Novel pathways to dinitrogen via iron-mediated anaerobic ammonium oxidation

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Novel pathways to dinitrogen via iron-mediated anaerobic ammonium oxidation

A thesis submitted in partial fulfillment of the requirement
for the degree of Bachelor of Science in Biology from
The College of William and Mary

by

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Accepted for Highest Honors

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Abstract

Nitrogen cycling in anoxic environments is fundamentally important to local and global fixed nitrogen budgets but remains poorly understood with respect to underlying biogeochemical mechanisms and controls. Following the discovery of anammox, evidence of additional novel N-cycling pathways rooted in the anaerobic oxidation of ammonium has been accumulating and investigations have expanded to focus on the potential interaction between nitrogen transformations and the geochemical cycles of common metals, notably iron and manganese. This study aimed to evaluate whether an iron-mediated anoxic ammonium oxidation process, or “feammox,” occurs in the coastal subterranean estuary, which is characterized by complex and highly interactive biogeochemical cycles. Iron-rich sediments from the subterranean estuary in Gloucester Point (VA, USA) were collected and incubated under anoxic conditions with ammonium-rich groundwater. Chemical concentrations were monitored over time, and isotope ratio mass spectrometry was used to determine production of enriched dinitrogen gas from $^{15}\text{NH}_4^+$ added to incubation bottles. Results of four sediment incubation experiments consistently indicated consumption of $^{15}\text{NH}_4^+$ in ammonium-spiked treatments and subsequent production of $^{29}\text{N}_2$ and $^{30}\text{N}_2$, which strongly suggests the occurrence of the hypothesized iron-mediated ammonium oxidation reaction. However, data on ammonium and ferrous iron concentrations revealed a number of competing biogeochemical processes that make it difficult to evaluate the quantitative importance of this reaction in situ. Importance of the feammox reaction appears to depend on availability of specific chemical substrates and active microbial populations, both of which may vary with biogeochemical conditions across different environmental settings (e.g. pH, seasonal
temperatures, oxygen saturation, magnitude and pattern of groundwater advection, redox cycle interactions).
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1. Introduction

The nitrogen cycle is an integral component of all environmental systems and maintains a principal role regulating ecosystem health, function and services. Investigation of nitrogen cycling under different biogeochemical conditions has led to vast changes in our understanding of fixed nitrogen conversions in aquatic systems (Brandes et al. 2007). While major processes such as nitrogen fixation, nitrification, and denitrification have long been recognized as critical features of the global nitrogen cycle, there is a growing body of evidence that suggests novel reaction pathways may have significant roles in myriad terrestrial and aquatic systems (Ward 2013; Hulth et al. 2005; Luther III et al. 1997). In particular, the recent discovery of anammox, a nitrogen transformation process now recognized as a major pathway in the global nitrogen cycle, suggests that the current paradigm may still be subject to drastic changes (Risgaard-Petersen et al. 2004).

In coastal marine environments, biogeochemical conditions strongly influence overall ecosystem productivity, health and function by governing the cycling of fixed nitrogen (Santoro 2009). While fixed nitrogen is an essential limiting nutrient for biological productivity, excess nutrient availability is increasingly implicated in widespread ecosystem deterioration through events such as harmful algal blooms and eutrophication (Diaz and Rosenberg 2008; Seitzinger 1988). Therefore, processes mitigating nutrient flux by the conversion of fixed nitrogen to biologically unavailable dinitrogen gas are of growing importance to aquatic systems.

Fixed nitrogen in the environment is primarily converted to dinitrogen gas via denitrification and anammox, which occur in suboxic environments (Engstrom et al. 2005;
Excess dinitrogen gas has also been detected under anoxic conditions (Bartlett et al. 2008; Grantz et al. 2012), but the geochemical mechanism behind \( \text{N}_2 \) formation in such settings is unclear because the oxidized nitrogen species that drive denitrification and anammox are largely unavailable. However, thermodynamic considerations indicate that fixed nitrogen loss could occur under anoxic conditions via several reaction pathways involving environmentally-abundant metals (Hulth et al. 2005; Luther III et al. 1997). In particular, reactive iron- and manganese-oxides have been identified as energetically favorable electron acceptors for \( \text{NH}_4^+ \), the most reduced species of fixed nitrogen (Thamdrup and Dalsgaard 2000; Weber et al. 2006; Clement et al. 2005; Luther III et al. 1997; Bartlett et al. 2007; Silver et al. 2009).

The subterranean estuary (STE) is the mixing zone of saline and fresh groundwaters in coastal permeable sediments, and is a biogeochemically active zone characterized by steep redox gradients and diverse, interactive elemental cycles (Kroeger and Charette 2008; Santoro 2009; Slomp et al. 2004). Given the high concentrations of potential reactants for unconventional nitrogen transformations in the STE (Charette and Sholkovitz 2006), it is an ideal system for examining the occurrence of novel nitrogen cycling pathways.

Biogeochemical activity in the STE largely governs the chemical composition of submarine groundwater discharge to the coastal ocean, which has been recognized as a major mode of chemical flux at the land-sea interface (Moore 2010). Submarine groundwater discharge is a key regulator of fixed nitrogen budgets in many coastal environments (Liefer et al. 2009; Giblin and Gaines 1990; Moran et al. 2013; Portnoy et al. 1998), indicating that nitrogen cycling processes occurring in the STE can have
substantial effects on nutrient flux to the coastal ocean (Slomp and Cappellen 2004; Kroeger and Charette 2008).

Preliminary results from an ongoing time-series of chemical profiles at a representative STE site at Gloucester Point, VA, show formation of substantial quantities of excess dinitrogen gas in anoxic zones despite the absence of oxidized N-species. The distribution of excess N\textsubscript{2} strongly correlates with redox cycling patterns of Fe and Mn and is coincident with consistently high concentrations of NH\textsubscript{4}+. Iron-oxides are present in STE sediments in a wide variety of mineral phases (e.g., amorphous ferrihydrite, goethite; Charette and Sholkovitz 2002; Testa et al. 2002) that can function as highly specific or versatile oxidants for different geochemical reactions. Thermodynamic calculations indicate that the reduction several of common iron-oxide forms could favorably be coupled to the oxidation of ammonium to N\textsubscript{2} (Table 1; Stumm and Morgan 1996). Collectively, these data are the basis of my hypothesis that iron-oxides mediate a novel anaerobic ammonium oxidation reaction pathway that ultimately yields dinitrogen gas, resulting in fixed nitrogen loss from the system.

