Fe-OM Coprecipitation and its Effects on Bioavailability of Cu and OM to Denitrifiers

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Introduction
Wetlands are amongst the most productive ecological systems, characterized by their efficiency in the treatment of contaminants, retention of organic matter (OM), and productive biota that drive nutrient cycling and retention (Carter 1996). These systems benefit the general public as they create aesthetic appeal, reduce flooding, encourage groundwater recharge, and keep rivers/lakes cleaner. Wetlands can improve downstream water quality due to their disproportionately high capacity to remove nutrients, metals, and other contaminants (Costanza et al. 1997; Zedler 2003). While these systems exist naturally, they are also recreated or constructed in various forms as the importance of these systems becomes more known. However, these reconstructed systems usually do not take in to account the biogeochemical processes occurring within, resulting in less functional systems that provide not as many benefits as they could. This is especially seen in situations where companies are required to build a wetland in replacement of the existing one they built on (Section 404 of the Clean Water Act and 1990 Memorandum of Agreement between the EPA and Army Corps of Engineers). A better understanding of wetland biogeochemical processes under the conditions seen in developed systems with natural or replacement wetlands will inform design or management to make them more functional and efficient.

Wetlands in developed landscapes or built replacement wetlands are exposed to different inlet concentrations of contaminants. With infiltration driving nitrogen species into groundwater from agricultural or heavily fertilized landscapes, stormwater runoff bringing excess nutrients and metals such as Cu and Fe to these systems, and urban runoff leaching larger than expected amounts of OM from suburban developments (Luan and Vadas 2015), these wetlands receive inputs that may alter their ability to function in the same way as natural systems. The inputs of Fe, Cu and OM in particular play a large role in controlling the forms and aqueous speciation of Cu and OM in the porewater (Seda et al. 2016), two components that are critical for optimal denitrification of the nitrate entering the system.

During denitrification, nitrate (NO_3^-) is converted to nitrous oxide (N_2O) and nitrogen gas (N_2) (Davis et al. 2008). N_2O is a greenhouse gas produced by both denitrification and nitrification, but denitrification dominates in reduced aquatic environments such as wetlands (Hartop 2017, Mathieu et al., 2005). It is preferred that N_2O release be minimized in wetland systems to minimize greenhouse gas effects. N_2O yield, expressed as [$N_2O/(N_2O + N_2)$], is commonly used to compare N_2O emissions across systems. However, the variance in N_2O yields seen in studies where water and O_2 levels have been kept constant has yet to be explained (Morse and Bernhardt, 2013). This suggests that other environmental controls determine N_2O yields. However, given limited experimental data and information available, the exact controls remain unclear.

Fe inputs and cycling controls the partitioning of Cu and OM in wetland systems, reducing bioavailability to denitrifying bacteria. Fe precipitation as ferrihydrite occurs in wetlands generating a large surface area for effective sorption of cations and organic carbon (Eusterhues et al., 2008, Moon & Peacock 2011, Saito 2005). As Fe
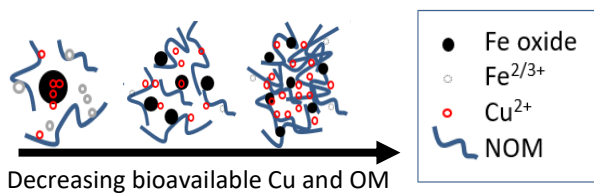

Figure 1. Ternary phase forms as Fe;OM ratios change

precipitation occurs, ternary phases of Fe oxides, OM, and Cu from through coprecipitiation, electrostatic, or chelation interactions. The mechanisms responsible for OM adsorption to mineral surfaces is largely due to ligand exchange- surface complexation and is a function of pH, the type of humic acid present, and electrolyte concentration (Gu 1995, Avena & Koopal 1999, Moon and Peacock 2011, Saito 2005). The presence of dissolved forms of organic matter during ferrihydrite precipitation can inhibit the aggregation (Figure 1), thereby decreasing the size of iron oxide particles and increasing binding sites for Cu (Eusterhues et al., 2008, Schwertmann et al., 2005). Cu may form a variety of bound species including isomorphic substitution, inner or outer sphere oxide surface complexes, and type A (oxide-Cu-OM) or type B (oxide-OM-Cu) ternary complexes (Hesterberg et al., 2011). There still remains, however, a competition for Cu binding from the often-stronger affinity to OM, which may either remain in solution or adsorb to the ferrihydrite surface. These interactions therefore dictate the partitioning of Cu and C between dissolved, colloidal, and bulk solid phases; where colloidal and dissolved make up the total dissolved phase but truly dissolved is what is typically considered bioavailable.

