

reaction (Eq. 14), goethite reduction (Eq. 15), and hypothesized Feammox reaction involving goethite (Eq. 16):

(14) $NH_4^+ + 2H_2O + 6Fe^{3+} \rightarrow NO_2^- + 6Fe^{2+} + 8H^+$ $\Delta G = -24.3$ kJ
(15) $FeOOH + 3H^+ + e^- \rightarrow Fe^{2+} + H_2O$ $\Delta G = -64.6$ kJ
(16) $NH_4^+ + 6FeOOH + 10H^+ \rightarrow NO_2^- + 6Fe^{2+} + 10H_2O$ $\Delta G = -30.9$ kJ

Although Feammox was defined by Clément et al. (2005), the work of Shrestha et al. (2009), Yang et al. (2012), Huang et al. (2014), Huang and Jaffé (2015) sought to support Feammox with empirical evidence. In a greenhouse mesocosm experiment, Shrestha et al. (2009) created anaerobic Fe(III) reducing conditions with the same acidic wetland soils (pH 3.5-4) used by Clément et al. (2005). The samples that were amended with NH_4Cl produced NO_2^- after 4 months, indicating a delayed reaction and Fe(II) increased with soil depth in treatments with and without NH_4Cl, indicating reducing conditions. This may not support Feammox because in anaerobic conditions other reactions produce NO_2^-, such as OM mineralization. In addition, as OM is decomposed, soil conditions become more reduced (DeLaune and Reddy, 2005). OM has an important role in Fe(III) reduction in these acidic wetland soils, which can minimize available Fe(III) for NH_4^+ oxidation. Therefore, the results of this incubation did not provide Feammox evidence. In another experiment in Shrestha et al. (2009), $^{15}NH_4Cl$ was added to NO_2^- and NO_3^--free slurries and ^{15}N was tracked for 50 days. The $^{15}NH_4^+$ loss corresponded to a gradual increase in $^{30}N_2$, with the $^{15}NH_4^+$ most likely first oxidized to $^{15}NO_3^-$ or $^{15}NO_2^-$ and then reduced to $^{30}N_2$. This occurred in Fe-reducing conditions, suggesting Feammox; however, denitrification or anammox may explain some, or all of the $^{30}N_2$ production (Shrestha et al., 2009). Furthermore, Fe(II) and $^{15}NO_2^-$ were not measured in the ^{15}N incubation (Shrestha et al., 2009), so Feammox is not fully supported.

Yang et al. (2012) conducted anaerobic soil slurry experiments using samples of Fe-rich weathered soils from the uplands of Puerto Rico with pH values of 4.3, 5.2, and 6.1. The soils were treated with NH_4Cl and/or ferrihydrite. All slurries with ferrihydrite produced NO_2^-, NO_3^-, and N_2. Co-amending the pH 4 soil sample with $^{15}NH_4^+$ and ferrihydrite produced more $^{30}N_2$ (e.g. 47 ± 27 to 72 ± 9% of the $^{30}N_2$) and $^{29}N_2$ relative to $^{15}NH_4^+$-only treatments. Co-amended treatments accounted for 60 ± 12% of the total ^{15}N-N_2 production. The NH_4^+ oxidation and N_2 production decreased as pH increased in the pH 5.2 and 6.1 samples. The $^{29}N_2$ production

suggests the microbes oxidized some soil-$^{14}NH_4^+$. The $^{30}N_2$ production was attributed to Feammox, including some Feammox direct-N_2 production. To minimize anammox and denitrification interference, Yang et al. (2012) added C_2H_2 (anammox and $N_2O \rightarrow N_2$ inhibitor) to the $^{15}NH_4^+$ + ferrihydrite co-amended treatment. The $^{30}N_2$ levels were lower in this C_2H_2 co-amended treatment than the C_2H_2-free co-amended treatment, but higher than the $^{15}NH_4^+$-only treatment. C_2H_2 inhibits $N_2O \rightarrow N_2$ by inhibiting nitrous oxide reductase and inhibits anammox to a greater degree (Jensen et al., 2007), likely by inhibiting the NH_4^+ activation step that uses NO_2^- as an oxidant (Huang and Jaffé, 2015). Therefore, these results imply that anammox and denitrification were responsible for some of the initial NH_4^+ oxidation prior to C_2H_2 addition. Yang et al. (2012) only studied the top 10 cm soil, which underestimates Feammox potential in deeper soils.

Feammox might explain 53 ± 27% of the N that formed NO_2^- or NO_3^- and did not become $^{30}N_2$ (Yang et al., 2012). Yang et al. (2012) propose that N_2 is the primary gas emitted in Feammox. They explain that as pH increases, Feammox becomes less thermodynamically favorable due to diminishing reactivity of Fe-oxide minerals. In a short 25-hour experiment, they compared Feammox in acidic soils (pH 4.3 and 5.2) and soils with a pH ~6.1 with the former soils exhibiting more $^{30}N_2$ production relative to pH 6 soils. This experiment was too short to estimate long-term effects of pH differences. Yang et al. (2012) propose the following Gibb's free energy and reactions with ferrihydrite that are energetically favorable at varying pH (Eq. 17), or less favorable at pH 6.5 (Eq. 18).

(17) $3Fe(OH)_3 + 5H^+ + NH_4^+ \rightarrow 3Fe^{2+} + 9H_2O + 0.5N_2$ $\Delta G = -245$ kJ mol^{-1}

(18) $6Fe(OH)_3 + 10H^+ + NH_4^+ \rightarrow 6Fe^{2+} + 16H_2O + NO_2^-$ $\Delta G = -164$ kJ mol^{-1}

Huang et al. (2014) propose an alternative pathway to Feammox in anammox bioreactors. In their experiment, elevated Fe^{2+} augmented anammox efficiency and anammox bacteria growth in the bioreactor. Huang et al. (2014) think the relationship is due to one or more of the following: 1) Fe promoted heme-C biosynthesis in anammox, which helps in energy metabolism, 2) anammox bacteria consumed Fe^{2+} which enhanced anammox efficiency, and 3) Fe^{2+} acted as an electron donor in anammox. Anammox bacteria are rich in heme, an electron carrier used in energy metabolism and cell synthesis. The biosynthesis of heme-C is dependent on essential nutrients Fe^{2+} and Mn (Huang et al., 2014). These three possibilities suggest Fe-enhanced

anammox rather than Feammox, which differs from previous work (Clément et al., 2005; Shrestha et al., 2009; Yang et al., 2012).

The first reliable Feammox evidence was presented in Huang and Jaffé (2015) with multiple anaerobic incubations that incorporated NH_4^+, different Fe(III) sources, inorganic C, ^{15}N isotopes, and PCR analysis. In a 30-day incubation, Huang and Jaffé (2015) attempted to stimulate Feammox with NH_4Cl, ferrihydrite, goethite, and ferric citrate amendments to acidic wetland soil (Clément et al., 2005; Yang et al., 2012) slurries (pH of 4-4.5). The ferric citrate + NH_4Cl treatment showed notable Fe reduction compared to the Fe(III)-oxides, but no appreciable NH_4^+ loss or changes (Huang and Jaffé, 2015). PCR analysis indicated that the communities in the ferric citrate + NH_4Cl treatment were predominantly Fe(III)-reducers, and not the proposed Feammox bacterium (*Acidimicrobiaceae* bacterium (A6)). Ferrihydrite, followed by goethite, proved most effective at oxidizing NH_4^+ in soil slurries. This may be because reactions with less-crystalline ferrihydrite are more thermodynamically favorable (larger negative ΔG value) than reactions with more-crystalline goethite (Kukkadapu et al., 2003; Clément et al., 2005). In the first 25 days of incubation, most of the Fe(III) reduction was attributed to Feammox and lesser so to dissimilatory Fe reduction because >3x more Fe(II) was produced in ferrihydrite + NH_4Cl slurries than in ferrihydrite-only slurries (Huang and Jaffé, 2015).

Due to the success of the ferrihydrite treatments, Huang and Jaffé (2015) extended the incubation to 180 days for a second experiment with continued NH_4Cl additions. In ferrihydrite + NH_4Cl treatments, the NH_4^+ was oxidized continuously with repeated NH_4Cl additions. The NH_4Cl additions also stimulated NO_2^- and NO_3^- production, but they disappeared rapidly which was likely due to denitrification (Huang and Jaffé, 2015). On day 125 the bottles were divided to study the effects of different inorganic C concentrations (0.20 mM and 1.20 mM $NaHCO_3$) on Feammox. The 1.20 mM $NaHCO_3$ amendment notably enhanced NH_4^+ oxidation and Fe reduction relative to 0.20 mM $NaHCO_3$ (Huang and Jaffé, 2015), indicating that Feammox is autotrophic (Jurtshuk Jr., 1996) like anammox (Kamp et al., 2019). Huang and Jaffé (2015) propose Eq. 19 as a Feammox pathway with ferrihydrite at pH 4.5.

$$(19)\ NH_4^+ + 3Fe_2O_3{\cdot}0.5H_2O + 10H^+ \rightarrow 6Fe^{2+} + 8.5H_2O + NO_2^- \qquad \Delta G_r \leq -145.1\ kJ\ mol^{-1}$$

Anammox may explain some of the initial 30-day incubation results in Huang and Jaffé (2015). Therefore, similar to Yang et al. (2012), Huang and Jaffé (2015) conducted a third experiment with C_2H_2. They incubated soils with $NaHCO_3$, NH_4Cl, and Fe(III) for 90 days before adding C_2H_2 and incubating another 20 days. In C_2H_2-amended samples there was less NH_4^+ oxidation, slow NO_2^- accumulation, greater $^{15}N_2O$ accumulation (when $^{15}NH_4Cl$ was added), and less NO_3^- production relative to C_2H_2-free samples. In summary, treatments with C_2H_2 produced NO_2^- and N_2O that were equivalent to the amount of NH_4^+ consumed, indicating that N_2 production via anammox and denitrification was inhibited. Therefore, denitrification and anammox likely explain some of the N_2 production in the initial 30-day incubation. Conversely, C_2H_2 did not appear to inhibit Fe reduction, and it is suggested that C_2H_2 does not inhibit NH_4^+ oxidation in Feammox (Huang and Jaffé, 2015).

Huang and Jaffé (2015) propose that PCR- and qPCR-isolated uncultured *Acidimicrobiaceae* bacterium (A6), whose closest cultivated relatives are heterotrophc acidophilic Fe-oxidizer *Ferrimicrobium acidiphilum* (with 92% identity) and autotrophic acidophilic Fe-oxidizer *Acidimicrobium ferrooxidans* (with 90% identity), may be responsible for the Feammox activity in their incubations (Huang and Jaffé, 2015). The A6 population was only detected in samples with ferrihydrite, NH_4^+, and $NaHCO_3$, with the A6 population increasing from 0.92% on day 0 to 14.8% on day 160. On day 180 of the incubation, the soils were used to enrich Feammox bacteria in a reactor. During the first 150 days in the reactor the NH_4^+ levels decreased by 64.5% and the A6 population increased to 40.2% on day 150 (Huang and Jaffé, 2015).

2.4.2 Feammox exploration and further characterization

The initial Feammox characterization period acknowledged that Feammox bacteria can reduce the Fe(III) in certain Fe(III)-bearing clay minerals, oxides, and hydroxides into soluble Fe^{2+} while oxidizing NH_4^+ into NO_2^-, NO_3^-, and/or N_2 (Huang and Jaffé, 2015). Acidic wetland soils are typically low in dissolved Fe(II) and NO_2^- and Feammox requires no dissolved oxygen, NO_2^-, or NO_3^- (Huang and Jaffé, 2018). Therefore, anaerobic acidic soils with thermodynamically favorable Fe(III) sources are ideal environments for Feammox. Follow up Feammox research explored the role of OM and electron shuttles (Zhou et al., 2016), organic and inorganic C (Yang et al., 2018), pH (Zhou et al., 2016) in Feammox, and further characterized

the Feammox bacteria (Huang and Jaffé, 2018). Zhou et al. (2016) studied how OM influenced Feammox by using ^{15}N tracers, DIRB cultures, electron shuttles (biochar and a model humic substance), and *ex situ* (from soil) and *in situ* (lab-synthesized) ferrihydrite in acidic paddy soils. The *in situ* and *ex situ* ferrihydrite may influence Feammox differently due to different structures and impurities. Zhou et al. (2016) experimented with biochar and a model humic substance compound, 9,10-anthraquinone-2,6-disulfonate (AQDS), which both contain quinone moieties that may act as electron shuttles between microbes and/or NH_4^+ and Fe-hydroxides to improve Feammox efficiency (Zhou et al., 2016). The samples in the 30-day incubation were: 1) abiotic treatment (without DIRB and sterilized) as the negative control and 2) abiotic treatment with AQDS and biochar, 3) biotic treatment (with DIRB inoculum) as the positive control, 4) biotic treatment with AQDS, 5) biotic treatment with biochar, 6) biotic treatment with AQDS and biochar (Zhou et al., 2016).

In the biotic treatments each organic amendment improved $^{30/29}N_2$ production and adding both AQDS and biochar produced the most $^{30}N_2$. The biotic treatment with AQDS and biochar amendments increased $^{15}NH_4^+$ use by 4.1-11.5% and had more Fe reduction than treatments with individual electron shuttles. The abiotic control with AQDS and biochar showed low levels of Fe(III) reduction, implying minimal abiotic Fe(III) reduction via these electron shuttles (Zhou et al., 2016). The $^{29}N_2$ was higher in all DIRB-enriched cultures relative to abiotic cultures, probably because microbes prefer lighter isotopes in the soil (Zhou et al., 2016) that have weaker bonds which require less energy to break. It is possible that ferrihydrite transformed to siderite ($FeCO_3$) and vivanite ($Fe_3(PO_4)_2$) in abiotic treatments. Overall, the Fe-related NH_4^+ oxidation increased by 17-340% when an electron shuttle was available, which may be related to Feammox (Zhou et al., 2016). Therefore, although OM is not necessary for autotrophic Feammox, it may enhance Feammox efficiency.

Biochar and AQDS both acted as electron shuttles that enhanced *nosZ* (a denitrification-related functional gene) and *hszB* (an anammox-related functional gene) expression, particularly in the *in situ* ferrihydrite treatments relative to *ex situ* ferrihydrite treatments. The *in situ* ferrihydrite had a smaller particle size and may have had more OM associations and impurities that enhanced its reactivity (Zhou et al., 2016). The NO_3^- and NO_2^- concentrations also decreased in all biotic treatments, which may indicate denitrification or anammox (Zhou et al., 2016). Although not mentioned in Zhou et al. (2016), DNRA may also explain the NO_3^- loss by

combining NO_3^- reduction to NH_4^+ to OM oxidation (Pillegaard, 2013; Bu et al., 2017). However, although gene expression indicated anammox and denitrification activity, minimal anammox bacteria were detected in any treatments, and $^{45}N_2O$ and $^{46}N_2O$ levels were below the detection limit and significantly lower than $^{29/30}N_2$ levels, which indicates minimal or no anammox and denitrification activity (Zhou et al., 2016). In biotic treatments with both ferrihydrite sources the Fe(III) reduction and $^{29/30}N_2$ accumulation had a significant positive correlation, which Zhou et al. (2016). Therefore, these results indicate the N transformation is primarily related to Feammox activity (Zhou et al., 2016). However, this result contrasts with denitrifier populations found in biotic AQDS and/or biochar treatments (Zhou et al., 2016), implying that there may have been an error in the overall result analysis. Furthermore, Feammox conclusion does not acknowledge the possibility that the Fe(III) reduction (e.g. a DIRB and SOM relationship) may be independent to NH_4^+ loss (e.g. N assimilation).

The electron shuttles enhanced NH_4^+ oxidation and Fe(III) reduction, especially in *in situ* ferrihydrite treatments; therefore, it appears that the shuttles enhanced Feammox (Zhou et al., 2016). The quinone components in the AQDS and biochar may have sorbed Fe(II), which could accelerate Fe(III) reduction in ferrihydrite treatments (Zhou et al., 2016) since excess Fe(II) can interfere with Fe(III) reduction (Adhikari et al., 2017). Upon further analysis of the $^{30}N_2$ generation, Zhou et al. (2016) clarify that most of the Fe reduction was coupled with OM oxidation rather than NH_4^+ oxidation, which was seen in Yang et al. (2012) and Clément et al. (2005). This conclusion was based on the abundance of acetate-oxidizing DIRB in the biotic treatments, which are responsible for these OM redox reactions (Zhou et al., 2016). Therefore, Zhou et al. (2016) believe both Feammox and acetate-oxidizing dissimilatory Fe(III) reduction worked concurrently and both were stimulated by electron shuttles. The electron shuttles also increased populations of unclassified fermenting, Fe-reducing bacteria related to *Pelobacteraceae* and denitrifier populations that imply potential fermentation and denitrification associated with these organic amendments (Zhou et al., 2016). Members of the genus *Geobacter* were isolated in the original paddy soils and likely acted as Feammox mediators by influencing Fe(III) reduction (Zhou et al., 2016). In conclusion, Zhou et al. (2016) believe that amending soils with electron shuttles will improve NH_4^+ oxidation rates and Fe reduction via Feammox and/or OM oxidation-Fe reduction pathways. However, the insinuation that Feammox was the primary pathway is debatable, since denitrification may have also been occurring in samples with

AQDS and/or biochar. Another important aspect of Zhou et al. (2016) is that the slurry pH ranged from 6.8-7.2, indicating that Feammox was still possible at neutral to slightly alkaline conditions.

Huang and Jaffé (2018) includes the first isolation and culturing of the *Acidimicrobiaceae* sp. A6 Feammox bacteria using the riparian wetland soils of New Jersey (Clément et al., 2005; Shrestha et al., 2009; Huang and Jaffé, 2015). The A6 bacteria were grown in soil slurries (pH 4.5) amended with ferrihydrite and NH_4Cl in an 80:20 N_2:CO_2 atmosphere, incubated for 300 days, enriched in batch cultures, and subcultured prior to PCR and qPCR analysis and 16S rRNA gene sequencing (Huang and Jaffé, 2018). Unidentified *Actinobacteria* and *Betaproteobacteria* were dominant during the 300-day incubation, with *Actinobacteria* increasing from 6.0 to 59.2%, and *Betaproteobacteria* (included denitrifiers *Bukholderia* and *Ralstonia*) decreasing from 43.2 to 32.6% of the total population (Huang and Jaffé, 2018). The presence of denitrifying *Betaproteobacteria* implies there was sufficient NO_2^-/NO_3^- for denitrification. The Fe reducer *Geothrix fermentans* (Huang and Jaffé, 2018), which often uses acetate for respiration, was found in the enrichment cultures, offering Fe-reducing potential. The A6 population increased from 7.5% to 47.6% over 300 days and had a doubling time of 8-10 days (Huang and Jaffé, 2018). Scanning Election Microscope (SEM) imagery showed the A6 cultures with visible accumulated Fe^{2+}. The cultured A6 oxidized 1.15 mmol/L NH_4^+, and produced 5.65 mmol/L of Fe(II) and 0.772 mmol/L NO_2^- during a 20-day incubation, similar to the hypothesized Feammox reaction in Huang and Jaffé (2015) (Eq. 19). Meanwhile, there was no NH_4^+ loss or Fe(II) reduction in controls without Fe(III) or NH_4^+, respectively (Huang and Jaffé, 2018).

Huang and Jaffé (2018) also investigated Feammox potential with A6 at pH values between 2 and 8, and noted maximum Feammox activity at pH 4. There was NH_4^+ loss and Fe reduction at pH 7-8 (Huang and Jaffé, 2018), so the reaction occurred less efficiently in alkaline conditions. However, this pH experiment lasted 14 days, which does not indicate long-term effects. Huang and Jaffé (2018) also performed three separate 14-day A6 incubations with AQDS, ^{13}C-labelled organic and inorganic C sources, and different Fe(III) sources. The A6 required inorganic C to grow and grew in organic C-free solid and liquid media. The $NaHCO_3$ and sodium acetate + $NaHCO_3$ treatments effectively stimulated Feammox unlike the organic C-only glucose treatment. When the potential electron shuttle AQDS was tested, the 25 and 50 mM AQDS treatments had relatively equivalent NH_4^+ loss, but losses were in the 4-6 mM range as

opposed to 0.75 mM NH_4^+ loss in the AQDS-free control. There was also substantial Fe reduction in the 25 and 50 mM AQDS treatments relative to the control, implying that the AQDS was related to Fe(III) reduction (Huang and Jaffé, 2018), which was also seen in Zhou et al. (2016). Similar to Huang and Jaffé (2015), the 6-line ferrihydrite, followed by goethite, were the most effective Feammox stimulants and had the greatest NH_4^+ loss and Fe reduction in the acidic slurry conditions. There was notable Fe reduction but minimal NH_4^+ loss in the ferric citrate treatments, similar to Huang and Jaffé (2015). Overall, NH_4^+, ferrihydrite, goethite, inorganic C, and AQDS amendments stimulated Feammox in A6 cultures (Huang and Jaffé, 2018).

Bao and Li (2017) indicate that other anaerobic pathways can provide electrons for Feammox, and may play a role in aerobic NH_4^+ oxidation. The sulfur cycle may be linked to Feammox because sulfur reduction is very common in environments where Feammox occurs, e.g. wetlands and upland soils (Bao and Li, 2017). Electrons often transfer from sulfide to Fe^{3+}, producing Fe^{2+} species and oxidized sulfur species (Bao and Li, 2017). Bao and Li (2017) view Feammox not as a single process performed by one bacteria, but instead co-opted by five bacteria that are part of a consortium labelled HJ-4. The HJ-4 is primarily three *Anaerospora hongkongensis* operational taxonomic units (OTUs) (85 %) and two ferrihydrite reducing *Comamonadaceae* OTUs (15%). *A. hongkongensis* can reduce sulfite, sulfide, and elemental sulfur, which act as electron shuttles for ferrihydrite reduction by *Comamonadaceae*, a facultative anaerobe which might also perform aerobic NH_4^+ oxidation to NO_2^- (Bao and Li, 2017). The HJ-4 only grew successfully if ferrihydrite, NH_4^+, and/or sulfide were all present, and grew best at pH 5.0. Bao and Li (2017) found evidence for Simultaneous Nitrification-Denitrification (SND) with N-isotope tracers in anaerobic conditions. The highest $^{29}N_2$ levels were in treatments amended with NH_4^+, ferrihydrite, and sulfide and the $^{29}N_2$ production was higher than $^{30}N_2$, possibly due to preference for the lighter isotope. The $^{29/30}N_2$ and $^{15}NO_2^-$ production were related to $^{15}NH_4^+$ oxidation and the NH_4^+ enhanced Fe(III) reduction (Bao and Li, 2017). Minimal NH_4^+ oxidation occurred when Fe(III) or sulfide were absent because sulfide was the favored electron shuttle. Sulfide and other sulfur species can regenerate to continue these reactions (Bao and Li, 2017). Therefore, Fe and sulfur have an important relationship in anaerobic conditions that may imply that Feammox is more complicated than previously assumed.