Iron-oxides are known to function as electron acceptors for a range of iron-reducing microorganisms abundant in coastal marine sediments, and repeated observation of iron-rich sediments at the STE site led me to investigate the potential occurrence of an iron-mediated ammonium oxidation reaction, or “feammox” (Weber et al. 2006; Yang et al. 2012). Preliminary laboratory experiments in which synthetic iron-oxides were incubated with ammonium-rich groundwater under sterile and anoxic conditions strongly suggest that an iron-mediated ammonium oxidation pathway, like most nitrogen transformations, occurs via microbial control (Santoro 2009; Van de Graaf et al. 2005).
While the occurrence of feammox has been investigated and hypothesized to be relevant in a range of environmental settings, occurrence in the STE and additional biogeochemical controls on the process have not been definitively identified.

The principal goals of this study were to verify whether feammox occurs in STEs, where dissolved reactants are transported with advecting porewater, and integrate this information with our current understanding of other chemical cycles at the Gloucester Point site to develop a framework of biogeochemical conditions relevant to the reaction. Controlled incubation experiments consisting of sediment and artificial seawater were used to investigate the process. Sediments collected from the STE field site were used to ensure that all biogeochemical constituents necessary to drive the reaction, such as particular microorganisms or iron-oxide mineral phases found in situ, were available within the incubation system. Artificial seawater was included to mimic circulating groundwater and to function as a medium for manipulating NH$_4^+$ availability and quantifying changes in dissolved chemical constituents over time due to feammox.

2. Methods

The sediment incubation experiment consisted of four consecutive trials that were based on the same basic experimental design. Each trial was comprised of incubation systems containing sediment from an Fe oxide-rich layer within the Gloucester Point STE and artificial brackish groundwater (Fig. 1). The groundwater composition was determined based on field measurements of porewater within the Fe-oxide layer (typically at ~65 cm depth; O’Connor et al. 2015, in press). The incubations included
controls and experimental treatments, which were manipulated to contain excess quantities of NH$_4^+$ and/or synthetic Fe-oxides.

Incubations were performed in glass serum vials that were sealed with air-tight butyl rubber stoppers. Damp sediment was partitioned and added to the serum vials along with anoxic artificial seawater. The artificial seawater was prepared according to the protocol of Harrison and Berges (2005) and diluted to adjust salinity. Solutions were sparged with an unreactive gas (Ar or He) to eliminate dissolved oxygen prior to the start of trial.

All treatment systems within each trial contained excess NH$_4^+$, and the time at which NH$_4^+$ was added to the serum vials represents the start of the incubation period. The four trials varied in duration but consisted of multiple time points at which groundwater samples were collected from the treatment and control systems, filtered (0.2 µm, PES filter) and subsampled for geochemical analyses including determination of NH$_4^+$, Fe$^{2+}$, O$_2$, NO$_3^-$ and NO$_2^-$ concentrations and N$_2$ production.

While the general preparation and procedure described above remained consistent for each trial, small changes were made with each consecutive trial. The details of individual trials are summarized in table 2 and discussed below.

2.1 Trial 1

The first trial consisted of 8 independent 60 mL incubation vials including 4 controls and 4 NH$_4^+$-spiked treatments. Sediment was added to the serum vials under
oxic conditions, after which the vials were placed in a glove bag under continuous flow with argon prior to inoculation with argon-sparged artificial seawater.

Inside the glove bag, 1 L of the artificial seawater solution was separated and half spiked with 1 mL of 50 mM NH₄Cl to obtain an ammonium concentration of 50 µM for the treatments. Aliquots of the original and NH₄⁺-spiked solutions were added to the control and treatment systems, respectively. All vials were sealed inside the Ar-flushed glove bag.

The incubation period was 44 days, during which a single control and a single treatment system were sacrificed in tandem at each of four time points for geochemical analyses. Sacrificial sampling of the incubation systems occurred at 0, 2, 30 and 44 days. The sacrificing procedure began by removing the butyl-rubber stopper from the serum vial and breaking the air-tight seal. Dissolved oxygen content was immediately measured using a Clark-type amperometric microsensor (Gieseke and de Beer 2004), after which the groundwater was filtered and subsampled. The geochemical parameters measured in the first trial included NH₄⁺, Fe²⁺, and the combined concentration of NO₃⁻ and NO₂⁻ (referred to hereafter as NOₓ).

2.2 Trial 2

The second trial consisted of 32 independent incubations systems, 16 controls and 16 ammonium-spiked treatments, prepared in 60 mL serum vials. In contrast to the previous trial, the incubation systems were assembled entirely under oxic conditions. Ten grams sediment and 45 mL argon-sparged artificial seawater were added to all 32 vials at the same time, after which they were left to incubate without further perturbation for 4
days. This period is referred to as the pre-incubation, during which any residual oxygen and/or NO₃ was consumed. This modification was made in effort to eliminate the potential for oxic processes to influence geochemical trends following the addition of NH₄⁺ and eliminated the need to assemble the incubation systems in a glove bag.

Following the pre-incubation period, the treatment systems were spiked with 300 μL of a 7.5mM (¹⁵NH₄)₂SO₄ solution, which was deoxygenated by sparging with argon. The use of ¹⁵NH₄⁺ corresponded with plans to measure dinitrogen gas production at the end of the incubation period via isotope-ratio mass spectrometry. The NH₄⁺ spike was added to the systems through the butyl rubber stopper using a 21-gauge needle and 1 cc syringe and raised the groundwater concentration to 50 μM. This method of adding excess NH₄⁺ to the treatment systems was used to preserve the effects of the pre-incubation by eliminating the need to re-open the vials.

The incubation period was 32 days and consisted of 8 sampling time points at which duplicate control and treatment vials were sacrificed for geochemical analyses. Samples were processed and analyzed after being sacrificed following procedures described for Trial 1.

Headspace gas samples were taken for N₂ analysis at the final time point (36 days). Prior to collecting gas samples from the vials, 5 mL helium was injected through the stoppers using a 21-gauge needle and a helium-flushed gas-tight syringe. The vials were then shaken vigorously and 5 mL gas from the vial headspace transferred to a helium-flushed 12 mL exetainer tube. Following gas sampling, the incubation systems were sacrificed and subsampled according to the traditional protocol.
N₂ measurements were made on a ThermoFisher Delta Plus isotope-ratio mass spectrometer (IRMS) with the Gas Bench introduction system, which directly extracted 0.2 mL samples from the exetainer tubes for analysis. Data on N₂ isotopic composition was generated using the Isodat Gas Isotope Ratio MS software (Version 3.0, Thermo Scientific 2008). δ¹⁵N values were used to compare ²⁹N₂ and ³⁰N₂ production in labeled ammonium-spiked treatments and control incubations.