The denitrification process entails a series of sequential reduction reactions that require organic carbon (C) as an electron donor and Cu as an enzyme cofactor. The final step of denitrification, N_2O is reduced to N_2 through nitrous oxide reductase (NosZ) acting as a catalyst (Tao 2017). NosZ has a unique multi-copper-sulfide center that is capable of activating N_2O, prior to the reduction reaction, by using electrons delivered by the Cu center (Felgate et al., 2012). Therefore, Cu availability could be a limiting factor to NosZ synthesis (Sullivan et al., 2013) as seen in laboratory culture studies (Granger and Ward 2003). Carbon availability also controls denitrification rates by increasing the amount of denitrification and decreasing N_2O yields (Weier et al., 1993, Mathieu et al. 2006). Carbon, used as an electron donor, stimulates microbial metabolism leading to an increase in the consumption of O_2 which creates favorable conditions for denitrification (Miller et al., 2008, Paul & Beauchamp et al., 1989, Kwon 2016). As both C and Cu are required for carrying out denitrification, it is important to further understand Cu and C availability in wetland systems in order to determine their effects on N_2O yields.

Preliminary data from denitrifier aqueous cultures suggest lower free Cu levels result in significantly higher N_2O yields (Felgate 2012, Sullivan et al. 2013). However, while ligand controlled metal availability affects yields in slurry cultures, studying how Fe-OM precipitation and coprecipitation affects OM and Cu availability illustrates a more complete picture of the system. This method incorporates the controls that are typically thought to impact N_2O yields (water content, O_2 concentrations), while also studying new possible environmental factors (OM, Cu, and pH) under environmentally relevant conditions and concentrations. We propose to

investigate microbial ecology in conjunction with geochemistry and OM interactions to address the following: **To determine if N_2O yield is a function of Cu and OM bioavailability.**

In a wetland environment, Fe cycling plays a major role in controlling Cu and OM availability. Research on the formation of these Fe-OM-Cu complexes from aqueous phase batch reactions has suggested trends in the partitioning of Cu and OM between dissolved, colloidal, and bulk solid phases. Within the pH range of 5-7, 90-100% of the Fe precipitated with the OM, resulting in lowered concentrations of Cu and OM remaining in the dissolved phase. Total dissolved concentrations of Cu are generally reduced as the ratio of Fe:OM increases and as pH increases. Conversely, dissolved concentrations of OM are reduced as pH decreases. At pH 7, we see the most dissolved OM and an increase in total dissolved Cu as Fe:OM ratio increases. In reality, within the dissolved phase as most researchers define it (based on a 0.45 µm filter size cutoff), there are still particulate phases of Fe-OM-Cu complexes in the colloidal size range (1-1000 µm). The bioavailability of these phases is uncertain. The free ion is typically considered the most bioavailable to organisms (Hamelink et al. 1994). Therefore relying on concentrations of the total dissolved Cu and OM may not accurately reflect the bioavailable fraction. Ongoing analysis is exploring trends in the colloidal data, allowing for a better understanding of the controls and limitations that will be utilized during this research.

The proposed project outlines research that will determine factors controlling N_2O yields in wetland environments. This research will utilize the completed experimental data mentioned above to determine conditions in which Cu and OM availability can be manipulated. These conditions will then be used to simulate an environment in which N_2O yields can be tested as a function of Cu and C availability. **We hypothesize that there will be a net decrease in denitrification when OM is coprecipitated with ferrihydrite and an increase in N_2O yield when Cu and OM availability is limited in the environment.** Our goal is to improve out understanding of organism's control on denitrifying rates and yield while connecting Cu, Fe, and OM geochemistry to microorganism functions. If it turns out that the incoming Fe leads to limited OM availability or too much Cu sequestration in the sediments, designed or replacement wetlands could be altered to target removal of Fe prior to exposure to the sediments where denitrifiers dominate.