Although Fe(III) and Fe(II) are often tracked in the dissolved fraction as an Feammox indicator (Shrestha et al., 2009; Yang et al., 2012; Huang and Jaffé, 2015, 2018; Zhou et al., 2016), spectroscopic characterization of C- and Fe(III)-species has received limited attention and remains as a gap in Feammox characterization in soil. Yang et al. (2018) used X-ray diffraction (XRD) and X-ray photoelectron spectroscopy to characterize crystalline Fe-species in an anaerobic incubation. The Feammox sludge slurry incubation showed an NH_4^+ removal efficiency of 69.5% in ferrihydrite treatments (Yang et al., 2018). The XRD analysis showed that endogenous slurry Fe-hydroxides (assumed to be ferrihydrite) and synthesized (pH 6.8-7.2) ferrihydrite amendments were both transformed into the Fe-oxide magnetite (Fe_3O_4) and the oxide-hydroxide akageneite (β-FeOOH) (Yang et al., 2018). This recrystallization could be due to Fe-oxides sorbing Fe(II) in solution and/or incorporating Fe(II) into their structure (Yee et al., 2006; Adhikari et al., 2017) or released Fe^{2+} causing ferrihydrite to transform into more crystalline phases (Adhikari et al., 2017). Furthermore, the Fe(II) may have been oxidized and regenerated Fe(III) resources via nitrate or nitrite-dependent Fe(II) oxidation (NDFO), which generates N_2 (Yang et al., 2018). Although suggested NDFO reaction mechanisms differ, Eq. 20 is an example with NO_3^-. There is debate about the existence of both nitrite- and nitrate-dependent Fe oxidation, with some research supporting only the latter pathway. However, although the reasoning for recrystallization/oxidation is debated, characterizing any Fe(III) mineral transformations via spectroscopy is relevant for *in situ* Feammox remediation applications in soil.

(20) $10Fe(II) + 2NO_3^- + 12H^+ \rightarrow 10Fe(III) + 2N_2 + 6H_2O$

To summarize, Feammox has been observed in acidic anaerobic Fe-rich sediments like tropical upland soils (Yang et al., 2012), paddy soils (Ding et al., 2014; Bao and Li, 2017), and wetland soils (Clément et al., 2005; Shrestha et al., 2009; Huang and Jaffé, 2015, 2018). The first cultured Feammox bacterium was *Acidimicrobiaceae* bacterium (A6) (Huang and Jaffé, 2015, 2018), which is shown in a conceptual Feammox diagram in Fig. 2.1. Most of the Feammox evidence is from laboratory experiments using field soils and this work indicates that ideal Feammox conditions are around pH ~4.5 (Huang and Jaffé, 2015) with a temperature optimum around 20°C (Ruiz-Urigüen, 2014). Yang et al. (2018) and Huang and Jaffé (2015) imply that 6-

line ferrihydrite is the most active Feammox Fe(III) resource. Conversely, ferric citrate is thought to be an indirect Feammox stimulant via two processes 1) rapid Fe(III) reduction by DIRB, using organic C as electron donor (if an anaerobic Fe-reducing community is present) 2) released citrate ($C_6H_5O_7^{-3}$) is consumed by microbial populations, creating poorly-crystalline minerals like ferrihydrite that can participate in Feammox (Huang and Jaffé, 2015). Although Feammox is autotrophic, its efficiency can be enhanced by SOM compounds like quinones that act as electron shuttles (Zhou et al., 2016; Ruiz-Urigüen et al., 2019). Furthermore, OM further complicates the implied Feammox relationship between Fe(III) reduction and NH_4^+ oxidation by influencing Fe(III) reduction (Zhou et al., 2016; Huang and Jaffé, 2018) and because OM, or components in OM, can be more thermodynamically favorable electron donors for NH_4^+ oxidation than Fe(III)-oxides (Yang et al., 2018).

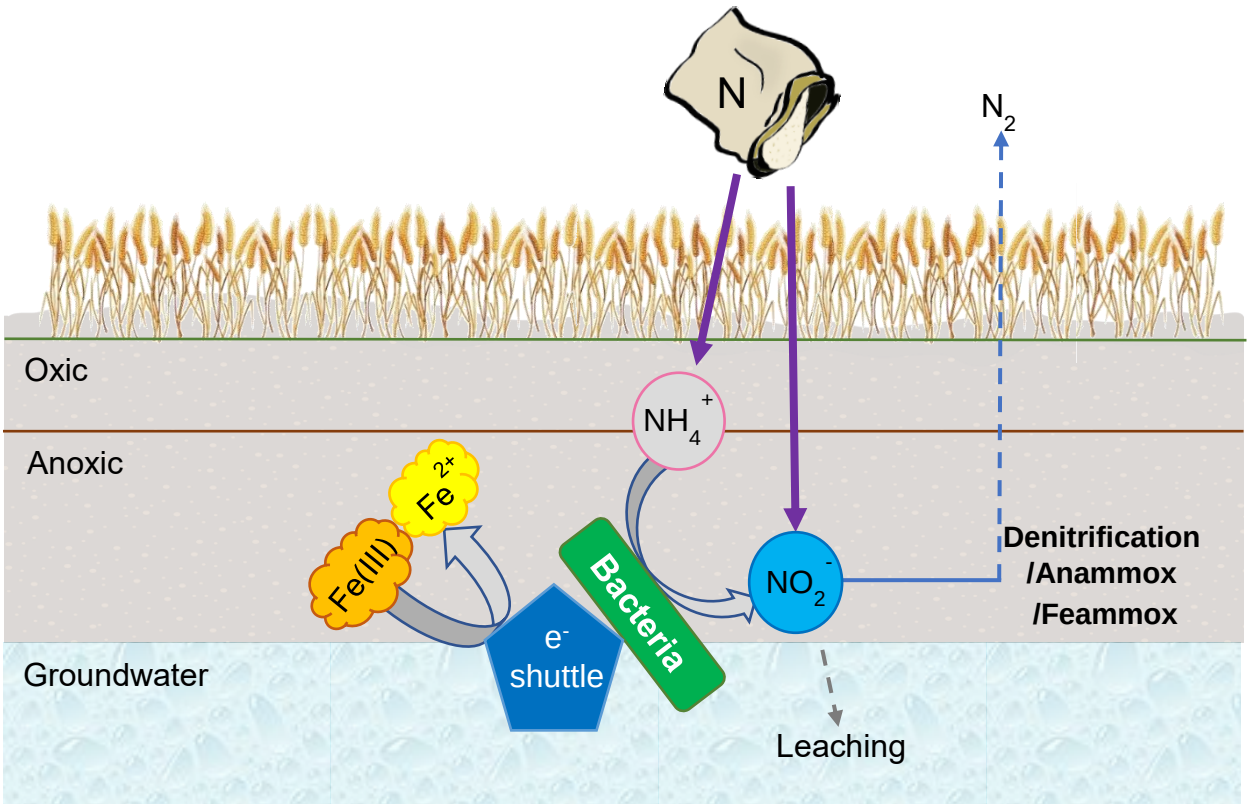

Fig. 2.1. Conceptual diagram of anaerobic iron ammonium oxidation (Feammox) where a bacterium uses an electron shuttle (e^- shuttle), e.g. a component in soil organic matter, to oxidize ammonium (NH_4^+) while reducing Fe(III) to Fe^{2+}, which yields nitrite (NO_2^-) that can be converted to nitrogen gas (N_2) via Feammox, anaerobic ammonia oxidation (anammox), and/or anaerobic denitrification after fertilizers are applied to the soil. The electron shuttle is not necessary for Feammox.

2.5 Feammox in Nitrogen pollution remediation

Feammox has been considered as a N-remediation method within WWTPs and *in situ* groundwater treatment. Thus far, the foreseeable limitations in applying Feammox in WWTPs include: 1) adding an Fe(III) source such as ferrihydrite because it is difficult and expensive to synthesize or locate, 2) adding an organic C source that may enhance efficiency but add to expenses, 3) the lower efficiency of Feammox relative to nitrification-denitrification and anammox, and 4) the unexplored aspects of Feammox that may also influence Feammox application in remediation. The following sections outline the initial remediation research involving Feammox.

2.5.1 Feammox in waste water treatment applications

Although data from nitrification-denitrification and anammox in WWTP studies does not typically acknowledge suspected Feammox activity, it is possible that Feammox also occurs in these reactors. Li et al. (2018b) conducted a WWTP-based 160-day cultivation experiment studying Feammox in inoculated anammox sludge. This experiment included two reactors with different treatments, both of which had a pH of ~ 6.5 since the optimum pH for anammox is between 6.5 and 8. Both contained NH_4^+-N via NH_4Cl, soluble Fe(III) via $FeCl_3$ (inflow concentrations 100 mg L^{-1} and 20 mg L^{-1}, respectively), but reactor 1 was microbe-free and reactor 2 contained microbes (Li et al., 2018b). There was simultaneous Fe(III) reduction and NH_4^+ oxidation and initiated Feammox in reactor 2, but no NH_4^+-transformation in reactor 1 (Li et al., 2018b). In reactor 2 Feammox was initiated and the NH_4^+ conversion rate was 80%, producing substantial NO_3^- and minimal NO_2^-, making the overall N-removal rate 63% (Li et al., 2018b). In the hypothesized anammox reaction in Li et al. (2018b), the ratio of NO_3^--N to total nitrogen removal is 0.126; however in reactor 2 the NO_3^--N production exceeded that ratio. Therefore, the ratio of NO_3-N to total N removal implies that another reaction may have generated some of the NO_3^-, for example 1) soil Fe(III) reacting with NH_3 to produce NO_3^--N as suggested by Yang et al. (2012) or 2) another pathway for NH_4^+-N oxidation generated NO_3- as an end product (Eq. 21) (Li et al., 2018b). It was assumed by Li et al., 2018b that nitrification could not explain this result; however, anaerobic nitrification may occur in some cases (Yang et al., 2012; Huang and Jaffé, 2015).

(21) $NH_4^+ + 1.32\ NO_2^- + 0.13\ H^+ \rightarrow 1.02\ N_2 + 0.26\ NO_3^- + 2.03\ H_2O$

The result that Fe(III) was an important electron acceptor for N conversion in the anammox sludge showed a possible Feammox-anammox relationship (Li et al., 2018b). However, Li et al. (2018b) do not clearly differentiate what amount of N was removed by anammox relative to Feammox which could have been supported by incorporating C_2H_2. The minimal NO_2^- generation in Li et al. (2018b) may be explained by NO_2^- consumption via anammox and denitrification, however, Li et al. (2018b) did not acknowledge the latter process as a possibility. There could also be a bacteria capable of performing coupled Feammox-anammox. Zhou et al. (2014) hypothesized that the anammox bacteria (*Keunenia stuttgartiensis*) can use the OM (e.g. from sludge) as an electron donor to reduce Fe(III) to Fe(II) while using NO_2^--N as the electron donor for anammox. Therefore, there may be a Feammox-anammox relationship that is useful in future WWTP applications. Unfortunately, Li et al. (2018b) note some practical limitations to applying Feammox in WWTPs, e.g. a solid $Fe(OH)_2$ precipitate via mineralization, which accumulated in the reactor and the lack of Fe-substrates appeared to decrease the anammox population. Overall, the solid Fe(II) precipitate accumulation greatly reduced N removal rates and reactor performance (Li et al., 2018b).

Accumulation of Fe precipitates may hinder Feammox application in wastewater treatment (Li et al., 2018b). However, the NDFO pathway can regenerate solid Fe(III) that may minimize this issue by stimulating Feammox. Feammox and NDFO have been explored in several studies (Bao and Li, 2017; Bao and Li, 2018b), and Li et al. (2018a) used a 63-day anaerobic incubation to further study the relationship. They inoculated Feammox sludge with NO_3^-, NH_4^+, and Fe(III) via $FeCl_3$ in an anaerobic 3.6 L bioreactor tank. They noticed a pattern, or cycle between anammox, Feammox, and NDFO in Fe and N cycling, with Fe acting as the catalyst. Li et al (2018a) believe Feammox oxidized 67.6% of the $^{15}NH_4^+$ to NO_2^- while reducing Fe(III) to Fe(II), with Fe(II) then acting as an electron donor in NDFO, reducing 58.8% of the NO_3^- into NO_2^- and N_2 and regenerating Fe(III). This was supported by an initial decrease in dissolved NO_3^--N and NH_4^+-N, which may have also been caused by simultaneous conversion of NO_3^- and NH_4^+ by denitrifier and anammox bacteria via OM (Li et al., 2018a). Minimal dissolved Fe(II) was detected, which was explained by coupled Fe(II) oxidation with NO_2^-, NO_3^-

(e.g. NDFO) or N_2 reduction (Li et al., 2018a). Therefore the added NO_3^- may have indirectly enhanced Feammox activity via NDFO (Li et al., 2018a). When NO_3^- was added a second time the NO_3^--N and NH_4^+-N concentrations decreased again; however, the Fe(III) values remained relatively stable, implying that Fe oxidation may have been more important in N-removal than Feammox in the second amendment period (Li et al., 2018a). Minimal NO_2^- was produced during the incubation, which may be explained by consumption via anammox activity (Li et al., 2018a). A link between anammox and Fe reduction which might also explain why Fe(II) decreased includes ferric reductases in anammox bacteria cell membranes that reduce Fe(III) to Fe(II) for assimilation and therefore could use Fe(III) as an anammox catalyzer (Li et al., 2018a). The bioreactor pH was kept at 7.0 throughout the experiment, showing that these relationships are possible in neutral conditions (Li et al., 2018a).

Although research with WWTP model bioreactors and soil slurries provides evidence of Feammox, the reported efficiencies are low, implying a low efficiency of electron transfer between NH_4^+ and Fe(III) (Yang et al., 2019). One solution to accelerate Feammox may include electron shuttles like AQDS and biochar (Zhou et al., 2016). An incubation experiment explored whether AQDS could improve Feammox efficiency for WWTP application (Yang et al., 2019). The results of the incubation of anaerobic sludge (pH adjusted to 4.5) showed NH_4^+ removal percentages of 82.6% (AQDS + Fe_2O_3 treatment), 64.3% (Fe_2O_3 treatment) and 46.0% in the AQDS- and Fe_2O_3-free control (Yang et al., 2019). The presence of Fe_2O_3 and AQDS increased the communities of iron reducing bacteria (IRB), and that IRB enrichment was related to N-loss and the Fe(II) content, which implies that the IRB performed Feammox (Yang et al., 2019). Furthermore, the results implied that AQDS acted as an electron shuttle between the Fe_2O_3 and NH_4^+ and ultraviolet-visible spectroscopy (UV/Vis) scanning spectra showed that NH_4^+ oxidation coupled to AQDS reduction (e.g. via IRB) to generate anthrahydroquinone-2,6-disulfonate (AH_2QDS) (Eq. 22) in the AQDS + Fe_2O_3 treatment. In a follow-up experiment the Fe_2O_3 reduction appeared to be related to the oxidation of AH_2QDS back to AQDS (Eq. 23) (Yang et al., 2019). Yang et al. (2019) imply that noted that there were populations in the samples capable of reducing both Fe(III) and AQDS.

(22) $AQDS + NH_4^+ \rightarrow AH_2QDS + N_2/NO_x^-$
(23) $AH_2QDS + Fe_2O_3 \rightarrow AQDS + Fe(II)$

Yang et al. (2019) saw an increase of NH_4^+ from 17 to 44 mg L^{-1} in the AQDS and Fe_2O_3-free control, and NH_4^+ also increased in the Fe_2O_3 and AQDS + Fe_2O_3 treatments during the initial 23 days. Yang et al. (2019) think the increase is explained by Fe_2O_3 and AQDS enhancing decomposition of sludge proteins, thus releasing sludge NH_4^+. After the initial 23 days Yang et al. (2019) indicate that the freed NH_4^+ stimulated Feammox and NH_4^+-N levels decreased in the AQDS-Fe_2O_3 and Fe_2O_3 treatments. There was also NH_4^+ loss in the AQDS and Fe_2O_3-free control, likely because of Feammox activity between sludge Fe and sludge NH_4^+ (Yang et al., 2019). The overall NO_2^- and NO_3^- production were minimal in all samples, but the highest NO_2^- production and NH_4^+ loss was in the AQDS + Fe_2O_3 treatments, which implies that AQDS enhanced Feammox activity (Yang et al., 2019). The NO_x^- can be readily reduced by OM or participate in NDFO. Yang et al. (2019) believe the NO_3^- and NO_2^- actively regenerated Fe(III) via NDFO, which may explain the FeOOH found in the Fe_2O_3 and AQDS+ Fe_2O_3 treatments. To supplement previous proposed reactions, Yang et al. (2019) propose a different Feammox pathway with NO_3^- as an end product (Eq. 24) and two NDFO mechanisms that regenerate Fe(III) while producing N_2 (Eqs. 25-26). Overall, Yang et al. (2019) implies that AQDS acted as a sustainable electron shuttle for Feammox and was regenerated by the Fe(III) source, but does not clarify if the NH_4^+ oxidation was connected to Fe reduction (Feammox), or if the two processes were independently supported by the AQDS.

(24) $8Fe(OH)_3 + 14H^+ + NH_4^+ \rightarrow 8Fe^{2+} + 21H_2O + NO_3^-$ $\Delta_rGm = -207$ kJ mol^{-1}
(25) $2NO_2^- + 6Fe^{2+} + 8H^+ \rightarrow 6Fe^{3+} + N_2 + 4H_2O$ $\Delta G^0 = -438.4$ kJ mol^{-1}
(26) $2NO_3^- + 10Fe^{2+} + 12H^+ \rightarrow 10Fe^{3+} + N_2 + 6H_2O$ $\Delta G^0 = -457.3$ kJ mol^{-1}

Ruiz-Urigüen et al (2019) used electrodes and AQDS to stimulate and explore Feammox for WWTP or *in situ* remediation applications. Some *in situ* work with electrodes in an acidic wetland involved a cathode in oxidized sediment and an anode in reduced sediment. Here, A6 successfully colonized the electrodes, resulting in Feammox-related current production (Ruiz-Urigüen et al., 2018). In a follow up reactor experiment, A6 colonized the anode of microbial electrolysis cells (MECs) and used it to oxidize NH_4^+ (Ruiz-Urigüen et al., 2019). The control was a treatment without an anode and cathode, and instead contained 2-line ferrihydrite. The number of A6 as similar between the MEC and 2-line ferrihydrite treatments (Ruiz-Urigüen et

al., 2019). Although AQDS facilitated the electron transfer between A6 and the anode and enhanced NH_4^+ oxidation, it may not be necessary once Feammox is established because after colonization the buildup of dead cells may functionally replace AQDS (Ruiz-Urigüen et al., 2019). This anode colonization result is not surprising, because the mechanisms bacteria use to transfer electrons to and from electrodes are often the same aid in the colonization of surfaces (Bird et al., 2011), e.g. Fe mineral surfaces in soil. The electrode is essentially replacing Fe(III) in this case (Ruiz-Urigüen et al., 2019), which may lower Fe input requirements and associated Fe waste accumulation (Bao and Li, 2018b) if Feammox were applied in WWTPs.

2.5.2 Feammox and co-degradation of other contaminants

Feammox may influence trichloroethylene (TCE) and tetrachloroethylene (PCE) degradation (Ge et al., 2019). When TCE and PCE were present in low concentrations (<10 mg L^{-1}) in a laboratory experiment they were degraded by 32-55% via reductive dehalogenation during Feammox (Ge et al., 2019). The degradation was only seen in NH_4^+ and ferrihydrite-amended treatments (Ge et al., 2019). In another study, enriching contaminated soils with A6 bacteria in a 100-day incubation experiment was associated with degradation of 60% of Per- and polyfluoroalkyl substances (PFAS) via reductive dehalogenation (Ruiz-Urigüen et al., 2019; Jaffé and Huang, 2019). Therefore, Feammox may help degrade other environmentally problematic contaminants.

2.5.3 Feammox and transformation of excess fertilizer nitrogen

Although Feammox is addressed in wastewater treatment, Yi et al. (2019) were the first to study Feammox in soils with different treatments of natural fertilizers and manufactured/commercial N fertilizers. They conducted a brief 10-day laboratory incubation using rice paddy soils with a pH between 6 and 7. The different soil treatments included: 1) unfertilized, 2) NPK fertilizer, 3) NPK fertilizer + manure, and 4) NPK fertilizer + crop straw. They amended $^{15}NH_4^+$ and C_2H_2 to these soils. Feammox related N-loss was between 3.6–24.9 kg N ha^{-1} y^{-1} in all treatments, with all treatments consuming $^{15}NH_4^+$ and generating $^{30}N_2$. Furthermore, the $^{30}N_2$ and $^{29}N_2$ production rates were highly correlated to Fe reduction rates. The anammox bacteria population decreased during the incubation while the A6 population increased. The soils with the NPK fertilizers had 3.4-5.8x more Feammox activity relative to

unfertilized soils and that difference was linked to higher reducible Fe(III) and A6 abundance in fertilized soils. In addition, the NPK fertilizer + manure, and NPK fertilizer + crop straw treatments had higher Feammox rates, as indicated by higher Fe(II) content and a larger A6 population (Yi et al., 2019). This is likely because the organic C acted as an electron shuttle which enhanced Feammox (Yi et al., 2019). Therefore, Feammox may reduce the negative impacts of N fertilizers, and co-amending soils with Fe(III) and organic residues may enhance Feammox in soil (Yi et al., 2019). However, Yi et al. (2019) also acknowledge that not all the Fe reduction was associated with Feammox in this study. Although the C_2H_2 and insufficient initial NO_x^- inhibited anammox and denitrification, the authors suspect that Feammox-denitrification may explain 20-45% of the N_2 losses (Yi et al., 2019). Yi et al. (2019) consider the following possibilities for why C_2H_2 does not inhibit Feammox, relative to anammox: (1) Feammox bacteria metabolic pathways do not use enzymes that are inhibited by C_2H_2 and (2) Feammox metabolism does not have the NH_4^+ activation step (Yi et al., 2019) that anammox require which is inhibited by C_2H_2 (Huang and Jaffé, 2015).