2.3 Trial 3

The third trial was assembled following the same protocol as Trial 2, and the sediment used in this trial was the same as that collected for use in Trial 2. The sediment was stored at 12°C in a plastic bag for 4 weeks and remained damp. This “aged” sediment was used in lieu of freshly collected sediment based on the assumption that substantial organic matter decomposition would have already occurred, so changes in NH₄⁺ concentration due to faamox may be more easily distinguishable. Additionally, only 5 grams were added to the incubation systems in effort to further minimize background NH₄⁺ production from sediment organic matter decomposition.

The artificial seawater solution was deoxygenated using helium, pH was adjusted to 6.5 using hydrochloric acid, and solutions were diluted to a salinity of 5 parts per thousand. Helium was used in lieu of argon to deoxygenate the solutions to facilitate quantification of labeled N₂ production. pH changes were made based on calculations of free energy yields for several possible metal-mediated ammonium oxidation reactions as well as evidence from the literature which indicated greater thermodynamic favorability under more acidic conditions (Table 2). Salinity was reduced to better mimic in situ
geochemical conditions characterized by ongoing field sampling at the Gloucester Point subterranean estuary site.

Trial 3 incubation vials included control, low $^{15}\text{NH}_4^+$-spike, and high $^{15}\text{NH}_4^+$-spike systems that were incubated for up to 62 days. Aliquots of 100 µL and 300 µL of 7.5 mM ($^{15}\text{NH}_4^+$)$_2\text{SO}_4^-$ were added to the low and high $^{15}\text{NH}_4^+$-spike treatment systems after the pre-incubation period by the same method used in trial 2 to achieve starting groundwater NH$_4^+$ concentrations of 30 µM and 50 µM, respectively.

A single control, low $^{15}\text{NH}_4^+$-spike, and high $^{15}\text{NH}_4^+$-spike system were sacrificed and analyzed at each of the ten sampling time points during the incubation period following the standard protocol described for previous trials. Headspace gas samples were taken at 0, 14, 35 and 62 days for N$_2$ analysis using IRMS.

Analysis of low and high $^{15}\text{NH}_4^+$-spike treatments at a greater number of sequential time points instead of duplicated treatments was intended to clarify whether general geochemical trends in the presence of different amounts excess ammonium were similar and eliminate potential system-to-system variability in reaction rates and substrate availability between experimental replicates.

2.4 Trial 4

In the fourth trial, the treatments and controls were assembled in 125-milliliter serum vials that were repeatedly subsampled over the course of the time series. This modification was made in effort to eliminate system-to-system variability that results from sacrificially sampling many different independent incubation vials.
Trial 4 consisted of 4 duplicated systems: controls, low-$^{15}$NH$_4^+$-spike, high $^{15}$NH$_4^+$-spike, and low $^{15}$NH$_4^+$-spike plus synthetic Fe-oxides. The starting concentration for low-$^{15}$NH$_4^+$-spike systems was 100 $\mu$M and 1000 $\mu$M for the high $^{15}$NH$_4^+$-spike treatment, both of which were higher than the respective starting concentrations in the previous trials. More $^{15}$NH$_4^+$ was added to all of the treatment systems compared with previous trials in effort to dampen the effect of $^{14}$NH$_4$ produced over the course of the time series on the enriched N$_2$ signal.

The 1000 $\mu$M $^{15}$NH$_4^+$ treatment was included in conjunction with the treatment containing excess Fe-oxides in effort to better evaluate potential limitation by different reactants. The feammox reaction should have been easier to distinguish in measurements of NH$_4^+$ concentration over the course of the incubation if the limiting substrate was made available in substantial excess.

Excess Fe-oxides were added in the form of synthethic ferrihydrite, an amorphous Fe$_{3+}$ mineral with the approximate formula Fe$_2$(OH)$_3$. Amorphous (2-line) ferrihydrite was synthesized according to an adaptation of the method of Schwertmann and Cornell (2007) that used ferric chloride in place of ferric nitrate. Prior to being filled with artificial seawater, the serum vials with the low $^{15}$NH$_4^+$-spike plus synthetic Fe-oxides treatment were inoculated with 50 mg of the freeze-dried iron-oxide precipitate. This was equivalent to approximately 90 $\mu$moles of additional reducible iron, a nearly 10-fold increase from the amount naturally present in the sediment.

Freshly collected sediment (25 g) was added to the incubation vessels, followed by 125 milliliters of helium-flushed artificial seawater. The serum vials were then left to
pre-incubate and spiked with the designated amounts of $^{15}$NH$_4^+$ after four days by the same method used in trials 2 and 3.

The incubation period was 57 days and groundwater samples were extracted from the serum vials for geochemical analyses at eight time points. 3 mL of helium was injected into the headspace to increase internal pressure and enable groundwater collection without sacrificing the airtight seal at each time point. 2 mL groundwater was extracted from the containers through the stopper using a helium-flushed 3-mL syringe and 21-gauge needle. Headspace gas samples were collected for analysis at 5 times points during the incubation following the previously used sampling protocol.

Because the same incubation vessels were sampled at each time point in sediment incubation experiment 4 rather than sacrificed as in the previous trials, it was necessary to flush the headspace with helium after each time point to avoid the build-up of negative pressure in the serum vials. The vials were flushed by introducing a needle connected to continuous helium flow and an out-flow needle. The additional step purged the headspace of any accumulated N$_2$, so IRMS data reflected production between time points rather than over the course of the entire time series. The $\delta^{15}$N values of experimental duplicates were averaged and then used to calculate the mean value for the different four incubation systems across the entire time series.

2.5 Geochemical analysis of groundwater

Concentrations of dissolved NH$_4^+$ and Fe$^{2+}$ were measured in incubation groundwater samples from all time points in all four trials using colorimetric methods. A
Shimadzu UV-1601 spectrophotometer was used to measure absorbance in analytical methods for these chemical parameters.

All groundwater samples were subsampled and processed for ferrous iron measurements immediately after filtration to minimize oxidation after exposure to air (Hopwood et al. 2014). Iron determination by the ferrozine method was used to quantify Fe$^{2+}$ concentrations, and a calibration curve prepared from a single stock solution of iron sulfate was run in with each time point analysis (Stookey 1970; Villoier et al 2000). The analytical uncertainty associated with Fe$^{2+}$ concentration measurements by this method was 3.3%.

Dissolved NH$_4^+$ was measured by the Koroleff phenol-hypochlorite method (1969). A matrix matched check standard solution of NH$_4$Cl was run with each analysis to control for variability in absorbance readings due to matrix effects (principally salinity) or day to day spectrophotometric drift. Concentration measurements obtained via this method had less than 5.7% analytical uncertainty below ~40 μM. Samples were diluted prior to analysis when concentrations were known to be above this threshold.

