ANAEROBIC AMMONIUM REMOVAL BY FEAMMOX BACTERIA IN
ELECTRODE BASED SYSTEMS
&
MICROBIAL PERFORMANCE IN A NITRIC OXIDE DENITRIFICATION
BIOREACTOR.

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Abstract

The coupling of natural nitrogen removal pathways into engineered systems helps minimize the detrimental effects of excessive nitrogen releases. The dominant anthropogenic nitrogen form is ammonium, which accumulates in soils and water, and undergoes nitrification and denitrification forming NO, N₂O and N₂ which are released to the atmosphere. The demand for clean resources requires the removal of N pollution, thus, we need to improve the existing technologies and development new ones with lower energy demand. Therefore, a core step is to understand the nitrogen cycle, the organisms involved, and their needs and interactions in different environments.

The motivation of this work is to contribute to: 1) the understanding of anaerobic ammonium oxidation by the Feammox bacterium Acidimicrobiaceae sp. A6 (A6), and its ability to use electrodes as terminal electron acceptor in lieu of iron oxides, its natural electron acceptor; and 2) the understanding of the denitrifying microbial community response to different nitrate and nitric-oxide loadings in a hollow fiber membrane (HFM) bioreactor.

Electrodes deployed in a forested riparian wetland soil where A6 thrives showed that A6 is capable of colonizing electrodes. Electrodes in soil columns in the laboratory showed that A6 preferably colonizes the anode over the cathode. Microbial electrolysis cells (MECs) showed that A6 is an electrode-reducing bacterium since current was produced, with the anode as the sole electron acceptor and ammonium as the sole electron donor. Taken together, this study expands our knowledge on electrogenic bacteria and
opens the possibility to develop Feammox-based technologies coupled to bioelectric systems for the treatment of ammonium and other contaminants in anoxic systems.

By tracking the relevant genes responsible for each denitrification step during different nitrate and nitric-oxide loading regimes in a HFM bioreactor, results showed that the denitrifying microbial community can adjust rapidly to changes in nitrogen loading. This rapid adjustment means that the nitrifying bacteria formed a vigorous microbial community which resulted in a robust performance of this type of nitric-oxide removal bioreactors.

The results presented in this dissertation provide information to build upon for the development of technologies based on microbial pathways for the removal of the above-mentioned nitrogen compounds.
To my beautiful family for making my life a journey of joy and love.

To my husband Juan-Carlos for being my best friend and my support. Walking together “arm in arm, we are so much more than two” *

To my son Gabriel, for being so patient while dad and I worked uncountable hours. Your smile, your love, your intelligence and curiosity has been my driver throughout these years.

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*Benedetti, M. Poemas de otros (1974).
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Nitrogen (N) is an essential molecule for life. Under natural conditions, N fixation is in balance with N losses. However, nowadays, N produced by humans has exceeded nature’s balance and as consequence, the equilibrium of the N cycle has been broken (Dentener et al., 2006), causing several adverse environmental and health impacts (US-EPA, 2016b). Before the Industrial era, the sources of all reactive nitrogen species (Nr), came primarily from natural sources which were in balance with N losses through denitrification, a process that returns nitrogen gas (N₂) back to the atmosphere. By the year 2010, the Nr formed by anthropogenic activity was at least two times larger than the rate of natural terrestrial formation, and the amount of N₂ produced by denitrification is only between 30 to 60% of the total Nr formed (Ciais and Sabine, 2013). Among the N compounds largely produced by human activity are N oxides (NOₓ), ammonium (NH₄⁺) or nitrite (NO₂⁻).

Emissions of NOₓ have increased exponentially over the past decades due to the rapid economic growth and energy demand from fossil fuels. Nitric oxide (NO) is among the forms of NOₓ produced by human activities such as power generation, industry, transit, and biomass burning (Dentener et al., 2006). These gases, NO and nitrous oxide (NO₂), are two major air pollutants responsible for the formation of urban and regional haze, acid rain, and ozone layer depletion, and can contribute to climate change (Molina and Molina, 2004; Wei et al., 2016). NOₓ pollution is a problem in many urban areas, and the exposure to these compounds can cause severe health problems such as respiratory illness (Weinberger
et al., 2001). NH$_4^+$ is a byproduct of animal metabolism, and it also comes from fertilizer application in crops, thus, NH$_4^+$ is found in high concentrations in wastewaters and in agricultural runoffs which can accumulate in soils and water (Ciais and Sabine, 2013). The accumulation of NH$_4^+$ or NO$_2^-$ in the environment can lead to eutrophication and low oxygen concentration in water logged systems, which in turn results in loss of biodiversity and poor health of ecosystems. Therefore, understanding the N cycle can aid the process of minimizing N releases by applying natural mechanism of N removal into engineered systems. Biological removal technologies draw attention because they can have lower operating costs, lower energy demands and absence of secondary pollutants requiring further treatment.

1.1 The Feammox process

Feammox is the name given to bacterial-mediated form of anaerobic ammonium (NH$_4^+$) oxidation under iron (Fe) reducing condition. It is a novel pathway in the nitrogen (N) cycle (Figure 1), a new linkage between the N and the Fe cycle, and a key process for alleviating NH$_4^+$ accumulation in anoxic soils and wetlands. Jaffé and collaborators first noted this process in riparian wetlands in New Jersey (Clement et al., 2005), and since then other researches have observed the existence of Feammox in different locations of the world. Sawayama (2006) detected the process in a bioreactor in Japan, and coined the term Feammox in reference to the similitude of the process to anammox, the only other known process to oxidize NH$_4^+$ under anoxic conditions, but in the presence of nitrite (NO$_2^-$) instead of Fe oxides. Feammox has also been observed in tropical highlands in Puerto Rico (Yang et al., 2012), in rice patties from China (Ding et al., 2014a) and in samples collected
from a series of local wetland-, upland-, as well as storm-water detention pond-sediments in New Jersey, river sediments from South Carolina, and forested soils near an acid mine drainage (Dabaoshan, Guangdong province) in China (Huang et al., 2016).

Figure 1. Nitrogen cycle. In green the Feammox process. Figure modified from Schnoor (1996.).

Feammox process is known to be carried out by an Actinobacterium named Acidimicrobiaceae sp. A6 (A6) (Huang and Jaffê, 2018), which is an iron reducer, a feature present in many electroactive bacteria. A6 are autotrophic anaerobic microorganisms that obtain their energy by oxidizing NH$_4^+$ to NO$_2^-$ and transferring the electrons to Fe(III), which acts as the final electron acceptor. Previous studies have shown that Feammox can
use more than one type of Fe(III) source. For example, Clement and collaborators (2005) showed that Feammox is feasible when using goethite (FeOOH) as the final electron acceptor (Equation 1).

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

Yang and collaborators (2012) used iron hydroxides Fe(OH)\(_3\) as the respirable iron (Equation 2).

\[
\text{NH}_4^+ + 6\text{Fe(OH)}_3 + 10\text{H}^+ \rightarrow \text{NO}_2^- + 6\text{Fe}^{2+} + 16\text{H}_2\text{O} \quad (\Delta G = -245 \text{ kJ mol}^{-1}) \quad (2)
\]

It has also been shown that A6 grows on solid Fe such as ferrihydrite (Fe\(_2\)O\(_3\)·0.5H\(_2\)O), but does not grow on dissolved Fe(III) such as ferric citrate or ferric chloride (Huang and Jaffê, 2018) (Equation 3).

\[
3\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O} + 10\text{H}^+ + \text{NH}_4^+ \rightarrow 6\text{Fe}^{2+} + 8.5\text{H}_2\text{O} + \text{NO}_2^- \quad (\Delta G \leq -145.08 \text{ kJ mol}^{-1}) \quad (3)
\]
Additionally, A6, can oxidize ammonium in the presence of nontronite, a swelling 2:1 phyllosilicate rich in Fe(III) (Keeling et al., 2000). Nontronite’s structural Fe(III) is available for microbial respiration (Dong, 2012), thus it can function as an electron acceptor for the Feammox reaction (Appendix A). A6 can also use anthraquinone-2,6-disulfonate (AQDS), a humic acid analogue, as an electron shuttle (Huang and Jaffé, 2018; Zhou et al., 2016a). Additionally, similarly to other metal reducing bacteria, A6 also has the ability to use other sources of electron acceptors such as uranium (Gilson et al., 2015). Therefore, these characteristics of A6 opened up the possibility of it also being an electrode reducing bacteria (ERB). Hence, harnessing A6’s ability to use multiple electron acceptors by facilitating the use of electrodes in bioelectrochemical systems (BES), such as Microbial Electrolysis Cells (MECs), became a subject of interest.

1.2 Feammox process as an option for ammonium removal in engineered systems.

Engineered systems such as wastewater treatment plants (WWTP) fully oxidize NH$_4^+$ to NO$_2^-$ and nitrate (NO$_3^-$) in a process known as nitrification. This is done to minimize the demand of dissolved oxygen (DO) in the receiving waters. 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 WWTP, constructed wetlands as well as in natural systems.
Autotrophic aerobic bacteria known as nitrifiers carry out nitrification. In WWTP, nitrification requires energetically costly oxygen inputs during the operation of aerators (Austin and Nivala, 2009; Leu et al., 2009). To lower energy consumption for wastewater treatment, anaerobic oxidation of NH$_4^+$ is a worthwhile endeavor. Anammox oxidizes NH$_4^+$ anaerobically by coupling it to NO$_2^-$ reduction, but some aeration is still required to form the needed NO$_2^-$. The oxidation of NH$_4^+$ via Feammox to NO$_2^-$ (Clement et al., 2005; Huang and Jaffé, 2018; Sawayama, 2006; Shrestha et al., 2009; Yang et al., 2012) occurs in the absence of molecular oxygen, and can take place at temperatures as low as 15 ºC, with the highest removal rate happening at 20 ºC (Ruiz-Urigüen, 2014), which makes it an attractive candidate for the development of an energy efficient NH$_4^+$ removal method. However, it requires iron oxides [Fe(III)] as electron acceptor in a stoichiometric ratio of 6:1 (Clement et al., 2005; Huang and Jaffé, 2015) (Equation 3).

Iron is abundant in the environment and thus Feammox can be enhanced in systems such as constructed wetlands to treat some wastewaters (Shuai and Jaffé, 2018). However, adding Fe(III) to WWTPs is technically inconvenient because iron build-ups are operationally limiting and their disposal is burdensome. To implement the Feammox process for large scale or continuous flow reactor applications, the Fe(III) phase needs to be replaced with a stable long-term terminal electron acceptor such as electrodes.
1.3 Ammonium oxidation by Feammox bacteria in Microbial Electrolysis Cells.

Electroactive bacteria, have been used in bioelectrochemical systems (BES) such as microbial fuel cells or microbial electrolysis cells (MECs) to extract energy from different types of electron donors and transfer the electrons to the electrodes (anode) (Logan, 2009; Lovley, 2008). The electron transfer process can be direct or with the aid of electron shuttles (Huang and Jaffé, 2018; Zhou et al., 2016b), resulting in the production of low-density electrical currents (Lovley, 2008).

Microbial electrolysis cells (MECs) are a type of low-cost, bioelectrochemical system that extract energy from a substrate, for example, wastewater or other contaminants (Call and Logan, 2011). This process harvests electrons biologically, which are then transferred to the anode that functions as the terminal electron acceptor for the microorganisms in the system. MECs utilize a small external voltage (0.2 - 0.8 V) for overcoming the thermodynamic barrier of electrolysis. The potential difference between the anode and the cathode is enough to drive the electron transfer from the NH$_4^+$ oxidation reaction. The possibility of a reaction can be determined by calculating its standard free energy ($\Delta G^\circ$) applying Nernst equation (Equation 4):

$$\Delta G^\circ = -nF \Delta E^\circ$$  \hspace{1cm} (4)

Where, $n$ is the number of electrons transferred during the reaction

$(n= 6$ for NH$_4^+ \rightarrow$ NO$_2$).

F is Faraday’s constant (96.485 kJ/V/mol).
\( \Delta E^o \) (Equation 5) is the difference in the potentials between two half reactions, in volts (V).

\[
(\Delta E^o = E_{anode} - E_{\text{substrate}}^o)
\]  

(5)

\( E_{\text{substrate}}^o \) is equal to 0.07 V vs for NH\(_4^+\) oxidation to NO\(_2^-\) (see section 3.1 for details). Hence, to make the reaction feasible, \( E_{anode} \) needs to be above 0.07 V vs. standard hydrogen electrode (SHE).

Facilitating A6 growth in MEC is an important step toward enhancing NH\(_4^+\) removal and a variety of other recalcitrant pollutants in bioelectrochemical reactors without the need to handle solid Fe(III) phases.

### 1.4 Denitrification and nitric oxide reduction.

Denitrification is a sequence of biological reaction carried out by microorganisms that use different nitrogen species as electron acceptors from the most to the least favorable thermodynamically, ultimately forming N\(_2\) (NO\(_3^-\rightarrow\)NO\(_2^-\rightarrow\)NO\(\rightarrow\)N\(_2\)O\(\rightarrow\)N\(_2\)). Denitrification involves a complex set of electron transport reactions catalyzed by four types of enzymes encoded by functional genes of the same name: nitrate reductase (\(Nar\)) responsible for NO\(_3^-\) reduction, nitrite reductase (\(Nir\)) for NO\(_2^-\) reduction, nitric oxide reductase (\(Nor\)) for NO reduction, and nitrous oxide reductase (\(Nos\)) which catalyzes N\(_2\)O reduction to N\(_2\). These series of steps are primarily done by heterotrophic bacteria which depend on organic carbon as their electron donor; however, there are some denitrifying lithoautothrophs such as *Thiobacillus denitrificans* (Claus and Kutzner, 1985).
Nitric oxide (NO) is among the forms of N produced by human activities including power generation, industry, transit, and biomass burning (Dentener et al., 2006). Typically, over 95% of nitrogen oxides (NO\textsubscript{x}) in a combustion flue gas contains NO. The reduction of NO forms N\textsubscript{2}O and ultimately N\textsubscript{2}. NO and NO\textsubscript{2}, are two major air pollutants responsible for the formation of urban and regional haze, acid rain, and ozone layer depletion, and can contribute to climate change (Molina and Molina, 2004; Wei et al., 2016). Furthermore, exposure to these compounds can cause severe health problems such as respiratory illness (Weinberger et al., 2001). Therefore, appropriate and urgent management of these gases is essential.

1.5 Objectives.

Nitrogen is essential for life, however, however, its overproduction by humans has caused N pollution in the environment, thus we need to understand the players in the N cycle in order to develop technologies that will aid the mitigation of N as a pollutant.

The discovery of Feammox and reports of this in multiple submerged sediments (Clement et al., 2005; Ding et al., 2014b; Huang and Jaffé, 2015; Li et al., 2015a; Yang et al., 2012) and the isolation of Acidimicrobiaceae sp. A6 from wetland sediments (Huang et al., 2016) has motivated continuing the study of this bacterium in site in order to better understand and characterize it. Therefore, the objectives for chapter 2 are:

(i) To investigate if Acidimicrobiaceae sp. strain A6, like other electrogenic bacteria, could colonize electrodes placed into sediments under natural or
controlled conditions, and thus could be enriched with respect to the surrounding sediments.

(ii) To better understand the interaction of the relative abundance of iron-reducing bacteria or nitrogen cycling bacteria found on the electrode’s surface, with respect to *Acidimicrobiaceae* sp. strain A6.

The conditions under which the Feammox process takes place makes it an attractive process for NH$_4^+$ oxidation in engineered systems if Fe can be replaced by stable and long-term electron acceptor such as electrodes, therefore in order to pave the way for the development of Feammox-based technologies, we studied *Acidimicrobiaceae* sp. strain A6 ability to function in Microbial electrolysis cells, thus the objectives for chapter 3 are:

(i) To harness *Acidimicrobiaceae* sp. strain A6 Feammox ability into MECs and to gain insights for the bioelectrochemical conditions required for such systems.

