CHARACTERIZING THE MICROBIALY MEDIATED FEAMMOX PROCESS:
TEMPERATURE EFFECT AND ELECTRON ACCEPTORS

Melany P. Ruiz-Urigüen

A THESIS PRESENTED TO THE FACULTY OF PRINCETON UNIVERSITY IN
CANDICACY FOR THE DEGREE OF MASTER OF SCIENCE

RECOMMENDED FOR ACCEPTANCE BY THE DEPARTMENT OF
CIVIL AND ENVIRONMENTAL ENGINEERING
ADVISOR: PETER R. JAFFE

June 2014
Abstract

Feammox is the name given to the bacterial-mediated form of anaerobic ammonium oxidation under iron reducing condition. Feammox is a novel pathway in the nitrogen cycle, and a key process for alleviating ammonium accumulation in anoxic soils. Biogeochemical processes that regulate the removal of compounds in the environment are affected by numerous factors such as temperature, and no such study has been previous conducted with enriched cultures of Acidimicrobiaceae-bacterium A6, the recently discovered bacteria group found to be responsible of Feammox (Huang and Jaffe, 2013). The research presented herein examines the effect of temperature on the bacterial-mediated Feammox process in batch experiments under controlled pH and nutrient concentration conditions, in order to determine its temperature dependent kinetics. It also takes a first approach to examine the effect of anthraquinone-2,6-disulfonate (AQDS) as a possible electron shuttle to enhance electron transfer from the oxidized ammonium to the iron particle. Finally, preliminary tests were carried out to determine if Fe$_2$O$_3$, and iron oxide formed from oxidizing Fe(0) could act as possible respirable iron. Results indicate that the highest ammonium removal occurs at 20°C. AQDS does not have an effect on the rate of ammonium removal; nonetheless, in its presence less iron is reduced suggesting that AQDS could act as an electron acceptor. Finally, the types of ferric iron analyzed showed to support Feammox; however, further studies are required to understand the
extent of their bioavailability. The results contribute to deepen our knowledge of the biogeochemical needs of the bacterial mediated Feammox process under cultured conditions in order to optimize it. Moreover, the temperature effect also allows us to establish the feasibility of the Feammox-bacteria application for wastewater treatment without the costly oxygen input and at temperatures less favorable for other anaerobic ammonium removal mechanisms.
To my beautiful family.
Acknowledgments

This work was possible with the support from the Secretaría de Educación Superior, Ciencia, Tecnología e Innovación del Ecuador and The Fulbright Commission. The funding for the research was available through The Project X Innovation Fund from Princeton University.

I am particularly grateful to my advisor, Peter Jaffé. Thank you for welcoming me into your Research Group and for guiding me through this amazing experience.

Shan Huang, many thanks for being my tutor and friend, I have learned so much from you.

Biogeochemistry Research Group members: Paul, Matt, Hagar, David, Zheyun, Allise and Emily, thank you all for your words of advice and for making our time in the lab unique and fun.

Thank you to my family for making my life full of joy. My Gabriel, thank you for being so patient while I studied and worked. Juank, thank you for being by my side in our adventure of life. Thank you Mami and Papi for being my inspiration. Thank you Edgar, Karina, Andrea and Becky for your constant support.
# Table of Contents

Abstract ........................................................................................................................................iii

Acknowledgments .........................................................................................................................vi

Table of Contents ..........................................................................................................................vii

List of Figures .................................................................................................................................ix

List of Tables ..................................................................................................................................x

1. Introduction ................................................................................................................................. 1

2. Materials and Methods ............................................................................................................... 8

   2.1. Temperature effect on the bacteria-mediated Feammox process ................................. 8

      2.1.1. Bacterial enrichment culture .................................................................................. 8
      2.1.2. Batch experiment at different temperatures ......................................................... 8
      2.1.3. Chemical analysis .................................................................................................... 9
      2.1.4. DNA isolation .......................................................................................................... 10
      2.1.5. Bacteria diversity and quantification analysis ....................................................... 10
      2.1.6. Computational framework ..................................................................................... 12

   2.2. Electron shuttle and acceptors approach ........................................................................ 13

      2.2.1. Batch culture experiments with AQDS ................................................................. 13
      2.2.2. Ferric iron sources batch culture experiments ...................................................... 14

3. Results and Discussion .............................................................................................................. 15
3.1. Temperature effect on the bacteria-mediated Feammox process .................. 15

3.1.1 Chemical analysis .................................................................................. 15

3.1.2 Bacterial diversity in the Feammox-bacteria batch experiment .............. 20

3.2 Electron shuttle and acceptors approach .................................................. 24

4. Conclusion .................................................................................................. 32

References ..................................................................................................... 35

Appendix ......................................................................................................... 39

A. 2-line ferrihydrite synthesis ........................................................................ 39

B. ANOVA analysis to determine the statistical significance for ammonium concentration change between temperatures ........................................ 41

C. Sequence alignment of Feammox-bacteria from batch culture with

Acidimicrobiaceae-bacterium (A6) sequence (GenBank accession number

KC581762.1) .................................................................................................. 42
List of Figures

Figure 1.1 Nitrogen Cycle. .................................................................................................................. 2
Figure 3.1 Fraction of NH$_4^+$ removed as a function of time and temperature.. .............. 16
Figure 3.2 Concentration of NH$_4^+$ (a) and NO$_2^-$ (b) at four different temperatures ......... 17
Figure 3.3 Acidimicrobiaceae-bacterium A6 detection by qPCR.................................................. 21
Figure 3.4 Reaction rate constants ($k$) for ammonium (NH$_4^+$) oxidation at 15ºC, 20ºC and 35ºC. ................................................................................................................................. 24
Figure 3.5 Measurements of NH$_4^+$ and Fe(II) in the Feammox-bacteria batch enrichment culture with AQDS................................................................................................................................. 26
Figure 3.6 Measurements of NH$_4^+$ and Fe(II) in the Feammox-bacteria batch enrichment culture without AQDS................................................................................................................................. 27
Figure 3.7 NH$_4^+$ oxidation, Fe(III) reduction using Fe$_2$O$_3$ as Fe(III) source................. 29
Figure 3.8 NH$_4^+$ oxidation, Fe(III) reduction using iron oxide formed from the oxidation of Fe.................................................................................................................................................... 30
List of Tables

Table 2.1 Primer sets used to target different bacteria groups in this study................. 11
1. Introduction

Characterizing the biochemical needs of the Feammox process is essential for understanding this bacterial-mediated form of anaerobic ammonium oxidation under iron reducing condition. Feammox is a novel pathway in the nitrogen cycle, and a new linkage between the nitrogen and the iron cycle. Moreover, Feammox represents a key process for alleviating nitrogen (N) accumulation in the form of ammonium (NH\textsubscript{4}\textsuperscript{+}) in anoxic soils, which otherwise can deteriorate the environment.