Methods

Amendment Set Up and Nomenclature

Soil slurries were set up to create scenarios in which Cu and OM controls of denitrification were isolated. A total of thirteen treatments were created that varied labile/binding carbon (humic acid), non-labile/non-binding carbon (glucose), Cu, and Fe. Each soil serum vial was spiked with 5ppm nitrate and amendments 24 hours prior to the start of the experiment, evacuated and flushed with helium, allowed to sit for 24 hours to ensure reduced conditions, then evacuated once more to begin sampling process. Soil from a Coventry wetland (41.75922, -72.27764) and Hebron wetland (41.67287, -72.39513) were utilized for these experiments. Both wetlands were forested emergent wetlands. The soil was sieved prior to use.

The 13 experimental treatments were broken into 3 groups based on the amendments, a control, carbon addition (3 treatments), copper addition (3 treatments), and iron addition (6 treatments). Within these experimental treatments, treatment 10 and treatment 13 should isolate the control of Cu on denitrification. Each treatment shown in this summary of results has been in run in triplicate.

Treatment #	Treatment Characteristics	Notation
T0	No Additions	NoA
T1	Nitrate	NO3
T2	Glucose and nitrate	G&NO3
T3	Humic Acid and nitrate	HA&NO3
T4	Copper and nitrate	Cu & NO3
T5	Copper, glucose, and nitrate	Cu, NO3, & G
T6	Copper, humic acid, and nitrate	Cu, HA, & NO3
T7	Iron and Nitrate	Fe & NO3
T8	Iron, glucose, and nitrate	Fe, G, NO3
T9	Iron, humic acid, nitrate	Fe, HA, NO3
T10	Iron, humic, glucose, nitrate	Fe, HA, G, NO3
T11	Iron, copper, nitrate	Fe, Cu, NO3
T12	Iron, humic, copper, nitrate	Fe, HA, Cu, NO3
T13	Iron, humic, glucose, copper, nitrate	Fe, HA, G, Cu, NO3

Each experiment was sampled for N_2O roughly every 30 minutes up to 120 mins. Initially, the sampling was done at 0, 30, 60, 90, and 120 but was changed to 0, 30, 60, 80, 90, 110 to see reaction under shorter time steps. At the final time step, an aliquot of the liquid was taken for N_2 analysis while the rest of the sample was filtered and analyzed for metal concentration, dissolved organic carbon, total nitrogen, nitrate, and ammonium.

Soil Initial Conditions

The soil was analyzed for initial soil conditions: total organic carbon, moisture content, pH, metal concentration, nitrate concentration, and total nitrogen. The initial concentration of the soil gave a basis of addition. The table below shows the initial parameters of each soil.

Table 1. Initial soil condition ranges based on 2 collections of Hebron soil and one from Coventry

Characteristic	Hebron Soil	Coventry Soil
Iron	200ppb-1000ppb	250ppb
Copper	9-12 ppb	10ppb
Total Organic Carbon	8-15 ppm	9ppm
Total Nitrogen	<1 ppm	2ppm
pH	5	5

Nitrate	3-16ppm	10ppm
Moisture Content	70-80%	70-80%

All experimental data shown will be referencing Hebron soil.