(ii) To determine if pure and enrichment cultures of the Feammox bacterium *Acidimicrobiaceae* sp. strain A6 can grow and can carry out anaerobic NH$_4^+$ oxidation in the absence of Fe(III) in MECs, and if so, if the rates of NH$_4^+$ oxidation and if A6 growth in MECs are comparable to those in a batch reactor with Fe(III) as the electron acceptor.
(iii) For A6 enrichment cultures in MECs, an additional goal was to analyze the overall microbial community to determine if there are other known NH$_4^+$ oxidizers present, and to get an assessment of the A6 relative abundance in such systems.

The removal of NO through the denitrification process requires organic carbon as the electron source, which can come from the organic matter present in wastewater. However, wastewater can have different loading of nitrate which can result in different concentrations of NO produced as denitrification proceeds. Therefore, it is important to understand the microbial community response to such changes, thus the objective for chapter 4 is:

(i) To examine the reactors microbial ecology in response to different NO$_3^-$ and NO loading regimes once steady state was reached.
CHAPTER 2. Electrode-colonizing Feammox Bacteria Acidimicrobiaceae sp. strain A6 in field experiments.

2.1 Overview

The Feammox process has been found in multiple submerged sediments (Clement et al., 2005; Ding et al., 2014b; Huang and Jaffé, 2015; Li et al., 2015a; Yang et al., 2012) and the bacterium associated to this process, Acidimicrobiaceae sp. strain A6 (A6), was isolated from wetland sediments (Huang et al., 2016). A6, belongs to the Actinobacteria phylum, and it is an iron-reducing bacterium. Commonly, electrode-reducing bacterium (ERB) are iron-reducing bacteria (FeRB) (Lovley, 2008), therefore A6 was studied as a potential ERB that may transfer electrons extracellularly onto electrodes while gaining energy from NH$_4^+$ oxidation. Most studies on electrogenic microorganisms have focused on the most abundant heterotrophs that dominate the bioelectrical systems community composition, these are the phyla Firmicutes, Acidobacteria, and Proteobacteria, the latter contains some of the most commonly present and extensively studied ERB: Geobacter spp. and Shewanella spp. (Jung and Regan, 2007; Logan, 2009; Lovley, 2008; Williams et al., 2010). While other microorganisms also commonly present in electrode microbial communities, such as Actinobacteria strains, they have been overlooked, and the importance of lithoautotrophic iron reducers as electrode-reducing bacteria at anodes has not been addressed.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

The objective of this study was to investigate if *Acidimicrobiaceae* sp. strain A6, like other electrogenic bacteria, could colonize electrodes placed into sediments under natural or controlled conditions, and thus could be enriched with respect to the surrounding sediments. This was achieved by installing electrodes in the soil of a forested riparian wetland where A6 thrives, and in soil columns in the laboratory. A6 population was analyzed and we compared its relative abundance and correlations with respect to other bacteria of importance in the system.

Results from this study indicate that A6 appears to be an electrode-reducing bacterium which can be enhanced on the electrode functioning as the anode. These findings expand the knowledge of the diversity of ERB by including a member of the previously overlooked Actinobacteria, and allow for the possibility of practical applications for *Acidimicrobiaceae* sp. strain A6 for the treatment of NH$_4^+$ contamination in anoxic systems using electrodes as stable, long-term terminal electron acceptors.

### 2.2 Materials and Methods

In this study, electrodes were installed in multiple locations of a forested riparian wetland as well as in laboratory to investigate A6 as a potential ERB. Results from electrodes deployed in the field provide insightful information regarding the natural and electrode-enhanced thriving conditions of A6, whereas electrodes deployed in the laboratory soil column provide controlled conditions to better understand the field findings.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

### 2.2.1 Field electrodes construction and setup

Electrodes consisted of graphite plates [7.5 (L) x 2.5 (W) x 0.32 cm (H)], with a surface area per face of 18.75 cm$^2$ (Grade GM-10; GraphiteStore.com Inc.). The plates were polished using sandpaper (grit type 400), sonicated to remove debris, cleaned by soaking in 1 N HCl overnight and rinsed three times in distilled water (Call and Logan, 2011). Each electrode set was connected by a titanium (Ti) wire cleaned with sandpaper (ultra-corrosion-resistant Ti wire, 0.08 cm in diameter, McMaster-Carr code 90455k32) by inserting the wire through two holes of size 0.08 cm drilled in each graphite plate to ensure a tight connection between the wire and the graphite plates to allow for low contact resistance <0.5 Ω. The Ti wire was long enough to allow for 10 or 30 cm separation between the graphite plates (Figure 2).

---

**Figure 2.** a) Picture of a shallow electrode, and b) Schematic of an electrode pair.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

Two pairs of electrodes were placed at three different sites. Each pair consisted of a shallow electrode placed no deeper than 5 cm into the soil, connected to another electrode with either 10 cm or 30 cm separation, i.e. a total of 6 sets (Table 1, a) in a temperate forested riparian wetland located at the Assunpink Wildlife Management Area (Assunpink WMA) in New Jersey, USA. This is the location were the Feammox reaction was first discovered (Clement et al., 2005), and later the Feammox bacteria A6, was identified in samples from this site (Huang and Jaffé, 2015) and isolated (Huang and Jaffé, 2018). Detailed physicochemical characteristic of the soil have been described in previous studies (Clement et al., 2005; Shrestha et al., 2009). Electrodes sets 1 and 2 were place in a fully flooded location, sets 3 to 6 were placed in a wet but unsaturated location. The electrode sets were left in the field for 52 days between June 13 and August 03, 2016.

A separate set of experiments was carried out as controls. First, electrode pairs with identical set up as the ones described above, were placed in triplicates at Assunpink WMA, additionally, triplicates of unconnected graphite plates at 10 cm and 30 cm deep were placed as controls, all of them were left in the field for 32 days (Table 1, b). Second, soil cores from the same location at the Assunpink WMA were brought to the laboratory to set up an identical experiment in which the water level was maintained saturated through the whole experiment by adding deionized water, to avoid possible redox fluctuations and changes in polarity due to drying of the soils (Table 1, c).
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Table 1.** Description of samples taken to analyze bacteria attached to electrodes (Elec., sample #, depth), on the control graphite plates (Plate., sample #, depth) and on soil (Soil, sample #, depth).

*a* Electrode sets placed at Assunpink WMA for 52 days.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode set and site sampled</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Elec. 1s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 1s</td>
</tr>
<tr>
<td>Set 1</td>
<td>Shallow electrode 1</td>
<td>Elec. 1.10</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 1</td>
<td>Soil 1.10</td>
</tr>
<tr>
<td>Site 1-2</td>
<td></td>
<td>Elec. 2s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 2s</td>
</tr>
<tr>
<td></td>
<td>Shallow electrode 2</td>
<td>Elec. 2.30</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 2</td>
<td>Soil 2.30</td>
</tr>
<tr>
<td></td>
<td>Site 1-2 Soil sample from site 1-2</td>
<td>Soil 1-2</td>
</tr>
<tr>
<td>Set 3</td>
<td></td>
<td>Elec. 3s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 3s</td>
</tr>
<tr>
<td></td>
<td>Shallow electrode 3</td>
<td>Elec. 3.10</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 3</td>
<td>Soil 3.10</td>
</tr>
<tr>
<td>Site 3-4</td>
<td></td>
<td>Elec.4s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 4s</td>
</tr>
<tr>
<td></td>
<td>Shallow electrode 4</td>
<td>Elec.4.30</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 4</td>
<td>Soil 4.30</td>
</tr>
<tr>
<td></td>
<td>Site 3-4 Soil sample from site 3-4</td>
<td>Soil 3-4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elec. 5s</td>
</tr>
<tr>
<td></td>
<td>Set 5 Shallow electrode 5</td>
<td>Soil 5s</td>
</tr>
</tbody>
</table>
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

<table>
<thead>
<tr>
<th>10 cm deep electrode 5</th>
<th>Elec. 5.10</th>
<th>Soil 5.10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shallow electrode 6</td>
<td>Elec. 6s</td>
<td>Soil 6s</td>
</tr>
<tr>
<td>30 cm deep electrode 6</td>
<td>Elec. 6.30</td>
<td>Soil 6.30</td>
</tr>
<tr>
<td>Site 5-6</td>
<td>Soil sample from site 5-6</td>
<td>Soil 5-6</td>
</tr>
</tbody>
</table>

b) Electrode sets and control sets at Assunpink WMA placed for 32 days.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode set and site sampled</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 7-8</td>
<td>Shallow electrode 7</td>
<td>Elec. 7s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 7s</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 7</td>
<td>Elec. 7.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 7.10</td>
</tr>
<tr>
<td>Set 8</td>
<td>Shallow electrode 8</td>
<td>Elec. 8s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 8s</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 8</td>
<td>Elec. 8.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 8.30</td>
</tr>
<tr>
<td>Control</td>
<td>10 cm deep control plate</td>
<td>Plate. C.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil C.10</td>
</tr>
<tr>
<td></td>
<td>30 cm deep control plate</td>
<td>Plate. C.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil C.30</td>
</tr>
<tr>
<td>Site 7-8</td>
<td>Soil sample from site 5-6</td>
<td>Soil 7-8</td>
</tr>
</tbody>
</table>
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

c) Electrode set and control set placed in soils cores in the laboratory, incubated for 52 days.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode set and site sampled</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Set 9</td>
<td>Shallow electrode 9</td>
<td>Elec. 9s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 9s</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 9</td>
<td>Elec. 9.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil 9.30</td>
</tr>
<tr>
<td>Lab</td>
<td>Shallow control plate</td>
<td>Plate. C.s</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>Soil C.s</td>
</tr>
<tr>
<td></td>
<td>30 cm deep control plate</td>
<td>Plate. C.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Soil C.30</td>
</tr>
</tbody>
</table>

### 2.2.2 Field electrodes recovery and sampling

After 52 days of deployment in the field for the first group of electrodes and 32 days for the second group, the electrode pairs were recovered by digging them out of the soil and placing each electrode individually in a sealed bag. All the electrodes were surrounded by soil. Furthermore, a soil sample was taken from a depth of about 20 cm from each site and placed in a sealed bag. All samples were transported to the laboratory within 2 hours and immediately stored at 4°C until processed for analysis. The same recovery procedure was applied to the electrodes placed in the laboratory soil cores, which were dismantled after 52 days.

The samples obtained from the electrodes and soil are enumerated in Table 1. To analyze the biomass attached to the electrodes, first, the loosely bound soil was removed...
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

by gently shaking the electrode. Second, duplicate samples were taken from the soil layer (< 2 mm thick) still surrounding the electrode. Third, duplicate samples of the biomass formed on the electrodes’ surface together with some graphite were removed using a sterile cutting blade (Details in Table 2). DNA was extracted from all samples and then used for determining, quantifying, and comparing their microbial composition, the latter only for the first group.

**Table 2.** Details of samples taken from the electrodes pairs, soil and control plates located at each field location and laboratory set up.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode set and site sample</th>
<th>Electrodes sample</th>
<th>Soil sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sample name</td>
<td>Electrode surface area sampled</td>
<td>Sample name</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sample 1</td>
<td>Sample 2</td>
</tr>
<tr>
<td>Site 1-2</td>
<td>Shallow electrode 1</td>
<td>Elec. 1s</td>
<td>9.375</td>
</tr>
<tr>
<td>Site 1-2</td>
<td>10 cm deep electrode 1</td>
<td>Elec. 1.10</td>
<td>9.375</td>
</tr>
<tr>
<td>Site 1-2</td>
<td>Shallow electrode 2</td>
<td>Elec. 2s</td>
<td>9.375</td>
</tr>
<tr>
<td>Site 1-2</td>
<td>30 cm deep electrode 2</td>
<td>Elec. 2.30</td>
<td>9.375</td>
</tr>
<tr>
<td>Site 1-2</td>
<td>Soil from site 1-2</td>
<td>Site 1-2</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode Depth</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Soil Sample 1</th>
<th>Soil Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-4</td>
<td>Shallow electrode 3</td>
<td>Elec. 3s</td>
<td>18.75</td>
<td>18.75</td>
<td>Soil 3s</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 3</td>
<td>Elec. 3.10</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 3.10</td>
</tr>
<tr>
<td>3-4</td>
<td>Shallow electrode 4</td>
<td>Elec. 4s</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 4s</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 4</td>
<td>Elec. 4.30</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 4.30</td>
</tr>
<tr>
<td>3-4</td>
<td>Soil from site 3-4</td>
<td>Site 3-4</td>
<td>0.55</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode Depth</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Soil Sample 1</th>
<th>Soil Sample 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-6</td>
<td>Shallow electrode 5</td>
<td>Elec. 5s</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 5s</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 5</td>
<td>Elec. 5.10</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 5.10</td>
</tr>
<tr>
<td>5-6</td>
<td>Shallow electrode 6</td>
<td>Elec. 6s</td>
<td>9.375</td>
<td>9.375</td>
<td>Soil 6s</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 6</td>
<td>Elec. 6.30</td>
<td>14 *</td>
<td>-</td>
<td>Soil 6.30</td>
</tr>
<tr>
<td>5-6</td>
<td>Soil from site 5-6</td>
<td>Site 5-6</td>
<td>0.56</td>
<td>0.55</td>
<td></td>
</tr>
</tbody>
</table>

* Deep electrode of set was only partially recovered, therefore only one sample was obtained from all faces.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

<table>
<thead>
<tr>
<th>Site</th>
<th>Electrode set and site sample</th>
<th>Electrodes sample</th>
<th>Soil sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sample name</td>
<td>Sample</td>
</tr>
<tr>
<td></td>
<td></td>
<td>name</td>
<td>surface area sampled</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(cm²)</td>
</tr>
<tr>
<td>Set 7</td>
<td>Shallow electrode 7</td>
<td>Elec. 7s</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>10 cm deep electrode 7</td>
<td>Elec. 7.10</td>
<td>37.5</td>
</tr>
<tr>
<td>Site 7-8</td>
<td>Shallow electrode 8</td>
<td>Elec. 8s</td>
<td>37.5</td>
</tr>
<tr>
<td>**</td>
<td>30 cm deep electrode 8</td>
<td>Elec. 8.30</td>
<td>37.5</td>
</tr>
<tr>
<td>Control</td>
<td>10 cm deep plate</td>
<td>Plate. C.10</td>
<td>37.5</td>
</tr>
<tr>
<td></td>
<td>30 cm deep plate</td>
<td>Plate. C.30</td>
<td>37.5</td>
</tr>
<tr>
<td>Site 7-8</td>
<td>Soil from site 7-8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Set 9</td>
<td>Shallow electrode 9</td>
<td>Elec. 9s</td>
<td>18.75</td>
</tr>
<tr>
<td></td>
<td>30 cm deep electrode 9</td>
<td>Elec. 9.30</td>
<td>18.75</td>
</tr>
<tr>
<td>Lab</td>
<td>Shallow control</td>
<td>Plate. C.10</td>
<td>18.75</td>
</tr>
<tr>
<td>Control</td>
<td>30 cm deep plate</td>
<td>Plate. C.30</td>
<td>18.75</td>
</tr>
</tbody>
</table>

** Each set is the average of triplicate samples
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

2.2.3 DNA extraction, *Acidimicrobiaceae* sp. A6 quantification and microbial community analysis

Total genomic DNA was extracted from each attached bacteria sample obtained in duplicate from a half face or full face of each electrode deployed in the field, except for the deep electrode of set 6, which was only partially recovered and only one sample could be obtained from all faces. DNA was also extracted from each soil sample. Extractions were done using the FastDNA® spin kit for soil (MP Biomedicals, USA) according to the manufacturer’s instructions. Total DNA was eluted in 100 µl of sterile water and its concentrations were measured using Qubit 2.0® (Invitrogen, USA). All DNA samples were preserved at -20 °C until further analysis.

Since at this point neither the mechanism by which A6 oxidizes NH$_4^+$ nor its functional gene has been identified, we use 16s rDNA sequence for two tasks, 1) to quantify A6 using specific primers designed using genomic sequence of A6 (Huang and Jaffé, 2018) and 2) for taxonomic annotation.