Nitrogen (N) is an essential molecule for life but in excess it is also a major pollutant. N is needed to promote primary production, and thus, to sustain life. Biological N fixation transforms dinitrogen gas (N\textsubscript{2}) into ammonia, a form of N available for plants and algae. Under natural conditions, N fixation is in balance with N losses through denitrification, a process that returns N in the form of N\textsubscript{2} to the atmosphere. Nonetheless, N produced by humans, mainly in the form of fertilizers, has exceeded nature’s balance, thus contributing to its accumulation in soils and water (Clais and Sabine, 2013). N is normally a limiting nutrient in the environments, but when it is found in excess it can lead to eutrophication and low oxygen concentration in waters, which in turn results in loss of biodiversity. Moreover, if N is not fully lost in the form of N\textsubscript{2}, it can result in other gaseous forms [e.g. nitrous oxide (N\textsubscript{2}O) or NO\textsubscript{x}], thus contributing negatively to climate change. N\textsubscript{2}O is a potent green house gas, and nitric oxide (NO) as well as nitrogen dioxide (NO\textsubscript{2}) cause ozone depletion (Clais and Sabine, 2013). Therefore, optimization of N removal mechanisms is required in order to maintain a healthy environment.
Understanding the nitrogen cycle (Figure 1.1) can aid the process of minimizing anthropogenic N releases (e.g. ammonium resulting from fertilizers) in order to maintain a healthy balance in the ecosystem. The biogeochemical cycle of N involves multiple transformations, most of which are carried out by microorganisms through enzymatic reactions (i.e. N fixation, nitrification, denitrification, and anaerobic ammonium oxidation). Ammonium (NH$_4^+$) is a byproduct of animal metabolism, and it also comes from fertilizer application in crops, thus, resulting in high concentrations of the compound in wastewaters and in agricultural runoffs. Only a few process (i.e. denitrification and anaerobic ammonium oxidation) fully remove N from soils and water back to its most stable form in the atmosphere, N$_2$.

Figure 1.1 Nitrogen Cycle. The Feammox process in shown in green. Note how Feammox links the nitrogen and the iron cycle (not fully drawn). Norg: organic nitrogen; NH$_4^+$: ammonium; N$_2$: dinitrogen gas; N$_2$O: nitrous oxide; NO: nitric oxide; NO$_2^-$: nitrite; NO$_3^-$: nitrate; Fe(III): ferric iron, and Fe(II): ferrous iron. Modified from Schnoor (1996).
The N removal processes are widely applied for treatment of N rich waters such as wastewater. Wastewater require that all N be in its most oxidized state, nitrate (NO$_3^-$), before being released back to the environment in order to ensure that no dissolved oxygen (DO) be consumed to further oxidize any N. DO is probably the single most important chemical parameter that is required to ensure the ecological health of a receiving water (Schnoor, 1996.). Low DO concentrations in water puts aquatic biodiversity under pressure, populations start to decrease and in some cases, it can lead to fauna kills and to general deteriorations of ecosystems. Ensuring the right conditions for NH$_4^+$ removal is thus essential in wastewater treatment plants (WWTP), constructed wetlands as well as in natural systems.

Ammonium is biologically removed in nature and in engineered systems. Nitrification is the main form of NH$_4^+$ removal in WWTP. Autotrophic aerobic bacteria known as nitrifiers mainly drive this process; for example, well-known nitrifiers like *Nitrosomonas* oxidize NH$_4^+$ to NO$_2^-$, and *Nitrobacter* oxidizes NO$_2^-$ to NO$_3^-$. In WWTP, these processes require energetically costly oxygen inputs. Nitrifiers derive their energy from ammonium oxidation and use atmospheric oxygen as their final electron acceptor. In the absence of oxygen, two autotrophic bacteria mediated processes have been described for NH$_4^+$ removal: Anammox (van der Star et al., 2007) and Feammox (Clement et al., 2005; Shrestha et al., 2009). The anammox process requires an initial aerobic nitrification step to form NO$_2^-$, which is combined with NH$_4^+$ on an equimolar basis to produce mainly N$_2$ (Van Hulle et al., 2007). The Feammox process is the only fully anaerobic NH$_4^+$
oxidation pathway known which couples NH₄⁺ oxidation to ferric iron [Fe(III)] reduction to form NO₂⁻ (Clement, et al. 2005), and possibly N₂ (Yang et al., 2012).

The Feammox process has been attributed to a group of uncultured Acidimicrobiaceae-bacterium named A6 which form part of the Actinobacteria phylum (Huang and Jaffe, 2013); referred throughout this document as Feammox-bacteria. It is thought that the microorganisms capable of this process obtain energy from NH₄⁺, which acts as the electron donor while they respire Fe(III), which acts as the electron acceptor. Clement and colleagues (2005) first found Feammox in forested riparian wetlands in New Jersey. They showed that this process is thermodynamically feasible when calculated for natural iron oxides such as goethite (FeOOH):

\[
\text{NH}_4^+ + 6\text{FeOOH} + 10\text{H}^+ \rightarrow \text{NO}_2^- + 6\text{Fe}^{2+} + 10\text{H}_2\text{O} \quad (\Delta G = -30.9 \text{ kJ mol}^{-1}) \quad (1)
\]

Yang and colleagues (2012) reported nitrogen losses from soil through anaerobic NH₄⁺ oxidation coupled to iron reduction in tropical upland soils. They show that Feammox can produce N₂ (equation 2) or NO₂⁻ (equation 3) when using iron (III) hydroxide [Fe(OH)₃] as the ferric iron source, which results in reactions even more thermodynamically favorable:

\[
\text{NH}_4^+ + 3\text{Fe(OH)}_3 + 5\text{H}^+ \rightarrow 0.5\text{N}_2 + 3\text{Fe}^{2+} + 9\text{H}_2\text{O} \quad (\Delta G = -164 \text{ kJ mol}^{-1}) \quad (2)
\]

\[
\text{NH}_4^+ + 6\text{Fe(OH)}_3 + 10\text{H}^+ \rightarrow \text{NO}_2^- + 6\text{Fe}^{2+} + 16\text{H}_2\text{O} \quad (\Delta G = -245 \text{ kJ mol}^{-1}) \quad (3)
\]
Biogeochemical processes that regulate the removal of nutrients in the environment, such as in wetlands, are affected by numerous factors such as pH and temperature. Water temperature is one of the most important cyclic stimuli, along with nutrient inflow rates and concentrations (Kadlec and Reddy, 2001). Different microorganisms show their unique minimum, maximum and optimum growing and activity temperatures, a basic characteristic not previously examined for Feammox-bacteria. The different bacteria responsible for NH$_4^+$ removal are active at different temperature ranges and show their own optimum temperature. Nitrifying bacteria, which convert NH$_4^+$ to NO$_2^-$ and NO$_3^-$ have an optimal temperature of 30-35 ºC (Kadlec and Reddy, 2001). Anammox, which convert NH$_4^+$ and NO$_2^-$ to N$_2$ show an optimal temperature between 30-33 ºC (van der Star et al., 2007). All previous studies on Feammox have been done at room temperature (Clement et al., 2005; Yang et al., 2012), at 35°C (Sawayama, 2006) or in a greenhouse where temperatures changed with ambient temperature (Shrestha et al., 2009). None of these studies were conducted with enriched cultures of the Feammox-bacteria. The lack of information on the effect that temperature has on Feammox-bacteria needs to be answered. Feammox have been found to occur at temperate temperatures, nonetheless, its optimal as well as it whole temperature range had not been previously studied.