2.6 Project Objectives

2.6.1 Study site – Lomond, Alberta

The research site is located in Lomond, Alberta. It is a bulk fertilizer storage and distribution area located on the Western side of Lomond and has been in operation since 1979 (Nichols Environmental, 2018). The site includes aboveground storage tanks for fertilizer storage, blending, and distribution. A historic retail fuel outlet is located east of the fertilizer storage area (Nichols Environmental, 2018). Between 2016 and 2018, Nichols Environmental (Canada) Ltd. completed Phase I and II environmental site assessments (ESAs) of the fertilizer site which included groundwater and soil assessments using 2016 Alberta Tier 1 Guidelines for Commercial, Residential and Agricultural Land Use for fine-grained soils (Nichols Environmental, 2018; Government of Alberta, 2017). The fertilizer site is currently an active remediation site with Alberta Tier 1 guideline exceedances for groundwater ammonia-N, NO_3^-, and NO_2^- (Alberta Environment and Parks, 2016; Table 2.2, 2.3). In the ESAs the nutrients on site were delineated vertically, but not horizontally (Nichols Environmental, 2018).

Table 2.2. Soil pH and Ammonium-N and Nitrate concentrations in milligrams per kilogram (mg/kg) at the fertilizer contaminated site in Lomond, Alberta. M17 indicates the sampling year (2017) and the second numeral indicates the sample number. Depth (m) is the depth below ground of the soil core extracted. The physical location of the sample is noted with the Easting and Northern geographic coordinates.

Location	Depth (m)	Easting	Northing	pH	Ammonium-N (mg/kg)	Nitrate (mg/kg)
M17-16†	4	383426	5578956	7.8	1	54
M17-17	5.5	383441	5578881	7.8	0.9	32
M17-18	2	383462	5578801	8.4	0.4	30
M17-19	5.5	383499	5578721	7.9	0.5	<2§
M17-20	4	383466	5578617	8.1	5.6	13
M17-21	2.5	383414	5578635	8	2.5	<2
M17-22B	2.5	383347	5578754	8.5	12	3
M17-23	2.5	383336	5578728	**8.7**‡	5.5	<2
M17-24	4.5	383311	5578721	**8.6**	<0.4	<2
M17-25	2	383307	5578799	7.6	1.1	24
M17-26	4.5	383280	5578851	7.6	<0.4	14
M17-27	3.5	383318	5578965	7.9	<0.3	16
M17-28	2	383336	5578940	8.2	<0.4	53
M17-29	1.5	383318	5578750	7.8	<0.4	35

† Grey shading indicates soil core samples selected for the present study.

‡ Bold numbers indicate values that exceed Alberta Tier 1 guidelines, but note that there are no soil guidelines for ammonium in Alberta.

§ "<" indicates values that were below the detection limit of the equipment.

Table 2.3. Groundwater samples from the fertilizer contaminates site in Lomond, Alberta. M17 indicates analysis year and second numeral indicates location. Data includes depth below ground in meters, pH, total organic C (TOC), dissolved iron (Fe), total Fe, dissolved manganese, total manganese, Ammonia-N, Nitrate, and Nitrite concentrations in milligrams per liter (mg L^{-1}).

Location	Depth below ground (m)	pH	TOC (mg L^{-1})	Dissolved Fe (mg L^{-1})	Total Fe (mg L^{-1})	Dissolved Mn (mg L^{-1})	Total Mn (mg L^{-1})	Ammonia-N (mg L^{-1})	Nitrate (mg L^{-1})	Nitrite (mg L^{-1})
M17-16†	4	7.6	58	<0.05	147	**880**‡	5.29	**0.29**	**225**	0.16
M17-17	5.5	-¶	-	-	-	-	-	-	-	-
M17-18	2	7.6	73	<0.05	175	**945**	6.08	**0.126**	**154**	<0.05
M17-19	5.5	7.6	18.1	<0.05	0.51	**447**	0.42	<0.03	1.5	0.03
M17-20	4	7.6	46.8	<0.05	0.58	**1180**	.601	**49**	**66.1**	**14**
M17-21	2.5	7.2	24.6	<0.05	0.03	**554**	1.97	**.411**	1.09	0.03
M17-22B	2.5	7.3	31.6	**9.52**	14	**381**	3.38	**1.57**	<0.05	<0.02
M17-23	2.5	-	-	-	-	-	-	-	-	-
M17-24	4.5	7.4	100	**1.9**	137	**433**	5.85	**0.36**	<0.05	<0.02
M17-25	2	7.4	74.7	<0.05	72.3	**429**	3.13	**0.297**	**304**	**0.65**
M17-26	4.5	7.3	54.3	<0.05	0.04	**823**	0.24	**0.075**	**89.9**	0.6
M17-27	3.5	-	-	-	-	**900**	0.03	-	-	-
M17-28	2	7.4	54.9	<0.05	0.66	**900**	0.03	**0.051**	**186**	0.14
M17-29	1.5	7.4	46.8	0.05	0.3	**1310**	.624	**0.154**	**159**	0.54

† Grey shading indicates soil core samples selected for the present study.

‡ Bold numbers indicate concentrations that exceed Alberta Tier 1 guidelines.

§ "<" indicates values that were below the detection limit of the equipment.

¶ "-" signifies samples without data. These samples were either not located on site, buried under ice, or had insufficient water for data analysis (Nichols Environmental, 2018).

The soils in Lomond originate from glacial moraine till. The subsoil stratification at the fertilizer site consists of gravel fill overlaying silty clay which extends further than the deepest borehole (> 6 meters below ground) (Nichols Environmental, 2018). The brown silty clay soil has coal and Fe inclusions. Generally, these soils are neutral to alkaline (pH 7-9), calcareous, and clay-rich (Nichols Environmental, 2018). Surface water drainage on site is primarily via infiltration or overland flow to the southeast (Nichols Environmental, 2018). The pH range for the ten groundwater monitoring wells was between 8.5 and 9.6 (Nichols Environmental, 2018).

2.6.2 Identifying and stimulating Feammox in a 118-day anaerobic incubation with biostimulation experiments

Feammox requires a Fe(III) source, inorganic C, anaerobic conditions, NH_4^+, and Feammox microbial community. Although Feammox is more active in acidic soils (Clément et al., 2005; Huang and Jaffé, 2015, 2018), other work shows Feammox activity in near-neutral to alkaline conditions (Zhou et al., 2016; Huang and Jaffé, 2018; Li et al., 2018a; Yi et al., 2019). Therefore, Feammox is thermodynamically possible in neutral to alkaline soils, although typically it with lower efficiency than in acidic soils. Stimulating Feammox in laboratory incubations using neutral-alkaline soils will provide insight into whether Feammox is a viable bioremediation method to apply *in situ* in alkaline N-polluted Canadian soils. Feammox could remediate these soils by enhancing N_2 production without generating N_2O.

In this study, six soils from the Lomond site (Table 2.2, 2.3) were selected and used in a preliminary incubation experiment to determine which soil had the greatest dissolved NH_4^+-N loss when supplied with NH_4Cl and 2-line ferrihydrite. Amendments were added to that soil, M17-22B, in a 118-day anaerobic incubation in an attempt to stimulate Feammox. The groundwater characteristics for the M17-22B sample location included high dissolved total Fe, Mn and NH_4^+ (Table 2.3). For the 118-day anaerobic incubation, soil slurries were made with the M17-22B soil and treatments included one or two of the following NH_4^+ and Fe(III) amendments: NH_4Cl, 2-line ferrihydrite, and ferric citrate. The 118-day incubation included four sequential biostimulation experiments to assess microbial response to periodic Fe(III), NH_4^+, and vitamin amendments, with each biostimulation experiment having a distinct hypothesis. It is important to note that the use of 2-line ferrihydrite differs from previous research that uses 6-line ferrihydrite (Huang and Jaffé, 2015, 2018). Meanwhile, some research used 2-line ferrihydrite

(Ruiz-Urigüen et al., 2018; Ruiz-Urigüen et al., 2019) while other research does not specify which ferrihydrite type was used (Zhou et al., 2016; Bao and Li, 2017; Yang et al., 2018). The 2-line and 6-line ferrihydrite can be differentiated by XRD characterization which shows the former having two broad peaks and the latter having 6 broad peaks (Kukkadapu et al., 2003). The 2-line ferrihydrite can be considered "protoferrihydrite" since it is less crystalline and more thermodynamically unstable than the mineral ferrihydrite, or 6-line ferrihydrite, which it can transform into depending on certain conditions (Kukkadapu et al., 2003). For example, the transformation tends to occur more rapidly in anaerobic conditions, and can happen when DIRB stimulate intra-aggregate transformation of 2-line ferrihydrite to 6-line ferrihydrite (Kukkadapu et al., 2003). Overall, this indicates that 2-line ferrihydrite may be more accessible to the microbial community in the present Feammox incubation study, and even if it transforms to 6-line ferrihydrite this is still a viable Feammox stimulant that is expected to outperform ferric citrate (Huang and Jaffé, 2015, 2018). Therefore, the overall hypothesis for the 118-day incubation was that the 2-line ferrihydrite, and not ferric citrate, would stimulate Feammox activity and dissolved NH_4^+-N loss in near-neutral to alkaline anaerobic soil slurries. The timeline of four sequential biostimulation experiments can be visualized in fig. 2.2.

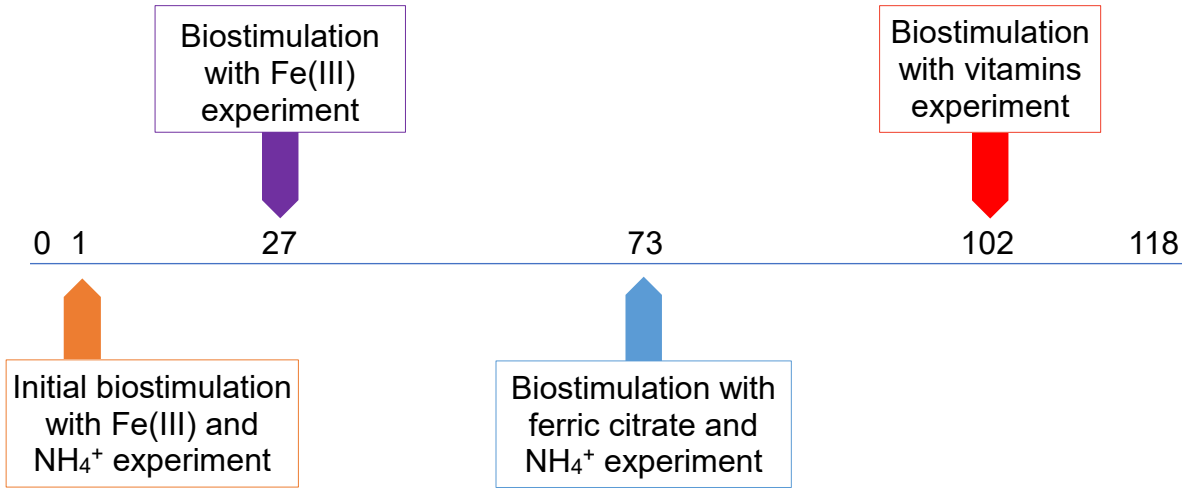

Fig. 2.2. Timeline of the four sequential Feammox biostimulation experiments during the 118-day anaerobic incubation. The numbers indicate the day of amendment and the beginning of the respective experiment.

In the first biostimulation experiment, labeled "Initial Biostimulation with Fe(III) and NH_4^+," the goal was to provide the microbial population in the samples with Fe(III) and NH_4^+ as key resources for Feammox activity. Ferric citrate, 2-line ferrihydrite, and NH_4Cl were added to respective treatments on day 1 of the 118-day anaerobic incubation. The hypothesis of this Initial Biostimulation with Fe(III) and NH_4^+ experiment was that the microbial population would reduce the Fe and consume or transform the NH_4^+, i.e. perform Feammox, and this would be evident in a decrease in dissolved Fe(III), increase in dissolved Fe(II), decrease in dissolved NH_4^+-N, and possibly dissolved NO_2^--N generation as an indicator of NH_4^+ oxidation. Colorimetry was used to detect dissolved Fe(II), Fe(III), NH_4^+, and NO_2^-. This colorimetry assessment was also used for the subsequent three biostimulation experiments.

The results of Initial Biostimulation with Fe(III) and NH_4^+ experiment included notable Fe reduction in the dissolved fraction, a statistically significant decrease in dissolved NH_4^+-N, and minimum dissolved NO_2^--N generation in the soil slurry treatment with ferric citrate + NH_4Cl. Therefore, the hypothesis of the first biostimulation experiment was partially supported by the results in the ferric citrate + NH_4Cl slurry treatment; however it was not clear if these results were evidence of Feammox without further experimental characterization. The ferrihydrite treatments with or without NH_4Cl showed minimal or negligible dissolved NH_4^+-N loss, NO_2^- production, and Fe reduction. The Fe in the ferric citrate slurries was reduced both visibly and in the dissolved fraction, but this was not seen in the ferrihydrite slurries. It is possible the Fe in the ferrihydrite treatments was not bioavailable for Feammox via transformation or due to pH constraints. In addition, there was some dissolved NH_4^+-N loss in the soil + ferric citrate and soil + ferrihydrite treatments, suggesting that soil endogenous NH_4^+ was consumed; therefore, Fe(III) alone may be capable of inducing soil NH_4^+-N loss without additional NH_4Cl. Therefore, in the second experiment, titled "Biostimulation with Fe(III)," 2-line ferrihydrite and ferric citrate were added to the Fe(III) treatments on Day 27 of the 118-day anaerobic incubation to assess whether Fe(III) was the primary limiting factor for dissolved NH_4^+-N loss, i.e. to see if NH_4^+-N loss could be sustained by only adding Fe(III). The hypothesis of this Biostimulation with Fe(III) experiment was that adding more Fe(III) would result in a decrease in dissolved NH_4^+-N without adding more NH_4Cl.

The results of the Biostimulation with Fe(III) experiment differed from the Initial Biostimulation with Fe(III) and NH_4^+ experiment. In the second experiment, the ferric citrate +

NH_4Cl treatment again had a statistically significant decrease in dissolved NH_4^+-N, but the loss was less than the first experiment. In this treatment the Fe(III) reduction was visible, but was not apparent in the dissolved fraction; instead, the dissolved Fe(II) notably decreased and Fe(III) notably increased during the experiment, suggesting potential Fe(III) production from the accumulated Fe(II) via oxidation or other pathways. Again, the ferrihydrite treatments showed no notable Fe reduction, decrease in dissolved NH_4^+-N, or dissolved NO_2^--N production, implying that ferrihydrite may not be active in these alkaline conditions despite repeated amendments. Therefore, the hypothesis for the Biostimulation with Fe(III) experiment was partially supported in the ferric citrate + NH_4Cl slurry treatment, given that there was a decrease in dissolved NH_4^+-N, but this did not explicitly correlate with Fe reduction. In addition, the dissolved NH_4^+-N loss in the ferric citrate + NH_4Cl treatment was less impressive than in the first biostimulation experiment, implying that the microbial population may need concurrent amendments of ferric citrate and NH_4Cl. Therefore, in the third experiment, titled "Biostimulation with Ferric citrate and NH_4^+," ferric citrate and NH_4Cl were added to respective treatments on day 73 of the 118-day anaerobic incubation. Ferrihydrite and/or NH_4Cl were not added to the ferrihydrite + NH_4Cl treatment, ferrihydrite treatment, and control since ferrihydrite showed little activity in previous biostimulation experiments. The hypothesis of this Biostimulation with Ferric citrate and NH_4^+ experiment was that fresh NH_4^+ and ferric citrate would stimulate Fe reduction and NH_4^+-N loss similar to the initial biostimulation experiment, particularly in the treatment with ferric citrate + NH_4Cl, with consideration that the residual Fe from the previous experiments might interfere with the reaction and Fe chemistry.

The results for dissolved NH_4^+-N in the Biostimulation with Ferric citrate and NH_4^+ experiment were similar to the first two biostimulation experiments. In the ferric citrate + NH_4Cl treatment, there was a statistically significant decrease in dissolved NH_4^+-N that was comparable to the initial biostimulation experiment. However, both the dissolved Fe(II) and Fe(III) notably decreased in this treatment, so Fe reduction was not as apparent as the first biostimulation experiment. It is possible that the dissolved Fe(III) was reduced, and the resultant Fe(II) partitioned or precipitated into the insoluble fraction, lowering the dissolved Fe(II) and Fe(III) levels. This could be tested with spectroscopic characterization of the solid Fe(III) phases, which was not included the present study. Therefore, the hypothesis of the Biostimulation with Ferric citrate and NH_4^+ experiment was partially supported in the ferric citrate + NH_4Cl treatment, as it

appeared that concurrent addition of NH_4^+ and Fe(III) via ferric citrate were necessary for a notable decrease in dissolved NH_4^+-N. However, these results imply that Fe bioavailability is now limited in the ferric citrate treatments but the slurries still contain dissolved NH_4^+. An alternative amendment could stimulate the microbes to use residual or regenerated Fe(III) to transform the residual NH_4^+. Since the incubation had been active for 102 days the soil microbes may require essential vitamins to stimulate Feammox activity. The hypothesis of the fourth and final biostimulation experiment, "Biostimulation with Vitamins", was that adding vitamin and molybdate solutions without adding Fe(III) or NH_4Cl would stimulate Fe reduction and a decrease in NH_4^+-N in the dissolved fraction. Vitamin and molybdate solutions were added to all treatments on day 102 of the 118-day anaerobic incubation. The four sequential biostimulation experiments in the 118-day anaerobic incubation are summarized below:

1) **Initial Biostimulation with Fe(III) and NH_4^+** - Add 2-line ferrihydrite, ferric citrate, NH_4Cl to respective treatments and controls that include ferrihydrite, ferric citrate, and/or ammonium on day 1 to determine if providing the microbial community with Fe(III) and NH_4^+ sources can stimulate Feammox.
2) **Biostimulation with Fe(III)** – Add 2-line ferrihydrite or ferric citrate to respective treatments and controls that included ferrihydrite or ferric citrate on day 27 to determine if adding more Fe(III) would stimulate a decrease in dissolved NH_4^+-N leftover from the initial biostimulation experiment.
3) **Biostimulation with Ferric citrate and NH_4^+** - Add ferric citrate and/or NH_4Cl to respective treatments and controls that included ferric citrate and/or ammonium on day 73 to determine if this concurrent addition of ferric citrate and NH_4Cl would reproduce results from the Initial Biostimulation with Fe(III) and NH_4^+ experiment.
4) **Biostimulation with Vitamins** – Add vitamin and molybdate solutions to all treatments and controls on day 102 to determine whether the microbial community needed vitamins to stimulate Feammox activity with accumulated NH_4^+ and Fe from previous biostimulation amendments.

2.6.3 Stimulating NH_4^+-N loss in a 94-day anaerobic incubation subculture experiment

Following the 118-day anaerobic incubation, the slurry treatment that showed the greatest decrease in dissolved NH_4^+-N and notable Fe reduction and/or oxidation—ferric citrate + NH_4Cl, was subcultured. Since this treatment exhibited the greatest decrease in dissolved NH_4^+-N in the 118-day incubation it was expected that this effect could be sustained once subcultured. Therefore, the ferric citrate + NH_4Cl slurries were subdivided, diluted, provided fresh NH_4Cl and ferric citrate, and incubated in anaerobic conditions for 94 days. The hypothesis of the subculture experiment was that the dissolved NH_4^+-N would decrease, i.e. the decrease in dissolved NH_4^+-N results from the 118-day incubation could be sustained in the subculture experiment.

3. MATERIALS AND METHODS

3.1 Soil preparation and preliminary incubation experiment

3.1.1 Lomond soil sample collection

The study site is in southern Alberta and has elevated groundwater ammonia-N and NO_3^- in some locations (Table 2.3). The soils on site are alkaline (pH 8-9) (Table 2.2) and clay-rich. Six soil cores (M17-16, -20,-21,-22B,-28, and -29) with elevated groundwater and/or soil NH_4^+-N were selected for a preliminary incubation. The soil cores were collected on site in September 2018 with polyethylene column containers (10 cm diameter, 1.5 m long) and transported to the laboratory within 7 days. The cores were frozen at -20°C until use. In August 2019, the cores were thawed and subsampled. Subsampling followed incremental sampling methodology from Hyde et al. (2018). Here, 2 g soil subsamples were collected with a Terracore device every 5 cm along the length of the core. The 2 g subsamples were combined, homogenized by hand, and frozen at -20°C until use in soil slurry preparation.

3.1.2 Preliminary incubation experiment to select soil for 118-day anaerobic incubation

A 30-day preliminary incubation experiment was conducted to identify the core with the greatest decrease in dissolved NH_4^+-N. The Lomond soil core subsamples (M17-16, -20, -21, -22B, -28, and -29) were used to create six anaerobic soil slurries (n=1). Slurries were created with a 1:20 ratio of soil:argon-purged MilliQ water (18.2 MΩ × cm purity), and one control was created which contained only argon-purged MilliQ water. All samples were prepared in 50 mL falcon tubes within an argon-filled anaerobic glovebox. All samples were stored in the dark for 6 days at room temperature (20-25°C) to stabilize prior to adding the initial amendments. Amendments were added to all samples until the concentrations were 12 mM 2-line ferrihydrite, 2 mM NH_4Cl, and 1.2 mM $NaHCO_3$. The 2-line ferrihydrite and $NaHCO_3$ were only added at the beginning of the incubation, but there were two spikes of 2 mM NH_4Cl, first on day 1 and again

on day 22. The 2-line ferrihydrite was synthesized in a three-step process. First a 0.4 M ferric chloride hexahydrite solution was prepared (pH adjusted to 6.5 with 1 M NaOH). Then the mixture was centrifuged with MilliQ water to extract solid ferrihydrite precipitate. Finally, the ferrihydrite pellet was suspended to create a 0.4 M stock solution of 2-line ferrihydrite which was used for amendments. The $NaHCO_3$ was added as a source of inorganic C to stimulate autotrophic activity. The samples were incubated within the glovebox at room temperature (20-25°C) in the dark when not actively sampling.