NOx concentrations were measured in only the first and second trials using the manual spongy cadmium reduction method (detection limit ~2 μM). All samples were processed and analyzed in with a calibration curve prepared from a stock solution of KNO$_3$. Concentration measurements by this method were associated with a 7.5% analytical uncertainty. After confirming that the pre-incubation period effectively eliminated residual NOx in the incubation systems in trial 2, regular NOx concentration measurements were omitted.
Samples from all time points in trials 1, 2 and 3 were measured for dissolved oxygen concentrations immediately after breaking the air-tight seal using a microsensor. In trial 4, oxygen measurements were not taken but samples were confirmed to be anoxic using supplementary data generated by the IRMS during headspace gas N₂ measurements.

2.6 Sediment characterization

Sediment samples used in the third and fourth trials were characterized with respect to organic matter content, reducible ferric iron, and adsorbed ammonium.

Organic matter content was estimated by weight loss on ignition (Dean, Jr. 1974; Luczak et al. 1997). Ten grams of sediment were dried in pre-weighed ceramic crucibles for 48 hours at 100 °C. The dried sands were weighed before and after ashing for 6 hours at 580 °C.

Reducible ferric iron was leached from the sediment using 0.25 M hydroxylamine hydrochloride in 0.25 M HCl (Lovley and Phillips 1987). One gram of sediment was shaken with 10 mL of the leaching solution for 12 hours in 10 experimental replicates, and reduced iron concentration in the supernatant measured by the ferrozine method.

Adsorbed NH₄⁺ on the sediments used in these experiments was measured using the KCl extraction method of Laima (1992). This was only quantified at the beginning of trials 3 and 4 to gauge the maximum potential increase in NH₄⁺ concentrations that could be attributed to remineralization of adsorbed ammonium from the initial sediment. Adsorbed ammonium was also measured on sediment from a sulfidic layer underlying the Fe-rich zone in the STE at the same time as collection of the Fe-rich samples for Trial 4. Briefly, sediments were shaken with 2 M KCl solution and 0.1% zinc acetate at a 1:1
mass to volume ratio on ice in 60 mL centrifuge tubes for one hour. Tubes were then centrifuged at 2600 rpm for 10 minutes, after which the supernatant was decanted and processed via the Koroleff method for ammonium analysis. The extraction process was repeated three times and ammonium concentration quantified for each sequential extraction. By the third extraction, no additional increase in ammonium concentration was observed, suggesting complete extraction of adsorbed ammonium. Adsorbed NH4 was calculated by summing the mass in all three extraction steps.

3. Results

3.1 Trial 1

Chemical analysis from the summer sediment and groundwater incubations demonstrated significant geochemical changes in concentrations of NH4+, NOx, and Fe2+ over the course of the series in both the NH4+-spiked treatment and corresponding control (Fig. 2). In the first two days, NH4+ decreased from a starting concentration of 45 μM to 26 μM in the treatment incubation but remained undetectable in the control. By day 30, NH4+ concentrations increased to 34 μM and 20 μM in the treatment and control incubations, respectively, and then decreased to 20 μM and 0 μM by 45 days. In both the treatment and control incubations, NOx was detected at the start of time series at concentrations of 12 μM and 6 μM. After the initial time point, significant concentrations of NOx were not detected in the control. In the treatment incubation, concentration decreased to 6 μM by day 2 and was undetectable by day 30.
Concentrations of Fe$^{2+}$ increased steadily over the course of the incubation in both the treatment and the controls to 100 µM and 120 µM, respectively. The groundwater in the treatments and controls was determined to be approximately 20% saturated with O$_2$ at time 0 but completely anoxic by day 2. Oxygen remained undetectable by microsensor measurement (detection limit = ~5 µM) in all incubations for the remainder of the time series.

3.2 Trial 2

In the second sediment incubation time series, $^{15}$NH$_4^+$ concentrations increased by approximately 15 µM in both the $^{15}$NH$_4^+$-spiked treatment and the corresponding control from starting concentrations (Fig. 3a). In the treatment incubation, concentrations increased from the starting concentration of 52 ± 3.8 µM to 64.6 ± 2.7 µM by day 17 and remained stable for the remainder of the time series. In the control, NH$_4^+$ concentration increased from <5 µM at the start of the incubation to 15 ± 9.7 µM by day 17 and then remained stable until the final time point. Between days 30 and 36, the concentration increased to 26 ± 2.7 µM. Over the course of the time series, NO$_x$ was undetectable in both treatment and control. Fe$^{2+}$ production occurred by day 4 of the time series and concentrations continuously increased over the subsequent 36 days to 125 µM and 145 µM in the treatment and control, respectively (Fig. 3b). Oxygen remained undetectable by microsensor measurement in all incubations over the course of the entire time series.

Isotopic composition of the N$_2$ at the final time point indicated greater enrichment in the $^{15}$NH$_4^+$-spiked treatment relative to the control. This is most evident in the
measurements of $\delta^{15}$N signature (Fig. 4). At 36 days, $\delta^{15}$N in the duplicated treatments was $61.945 \pm 42.0$ per mil but only $4.331 \pm 0.16$ per mil in the controls.

3.3 Trial 3

The aged-sediment and groundwater incubation experiment revealed a similar trend of ammonium production in ammonium-spiked treatments as well as the control (Figure 5a). Concentrations of $^{15}$NH$_4^+$ in the control, low $^{15}$NH$_4^+$-spike, and high- $^{15}$NH$_4^+$ spike incubations remained constant within the range of uncertainty of their starting concentrations through 45 days. Significant increases in ammonium concentration occurred in the control and the high-spike treatment between days 45 and 62: from $0 \mu$M to $23 \mu$M and $50 \mu$M to $75 \mu$M, respectively. However, the ammonium concentration in the low-spike treatment remained unchanged at $30 \mu$M. NO$_x$ remained undetectable in all treatments and controls over the course of the time series. Fe$^{2+}$ production was detected beginning at day 7 in all incubation systems (Figure 5b). In the control and the high $^{15}$NH$_4^+$-spike incubation, Fe$^{2+}$ concentrations increased to $111 \mu$M and $95 \mu$M, respectively. In the low $^{15}$NH$_4^+$-spike treatment, ferrous iron production occurred at the same rate through day 35, after which the concentration of Fe$^{2+}$ was constant at $64 \mu$M.

Isotope ratio data from days 0, 14, 35, and 62 indicated consistently higher quantities of enriched N$_2$ production in both $^{15}$NH$_4^+$-spiked treatments relative to the controls (Fig. 6). $\delta^{15}$N values show a rapid initial increase production of N$_2$ containing $^{15}$N relative to $^{14}$N. In the subsequent time points, $\delta^{15}$N measurements indicated consistent enrichment of N$_2$ in the treatment, while the controls showed no change in $\delta^{15}$N over time. However, the $\delta^{15}$N in the treatments peaked at 14 days and then declined slowly between
the remaining time points, which is likely a result of dilution of the $^{15}$NH$_4$+ label by $^{14}$NH$_4$+ released by organic matter decomposition.