Bacteria quantification was carried out via Quantitative PCR (qPCR) using the Applied Biosystems StepOnePlus™ Real Time PCR system. A6 quantification was done by amplifying a section of the 16S rRNA gene between the variable regions V1 and V4 using primer set 33F/232R (33F: 5’-GGCGGCGTGCTTAACACAT-3’ / 232R: 5’-GAGCCCGTCCCAGAGTGATA-3’). *Geobacter* spp., an electrogenic bacteria known for its ability colonize electrodes, were quantified by amplifying a region of the 16S rRNA gene using primer set 561F/825R (561F: 5’-GCCTGATAGGCGGTTTCTTAA-3’ / 825R: 5’ATCTACGGATTTCACCTCCTACA-3’).
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

Each qPCR mixture (20 µL) was composed of 10 µL of SYBR Premix Ex Taq® II 2X (Takara, Japan), 0.8 µL of each forward and reverse 10 µM primer, and DNA template. Thermal cycling conditions were initiated for 30 s at 95 ºC, followed by 40 cycles with varying times and temperature depending on the amplicon being generated, and ended with a melting curve analysis for SYBR Green assay used to distinguish the targeted PCR product from the non-targeted PCR products. For A6 amplification, the cycling consisted of 10 s at 95 ºC, 15 s of annealing at 59 ºC and 15 s at 72 ºC. For total bacteria quantification, each cycle consisted of 5 s at 94 ºC, 30 s at 55 ºC and 30 s at 70 ºC. Each qPCR reaction was run in duplicate or triplicate per sample and included negative controls and a standard curve; the last one consisting of serial dilutions of known numbers of copies of DNA of the gene per volume. Finally, the results were converted into copies of DNA / m² by dividing the total gene copies obtained from qPCR by the surface area of sediments for soil samples or by the surface area of the electrode for the samples of attached bacteria on electrodes.

In order to determine the microbial community composition and abundance in the sediments (field and CW) and compare it to that formed on the electrodes, sequencing and microbial community analysis was performed by Novogene (Beijing, China) as described in detail in Appendix B. A total of 24992 - 40037 sequences were obtained, from which 3206 operational taxonomic units (OTUs) were produced across all field samples, with a range between 1422-1794 OTUs per sample.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiiaceae* sp. strain A6 in field experiments.

### 2.2.4 Soil surface area analysis

Nitrogen sorption was used to determine the surface area of the soil samples. Prior to the analysis, samples were oven-dried at 56°C until the mass stabilized. Subsequently, the samples were degassed at 60°C and 0.1 mmHg using a Smart Micrometrics VacPrep (Norcross, GA, USA). The nitrogen sorption measurements were conducted using a Micromeritics 3FLEX (Norcross, GA, USA), using the BET method (Brunauer–Emmett–Teller) to calculate the surface area of the soil. The measurements obtained were used to normalize the bacterial count data by surface area.

### 2.2.5 Analytical Methods

The sediment’s iron concentrations were analyzed using the ferrozine method (Gibbs, 1979): 0.5 g sediment sample was added to 9.5 mL 0.5 M HCl to extract Fe(II). Total Fe concentration was obtained by adding 60 µL 6.25 M NH$_2$OH·HCl to 3 mL of extraction solution and incubated for 24 hours to reduce Fe(III) to Fe(II). For the chromogenic reaction, 60 µL of extraction solution was added to 3 mL 1 g/L ferrozine solution (pH 7.0) and reacted for 30 minutes. The concentrations of Fe(II) and total Fe were measured by reading the absorbance at the 562-nm wavelength using a Spectronic® Genesys$^\text{TM}$ 2 instrument, and the Fe(III) concentration was calculated from the difference.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

2.2.6 Statistical Analysis.

The Welch *t*-test statistical analysis was used to determine if there were statistical differences in bacterial counts per surface area between electrode and soil samples.

2.3 Results and Discussion

2.3.1 *Acidimicrobiaceae* sp. strain A6 quantification.

Bacterial count by qPCR confirmed our initial hypothesis that the count of A6 could be enhanced on the electrodes’ surface because the bacteria may have the ability to use electrodes as electron acceptors in the same manner that other FeRB do. The average count of A6 among the attached bacteria on the electrodes from the first group incubated for 52 days was orders of magnitude higher than the average count of the bacterium in the soil (4.13 x 10^6 copies of DNA/m^2 on the electrode and 7.46 x 10^3 copies of DNA/m^2 in the soil). The second group was maintained for 32 days, and the results show that A6 quantification from attached bacteria on the electrode was significantly higher than from soils (Welch *t*-test, *p* < 0.05), unlike the controls, where no significant difference was found (Figure 3a and b).
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

Field operation: 52 days

(a) Acidimicrobiaceae sp. strain A6 quantification after 52 days (a), and after 32 days of field operation (b).

(c) Geobacter spp. quantification after 52 days (c), and after 32 of field operation (d). Bars represent the mean and error bars the standard deviation (SD) (n=3).

**Figure 3.** Quantification of *Acidimicrobiaceae* sp. strain A6 and *Geobacter* spp. from bacteria attached to connected electrodes, on control unconnected graphite plates, and from soil samples. a and b) *Acidimicrobiaceae* sp. strain A6 quantification after 52 days (a), and after 32 days of field operation (b). c and d) *Geobacter* spp. quantification after 52 days (c), and after 32 of field operation (d). Bars represent the mean and error bars the standard deviation (SD) (n=3).
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Table 3.** Average number of copies of DNA /m$^2$ quantified for *Acidimicrobiaceae* sp. *strain A6* and *Geobacter* spp. from attached bacteria on the electrodes and on soil samples from field and lab sets, and current (I) measured for each set at the time of their retrieval. C refers to controls.

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* Average from triplicate samples.

C: Control, unconnected graphite plates.
In order to confirm that the electrodes were also being colonized by other electrogenic bacteria, we choose to quantify the genus *Geobacter*, which has become a model organism for the study of electrogenic organisms. *Geobacter* spp. also had significant higher populations among the attached bacteria on the surface of the electrodes than on the surrounding soil (Figure 3 d and e) (Welch t-test, p < 0.001). On average, across all sites, the quantification for A6 and *Geobacter* resulted in higher bacterial counts on the electrodes than on the surrounding soil, but not on the unconnected graphite plates (Table 3). These results indicate that the number of A6 was clearly enhanced by the electrodes.

However, we could not always see a trend in biomass being higher on the deeper electrodes than on the shallow electrodes in the field, as hypothesized. We had initially assumed that the surroundings of the shallow electrode would be the more oxidized, thus acting as the cathode, and the deeper the more reduced, hence acting as an anode. Nonetheless, because the first group of electrodes were placed in the field for 52 days, and the second group for 32 days, without any interference, during which time multiple days of rain were recorded with as much as 21.8 mm in July 2016, and 81.5 mm in May 2018 (Figure 4) the redox state of the soils could have shifted, thus inverting the polarity of the electrodes due to the fluctuation in the water table. Such conditions could have favored the colonization of electrode-reducing bacteria on both electrodes, the deep as well as on the shallow ones at different times. Current measurements were taken between the deep and shallow electrodes at the time of their retrieval (Table 3). All sets showed positive current, except set 6, which had a current of -0.50 µAmps, thus indicating that electrode polarity
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

could have shifted during the time when the electrodes were in the field. Therefore, the soil cores taken from the field to the lab were used in soil columns to test a set of connected electrodes (set 9) and a set of unconnected graphite plates as controls. Under laboratory conditions, the soil columns were kept saturated with water for the duration of the incubations to avoid changes in the redox conditions due to water level fluctuation. The incubations carried out in the laboratory resulted in higher A6 counts on the deep electrode compared to the shallow one, and both electrodes had higher biomass than on the surrounding soil (Welch *t*-test, *p* < 0.01) (Figure 5). The control graphite plates had no statistical significant difference between the amount of A6 attached on the unconnected electrodes and the amount in the surrounding soil.

**Figure 4.** Daily rainfall record near Assunpink Wildlife Management Area during the time electrodes were deployed at the field site. **a)** Station US1NJMC0016 in Robbinsville Township 1.7 WSW, NJ. (GHCN-NOAA, 2016). **b)** Station: US USC00283951 in Hightstown 2 W, NJ (GHCN-NOAA, 2018).
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Figure 5.** *Acidimicrobiaceae* sp. strain A6 quantification from attached bacteria on the connected electrodes and control unconnected graphite plates during 52 days of incubation in soil columns in the laboratory. Bars represent the mean and error bars the SD (n=4).

The number of A6 quantified on the electrodes as opposed to the surrounding soils is consistent with the hypothesis that this bacterium is able to colonize electrodes. Furthermore, their higher bacterial count on the deeper electrode in the laboratory set up (Figure 5) is consistent with work published together with this information, where it was found that there was higher bacterial count on the electrodes located in the more reduced soil of constructed wetlands mesocosms (Work done by Shuai, W. which is part of Ruiz-Urigüen *et al* 2018). Additionally, although the field experiments and the laboratory soil columns were not designed to control Fe availability, we found that in general there was higher A6 enrichment at sites with lower soil Fe(III) (Table 4), which is consistent with
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

Shuai’s work where she found that CW had higher current production under lower bioavailable Fe conditions. Furthermore, in the soil columns that were kept saturated throughout the incubation, where there was no polarity switch in the electrodes, higher enrichment was found on the anode, and no A6 enrichment was found on the unconnected graphite plates placed in the field or in the laboratory’s soil columns. These results together with the ones produced by Shuai and the ones presented in Chapter 3 which show the production of current linked to *Acidimicrobiaceae* sp. strain A6, confirm the affinity of A6 for the electrode working as an anode. This means A6 is able to use the anode as an alternative electron acceptor to Fe(III), and thus promote their biomass enhancement compared to its surrounding soil.

**Table 4.** Soil Fe (III) concentration at the different field sites.

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Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

2.3.2 Microbial community analysis.

The microbial diversity at the phylum level found in the attached bacteria on the electrodes and soil samples taken from the field. (Figure 6; archaea data in Table 5) show that Proteobacteria and Acidobacteria represent on average more than 70% of the diversity found in all samples, followed in abundance by Chloroflexi and Bacteroidetes. These highly abundant groups make up more than 80% of the population found in all samples. All these phyla are commonly found in soil and in bioelectrochemical systems due to their electrode-reducing ability (Ahn et al., 2014; Holmes et al., 2004b; Lu et al., 2015; Zhu et al., 2014), therefore, they are common subjects of study. Actinobacteria, the phylum to which *Acidimicrobiaceae* sp. strain A6 belongs, represents as little as 2, 4, 5% of the relative abundance found at the three different sites. Still, Actinobacteria ranks in the top 5 most abundant phyla found in each field site and makes it to the third position for some samples of attached bacteria on the electrode from the field study.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Figure 6.** Microbial community composition at the phylum level of attached bacteria on electrodes and on soil samples from electrode pairs from 3 different field locations. Actinobacteria phylum, highlighted in green, to which *Acidimicrobiaceae* sp. strain A6 belongs, is amongst the most abundant phyla in all samples.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Table 5.** Relative abundance of Archaea at phylum level in field samples.

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Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

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<tr>
<td>5</td>
<td>Elec. 5s</td>
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<td>0</td>
<td>2.00×10^{-4}</td>
<td>4.00×10^{-5}</td>
<td>7.20×10^{-4}</td>
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<tr>
<td></td>
<td>Soil 5s</td>
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<td>0</td>
<td>2.00×10^{-4}</td>
<td>0</td>
<td>4.80×10^{-4}</td>
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<td>0</td>
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<td>8.00×10^{-5}</td>
<td>5.56×10^{-3}</td>
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<tr>
<td></td>
<td>Soil 5.10</td>
<td>4.00×10^{-5}</td>
<td>0</td>
<td>3.60×10^{-4}</td>
<td>8.00×10^{-5}</td>
<td>5.56×10^{-3}</td>
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<td>6</td>
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<td>5.72×10^{-3}</td>
<td>1.60×10^{-4}</td>
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<td>2.00×10^{-4}</td>
<td>5.20×10^{-4}</td>
<td>4.00×10^{-5}</td>
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<tr>
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<td>Elec. 6.30</td>
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<td>0</td>
<td>1.20×10^{-4}</td>
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</table>

The Actinobacteria phylum contains *Acidimicrobiaceae* sp. strain A6 is described as an unidentified *Acidimicrobiales* at the genus level because its 16S rDNA sequence was not available in the public data bases at the time of the field study. Currently it’s GenBank accession number is MG589453.1. The OTU annotated as unidentified *Acidimicrobiales* had ≥ 97% sequence identity with A6, thus confirming the presence of this Feammox bacterium in our samples. A total of 316 genera were annotated in the microbial community analysis, however, between 51% (site 1-2) to as much as 69% (site 5-6) of the OTUs could not be classified at this level, thus, they were added to the “others” category. Among the top 100 most abundant genera, the unidentified *Acidimicrobiales* ranked 56th (Figure 7). The genera with the highest relative abundance at site 1-2, characterized by its waterlogged condition, were *Sideroxydans* (Proteobacteria), an Fe(II) oxidizer (Weiss et al., 2007), and
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

*Geothrix* (Acidobacteria), a known ERB (Bond and Lovley, 2005). At sites 3-4 and 5-6, the most relative abundant genera were the *Bryobacter* (Acidobacteria) an aerobic heterotroph, candidatus *Solibacter* (Acidobacteria), *Acidibacter* (Proteobacteria) an FeRB, the autotroph *Acidotherrmus* (Actinobacteria), and *Sorangium* (Proteobacteria). Other Fe cycling bacteria found among the top 100 most abundant genera are *Acidiferrobacter, Anaeromyxobacter, Ferritrophicum, Geobacter, Gallionella, Desulfobulbus,* and *Georgfuchia*, all from the Proteobacteria phylum.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

**Figure 7.** Relative abundance of the 100 most abundant genera from attached bacteria on the electrodes and on soil samples from the first group of electrode sets placed in the field. Acidimicrobiaceae sp. A6 had $\geq 97\%$ identity with the unidentified Acidimicrobiales which ranked 56th in abundance. In bold other FeRB.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

### 2.3.3 Correlation of relative abundance between *Acidimicrobiaceae* sp. strain A6 and other bacteria

*Acidimicrobiaceae* sp. strain A6 ranked 56th in the relative abundance at the genera level in the field study soil and electrode samples, being outranked by other FeRB (Figure 7). It is not surprising that other FeRB, such as *Geobacter* spp. are more abundant than A6. This could be due to the fact that *Geobacter* is a heterotroph and thus has a much faster doubling time (5 hours to less than a day depending on the species) compared to litoautotrophs like A6, which has a doubling time of 8-10 days (Huang and Jaffé, 2018).

A6 showed negative correlations between their relative abundance in the field soil and electrode samples with other FeRB (Figure 8 a). This is not the case for the relative abundance of A6 with other non-metal reducing bacteria, also from field soil and electrode samples where it showed a positive or no correlation ($r \approx 0.0$) (Figure 8 b). When only the relative abundances of attached bacteria on the electrodes are analyzed for correlations, the correlation between A6 and *Collimonas* shifts from slight negative ($r = -0.02$) to a positive correlation ($r = 0.45$) ($p > 0.1$). For all the other genera, the trends of their correlations are maintained when all the data (bacteria from soil and electrodes) is either pooled for analysis or separated by bacteria on the electrodes or soil samples only. This indicates, that in soils and on the electrodes, A6 presence is negatively affected by most other Fe-cycling bacteria found in our samples. Whereas when the relative abundance of *Geobacter* is correlated with the other Fe-cycling bacteria, it shows a positive correlation with all except *Acidibacter* and *Acidiferro bacter*. These findings open up the need for further research to
understand what drives these correlations and if they may indicate a competition for the electron acceptor (iron and electrode) between A6 and other FeRB.

**Figure 8.** Correlation of the relative abundance between *Acidimicrobiaceae* sp. strain A6 (unidentified *Adicimicrobiales*) and a) Fe-cycling bacteria attached, and b) other non-metal-reducing bacteria on the field-deployed electrodes and soil samples (n=27). In a) Fe-oxidizing bacteria are in italics and Fe-reducing bacteria in bold italics. † Anode colonizer (Ahn et al., 2014; Bond and Lovley, 2005; Gregory et al., 2004; Strycharz-Glaven et al., 2013), †† Cathode colonizer (Ahn et al., 2014), in b) in bold N cycling bacteria. ***p < 0.01, **p < 0.5, *p < 0.1.
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

2.4 Conclusions

The results from this study show that *Acidimicrobiaceae* sp. strain A6, which is an iron reducer, is capable of colonizing electrodes in the field and in soil columns in the laboratory. Under controlled conditions, like in the soil columns in the laboratory, A6 is enriched on the anode, although in some cases A6 was also detected in smaller numbers on the cathode.