Results
Soil Slurry Sampling Data

Controls were run for the experiment to see what the initial measurements of N_2O would be for a just water only scenario, a soil slurry with no amendments, and a soil slurry with nitrate. In the Figure 1 below, we see that in both the "water only" treatment (W) and "no additions" (NoA) that the measurements are below the helium and air blanks. These blanks show the experimental detection limit for GC analysis. Therefore, the used the "nitrate addition" (NO3) as the control for the rest of the treatment comparisons.
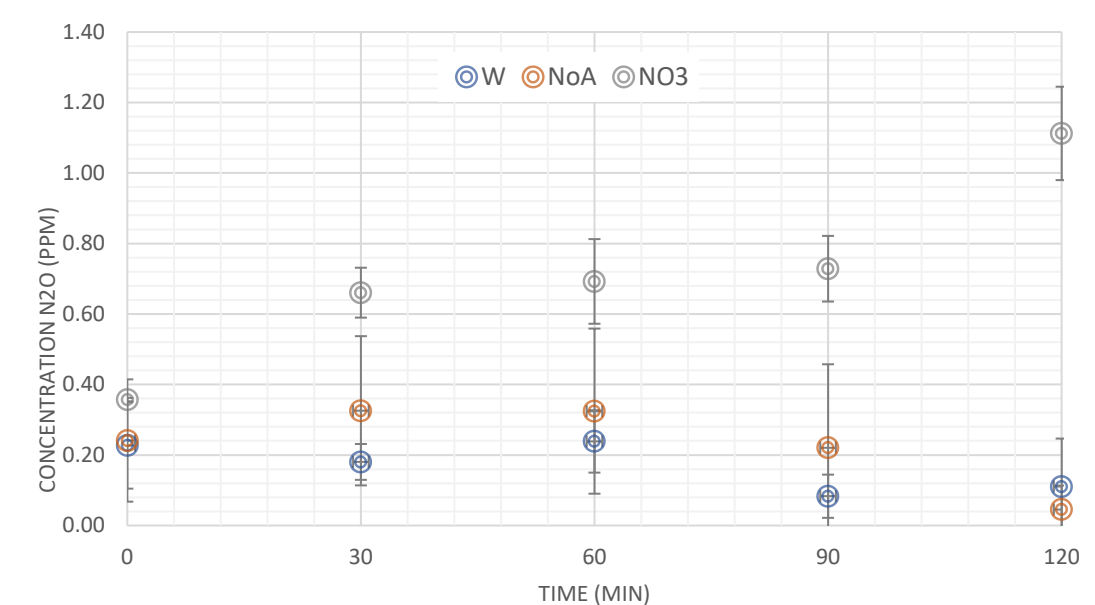
Figure 2. Shows a comparison of the control scenarios used for the soil slurries. As helium (He) concentrations were roughly 0.2ppm and air blanks were 0.5 ppm the nitrate control will be used as basis of comparison in further figures. Where W is water only, NoA is a soil slurry with no other additions, and NO3 is a soil slurry with a nitrate only spike.

The next series of treatments looked at carbon additions. In these treatments the slurry was spiked with nitrate and 5ppm as carbon of either glucose or humic acid. It was hypothesized that the addition of a carbon source would show more total denitrification within the system as both nitrate and carbon were readily available to the denitrifiers. In addition, it was expected that the glucose treatment would produce lower denitrification yields than the humic acid due to the binding versus non-binding carbon affecting both carbon and copper availability within the system. In Figure 2, the comparison of adding humic acid versus glucose shows that more N_2O was measured at each time point for humic acid versus glucose. It shows the humic acid and nitrate (HA&NO3) treatment following the nitrate plot initially then a drop below detection.
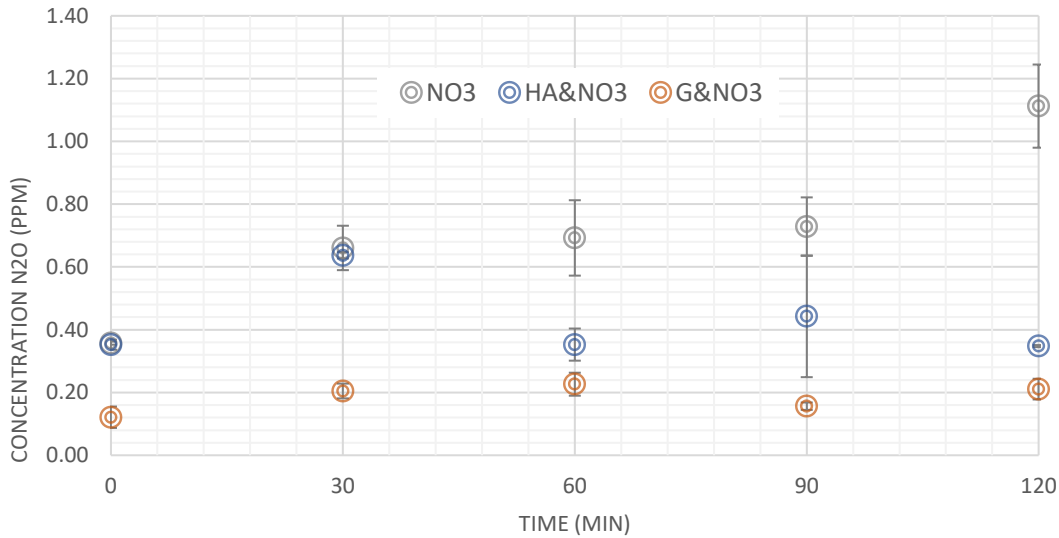
Figure 3. N2O concentration at each sampling point for treatments with a carbon addition. Where NO3 is the nitrate only addition, HA&NO3 is humic acid and nitrate addition, and G&NO3 is glucose and nitrate addition.