Inside the argon-purged glovebox 1 ml subsamples were periodically extracted from the samples into 1.5 mL microcentrifuge tubes, centrifuged, and the supernatant and pellet were separated. The supernatant was used to assess dissolved Fe(II) inside the glovebox, and used to assess total dissolved Fe(III), NO_2-N, NO_3-N, and NH_4^+-N outside the glovebox. All concentrations were assessed using an iMark™ Microplate Absorbance Reader (Bio-Rad, California, USA). The extractable dissolved NH_4^+-N was analyzed with the ammonium nitrogen extraction colorimetric method (Baethgen and Alley, 1989) at 655 nm within 4 hours of subsampling. The dissolved NO_2^--N and NO_3^--N were analyzed with a vanadium chloride method (Doane and Horwath, 2003) and measured at 545 nm. Dissolved total Fe and Fe(II) were assessed with the ferrozine colorimetric method (Viollier et al., 2000) at 570 nm and the dissolved Fe(III) was calculated by subtracting dissolved Fe(II) from total dissolved Fe.

In all soil slurries the pH was between 8 and 9 on day 1 and between 7.5 and 9 day 30, indicating minimal change. Conversely, the pH in the control changed notably, from 6.4 on day 1 to 9 on day 30. After both NH_4Cl spikes, the greatest decrease in dissolved NH_4^+-N was in the sample with soil M17-22B (13% loss after the first spike, 23% loss after the second spike). When the 2-line ferrihydrite was added the Fe(III) slightly decreased by 0.85 mg L^{-1} and Fe(II) slightly decreased by 0.45 mg L^{-1} relative to the unamended slurries at the beginning of the incubation. This may indicate rapid Fe reduction immediately after the ferrihydrite amendment; however, the dissolved Fe-species were not tracked subsequently due to time constraints, so further changes in Fe(III) and Fe(II) cannot be identified. However, it is still possible that the added Fe(III) and/or the soil Fe(III) minerals influenced the changes in dissolved Fe and the decrease in dissolved NH_4^+-N. In the M17-22B sample the pH decreased from 8.3 on day 1 to 7.6 on day 30, which may indicate proton generation via redox reactions like denitrification (Bernhard, 2010) and Feammox (Huang and Jaffé, 2015). Furthermore, the M17-22B sample produced the most NO_2^--

N relative to the other samples, up to 0.286 mg L^{-1}, which is a common Feammox end product (Clément et al., 2005; Yang et al., 2012; Huang and Jaffé, 2015, 2018). However, this suspected Feammox evidence with the M17-22B sample cannot be confirmed without more replication in a follow-up experiment. Therefore, since the M17-22B sample exhibited suspected Feammox activity this soil was chosen to conduct the long-term 118-day anaerobic incubation with Feammox biostimulation experiments.

3.2 118-day incubation with sequential biostimulation experiments

A 118-day anaerobic incubation was conducted from October 2019-February 2020 and included four sequential biostimulation experiments: 1) Initial Biostimulation with Fe(III) and NH_4^+, 2) Biostimulation with Fe, 3) Biostimulation with Ferric citrate and NH_4^+, and 4) Biostimulation with Vitamins (fig. 2.2). For the 118-day anaerobic incubation, 2 g moist homogenized soil from the M17-22B soil core was subdivided among sterilized 75 mL serum bottles. Then 60 mL MilliQ water was added to create soil slurries with a 1:30 soil to water ratio. The controls consisted of sterilized 75 mL serum bottles with 60 mL MilliQ water and no soil. All serum bottles were sealed with septa and purged thoroughly with an 80:20 N_2/CO_2 mixture for 30 minutes, resulting in a final pH of ~6.5 in soil slurries and pH of ~4.5 in control samples. The CO_2 and N_2 are both inert gases and 80% N_2 is meant mimic the 78% N_2 in the earth's atmosphere. All samples were stored in the dark for one week at room temperature (20-25°C) to stabilize prior to the initial sampling and four experiments. Throughout the incubation, the anaerobic samples were stored in the dark at room temperature when not actively sampling or adding amendments.

Soil slurries and controls were treated and incubated with two different Fe(III) sources to stimulate Feammox: 2-line ferrihydrite and ferric citrate. The 2-line ferrihydrite was synthesized following the same method as the 30-day preliminary incubation experiment, but the solution pH was adjusted to ~5 with 1 M NaOH while the pellet was suspended in the 0.4 M stock solution. A 0.4 M ferric citrate solution was with solid ferric citrate and the pH was adjusted to ~5.5 with 1 M NaOH. The pH of the Fe(III) sources was buffered to minimize the pH change in the samples during amendment, because the original ferrihydrite and ferric citrate solution pH was 1-3 but the goal of this incubation was to assess Feammox activity in near-neutral to alkaline conditions. A 0.4 M NH_4Cl stock solution was made without buffering since the solution pH was

5-5.5. All amendment solutions were then thoroughly purged with an 80:20 N_2/CO_2 mix. There was one replicate of the controls (1-3) and three replicates of the soil slurry treatments (4-8) using the M17-22B soil, with 2-line ferrihydrite abbreviated as FH, ferric citrate abbreviated as FC, and NH_4Cl abbreviated as A:

1) **NS-FCA**: No soil + ferric citrate + NH_4Cl
2) **NS-FHA**: No soil + 2-line ferrihydrite + NH_4Cl
3) **NS-A**: No soil + NH_4Cl
4) **S-A**: Soil + NH_4Cl
5) **S-FH**: Soil + 2-line ferrihydrite
6) **S-FC**: Soil + ferric citrate
7) **S-FHA**: Soil + 2-line ferrihydrite + NH_4Cl
8) **S-FCA**: Soil + ferric citrate + NH_4Cl

Before the first experiment (day 0) in the 118-day anaerobic incubation, the dissolved NH_4^+-N, NO_2^--N, Fe(II) and Fe(III) were measured for all samples. The first experiment, Initial Biostimulation with Fe(III) and NH_4^+, consisted of adding amendments to the anaerobic samples on day 1 so the concentrations were as follows: 12 mM Fe(III) via 2-line ferrihydrite in S-FHA, S-FH, and NS-FHA, 12 mM Fe(III) via ferric citrate in S-FCA, S-FC, and NS-FCA, and 2 mM NH_4^+ via NH_4Cl in S-A, S-FHA, S-FCA, and NS-A. Amendments were added inside the N_2-purged anaerobic glovebox. The septa were temporarily removed to add amendments, so the bottles were then thoroughly purged with an 80:20 N_2/CO_2 mixture for 30 minutes.

The second experiment, Biostimulation with Fe(III), consisted of adding amendments to the anaerobic samples on day 27 so the concentrations were as follows: 12 mM Fe(III) via 2-line ferrihydrite in S-FHA, S-FH, and NS-FHA and 12 mM Fe(III) via ferric citrate in S-FCA, S-FC, and NS-FCA. Amendments were added inside the N_2-purged anaerobic glovebox and the bottles were then thoroughly purged with an 80:20 N_2/CO_2 mixture.

The third experiment, Biostimulation with Ferric citrate and NH_4^+, consisted of adding amendments on day 73 so the concentrations were as follows: 12 mM Fe(III) via ferric citrate in S-FCA, S-FC, and NS-FCA and 2 mM NH_4^+ via NH_4Cl in S-A, S-FCA, and NS-A. Amendments were added inside the N_2-purged anaerobic glovebox. The bottles were then thoroughly purged with an 80:20 N_2/CO_2 mixture.

The fourth experiment, Biostimulation with Vitamins, involved spiking all anaerobic treatments and controls on day 102 with 20 µLof 1.15% (w/v) sodium molybdate solution and 40 µL of vitamin solution (the stock vitamin solution consisted of 20 mg L^{-1} biotin, 20 mg L^{-1}

folic acid, 50 mg L^{-1} riboflavin, 50 mg L^{-1} pantothenic acid, 50 mg L^{-1} para-aminobenzoic acid, 50 mg L^{-1} vitamin B12, 50 mg L^{-1} nicotinamide, 100 mg L^{-1} pyridoxine HCl, 50 mg L^{-1} thiamine HCl, and 50 mg L^{-1} thioctic acid). Amendments were added inside the N_2-purged anaerobic glovebox. The bottles were then thoroughly purged with an 80:20 N_2/CO_2 mixture.

3.2.1 Analysis of pH and dissolved NH_4^+-N, Fe(II), Fe(III), and NO_2^--N

The solution pH was analyzed in all the anaerobic samples on day 1, day 27, and day 119 using a handheld pH probe inside the N_2-purged glovebox. The samples were periodically subsampled for chemical analysis of dissolved N- and Fe-species using the same methodology as the preliminary 30-day incubation experiment, except in a N_2-purged glovebox. Following each sampling session the headspace of all samples was purged with an 80:20 N_2/CO_2 mixture. Dissolved total Fe and Fe(II), NO_2^--N, and NH_4^+-N were assessed with the same methodology as the 30-day preliminary incubation experiment. After analysis, the separated supernatant and pellets were frozen and preserved at -20°C. The NO_2^- was analyzed with the vanadium chloride method (Doane and Horwath, 2003) on the subsampling day, or analyzed on thawed supernatant later. All absorbance readings were converted to mM concentrations using the standard curve and then converted to mg L^{-1}.

3.2.2 Statistical analysis

When assessing the significance of the decrease in dissolved NH_4^+-N for each experiment, a two-sample T-test assuming unequal variances was performed with Excel 2016 (Microsoft, 2016) to assess if the change in dissolved NH_4^+-N lost in each treatment (n=3) was significantly different from zero in each experiment.

3.2.3 PCR analysis

A PCR analysis was used to amplify 16S rRNA genes from pellets periodically extracted during the 118-day anaerobic incubation. The DNA was extracted from the pellets using the FastDNA spin kit for soil (Qiagen, Hilden, Germany). The DNA concentration was quantified using a Qubit fluorometer (dsDNA assay kit). The initial PCR was performed with 16s primers (342F and 806R) using a PCR mastermix consisting of dreamtaq buffer, primers, nucleotides, dreamtaq hot start, and nuclease-free water. The PCR was run on the Thermocycler under the

following conditions: 2 min at 94°C, 4 sec at 94°C, 25 cycles of 1 min at 50°C, 1.3 min at 72°C, 10 min at 72°C, and held at 4°C until removed. All products were run on a 1.5% agarose gel and imaging was done with a gel documentation system (Axygen, Corning Inc. California, USA).

A follow-up PCR analysis was conducted to amplify any 16S rRNA genes belonging to *Acidimicrobiaceae* sp. A6, i.e. the Feammox bacteria. The A6 primers, 1055F and 1392R (Huang and Jaffé, 2018), and mastermix mentioned previously were used for the PCR run on the Thermocycler under the following conditions: 30 sec at 95°C, 5 sec at 94°C, 40 cycles of 30 sec at 55°C, 30 sec at 70°C, and 4°C until removed. All products were run on 1.5% agarose gel.

3.3 Subculture experiment of ferric citrate + NH_4Cl slurry treatment from the 118-day anaerobic incubation

A follow-up 94-day subculture experiment was performed using the slurry treatment that was the most active throughout the 118-day incubation, the S-FCA treatment. Each of the three S-FCA samples were subdivided into three subcultures 7 days after the end of the 118-day incubation. Three S-FCA treatments (n=3) and no-soil controls (n=3) were created with a 1:30 soil to MilliQ water ratio for treatments, or just milliQ water (18.2 MΩ × cm purity) for the controls. The twelve bottles were purged with an 80:20 N_2/CO_2 mix for 1 hour, spiked with 12 mM ferric citrate and 2 mM NH_4Cl, and incubated at room temperature (20-25°C) in a dark place for 94 days. The dissolved NH_4^+-N, Fe(II), and Fe(III) was periodically analyzed using the same methodology as the preliminary incubation and 118-day incubation.

4. RESULTS

4.1 118-day Anaerobic Incubation with sequential biostimulation experiments

4.1.1 pH in the 118-day anaerobic incubation

The pH measurements are available in Table 4.1. There was some variation in pH between the soil slurry treatments, where the pH in S-A remained consistently lower than other treatments. The slurry pH fluctuated minimally throughout the incubation, and there was a slight increase in pH in all soil slurries at the end of the anaerobic incubation (day 119) relative to initial pH values. The controls had a lower pH than the soil slurries and the pH increased in NS-A, but decreased in NS-FCA and NS-FHA (Table 4.1).

Table 4.1. Average pH results (mean ± SE for soil slurries (n=3), n=1 for no-soil controls) in no-soil controls (NS-) and soil slurries (S-) in the 118-day anaerobic incubation when treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). The initial column indicates the pH results before any amendment additions. Between day 0 and day 27 an Initial Biostimulation with Fe(III) and NH_4^+ experiment was conducted where FC, FH, and A were added to respective treatments. After day 27 a two experiments were conducted, first a Biostimulation with Fe(III) experiment where FC and FC were added to respective treatments on day 27 followed by a Biostimulation with Ferric citrate and NH_4^+ experiment where FC and A were added to respective treatments on day 73. On day 102 a vitamin and molybdate solution was added to all samples.

	Initial	**Before Biostimulation with Fe(III) experiment**	**After Biostimulation with Vitamins experiment**
Day	*0*	*27*	*119*
NS-A	4.5	4.2	5.5
NS-FCA	4.7	4.3	3.9
NS-FHA	4.6	4.1	4.3
S-A	6.4 ± 0.06	6.2 ± 0.16	6.5 ± 0.07
S-FC	6.4 ± 0.02	6.2 ± 0.06	6.8 ± 0.08
S-FH	6.5 ± 0.02	6.6 ± 0.09	6.8 ± 0.03
S-FCA	6.5 ± 0.05	6.5 ± 0.13	6.8 ± 0.10
S-FHA	6.5 ± 0.01	6.6 ± 0.07	6.8 ± 0.09

4.1.1 Observations of the Initial Biostimulation with Fe(III) and NH_4^+ Experiment

In this initial experiment, I ensured that there was available Fe(III) and/or NH_4^+ in the samples since the goal was to stimulate Feammox by adding 12 mM of Fe(III) via ferric citrate to NS-FCA, S-FC, and S-FCA, 12 mM Fe(III) via 2-line ferrihydrite to NS-FHA, S-FH, and S-FHA, and 2 mM NH_4^+ via NH_4Cl to NS-A, NS-FCA, NS-FHA, S-A, S-FCA, and S-FHA. There was a notable decrease in dissolved NH_4^+-N and Fe reduction observed in the S-FCA treatment.

4.1.2.1 Dissolved NH_4^+-N

In the Initial Biostimulation with Fe(III) and NH_4^+ experiment, the decrease in dissolved NH_4^+-N in S-FC, S-FH, and S-FCA were all significant (Table 4.2, A-4). The initial soil NH_4^+-N levels before amendment were only 1 ± 0.3 mg L^{-1} in S-FC and 1 ± 0.5 mg L^{-1} in S-FH; therefore, the significant decrease in dissolved NH_4^+-N implies that the ferrihydrite and ferric citrate amendments depleted soil endogenous NH_4^+. Conversely, the average decrease in dissolved NH_4^+-N in S-FCA was both significant and substantial, changing from 28 ± 0.6 mg L^{-1} to 14 ± 2.1 mg L^{-1} dissolved NH_4^+-N. The S-FHA treatment exhibited minimal dissolved NH_4^+-N loss. The NH_4^+-N loss for the three controls varied, with a notable dissolved NH_4^+-N increase in NS-A which was similar to S-FCA and less notable changes in NS-FCA and NS-FHA.

Table 4.2. Average change in dissolved NH_4^+-N (mean ± SE for slurries (n=3), n=1 for no-soil controls) in no-soil controls (NS-) and soil slurries (S-) in milligrams per liter (mg L^{-1}) the anaerobic incubation when treated with ammonium (A), ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric Citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (and no Fe(III) or NH_4Cl) were added to all samples.

	Initial Biostimulation with Fe(III) and NH_4^+	**Biostimulation with Fe(III)**	**Biostimulation with Ferric citrate and NH_4^+**	**Biostimulation with Vitamins**
NS-A	*13.8*† ‡	4.2§	*1.1*	16.1
NS-FCA	***-1.2*** ¶ #	**-1.6**††	***-2.0***	-16.4
NS-FHA	***3.4***	**-7.3**	-7.7	5.7
S-A	*-0.8* ± *3.38*	1.4 ± 5.03	*-5.9* ± *5.77*	-4.5 ± 10.17
S-FC	**-4.2**‡‡ **±** **0.21**	**0.7** **±** **1.16**	**-3.9** **±** **8.61**	1.6 ± 0.94
S-FH	**-1.9** **±** **0.79**	**-0.6** **±** **0.15**	-1.6 ± 1.92	0.7 ± 0.99
S-FCA	*-13.5* ± *1.70*	**-4.6** **±** **2.02**	***-13.0*** **±** ***6.51***	13.0 ± 11.51
S-FHA	*1.2* ± *1.46*	**-1.0** **±** **2.59**	-2.0 ± 2.51	5.6 ± 6.83

† Numbers with italic font indicates that the samples were amended with NH_4Cl (A) in the experiment.
‡ Positive numbers indicate a gain in dissolved NH_4^+-N.
§ Numbers with plain font indicates that the samples were not amended with Fe(III) or A in the experiment.
¶ Negative numbers indicate a loss in dissolved NH_4^+-N.
# Numbers with bold and italic font indicates that the samples were amended with that Fe(III) and A in the first three experiment.
†† Numbers with bold font indicates that the samples were amended with Fe(III) in the first three experiments.
‡‡ Grey shading indicates a change in dissolved NH_4^+-N that was significantly different than zero ($p<0.05$) in a two-sample T-test assuming unequal variances for the treatments (n=3). All other values were not significantly different ($p>0.05$).

4.1.2.2 Dissolved NO_2^-, Fe(III), and Fe(II)

The average dissolved NO_2^--N values were low (all <1 mg L^{-1}) and decreased slightly in all treatments during the Initial Biostimulation with Fe(III) and NH_4^+ experiment (Table 4.3, A-3). However, it is important to note that some of the dissolved NO_2^--N values were negative, which indicates that the standard curve blank contained trace NO_2^- or that the values fell below the equipment's detection limit (Table 4.3). Furthermore, a similar issue occurred in the dissolved Fe(II) and Fe(III) absorbance readings. The S-FCA treatment had notable dissolved

NH_4^+-N loss and a slight decrease in NO_2^--N in this treatment, indicating that the decrease in dissolved NH_4^+-N did not correspond to NO_2^- production.

For all treatments a decrease in dissolved Fe(III) corresponded to an increase in dissolved Fe(II). The S-FC, S-FCA, and NS-FC treatments had the largest decrease in dissolved Fe(III) (Table 4.4, A-2) and the largest increase in dissolved Fe(II) (Table 4.5, A-1), indicating Fe reduction. In this experiment the S-FCA and S-FC also exhibited visible Fe reduction, where the yellow solution turned to grey. This color change was not seen in NS-FCA. The S-FH, S-FHA, and NS-A treatments exhibited minimal change in dissolved Fe(II) and Fe(III) concentrations (Table 4.4, 4.8). Lab synthesized ferrihydrite can turn from dark red to dark grey when the Fe is reduced (Huang and Jaffé, 2018), or other colors like orange and light red when another mineral like hematite or goethite is formed. In this experiment the ferrihydrite treatments changed from a dark red to a dark orange color after amendment, which may indicate some Fe reduction or transformation into hematite or goethite, which can occur with reactive ferrihydrite (Adhikari et al., 2017). The ratios of NH_4^+-N loss to NO_2^- produced and Fe(II) produced in S-FCA (Table 4.6) imply that there is evidence of Feammox in the dissolved fraction, with Fe reduction appearing to correspond with NH_4^+ oxidation and minimal NO_2^- production.

Table 4.3. Average dissolved NO_2^--N (mg L^{-1}) (mean ± SE for soil slurries (n=3) n=1 for no-soil controls) in no-soil controls (NS-) and soil slurries (S-) in the anaerobic incubation when treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric Citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (and no Fe(III) or NH_4Cl) were added to all samples.

	Initial Biostimulation with Fe(III) and NH_4^+		**Biostimulation with Fe(III)**		**Biostimulation with Ferric Citrate and NH_4^+**		**Biostimulation with Vitamins**	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
NS-A	*0.1*†	*-0.5*‡	-0.5§	-0.7	*-0.1*	*0.1*	0.0	0.1
NS-FCA	***0.7***¶	***0.3***	**-0.2**#	**0.1**	***-0.1***	***0.0***	-0.1	0.2
NS-FHA	***0.0***	***-0.4***	**-0.4**	**-0.8**	-0.1	0.1	0.0	0.4
S-A	*0.0 ± 0.11*	*-0.6 ± 0.17*	-0.7 ± 0.72	-0.6 ± 0.22	*-0.1 ± 0.01*	*0.2 ± 0.06*	-0.1 ± 0.05	0.2 ± 0.65
S-FC	**0.6 ± 0.07**	**0.1 ± 0.24**	**0.6 ± 0.23**	**-0.5 ± 0.20**	**0.0 ± 0.05**	**0.1 ± 0.05**	0.0 ± 0.05	0.3 ± 0.09
S-FH	**0.0 ± 0.03**	**-0.6 ± 0.16**	**-0.6 ± 0.27**	**-0.4 ± 0.14**	-0.1 ± 0.02	0.3 ± 0.12	-0.1 ± 0.03	0.3 ± 0.19
S-FCA	***0.6 ± 0.03***	***0.0 ± 0.11***	**0.3 ± 0.38**	**-0.8 ± 0.02**	***0.0 ± 0.05***	***0.1 ± 0.05***	-0.2 ± 0.08	0.3 ± 0.05
S-FHA	***0.0 ± 0.03***	***-0.6 ± 0.14***	**-0.6 ± 0.16**	**-0.7 ± 0.11**	-0.1 ± 0.04	0.2 ± 0.08	-0.2 ± 0.06	1.3 ± 1.06

† Numbers with italic font indicates that the samples were amended with NH_4Cl (A) in the experiment.
‡ Negative numbers were below the equipment detection limit or the standard curve (0 mg L^{-1})
§ Numbers with plain font indicates that the samples were not amended with Fe(III) or A in the experiment.
¶ Numbers with bold and italic font indicates that the samples were amended with that Fe(III) and A in the first three experiment.
# Numbers with bold font indicates that the samples were amended with Fe(III) in the first three experiments.