The relative abundance analysis indicates that *Acidimicrobiaceae* sp. strain A6 is present in the system, however it is not amongst the top most abundant, unlike other FeRB, which is not surprising due to the fact that A6 is a slow grower compared to other FeRB, which would compete for the same electron acceptors, which can explain the negative correlation found between A6 and most of the other FeRB found, and the mostly positive or insignificant correlation with other non-metal reducing bacteria. The findings from this chapter confirm that *Acidimicrobiaceae* sp. strain A6 biomass can be enhanced on electrodes driven by the natural redox potential of the soils, in both, its natural habitat, as well as in soil column setups in the laboratory. A6 has a clear preference for the more reduced electrode which functions as the anode as confirm with the laboratory setups. Thus, *Acidimicrobiaceae* sp. strain A6 is a novel anaerobic lithoautotroph from the Actinobacteria phyla capable of colonizing the anode and using it as its terminal electron acceptor, which will be further discussed in the next chapter. Altogether, this work expands the knowledge of the diversity of electrogenic microorganisms beyond the commonly studied groups and opens up the possibility for applications of this bacteria in
Chapter 2. Electrode-colonizing Feammox Bacteria *Acidimicrobiaceae* sp. strain A6 in field experiments.

Bioelectrochemical systems (BES), however, further research is needed to elucidate what drives the different interactions between A6 and other FeRB and ERB in order to optimize its applications in BES.
CHAPTER 3. Oxidation of Ammonium by Feammox Acidimicrobiaceae sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

3.1 Overview

The most extensively method of oxidizing ammonium ($\text{NH}_4^+$) in engineered systems is through nitrification ($\text{NH}_4^+ \rightarrow \text{NO}_2^- \rightarrow \text{NO}_3^-$); however, nitrification is energetically intensive as it requires oxygen inputs, which can account for a substantial amount of energy usage in wastewater treatment plants (WWTPs) during the operation of aerators (Austin and Nivala, 2009; Leu et al., 2009). To lower energy consumption for wastewater treatment, anaerobic oxidization of $\text{NH}_4^+$ is a worthwhile endeavor. The oxidation of $\text{NH}_4^+$ via Feammox to $\text{NO}_2^-$ (Clement et al., 2005; Huang and Jaffé, 2018; Sawayama, 2006; Shrestha et al., 2009; Yang et al., 2012) occurs in the absence of molecular oxygen, which makes it an attractive candidate for the development of an energy efficient $\text{NH}_4^+$ removal method. However, it requires iron oxides [Fe(III)] as electron acceptor in a stoichiometric ratio of 6:1 (Clement et al., 2005; Huang and Jaffé, 2015) (Equation 6).

$$\text{NH}_4^+ + 3\text{Fe}_2\text{O}_3 \cdot 0.5\text{H}_2\text{O} + 10\text{H}^+ \rightarrow \text{NO}_2^- + 6\text{Fe}^{2+} + 8.5\text{H}_2\text{O} \quad (6)$$

To implement the Feammox process for large scale reactor applications, the Fe(III) phase needs to be replaced with an electrode that can act as the electron acceptor.
Acidimicrobiaceae sp. A6 (Huang and Jaffé, 2018), has been shown to be an electrode (anode) colonizing bacterium (Ruiz-Urigüen et al., 2018). The required difference in potential between electrodes in wetlands is driven by the natural redox potential in the soil, and in fully mixed reactors it is driven by an applied voltage.

The anaerobic NH$_4^+$ oxidation reaction that takes place in the absence of iron oxides in MECs is NH$_4^+$ + 2H$_2$O $\rightarrow$ NO$_2^-$ + 3H$_2$ + 2H$^+$ (Figure 9), where the anode functions as the electron acceptor. MECs utilize a small external voltage (0.2 - 0.8 V) for overcoming the thermodynamic barrier of electrolysis. The potential difference between the anode and the cathode is enough to drive the electron transfer from the NH$_4^+$ oxidation reaction. The possibility of a reaction can be determined by calculating its standard free energy ($\Delta G^\circ'$) applying Nernst equation ($\Delta G^\circ' = -nF \Delta E^\circ$), where $n$ is the number of electrons transferred during the reaction ($n$ = 6 for NH$_4^+$ $\rightarrow$ NO$_2^-$), F is Faraday’s constant (96.485 kJ/V/mol), and $\Delta E^\circ$ is the difference in the potentials between two half reactions, measured in volts (V) ($\Delta E^\circ = E_{\text{anode}} - E_{\text{substrate}}^\circ$). $E_{\text{substrate}}^\circ$ is equal to 0.07 V for NH$_4^+$ oxidation to NO$_2^-$ (Equation 7). Hence, to make the reaction feasible, $E_{\text{anode}}$ needs to be above 0.07 V vs. SHE.
Chapter 3. Oxidation of Ammonium by Feammox \textit{Acidimicrobiaceae} sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

\[ E^{o'} = E^{o'} \text{acceptor} - E^{o'} \text{donor} \quad (7) \]

<table>
<thead>
<tr>
<th>Half reduction reaction</th>
<th>( E^{o'} )</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{NO}_3^- + 10\text{H}^+ + 8\text{e}^- \rightleftharpoons \text{NH}_4^+ + 3\text{H}_2\text{O} )</td>
<td>0.36 V</td>
<td>(Schwarzenbach et al., 2003)</td>
</tr>
<tr>
<td>( \text{NO}_3^- + 2\text{H}^+ + 2\text{e}^- \rightleftharpoons \text{NO}_2^- + \text{H}_2\text{O} )</td>
<td>0.43 V</td>
<td>(Schwarzenbach et al., 2003)</td>
</tr>
<tr>
<td>( \text{NH}_4^+ + 2\text{H}_2\text{O} \rightleftharpoons \text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+ )</td>
<td>0.07 V</td>
<td></td>
</tr>
</tbody>
</table>

The objective of this study is to harness A6’s ability to use electrodes in BES, specifically in Microbial Electrolysis Cells (MECs) for the removal of \( \text{NH}_4^+ \) and to gain new insights into the bioelectrochemical conditions required for such systems. A specific goal was to determine if pure and enrichment cultures of the Feammox bacterium \textit{Acidimicrobiaceae} sp. strain A6 can grow and can carry out anaerobic \( \text{NH}_4^+ \) oxidation in the absence of Fe(III) in MECs; and if so, if the rates of \( \text{NH}_4^+ \) oxidation and the A6 growth in MECs are comparable to those in a batch reactor with Fe(III) as the electron acceptor. For A6 enrichment cultures in MECs, an additional goal was to analyze the overall microbial community to determine if there are other known \( \text{NH}_4^+ \) oxidizers present, and to get an assessment of the A6 relative abundance in such systems. This was achieved by continuously monitoring current production, measuring \( \text{NH}_4^+ \) removal and A6 biomass concentration in MECs with pure A6 and A6 enrichment cultures.

Results presented here demonstrate that A6, either in a pure or enrichment culture, can thrive in microbial electrolysis cells (MEC) by oxidizing ammonium, the only electron
Chapter 3. Oxidation of Ammonium by Feammox Acidimicrobiaceae sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

donor provided, while using the anode as the electron acceptor. Results have shown that the current produced increases with the concentration of 9,10-anthraquinone-2, 6-disulfonic acid (AQDS), an electron shuttling compound, and that this effect is especially noticeable for the pure A6 culture. Although electron microscopy of the anode’s surface reveals attached cells in the pure culture MEC, it is clear that over the setup/time of operation there is no formation of a biofilm on the anode’s surface and that the majority of cells are in the bulk liquid, explaining the observed effect of AQDS. Maximum current density measured was 4.2 A/m$^3$ and a coulombic efficiencies (CE) of 16.4%. These results show that ammonium removal by Feammox bacteria Acidimicrobiaceae sp. A6 can be carried out in bioelectrochemical reactors without the need to supply a solid iron phase as electron acceptor and that Acidimicrobiaceae sp. A6 population can be maintained stable during this process. This is a first step towards the development of a Feammox bacteria-based system for anaerobic ammonium oxidation while reducing electrodes.

3.2 Materials and Methods

3.2.1 Experimental setup

MECs were constructed and run in parallel as described by Call and Logan (2011) (Figure 9), using a stainless steel mesh as the cathode, and a graphite plate as the anode since it is chemically stable (Logan et al., 2006). The headspace of each MEC was purged with an 80% N$_2$, 20% CO$_2$ gas mixture, and autoclaved. MECs were connected in parallel to a programmable power supply (model 3645A; Circuit Specialists Inc.) with a constant external applied voltage ($V_{app}$) set at 0.3 V. Voltage was recorded hourly with a multimeter.
Chapter 3. Oxidation of Ammonium by Feammox *Acidimicrobiaceae* sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

(model 2750; Keithley Instruments Inc.) across a 10 Ω resistor placed between the lead connecting the anode and the positive terminal of the power supply. Current ($I$) was calculated using Ohm’s law ($I = V/R$), were $V$ is voltage and $R$ the resistance. Data are reported as volumetric current density ($I_d = A/m^3$) which was obtained by dividing current by the volume of the culture.

**Figure 9.** Schematic of a biotic MEC with Feammox bacteria *Acidimicrobiaceae* sp. A6 culture. $\text{NH}_4^+$ is oxidized to $\text{NO}_2^-$ at the anode and $\text{H}^+$ reduced to form $\text{H}_2$ at the cathode. P is the power supply and M the multimeter.

### 3.2.2 Cyclic voltammetry

Cyclic voltammetry (CV) was conducted on the anode with the cathode as the counter electrode, and a 1 mm thick Ag/AgCl 3.5M KCl reference electrode (model ET072-1mm, EDaq Inc.) placed between the working and counter electrodes (Figure 10).
MECs were cycled using an Ivium potentiostat, using the Ivium software. Three consecutive scans were conducted, which ranged from $-1 \, \text{V}$ to $+1 \, \text{V}$ at a rate of $1 \, \text{mV/second}$. Only the last 2 scans are shown to avoid overcrowding of the figure. CVs were conducted on MECs with live pure and A6 enrichment culture with and without 9,10-anthraquinone-2, 6-disulfonic acid (AQDS), which is an electron shuttling compound, to determine possible effects of AQDS on the system’s required voltage. CV was also conducted on MECs with dead A6 culture with AQDS, and on abiotic control MECs with and without AQDS to confirm the effect of A6.

**Figure 10.** Picture of a MEC connected to an Ivium potentiostat for CV. The red lead is connected to the graphite anode, the black lead to the stainless steel cathode and the blue lead to the Ag/AgCl reference electrode.
3.2.3 MEC operating conditions

Each MEC was inoculated with an A6 pure or enrichment culture in Feammox enrichment medium. The medium contained: 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, vitamin supplement (ATCC® MD-VS) 0.1µl/l, and trace element solution as described by Sawayama (2006). AQDS 0.15 mM was included after determining that its addition facilitated electron transfer to the anode from NH$_4^+$ oxidation, enhancing both, the amount of ammonium oxidized and hence current produced (Figure 11). Additionally, it was shown that AQDS is required to grow the pure A6 culture when Fe(III) is the electron acceptor (Huang and Jaffé, 2018), while for long-term growth of A6 enrichment cultures, AQDS is not needed (Huang and Jaffé, 2015). Vials contained resazurin (1 mg/L) as an indicator of anaerobic conditions. The pH of the medium was initially set to 5 - 5.5 because Feammox process works best at acidic conditions with pH below 6.3 (Huang and Jaffé, 2015; Huang and Jaffé, 2018).
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### a.

**Figure 11.** a) Average current density measured in MECs containing a pure A6 culture and different concentrations of AQDS added as an electron shuttle ($V_{app}$ 0.7 V). The points show the mean and the bars the standard error (n=3). b) Average amount of NH$_4^+$ removed at each AQDS concentration. Notice that in the absence of AQDS no NH$_4^+$ change was detected, thus the current measured is the result of electrons extracted from NH$_4^+$ oxidation and transferred to the anode aided by AQDS.

MECs with working Feammox cultures were run (n=2 for pure cultures and n=4 for enrichment cultures). Three types of controls were set up to confirm that current production and NH$_4^+$ removal were the result of biotic activity: 1) MECs with dead bacteria by autoclaving, 2) Abiotic MECs with enrichment medium without microbial inoculum, and 3) MECs with live bacteria in Feammox medium without NH$_4^+$. Furthermore, positive controls with live Feammox A6 pure culture were incubated in batch reactors containing Fe(III) in the form of 2-line ferrihydrite as the electron acceptor (Schwertmann and Cornell, 2010).
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2000) for NH$_4^+$ oxidation and biomass change comparison. All MECs were placed on a mixing plate at 240 rpm. The MECs that were run for over 3 weeks with the pure culture were placed on a magnetic plate with a stirrer to avoid noisy readings generated by movement caused by the mixing on the shaker. When current production decreased, headspace was flushed with an 80% N$_2$, 20% CO$_2$ gas mixture. Coulombic efficiency (CE = $C_p/C_T \times 100\%$) was determined as the percentage ratio between the coulombs ($C$) calculated by integrating the current over time ($C_p = \int I \, dt$) divided by the theoretical amount of C produced from the amount of NH$_4^+$ oxidized to NO$_2^-$ ($C_T = \Delta$NH$_4^+$ n F), where $\Delta$NH$_4^+$ is the change in moles NH$_4^+$ measured, n is the amount of electrons produced from NH$_4^+$ oxidation to NO$_2^-$ (6 mM of electrons per mM of NH$_4^+$), and F is Faraday’s’s constant (96,485.33 C mol$^{-1}$).

### 3.2.4 Chemical analyses

Initial and final 1 ml-samples were taken from the MECs. Each sample was filtered using a 0.2 μm pore size syringe filter and used to measure NH$_4^+$ and NO$_2^-$ concentrations in a Dionex™ Ion Chromatograph ICS3000, with a CS-16 column, CS-16 guard column, and a CERS 500 (4 mm) suppressor for cations; and with an AS-22 column, AG-22 guard column, and an ASRS 300 (4 mm) suppressor for anions. Iron was analyzed to quantify the small amount of Fe that was transferred with the culture seed to the MECs (Table 6). Total Fe was analyzed by adding 100 μl of the MEC or control culture to 4.8 ml of 1N HCl and 100 μl of 6.25M NH$_2$OH-HCl, then Fe was quantified photometrically using the ferrozine method analysis (Stookey, 1970), as adapted by Komlos and Jaffé (2004). Ferrous iron [Fe(II)] was quantified by direct ferrozine method.
Chapter 3. Oxidation of Ammonium by Feammox Acidimicrobiaceae sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

Table 6. Fe content in MECs

<table>
<thead>
<tr>
<th>MEC condition</th>
<th>Fe (II) (mM)</th>
<th>Fe (III) (mM)</th>
<th>Amount of NH$_4^+$ (mM) that could be removed by Fe (III) available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure A6 mixed on stirrer</td>
<td>1.94 ± 0.1</td>
<td>0.59 ± 0.035</td>
<td>0.1 ± 0.006</td>
</tr>
<tr>
<td>Pure A6 mixed on shaker</td>
<td>0.46 ± 0.1</td>
<td>0.59 ± 0.025</td>
<td>0.1 ± 0.004</td>
</tr>
<tr>
<td>A6 enrichment mixed on shaker</td>
<td>0.5</td>
<td>0.5</td>
<td>0.0</td>
</tr>
</tbody>
</table>

3.2.5 DNA extraction, quantification and microbial community composition

Total genomic DNA was extracted from 5 ml of working culture of MECs and positive live controls at the end of each operational period. DNA was extracted using FastDNA® spin kit for soil (MP Biomedicals, USA) following the manufacturer’s instructions with an additional first step in which the bacteria were concentrated by centrifuging the liquid medium for 10 min at 14,000 relative centrifugal force (RCF); the pellet was resuspended in 500 µl of the supernatant and used as the initial substrate for extraction. Total DNA was eluted in 100 µl of sterile water and its concentrations were measured using Qubit 2.0® (Invitrogen, USA). All DNA samples were preserved at -20 °C until further analysis.