3.4 Trial 4

Concentration data from the fourth trial revealed distinctly different trends for ammonium between the treatments and controls (Fig. 7). The duplicated control incubations showed a linear increase of 21 ± 3.0 µM NH$_4$+ (Fig. 7a). However, $^{15}$NH$_4$+ concentrations in all three $^{15}$NH$_4$+ -spiked treatments oscillated in consistent temporal and directional patterns over the course of the 57-day time series (Fig. 7b, 7c and & 7d).

Between days 0 and 9, concentrations increased in all treatments by an average of 17% (range 13.4-23.4%) and then decreased from day 9 to 22 by an average of 15% (range 5.7%-26%). Between days 22 and 35, $^{15}$NH$_4$+ concentrations were constant in all three treatments. By 50 days, the concentrations in all treatments had increased from their lowest measured concentrations between days 22 and 35 and then subsequently decreased again to levels between the starting and peak-high measured concentrations. Given the precision of the NH$_4$+ analytical method, observed changes in $^{15}$NH$_4$+ concentrations are significant with the exception of the decrease between days 50 and 57 measured in one of the experimental duplicates for treatment 3.

Ferrous iron was produced in all treatments and controls (Fig. 8). Fe$^{2+}$ concentration increased at a rate of 6.7 ± 3.3 µM/day in the controls for the first 9 days and then 1.27 ± 0.06 µM/day through day 43 to a peak concentration of 136.8 ± 0.17 µM. Between days 43 and 57, concentration decreased at a rate of 0.96 ± 0.36 µM/day.
In the low $^{15}$NH$_4^+$-spike treatment, Fe$^{2+}$ concentration increased at a rate of 5.25 ± 0.54 µM/day between the start of the incubation and day 14. After day 14, the concentration remained stable at 123 ± 7.02 µM until the end of the 57-day time series.

In the high $^{15}$NH$_4^+$-spike treatment, the concentration of Fe$^{2+}$ increased at an average rate of 2.41 ± 0.26 µM/day between days 0 and 43 to a peak concentration of 132.7 ± 19.5 µM. Between days 43 and 50, the concentration decreased to 95.1 ± 9.8 µM but then increased again to 128.6 ± 32.3 µM by day 57.

In the low $^{15}$NH$_4^+$-spike treatment supplemented with ferrihydrite, measurements of Fe$^{2+}$ concentration show continuous production of ferrous iron. Over the course of the 57-day incubation period, the concentration increased at an average rate of 2.03 ± 0.3 µM/day to 150.3 ± 36.4 µM.

N$_2$ analysis by IRMS demonstrated production of enriched N$_2$ in all $^{15}$NH$_4^+$-spiked treatments. After correcting for the starting δ$^{15}$N values measured at day 0, mean enrichment relative to the control in the low $^{15}$NH$_4^+$-spike, high $^{15}$NH$_4^+$-spike, and low $^{15}$NH$_4^+$-spike plus excess Fe treatments was 0.745 ± 0.565, 3.461 ± 0.964 and 4.201 ± 0.756 per mil, respectively.

3.5 Sediment Characterization

Sands within the Fe-rich layer contained 0.17% organic matter. This value was used to calculate the maximum potential NH$_4^+$ production due to organic matter decomposition and subsequent ammonification. The mass lost on ignition was assumed to be 58% organic carbon (Allen et al. 1974), and the Redfield ratio was used to estimate the equivalent mass of nitrogen. Assuming all available organic nitrogen was converted to
dissolved ammonium, 13.96 \mu\text{moles of NH}_4^+ could be produced from each gram of sediment included in the incubation systems. Therefore, the maximum potential increase in dissolved NH$_4^+$ concentration due to organic matter decomposition was 1.55 mM in trial 3 and 2.79 mM in trial 4.

Adsorbed NH$_4^+$ represented approximately 0.0076 ± 0.0003 \mu\text{mol/g sediment in the Fe-rich sediments used in the third trial and 0.0126 ± 0.002 \mu\text{mol/g of sediment in the fourth trial. These measurements correspond to a maximum potential increase in groundwater concentration of 0.84 \mu\text{M and 2.52 \mu\text{M in trials three and four, respectively.}} $0.0738 ± 0.0023 \mu\text{mol/g of adsorbed NH}_4^+$ was extracted per gram of sediment from the sulfidic zone underlying the Fe-rich layer (Fig. 9).

Sediments used in both the third and fourth trials contained approximately 7.47 ± 0.34 \mu\text{mol/g of reducible iron. This corresponds to a maximum potential increase in groundwater Fe}^{2+} \text{ concentration of 830 \mu\text{M in the third trial and 1.49 mM in the fourth trial if all available ferric iron was consumed on the sediments.}}$

Additionally, the sediment contained in the incubation vessels progressively changed from a dark orange to light grey color over the course of trial 4 (Fig. 10). This occurred in all duplicated treatments and controls.

**Discussion**

These experiments were designed using the NH$_4^+$-spike and spike-free control approach under the assumption that the reaction was NH$_4^+$-limited and the reactant would be absent from the control for the duration of the time series. Therefore, any
geochemical trends attributable to feammox would clearly stand out against the background of any other competing processes in the control incubations. However, $\text{NH}_4^+$ production occurred in the control incubations in all four trials. While this conflicted with the purpose behind the experimental design, the control incubations were still used as the basis for distinguishing geochemical trends resulting from experimental manipulation from background processes in the treatment systems for each trial. Unfortunately, no enriched culture exists for feammox-performing bacteria, so any addition of natural microbial communities also introduces organic matter and solid-phase Fe.

Substantial decreases in $\text{NH}_4^+$ concentrations under anoxic conditions in the spiked treatment and control in the first trial indicated the occurrence of an anaerobic ammonium oxidation process, and production of $\text{Fe}^{2+}$ simultaneous with $\text{NH}_4^+$ consumption between 30 and 45 days indicated the potential for $\text{NH}_4^+$ oxidation to be coupled to iron-oxide reduction. However, the geochemical trends in the first trial were complicated by the presence of residual oxygen and NO$_x$ in the sediments inoculated into the incubation vessels. The rapid decrease in $\text{NH}_4^+$ concentration from its starting value in the first two days of the time series was coincident with consumption of all remaining oxygen, likely reflecting oxic nitrification. NO$_x$ produced from this process as well as any residual NO$_x$ present at the start of the incubation was removed by day 30 of the time series, suggesting the occurrence of a nitrification-denitrification coupling while both $\text{NH}_4^+$ and NO$_x$ substrates were available (Pochana and Keller 1999).