Table 4.4. Average dissolved Fe(III) (mg L^{-1}) (mean ± SE for soil slurries (n=3) n=1 for no-soil controls) in no-soil controls (NS-) and soil slurries (S-) in the anaerobic incubation when treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (and no Fe(III) or NH_4Cl) were added to all samples.

	Initial Biostimulation with Fe(III) and NH_4^+		**Biostimulation with Fe(III)**		**Biostimulation with Ferric citrate and NH_4^+**		**Biostimulation with Vitamins**	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
NS-A	*2*†	*3*	6‡	4	*11*	*-5*§	4	1
NS-FCA	***105***¶	***50***	**384**#	**357**	***620***	***495***	523	500
NS-FHA	***2***	***2***	**7**	**6**	13	-4	6	-165
S-A	*3 ± 0.3*	*38 ± 65.7*	5 ± 0.3	3 ± 0.5	*12 ± 0.4*	*-4 ± 0.7*	5 ± 0.2	1 ± 0.1
S-FC	**72 ± 18.5**	**-26 ± 5.1**	**38 ± 3.5**	**10 ± 1.4**	**261 ± 35.2**	**-3 ± 0.6**	13 ± 3.3	5 ± 1.6
S-FH	**3 ± 0.2**	**3 ± 0.1**	**5 ± 0.3**	**4 ± 0.5**	10 ± 0.3	-7 ± 4.7	5 ± 1.9	1 ± 0.3
S-FCA	***82 ± 27.8***	***-19 ± 4.3***	**-104 ± 4.5**	**-13 ± 41.9**	***204 ± 20.2***	***-4 ± 1.2***	16 ± 4.8	7 ± 2.0
S-FHA	***3 ± 0.7***	***6 ± 5.1***	**5 ± 0.8**	**2 ± 1.9**	10 ± 2.1	-6 ± 1.2	3 ± 2.6	1 ± 1.4

† Numbers with italic font indicates that the samples were amended with NH_4Cl (A) in the experiment.
‡ Numbers with plain font indicates that the samples were not amended with Fe(III) or A in the experiment.
§ Negative numbers were below the equipment detection limit or the standard curve (0 mg L^{-1}).
¶ Numbers with bold and italic font indicates that the samples were amended with that Fe(III) and A in the first three experiment.
# Numbers with bold font indicates that the samples were amended with Fe(III) in the first three experiments.

Table 4.5. Average dissolved Fe(II) (mg L^{-1}) (mean ± SE for soil slurries (n=3) n=1 for no-soil controls) in no-soil controls (NS-) and soil slurries (S-) in the 118-day anaerobic incubation when treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (and no Fe(III) or NH_4Cl) were added to all samples.

	Initial Biostimulation with Fe(III) and NH_4^+		**Biostimulation with Fe(III)**		**Biostimulation with Ferric citrate and NH_4^+**		**Biostimulation with Vitamins**	
	Initial	*Final*	*Initial*	*Final*	*Initial*	*Final*	*Initial*	*Final*
NS-A	*-2*†‡	*-17*	-13§	-7	*-38*	*-17*	-16	-12
NS-FCA	***17***¶	***35***	**196**#	**80**	***62***	***91***	100	89
NS-FHA	***-2***	***-17***	**-13**	**-8**	-40	-17	-15	154
S-A	*-2 ± 0.5*	*-18 ± 0.4*	-13 ± 0.2	-7 ± 0.4	*-41 ± 0.3*	*-16 ± 0.7*	-15 ± 1.8	-12 ± 0.3
S-FC	**2 ± 3.5**	**182 ± 6.5**	**576 ± 14.1**	**65 ± 29.6**	**48 ± 38.6**	**10 ± 5.0**	-13 ± 1.1	-12 ± 0.8
S-FH	**-2 ± 0.2**	**-18 ± 0.1**	**-13 ± 0.0**	**-8 ± 0.4**	-42 ± 0.6	-14 ± 6.1	-15 ± 3.1	-12 ± 1.2
S-FCA	***3 ± 0.5***	***181 ± 6.0***	**723 ± 8.8**	**38 ± 13.8**	***42 ± 17.7***	***10 ± 3.9***	-9 ± 3.2	-8 ± 1.7
S-FHA	***-2 ± 0.2***	***-17 ± 0.7***	**-13 ± 0.4**	**-5 ± 2.9**	-42 ± 2.0	-13 ± 1.5	-13 ± 2.6	-12 ± 0.8

† Numbers with italic font indicates that the samples were amended with NH_4Cl (A) in the experiment.

‡ Negative numbers were below the equipment detection limit or the standard curve (0 mg L^{-1})

§ Numbers with plain font indicates that the samples were not amended with Fe(III) or A in the experiment.

¶ Numbers with bold and italic font indicates that the samples were amended with that Fe(III) and A in the first three experiment.

Numbers with bold font indicates that the samples were amended with Fe(III) in the first three experiments.

Table 4.6. Ratio of dissolved NH_4^+ loss that corresponds to dissolved NO_2^- and Fe(II) produced for the most active anaerobic soil slurry treatment: soil with ferric citrate and ammonium chloride (S-FCA) in the 118-day anaerobic incubation. Ratios were calculated using the average initial and final concentrations (mg L^{-1}) in each experiment. In the Initial Biostimulation with Fe(III) and NH_4^+ experiment, NH_4Cl and ferric citrate were added to S-FCA; in the Biostimulation with Fe(III) experiment ferric citrate was added to S-FCA; in the Biostimulation with Ferric citrate and NH_4^+ experiment NH_4Cl and ferric citrate were to S-FCA; and in the Biostimulation with Vitamins experiment vitamin and molybdate solutions (and no Fe(III) or NH_4Cl) were added to S-FCA.

	Initial Biostimulation with Fe(III) and NH_4^+	**Biostimulation with Fe(III)**	**Biostimulation with Ferric citrate and NH_4^+**	**Biostimulation with Vitamins**
	NH_4^+ loss : NO_2^- produced	*NH_4^+ loss : NO_2^- produced*	*NH_4^+ loss : NO_2^- produced*	*NH_4^+ loss : NO_2^- produced*
NS-FCA	1 : 0.0	1 : -0.2	1 : 0.0	-1 : 0.0
	NH_4^+ loss : Fe(II) produced	*NH_4^+ loss : Fe(II) produced*	*NH_4^+ loss : Fe(II) produced*	*NH_4^+ loss : Fe(II) produced*
NS-FCA	1 : 13.1	1 : -148.0	1 : -2.5	-1 : -0.1

† A negative value for Fe(II)/NO_2^- produced signifies a decrease in dissolved NO_2^--N or Fe(II) relative to an decrease in dissolved NH_4^+-N. A negative value for NH_4^+ loss signifies an increase in dissolved NH_4^+-N.

4.1.2 Observations of the Biostimulation with Fe(III) Experiment

In this experiment the goal was to determine if adding fresh Fe(III) would be sufficient to stimulate loss of residual NH_4^+-N from the Initial Biostimulation with Fe(III) and NH_4^+ experiment, i.e. if Fe(III) was the primary limiting resource for continued dissolved NH_4^+-N loss. Therefore, 12 mM Fe(III) via ferric citrate was added to NS-FCA, S-FC, and S-FCA and 12 mM Fe(III) via 2-line ferrihydrite was added to NS-FHA, S-FH, and S-FHA. In S-FCA there was some a significant decrease in dissolved NH_4^+-N after amending with fresh Fe(III), but the amount was less than in the Initial Biostimulation experiment.

4.1.3.1 Dissolved NH_4^+-N

In the Biostimulation with Fe(III) experiment, ferrihydrite and ferric citrate addition caused some changes in dissolved NH_4^+-N. There was minimal change in dissolved NH_4^+-N in S-FH and S-FC (Table 4.2), suggesting the endogenous NH_4^+ was exhausted in the first biostimulation experiment. The S-FCA treatment had a significant decrease in dissolved NH_4^+-N, decreasing from 18 ± 0.6 to 14 ± 1.4 mg L^{-1} dissolved NH_4^+-N, which is less than the first biostimulation experiment (Table 4.2). Therefore, adding only ferric citrate and not NH_4Cl appeared to negatively impact the dissolved NH_4^+-N loss relative to the first experiment when both amendments were added to S-FCA. The S-FHA treatment exhibited a slight increase in dissolved NH_4^+-N (Table 4.2), indicating that this treatment was not capable of stimulating dissolved NH_4^+-N in the first or second biostimulation experiments compared to S-FCA. During this Biostimulation with Fe(III) experiment, some gas bubbles began rising from the soil in S-FC and S-FCA treatments, creating enough pressure that the 1 mL sampling syringe plunger popped out of the barrel within the glovebox. These bubbles continued to form throughout the remainder of the 118-day incubation but their composition was not characterized.

4.1.2.2 Dissolved NO_2^-, Fe(III), and Fe(II)

Similar to the Initial Biostimulation with Fe(III) and NH_4^+ experiment, in the Biostimulation with Fe(III) experiment the average dissolved NO_2^--N values were low (<1 mg L^{-1}) and there was a slight decrease in dissolved NO_2^--N in nearly all treatments (Table 4.3). The decrease in dissolved Fe(III) in S-A, S-FH, and S-FHA (Table 4.4) corresponded to a slight increase in dissolved Fe(II) (Table 4.5). However, this trend was not seen in FC treatments,

unlike in the Initial Biostimulation with Fe(III) and NH_4^+ experiment. The largest increase in dissolved Fe(III) was in S-FCA, while the largest decrease in Fe(III) was in S-FC and NS-FCA. However, the Fe(III) first increased by ~140 mg L^{-1} in the first 16 days after amendment and then decreased by ~60 mg L^{-1} in the following 24 days to above the initial Fe(III) value in this experiment (Table A-2). The largest decrease in Fe(II) was seen in S-FCA, S-FC, and NS-FCA. Similar to the Initial Biostimulation with Fe(III) and NH_4^+ experiment, the added ferric citrate quickly turned from yellow to grey in the S-FCA and S-FC treatments, indicating Fe reduction. However, the notable increase and then decrease in dissolved Fe(III) and the decrease in dissolved Fe(II) in S-FCA implies initial Fe reduction in S-FCA and that the dissolved Fe(II) was consumed by another reaction. In S-FH and S-FHA, the added ferrihydrite changed from a dark red to a dark orange, similar to the Initial Biostimulation with Fe(III) and NH_4^+ experiment. This color change corresponded to some Fe reduction (Table 4.4, 4.5) and may represent mineral transformation (Adhikari et al., 2017). The ratios of NH_4^+-N loss to NO_2^- produced and Fe(II) produced in S-FCA (Table 4.6) indicates no clear evidence of Feammox in the dissolved fraction, since NH_4^+-loss appeared to correspond with Fe oxidation and minimal NO_2^- production.

4.1.3 Observations of the Biostimulation with Ferric citrate and NH_4^+ Experiment

In this experiment the goal was to supply the microbial community in some samples with NH_4^+ via NH_4Cl and Fe(III) via ferric citrate, not ferrihydrite. Ferrihydrite was excluded because adding ferrihydrite in each of the previous biostimulation experiments did not result in a significant decrease in dissolved NH_4^+-N. The ferric citrate and NH_4Cl were added concurrently because adding Fe(III) alone in the Biostimulation with Fe(III) experiment did not yield impressive NH_4^+-N loss results, contrary to the Initial Biostimulation with Fe(III) and NH_4^+ experiment. Therefore, 12 mM of Fe(III) via ferric citrate was added to NS-FCA, S-FC, and S-FCA and 2 mM NH_4^+ via NH_4Cl was added to S-A, S-FCA, and NS-A. As a result, the dissolved NH_4^+-N loss in S-FCA was similar to the loss in the Initial Biostimulation with Fe(III) and NH_4^+ experiment.

4.1.4.1 Dissolved NH_4^+-N

In the Biostimulation with Ferric citrate and NH_4^+ experiment, ferric citrate and NH_4Cl amendments influenced dissolved NH_4^+-N levels, particularly in S-FCA. In S-FCA there was a significant decrease in dissolved NH_4^+-N, from 22 ± 5.8 to 9 ± 3.9 mg L^{-1} dissolved NH_4^+-N. This loss was greater than in the second biostimulation experiment when only Fe(III) via ferric citrate was added to S-FCA, implying that concurrent addition of NH_4Cl and Fe(III) via ferric citrate stimulates a greater loss in dissolved NH_4^+-N. All other samples had non-significant changes in dissolved NH_4^+-N, with NS-A exhibiting a slight increase and the other samples exhibiting a slight decrease in dissolved NH_4^+-N (Table 4.2).

4.1.4.2 Dissolved NO_2^-, Fe(III), and Fe(II)

There was a slight increase in dissolved NO_2^--N in all treatments during the Biostimulation with Ferric citrate and NH_4^+ experiment, but similar to the previous biostimulation experiments the dissolved NO_2^--N values were low (<1 mg L^{-1}) for all samples (Table 4.3). There was an increase in dissolved Fe(III) in NS-A and NS-FCA, but a decrease in all other treatments, especially in S-FC and S-FCA (Table 4.4). There was an increase in dissolved Fe(II) in all treatments except S-FCA and S-FC (Table 4.5). In particular the dissolved Fe(II) first increased by ~86 mg L^{-1} in the first 6 days, followed by a decrease of ~118 mg L^{-1} in the next 16 days of the experiment (Table A-1). There was a color change from yellow to grey in S-FCA and S-FC like in previous experiments. These results indicate that Fe(III) reduction may have occurred in S-FCA and initially generated some dissolved Fe(II), but that the Fe(II) was then consumed or otherwise excluded from the dissolved fraction. Similar to previous experiments there was no perceptible color change in NS-FCA which remained yellow; however, there was minimal Fe reduction in this control (Table 4.4, 4.5). The S-FH and S-FHA treatments remained a dark orange, likely because no ferrihydrite or NH_4Cl were added which may have stimulated a mineral transformation and/or color change. Throughout the first three biostimulation experiments, S-FCA exhibited the most substantial decrease in dissolved NH_4^+-N (Table 4.2), minimal dissolved NO_2^--N production (Table 4.3), and suspected Fe reduction in the dissolved fraction (Table 4.4, 4.5). Conversely, the S-FHA treatment exhibited minimal dissolved NH_4^+-N loss, minimal dissolved NO_2^--N production, and minimal changes in dissolved Fe. The ratios of NH_4^+-N loss to NO_2^- produced and Fe(II) produced in S-FCA (Table 4.6)

indicates no clear evidence of Feammox in the dissolved fraction, since it was unclear if the NH_4^+-loss did not appear to correspond with Fe reduction due to the Fe(II) loss.

4.1.4 Observations of the Biostimulation with Vitamins Experiment

The goal of this experiment was to determine if adding vitamin and molybdate solutions to all samples without adding Fe(III) or NH_4Cl could stimulate dissolved NH_4^+-N loss. The greatest change in NH_4^+-N was expected in S-FCA, which had been the most active treatment thus far and had accumulated Fe(III) and residual NH_4^+ from previous biostimulation experiments that could support Feammox activity. However, after adding these solutions there was an increase in dissolved NH_4^+-N loss in nearly all the samples.

4.1.5.1 Dissolved NH_4^+-N

Adding molybdate and a vitamin solution to all the samples in the Biostimulation with Vitamins experiment influenced dissolved NH_4^+-N levels differently than the previous biostimulation experiments. Almost all treatments exhibited an increase in dissolved NH_4^+-N except NS-FCA and S-A. There a significant increase in dissolved NH_4^+-N in S-FCA and S-FHA (Table 4.2). In S-FCA the dissolved NH_4^+-N increased from 11 ± 0.3 to 24 ± 7.2 mg L^{-1} and in S-FHA the dissolved NH_4^+-N increased from 17 ± 2.1 to 23 ± 5.9 mg L^{-1}.

4.1.5.2 Dissolved NO_2^-, Fe(III), and Fe(II)

There was a slight increase in dissolved NO_2^--N in all treatments, but the NO_2^--N remained at ~0-1 mg L^{-1} in most samples, similar to previous experiments (Table 4.6). There was a slight decrease in dissolved Fe(III) all treatments except NS-FHA which exhibited an uncharacteristic substantial Fe(III) loss (Table 4.4) and substantial Fe(II) increase (Table 4.5), indicating abiotic Fe(III) reduction. There was a slight increase in dissolved Fe(II) in all other treatments except NS-FCA which exhibited a decrease in dissolved Fe(II) (Table 4.5). This could indicate minimal abiotic Fe(III) reduction. These results in S-FHA and S-FCA imply that the amendments may have influenced the Fe chemistry in abiotic conditions. The S-FC and S-FCA treatments remained grey, NS-FCA remained yellow, and FH treatments remained a dark orange since no Fe(III) was added. The ratios of NH_4^+-N loss to NO_2^- produced and Fe(II) produced in

S-FCA (Table 4.6) indicates no evidence of Feammox in the dissolved fraction, since there was both a gain in NH_4^+-N and minimal evidence of Fe reduction.

4.1.6 PCR Analysis

The results of the initial DNA amplification (342F and 806R) of the diluted DNA from the pellets that were extracted from the anaerobic samples during the 118-day incubation included faint bands. The results of the follow-up PCR (1055F and 1392R) were inconclusive. Only the positive control band was visible. Furthermore, when the DNA was spiked with a positive control all the bands were visible, indicating that the sequence which the A6 primers were searching for was not present in any of the pellet subsamples from the 118-day anaerobic incubation.

4.2 Subculture experiment of ferric citrate + NH_4Cl slurry treatment from the 118-day anaerobic incubation

4.2.1 Dissolved NH_4^+-N, Fe(III), and Fe(II)

The results for dissolved NH_4^+-N in the 94-day S-FCA subculture experiment can be visualized in fig. 4.1. The dissolved Fe(II) and Fe(III) results can be visualized in fig. 4.2; however, the Fe-species were infrequently tracked and only tracked until day 48 because an unforeseen circumstance caused limited laboratory access which only allowed time for periodic NH_4^+-N analysis. The dissolved NH_4^+-N values are generally higher for the S-FCA treatments relative to the soil-free control. In all samples the dissolved NH_4^+-N values initially decreased prior to day 12 but steadily increased during the remaining 82 days (Fig. 4.1). In the NS-FCA control, the Fe(II) and the Fe(III) steadily increased before decreasing slightly, which indicates that some abiotic Fe(III) reduction occurred (Fig. 4.2). The dissolved Fe(III) in all three S-FCA treatments followed a similar pattern, where Fe(III) initially increased or slightly decreased (S-FCA3) in the first 3 days, and then decreased to almost ~0 mg L^{-1} by day 48. The dissolved Fe(II) followed a similar pattern in all S-FCA treatments, marked by an substantial increase by day 3 which was followed by a substantial decrease by day 48. The relationship between the dissolved NH_4^+-N and Fe-species is worth noting, since the decrease in dissolved NH_4^+-N prior to day 12 corresponds to Fe(III) reduction in the S-FCA treatments. However, this effect appears

to have not been sustained given the subsequent increase in dissolved NH_4^+-N and decrease in dissolved Fe(II) after day 12 and 3, respectively.

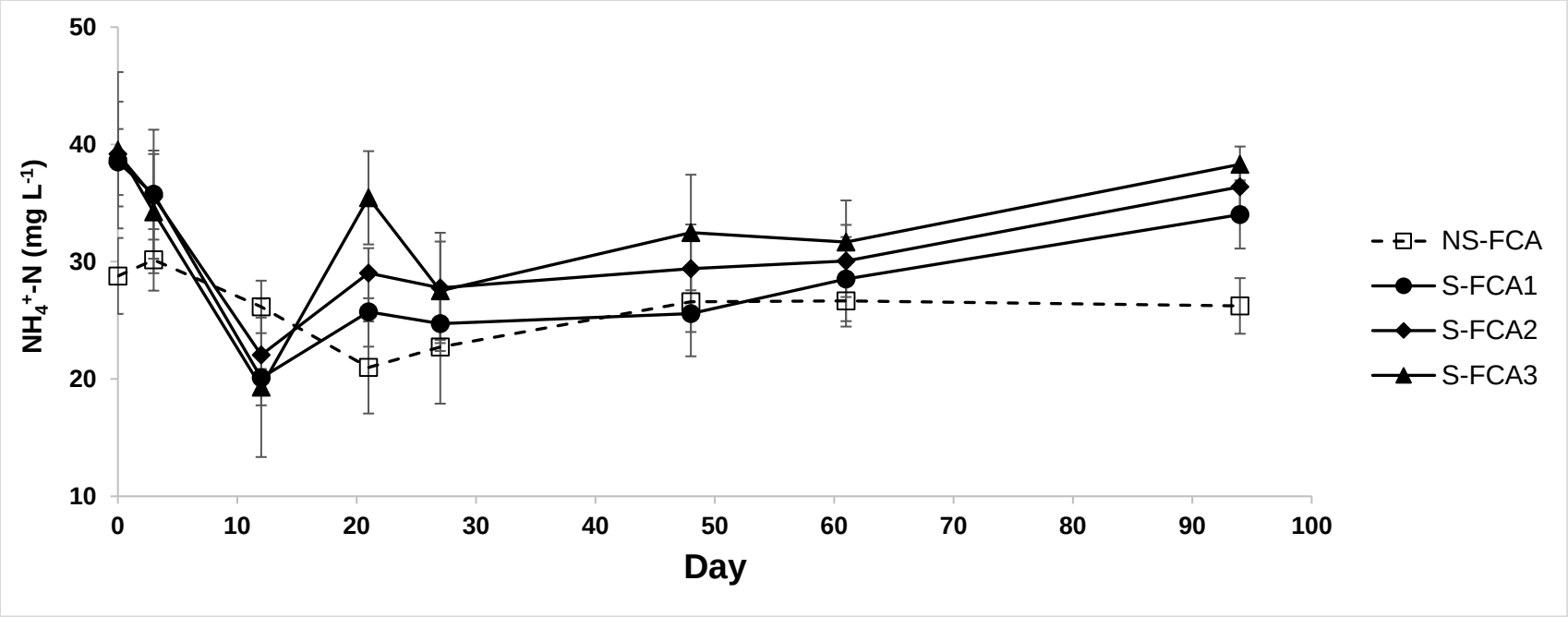

Fig. 4.1. Results of average dissolved NH_4^+-N in milligrams per liter (mg L^{-1}) with standard error bars for the control with no soil (NS-) (n=3) and soil (S-) with ferric citrate (FC) and ammonium (A) treatments (n=3) in a 94-day subculture experiment. Ferric citrate and ammonium were amended all samples on the first day. The 1, 2, and 3 correspond to S-FCA treatment replicates 1, 2, and 3 from the 118-day anaerobic incubation.