Quantification of A6 in the pure culture was carried out via qPCR using the Applied Biosystems StepOnePlus real-time PCR system by amplifying a section of the 16S rRNA gen using primer set 1055F/1392R (1055F, 5’-ATGGCTGTCGTCGCTAGCT-3’; 1392R, 5’-
ACGGGGCGGTGTGTAC-3’). Each qPCR mixture (20 µl) was composed of 10 µl of
SYBR Premix Ex Taq II 2X (TaKaRa, Japan), 0.8 µl of each forward and reverse primer
at 10 µM, and the DNA template. Thermal cycling was initiated with 30 s at 95°C, followed
by 40 cycles, each cycle consisted of 5 s at 94°C, 30 s at 55°C, and 30 s at 70°C. Each
qPCR assay was run in triplicate for each sample and included negative controls and a
standard curve; the latter consisted of serial dilutions of known numbers of copies of DNA.

In order to determine the microbial community composition, sequencing and
phylogenetic analysis was performed by Novogene (Beijing, China) as described in detail
in Appendix B. A total of 31,969 sequences were obtained which were clustered into
OTUs. A total of 995 OTUs were produced.

3.2.6 Environmental scanning electron microscopy of the MECs’ anode.

The graphite plate working as an anode of a MECs containing live A6, and one from
the autoclaved MEC, both from MECs operated under stirring for over 1 month, were
analyzed using and Environmental scanning electron microscope (Quanta 200 FE-ESEM)
at the Imaging and Analysis Center of Princeton University.

3.3 Results and Discussion

3.3.1 Cyclic voltammetry analysis

Cyclic voltammetry (CV) results (Figure 12) show oxidation peaks for all biotic
MECs containing AQDS at -0.03 ± 0.025 V vs. Ag/AgCl (3.5 M KCl, + 205 mV vs. the
standard hydrogen electrode, SHE), while for the experiments without AQDS the peak was
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shifted slightly toward more negative values, $-0.11 \pm 0.035$ V vs. Ag/AgCl. Applying these voltages to calculate the $\Delta G^\circ$ for the biotic reactions, using Nernst equation, results in $-60.78$ kJ/mol with AQDS, and $-14.47$ kJ/mol without AQDS. Moreover, initial MECs analysis with pure and A6 enrichment culture ($V_{\text{app}}$ of 0.7 V), clearly showed that AQDS has a greater effect when added to pure A6 culture than to the A6 enrichment culture (Figure 13). These results are consistent with other studies which have demonstrated that electron shuttling compounds facilitate electron transfer, for example natural organic matter (Lovley et al., 1996; Rios-Del Toro et al., 2018) or certain bacterial cells which can enable cell-to-cell electron transfer (Summers et al., 2010). In the absence of available organic matter, analogues, such as quinones, aid the electron transport between the cell and the final electron acceptor (Orsetti et al., 2013) and facilitate $\text{NH}_4^+$ oxidation by aiding electron transfer to the anode (Figure 11). Therefore, in the absence of natural electron shuttles, AQDS is good option to facilitate electron transfer, including in BES, as it has been shown that graphite anodes incorporated with microbial oxidants, such as AQDS, can increase their $I_d$ performance (Feng et al., 2010; Lowy et al., 2006), as seen in our system. Given the decreased effect of AQDS on current production in the A6 enrichment culture, it is unlikely that AQDS will be needed in A6 enrichment cultures grown in MECs or other bioelectrochemical reactors for extended time, since organic matter will accumulate due to cell turnover.
Figure 12. Cyclic voltammetry scans (1mV/sec) for MECs with and without Acidimicrobiaceae sp. strain A6, and with and without AQDS. Optimal V_{app}: \sim -0.03 \pm 0.025 \text{ V vs. Ag/AgCl} for cultures with AQDS, and -0.11 \pm 0.035 \text{ V vs. Ag/AgCl} for MECs without AQDS.
Figure 13. Average current density measured in MECs containing live pure and A6 enrichment culture, with and without AQDS, and in dead culture with and without AQDS ($V_{\text{app}}$ 0.7 V). The points show the mean and the bars the standard error. Notice that the addition of AQDS has a greater effect on pure A6 cultures than in A6 enrichment cultures, and no significant effect of AQDS on current production is found in dead cultures.

Other studies on $\text{NH}_4^+$ removal using BES inoculated with sludge from WWTPs reported current oxidation peaks of 0.59 V vs. Ag/AgCl (Zhan et al., 2014) and 0.53 V vs. Ag/AgCl (Vilajeliu-Pons et al., 2018). The conditions used in those studies, such as pH of 7.7 (Feammox reaction requires acidic pH), and the associated microbial community, are different from the ones presented here. The anaerobic $\text{NH}_4^+$ removal reported by those studies does not appear to be associated to the Feammox reaction. Although similar oxidation peaks (-0.08 vs. Ag/AgCl) to the ones found for the MECs have been reported for other bioelectrogenic system, their system set up and microbial community composition were different to the one reported here (Zhu et al., 2014; Zhu et al., 2012).
Chapter 3. Oxidation of Ammonium by Feammox *Acidimicrobiaceae* sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

The CV on abiotic and dead controls did not show a peak near the oxidation peak for the biotic reactors. The amplitude of the voltagram on the reverse scans is believed to be due to a pseudocapacitance behavior of the system. The proposed NH$_4^+$ oxidation reaction in the MECs ($\text{NH}_4^+ + \text{H}_2\text{O} \rightarrow \text{NO}_2^- + 3\text{H}_2 + 2\text{H}^+$) supports the increased amplitude of the voltagram during reverse scanning, because protons can undergo adsorption and desorption prior to H$_2$ formation during scans (Eftekhari, 2017).

### 3.3.2 MECs with pure *Acidimicrobiaceae* sp. strain A6 culture.

Results show that A6 has the ability to be active in MECs, under constant mixing and with a $V_{\text{app}}$. Different replicas of the MECs run with the pure A6 culture had $I_d$ peaks at different time points (Figure 14). For example, some peaked in the first couple of days, with a maximum $I_d$ of up to 3.2 A/m$^3$, while other live A6 culture ramped up slower and never peaked, instead they showed a stable increase of $I_d$ and then leveled out. Although MECs were operated for 3 weeks, $I_d$ data is shown only for a 2-week period, because after that, the connections to the electrodes became loose due to the constant shaking, resulting in noisy data. These MECs with pure culture had a CE of 4.33%. All control conditions, including MECs with dead A6, abiotic MECs, and MECs with live A6 without NH$_4^+$ showed negligible $I_d$(Figure 15).
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Figure 14. a-b) Average current density ($I_d$) measured in MECs replicas with pure A6 culture. Live A6-a peaked on day 1 while b only ramped up on day 5. Dead A6, as well as other control conditions, not shown here to avoid overcrowding of figure, show negligible $I_d$. Arrow represents the time of headspace flushing and injection of an 80% N$_2$, 20% CO$_2$ gas mixture. c) Average NH$_4^+$ removal after 3 weeks of operation, measured from MECs containing dead A6, MECs without Fe(III) with live A6, batch incubations with live A6 cultures using Fe(III) as the electron acceptor, and from abiotic control. Bars represent the mean and the marks the maximum and minimum amount removed.
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**Figure 15.** Average current density measured in MECs with pure live A6 in Feammox medium without NH$_4^+$ under stirring conditions. Marks show the mean and lines the standard error (n=3).

All MECs containing the live A6 culture removed on average 0.6 ± 0.25 mM of NH$_4^+$, which is similar to the amount removed for cultures grown using Fe(III) as the electron acceptor, over the same time period, i.e. 0.63 ± 0.14 mM of NH$_4^+$. The control conditions showed no removal of NH$_4^+$, on the contrary a slight increase in NH$_4^+$ concentration was detected (+ 0.11 ± 0.09 mM of NH$_4^+$), which could be due to desorption of NH$_4^+$ from the solid phase (Figure 14, c). NO$_2^-$ concentration measurements in MECs were below the detection limit. Low detection of formed NO$_2^-$ with respect to NH$_4^+$ removed via Feammox process has been previously reported (Huang and Jaffé, 2015; Huang and Jaffé, 2018); however, a N mass balance in mixed cultures was observed when acetylene gas (C$_2$H$_2$) was added (Huang and Jaffé 2015), which stops the loss of N in the form of N$_2$, by inhibiting the
reduction of N₂O to N₂. Abiotic Fe(II) oxidation by NO₂⁻ has been observed under similar conditions as the ones used during the Feammox process and in the MECs (Klueglein and Kappler, 2013; Klueglein et al., 2014), which results in the reduction of NO₂⁻ to different N-gas forms. Hence, a control test incubating NO₂⁻ with Fe(II) while tracking NO₂⁻ concentration over time was performed. Results show that NO₂⁻ disappeared while in presence of Fe(II) (Figure 16). The presence of small amounts of Fe that are transferred to the MECs with the bacterial seed (Table 6), explains why NO₂⁻ was not detected, as it would have reacted with the transferred Fe(II).

**Figure 16.** NO₂⁻ incubations with Fe(II). a) NO₂⁻ measurements over time. b) Fe(II) measurements over time.

Quantification of biomass from MECs with the pure live A6 culture, revealed that A6 numbers can be sustained as well as when grown as a pure culture with Fe(III) as the electron acceptor over 3 weeks of operation (2.90 x 10⁹ ± 2.8 x 10⁹ copies of DNA/ml in MECs and 3.35 x 10⁹ ± 1.97 x 10⁹ copies of DNA/ml in batch culture with Fe(III)).
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Mixing of the medium in the MECs facilitates transport of NH$_4^+$ to cells on the electrode and/or of reduced AQDS to the electrode. However, using a shaker for this purpose resulted in deterioration of the connections in the MECs. To expand the operational time of the MECs, magnetic stirring bars were placed in each reactor, which were placed on a stirring plate. This change resulted in MECs with pure A6 culture to operate for over 1 month, with continuous increase in $I_d$ over time, with up to 4.2 Amp/m$^3$ (Figure 17). MECs removed an average of 0.66 ± 0.03 mM of NH$_4^+$, comparable to the bench pure A6 cultures containing Fe(III) which removed 0.64 mM of NH$_4^+$. Both, MECs and bench cultures sustained similar amount of A6 biomass (2.11 x 10$^9$ ± 1.33 x 10$^9$ copies of DNA/ml in MECs and 6.97 x 10$^9$ copies of DNA/ml in batch culture with Fe(III)). This set up resulted in a CE of 16.4%.

![Figure 17](image)

**Figure 17.** Average current density measured in MECs with pure A6 under stirring conditions. Marks show the mean and lines the standard error. A representative control MEC containing dead A6 is shown in the figure. Arrows represent the times of headspace flushing and injection of an 80% N$_2$, 20% CO$_2$ gas mixture.
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In MECs operated with the pure A6 culture, $I_d$ of up to 3.2 Amps/m$^3$ were measured in MECs within 2 weeks, and up to 4.2 Amps/m$^3$ were measured in MECs that operated for over 1 month. These values, are on the lower side of $I_d$ measured in other MEC systems (Lu and Ren, 2016), however, their performance cannot be directly compared since their substrates, microbial communities and reactor configurations are different. The highest Coulombic efficiency achieved in the MECs was 16.4%, which is similar to values found in other studies (CE 9.6 – 26.4%) (Ditzig et al., 2007), but is on the low side for CE reported for other systems (CE ≥ 23%) (Liu et al., 2010), however; none of those systems are comparable to the ones described here as they contain organic C as their electron donor. CE for anaerobic NH$_4^+$ removal in MECs from other studies report CE of 32.7- 50% (Qu et al., 2014) (Vilajeliu-Pons et al., 2018), which are higher than those reported in this study, however their systems also differ from the one described here in that their microbial communities are composed of a high bacterial diversity, many of which are involved in various steps of the N cycle. Low CE means that the electron from NH$_4^+$ are not recovered as current, this could be due to use of these electrons for the formation of secondary metabolites (Yang et al., 2014) or mediators that can be stored and used later (Ditzig et al., 2007). Low CE is also associated to low $V_{app}$, which should be further analyzed. Additionally, it is important to point out that although it has been shown that A6 can use H$_2$ as electron acceptor (Huang and Jaffé, 2018), neither the pure A6 culture nor the enrichment culture MECs produced current in the absence of NH$_4^+$.

Through E-SEM, we found rod-shaped, approximately 1.5–3 µm long by 0.5 µm wide bacteria cells, as described for A6 (Huang and Jaffé, 2018). These cells were found attached to the surface of the anode of the MEC with the pure live A6 culture, while no
cells could be found on the electrode’s surface of the control MEC with dead bacteria (Figure 18). This result is consistent with a previous study in natural and constructed wetlands showing that the A6 population was enhanced on biofilms formed on electrodes working as anodes while no A6 enrichment was found on unconnected plates, thus indicating the need of a voltage difference to promote A6 preference for the anode’s surface (Ruiz-Urigüen et al., 2018).

![E-SEM images of the graphite anode of a control MEC operated with dead A6, no bacterial cells were found.](image1)
![Graphite anode of MEC operated with live A6, bacterial cells are encircled.](image2)

**Figure 18.** a) E-SEM images of the graphite anode of a control MEC operated with dead A6, no bacterial cells were found. b) Graphite anode of MEC operated with live A6, bacterial cells are encircled.

### 3.3.3 MECs with *Acidimicrobiaceae* sp. strain A6 enrichment culture.

Since maintaining pure cultures in a biological reactor for extended time periods is a challenge, and also, A6 might not remain active in pure cultures for long periods (Huang
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and Jaffé, 2018), and since in real-world application, it would perform in mix microbial population conditions, we also tested the performance of an A6 enrichment culture in MECs. The goal was to determine if, as for the case of the pure culture, the enrichment culture could also produce current as a result of NH$_4^+$ removal. Results for the MECs with the A6 enrichment culture show that MECs produced an average current density ($I_d$) of 2.5 A/m$^3$ resulting in a CE of 5.4%, and removed a total of 0.52 mM NH$_4^+$ after 3 weeks of operation (Figure 19).

![Figure 19](image)

**Figure 19.** Average current density measured in MECs containing A6 enrichment culture. Arrow represents the point of headspace flushing and injection of an 80% N$_2$, 20% CO$_2$ gas mixture.

The microbial community was composed of 47.6% Actinobacteria, the genus to which A6 belongs to, and Feammox bacteria had a relative abundance of 61.6% of the total diversity of all Fe-reducing bacteria present in the culture (Figure 20). The relative abundance of A6 in these MECs is similar to that obtained by Huang and Jaffe (2015) for
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an A6 enrichment culture in a membrane reactor to which ferrihydrite was provided as the electron acceptor.

**Figure 20.** Microbial community composition from a MEC with the A6 enrichment culture. **a)** Phylum level. **b)** Genera in the Actinobacteria phylum, within this phylum, *Acidimicrobiaceae* sp. strain A6 has a relative abundance of 47.6%. **c)** Relative abundance of A6 and other Fe-reducer genera found in the MECs.
The composition of the microbial community of a working MEC culture consisted of 3.83% of Actinobacteria phylum, of which 47.6% was classified at the genus level as Feammox bacterium A6, i.e. *Acidimicrobiaceae* sp. strain A6 (For complete taxonomy of the microbial community composition of the top 100 most abundant genus from a MEC with an A6 enrichment No other NH$_4^+$ oxidizing bacteria were detected at the genus level culture see Appendix C). Actinobacteria is a phylum frequently present in BES (Ruiz-Urigüen et al., 2018) but normally not considered in the microbial analysis of such systems. Feammox bacteria A6 made up 61.6% of the total diversity of all other Fe-reducing bacteria (FeRB) present in the culture. Other present FeRB that are also known to be electrogenic bacteria that can power BES (Logan, 2009) include *Geobacter* (7.8%), *Geothrix* (7.2%), and *Desulfobulbus* (4.7%), however, they require organic C as their electron source (Bond and Lovley, 2003, 2005; Holmes et al., 2004a) and this was not provided at all in the system. The only source of C added was CO$_2$ because A6 is an autotroph (Huang and Jaffé, 2018) and we attribute the presence of other FeRB to be remnants of the initial microbial community, and any carbon they might be using would come from biomass turnover. Iron reducers such as *Geobacter sp.* (Caccavo et al., 1994) as well as A6 (Huang and Jaffé, 2018) can also use H$_2$ as electron donor, for which reason the headspace of MECs was flushed periodically.