Nonetheless, production of substantial quantities of isotopically labeled $\text{N}_2$ from $^{15}\text{NH}_4^+$ under anoxic conditions in the treatment incubations from trials 2 and 3 provides
unequivocal evidence for occurrence of aerobic ammonium oxidation. However, total \( \text{NH}_4^+ \) concentration drawdown in the first three trials indicate that rates were probably low and therefore difficult to detect, especially due to production of unlabeled \( \text{NH}_4^+ \).

The pre-incubation period in the second and third trials ensured all oxygen and residual \( \text{NO}_x \) were eliminated prior to the \( \text{NH}_4^+ \) spike addition so that any subsequent changes could definitively be attributed to anoxic processes. Additionally, oxygen and \( \text{NO}_x \) were undetectable after day two of the first trial in the treatments and controls. Additional production of \( \text{NH}_4^+ \) in all \( \text{NH}_4^+ \)-spiked treatments as well as the corresponding controls in all four experiments under anoxic conditions is suggestive of one or more processes occurring parallel to novel anaerobic ammonium oxidation reactions.

Given the amount of sediment and corresponding organic matter added to the incubations, \( \text{NH}_4^+ \) production is most likely the result of organic matter oxidation and subsequent ammonification. Substantial increases in Fe\(^{2+}\) concentration in both the treatments and controls with no detectable consumption of ammonium suggest the coupling of iron-oxide reduction to organic matter oxidation, as would be expected during sub-oxic diagenesis (Lovley and Phillips 1986).

The stoichiometry of iron-mediated organic matter oxidation in early diagenesis indicates that \( \text{NH}_4^+ \) and Fe\(^{2+}\) are produced at molar ratio of 1:4 (Berner 1980). However, measurements of net increases in Fe\(^{2+}\) and \( \text{NH}_4^+ \) concentrations observed in the incubation trials were not consistent with this ratio. For example, the 147± 4.4 \( \mu \)M net increase in Fe\(^{3+}\) concentration in the treatment incubations over the course of the second
trial would be expected to correspond to an increase in NH$_4^+$ concentration of approximately 36.5 µM if iron-mediated organic matter oxidation was the only relevant process. However, over the course of the second trial, NH$_4^+$ concentration only increased by a total of 13 ± 2.7 µM, suggesting the simultaneous occurrence of other processes that consume NH$_4^+$ and/or yield Fe$^{2+}$, such as feammox.

Changes in concentrations of Fe$^{2+}$ and NH$_4^+$ were hard to interpret definitely as feammox because the magnitude of expected NH$_4^+$ consumption was small relative to the magnitude of NH$_4^+$ production occurring due to background processes. Theoretically, any trend changes should be detectable so long as the control serves as a suitable comparison for the background of competing processes. However, the analytical methods used to quantify most geochemical parameters lack the necessary precision to distinguish small concentration differences and changes in concentration within the ranges of the analytical uncertainties could go undetected or be considered insignificant.

Additionally, the experimental design included several variables that may have affected analytical uncertainty. The first three trials were made up of independent incubation vials that were sampled and sacrificed at various times to provide data that made up a time series. While the incubations were assembled and treated as uniformly as possible, the added sediment is inherently heterogeneous with respect to quantities of reducible iron, organic matter, and solid-phase ammonium. Therefore, many changes in geochemical parameters that occur between time points may not be representative of the passage of time but rather the occurrence of the same processes at slightly different rates in independent systems.
Data from all four trials were supportive of simultaneous occurrence of iron-mediated organic matter oxidation and feammox under two possible scenarios: 1) feammox occurs at such low rates that resulting geochemical changes are indistinguishable from other processes, or 2) feammox and iron-mediated organic matter oxidation occur at similar rates such that the net effect of the two processes causes only small changes in the concentration in groundwater.

The latter scenario is strongly supported by the data in trials two and three, which consistently showed substantial production of Fe\(^{2+}\) in all incubation vials in the absence of detectable changes in NH\(_4^+\) concentration between time points. For example, data from the third sediment incubation experiment showed an increase in Fe\(^{2+}\) groundwater concentration from 4 µM to 78 µM in the control between days 17 and 44 but less than 5 µM change in ammonium concentration.

Additionally, N\(_2\) produced in the \(^{15}\)NH\(_4\)\(_\text{spiked treatments in the third trial was highly enriched relative to the controls for the duration of the incubation. Importantly, δ}^{15}\text{N values were highest at early time points rather than at the conclusion of the experiment, indicating that enriched dinitrogen gas formed from the isotopically labeled ammonium was progressively diluted by }^{28}\text{N}_2\). This can be explained by progressive organic matter oxidation yielding \(^{14}\text{NH}_4^+\), which was eventually converted into dinitrogen gas via anaerobic ammonium oxidation. Our current understanding of the reaction rates of all possible competing transformations in the incubation systems is limited, so the ratio data alone could favor either of the described scenarios.
The N₂ data from trial 4 showed higher enrichment in all ¹⁵NH₄⁺-spiked treatments relative to the controls, which is supportive of feammox. However, the δ¹⁵N values are much lower compared with those from the second and third trials. While the underlying reason for this change is not immediately clear, it could in part be a result of using the mean δ¹⁵N value from the measurements at each time point to quantify enrichment over the entire incubation period. The sequential subsampling approach entailed repeatedly purging the incubation systems of accumulated N₂, so averaging the δ¹⁵N values may obscure rate differences between time points due to loss of the accumulated N₂ between each analysis. Another possible explanation for reduced δ¹⁵N values in trial 4 is that the ¹⁵NH₄⁺ pool in the treatments was diluted with higher amounts of unlabeled NH₄⁺ by organic matter decomposition than expected based on results of previous trials. It appears that ¹⁴NH₄⁺ production in the treatment incubations was proportional to the amount of ¹⁵NH₄⁺ added (Fig. 7b, 7c, 7d), although the mechanism for this remains unclear. Consistent rates of ¹⁴NH₄⁺ across the treatment systems would have corresponded with nearly 10-fold higher δ¹⁵N values in the high ¹⁵NH₄⁺-spiked treatment than the low ¹⁵NH₄⁺-spiked treatments, given the order-of-magnitude difference between the amounts of labeled substrate added. This is supported by the NH₄⁺ concentration data from trial 4, which indicated consistent relative increases and decreases between the low ¹⁵NH₄⁺-spiked treatments and the high ¹⁵NH₄⁺-spiked from their respective starting concentrations rather than similar magnitudes of NH₄⁺ production and consumption.

While the second and third sediment trials were carried out in effort to demonstrate the same ammonium oxidation trend depicted in the first experiment, there was no apparent decrease in NH₄⁺ following a period of production due to organic matter
oxidation. However, this can in part be attributed to the variable lengths of the incubation periods as well as amount of sediment used in the trials and is not strong evidence against the occurrence of iron-mediated anaerobic ammonium oxidation.