The Feammox-bacteria are iron respiring bacteria, and as shown in equations 1-3, the reaction is thermodynamically favorable with different types of Fe(III) (i.e. FeOOH or Fe(OH)$_3$). Nonetheless, iron bioavailability (i.e. iron used by the microorganisms) can be a limiting factor, and the extend to which ferric iron is bioavailable depends on many
factors: (1) Fe(III) chemical structure ranging from more bioavailable when iron has an amorphous structure, to less bioavailable when in a crystalline structure; (2) Fe(III) concentration in the system; and (3) Accessibility to the Fe(III) phase, since it could be blocked by Fe(II) being formed and then sorbed to Fe(III) phase (Komlos and Jaffe, 2004). Moreover, it is known that Fe(III) reduction can be stimulated by humic substances and humic analogues such as anthraquinone-2,6-disulfonate (AQDS) by serving as electron shuttles from the Fe(III)-reducing microorganisms and Fe(III) oxides; presumably because the electron shuttling alleviates the need for direct contact between the microorganism and Fe(III) particles (Komlos and Jaffe, 2004; Nevin and Lovley, 2000). Therefore, the ability of Feammox-bacteria to use more than one form of Fe(III) opens the questions of whether they could use other, more abundant sources of Fe(III) such as iron oxides formed from Fe(0) and if the AQDS, could act as electron acceptor and shuttle in a Feammox-bacteria enrichment culture, thus facilitating NH$_4^+$ oxidation.

The research presented herein primarily examines the effect of temperature on the bacterial-mediated Feammox process in batch experiments under controlled pH, and nutrient concentration conditions, in order to determine its temperature dependent kinetics. Secondly, it seeks a preliminary determination of AQDS’s role in the Feammox-bacteria enrichment culture, as well as a preliminary determination of other forms of bioavailable Fe(III) sources, particularly iron oxide formed by oxidizing Fe(0) because of its low cost and abundance. The results of this research will allow us to establish the feasibility of the Feammox-bacteria consortium application for wastewater treatment in constructed wetlands or in activated sludge process in WWTPs without the costly oxygen
input and at temperatures less favorable for other anaerobic NH$_4^+$ removal mechanisms. Furthermore, the results will help us optimize the culture conditions for higher Feammox-bacteria yield in a laboratory facility.
2. Materials and Methods

2.1. Temperature effect on the bacteria-mediated Feammox process

2.1.1. Bacterial enrichment culture

The seed sludge for the Feammox-bacteria used in the incubation experiments came from a continuous membrane reactor with constant nutrient supply. The reactor favored Acidimicrobiaceae-bacterium A6, previously reported as responsible for the anaerobic ammonium oxidation process under iron reducing conditions (Huang and Jaffé, 2013). The seed was transferred to a batch culture kept at room temperature (20-25°C) in an oxygen-free glove box, with constant stirring and nutrients supplied every 10 days. The enrichment medium for the culture contained: 2-line ferrihydrite (Fe$_2$O$_3$·0.5H$_2$O) 30mM (see Appendix 1 for preparation), NH$_4$Cl 5mM; NaHCO$_3$ 0.24mM, KHCO$_3$ 0.71mM, KH$_2$PO$_4$ 0.052mM, MgSO$_4$·7H$_2$O 0.41mM, CaCl$_2$ 0.54mM, AQDS (9,10-anthraquinone-2, 6-disulfonic acid) 0.05mM, vitamin supplement (ATCC®) 0.1µl/l, and trace element solution; the last one as describe in Sawayama (2006).

2.1.2. Batch experiment at different temperatures

The bacteria grown in the batch enrichment culture (section 2.1.1) were given enrichment medium with increased amounts of Fe(III) to 90mM; this is 3 times more Fe(III) than
stoichiometrically predicted (equation 1). The excess Fe(III) was given in order to avoid iron limitations during incubations. The pH was set at 4.5 using HCl 2N. The bacteria were allowed to acclimate overnight to the medium before 10ml of the completely mixed bacteria culture were dispensed into borosilicate-glass scintillation vials. A set of control vials was prepared with enrichment medium containing 90mM of Fe(III), but without adding bacteria (i.e. without enrichment culture). Before being taken out the glove box, each vial was capped and sealed with parafilm® to ensure anoxia in the system. The vials were kept at 4°C until each incubation cycle began, and none of the vials exceed 6 days at 4°C. The vials were separated into four groups including control vial, and each group was incubated with horizontal rotation under one temperature; i.e. 10°C, 15°C, 20°C or 35°C. Once the desired incubation temperature was reached (approximately in 1 hour), two 8-hours sampling rounds took place at each temperature. On the first day, 2 or 3 vials were destructively sampled every hour for 8 consecutive hours, and one control vial was sampled every 2 hours (i.e. at time = 0, 2, 4, 6 and 8 hours). On the second day, 24 hours since the incubation began (i.e. 24 hours after the first vials were sampled), another sampling round took place, exactly as described above. Each vial was analyzed for NH$_4^+$, NO$_2^-/NO_3^-$, Fe(II) and bacteria biomass diversity and concentrations.

2.1.3. Chemical analysis

From each incubated vial, 1.5ml of the culture were filtered using a 0.2µm pore size syringe filter. The filtered solution was used to determine NH$_4^+$, NO$_2^-$ and NO$_3^-$ concentrations using a Dionex™ Ion Chromatograph ICS3000. For analysis of cations, i.e. NH$_4^+$, a CS-16 column with CS-16 guard column, and a CERS 500 (4mm) suppressor
were used. For anion analysis, i.e. NO$_2^-$ and NO$_3^-$, an AS-22 column with an AG-22 guard column, and an ASRS 300 (4mm) suppressor were used. Ferrous iron [Fe(II)] was extracted by adding 1ml of the incubated bacterial culture to 1ml of 2N HCl (i.e. HCl final concentration: 1N), then the HCl-extracted iron was quantified photometrically using the ferrozine method analysis (Komlos and Jaffe, 2004; Stookey, 1970).