There are a limited number of studies on bioelectrochemical NH$_4^+$ oxidation using electrodes as the electron acceptor, and they conclude that the key organism responsible for this process is *Nitrosomas* (Proteobacteria) (Qu et al., 2014; Vilajeliu-Pons et al., 2018; Zhan et al., 2014), a group not found in our MECs. *Nitrosomas* are common aerobic nitrifiers present in soil, freshwater and wastewater. Qu et. al. (2014) inoculated their
system with freshwater sediments, and Zhan et. al (2014) and Vilajeliu-Pons et. al. (2018) inoculated the anode chamber, of a dual-chamber bioelectrochemical system, with sludge from wastewater treatment plants. It is not surprising that they encountered nitrifiers in their systems; however, the mechanism used for anaerobic NH$_4^+$ oxidation by organisms that are normally aerobes, and how they produce current it still unknown. It has been shown that commonly FeRB, such as *Geobacter* or *Geothrix*, have the ability to be electrogenic and thus transfer the electron to an anode, thus producing current. A6 is an FeRB that carries out Feammox, the only fully anaerobic NH$_4^+$ oxidation process known, hence, we attribute the current production and ammonium oxidation in the MECs in part to A6 activity, especially since no other organism was present in the pure A6 culture MECs. No current was observed in the control MECs with dead A6, abiotic MECs nor in the MEC operated with live A6 but without NH$_4^+$. 

### 3.4 Potential application of *Acidimicrobiaceae* sp. strain A6 in MECs.

The coupling of Feammox bacteria A6 in MECs allows for the advancement of potential application such as analyzing and treating other compounds of environmental concern. For example, per- and polyfluoroalkoxy alkanes (PFAS), which are of environmental concern due to their persistence in the environment owed to their resistance to environmental and biodegradation, and their linkage to possible adverse effects to human health (US-EPA, 2016a, 2018).
Figure 21. Chemical structure of a) perfluorobutanesulfonic acid (PFBS) and b) perfluoroctanoic acid (PFOA)

Preliminary results show that perfluorobutanesulfonic acid (PFBS) (C₄HF₉O₃S) (Figure 21 a) or perfluoroctanoic acid (PFOA) at 100 ppm (C₈HF₁₅O₂) (Figure 21 b), two types of PFAs, are degraded in MECs containing pure A6 culture, but no change was seen in the absence of A6. The results were obtained in MECs operated under the same conditions as described above (Section 3.2.3). PFBS and PFOA were degraded, current production was detected (Figure 22) and NH₄⁺ removed (Figure 23 a). Fluoride (F⁻) and acetate (CH₃COO⁻), ions not provided in the culture medium, are degradation byproducts of these PFAS, and thus, they were measured as mean to detect the PFAS degradation. F⁻ and CH₃COO⁻ concentration increased over the course of operation in MECs with live A6 (Figure 23 b and c). Although in traditional Feammox incubations it appears that the defluorination of PFAS is catalyzed by a reductive dehalogenase, and organic fluorine acts as electron acceptor (Huang and Jaffé, 2019), the mechanism of action in the MECs is not yet known; thus further studies are required to understand the mechanism of the PFAS degradation with A6 in bioelectrochemical systems. However, the initial results presented
here show that NH$_4^+$ is removed in the presence of PFAS, thus current is detected. Nonetheless, given that PFAS function as electron acceptors (Huang and Jaffé, 2019), it would be expected that it would compete with the electrode for electrons, thus, the amount of current produced would be less than the amount produced in the absence of PFAS. Although the MECs containing PFAs are not replicas of the above mentioned MECs with pure A6 and without PFAS (Figure 14 and Figure 17), it is still clear that higher $I_d$ was measure in the ones without PFAS, however, further controls are needed to verify this argument. Additionally, the amount of F$^-$ produced, 0.14 and 0.28 mM for PBSA and PFOA respectively, is consistent with the amount of F$^-$ ions produced during comparable incubation periods of experiments run with A6 using ferrihydrite as the main electron acceptor (Huang and Jaffé, 2019). Finally, in the same study by Huang and Jaffé (2019), they show that incubations of A6 with PFAS but without ferrihydrite did not result in NH$_4^+$ or PFAS removal, thus indicating that the presence of Fe(III) as the electron acceptors is required and that PFAS cannot function as the sole electron acceptor for the Feammox process. However, the results presented here indicate, once again, that Fe(III) can be replaced by an electrode and thus facilitate PFAS removal.
Figure 22. Current density ($I_d$) measured in MECS containing PFAS: perfluorobutanesulfonic acid (PFBS) or perfluorooctanoic acid (PFOA 100 ppm. Closed circles show MECs with active pure A6 culture, open circles show MECs without A6 culture. Marks show the mean and the error bars the standard deviation ($n=3$ for PFBS and $n=2$ for PFOS).
Chapter 3. Oxidation of Ammonium by Feammox *Acidimicrobiaceae* sp. strain A6 in Anaerobic Microbial Electrolysis Cells.

a)

![NH₄⁺ removed](image)

b)

![F⁻ produced](image)

c)

![CH₃COO⁻ produced](image)

**Figure 23.** Amount of a) ammonium (NH₄⁺) removed, b) fluoride (F⁻) and c) acetate (CH₃COO⁻) formed, over 3 weeks of operation of MECs with perfluorobutanesulfonic acid (PFBS) or perfluorooctanoic acid (PFOA) 100 ppm, and in control MEC with pure A6 without PFAS. Bars show the mean and the error bars the standard deviation (n=3 for PFBS and n=2 for PFOA).
3.5 Conclusions

The Feammox bacterium *Acidimicrobiaceae* sp. strain A6 can be grown in MECs as a pure culture or an enrichment culture, utilizing NH$_4^+$ as the electron donor and the anode as the electron acceptor in the absence of an Fe(III) source, which under natural conditions is the electron acceptor.

A6 biomass can be sustained in equal concentration in MEC reactors as in batch reactors containing Fe(III), thus, for very similar A6 numbers in MECs vs. incubations with Fe(III), similar amounts of NH$_4^+$ removal were observed. Furthermore, the relative numbers of A6 in the MECs with the A6 enrichment culture were similar to those observed in a Feammox membrane reactor to which ferrihydrite was supplied as electron acceptor. This indicates that A6 grows as well in bioelectrochemical reactors without Fe(III) as in reactors to which Fe(III) is supplied.

E-SEM revealed A6 cells attached to the electrode surface, however, the formation of a clear biofilm could not be detected, thus the majority of the cell were in the bulk liquid, therefore, there was a significant improvement in current production when an electron shuttling compound (AQDS) was added.

Maximum $I_d$ value of 4.2 A/m$^3$ and CE of 16.4% were found. Although these values are on the lower side of other values reported, none of the other systems can be directly compared to the system described here because the work presented here couples the Feammox pathway to MECs, which has not been addressed in other studies.
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Energy production is not the objective of oxidizing NH$_4^+$ in the MECs, the objective is rather to develop an energy efficient method for oxidizing NH$_4^+$ without the need for aeration and as an alternate method to Anammox, and results presented here show that this is feasible and warrants further development.
CHAPTER 4. Effect of Nitrate and Nitric Oxide Loadings on the Microbial Performance in a Nitric Oxide Denitrification Bioreactor.

4.1 Overview

Nitric oxide (NO) is a form of reactive nitrogen produced by human activities such as power generation, industry, transit, and biomass burning (Dentener et al., 2006). Typically, over 95% of nitrogen oxides (NO\textsubscript{x}) in a combustion flue gas contains NO, while nitrogen dioxide (NO\textsubscript{2}) is mostly formed via photochemical reactions in the atmosphere. These flue gases, NO and NO\textsubscript{2}, are two major air pollutants responsible for the formation of urban and regional haze, acid rain, and ozone layer depletion, and can contribute to climate change (Molina and Molina, 2004; Wei et al., 2016). Furthermore, exposure to these compounds can cause severe health problems such as respiratory illness (Weinberger et al., 2001). NO\textsubscript{x} pollution is a problem in many urban areas. Some megacities such as Beijing, Mexico and New York city have had NO\textsubscript{x} concentration far beyond the WHO standards (Molina and Molina, 2004), therefore, NO\textsubscript{x} pollution requires urgent remediation and appropriate management. Conventional chemical and physical methods used to treat NO\textsubscript{x} are cost intensive and produce large amounts of hazardous by-products such as ammonia and particulate matter (Li et al., 2015b); therefore, biological NO\textsubscript{x} removal technologies have recently drawn much attention due to the lower operating costs, lower energy demands and absence of secondary pollutants requiring further treatment (Chen et al., 2009).
Chapter 4. Effect of Nitrate and Nitric Oxide Loadings on the Microbial Performance in a Nitric Oxide Denitrification Bioreactor.

NO is fairly insoluble in water and cannot be separated easily (Pandey and Chandrashekhar, 2014). To overcome the limitations of the low solubility of NO in water, membrane bioreactors have been employed, and among the different types, the hollow fiber membrane (HFM) bioreactors possesses a number of advantages over the other forms of membranes such high packing density and large surface contact area (Zhang et al., 2013). Microbial studies on the denitrification of NO linked to the performance of HFM bioreactor when altering the N composition and concentrations have yet to be investigated. Therefore, the objective of this study was to investigate the effects of NO$_3^-$ and NO loading on the biological NO removal and its link to the microbial ecology once steady state was reached. This was achieved by tracking the relevant genes responsible for each denitrification step during each different NO:NO$_3^-$ loading regime to the reactor. Results showed that the denitrifying microbial community adjust rapidly to changes in the different N loadings and that the overall the performance of the reactor is robust which we link to a stable and vigorous microbial community.

4.2 Materials and Methods

4.2.1 Bioreactor setup and operation

The bioreactors setup, operation and feeding protocols were carried out by Vahid Razaviarani, as described in Razaviarani et al (2018). The experiment was conducted in parallel in two separate bioreactors: 1) a hollow fiber membrane bioreactor (HFM) which contained a mixing tank which fed the hollow fiber membrane, and 2) a semi-continuous bioreactor (BR), both operated at room temperature (25 ± 1 °C). The BR was used as a
control reactor which received NO$_3$-N as the only source of N during the whole operation period, and the HFM followed different N feeding protocols, with different loads of NO$_3$-N and NO as N sources in 6 different stages as shown in Table 7. Initially, both bioreactors were filled with 500 ml of acclimated biomass containing denitrifying bacteria collected from the effluent of a landfill leachate treatment facility in Princeton, New Jersey. The composition of the nutrient solution fed to the bioreactors was as follows: 150 mg/L CH$_3$COONa, 100 mg/L KNO$_3$, 17 mg/L KH$_2$PO$_4$, and 8 mg/L K$_2$HPO$_4$. Simulated flue gas was a mixture of NO (500 ppm in N$_2$, v/v) and N$_2$ (99.95%).

**Table 7.** Bioreactors substrate loading characteristics from Razavariani et al (2018).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>BR</th>
<th>HFM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>NO$_3$-N (g/l)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4.6</td>
<td>4.6</td>
<td>4.6</td>
</tr>
<tr>
<td>NO* (l/d)</td>
<td>---</td>
<td>144</td>
</tr>
<tr>
<td>TOC (g/l)</td>
<td>17.8</td>
<td>17.8</td>
</tr>
<tr>
<td>Duration (day)</td>
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<td>20</td>
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</table>


* The concentration of NO was maintained at 500±30 ppm using N$_2$ as the makeup gas.
4.2.2 Sludge sampling and DNA extraction for Denitrifiers composition and quantification analysis

A 5 ml sludge sample was collected from the acclimated biomass prior to starting the reactors operations. From the HFM bioreactor, 13 ml were sampled from the mixing tank during stages 1 to 6. At the end of the operation period, 6.5 ml from the biofilm in the HFM compartment were obtained via destructive sampling of the HFM. From the BR, 13 ml of sludge sample were obtained at the end of the last stage. Total genomic DNA was extracted using Fast DNA® Spin kit for soil (Biomedical, USA) according to the manufacturer’s instructions. The sludge samples were initially centrifuged at 10,000 rpm for 10 minutes and the supernatant was decanted carefully to obtain the pellet of biomass for DNA extraction. All the DNA samples were preserved at -20 °C until further analysis.

Gene abundances of relevant functional genes have been shown to serve as a tool to predict the capacity of an ecosystem to carry out a given process such as denitrification (Petersen et al., 2012). In this study we chose to detect denitrifying bacteria by targeting relevant genes responsible for each denitrification step: narG for nitrate reduction, nirS for nitrite reduction as it has been shown to be the gene present in denitrifiers better adapted to waterlogged systems (Petersen et al., 2012), CnorB for NO oxidation, and nosZ for N2O oxidation. Quantification of total and denitrifying bacteria present in the samples were determined by Quantitative PCR (qPCR) using specific primer sets reported in the literature (Table 8) (Braker et al., 1998; Braker and Tiedje, 2003; Harms et al., 2003; Reyna et al., 2010; Scala and Kerkhof, 1998a) which amplify the genes encoding narG, nirS, CnorB and nosZ enzymes. Furthermore, to compare biomass production and sustainability over time, a total bacteria count was conducted by amplifying a region of the 16S rDNA gene using
the primer set 1055F/1392R. The qPCR assays were carried out using the Applied Biosystems StepOnePlus™ Real Time PCR system.

**Table 8.** Primer sets used to target different bacteria groups.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers*</th>
<th>Primer sequence (5’-3’)</th>
<th>Annealing temp. (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>narG</em></td>
<td>narG328F</td>
<td>GACAAACTTCGCAGCGG</td>
<td>61</td>
<td>(Reyna et al., 2010)</td>
</tr>
<tr>
<td></td>
<td>narG497R</td>
<td>TCACCCAGGACGCTGTTTC</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td><em>nirS</em></td>
<td>nirS1F</td>
<td>CCTAYTGCGCCGCCRACART</td>
<td>57</td>
<td>(Braker et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>nirS3R</td>
<td>GCCGCGGTGRTGVAGGAA</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td><em>CnorB</em></td>
<td>cnorB2F</td>
<td>GACAAGNNNTACTGGTGTT</td>
<td>57</td>
<td>(Braker and Tiedje, 2003)</td>
</tr>
<tr>
<td></td>
<td>cnorB7R</td>
<td>TGNCCRTGNGCNGCNGT</td>
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<tr>
<td><em>nosZ</em></td>
<td>nos1527F</td>
<td>CGCTGTTCAGACAGYCA</td>
<td>56</td>
<td>(Scala and Kerkhof, 1998b)</td>
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<td>nos1773R</td>
<td>ATRTCGATCARCTGBTCGT</td>
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<td>1055F</td>
<td>ATGGCTGTCGTCAGCT</td>
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<td>(Harms et al., 2003)</td>
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<tr>
<td><strong>16s rDNA</strong></td>
<td>1392R</td>
<td>ACGGGGCAGGTGACGCT</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

* F = Forward and R = Reverse primers.

Each qPCR mixture (20 µl) was composed of 10 µl of SYBR Premix Ex Taq® II 2X (Takara, Japan), 0.8 µl of each forward and reverse 10 µM primer, and DNA template. Thermal cycling conditions were initiated for 30 s at 95 °C, followed by 40 cycles of 5s at 94 °C, 30s of annealing according to primers’ specific temperature indicated in Table 8, and 30s at 70 °C. Finally, a melting curve analysis for SYBR Green assay was run to distinguish the targeted PCR product from the non-targeted PCR product. Each qPCR
Chapter 4. Effect of Nitrate and Nitric Oxide Loadings on the Microbial Performance in a Nitric Oxide Denitrification Bioreactor.

reaction was run in triplicate and included negative controls and a standard curve; the last one consisting of serial dilutions of known numbers of copies of DNA of the gene per volume. Standards were generated via PCR using TaKaRa Ex Taq® (Takara, Japan) following the manufactures protocol, by amplification of each targeted gene using the primers indicated in Table 8 and total genomic DNA from *Thiobacillus denitrificans* (ATCC 25259C-5) as the template. The standards’ single amplicon purity and size was confirmed by 0.8% agarose gel electrophoresis and then purified using QIAquick® PCR Purification Kit (Qiagen). Qubit 2.0® (Invitrogen, USA) was used to measure DNA concentration which was then used to calculate the standards concentration according to each amplicon size.