In trial 2, the time series was 12 days shorter than the first trial. It is likely that this time period was too short to detect significant changes in geochemical parameters resulting from feammox. In trial 1, significant NH₄⁺ consumption was not detected until day 45. However, trial 2 was planned to run for a shorter period of time because we believed the pre-incubation would eliminate geochemical changes associated with residual oxygen and NOₓ and therefore reduce the amount of time necessary to establish ideal anoxic conditions for feammox.

In the third trial, NH₄⁺ oxidation was not detected despite the 62-day incubation period. In part, this may be a result of the “aged” state of the sediment used in the incubations. Whereas sediment used in the previous two trials was collected fresh on the day the experiment was assembled, sediment used for the third trial was leftover from the batch collected for the second trial and had been stored in the fridge for approximately 6 weeks. We opted to use this sediment instead of collecting a new batch for the third trial in the hope that some of the background processes seen in the previous trials may been occurring during the storage period and thus the associated geochemical changes in the incubations would be substantially reduced in the third trial. Conversely, it appears that the aged sediment was depleted of substrates that are characteristic of freshly collected sands and critical to the occurrence of feammox, such as specific Fe-oxide minerals or microbial populations.
Additionally, the mass of sediment used in the third trial was reduced from 10 grams, which was the quantity used in previous trials, to 5 grams in effort to further reduce background signals from competing geochemical processes. It is possible that reducing the mass of sediment added to the incubations consequently reduced the abundance of some biogeochemical parameter (e.g., bacteria) in the vials, effectively reducing the reaction rates and reactant mass.

The effects of using smaller quantities of aged sediment on the occurrence and detection of feammox is supported by the \( \text{NH}_4^+ \) data in the third trial. In the previous two experiments, significant \( \text{NH}_4^+ \) production had been detected by approximately 30 days into the incubation. However, the third trial showed no substantial increases in \( \text{NH}_4^+ \) concentration in the high-spike treatment or control until day 62.

The lack of \( \text{NH}_4^+ \) production in the low-spike treatment at the end of the third trial is unremarkable due to the detection of dissolved oxygen at 62 days (i.e., contamination). Any excess \( \text{NH}_4^+ \) produced as a result of organic matter decomposition may have been oxidized, resulting in no net change in concentration. Iron data from day 62 in the low-spike treatment show a decrease in ferrous iron concentration from the previous time point, which is consistent with iron oxidation following oxygen contamination.

\( \text{NH}_4^+ \) concentration changes in the fourth trial provided support for the occurrence of feammox. In the experimental treatments with added \( ^{15}\text{NH}_4^+ \), significant decreases in concentration followed a period of initial increase that was consistent with data from the previous trials. Oxygen remained undetectable in IRMS measurements after the pre-incubation, which indicates that these decreases resulted from feammox.
The oscillating $\text{NH}_4^+$ concentrations in the treatment incubations from trial 4 suggest that feammox is only one of several co-occurring reaction pathways. The non-monotonic or oscillatory pattern consistently observed in the four trials indicates a series of reaction periods associated with feammox and organic matter diagenesis (Figure 11). The initial increase $\text{NH}_4^+$ was likely due to organic matter oxidation coupled to ferric iron reduction. During this phase, feammox may have co-occurred, but was undetected against the background of Fe-mediated organic matter decomposition and subsequent ammonification. However, as organic matter was depleted, ammonification declined and feammox became dominant. This is represented by the peak in $\text{NH}_4^+$ concentration and marks the transition to a feammox-dominated second phase, during which the remaining iron-oxides were consumed.

The third phase began once iron-oxides became limiting and feammox rates became progressively slower. At this time, $\text{NH}_4^+$ concentrations began to increase again as organic matter oxidation by sulfate reduction became dominant. Sulfate reduction led to hydrogen sulfide accumulation, which formed grey and black precipitates on the sediment, consistent with the progressive color change noted at later time points in the experiment (Fig. 11). Sulfidic conditions and sulfide mineral precipitates promote increased ammonium adsorption. Thus, the latter period of decreasing ammonium concentrations appears to reflect transfer of ammonium from the dissolved phase to solid surfaces due to increasingly sulfidic sediments, which is supported by the results from adsorbed $\text{NH}_4^+$ sediment extraction (Fig. 9).
Ammonium was low but present in the control incubations at the start of the time series, suggesting that feammox was not limited, but measurements of NH$_4^+$ concentration showed no decrease over the course of the time series as would be expected. Rather, the data showed an increasing linear trend in NH$_4^+$ concentration for the duration of the time series. Comparison of these data with the trends in the treatment incubations suggests a potential threshold concentration of ammonium below which the rate of feammox is too low for detection against the background of NH$_4^+$ increase caused by organic matter decomposition. Additionally, NH$_4^+$ production due to organic matter composition is associated with smaller changes in concentration between time points in the controls relative to the treatments, which would be expected if feammox is occurred simultaneously at a low rate, buffering the associated increase in concentration.

Results from trials 2 and 3 were consistent with this explanation, as starting NH$_4^+$ concentrations were only approximately 50 μM in the treatment systems. However, the results from the first sediment experiment appeared to show feammox despite equally low starting NH$_4^+$ concentration in the treatments. The apparent differences in reaction rates may be a function of seasonal changes in microbial community composition or activity, as sediment used in the first trial was collected in mid-summer while samples for the other experiments were obtained in fall and winter.

It is also important to note that the addition of excess Fe$^{3+}$ in the form of synthetic 2-line ferrihydrite did not appear to stimulate feammox in trial 4. If availability of this form of Fe$^{3+}$ limited the reaction, NH$_4^+$ concentrations would be expected to show greater
decreases in the low $\text{NH}_4^+$ spike treatment supplemented with ferrihydrite than in the treatment system containing only the low $\text{NH}_4^+$-spike.

**Conclusions and Future Directions**

The results of this study strongly suggest that a novel anoxic ammonium-oxidation reaction mediated by iron-oxide reduction occurs in the Gloucester Point subterranean estuary. However, under the experimental conditions tested, feammox did not appear to cause substantial removal of dissolved $\text{NH}_4^+$. Because of this, conclusions about its quantitative relevance to the nitrogen cycle on a local or global scale are not currently possible. In particular, there remain important questions about availability of the optimal chemical substrates, environmental variables, and microbial metabolic processes that enable the reaction to proceed. Further investigation of these components would enable us to develop a clearer geochemical framework for the conditions favoring the feammox reaction.