2.1.4. DNA isolation.

DNA was extracted from 5ml of the bacterial culture belonging to samples taken at the beginning (time = 0 hours) and at the end (time = 8 hours) of each incubation cycle, i.e. on the first and second day of sampling; therefore, four DNA samples were obtained from each group of vials incubated at each temperature. DNA was extracted using FastDNA® spin kit for soil from MP Biomedicals (USA); the protocol used was as described by the manufacturer, being the initial substrate a pellet formed from the bacterial culture. The pellet was obtained by centrifuging the 5ml of the bacteria culture at 10,000 rpm for 2 minutes. All DNA samples were preserved at -20ºC for subsequent analysis.

2.1.5. Bacteria diversity and quantification analysis

Quantitative PCR (qPCR) assays were carried out using the Applied Biosystems StepOnePlus™ Real Time PCR system. The presence of Feammox-bacteria, other groups of ammonium oxidation bacteria and denitrifiers, as well the count of total bacteria present in the system were determined by qPCR using specific primer sets reported in the literature (Table 2.1). Feammox-bacteria detection and quantification were determined
using the primer set acm432F/acm439R (Personal communication by Shan Huang 2013). Ammonium-oxidizing bacteria, also known as AOB, responsible for nitrification were detected using the primer set AmoA-1F/AmoA-2R that targets part of the functional gene ammonia monooxygenase (Rothhauwe et al., 1997). Anaerobic ammonium-oxidation bacteria, i.e. Anammox, were detected using the primer set Amx368F/Amx820R which targets a region of the 16S rRNA gene of Anammox (Schmid et al., 2003). Denitrifiers were detected by using the primer set NirS3F/NirS5R that amplifies a specific fragment of the nitrite reductase gene (Braker et al., 1998). Finally, in order to determine the percentage of Feammox-bacteria in the system relative to the total biomass, a total bacteria count was achieved by amplifying a region of the 16S rRNA gene using the primer set 1055F/1392R (Harms et al., 2003).

Table 2.1 Primer sets used to target different bacteria groups in this study.

<table>
<thead>
<tr>
<th>Targeted group</th>
<th>Target gene</th>
<th>Primer name&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feammox-bacteria</td>
<td>16s</td>
<td>acm432F</td>
<td>GCA ATG GGG GAA ACC CTG AC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acm439R</td>
<td>ACC GTC AAT TTC GTC CCT GC</td>
</tr>
<tr>
<td>AOB</td>
<td>AmoA</td>
<td>AmoA-1F</td>
<td>GGG GTT TCT ACT GGT GGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AmoA-2R</td>
<td>CCC CTC (G/T)G(G/C) AAA GCC TTC TTC</td>
</tr>
<tr>
<td>Anammox-bacteria</td>
<td>16S</td>
<td>Amx368F</td>
<td>CCT TTC GGG CAT TGC GAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Amx820R</td>
<td>AAA ACC CCT CTA CTT AGT GCC C</td>
</tr>
<tr>
<td>Denitrifiers</td>
<td>NirS</td>
<td>NirS3F</td>
<td>TTC CT(C/G/T) CA(C/T) GAC GGC GGC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NirS5R</td>
<td>CTT GTT G(A/T)A CTC G(C/G)(C/G) CTG CAC</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>16S</td>
<td>1055F</td>
<td>ATG GCT GTC GTC AGC T</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1392R</td>
<td>ACG GGC GGT GTG TAC</td>
</tr>
</tbody>
</table>

<sup>a</sup>Primers’ names where F = forward and R = reverse.
Sequencing of two samples of DNA extracted and amplified from the batch enrichment cultures was performed by Genewiz Inc. (USA) in order to further confirm that the DNA targeted and amplified sequences belonged to Feammox-bacteria as reported by Huang and Jaffé (2013). Using Clustal Omega©, the resulting sequences were aligned against the original Feammox-bacteria partial sequence available in GenBank under the accession number KC581762.1.

2.1.6. Computational framework.

The statistical significance of differences among NH₄⁺ concentration changes between temperatures was determined by one-way ANOVA. The average value for each for each time point was used for the analysis.

The NH₄⁺ concentration data obtained was used to determine the reaction rate constant \( k \) in units of hour⁻¹.

\[
\frac{d[NH_4^+]}{dt} = -k[NH_4^+] \quad (4)
\]

The modified form of the van’t Hoff-Arrhenius relationship was applied to adjust the removal rate coefficient to the temperature effects on the Feammox process.

\[
k = k_{20} \theta^{(T-20)} \quad (5)
\]

where,  
- \( k \) = ammonium oxidation rate constant at \( T \)°C (mM/h)  
- \( k_{20} \) = ammonium oxidation rate constant at 20°C (mM/h)  
- \( \theta \) = temperature coefficient  
- \( T \) = temperature (°C)
2.2. Electron shuttle and acceptors approach

2.2.1. Batch culture experiments with AQDS

AQDS (antraquinone-2,6-disulfonate) was used as a humic acid analogue. Two 200ml bottles were prepared with the bacterial enrichment culture from the continuous membrane reactor. Both bottles were maintained as described in section 2.1.1, except for AQDS. One bottle was not provided with AQDS and the second one did have AQDS. Sampling took place over 20 days period, sampling consisted of taking two replicas of 2.5ml of the culture to be analyzed for NH$_4^+$ and Fe(II) as described in section 2.1.3 (total n=10, two initial replicas and 3 replicas when ammonium removal was detected). Control vials without Feammox culture and containing the enrichment media with AQDS and without AQDS were set up at the beginning of the incubation and they were sampled at the beginning and end of the sampling period in the same way as the culture medium.

Analysis of the enrichment cultures with and without AQDS were performed by reversed-phase, ion–pairing liquid chromatography coupled by electrospray ionization to a high-resolution, high-mass-accuracy mass spectrometer (LC-MS) (Exactive; ThermoFisher). Personnel at the Lewis-Sigler Institute for Integrative Genomics at Princeton University carried out the analysis and provided the results.
2.2.2. Ferric iron sources batch culture experiments

Ferric iron in the form of Fe₂O₃ powder, and iron oxide formed from the oxidation of Fe(0) were used in the bacterial enrichment culture as the electron acceptor source. Metal iron of zero valence [Fe(0)] was hydrated with deionized water and left to dry at room-ambient conditions until it was oxidized, which was determined by the change of color, from original metal grey, to black and finally rusty orange. The amount of total Fe(II) and Fe(III) formed from the oxidation reaction was determined by quantifying the total extracted iron using the ferrozine method and the hydroxylamine hydrochloride - ferrozine method respectively, as described in Komlos and Jaffe (2004). Two 200 ml bottles (i.e. two replicas) were set up for each type of ferric iron provided (total = 4 bottles), and they were maintained as described in section 2.1.1. Sampling took place over 20 days period, sampling consisted of taking 2.5ml of the culture from each bottle to be analyzed for NH₄⁺ and Fe(II) as described in section 2.1.3 (total n=10, tow initial replicas and 3 replicas when ammonium removal was detected). A control vial containing the culture medium with Fe₂O₃ or iron oxide form from Fe(0), and without Feammox culture was set up at the beginning of the incubation cycle. Controls were sampled at the beginning and end of the sampling period in the same way as the culture medium.
3. Results and Discussion

3.1. Temperature effect on the bacteria-mediated Feammox process

Determination of the optimal temperature for the Feammox-bacteria facilitates its cultivation and allows the determination of its possible applications. Therefore, the effect of temperature on the bacterially mediated Feammox process was analyzed in order to determine its temperature dependent kinetics. This type of study has not been reported previously for the bacteria responsible for the Feammox process, the Acidimicrobiaceae-bacterium named A6 (Huang and Jaffe, 2013).