However, both glucose and humic acid additions were below detection at t=60 min and beyond for humic acid and the entire plot for glucose. The N_2 measured at the final time point for NO3, HA&NO3, and G&NO3 were 1.38ppm, 0.47ppm, and 1.18 ppm respectively. This indicates that complete denitrification was occurring in each treatment and the shift from N_2O production to N_2 may have caused the below detection values.

The below two figures, Figure 3 and 4 show the change in mass of Fe, Cu, OM, and NO_3^- from the initial time step and the final time step respectively. The initial time step was determined by using sacrificing 5 soil slurries with no additional amendments at the start of the experiment. The slurries were analyzed for each component on their respective instruments, then the average value of these 5 slurries was added to the amendments for each treatment to get a total initial mass.
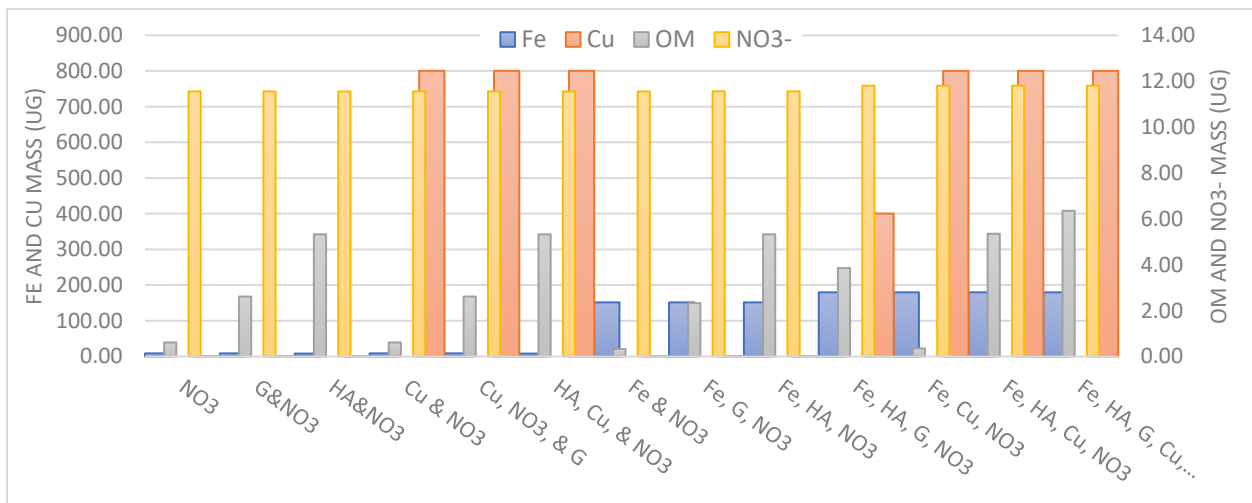
Figure 4 shows the initial Fe, Cu, OM, and NO3- masses for the amendments