4.2.3 RNA extraction, RT-qPCR and gene expression quantification

To determine shifts on gene transcripts due to NO\textsubscript{3}\textsuperscript{-}-N loading changes (4.6, 2.3, 0 and 9.2 g/l) under constant NO loading (288 l/d), RNA was extracted from the HFM Reactor’s Mixed Tank samples obtained at the end of stages 4-6. Additionally, RNA analysis was carried out on a sample of the biofilm formed on the membrane fibers, for this purpose the reactor had to be destructively sampled. PureLink® RNA Mini Kit (Invitrogen, USA) was used to extract RNA from 15 ml of sludge from the mixing tank. The sludge samples were centrifuged at 10,000 rpm for 15 minutes, the supernatant was discarded and the formed pellet was used as the substrate for the RNA extraction, according to the manufacturer’s instructions. Immediately after the RNA extraction, cDNA of *nirS* and *nosZ* genes were produced via reverse transcription using qScript™ One-Step SYBR®
Green qRT-PCR kit, ROX™ (Quanta Biosciences, USA), and corresponding primer sets indicated in Table 8.

4.2.4 Statistical Analysis.

To determine if the samples show a significant difference between gene quantity and gene expression at each stage, ANOVA and MANOVA-Pillai’s trace statistical analysis were run using R software (R Development Core Team, 2014).

Results and Discussion

4.3.1 Denitrifier quantification analysis due to N loading effect.

Denitrification is a sequential reaction carried out by various microorganisms, catalyzed by four types of enzymes: nitrate reductase (Nar), nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) as summarized in Figure 24,a. In the HFM bioreactor set up, in the absence of biomass, NO removal only reached a maximum of 6.22%, while with biomass it reached a removal efficiency of up to 92% (Razaviarani et al., 2018). Hence, an investigation on how these genes respond when the NO and NO$_3^-$-N loading to the reactors changed during each stage can provide insights on the reactor’s performance. The dynamics of these genes during the different phases of the reactor’s performance are shown in Figure 24,b.
Chapter 4. Effect of Nitrate and Nitric Oxide Loadings on the Microbial Performance in a Nitric Oxide Denitrification Bioreactor.

a) Denitrification pathway and enzymes analyzed.

\[
\begin{align*}
narG & \rightarrow nirS & \rightarrow CnorB & \rightarrow nosZ \\
\text{NO}_3^- & \rightarrow \text{NO}_2^- & \rightarrow \text{NO} & \rightarrow \text{N}_2\text{O} & \rightarrow \text{N}_2
\end{align*}
\]

b) Denitrifying genes dynamics

Figure 24. a) Denitrification pathway, substrates and enzymes. b) Dynamics of gene families involved in denitrification during the different stages of NO and NO\textsubscript{3}-N loading. Values show the mean and SD (n=3).

The effects of NO\textsubscript{3}-N and NO loadings on the microbial community structure was studied on the samples collected from the Mixing Tank (MT) at the end of each stage (1-6), from the HFM compartment at the end of the operation period via destructive sampling, and from the BR, the latter only received NO\textsubscript{3}-N as N source.
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Results show that all the denitrifying genes vary significantly (MANOVA p<0.01) between stages. However, the statistical analysis cannot determine the precise effect of each loading regime. Therefore, we also analyze them separately.

During stages 1 to 3, the results of the specific gene DNA quantification showed that changes in NO loading while keeping the NO$_3^-$-N inflow unchanged did not significantly alter the dynamics of the denitrifying genes as the experiment progressed. Additionally, during stages 1-3, there was no change in the NO$_3^-$-N removal capacity, suggesting that NO was not a competing substrate over NO$_3^-$-N. Moreover, Figure 24 shows that the levels of CnorB, the gene responsible for NO reduction, remained fairly constant during the experiment, even in the absence of NO. This suggests that NO reduction was also occurring in response to the NO$_3^-$-N denitrification.

Throughout stages 4 to 6, when NO flow was kept constant and NO$_3^-$-N input concentrations changed from 4.6 g/l to 2.3 g/l and 0 g/l at stages 4, 5 and 6 respectively, the genes codifying for narG and nirS decreased at each stage (MANOVA p < 0.001). Quantified narG showed a decrease of over 90% from stage 4 to 5 and 55% from stage 5 to 6. NirS DNA quantification showed a decrease of 20% going from stage 4 to 5 and of 33% from 5 to 6.

Quantifying functional genes using DNA amplification is a useful method to determine the overall microbial composition at different stages and in the different reactors. However, RNA quantification is a more optimal approach to capture the immediate microbial response to changes in the reactor’s operation. The genes nirS and nosZ have been shown to be a good predictor of denitrification potential rates of the systems (Petersen
et al., 2012). Therefore, we quantified the amount of transcripts of $nirS$ and $nosZ$ genes during stages 4 to 6 from HFM Reactor’s Mixing Tank samples. Decrease of NO$_3$-N resulted in the decrease of $nirS$ and $nosZ$ gene transcripts (Figure 25). The major change is captured when no NO$_3$-N is provided in the feed at stage 6, $nirS$ and $nosZ$ mRNA decrease by 31% and 17% respectively. Statistical analysis denotes that NO$_3$-N loading regimes affected both $nirS$ and $nosZ$ expression (MANOVA p <0.1); and the ANOVA analysis showed equal significant response from both $nirS$ and $nosZ$ expression (p<0.05). This indicates that the changes in NO$_3$-N loading have similar effect on the activity of both genes. These results suggest that the microbial community can rapidly adjust gene transcription as a response to changes in their environment, and that denitrification of the N gas forms are driven by the NO inflow and by the byproducts of NO$_3$- denitrification. The ratio of $nirS:nosZ$ during stages 4, 5, and 6 was 2.839, 2.829, and 2.333 respectively.

**Figure 25.** $NirS$ and $nosZ$ RNA response to NO$_3$- loading changes under constant NO load. Number of copies of $nirS$ and $nosZ$ cDNA/mL of sample, used for quantifying the effect of NO$_3$-N loading changes, stage 4: 4.6 g/L, stage 5: 2.3 g/L, and stage 6: 0 g/L, while at constant NO loading of 288 L/d. Values show the mean and SD (n=3).
4.3.2 Denitrifier quantification analysis in HFM and BR.

The difference in loading and type of the N source introduced to the HFM compartment, the HFM Reactor’s Mixed Tank and the BR is translated to the difference in the number of denitrifying genes detected. The acclimated biomass at the initial stage, used as the seed for the HFM and BR, had a slight higher amount of genes responsible for NO$_3^-$ and NO$_2^-$ reduction ($narG + nirS = 5.53 \times 10^{10}$ copies of DNA/ml) than the number of genes responsible of NO and N$_2$O reduction ($CnorB + nosZ = 1.98 \times 10^{10}$ copies of DNA/ml) (Figure 26, a). At the end of the operation, the HFM had over 6 times more total biomass than the initial seed and 3 times more total quantified biomass than in the BR (Figure 26, b). Furthermore, the HFM bioreactor showed a higher density of all denitrifying genes compared to the initial biomass and the BR. The HFM bioreactor had 7 times more DNA copies of $CnorB$ and $nosZ$ than the initial biomass, and $1.9 \times 10^3$ more than the BR. These results show a clear difference in the microbial composition between the two types of reactors, HFM bioreactor and BR, and between the two compartment that make up the HFM bioreactor, in which the HFM compartment is where most of the biomass accumulates. It is also at HFM where the genes responsible for the reduction to the N gas forms are enriched. Data shows a clear connection between the type of N.
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a) Denitrifying genes quantification

![Bar chart showing DNA copies/mL for NarG+NirS, CnorB+NosZ, Aclimatized seed, MT, HFM, BR.](chart1)

b) Total 16s rRNA gene quantification

![Bar chart showing DNA copies/mL for Aclimatized seed, MT, HFM, BR.](chart2)

**Figure 26.** Number of amplified genes per mL of sludge sampled from the acclimated biomass used as a seed for the reactor at the initial stage, and at the end of the operation from the HFM bioreactor’s Mixing Tank, from the HF membranes compartment, and from the Batch Reactor (BR), the latter was run in parallel without NO loadings. a) Denitrifying genes quantification, and b) 16s rDNA quantification. Values show the mean and SD (n=3).
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NO was directly delivered via the gas phase to the HFM compartment through the hollow part of each membrane thread, where it was transferred to the aqueous phase across the membrane. A biofilm can then develop on the wet surface of each thread, and as the NO diffuses away from the surface, it allows for the biofilm to grow, reducing the NO that diffuses from the membrane and utilizing the organic carbon that diffuses into the biofilm from the bulk liquid. NO$_3^-$-N from the bulk liquid may also diffuse into the biofilm (Figure 27). This explains why the microbial composition of the biofilm formed on the HF membranes is very different from that in the HFM Reactor’s Mixing Tank (Figure 26, a).

Figure 27. a) Schematic of NO flow through HFM thread and biofilm formed over it. b) Representation of NO and NO$_3^-$ concentration change over distance.
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4.4 Conclusions

The microbial population colonizing the membrane on the wet side of the membrane, responsible for reducing the NO, is required to maintain a diffusion gradient needed for NO transfer across the membrane, without which, only a maximum of 6.22% NO transfer from the gas to the liquid phase was achieved (Razaviarani et al., 2018). Additionally, NO removal benefits from higher NO$_3^-$-N loads, which rather than acting as a competing substrate with NO, stimulates denitrifiers’ activity and enhances the overall NO removal rate.

RNA captured the quick response in the decrease of both nirS and nosZ where nosZ decreased faster (as shown by the decreasing nirS:nosZ) due to the decreasing of NO$_3^-$-N in the feed. However, since during stage 6 the NO$_3^-$-N was discontinued we expected not to detect nirS. Its detection could be because the reactor did not run long enough at these stages for nirS to reach steady state, or transcription is not shut down completely while denitrifiers are active even in the absence of NO$_3^-$. Overall, we show that the Mixing Tank of the HFM bioreactor can support a stable and active microbial population.

Finally, the results presented here show that denitrifiers can sustain their population under different NO$_3^-$-N and NO loadings, including in the absence of NO or NO$_3^-$-N, and the HFM bioreactor’s microbial community adjusts rapidly to changes in the different N loadings, hence, the varying loadings of NO/NO$_3^-$ does not affect the overall the perforce of the reactor.
CHAPTER 5. Summary and Future directions.

Nitrogen pollution is greatly caused by human activity and the dominant form of reactive nitrogen produced is NH$_4^+$, mainly for fertilizers (Ciais and Sabine, 2013). NH$_4^+$ goes through nitrification and denitrification causing the formation of NO$_x$ released to the atmosphere. Some forms of gaseous N compounds such as N$_2$O and NO are atmospheric pollutants and N$_2$O is a powerful greenhouse gas. Therefore, there is constant growing need to take action to remediate the amount of N being fixed by humans in an energy efficient manner. Biological N removal technologies have been extensively used due to their effectiveness, and they keep drawing attention due to the lower operating costs and absence of secondary pollutants requiring further treatment. Nonetheless, there is the continuous need to optimize pollutant removal process making them more energy efficient, and for that, we need a thorough understanding of the biological processes involved, the organisms taking part of it, their interactions, and responses to their environment under natural and engineered conditions. Therefore, the studies presented in this dissertation aimed to analyze the biological remediation of NH$_4^+$ with Acidimicrobiaceae sp. strain A6 using electrodes as alternative electron acceptor, and the microbial response during NO denitrification in a hollow fiber membrane bioreactor.

The results presented in chapter 2 and 3 from this dissertation show that Acidimicrobiaceae sp. strain A6, which is an iron reducer, is capable of colonizing electrodes in the field and under controlled conditions in soil columns in the laboratory. In
soil columns, where the soils were maintained saturated throughout the experiment so that the redox potential gradient of the soil remained oxidized at the surface and reduced at depth, resulted in A6 being enriched on the anode, as hypothesized. A6 would colonize the anode to use it as an alternative electron acceptor.

An important aspect of understanding a biological pathway, is learning about the organisms involved and their interactions with others. The relative abundance of the microbial community from electrodes deployed in the field showed that A6 appears to be negatively correlated with other FeRB, which is not the case when A6 is correlated with other non FeRB. This result is understandable due to the autotrophic nature of A6 (Huang and Jaffé, 2018) compared to the heterotrophic nature of other FeRB. Organic compounds oxidation such as acetate used by Geobacter spp. has a more negative half reaction redox potential \(E^{\circ} = -0.29\ V\) than inorganic compounds such as ammonium \(E^{\circ} = 0.07\ V\), and when coupled to the same electron acceptor, such as when coupled to Fe reduction \(E^{\circ} = 0.77\ V\), the bigger the difference between the two half reactions coupled, the higher the energy yielded (Jørgensen, 2000). Therefore, it would be expected that A6 would have less energy available for its metabolism and thus it could result in A6 growing slower than other FeRB. In order to implement A6 in BES containing mix cultures, further analysis is required to understand bacterial interaction and effects on A6 population and with the anode surface. An initial approach would be introducing co-cultures of A6 with Geobacter sp. and A6 with other ERB in BES and bench cultures to understand their interactions, for this, different feeds should be analyzed, such as feeds containing organic carbon in addition to ammonium. Moreover, it would be useful to understand the effect of other bacteria that would normally thrive under ammonium rich conditions, such as nitrifying bacteria, thus
similar co-culture analysis is recommended. The results obtained would more closely resemble real world conditions which would consist of mixed culture with more complex feeds. Although, other organisms might be faster and more robust growers, their presence might not necessarily have a negative effect on A6. In fact, maintain pure A6 cultures for long periods of time have not been possible, thus pointing to the possibility that something is missing in the growing medium (Huang and Jaffé, 2018), which could be some metabolite produced by other organism.

*Acidimicrobiaceae* sp. strain A6 can be sustained in MECs as a pure culture or an enrichment culture while utilizing NH$_4^+$ as the electron donor and the anode as the electron acceptor in the absence of an Fe(III) source. Additionally, although A6 has shown to be a difficult bacteria to culture (Huang and Jaffé, 2018), results show that A6 biomass can be well sustained in MEC reactors, in equal concentration as in batch reactors containing Fe(III), and similar amounts of NH$_4^+$ removal were observed.

E-SEM revealed A6 cells attached to the electrode surface, however, the formation of a clear biofilm could not be detected under the period and operating conditions of the MECs. Quantification of A6 through qPCR showed that the majority of the cell were in the bulk liquid. Therefore, it explains why the addition of an electron shuttle like AQDS aided current production, as it facilitated the transfer of electron between the bacterial cell and the electrode surface. Further studies are recommended to understand what is required to build a robust biofilm on the electrode. For example, as indicated above, co-culture analysis could help answer questions of whether the presence of biofilm forming organisms in BES could have a positive or negative impact.
Current density \( (I_d) \) for MECs with A6 obtained a maximum value of 16.4% when a subtler mixing condition by stirring was adopted. Lower CE percentages were measured when mixing was carried out with a shaker. This suggest that there is still the need to optimize the configuration of MECs to allow for a better electron transfer. Although the obtained CE values are on the lower side of other values reported, they cannot be directly compared because none of the other systems have coupled the Feammox pathway to MECs. A double chamber MECs with a proton exchange membrane between the anodic and cathodic chamber could facilitate \( \text{NH}_4^+ \) oxidation in the anodic chamber and would keep \( \text{H}_2 \) formed at the cathodic chamber separated from A6 in the anodic side. This should promote \( \text{NH}_4^+ \) utilization without \( \text{H}_2 \) interference, since A6 is known to be able to also oxidize \( \text{H}_2 \) (Huang and Jaffé, 2018).

The results from the enrichment of A6 using electrodes deployed in the field, and the current production linked to \( \text{NH}_4^+ \) removal in MECs with A6 indicates that Acidimicrobiaceae sp. strain A6 is a novel anaerobic lithoautotroph from the Actinobacteria phyla capable of using electrodes as its terminal electron acceptor; thus, expanding our knowledge of the diversity of electrogenic microorganisms beyond the commonly studied groups and reaffirming that BES technology coupled to the Feammox pathway can be used for the treatment of \( \text{NH}_4^+ \). Furthermore, A6’s ability to thrive in MECs, opens up the possibility of analyzing and treating other compounds of environmental concern, such as perfluoroalkoxy alkanes (PFAS), for which preliminary results show A6 is able to degrade perfluorobutanesulfonic acid and perfluorooctanoic acid in MECs. Although the mechanism of action of the PFAS degradation with A6 is not yet
known, it is encouraging to learn that A6 coupled to MECs can help answer such type of questions.