3.1.1 Chemical analysis

Ammonium (NH$_4^+$) concentration change was measured as a function of time and temperature. The results show a clear temperature effect on the kinetics of Feammox (p<0.001) (Figure 3.1) (ANOVA input data and results shown in Appendix B). The data points obtained from NH$_4^+$ concentration were plotted (Figure 3.2 a), and a linear regression was applied in order to get their slope values. At 20ºC about 20% of the NH$_4^+$ was removed over an 8-hour period (slope: -0.26 mMh$^{-1}$ on the first cycle of sampling and -0.21 mMh$^{-1}$ on the second cycle). The second highest removal occurred at 35ºC at which temperature 13% of the NH$_4^+$ was removed after 8 hours (slope: -0.1 mMh$^{-1}$ on the first day, and -0.16 mMh$^{-1}$ on the second sampling day). At 15ºC, 7.2% of the NH$_4^+$
was removed, most of it on the second day (slope: -0.075 mMh\(^{-1}\) obtained from the second set of sampling). No removal was detected at 10ºC.

Figure 3.1 Fraction of NH\(_4^+\) removed as a function of time and temperature. The graph shows that the highest fraction of ammonium removed over 8-hour period was measured at 20ºC. The values represent the mean, where n=6 for 10, 20 and 35ºC. At 15ºC, n=3.

The initial NH\(_4^+\) concentration for all treatments is almost double the concentration given in the enrichment medium (5mM). This increment in concentration is originated from the Feammox-bacteria seed sludge from a reactor that had a constant NH\(_4^+\) 5mM influent. This effect was not found for nitrite (NO\(_2^-\)), which concentration was undetectable at the beginning of the first set of incubations. However, throughout both sampling cycles (i.e. day 1 and 2), some NO\(_2^-\) was detected (Figure 3.2 b).
Figure 3.2 Concentration of NH$_4^+$ (a) and NO$_2^-$ (b) at four different temperatures (10, 15, 20 and 35°C). Measurements were taken on two days for 8 hours each day. The values represent the mean and standard error, (n=3).
Reaction rates are strongly affected by temperature changes, but reaction stoichiometry is relatively unaffected (Tchobanoglous, 1987). According to the stoichiometry of the reaction (equation 1), for each mol of NH$_4^+$ removed, a mol of NO$_2^-$ must be formed. The NO$_2^-$ measurements presented in this study show little of it in the system. The only known forms of NO$_2^-$ formation are through nitrification and denitrification, both of which are microbially mediated processes. Nitrification is an aerobic process that converts NH$_4^+$ to NO$_2^-$. Denitrification transforms NO$_3^-$ to NO$_2^-$ by heterotrophic bacteria. The Feammox-culture was kept at anaerobic condition (Dissolved oxygen concentration between 0.1mg/l and below detection limits), and did not have any organic carbon input. Therefore, the NO$_2^-$ peaks found in our system cannot be attributed to nitrification nor denitrification. On the other hand, the Feammox pathway oxidizes NH$_4^+$ to NO$_2^-$ in the absence of oxygen and organic carbon. Nitrite measurements from the batch experiments showed concentrations that were too low to be detected at all times. Nonetheless, as shown in figure 3.2 b, some NO$_2^-$ peaks appear at different times in the incubations at 20°C and 35°C. In the incubation at 15°C, NO$_2^-$ peaks appear on the second day of the sampling cycle, which coincides with NH$_4^+$ concentration decrease. Moreover, at 10°C no NH$_4^+$ removal was detected, which explains why no NO$_2^-$ was detected at 10°C, except at 8 hours on the second day of sampling where a small amount was measured in 1 out of 3 replicas.

NO$_2^-$ is considered an intermediate molecule between NH$_4^+$ and NO$_3^-$, or between NO$_3^-$ and a form of reduced N (e.g. N$_2$). NO$_2^-$ is known to be rapidly consumed and thus, hard to measure. However, other studies (Clement et al., 2005; Shrestha et al., 2009) have
measured µM concentration of NO$_2^-$ when conditions were appropriate for Feammox. Such measurements are consistent with the ones found in this study. Moreover, the amount of NO$_3^-$ measured was stable throughout the entire sampling period, with an average concentration difference between samples of 0.01mM. This is an indicator that denitrification is not responsible for the NO$_2^-$ measured, but perhaps for the lack of NO$_2^-$ build up.

Iron is the electron acceptor in the Feammox reaction and in this study we provided excess Fe(III) in order to avoid any electron acceptor limitation. Fe(III) is soluble at low pH but its solubility decreases as the pH increases. Therefore, Fe(III) forms small visible particles when in solution at pH 7, which is the same pH used for the ferrozine analysis. The ferrozine method used was useful for detecting the presence of Fe(II) but did not permit its quantification. The high concentration of the particulated Fe(III) formed when in contact with ferrozine interfered with the spectrophotometer reading. Consequently, ferrozine analysis was used to determine the presence of Fe(II), which was confirmed in all samples, and the control samples showed no visible color reaction. The presence of Fe(II) is the result of Fe(III) accepting the electron released from NH$_4^+$ during the Feammox process. Under lower iron concentration conditions, the amount of ferrous iron has been easily quantified; moreover, previous studies (Clement et al., 2005) have shown that the NO$_2^-$/Fe(II) ratios measured are relatively consistent with the stoichiometry of the reaction (equations 1 and 3).
3.1.2 Bacterial diversity in the Feammox-bacteria batch experiment

All batch experiments in this study were carried out with Feammox-bacteria enrichment culture and its sludge seed came from a continuous membrane reactor. The reactor conditions as well as the batch incubation conditions favored Feammox-bacteria population. Nonetheless, other microorganisms are known to be present in the system.