The mass at the final time point was given by centrifuging the soil slurry, pipetting the liquid off the top and analyzing for each component. These values represent total dissolved values of each component and help demonstrate how copper and carbon availability is altered in each scenario. This can be seen when comparting treatments with copper and humic acid versus copper and glucose. The humic acid and copper have a strong affinity for one another and therefore less copper is seen in the dissolved phase. Once iron is added to the treatments you see an additional reduction in copper as possible Fe-C-Cu ternary complexes are forming.
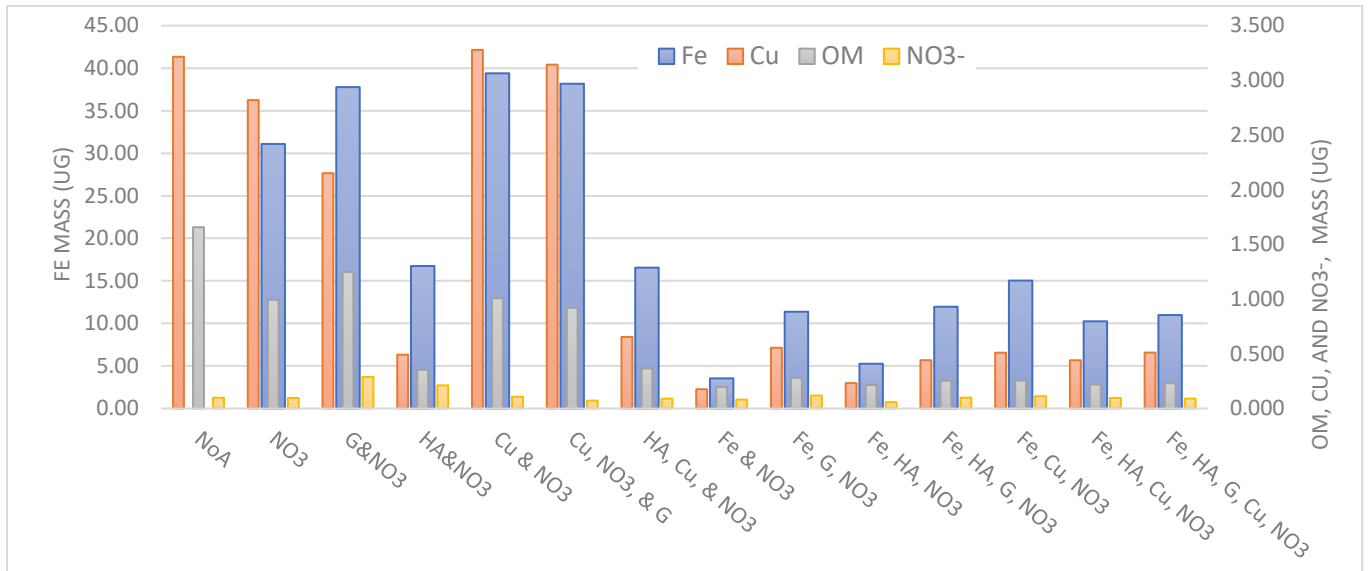

Figure 5 shows the mass of Fe, Cu, OM, and NO3- in the final time step

The set of treatments changed the copper availability within the system and combines how copper not only alone affects the system but how copper mixed with a carbon source changes the dynamics of the system. It was hypothesized in these scenarios that any addition of Cu would facilitate complete denitrification within the system meaning that in amendments with more bioavailable Cu, lower denitrification yields would be seen due to either less N_2O being produced or a quicker shift to N_2 production. The addition of and additional carbon source were expected to alter the availability of Cu in the slurry. The humic acid as a binding source of carbon would limit the copper while the glucose as a non-binding source would not. Figure 3 depicts the N_2O concentrations at each time step comparison amongst these 3 copper treatments. It shows that the Cu&NO3 treatment had N_2O concentrations at each time step below the nitrate control treatment. The Cu, NO3, & G treatment had higher N_2O concentrations at each time step compared to the nitrate control while the Cu, NO3, & HA treatment were the lower.
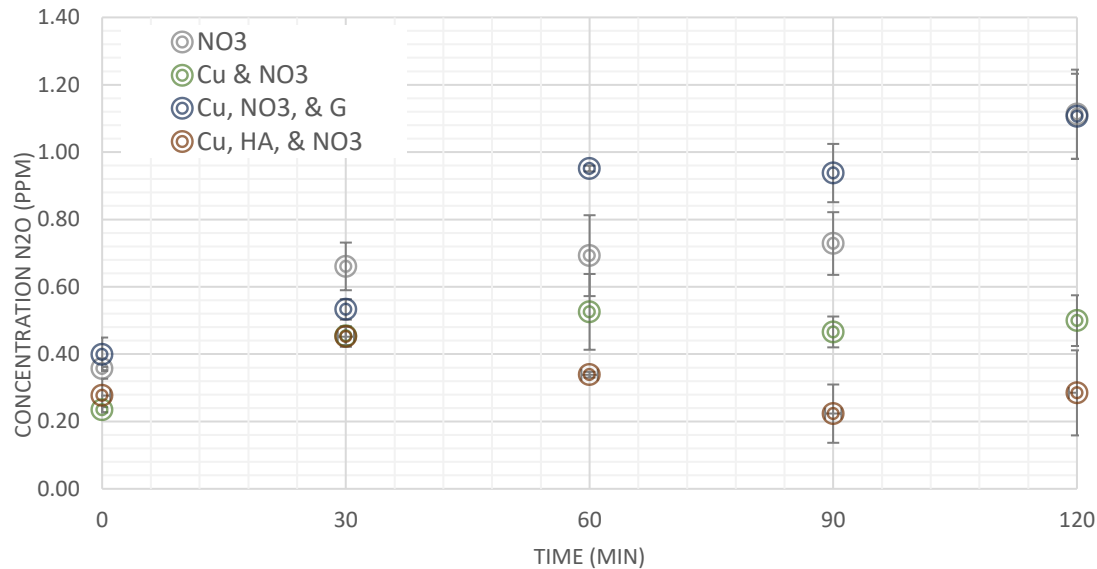
Figure 6. Shows the measured N_2O at each time set for the copper treatments compared to the nitrate control treatment. Where Cu &NO3 is a copper and nitrate amendment, Cu, NO3, & G is copper, nitrate, and glucose amendment, Cu, NO3, &HA is copper, nitrate, and humic acid amendment