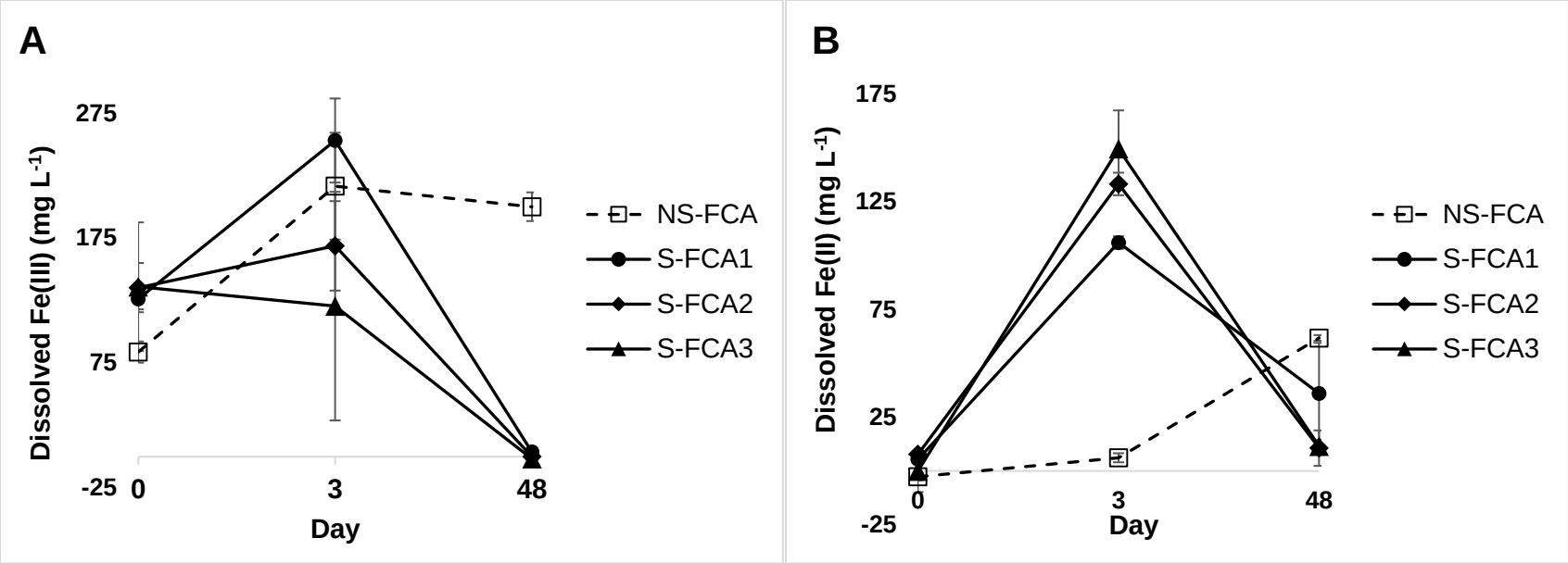

Fig. 4.2. Results of average dissolved Fe(III) (A) and dissolved Fe(II) (B) in milligrams per liter (mg L^{-1}) with standard error bars for the control with no soil (NS-) (n=3) and soil (S-) with ferric citrate (FC) and ammonium (A) treatments (n=3) in a 94-day subculture experiment. Ferric citrate and ammonium were amended all samples on the first day. The 1, 2, and 3 correspond to S-FCA treatment replicates 1, 2, and 3 from the 118-day anaerobic incubation.

5. DISCUSSION

5.1 Biostimulating Feammox During a 118-day Anaerobic Incubation

Over a 118-day anaerobic incubation period, I attempted to stimulate Feammox by first adding NH_4^+ via NH_4Cl and Fe(III) via ferric citrate and 2-line ferrihydrite, then Fe(III) via ferric citrate and 2-line ferrihydrite, then NH_4^+ via NH_4Cl and Fe(III) via ferric citrate, and finally vitamin and molybdate solutions to anaerobic soil slurries made with alkaline soils from a N-polluted site in Alberta. Certain amendments and amendment combinations stimulated a decrease in dissolved NH_4^+-N, especially when ferric citrate and NH_4Cl were added concurrently in the S-FCA treatments. Furthermore, Fe reduction occurred in S-FCA but there also seemed to be Fe(II) oxidation and mechanisms that removed Fe(II) from the dissolved fraction following reduction. Although these results imply Feammox activity in S-FCA, both results were insufficient to conclusively demonstrate that this NH_4^+-N loss was due to only Feammox. For example, the dissolved NH_4^+-N loss may have generated N end products beside NO_2^-, the Fe end products were not characterized, and the composition of the gas generated in the S-FC and S-FCA treatments was not identified. Therefore, more research is required to explore the relationship between ferric citrate and NH_4^+-N loss in near-neutral to alkaline anaerobic conditions. The goal of the 118-day incubation was to stimulate Feammox; however, from a biostimulation point of view Feammox isolation may not be essential as typically multiple microbial N processes occur simultaneously. For example, other Feammox research discovered that Feammox and other pathways such as anammox, denitrification (Shrestha et al., 2009; Yang et al., 2012; Hang and Jaffé, 2015; Zhou et al., 2016; Li et al., 2018a; Li et al., 2018b; Yi et al., 2019), and NDFO (Li et al., 2018a; Yang et al., 2019) were occurring simultaneously. Therefore, when addressing future bioremediation work it may be relevant to quantify Feammox rates as they contribute to overall N-removal in technologies like WWTPs and PRBs. Furthermore, the SOM and ferric citrate may have significantly impacted the results in S-FCA via processes such as oxidation, reduction, and methanogenesis, and are worth further exploration in future experiments.

5.1.1 pH in the 118-day anaerobic incubation

The pH measurements collected periodically throughout the 118-day incubation do have some implications. The pH was higher in the soil slurries than the soil-free controls, which is likely due to the buffering capacity of the neutral-alkaline Lomond soil samples used (pH 7-9) (Table 2.1). The pH slightly increased in the soil slurries during the incubation, which may not be a significant result. However, this slight increase may represent the occurrence of chemical reactions that consume protons, like anammox (Gonzalez-Martinez et al., 2018) which occurs most efficiently when the pH is between 6.7 and 8.3 (Tomaszeski et al., 2017), Feammox (Clément et al., 2005; Yang et al., 2012; Huang and Jaffé, 2015; Yang et al., 2019), denitrification (Bernhard, 2010), or NDFO (Jamieson et al., 2018; Yang et al., 2019). The pH decrease of 0.8 in NS-FCA and increase of 1.0 in NS-A may be due to chemical changes in abiotic conditions. NH_4Cl, a weak acid, was added on day 1 and may explain the pH decrease between days 0 and 27 in NS-A. The higher pH in NS-A after the Biostimulation with Vitamins experiment (day 119) (Table 4.1) may be explained by: 1) the NH_4Cl amendment on day 73 influenced the pH, 2) NH_4^+ may have transformed to slightly basic NH_3 given that the pH was near neutral and the samples were incubated at 20-25°C (CCME, 2010), or 3) chemical reactions between NH_4^+ and/or Cl^- with other ions that form basic compounds or consume protons. In future experiments the pH should be monitored more consistently throughout the incubation to see clearer trends in pH. Furthermore, the controls should be created with at least 3 replicates for more accurate average pH results and also for more accurate average dissolved Fe(III), Fe(II), NH_4^+-N and NO_2^--N results.

5.1.2 Initial Biostimulation with Fe(III) and NH_4^+ experiment

In the Initial Biostimulation with Fe(III) and NH_4^+ experiment, the goal was to provide the microbial population with Fe(III) via ferric citrate or 2-line ferrihydrite and NH_4^+ via NH_4Cl as key resources to stimulate Feammox activity. The hypothesis was that the microbial population would reduce the Fe(III) and consume or transform the NH_4^+, i.e. perform Feammox, and this would result in a decrease in dissolved Fe(III), increase in dissolved Fe(II), decrease in dissolved NH_4^+-N, and potentially an increase in dissolved NO_2^- as one Feammox end product from NH_4^+ oxidation. This hypothesis was supported, in part, by the S-FCA treatment which had

notable Fe(III) reduction in the dissolved fraction, a statistically significant decrease in dissolved NH_4^+-N, and minimal dissolved NO_2^- generation. Overall, although the S-FCA results indicate a Fe reduction-NH_4^+ oxidation relationship that implies Feammox activity (fig. 5.1E), it was not clear evidence of Feammox since other microbial communities can independently reduce Fe and oxidize NH_4^+, e.g. DIRB (fig. 5.1G) (Schröder et al., 2003; Huang and Jaffé, 2015; Zhou et al., 2016; Adhikari et al., 2017) and anammox bacteria (fig. 5.1B) (Huang et al., 2014), respectively. Furthermore, in the near-neutral to alkaline conditions of S-FCA at room temperature (20-25°C), some of the NH_4^+ may have transformed to NH_3, which can be readily assimilated by microbes (CCME, 2010), reducing the amount of dissolved NH_4^+-N (fig. 5.1A). In addition, N_2, N_2O, and NO_3^- were not tracked as other NH_4^+ oxidation end products or intermediates and would influence conclusions about other processes like NDFO (Yang et al., 2018; Bao and Li, 2018a; Yang et al., 2019), anammox, and denitrification (fig. 5.1C) (Clément et al., 2005; Shrestha et al., 2009; Shapleigh, 2009; Yang et al., 2012; Huang and Jaffé, 2015, 2018; Zhou et al., 2016; Kamp et al., 2019). The dissolved Fe(II) produced in S-FCA could not continue to participate in Feammox unless more ferric citrate is added, or the Fe(II) is oxidized or regenerated as bioavailable Fe(III) via a process like NDFO or chemodenitrification (fig. 5.1D) (Jamieson et al., 2018). In contrast to treatments with ferric citrate, the 2-line ferrihydrite treatments showed minimal or negligible amounts of dissolved NH_4^+-N loss, dissolved NO_2^- production, and Fe reduction, unlike the results with 6-line ferrihydrite in Huang and Jaffé (2015) and Huang and Jaffé (2018).

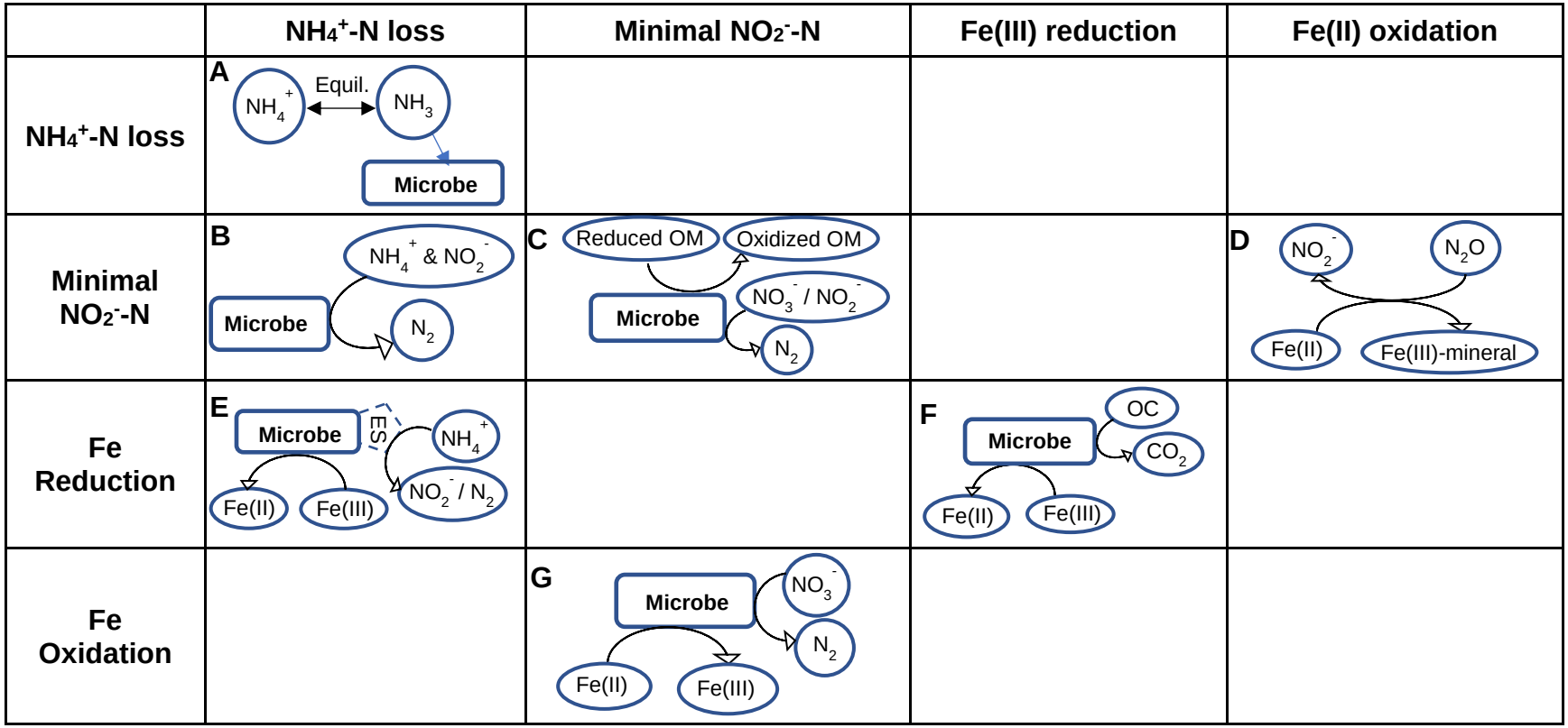

Fig. 5.1. Pathways that may explain results for dissolved ammonium-N (NH_4^+-N) loss, dissolved nitrite (NO_2^-) production, ferric iron (Fe(III)) reduction, and ferrous iron (Fe(II)) oxidation in the soil slurry with ferric citrate + NH_4Cl treatment in the 118-day anaerobic incubation. **A**. Nitrogen assimilation where the near-neutral conditions facilitated NH_3 generation and assimilation. NO_3^- can also be converted via dissimilatory nitrate reduction to ammonium (DNRA), for example, to NH_3/NH_4^+ which can used for assimilation. **B**. Anaerobic ammonium oxidation (anammox) where NH_4^+ oxidation and nitrite NO_2^- reduction generates nitrogen gas (N_2). **C**. Denitrification, where organic matter (OM) oxidation is coupled to nitrate (NO_3^-) and/or NO_2^- reduction. **D**. Chemodenitrification, an abiotic reaction that couples NO_2^- reduction to Fe(II) oxidation, generating Fe(III) minerals and nitrous oxide (N_2O) while. **E**. Iron ammonium oxidation (Feammox) where Fe(III) reduction couples with NH_4^+ oxidation to generate NO_2^- or N_2, with or without the help of an electron shuttle (ES). **F**. Anaerobic respiration with dissimilatory iron reducing microbes which couples Fe(III) reduction with organic C (OC) oxidation. **G**. Nitrate or nitrite depended Fe oxidation (NDFO) where Fe(II) oxidation couples with (NO_3^-) and/or NO_2^- reduction to N_2.

These results suggest that ferric citrate was active in these anaerobic soil slurries and may stimulate Feammox more effectively than ferrihydrite at near-neutral to alkaline pH, unlike in previous experiments where ferrihydrite was more successful in acidic conditions (Huang and Jaffé, 2015, 2018). This implies that ferric citrate was more bioavailable than the 2-line ferrihydrite at a higher pH than previous studies, which may be explained by the different E_hs of the respective Fe(III) sources. The E_{env} of Fe(III)-citrate/Fe(II)-citrate at pH 7 is +0.385 V, which is notably higher than the E_{env} of solid ferrihydrite/Fe^{2+} at pH 7 (+0.1 to -0.1 V) (Bird et al., 2011). However, the specific E_h of the laboratory synthesized 2-line ferrihydrite and ferric citrate was not determined in the present study, so these values only offer general insight into the bioavailability of the respective Fe(III) sources in neutral to alkaline pH.

The difference in bioavailability could also be due to the nature of each compound and how it interacts with SOM and the microbial community. 6-line ferrihydrite is a poorly crystalline mineral that is thermodynamically unstable (Kukkadapu et al., 2003; Tang et al., 2016). Ferrihydrite's success in previous experiments (Huang and Jaffé, 2015, 2018) may be related to the more favorable E_h in acidic conditions, as well as ferrihydrite's high surface area (Vodyanitzkii and Shoba, 2016) and relationship with OM which involves OM sorption to Fe (Eusterhues et al., 2008), Fe sorption to OM (Cooper et al., 2017; Adhikari et al., 2017), OM enhancing (by acting as an electron shuttle) or inhibiting ferrihydrite redox activity (Eusterhues et al., 2008; Cooper et al., 2017;), and ferrihydrite protecting OM (Eusterhues et al., 2008) and/or influencing OM mineralization (Adhikari et al., 2017). For example, OC can complex with ferrihydrite to limit Fe(III) reduction and OC can chelate Fe(II) which inhibits microbial ferrihydrite-Fe(III) reduction, Fe oxidation, and ferrihydrite dissolution and recrystallization (Adhikari et al., 2017). Relative to 6-line ferrihydrite the less thermodynamically stable 2-line ferrihydrite (Kukkadapu et al., 2003) was used in the present study. The 2-line ferrihydrite may have also had an important relationship with OM; for example, the OM may have influenced its transformation into 6-line ferrihydrite, which can occur via DIRB activity and intra-aggregate transformation (Kukkadapu et al., 2003).

Another difference in bioavailability between ferric citrate and ferrihydrite is in assimilation. The success in S-FCA contrasts with results in Ruiz-Urigüen et al. (2019) which note that Feammox bacteria like A6 cannot colonize ferric citrate, but could colonize ferrihydrite, there may have been other microbes capable of colonizing and using the ferric

citrate in the present study. Although some microbes can assimilate ferric citrate (Pierre and Gautier-Luneau, 2000; Schröder et al., 2003) or the Fe(III) in ferric citrate (Schröder et al., 2003), Fe(III) oxides like ferrihydrite and OM-mineral complexes cannot pass through the outer membrane of Fe(III)-reducing microorganisms (Cooper et al., 2017). Therefore, there are two characterized electron transfer methods, e.g. 1) direct transfer of electrons via enzymes anchored to the outer cell membrane and 2) mediated electron transfer via exogenous electron-shuttling compounds found in OM, endogenous electron-shuttling compounds, or via Fe(III)-chelating compounds like siderophores (Cooper et al., 2017). If ferrihydrite had exhibited notable Fe(III) reduction in the present study these processes may have occurred.

The apparent lack of ferrihydrite bioavailability for Feammox in the present study could also be explained by a transformation into less thermodynamically favorable goethite (Yee et al., 2006; Adhikari et al., 2017) or hematite; however, these transformations can take a long time (months to years) and are highly dependent on environmental conditions like pH and SOM presence (Adhikari et al., 2017). However, if a mineral transformation did occur, the conditions in S-FHA favor hematite formation which typically occurs in pH<8 conditions and minimal soluble Fe(II) Meanwhile, goethite formation from 6-line ferrihydrite favors a pH of 2-5 or 10-14 and sufficient soluble Fe(II), neither of which occurred in S-FHA. Without characterization of the solid Fe(III) via technologies like spectroscopy or XRD, it cannot be assumed that the 2-line ferrihydrite transformed into 6-line ferrihydrite or other minerals, which would lower its thermodynamic favorability and bioavailability (Adhikari et al., 2017). Finally, the present study used laboratory-synthesized ferrihydrite, while in nature ferrihydrite is rarely chemically pure and this influences it's reactivity (Vodyanitzkii and Shoba, 2016) and may make it more bioavailable in alkaline field soils than implied in the present study. Therefore, more research is required to understand why the 2-line ferrihydrite did not stimulate bioavailability.

The bioavailability of ferric citrate in the present study may have influenced the change in dissolved NH_4^+-N; however, this influence may not be solely due to the Fe(III). The ferric citrate, Fe(III), and citrate may have stimulated microbial activity, NH_4^+-N loss, and Fe reduction. As mentioned, ferric citrate is a soluble Fe(III)-C complex which can be assimilated (Pierre and Gautier-Luneau, 2000; Schröder et al., 2003). When the environmental pH is near-neutral to alkaline, Fe(III) bioavailability is typically limited and certain microbes overcome this by secreting citrate (a molecule that is more prevalent at pH ~7 relative to citric acid (Martell and

Motekaitis, 1992)) to extract and assimilate Fe(III) and/or uptake ferric citrate (Schröder et al., 2003). Assimilating Fe(III) can stimulate biosynthesis of proteins like heme proteins (Huang et al., 2014), and dissimilatory reduction of Fe(III) (fig. 5.1F) can generate energy that stimulates population growth and the need for additional Fe (Schröder et al., 2003) and C. Since autotrophic anaerobic respiration can reduce Fe(III) while oxidizing OC (Straub, 2011; Bird et al., 2011), i.e. perform dissimilatory Fe reduction, then the Fe(III) may have stimulated autotrophic growth and it is possible that the citrate participated in those reactions as an organic C source.

The soil OM also may have also influenced ferric citrate bioavailability, Fe reduction, and Fe assimilation. Soils with high OM, including humic substances, tend to render Fe^{3+} more available via Fe^{3+} chelation (Schröder et al., 2003). Humic substances can also act as electron shuttles for Fe^{3+} oxides, and the oxidized active components of humic substances can be electron acceptors (Schröder et al., 2003). Therefore, OM can facilitate Fe reduction (Shrestha et al., 2009; Zhou et al., 2016) and microbes can reduce Fe to aid in OM breakdown (Nichols Environmental, 2018). The soil OM likely influenced the results in the 118-day anaerobic incubation, which could be tested in future experiments with treatments that use AQDS in place of soil.