The results from chapter 4 of this dissertation show that the microbial population colonizing the wet side of the membrane of the HFM bioreactor is required to reduce NO as it maintains the diffusion gradient needed for high NO transfer across the membrane, without the biofilm, there is only very basal NO removal (Razaviarani et al., 2018). By focusing on the microbial community, the results show that denitrifiers can sustain their population under different NO$_3^-$-N and NO loadings. In the absence of one of the forms of N, i.e. NO or NO$_3^-$, the microbial population still maintains its robustness as it continues with denitrification. By using RNA analysis, we were able to captured the quick response in the decrease of both nirS and nosZ. In response to decreasing NO$_3^-$ in the feed, nosZ decreased faster, however, nirS was always detected even at the stage when NO$_3^-$-N was discontinued, perhaps because transcription is not shut down completely while denitrifiers are active even in the absence of its substrate. The results suggest that higher loads of NO$_3^-$, rather than acting as a competing substrate with NO for denitrification, it stimulates denitrifying activity which results in better NO removal. The HFM bioreactor’s microbial community adjust rapidly to changes in the different N loadings. Therefore, biological removal of NO in biological reactors is feasible and the microbial community robust to tolerate fluctuation in the amount of NO provided without having a major effect on the removal efficiency. NO biological reduction happens at ambient temperatures, but NO originated from combustion processes are emitted at high temperatures (i.e. ~ 200 °C); therefore, a major challenge is to bring the NO temperature to one that is suitable for its biological reduction. Therefore, to make the biological process attractive for industrial
purposes, a cost effective and energetically efficient method to quickly change the gas temperature is needed, and thus, it should be the focus of future studies in order to couple it to the HFM bioreactor for NO removal.
Appendix

A Nontronite as Fe(III) source for Feammox reaction.

*Acidimicrobiaceae* sp. strain A6 can oxidize ammonium in the presence of nontronite, a swelling 2:1 phyllosilicate rich in Fe(III) (Keeling et al., 2000). Nontronite’s structural Fe(III) is available for microbial respiration (Dong, 2012), thus it can function as an electron acceptor for the Feammox reaction (Figure 28, a). Furthermore, when its Fe(III) is reduced most of it stays in the clay and doesn’t go into solution, thus the Feammox reaction from being inhibited by high concentrations of aqueous Fe$^{2+}$ formed as the reaction proceeds. Moreover, nontronite as well as ferrihydrite can undergo aeration periods to reoxidize de Fe which can be reutilized by A6 which don’t seem to be affected by short aeration periods (Figure 28, b).
Figure 28. a) Comparison of NH$_4^+$ removal by *Acidimicrobiaceae* sp. strain A6 using Ferrihydrite and Nontronite as Fe(III) sources. Total amount removed after 2 weeks of incubations. Nontronite only had no dead bacteria. Bars show the mean and error bars show the highest and lowest average values obtained, n=4. b) Amount of ammonium removed and Fe(II) formed using fresh nontronite (pre-aeration) and reoxidized nontronite (post-aeration). A6 was incubated in nontronite for 2 weeks, and then subjected to aeration to reoxidize the nontronite to carry incubation for another 2 weeks (post-aeration). Bars show the mean and error bars show the highest and lowest average values obtained, n=3.
B Methods for microbial community composition analysis.

In order to determine the microbial community composition, sequencing and phylogenetic analysis was performed by Novogene (Beijing, China) as follows: From total genomic DNA, the variable region V4 of the 16s rRNA gene was amplified using the primer set 515F/806R (515F: 5′-GTGCCAGCMGCGCGGTAA-3′ / 806R: 5′-GGACTACHVGGGTWTCTAAT-3′) with a barcode following the method of Caporaso et al (2011). All PCR reactions were carried out with Phusion® High-Fidelity PCR master mix (New England Biolabs). PCR products quantification and qualification were determined by electrophoresis on 2% agarose gel. The resulting amplicons were pooled, purified, quantified. Sequencing libraries were generated using TruSeq® DNA PCR-free sample preparation kit (Illumina, USA) following the manufacturer’s protocol and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. Finally, sequencing was performed on an IlluminaHiSeq2500 platform and 250 bp paired-end reads were generated.

Paired-end reads were assembled by using FLASH V.1.2.7 (Magoc and Salzberg, 2011). Raw reads were processed according to QIIME V1.7.0 quality controlled process (Caporaso et al., 2010) and chimeric sequences were filtered out using the UCHIME algorithm (Edgar et al., 2011). These resulting sequences were clustered into operational taxonomic units (OTUs) using Uparse V7.0.1001 (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for taxonomic annotation using the Ribosomal Database Project (RDP) Classifier (Cole et al., 2014; Wang et al., 2007) using GreenGene database (DeSantis et al., 2006) at a minimum of 80% confidence threshold for all OTUs. For the top 100 most abundant
family and genus annotation, the blastn algorithm against the 2016 NCBI’s 16s ribosomal RNA sequences for bacteria and archaea was used at an e-value of $1e^{-5}$. A6’s 16s rRNA gene sequence was included to NCBI’s database for such purpose. Finally, samples were standardized using the least sequence number obtained from all samples so that the same number of sequences were used for calculating the relative abundance of OTUs.
C Microbial community composition taxonomy of MEC with enriched A6.

Table 9. Taxonomy of the microbial community composition of the top 100 most abundant genus from a MEC with an A6 enrichment culture. Kingdom (k), phylum (p), class (c), order (o), family (f) and genus (g). Feammox bacteria A6 shown in bold.

<table>
<thead>
<tr>
<th>Relative abundance</th>
<th>Taxonomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.21684</td>
<td>k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Burkholderiaceae; g: Ralstonia</td>
</tr>
<tr>
<td>0.21111</td>
<td>k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Rhodospirillaceae; g: Telmatospirillum</td>
</tr>
<tr>
<td>0.06897</td>
<td>k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Hyphomicrobiaceae; g: Hyphomicrobium</td>
</tr>
<tr>
<td>0.03672</td>
<td>k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Lysobacter</td>
</tr>
<tr>
<td>0.02925</td>
<td>k: Bacteria; p: Bacteroidetes; c: Sphingobacteria; o: Sphingobacteriales; f: Chitinophagaceae; g: Sediminibacterium</td>
</tr>
<tr>
<td>0.02515</td>
<td>k: Bacteria; p: Actinobacteria; c: Acidimicrobiia; o: Acidimicrobiales; f: unidentified Acidimicrobiales; g: Feammox bacterium A6</td>
</tr>
<tr>
<td>0.02258</td>
<td>k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Pseudomonadales; f: Pseudomonadaceae; g: Pseudomonas</td>
</tr>
<tr>
<td>0.02124</td>
<td>k: Bacteria; p: Bacteroidetes; c: Bacteroidia; o: Bacteroidales; f: Prevotellaceae; g: Prevotellaceae:UCG-00</td>
</tr>
<tr>
<td>0.01720</td>
<td>k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Thermomonas</td>
</tr>
<tr>
<td>0.00801</td>
<td>k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Bradyrhizobiaceae; g: Bradyrhizobium</td>
</tr>
<tr>
<td>0.00588</td>
<td>k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: unidentified Xanthomonadaceae; g: Acidibacter</td>
</tr>
<tr>
<td>0.00538</td>
<td>k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Burkholderiaceae; g: Burkholderia</td>
</tr>
<tr>
<td>0.00510</td>
<td>k: Bacteria; p: Acidobacteria; c: unidentified Acidobacteria; o: Subgroup 3; f: unidentified Acidobacteria; g: Bryobacter</td>
</tr>
</tbody>
</table>
Appendix

0.00435  k: Bacteria; p: Acidobacteria; c: unidentified Acidobacteria; o: Subgroup 3; f: unidentified Acidobacteria; g: Candidatus Solibacter

0.00397  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: unidentified Rhizobiales; g: Rhizomicrobium

0.00394  k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Rhodanobacter

0.00360  k: Bacteria; p: Actinobacteria; c: unidentified Actinobacteria; o: Frankiales; f: Acidothermaceae; g: Acidothermus

0.00341  k: Bacteria; p: Firmicutes; c: Bacilli; o: Bacillales; f: Alicyclobacillaceae; g: Alicyclobacillus

0.00338  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Gallionellaceae; g: Sideroxydans

0.00322  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Desulfuromonadales; f: Desulfomicrobiaceae; g: Desulfomicrobium

0.00297  k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae:1; g: Clostridium sensu stricto 12

0.00225  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Phyllobacteriaceae; g: Mesorhizobium

0.00210  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Desulfobacterales; f: Desulfobulbaceae; g: Desulfobulbus

0.00181  k: Bacteria; p: Bacteroidetes; c: Bacteroidia; o: Bacteroidales; f: Bacteroidaceae; g: Bacteroides

0.00178  k: Bacteria; p: Firmicutes; c: Bacilli; o: Bacillales; f: Bacillaceae; g: Bacillus

0.00141  k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Enterobacteriales; f: Enterobacteriaceae; g: Escherichia-Shigella

0.00135  k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Family XI; g: Sedimentibacter

0.00131  k: Bacteria; p: Acidobacteria; c: unidentified Acidobacteria; o: Acidobacteriales; f: Acidobacteriaceae Subgroup 1.; g: Telmatobacter
Appendix

0.00125 k: Bacteria; p: Acidobacteria; c: unidentified Acidobacteria; o: Acidobacteriales; f: Acidobacteriaceae Subgroup 1; g: Ca. Koribacter

0.00122 k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Rhodocyclales; f: Rhodocyclaceae; g: Dechloromonas

0.00116 k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Dyella

0.00109 k: Bacteria; p: Bacteroidetes; c: Bacteroidia; o: Bacteroidales; f: Rikenellaceae; g: vadinBC27 wastewater-sludge group

0.00106 k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Xanthomonadales; f: Xanthomonadaceae; g: Dokdonella

0.00100 k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Caulobacterales; f: Caulobacteraceae; g: Phenyllobacterium

0.00097 k: Bacteria; p: Proteobacteria; c: Gammaproteobacteria; o: Chromatiales; f: Ectothiorhodospiraceae; g: Acidiferrobacter

0.00091 k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae; g: Simplicispira

0.00091 k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae:1; g: Clostridium sensu stricto1

0.00088 k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiacea; g: Clostridium

0.00081 k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Christensenellaceae; g: Christensenellaceae R-7 group

0.00081 k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Syntrophobacterales; f: Syntrophaceae; g: Syntrophus

0.00078 k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Peptococcaceae; g: Desulfosporosinus

0.00075 k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Anaerolineales; f: Anaerolineaceae; g: Leptolinea

0.00072 k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Myxococcales; f: Cystobacteraceae; g: Anaeromyxobacter

0.00066 k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Nitrosomonadales; f: Gallionellaceae; g: Candidatus Nitrotoga

0.00066 k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Acidocella

0.00053 k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Anaerolineales; f: Anaerolineaceae; g: Longilinea

0.00053 k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Xanthobacteraceae; g: Pseudolabrys

0.00050 k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Peptococcaceae; g: Desulfotobacterium
0.00050  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Collimonas

0.00047  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Desulfobacteriales; f: Desulfobulbaceae; g: Desulfocapsa

0.00047  k: Bacteria; p: Thermotogae; c: unidentified:Thermotogae; o: Thermotogales; f: Thermotogaceae; g: GAL15

0.00044  k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae:1; g: Clostridium sensu stricto 9

0.00041  k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae:1; g: Clostridium sensu stricto 10

0.00041  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae; g: Aquabacterium

0.00041  k: Bacteria; p: Bacteroidetes; c: Sphingobacteriia; o: Sphingobacteriales; f: Sphingobacteriaceae; g: Mucilaginibacter

0.00038  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Myxococcales; f: Haliangiaceae; g: Haliangium

0.00034  k: Bacteria; p: Proteobacteria; c: Epsilonproteobacteria; o: Campylobacteriales; f: Helicobacteraceae; g: Sulfovirum

0.00034  k: Bacteria; p: Firmicutes; c: Erysipelotrichia; o: Erysipelotrichales; f: Erysipelotrichaceae; g: Erysipelothrix

0.00034  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Desulfobacteriales; f: Desulfobulbaceae; g: Desulfurvibrio

0.00034  k: Bacteria; p: Spirochaetes; c: unidentified:Spirochaetes; o: Spirochaetales; f: Spirochaetaceae; g: Spirochaeta:2

0.00034  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Syntrophobacteriales; f: Syntrophobacteraceae; g: Syntrophobacter

0.00031  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Burkholderiaceae; g: Chitinimonas

0.00031  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Oxalobacteraceae; g: Pseudoduganella

0.00028  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Rhodospirillaceae; g: Inquilineus

0.00028  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhizobiales; f: Bradyrhizobiaceae; g: Rhodoblastus

0.00025  k: Bacteria; p: Proteobacteria; c: Deltaproteobacteria; o: Desulfobacteriales; f: Desulfobacteriaceae; g: Desulfativirbium

0.00022  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Rhodospirillaceae; g: Elstera

0.00022  k: Bacteria; p: Acidobacteria; c: unidentified Acidobacteria; o: Acidobacteriales; f: Acidobacteriaceae Subgroup 1.; g: Granulicella
Appendix

0.00019  k: Bacteria; p: Planctomycetes; c: Planctomycetacia; o: Planctomycetales; f: Planctomycetaceae; g: Planctomyces

0.00016  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: Comamonadaceae; g: Polaromonas

0.00016  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Hydrogenophilales; f: Hydrogenophilaceae; g: Thiobacillus

0.00016  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Sphingomonadales; f: Sphingomonadaceae; g: Novospingobium

0.00016  k: Bacteria; p: Proteobacteria; c: Alphaproteobacteria; o: Rhodospirillales; f: Acetobacteraceae; g: Rhodovastum

0.00016  k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Anaerolineales; f: Anaerolineaceae; g: Anaerolinea

0.00016  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Burkholderiales; f: unidentified Burkholderiales; g: Thiomonas

0.00016  k: Bacteria; p: Chloroflexi; c: Anaerolineae; o: Anaerolineales; f: Anaerolineaceae; g: unidentified Anaerolineaceae

0.00016  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Rhodocyclales; f: Rhodocyclaceae; g: Denitratisoma

0.00013  k: Bacteria; p: Chloroflexi; c: Ktedonobacteria; o: Ktedonobacterales; f: Ktedonobacteraceae; g: Ktedonobacter

0.00013  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Nitrosomonadales; f: Gallionellaceae; g: Gallionella

0.00013  k: Bacteria; p: Bacteroidetes; c: Bacteroidia; o: Bacteroidales; f: Porphyromonadaceae; g: Paludibacter

0.00013  k: Bacteria; p: Planctomycetes; c: Planctomycetacia; o: Planctomycetales; f: Planctomycetaceae; g: Isosphaera

0.00013  k: Bacteria; p: Firmicutes; c: Bacilli; o: Bacillales; f: Alicyclobacillaceae; g: Tumebacillus

0.00009  k: Bacteria; p: Proteobacteria; c: Epsilonproteobacteria; o: Campylobacterales; f: Helicobacteraceae; g: Sulfuricurvum

0.00009  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Rhodocyclales; f: Rhodocyclaceae; g: Candidatus Accumulibacter

0.00006  k: Bacteria; p: Firmicutes; c: Clostridia; o: Clostridiales; f: Clostridiaceae:1; g: Clostridium sensu stricto 3

0.00006  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Hydrogenophilales; f: Hydrogenophilaceae; g: Ferritrophicum

0.00006  k: Bacteria; p: Verrucomicrobia; c: OPB35 soil group; o: unident. OPB35 soil g.; f: unident. OPB35 soil g.; g: unident. OPB35 soil group

0.00003  k: Bacteria; p: Proteobacteria; c: Betaproteobacteria; o: Rhodocyclales; f: Rhodocyclaceae; g: Georgfuchsia
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References


References


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