Future studies should especially endeavor to assess the reaction with respect to different mineral phases of oxidized iron. The thermodynamic favorability of feammox is highly contingent on the chemical composition of the iron-oxide substrate, and the relative abundance of different forms can vary widely among different subterranean estuary sites and even within individual sites (Charette et al., 2005). Thus, the occurrence and relative importance of feammox could largely be a function of site-specific mineral composition. A major component of my future research will be characterizing the mineral composition of different subterranean estuary sites in the Chesapeake Bay watershed.
using x-ray crystallography to assess Fe mineral variability in the STE and its association with the distribution of nitrogen species at different STE sites.

Similar site-specific limitation may apply to feammox with respect to microbial mediation. The occurrence of this reaction could be contingent on the availability of an active population of a specific microorganism across different geochemical environments. Characterizing the microbial community at sites where feammox is known to occur may enable us to devise a biogeochemical marker that could be widely used in site comparisons as an initial indicator of the process. Improved understanding of the relevant microorganisms and their metabolic needs will provide insight into temporal and spatial variability in excess N$_2$ produced via feammox.

Although the results of this investigation are insufficient to immediately evaluate the importance of feammox *in situ*, they demonstrate that the reaction does occur under anoxic conditions in STE sediments and warrants additional study as a potentially important pathway in the global nitrogen cycle. The results of the current study are a timely addition to our understanding of the coastal N cycle, as rapidly changing hydrologic and climatic regimes call for improved understanding of nitrogen cycling in aquatic systems.
Works Cited


<table>
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<tr>
<th>Iron-oxide mineral</th>
<th>Feammox reaction pathway</th>
<th>$\Delta G_r$ (kJ mol$^{-1}$ N)</th>
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<td>Amorphous Fe precip.</td>
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**Table 1.** Free energy yields of anaerobic ammonium oxidation reactions limited by different iron-oxide mineral phases occurring at pH 7. Chemical concentrations used in $\Delta G_r$ calculations are based on average levels reported for *in situ* conditions: $pN_2 = 0.781$ atm, $[NH_4^+] = 100$ μM, $[Fe^{2+}] = 50$ μM.
<table>
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<tr>
<th>Trial #</th>
<th>Duration (days)</th>
<th># of time points</th>
<th>Type of time point sampling</th>
<th>Initial volume ASW (mL)</th>
<th>Sediment mass (g)</th>
<th>Serumial volume (mL)</th>
<th>Incubation systems</th>
<th>Deoxygenating gas</th>
<th>Parameters measured</th>
<th>N₂ analysis</th>
<th>Sediment collection date</th>
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<td>(¹⁵N)NH₄SO₄</td>
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<td>60</td>
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<td>(¹⁵N)NH₄SO₄</td>
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Table 2. Detailed description of all four incubation trials with respect to specific aspects of experimental design and methodology that varied between attempts.

1 "ASW" = artificial seawater.
Figure 1. Map showing the location of Gloucester Point subterranean estuary site. Sediment samples used in experiment were collected from a depth of approximately 65 cm at the mid-tide line. The image depicts the deep orange color of the sands found in this zone that results from high quantities of iron-oxides.
**Figure 2.** Concentrations of NH$_4^+$, NO$_x$, and Fe$^{2+}$ over the course of Trial 1. Detection of NO$_x$ in the initial time points indicates that corresponding changes in ammonium are likely due to oxic processes. The highlighted area represents the period that corresponds to the so-called pre-incubation period in future trials.
Figure 3. Trial 2. Average NH$_4^+$ and Fe$^{2+}$ concentration measurements and standard deviations of experimental duplicates sacrificed at each time point. NH$_4^+$ concentrations increase to 64.5 µM in the treatments and 26 µM in the control over the course of the time series. Fe$^{2+}$ was detected in the treatments and controls by day 7, after which concentration increases through the end of the time series. The apparent fluctuations in the concentration over time are believed to result from NH$_4^+$ and Fe$^{2+}$ production occurring at slightly variable rates across independent incubation systems.
Figure 4. Isotope ratio mass spectrometry calculations of δ\textsuperscript{15}N at the final time point of sediment incubation experiment 2. Substantially higher δ\textsuperscript{15}N values in treatment systems demonstrate the consumption of \textsuperscript{15}NH\textsubscript{4} introduced at the start of the time series in a process that ultimately yields dinitrogen gas.
**Figure 5.** Concentrations of Fe$^{2+}$ and NH$_4^+$ over the course of trial 3. NH$_4^+$ concentrations remain constant in all three systems until the final time point, at which production in the control and high-spike treatment is likely indicative of organic matter decomposition. Lack of NH$_4^+$ production as well as a non-linear trend in Fe$^{2+}$ production is suggestive of substantial air contamination in the low-spike treatment by 62 days.
Figure 6. $\delta^{15}\text{N}$ of $\text{N}_2$ produced over the course of sediment incubation experiment 3. The controls remain unchanged while the ammonium-spiked treatments demonstrate consistent enrichment despite decreasing ratios over time. The decreasing trend can likely be attributed to the dilution of enriched $\text{N}_2$ with $^{28}\text{N}_2$ as $^{14}\text{NH}_4$ becomes increasingly available due to progressive organic matter decomposition.
Figure 7. Concentrations of NH$_4^+$ in control and treatment systems over the course of trial 4. Values depicted represent mean concentration between system duplicates at each time point. Oscillating concentrations are consistent among the treatment systems, while the control maintains an increasing trend over the course of the 57-day incubation period.
Figure 8. Concentrations of Fe$^{2+}$ for trial 4 in the control and treatment systems. Values depicted represent the mean concentration between system duplicates at each time point. All treatments and the control demonstrate high rates of production through day 22, after which concentrations remained stable in the incubations with the exception of a decline between days 43 and 50 in the high NH$_4^+$-spiked treatment.
Figure 9. Quantity of adsorbed NH$_4^+$ extracted with KCl per gram of sediment in the Fe-rich zone of the Gloucester Point STE and the underlying sulfidic layer. The sulfidic sands contain higher amounts of solid phase NH$_4^+$ despite the immediate spatial proximity of the two distinct layers.
Figure 10. Fe-rich sediments frozen and preserved from the day of collection (left) compared with leftover sediment in an incubation vial from trial 4 after the 57-day incubation period (right). The sediment used in the trial shows a clear transition from the bright-orange coloration characteristic of the Fe-rich zone to dark grey, which is consistent with production of sulfide precipitates.
Figure 11. Hypothesized progression of the trial 4 incubation systems through different phases distinguished by dominance by one of four geochemical processes associated with feammox and/or organic matter diagenesis. The phases correspond sequential periods of increasing and decreasing NH$_4^+$ concentration and include descriptions of expected patterns of other relevant geochemical parameters, such as Fe$^{2+}$ and HS$^-$. 