The citrate may have functioned as redox active OC in those reactions. Although studies related to citrate reactivity in soils are limited, the anion citrate contains one hydroxyl group and three carboxylate groups ($-COO^-$), which may influence redox activity, e.g. by potentially acting as an electron shuttle in Feammox. For example, the carboxylate group could be reduced and the hydroxyl group is a pH dependent functional group that can sorb organic compounds (Eusterhues et al., 2008) and is important in citrate biodegradation (Francis and Dodge, 1993). This Fe(III) reduction-organic C electron donor phenomenon was indicated by Huang and Jaffé, (2015). Huang and Jaffé, (2015) suggest that ferric citrate could stimulate Feammox in different conditions via rapid Fe(III) reduction by dissimilatory Fe reducers (fig. 5.1F), using organic carbon as the electron donor, or by citrate uptake. The citrate may have been the organic C source in this scenario considering that it may be considered bioavailable labile C relative to the native SOM. Zhou et al. (2016) studied Feammox activity with DIRB enrichments and electron shuttles and noted that there appeared to be concurrent Feammox activity and OM oxidation-Fe reduction, as supported by an abundance of acetate-oxidizing DIRB (Geobacter) in their neutral-slightly alkaline anaerobic samples. If citrate can be used in place of acetate in this OM

oxidation-Fe reduction pathway, then citrate may have stimulated Fe(III) reduction in the present study. Therefore, adding ferric citrate may have satisfied and also stimulated microbial needs for Fe(III) and C. Concurrent addition of Fe(III) and labile organic C (e.g. citrate) can enhance microbial activity in anaerobic conditions (Chen et al., 2020). Citrate can also be consumed by certain microbial populations (Huang and Jaffé, 2015), which could have stimulated growth of certain microbial populations, e.g. citrate fermenters.

Citrate is also important in microbial metabolism (Starrenburge and Hugenholtz, 1991; Jurtshuk Jr., 1996) outside of Fe redox reactions and is a ubiquitous natural compound found in all living cells (Chen et al., 2009). The citric acid cycle, or Kreb's cycle, involves citrate as a metabolic intermediate in cellular respiration, so citrate is involved in cellular energy production (Jurtshuk Jr., 1996). Each amendment of ferric citrate to S-FC and S-FCA created a 12 mM citrate environment in the slurries and that citrate may have been used in anaerobic metabolism besides pathways that involve Fe(III) reduction, e.g. citrate fermentation (Starrenburge and Hugenholtz, 1991; Chen et al., 2009; Gámez et al., 2009), methanogenesis (Gámez et al., 2009), and methane oxidation (Ettwig et al., 2016). Although citrate fermentation is poorly understood, particularly in soils, research indicates that bacteria are capable of fermenting citrate if they can grow on citrate and if they possess both an enzyme to degrade citrate and a citrate carrier that allow them to use citrate as a C source (Chen et al., 2009). Citrate fermentation can generate products like oxaloacetate, acetate, pyruvate, formate, and CO_2 via anaerobic catabolism (Chen et al., 2009) and this fermentation can stimulate methanogenesis to generate CH_4, acetate, CO_2, and H_2, with oxaloacetate and pyruvate as intermediates (Gámez et al., 2009). Furthermore, the CH_4 can stimulate anaerobic methane oxidation to generate CO_2 from CH_4 (Ettwig et al., 2016). Some methanogenic bacteria are inhibited by Fe(III) while others can also reduce Fe(III) (Sivan et al., 2016; Prakash et al., 2019). Overall, the abundance of Fe(III), citrate, or ferric citrate in S-FC and S-FCA in the present study may have influenced microbial energy and growth, Fe(III) reduction, OM stability and redox activity, citrate degradation, and other important processes. These important Fe and C relationships need to be explored because they impact the microbial community and microbial N requirements (Kuzyakov et al., 2000; Barrett and Burke, 2000), which may explain the NH_4^+-N results.

5.1.3 Biostimulation with Fe(III) experiment

The results of the Initial Biostimulation with Fe(III) and NH_4^+ experiment indicated that ferric citrate, not ferrihydrite, exhibited a significant and notable decrease in dissolved NH_4^+-N and signs of Fe reduction that could support Feammox. The Initial Biostimulation with Fe(III) and NH_4^+ experiment also indicated that soil endogenous NH_4^+ may be influenced by Fe(III), given the loss of dissolved NH_4^+-N in both S-FC and S-FH. Therefore, the rationale for adding Fe(III) in the Biostimulation with Fe(III) experiment was to assess whether Fe(III) alone is the limiting resource for Feammox, since both Fe(III) sources may induce NH_4^+-N loss without adding additional NH_4^+. The hypothesis of the Biostimulation with Fe(III) experiment was that adding more Fe(III) would result in dissolved NH_4^+-N loss without adding more NH_4Cl. After this Fe(III) addition, ferrihydrite treatments showed almost no activity, similar to the first biostimulation experiment, which may be related to reduced bioavailability (Bird et al., 2011; Cooper et al., 2017; Adhikari et al., 2017). The S-FCA treatment showed statistically significant dissolved NH_4^+-N loss, but the loss was less than the first experiment when both ferric citrate and NH_4Cl were added to S-FCA. This implies that concurrent addition of ferric citrate and NH_4Cl stimulates greater dissolved NH_4^+-N loss in S-FCA. Furthermore, the dissolved NH_4^+-N loss may also be explained by anammox (fig. 5.1B) (Huang et al., 2014) or NH_3 assimilation (fig. 5.1A) (Burkovski, 2003).

In S-FCA the dissolved Fe-species showed an interesting pattern, with the Fe(II) steadily decreasing and the Fe(III) initially increasing and then decreasing to above the initial Fe(III) value (Table A-1). Therefore, although the dissolved Fe(III) values indicate reduction, it appears that the Fe(II) was somehow being excluded from the dissolved fraction. There are several reasons why this may have occurred. For example, the overall change in Fe(III) in this experiment (~-104 mg L^{-1} to -13 mg L^{-1}) could imply a reaction that generates soluble Fe(III) and consumes Fe(II), i.e. Fe oxidation, such as NDFO (fig. 5.1G) (Yang et al., 2018; Bao and Li, 2018a; Yang et al., 2019) and abiotic chemodenitrification that can generate Fe(III) minerals (fig. 5.1D) (Jamieson et al., 2018). Furthermore the Fe(II) can also precipitate. Bao and Li, (2018b) noted that after Fe(III) reduction the free Fe(II) formed a solid $Fe(OH)_2$ precipitate that inhibited anammox/Feammox activity. These potential relationships could be tested by incorporating ^{15}N and ^{57}Fe in future experiments with and without soil. There may have also been SOM interactions that influenced Fe(II) availability, e.g. OM can sorb or complex with available Fe(II)

and Fe(III) (Cooper et al., 2017; Adhikari et al., 2017), and OM can influence Fe(II) oxidation into Fe(III), e.g. autotrophic Fe(II) oxidizers perform CO_2 fixation, therefore reducing OC (Bird et al., 2011). Furthermore, it is possible the dissolved Fe(II) was assimilated by microbes (Schröder et al., 2003) or used in other anaerobic reactions since it is an important reduced compound in anaerobic conditions (Beck and Mann, 2010; DeLaune and Reddy, 2005; Nichols Environmental, 2018). Therefore, the hypothesis in the Biostimulation with Fe(III) experiment was partially supported in the S-FCA treatment, given that dissolved NH_4^+-N loss occurred; however, the evidence for Feammox was unclear due to notable decrease in dissolved Fe(II).

5.1.4 Biostimulation with Ferric citrate and NH_4^+ experiment

The results of the Biostimulation with Fe(III) experiment indicated that S-FCA exhibited a statistically significant decrease in dissolved NH_4^+-N, but the NH_4^+-N loss was less than the first biostimulation experiment when both NH_4Cl and ferric citrate were added. The ferrihydrite treatments showed no notable Fe reduction, dissolved NH_4^+-N loss, or dissolved NO_2^- production; therefore, adding additional ferrihydrite did not induce a response. Therefore, the Biostimulation with Fe(III) experiment indicated that the microbial population may need a co-amendment of NH_4Cl and ferric citrate and that adding ferrihydrite is unlikely to influence the dissolved NH_4^+-N. Therefore, in the next experiment, Biostimulation with Ferric citrate and NH_4^+, additional NH_4Cl and Fe(III) via ferric citrate were added to respective samples and no ferrihydrite or NH_4Cl was added to S-FHA or NS-FHA. The hypothesis of the Biostimulation with Ferric citrate and NH_4^+ experiment was that this co-amendment would stimulate Fe reduction and dissolved NH_4^+-N loss in S-FCA similar to the initial biostimulation experiment, with consideration that the residual Fe from the previous experiments might influence Fe chemistry like in the second biostimulation experiment. This hypothesis was somewhat supported in the S-FCA treatment, as it appeared both Fe and NH_4^+ resources were necessary for improving dissolved NH_4^+-N loss.

In the S-FCA treatment there was statistically significant NH_4^+-N loss that was similar to the initial biostimulation experiment, indicating that co-amendment was successful. However, the dissolved Fe-species showed an unusual trend in S-FCA where dissolved Fe(III) steadily decreased, but the dissolved Fe(II) first notably increased and then decreased substantially. Therefore, the Fe(III) results and initial Fe(II) results indicate Fe reduction, but the dissolved

Fe(II) then decreased, suggesting that the Fe(II) was consumed in another reaction without generating additional dissolved Fe(III). Therefore, there may been reactions occurring that generated insoluble Fe(III) minerals, such as chemodenitrification (fig. 5.1D) (Jamieson et al., 2018) or the formation of solid $Fe(OH)_2$ precipitate (Bao and Li, 2018a). SOM interactions (Bird et al., 2011; Adhikari et al., 2017; Cooper et al., 2017), Fe(II) assimilation (Schröder et al., 2003), and other anaerobic reactions may also explain the decrease in dissolved Fe(II). This result in S-FCA indicates that there may be constraints in Fe(III) availability for Feammox activity, probably due to accumulation of Fe-species from repeated ferric citrate additions. There was evidence of some consistent Fe reduction in all treatments except S-FC and S-FCA, which may indicate delayed Fe reactivity. Feammox activity is not fully supported with these results in S-FCA, and it is important to note that the dissolved NH_4^+-N loss may also be explained by anammox (fig. 5.1B) (Huang et al., 2014), NH_3 assimilation (fig. 5.1A) (Burkovski, 2003) or SOM interactions.

5.1.5 Biostimulation with Vitamins experiment

The results in the Biostimulation with Ferric citrate and NH_4^+ experiment indicated that concurrent addition of ferric citrate and NH_4Cl stimulated dissolved NH_4^+-N loss in S-FCA similar to the Initial Biostimulation with Fe(III) and NH_4^+ experiment. Therefore, this co-amendment appears to be necessary for substantial NH_4^+-N loss in S-FCA. However, both dissolved Fe(II) and Fe(III) notably decreased in S-FCA during this experiment, so consistent Fe reduction was not as apparent in this treatment. These results imply that Fe(III) bioavailability may be limited and S-FCA contains residual dissolved NH_4^+. Therefore in the fourth biostimulation experiment, Biostimulation with Vitamins, the hypothesis was that adding vitamin and molybdate solutions would stimulate dissolved NH_4^+-N loss by stimulating the microbial community to use residual Fe and NH_4^+. This hypothesis was not supported by the results. Adding vitamin and molybdate solutions did not stimulate notable dissolved NH_4^+-N loss or oxidation, even in the S-FCA treatment (Table 4.2, 4.9). Instead, the dissolved NH_4^+-N was higher in most of the soil slurries at the end of the experiment compared to initial values, with a significant change, or gain in dissolved NH_4^+-N in S-FCA and S-FHA (Table 4.2). Given the notable dissolved NH_4^+-N loss when ferric citrate was added in the previous three biostimulation experiments, the vitamin and molybdate solutions may have improved dissolved NH_4^+-N loss in

S-FCA if fresh ferric citrate was added. If bioavailable Fe(III) sources in S-FCA were exhausted, or partitioned in the insoluble fraction via processes like chemodenitrification, that may have inhibited NH_4^+ oxidation in this Biostimulation with Vitamins experiment. These dissolved NH_4^+-N results also indicate that the vitamins stimulated NH_4^+ availability via mineralization or another pathway.

There was some dissolved NH_4^+-N loss in the NS-FHA and NS-FCA controls throughout the 118-day incubation (Table 4.2), which could indicate some abiotic NH_4^+-N oxidation, or microbial contamination in the controls. However, since only one replicate was used for each control, it is difficult to make assumptions about contamination or abiotic reactions. An unexpected result in dissolved Fe occurred in NS-FHA, which exhibited substantial Fe(III) reduction in the dissolved fraction during the Biostimulation with Vitamins experiment. This was unexpected because it was an abiotic control and had not shown any previous notable Fe reduction activity. However, this result may be explained by the reactivity of ferrihydrite in abiotic conditions. Adhikari et al. (2017) indicate that abiotic ferrihydrite reduction can occur, and that this reduction can generate secondary minerals like magnetite or goethite. Furthermore, SOM can inhibit ferrihydrite redox reactions and mineral transformations (Adhikari et al., 2017), which may explain why the Fe(III) reduction occurred in the soil-free control. Therefore, the vitamin and molybdate solutions may have stimulated abiotic reactions in NS-FHA.

The Fe-reduction result in NS-FHA and the increase in dissolved NH_4^+-N in most treatments may also be explained the vitamin and molybdate solutions. Zhou et al. (2016) indicates that vitamin solutions can stimulate electron transfer reactions in both biotic and abiotic conditions. Therefore, the vitamin solution may have influenced chemical reactions that reduced the Fe(III) in S-FHA and increased the dissolved NH_4^+-N. It is also possible that these solutions stimulated microbial growth (Omotani et al., 2017) and enhanced ammonification/mineralization (Barak et al., 1997; Kuzyakov et al., 2000; Shaffer et al., 2001), assimilation (fig. 5.1A), or OM decomposition which released NH_4^+ into solution (Dai et al., 2018). For example, adding commercial N fertilizers (which include NH_4^+) can stimulate N immobilization via microbes, which can enhance SOM mineralization to satisfy C and N needs (Kuzyakov et al., 2000), which may explain additional microbial N assimilation. Furthermore, Fe and molybdenum (Mo) are important in the nitrogenase enzyme that is essential for NO_3^- assimilation (Čorić and Holland, 2016; Macleod and Holland, 2013) and Mo is essential in the nitrate reductase enzyme used for

N fixation (Glass et al., 2012). The active site of the Mo-dependent nitrogenase is the Fe−Mo cofactor (Čorić and Holland, 2016; Macleod and Holland, 2013). Furthermore, DNRA can reduce NO_3^- to NH_4^+ in anaerobic conditions (Pilegaard, 2013) and is influenced by the presence of OM (Bu et al., 2017), which may explain the increase in dissolved NH_4^+-N. In the present study the Mo and Fe could have influenced N assimilation (Herrero et al., 2018) or DNRA; however, this cannot be determined since NO_3^- was not tracked. Therefore, assimilation and mineralization of NH_4^+ may have occurred in the present study but there is not enough data to support this possibility.

The important implication of the Biostimulation with Vitamins experiment is that the dissolved NH_4^+-N increase, e.g. in S-FCA where the increase was 13 mg L^{-1}, indicates that the suspected NH_4^+-N oxidation in previous experiments in S-FCA may actually be the result of immobilization via assimilation, which is a process that is reversible by mineralization (Barak et al., 1997; Kuzyakov et al., 2000; Shaffer et al., 2001). Furthermore, other reactions can generate NH_4^+ such as DNRA (Pillegaard, 2013; Bu et al., 2017). Therefore, if these relationships occurred in S-FCA then it is important to note that assimilation is not equivalent to Feammox, and the previous three biostimulation experiments may actually indicate independent Fe(III) reduction and/or Fe(II) oxidation and NH_4^+ consumption. Therefore, future work should incorporate ^{15}N isotopes and other methods for assessing the effects of the vitamin and molybdate solutions on NH_4^+-N and Fe with and without soil.

5.1.6 Nitrite behavior in the biostimulation experiments

Minimal dissolved NO_2^- was produced in all treatments and controls (Table 4.3) throughout the four biostimulation experiments within the 118-day anaerobic incubation, indicating that the decrease in dissolved NH_4^+-N did not correspond to substantial dissolved NO_2^- production. Feammox can produce NO_2^- alongside N_2, and may explain some of the dissolved NO_2^- production. However the low dissolved NO_2^- may also be explained by other anaerobic activity. For example, anammox consumes NO_2^- to produce N_2 (fig. 5.1B) (Gonzalez-Martinez et al., 2018), anaerobic DNRA involves NO_3^- reduction to NO_2^- (Pilegaard, 2013; Bu et al., 2017), denitrification reduces NO_2^- to N_2 (fig. 5.1C) (Clément et al., 2005; Huang and Jaffé, 2015), NDFO can convert NO_2^- to N_2 while oxidizing Fe^{2+} (Fig. 5.1G) (Bao and Li, 2017; Bao and Li, 2018b; Yang et al., 2018; Yang et al., 2019), and chemodenitrification reduces NO_2^- to

N_2O (fig. 5.1D) (Jamieson et al., 2018). Characterization of N_2, N_2O, and NO_3^- production in future experiments as well as incorporating ^{15}N would help elucidate other N intermediates or end products and provide evidence for Feammox or other reactions.

5.1.7 Feammox presence in the 118-day anaerobic incubation

The different results in the S-FCA and S-FHA treatments do not support the hypothesis that only ferrihydrite can stimulate NH_4^+ oxidation/loss in near-neutral to alkaline anaerobic soil slurries in the 118-day anaerobic incubation. Instead, the most significant and consistent decrease in dissolved NH_4^+-N was in the S-FCA treatment. This result conflicts with previous anaerobic incubation experiments involving acidic conditions where the ferric citrate + ammonium treatments exhibited Fe reduction but no NH_4^+ oxidation (Huang and Jaffé, 2015, 2018). The loss of dissolved NH_4^+-N, minimal dissolved NO_2^--N, and the Fe(III) reduction and/or oxidation in S-FCA may be explained by a relationship between the N, Fe, and C. e.g. via N assimilation (Burkovski, 2003), Fe(III) reduction (e.g. via Feammox or other pathways), and citrate consumption and/or degradation. The results in S-FCA may also be explained by anammox, denitrification, or other pathways like chemodenitrification or NDFO. Without characterization of other N species like NO_3^-, N_2O, and N_2 (e.g. via ^{15}N) and incorporating C_2H_2, AQDS, general microbial community characterization, and Fe(III) spectroscopy, it is difficult to determine if these results in S-FCA are strictly related to Feammox.

It is possible that the Fe(III) reduction and dissolved NH_4^+-N loss are independent processes in S-FCA, or are related but not due to Feammox. For example, the Fe(III) reduction could be mediated by DIRB (Huang and Jaffé, 2015; Zhou et al., 2016), or influenced by OM oxidation-Fe reduction (Zhou et al., 2016), while the dissolved NH_4^+-N decrease could be explained by OM interactions or anammox (Gonzalez-Martinez, 2018). Huang and Jaffé (2015) suggest that released citrate ($C_6H_5O_7^{-3}$) is consumed by microbial populations, creating poorly-crystalline minerals like ferrihydrite that participates in Feammox (Huang and Jaffé, 2015). Huang and Jaffé (2015) do not provide evidence or details on how the latter process may have worked, and it was difficult to assess in the present study because mineral spectroscopy was not incorporated. However, in the Biostimulation with Fe(III) and Biostimulation with Ferric citrate and NH_4^+ experiments, the changes in dissolved Fe(III) and dissolved Fe(II) in S-FCA may

represent the generation of insoluble Fe(III) or Fe(II) precipitates that may have been influenced by Fe(III) reduction and oxidation activity.

As mentioned in section 5.1.2, citrate may be an important organic C source in the anaerobic conditions of the present study. When considering the NH_4Cl and ferric citrate amendments in S-FCA a potential N-Fe-C relationship can be seen. For example, the NH_4^+ can readily change into NH_3 in near neutral to alkaline conditions and at room temperature (CCME, 2010), which might trigger NH_3 assimilation (fig 5.1A) that aids in biosynthesis and cell growth (Burkovski, 2003). Assimilation NO_3^-, NO_2^- (Burkovski, 2003; Sparacino-Watkins, 2014; Herrero et al., 2018) may have also occurred. This assimilation could also stimulate the microbe's need for Fe(II), e.g. for enzymes (Čorić and Holland, 2016; Macleod and Holland, 2013) or proteins (Huang et al., 2014), which it can access in ferric citrate or soil minerals via dissimilatory reduction or assimilation that then improves microbial growth and/or reproduction (Schröder et al., 2003). For example, Fe^{2+} assimilation can enhance anammox efficiency (and therefore NH_4^+-N consumption) (Huang et al., 2014). The citrate, if assimilated, can also aid in energy generation (Jurtshuk Jr., 1996). Therefore, in S-FCA the Fe(III) in ferric citrate may have been rapidly reduced, and the citrate could participate in microbe-mediated Fe(III)-extraction (Schröder et al., 2003) and/or C cycling (Jurtshuk Jr., 1996). Furthermore, these positive effects of the C, N, and Fe on microbial growth and/or energy can improve the microbial population's need for additional N, Fe, and C, thus potentially inducing priming of native SOM (Kuzyakov et al., 2000). The increase in dissolved NH_4^+-N in S-FCA in the Biostimulation with Vitamins experiment may be due to OM priming, leading to mineralization and re-release of NH_4^+ from dead cell material (Barak et al., 1997; Kuzyakov et al., 2000; Shaffer et al., 2001).

This N-Fe-C relationship may have been stimulated by NH_4Cl and ferric citrate additions in S-FCA, but anammox, Feammox, and denitrification are still possible explanations for the results. Fe^{2+} assimilation can enhance anammox efficiency (Huang et al., 2014); therefore, the Fe^{2+} generated from bioavailable ferric citrate may have enhanced anammox efficiency, resulting in a greater dissolved NH_4^+-N loss and minimal dissolved NO_2^--N accumulation. The change in dissolved NH_4^+-N concentration, minimal dissolved NO_2^--N production, and Fe(III) reduction in S-FCA may also be explained by Feammox activity (Clément et al., 2005; Huang and Jaffé, 2015). Citrate may have also stimulated denitrification, a heterotrophic process, which could explain the minimal NO_2^--N in S-FCA in the present study. Since C_2H_2 was not used in the

present study and N_2O and N_2 were not measured it is difficult to isolate anammox and denitrification (which can produce N_2O and N_2) from Feammox (produces N_2 and no N_2O) in S-FCA. Therefore, reaction coupling could be occurring in S-FCA, e.g. Feammox-denitrification (Huang and Jaffé, 2015; Yi et al., 2019) or Feammox-anammox (Huang and Jaffé, 2015). More experiments that incorporate components like ^{13}C, ^{15}N and C_2H_2 are needed to confirm which reactions were occurring.