Real-time quantitative PCR (qPCR) analysis confirmed the presence of Feammox-bacteria in all of the samples analyzed (i.e. vials sampled at time = 1 and 8 hours of incubation on the first and second incubation cycle) (Figure 3.3). Most importantly, the quantity of DNA copies per ml of culture was found to be fairly stable at 10 and 15ºC. Interestingly, at 20ºC, there appears to be a slight increase in the bacteria biomass over time, which could be linked with the amount of NH$_4^+$ consumed by Feammox-bacteria. At 35ºC, Feammox-bacteria also show a number of DNA copies per ml of culture comparable to the amount found mainly at 20ºC, however, the NH$_4^+$ consumption was slower. Overall, the amount of biomass does not have a great change among samples; therefore, temperature appears to have an effect on the Feammox-bacteria NH$_4^+$ oxidation metabolism, more than in biomass buildup as such.
Anammox were detected in the system in the initial sample at 10°C, and in the rest of the samples at 20°C and 35°C, however the detection was below 5 DNA-copies/ml. Nitrifiers (i.e. AOB: aerobic ammonium oxidation bacteria) were also detected in the samples at low concentration (<10 DNA-copies/ml). Although AOBs were found in the system it is known that they aerobic organisms; therefore, under the anaerobic conditions at which our system was maintained, it is unlikely that they were active. Denitrifiers were also detected in the analyzed vials, in most cases the concentration was below 50 DNA-copies/ml, except in the first vials sampled from the incubations at 10 and 15°C that had DNA-copies numbers nearly an order of magnitude higher than the rest of the tested
vials. Finally, the total bacteria quantification follows the same pattern as shown in figure 3.3 for the Feammox-bacteria, however, with higher DNA copy numbers. Feammox-bacteria DNA copy number represented in average 79% of the total DNA copy per ml of culture.

The presence of different types of bacteria in the Feammox enrichment culture is a required feature in order to maintain adequate chemical conditions of the system. When NO$_2^-$ concentration increases above 1mM in the culture, Feammox reaction is inhibited (Personal communication by Shan Huang 2013). Denitrifiers are NO$_2^-$ consuming organisms that were detected in all of the incubations and very likely, responsible for the NO$_2^-$ low and even undetectable levels in our samples, as shown in figure 3.2b. Therefore, denitrifiers are an essential component of the Feammox-enrichment culture necessary to sustain the proper chemical environment for Feammox to proceed.

In our incubations, Feammox-bacteria were detected with a primer set developed to target a variable region within their 16s rRNA gene (Personal communication by Shan Huang 2013). However, as an step further to confirm that the bacteria responsible for the Feammox reaction were the Acidimicrobiaceae-bacterium A6 reported by Huang and Jaffe (2013), the sequences obtained were aligned with the original sequence available on GenBank (accession number KC581762.1). One of the sequences aligned 100% with the original Acidimicrobiaceae-bacterium A6, the second sequence aligned 98% of its extension, showing only 3 point mismatches out of 154bp (Appendix C). Therefore, the
Feammox reaction detected in our incubations is indeed attributed to Acidimicrobiaceae-bacterium A6.

Iron is a highly abundant element and as such, Fe(III) reducing microorganism are ubiquitous and phylogenetic diverse (Weber et al., 2006). Therefore, it is possible that the variety of microorganisms able to carry out Feammox goes further beyond Acidimicrobiaceae-bacterium A6. Nonetheless, the sequences repeatedly obtained for this study and previous ones in our laboratory (other data not shown in this manuscript) confirm that Acidimicrobiaceae-bacterium A6 is so far the only reported organism known responsible for Feammox and the one present in our incubations.

This study addresses the effect of temperature of the Feammox process; more precisely, the effect of temperature on the Feammox-bacteria metabolism and thus, its rate of NH$_4^+$ removal. The reaction rate ($k$) obtained by applying equation 4, showed a higher $k$ value at 20ºC ($k$= 0.03hr$^{-1}$), followed by 35ºC ($k$=0.019$^{-1}$) and 15ºC ($k$=0.01$^{-1}$) (Figure 3.4). Finally, the temperature coefficients for temperatures above and below 20ºC were calculated using equation 5. Hence, for temperatures above 20ºC, $\theta$ = 0.97, and for temperatures below 20ºC, $\theta$ = 0.79. This results indicate that the rate removal rapidly increase as it approaches 20ºC and it starts decreasing between 20 and 35ºC. Shrestha and colleagues (2009) determined that the NH$_4^+$ removal rate in forested riparian wetland during the month of October, 2006 in New Jersey area was of 0.0026 min$^{-1}$. The reported value is higher than the reaction rate measured in our batch culture, which indicates that
other factors beyond temperature ought to be taken into account to optimize the process to the scale measured in the environment.

![Graph showing reaction rate constants](image)

Figure 3.4 Reaction rate constants ($k$) for ammonium ($\text{NH}_4^+$) oxidation at 15°C, 20°C and 35°C. The highest rate was measured at 20°C, $k=0.03\text{hr}^{-1}$, followed by 35°C, $k=0.019\text{hr}^{-1}$, and 15°C, $k=0.01\text{hr}^{-1}$. No removal was measured at 10°C. The marks on the graph represent the mean $\text{NH}_4^+$ concentration.

### 3.2 Electron shuttle and acceptors approach

Feammox-bacteria seem to use anthraquinone-2,6-disulfonate (AQDS) as an electron shuttle and perhaps as an electron acceptor. This bacteria also appear to use $\text{Fe}_2\text{O}_3$ and iron oxide formed from Fe(0) as their electron acceptor. Iron respiration has been proposed as one of the first forms of microbial metabolism to have evolved; furthermore,
Fe(III) respiration by extracellular electron transfer has been identified in a number of Bacteria, Archaea and Fungi (Lovley, 1991; Weber et al., 2006). The bioavailability of Fe(III) in inversely proportional to its degree of crystallization (i.e., the more crystalline, the less bioavailable) (Komlos and Jaffe, 2004). However, a review by Lovley et al. (2004) indicates that culturing microorganisms in rich media may permit the reduction of crystalline Fe(III) oxides.

Fe(III) becomes insoluble with increasing pH and this means that it become less accessible for microorganism. However, many forms of possible utilization of insoluble Fe(III) phases have been proposed. For example, some iron reducing bacteria require direct contact with the Fe(III) particle. In other cases, such as in Geobacter spp., indirect contact through nanowires facilitate the electron transfer by creating a bridge between the cell and the physically unavailable iron (Reguera et al., 2005). To alleviate the need for contact, extracellular electron shuttles such as humic acids aid the transfer of the electron to the iron particle (Weber, et al 2006 and references therein), as possibly done by Feammox-bacteria.

In the presence of AQDS, a humic acid homologue, Feammox-bacteria maintain a similar rate of NH$_4^+$ removal as in the culture without AQDS. However, the amount of Fe(II) formed as a result of the reaction is much less than when in culture without AQDS (Figure 3.5 and 3.6).
a. Incubations with AQDS.