The N_2 concentrations at the final time step for NO3, Cu & NO3, Cu, NO3, & G, and Cu, HA, & NO3 are 1.38 ppm, 1.33 ppm, 1.24ppm, and .488 ppm respectively. The low concentration of N_2 and near below detection limit for N_2O for the Cu, NO3, &HA treatment may suggest that denitrification within the system was limited under these conditions due to the limited availability of a carbon and copper source.

The next six treatments look at how adding iron to the system changes the availability of the carbon and copper to the denitrifiers. In Figure 3 and 4 we see that the these treatments do reduce the carbon and copper concentrations seen in the initial time step to the end. However, we can also measure this change by looking at the N_2O and N_2 produced. The six treatments are being broken into two groups of three where the first group, seen in Figure 6, shows how iron addition affects the slurry without any additional copper addition. The last three show a more complete picture where all components are added. It is hypothesized that in these scenarios with iron we will see a decrease in carbon and copper availability due to the precipitation of iron with carbon and available copper as well as the interactions of copper and carbon alone. As no other additional copper is added the treatment with Fe, HA, and NO_3^- should show the highest yield, potentially more N_2O production, and/or interfere with the ability to perform denitrification altogether.
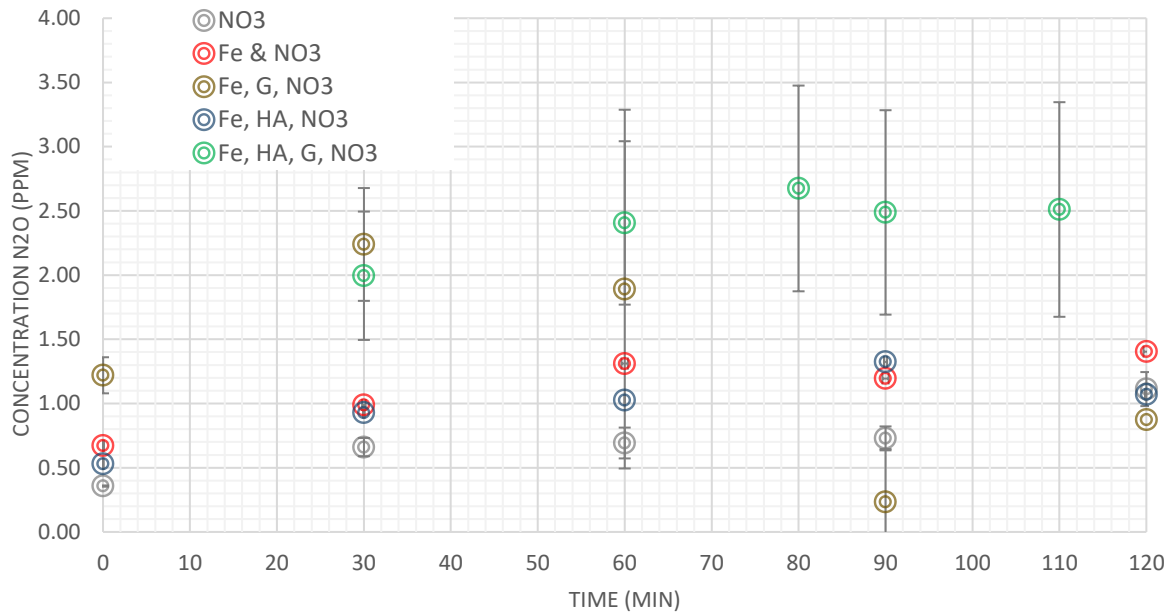
Figure 7 includes the first 3 treatments of the 6 iron group treatments. In this set, the effects of Fe affecting carbon and copper availability is compared through N2O concentrations at each time step. Similar notation is used as previous groupings.