In the S-FC and S-FCA treatments gas bubbles began arising from the soil around day 30 of the 118-day anaerobic incubation during the Biostimulation with Fe(III) experiment. The samples continued generating gas for the remainder of the incubation, however their composition was not determined. These bubbles could potentially include products of anaerobic NH_4^+ oxidation or NO_2^- reduction, such as N_2O, N_2, NO (Huang and Jaffé, 2015; Yang et al., 2020). However, there was minimal dissolved NH_4^+-N in S-FC; therefore the gas bubbles in S-FC and S-FCA could also be the result of SOM-ferric citrate interactions, products of a Fe(III) reduction by DIRB, or products of citrate consumption, such as CH_4, H_2, and CO_2 (Gámez et al., 2009; Ettwig et al. 2016). Although this gas generation would support a N-Fe-C relationship, no assumptions can be made without gas composition data and ideally a characterization of the microbial community.

Furthermore, the role of OM is important in ferric citrate and ferrihydrite bioavailability, as well as Feammox, anammox, denitrification, dissimilatory Fe(III) reduction, assimilation, and other soil reactions. Clay surfaces (Sieczka and Koda, 2016) and OM can sorb NH_4^+ (Wen-Zhao et al., 2013) and influence NH_4^+ oxidation and reduction (Yang et al., 2019) in Feammox and anammox. Given that the soils from the Lomond site are clay-rich (Nichols Environmental, 2018), this may have influenced the dissolved NH_4^+-N results in the 118-day anaerobic incubation. In future experiments characterizing the clay composition, OM composition, and Fe(III)-mineral composition may influence the interpretation of results in S-FCA. Soil OM mineralization can produce NH_4^+, NO_2^-, and NO_3^- (Barak et al., 1997; Kuzyakov et al., 2000; Shaffer et al., 2001) that can stimulate Feammox, anammox, and denitrification. Furthermore, OM can also influence Feammox by influencing Fe(III) reduction (Clément et al., 2005; Yang et al., 2012) and by acting as an electron shuttle (Zhou et al., 2016). Although an OM proxy such as AQDS was not used in the present study, the presence of soil OM in S-FCA versus NS-FCA may explain the difference in dissolved NH_4^+-N loss between the treatments in the first three

biostimulation experiments (Table 4.2). These results indicate the importance of soil OM and the microbial community in Fe(III)-reduction and NH_4^+-N loss, particularly in S-FCA. Future experiments could study the influence of OM as a redox active component and electron shuttle by incorporating AQDS (Zhou et al., 2016).

5.2 Subculture experiment of ferric citrate + NH_4Cl slurry treatment from the 118-day anaerobic incubation

The hypothesis of the subculture was that the dissolved NH_4^+-N would decrease in the S-FCA treatments, i.e. the NH_4^+-N loss results from S-FCA in the 118-day incubation could be sustained in the subculture experiment. Although this hypothesis was not supported for the duration of the incubation, there were signs of a relationship between Fe(III) reduction and NH_4^+ loss in S-FCA at the beginning of the incubation. In the S-FCA treatments there was a brief decrease in dissolved NH_4^+-N prior to day 12 (fig. 4.1) which corresponded to Fe(III) reduction prior to day 3 (fig. 4.2), which may indicate Feammox activity. However, this suspected Feammox activity was temporary. After day 12 the dissolved NH_4^+-N steadily increased and the Fe(III) and Fe(II) substantially decreased after day 3 in the S-FCA treatments. The result in dissolved NH_4^+-N implies that the ferric citrate may have stimulated immobilization followed by mineralization that released NH_4^+ (Barak et al., 1997; Kuzyakov et al., 2000; Shaffer et al., 2001), as suggested in the Biostimulation with Vitamins experiment in the 118-day incubation. The result for the dissolved Fe-species in the S-FCA treatment in the 94-day incubation was similar to the result in the S-FCA treatment during the Biostimulation with Ferric citrate and NH_4^+ experiment in the 118-day anaerobic incubation. The decrease in dissolved Fe(III) and Fe(II) in both incubations indicates that the Fe(III) reduction was proceeded by a mechanism that removed Fe(II) from the insoluble fraction without generating soluble Fe(III), e.g. via chemodenitrification (Jamieson et al., 2018) or SOM interactions (Bird et al., 2011; Adhikari et al., 2017; Cooper et al., 2017). However, it is important to note that the dissolved NH_4^+-N loss was sustained in the 118-day anaerobic incubation by repeatedly amending the S-FCA treatment with ferric citrate and NH_4Cl, whereas they were only added once the beginning of the 94-day incubation. The lack of fresh NH_4Cl and/or ferric citrate amendments may explain why the dissolved NH_4^+-N loss was not sustained. Another possible explanation for the subculture results was that the hypothesis was based on the assumption the microbial community in the S-FCA

treatments could propagate once subcultured. It is possible that diluting the microbial community and soil for the subculture experiment adversely affected the population's ability to oxidize or otherwise transform the NH_4^+. With less soil there is also less SOM that can serve as an electron shuttle and participate in anaerobic microbial reactions such as NH_4^+ oxidation and Fe(III) reduction. Therefore, dilution or lack of additional NH_4Cl and ferric citrate amendments may have hindered dissolved NH_4^+-N loss in the 94-day subculture experiment.

6. SUMMARY AND CONCLUSIONS

The results from the current experiment imply that adding ferric citrate and NH_4Cl to anaerobic soil slurries with near-neutral pH conditions stimulated significant dissolved NH_4^+-N loss, production of minimal dissolved NO_2^-, and generation of gas bubbles with an unknown composition. Furthermore, in the S-FCA treatment there was evidence of Fe(III) reduction in after each ferric citrate addition; however, in some cases Fe(II) oxidation and insoluble Fe-species formation also appeared to be occurring in S-FCA. Despite the changes in dissolved Fe-species, the dissolved NH_4^+-N loss was sustained and improved when both ferric citrate and NH_4Cl were added, and was slightly lower but sustained when only ferric citrate was added to the S-FCA treatment. The only treatment that did not stimulate dissolved NH_4^+-N loss in S-FCA was the vitamin and molybdate solutions, possibly due to the lack of fresh Fe(III) and NH_4Cl, or possibly due to N mineralization. Significant work remains to understand why ferric citrate stimulated this response.

The success of ferric citrate over ferrihydrite in the present study may be due to a difference in Fe(III) bioavailability in near-neutral conditions, and the ability of citrate to stimulate microbial activity and deserves further experimental exploration. These results in S-FCA are not necessarily evidence of Feammox, since anammox and denitrification are still possible and other reactions also influence Fe and N chemistry. Future experiments should assess Feammox in these soils and isolate Feammox from these other N-removal pathways before considering *in situ* remediation applications. Incorporating ferric citrate in *in situ* bioremediation applications in near-neutral to alkaline soils might not have adverse effects, depending on the nature of the unidentified gas bubbles which could contain GHGs. However, further laboratory experiments should be conducted as a precautionary method. The biostimulation experiments indicated that if the S-FCA treatment was most active when both ferric citrate and NH_4Cl were added. Therefore, if ferric citrate was applied periodically to a N-polluted soil, the communication between soil and groundwater may provide continuous NH_4^+ to stimulate NH_4^+ loss. The results from this study indicate that ferric citrate might stimulate denitrification,

anammox, and Feammox to reduce the negative impacts of N pollution in neutral-alkaline anaerobic soils. Incorporating C_2H_2, ^{15}N isotope tracing, general microbe community characterization, mineral spectroscopy, and testing the influence of OM via AQDS addition are examples of components to incorporate into future Feammox experiments with ferric citrate prior to *in situ* applications.

7. REFERENCES

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APPENDIX

8.1 Appendix A: Tables for dissolved NO_2^-, Fe(III), Fe(II), and NH_4^+ for the 118-day anaerobic incubation

Table A1. Average dissolved Fe(II) (mg L^{-1}) (mean ± SE for soil (S-) slurries (n=3) n=1 for no-soil (NS-) controls) for samples treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (without Fe(III) and NH_4Cl) were added to all treatments.

	Initial	**Initial Biostimulation with Fe(III) and NH_4^+**				**Biostimulation with Fe(III)**			
Day	*0*	*1*	*7*	*12*	*22*	*33*	*49*	*68*	*73*
NS-A	-3	-2	-12	-19	-17	-13	10	-14	-7
NS-FCA	-4	17	39	47	35	196	80	78	80
NS-FHA	-4	-2	-11	-19	-17	-13	10	-13	-8
S-A	-4 ± 0.1	-2 ± 0.5	-12 ± 0.4	-20 ± 0.3	-18 ± 0.4	-13 ± 0.2	10 ± 0.2	-14 ± 0.5	-7 ± 0.4
S-FC	-3 ± 0.4	2 ± 3.5	190 ± 6.2	221 ± 19.4	182 ± 6.5	576 ± 14.1	240 ± 8.3	114 ± 46.9	65 ± 29.6
S-FH	-3 ± 0.2	-2 ± 0.2	-12 ± 0.2	-20 ± 0.1	-18 ± 0.1	-13 ± 0.0	10 ± 0.8	-14 ± 0.7	-8 ± 0.4
S-FCA	-4 ± 0.2	3 ± 0.5	206 ± 4.9	199 ± 5.2	181 ± 6.0	723 ± 8.8	254 ± 9.1	44 ± 5.5	38 ± 13.8
S-FHA	-4 ± 0.1	-2 ± 0.2	-13 ± 0.6	-19 ± 0.4	-17 ± 0.7	-13 ± 0.4	10 ± 0.4	-13 ± 2.6	-5 ± 2.9

	Biostimulation with Ferric citrate and NH_4^+				**Biostimulation with Vitamins**	
Day	*74*	*80*	*82*	*96*	*104*	*112*
NS-A	-38	-15	-20	-17	-16	-12
NS-FCA	62	77	88	91	100	89
NS-FHA	-40	-15	-19	-17	-15	154
S-A	-41 ± 0.3	-15 ± 1.1	-20 ± 0.2	-16 ± 0.7	-15 ± 1.8	-12 ± 0.3
S-FC	48 ± 38.6	118 ± 1.5	68 ± 10.8	10 ± 5.0	-13 ± 1.1	-12 ± 0.8
S-FH	-42 ± 0.6	-14 ± 0.6	-20 ± 0.9	-14 ± 6.1	-15 ± 3.1	-12 ± 1.2
S-FCA	42 ± 17.7	128 ± 11.9	81 ± 17.3	10 ± 3.9	-9 ± 3.2	-8 ± 1.7
S-FHA	-42 ± 2.0	-14 ± 2.0	-19 ± 0.7	-13 ± 1.5	-13 ± 2.6	-12 ± 0.8

Table A2. Average dissolved Fe(III) (mg L^{-1}) (mean ± SE for soil (S-) slurries (n=3) n=1 for no-soil (NS-) controls) for samples treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (without Fe(III) and NH_4Cl) were added to all treatments.

	Initial	**Initial Biostimulation with Fe(III) and NH_4^+**				**Biostimulation with Fe(III)**			
Day	*0*	*1*	*7*	*12*	*22*	*33*	*49*	*68*	*73*
NS-A	2	2	3	-6	3	6	1	-13	4
NS-FCA	3	105	110	58	50	384	129	358	357
NS-FHA	2	2	4	-2	2	7	-1	-12	6
S-A	3 ± 0.3	3 ± 0.3	3 ± 0.2	-4 ± 1.6	38 ± 65.7	5 ± 0.3	0 ± 0.7	-12 ± 1.0	3 ± 0.5
S-FC	3 ± 0.2	72 ± 18.5	-18 ± 15.4	-23 ± 16.7	-26 ± 5.1	38 ± 3.5	54 ± 36.6	-9 ± 2.9	10 ± 1.4
S-FH	3 ± 0.2	3 ± 0.2	3 ± 0.1	-4 ± 0.1	3 ± 0.1	5 ± 0.3	1 ± 1.0	-12 ± 1.3	4 ± 0.5
S-FCA	3 ± 0.1	82 ± 27.8	-41 ± 6.8	-4 ± 3.6	-19 ± 4.3	-104 ± 4.5	47 ± 7.8	-7 ± 1.8	-13 ± 41.9
S-FHA	3 ± 0.2	3 ± 0.7	4 ± 0.5	-3 ± 0.4	6 ± 5.1	5 ± 0.8	-1 ± 1.6	-14 ± 2.4	2 ± 1.9

	Biostimulation with Ferric citrate and NH_4^+				**Biostimulation with Vitamins**	
Day	*74*	*80*	*82*	*96*	*104*	*112*
NS-A	11.2	-2.3	10.9	-4.6	3.9	0.6
NS-FCA	620.1	459.0	558.0	494.7	522.8	499.6
NS-FHA	13.4	-1.9	12.0	-4.0	6.0	-165.0
S-A	12 ± 0.4	-2 ± 0.9	5 ± 0.23	-4 ± 0.7	5 ± 0.23	1 ± 0.1
S-FC	261 ± 35.2	4 ± 10.4	13 ± 3.34	-3 ± 0.6	13 ± 3.34	5 ± 1.6
S-FH	10 ± 0.3	-2 ± 0.4	5 ± 1.86	-7 ± 4.7	5 ± 1.86	1 ± 0.3
S-FCA	204 ± 20.2	4 ± 0.7	16 ± 4.79	-4 ± 1.2	16 ± 4.79	7 ± 2.0
S-FHA	10 ± 2.2	-3 ± 1.8	3 ± 2.58	-6 ± 1.2	3 ± 2.58	1 ± 1.4

Table A3. Average dissolved NO_2^--N (mg L^{-1}) (mean ± SE for soil (S-) slurries (n=3) n=1 for no-soil (NS-) controls) for samples treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (without Fe(III) and NH_4Cl) were added to all treatments.

	Initial	**Initial Biostimulation with Fe(III) and NH_4^+**					
Day	*0*	*1*	*3*	*5*	*7*	*12*	*22*
NS-A	0.0	0.1	0.1	0.1	0.0	-0.6	-0.5
NS-FCA	-0.1	0.7	1.0	0.6	0.5	0.2	0.3
NS-FHA	-0.1	0.0	0.0	0.1	0.0	-0.6	-0.4
S-A	0.0 ± 0.04	0.0 ± 0.11	0.0 ± 0.01	0.1 ± 0.16	0.1 ± 0.04	-0.5 ± 0.05	-0.6 ± 0.17
S-FC	-0.1 ± 0.02	0.6 ± 0.07	0.7 ± 0.03	0.6 ± 0.02	0.5 ± 0.02	-0.2 ± 0.15	0.1 ± 0.24
S-FH	0.2 ± 0.18	0.0 ± 0.03	0.0 ± 0.01	0.1 ± 0.02	0.1 ± 0.02	-0.4 ± 0.14	-0.6 ± 0.16
S-FCA	-0.1 ± 0.03	0.6 ± 0.03	0.8 ± 0.15	0.8 ± 0.13	0.5 ± 0.04	-0.3 ± 0.30	0.0 ± 0.11
S-FHA	-0.1 ± 0.01	0.0 ± 0.03	0.0 ± 0.02	0.1 ± 0.02	0.0 ± 0.03	-0.6 ± 0.15	-0.6 ± 0.14

	Biostimulation with Fe(III)				**Biostimulation with Ferric citrate and NH_4^+**				
Day	*33*	*49*	*68*	*73*	*74*	*80*	*82*	*89*	*97*
NS-A	-0.5	-0.3	-0.6	-0.7	-0.1	0.4	0.1	0.0	0.1
NS-FCA	-0.2	-0.4	-0.3	0.1	-0.1	0.1	-0.6	-0.1	0.0
NS-FHA	-0.4	0.0	-0.5	-0.8	-0.1	0.5	0.2	-0.1	0.1
S-A	-0.7 ± 0.72	0.0 ± 1.89	-0.6 ± 0.29	-0.6 ± 0.22	-0.1 ± 0.01	0.3 ± 0.33	0.1 ± 0.13	0.1 ± 0.21	0.21 ± 0.06
S-FC	0.6 ± 0.23	1.6 ± 0.94	0.3 ± 0.50	-0.5 ± 0.20	0.0 ± 0.05	0.6 ± 0.27	0.5 ± 0.25	-0.3 ± 0.22	0.08 ± 0.05
S-FH	-0.6 ± 0.27	-0.6 ± 0.60	-0.5 ± 0.20	-0.4 ± 0.14	-0.1 ± 0.02	0.3 ± 0.38	0.1 ± 0.03	0.3 ± 0.16	0.25 ± 0.12
S-FCA	0.3 ± 0.38	0.0 ± 0.13	-0.3 ± 0.28	-0.8 ± 0.02	0.0 ± 0.05	0.8 ± 0.47	0.3 ± 0.14	0.0 ± 0.22	0.1 ± 0.05
S-FHA	-0.6 ± 0.16	0.2 ± 1.00	-0.1 ± 0.18	-0.7 ± 0.11	-0.1 ± 0.04	0.2 ± 0.36	0.1 ± 0.08	0.2 ± 0.17	0.2 ± 0.08

	Biostimulation with Vitamins		
Day	*102*	*104*	*108*
NS-A	0.0	0.2	0.1
NS-FCA	-0.1	-0.1	0.2
NS-FHA	0.0	0.2	0.4
S-A	-0.1 ± 0.05	0.1 ± 0.22	0.2 ± 0.65
S-FC	0.0 ± 0.05	0.2 ± 0.13	0.3 ± 0.09
S-FH	-0.1 ± 0.03	0.3 ± 0.12	0.3 ± 0.19
S-FCA	-0.2 ± 0.08	0.1 ± 0.04	0.3 ± 0.05
S-FHA	-0.2 ± 0.06	0.3 ± 0.04	1.3 ± 1.06

Table A4. Average dissolved NH_4^+-N (mg L^{-1}) (mean ± SE for soil (S-) slurries (n=3) n=1 for no-soil (NS-) controls) for samples treated with ammonium chloride (A), and/or ferric citrate (FC, FCA), and/or ferrihydrite (FH, FHA). In the Initial Biostimulation with Fe(III) and NH_4^+ experiment A, FC, and FH were added to respective treatments; in the Biostimulation with Fe(III) experiment FC and FH were added to respective treatments; in the Biostimulation with Ferric citrate and NH_4^+ experiment A and FC were added to respective treatments; and in the Biostimulation with Vitamins experiment, vitamin and molybdate solutions (without Fe(III) and NH_4Cl) were added to all treatments.

	Initial	**Initial Biostimulation with Fe(III) and NH_4^+**					
Day	*0*	*1*	*3*	*5*	*7*	*12*	*22*
NS-A	0	16	29	30	24	28	30
NS-FCA	0	28	27	29	28	26	27
NS-FHA	0	27	28	31	28	28	30
S-A	1 ± 0.7	26 ± 3.0	23 ± 0.9	24 ± 3.1	20 ± 2.4	22 ± 1.1	25 ± 2.5
S-FC	1 ± 0.5	4 ± 0.3	2 ± 0.4	1 ± 0.4	0 ± 0.4	0 ± 0.3	-1 ± 0.1
S-FH	1 ± 0.3	3 ± 0.5	1 ± 0.8	1 ± 1.0	1 ± 0.5	1 ± 0.2	1 ± 0.8
S-FCA	1 ± 0.4	28 ± 0.6	23 ± 2.2	24 ± 2.4	16 ± 3.4	14 ± 2.3	14 ± 2.1
S-FHA	1 ± 0.6	27 ± 1.6	24 ± 1.4	30 ± 3.7	22 ± 2.1	23 ± 1.2	28 ± 0.2

	Biostimulation with Fe(III)			**Biostimulation with Ferric citrate and NH_4^+**					
Day	*33*	*49*	*68*	*74*	*76*	*80*	*82*	*89*	*97*
NS-A	27	27	31	59	45	51	40	40	60
NS-FCA	30	25	28	44	43	44	35	31	42
NS-FHA	31	25	24	26	23	18	19	21	18
S-A	23 ± 2.0	19 ± 4.4	25 ± 3.5	43 ± 11.3	40 ± 3.7	45 ± 5.0	26 ± 0.9	34 ± 0.7	37 ± 5.5
S-FC	-1 ± 0.9	-2 ± 0.9	-1 ± 0.6	1 ± 9.4	-5 ± 1.2	-2 ± 2.0	-1 ± 0.3	-2 ± 0.5	-3 ± 0.9
S-FH	0 ± 0.7	-1 ± 0.3	0 ± 0.4	-1 ± 1.1	-5 ± 0.9	-2 ± 0.6	-2 ± 0.8	-2 ± 1.2	-3 ± 0.9
S-FCA	18 ± 0.6	8 ± 2.3	14 ± 1.4	22 ± 5.8	19 ± 8.9	28 ± 4.1	20 ± 1.2	19 ± 1.4	9 ± 3.9
S-FHA	24 ± 1.2	20 ± 2.0	24 ± 2.4	17 ± 1.4	17 ± 7.1	16 ± 1.2	14 ± 0.8	14 ± 0.1	15 ± 1.7

	Biostimulation with Vitamins				
Day	*102*	*104*	*108*	*112*	*118*
NS-A	49	54	68	66	65
NS-FCA	55	45	65	27	38
NS-FHA	26	30	30	29	31
S-A	35 ± 13.2	48 ± 4.4	48 ± 1.9	42 ± 2.9	30 ± 9.0
S-FC	-1 ± 1.1	-1 ± 0.6	0 ± 1.4	1 ± 0.8	1 ± 0.6
S-FH	0 ± 1.1	-1 ± 0.4	0 ± 0.4	2 ± 0.6	1 ± 0.6
S-FCA	11 ± 0.3	22 ± 13.8	20 ± 3.5	19 ± 10.6	24 ± 7.2
S-FHA	17 ± 2.1	23 ± 1.1	25 ± 2.8	18 ± 4.8	42 ± 8.0