Figure 3.5 Measurements of NH$_4^+$ and Fe(II) in the Fammox-bacteria batch enrichment culture with AQDS. Plot (a) shows the change of concentration of NH$_4^+$ and Fe(II) over 20 days of sampling, 2 samples per day (n=10). Notice the break in the time axis between the initial and final concentrations measurements. Graph (b) summarizes the amount of NH$_4^+$ (gray) and Fe(II) (white) measured at the beginning (solid color) and end (textured color) of the 20 days incubation.
a. Incubations without AQDS.

Figure 3.6 Measurements of NH$_4^+$ and Fe(II) in the Feamnox-bacteria batch enrichment culture without AQDS. Plot (a) shows the change of concentration of NH$_4^+$ and Fe(II) over 20 days of sampling, 2 samples per day (n=10). There is break in the time axis between the initial and final concentrations measurements. Graph (b) summarizes the amount of NH$_4^+$ (gray) and Fe(II) (white) measured at the beginning (solid color) and end (textured color) of the 20 days incubation.
Whether AQDS is present or not in the system did not appear to affect the amount of NH$_4^+$ oxidized (slope with AQDS: 0.063 mM d$^{-1}$; slope without AQDS: 0.059 mM d$^{-1}$). Nonetheless, the amount of reduced iron showed significant difference between samples. The control samples, in both cases, did not change the amount of initial NH$_4^+$. The amount of NH$_4^+$ removed over 20 days in these cultures was not optimal due to low Feammox-bacteria concentration in the system at that time. LC-MS analysis of the cultures showed the presence of AQDS at three different oxidation states, being the most reduced state present at much higher concentration than the less reduced AQDS molecules (Personal communication Zheyun Zhan, 2014). Therefore, it is reasonable to believe that AQDS is keeping the electrons and not readily delivering them to Fe(III), thus explaining why Fe(II) formation is almost negligibly in comparison to the analysis done without AQDS, where Fe(III) must be the only electron acceptor available.

Ferric iron, is abundant in nature under different configurations, thus microorganism have evolved to respire more than one type of Fe(III). The use of Fe$_2$O$_3$, a crystalline compound, as well as iron oxides formed from the oxidation of Fe(0), both of which are Fe(III), appeared to support Feammox-bacteria respiration (Figure 3.7 and 3.8). The amount of ammonium removal was similar with either type of Fe(III) source (slope: 0.026 and 0.025mM d$^{-1}$ respectively). Nonetheless, the amount of Fe(II) detected for iron oxide was almost double the amount compared to the culture with Fe$_2$O$_3$. The oxidation of Fe(0) initially forms magnetite, a combination of Fe(II) and Fe(III), which explains the higher amount of Fe(II) measured in the system. Nevertheless, the amount of initial Fe(III) provided was measured at 20.1mM and the amount of Fe(II) in the first sample
was of 6.1mM, which later clearly increased with incubation time. The amount of NH₄⁺ did not change in any of the control vials.

**a. Incubations with Fe₂O₃.**

![Graph showing NH₄⁺ and Fe(II) concentrations over time](image)

**b.**

![Bar graph showing NH₄⁺ and Fe(II) concentrations](image)

Figure 3.7 NH₄⁺ oxidation, Fe(III) reduction using Fe₂O₃ as Fe(III) source. Plot (a) shows the change of concentration of NH₄⁺ and Fe(II) over 20 days of sampling, 2 samples per day. There is break in the time axis between the initial and final concentrations measurements. Graph (b) summarizes the amount of NH₄⁺ and Fe(II) measured at the beginning (solid color) and end (textured color) of the 20 days incubation.
a. Incubations with iron oxide from oxidized Fe(0).

Figure 3.8 NH$_4^+$ oxidation, Fe(III) reduction using iron oxide formed from the oxidation of Fe(0). Plot (a) shows the change of concentration of NH$_4^+$ and Fe(II) over 20 days of sampling, 2 samples per day. There is break in the time axis between the initial and final concentrations measurements. Graph (b) summarizes the amount of NH$_4^+$ and Fe(II) measured at the beginning (solid color) and end (textured color) of the 20 days incubation.
Fe$_2$O$_3$ is a crystalline compound, therefore; its bioavailability can be expected to be very low. Thermodynamic calculations for the Feammox process using Fe$_2$O$_3$ do not result in a reaction that is energetically feasible. There is very literature defining if Fe$_2$O$_3$ forms a more bioavailable compound when in contact with water, for example some type of hydrated iron oxide. The formation of a hydrated iron oxide would make the Feammox reaction thermodynamically feasible, thus explaining the ammonium removal detected in the system (figure 3.6.a). Nonetheless, the results presented herein are an initial approach towards understanding the ability that Feammox-bacteria have to use many forms of ferric iron. Undoubtedly, further studies should be carried out to fully understand the bioavailability of Fe$_2$O$_3$.

Fe oxide formed from zero valence iron Fe(0) was obtained by a reaction in presence of water and oxygen, this reaction forms Fe(II) and Fe(III) (Li et al., 2006). Our results from the incubations indicate that NH$_4^+$ decreased and Fe(II) increased over time. As in the case of Fe$_2$O$_3$, the amount of NH$_4^+$ removed was small; however, in both cases, this can be attributed to the low Feammox-bacteria number at the time of the experiment. Fe(0) has been tested as an electron source for anaerobic wastewater treatment to facilitate organic matter degradation (Feng et al., 2014). Oxidized zero valence iron forms Fe(III) which could then be used by Feammox-bacteria, thus future studies could analyze the possibility of using this highly abundant and low prize type of iron for redox reactions to enhance anaerobic wastewater treatment coupling organic matter degradation with ammonium removal by Feammox-bacteria.
4. Conclusion

The activities of microorganisms are greatly affected by the physicochemical state of their environments, within which nutrient availability; pH and temperature are key factors (Madigan et al., 2012). The research presented here analyzed the effect of temperature on Feammox-bacteria enrichment culture. It also shows the results of an initial analysis of the use of AQDS as an electron shuttle and of two types of Fe(III) sources in the same Feammox-bacteria enrichment culture. It is expected that under ideal nutrient conditions, and as temperature rises, chemical and enzymatic reactions in the cell proceed at more rapid rates and biomass production increases up to a maximum temperature above which the cell may become inactive and die (Madigan et al., 2012). The optimization of any biological process is fundamental prior to its application. Feammox, beyond representing a whole new mechanism of fully anaerobic ammonium oxidation in soils, it is also an attractive candidate to by applied for ammonium removal in engineered systems, and perhaps for bioremediation of other compounds.

In this study we have demonstrated that the Acidimicrobiaceae-bacteria A6 enrichment culture has an optimal temperature at around 20°C, decreasing but not ceasing when moving towards 35°C or 15°C. Aerobic nitrification process is so far the most widely process used for ammonium removal at large scale in WWTP. Nitrifiers increase their activity with increasing temperature ($\theta = 1.04$) (Schnoor, 1996), reaching maximum nitrification at 30-35°C and stopping when reaching temperatures of 45°C (Kadlec and Reddy, 2001). Anammox for wastewater treatment in full-scale reactors require an
optimal temperature of 32-33°C (van der Star et al., 2007). Feammox, unlike other ammonium removal, are active at temperatures less adequate for other NH$_4^+$ oxidation processes. Even at 15°C, the Feammox-bacteria were able to oxidize NH$_4^+$, however, they seem to require a longer acclimation period than when at higher temperature. Feammox-bacteria ought to be studied into more detail in order to develop a mixed culture system for large scale NH$_4^+$ removal in which Feammox could be active when other mechanisms slow down or cease.

In general, organisms live in mixed communities in natures. Mixed cultures mainly formed by nitrifies and denitrifiers are responsible for N removal in nature and in activated sludge. Anammox reactors have already been successfully installed (van der Star et al., 2007), though they also need an initial nitrification process to provide them with NO$_3^-$ needed to couple it to NH$_4^+$ for oxidation. On the other hand, Feammox is inhibited at NO$_2^-$ concentration above 1mM (Personal communication by Shan Huang 2013); thus, NO$_2^-$ uptake by other organism is a required process for Feammox to continue active. Therefore, coupling a succession of optimal N removal setups, from the required aerobic ammonium oxidation to nitrite, to denitrification that requires organic carbon but not oxygen, to anammox that depends on an initial NO$_3^-$ pool, to a final Feammox step that does not require oxygen nor organic carbon, is a promising approach to enhance N losses in engineered systems.

Furthermore, the optimization of Feammox by adequate provision of electron acceptors is yet to be determined. Previous studies have shown that Feammox can use more than one
type of Fe(III) source. For example, goethite (FeOOH) (Clement et al., 2005), iron hydroxides Fe(OH)$_3$ (Yang et al., 2012), or ferrihydrite (Fe$_2$O$_3$·0.5H$_2$O), which is the current iron provided to the Feammox-bacteria enrichment culture reactor. In this study we see that other iron oxides in solution [i.e. Fe$_2$O$_3$ and iron oxide formed from Fe(0)], as well as AQDS can function as electron acceptors. A more recent study suggest that Feammox can possibly use an anode as the terminal electron acceptor (Qu et al., 2014). In this sense, finding the most abundant, low cost form of Fe(III) source or other form of electron acceptor is an import part of optimizing the Feammox process and the Feammox-bacteria production. Furthermore, knowing that Feammox-bacteria can use other sources as electron acceptors opens another research question about their ability for dissimilatory metal reduction of toxic compounds.

Finally, the Feammox process is still a novel pathway that requires extensive study to elucidate its abundance in nature, the extent to which it is responsible for N losses from soils and to determine its efficiency in engineered systems. This study has contributed to the understanding of temperature dependence kinetics of the Feammox and has given initial insights for a possible diversity in electron acceptors. These findings aim to contribute to the optimization of the Feammox process for future applications in the remediation of ammonium pollution and perhaps in the immobilization of other toxic compounds.
References


Appendix

A. 2-line ferrihydrite synthesis

This protocol was adapted from (Schwertmann and Cornell, 2000)

Material:

Fe(NO$_3$)$_3$·9H$_2$O

KOH 1M

Dionized water

Dialysis membrane

Preparation:

1. Add 100ml dionized water in a 250ml flask.

2. Dissolve 8g Fe(NO$_3$)$_3$·9H$_2$O with a stirring speed of 500rpm

3. Add 60ml 1M KOH while stirring, to bring pH to 7-8. Slowly add approximately 6ml 1M KOH to bring pH to 7.0<final pH<7.5.

4. Stir vigorously overnight with a parafilm top.

5. Centrifuge everything at 8000rpm for 15 min and discard supernatant. Resuspend pellet with leftover solution in tube or by adding about 5ml of dionized water.

6. Transfer into a dialysis tubing and perform a 3-5 days dialysis in dionized water by leaving the tubes submerged and with dionized water slowly running from the bottom of container.

7. Transfer the dialysis tube content to a new bottle. Use dionized water to get everything out of the bag.
8. Mix it over night until all there are no aggregated big particles.

9. Measure concentration using the Ferrozine method.
   a. Dilute ferrihydrite sample to a concentration below 0.5mM.
   b. Add 100ul 6.25M hydroxylamine-hydroxychloride to 5ml of dissolved Fe(III) to reduce to Fe(II) to measure total Fe. Let react over night to get 100% Fe concentration in the solution.
   c. Mix 3ml Ferrozine and add 100ul Fe from point b, let react for 30 minutes exactly. Read at 562nm in a spectrophotometer.
B. ANOVA analysis to determine the statistical significance for ammonium concentration change between temperatures.

<table>
<thead>
<tr>
<th>Sampling cycle</th>
<th>Time (hours)</th>
<th>Temperature (ºC)</th>
<th>Mean [NH₄⁺] (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>15</td>
</tr>
<tr>
<td>Day 1</td>
<td>0</td>
<td>9.53</td>
<td>10.003</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.45</td>
<td>9.89</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.43</td>
<td>9.95</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.54</td>
<td>9.91</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.60</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.68</td>
<td>10.00</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>9.55</td>
<td>9.89</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>9.71</td>
<td>10.00</td>
</tr>
<tr>
<td>Day 2</td>
<td>0</td>
<td>9.71</td>
<td>9.29</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>9.75</td>
<td>9.01</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9.68</td>
<td>8.96</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>9.75</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9.61</td>
<td>9.01</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>9.92</td>
<td>8.91</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.94</td>
<td>8.74</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>9.94</td>
<td>8.61</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>9.86</td>
<td>8.57</td>
</tr>
<tr>
<td>n</td>
<td>18</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>mean</td>
<td>9.681</td>
<td>9.431</td>
<td>7.401</td>
</tr>
<tr>
<td>s</td>
<td>0.159</td>
<td>0.560</td>
<td>1.307</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>source</th>
<th>Degrees of freedom</th>
<th>Sum of Squares</th>
<th>Mean Square</th>
<th>F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>treatments</td>
<td>3</td>
<td>60.502</td>
<td>20.167</td>
<td>27.1050</td>
<td>0.0001</td>
</tr>
<tr>
<td>error</td>
<td>68</td>
<td>50.595</td>
<td>0.744</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>71</td>
<td>111.096</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
C. Sequence alignment of Feammox-bacteria from batch culture with *Acidimicrobiaceae*-bacterium (A6) sequence (GenBank accession number KC581762.1).

Sample 1 and 2 are sequences were obtained from DNA extracted and amplified from the Feammox-bacteria batch enrichment culture. Primer set: acm432F/acm439R