In Figure 6, all treatments were above the control nitrate treatment with the Fe, HA, G, NO3 treatment having the highest concentrations at each time step. The Fe & NO3 and Fe, HA, NO3 treatments were similar to one another as well as close in value to the nitrate control. The N_2 concentrations for Fe& NO3, Fe,G,NO3, Fe, HA, NO3, and Fe, G, HA, NO3 were 0.40pppm, 0.51ppm, 0.429ppm, and 0.55ppm respectively.

Figure 7 shows the final 3 treatments compared to the nitrate control and the Fe, G, HA, and NO3 treatment. It was hypothesized that in treatment 10, Fe, HA, G, NO3 and treatment 13 Fe, HA, G, Cu, NO3 the true effects of copper and carbon control on denitrification would be seen. It was expected that in treatment 10, denitrification should show higher yields as carbon and nitrate are available, but copper is limited and that in treatment 13 yields would be lowest.
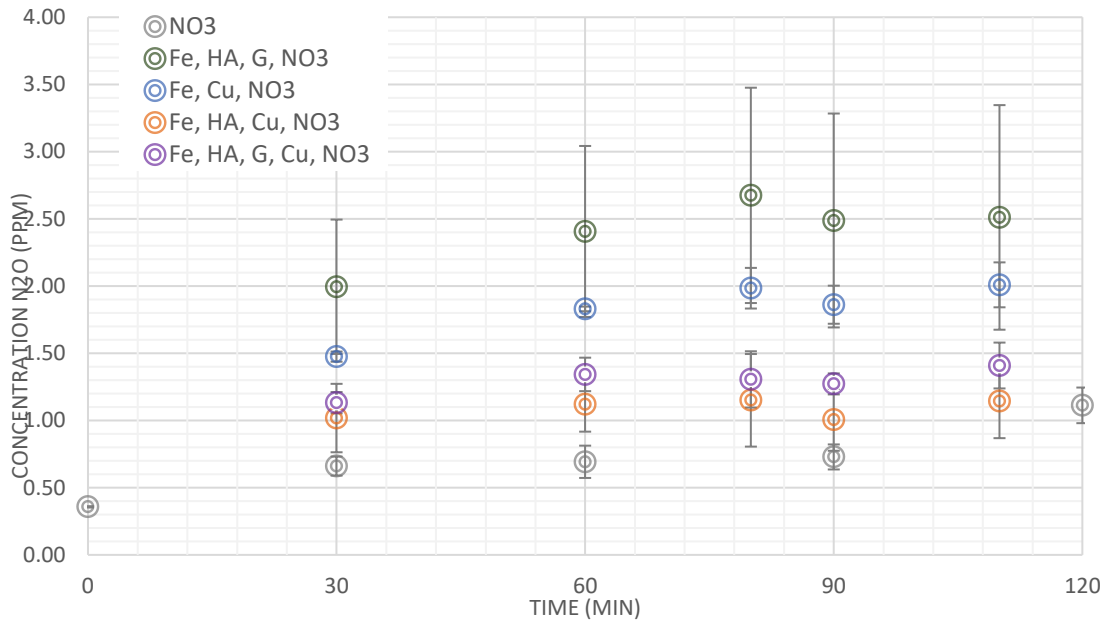
Figure 8 compares the last of the treatments which compare how adding Cu to the previous Fe added treatments changes denitrification.

Treatment 10 (Fe, HA, G, NO3) does have the highest N_2O concentrations at each time step than the following 3 treatments with Fe and Cu amendments. Treatment 12 (Fe, HA, Cu, NO3) shows slightly lower concentrations of N_2O than Treatment 13(Fe, HA, G, Cu, NO3). The N_2 concentrations of these treatments is not yet available therefore it is not possible at this time to discern if this difference in N_2O concentrations is referencing yield or total denitrification.

Denitrification Rates and Fluxes

Using the sampling times and concentrations of N_2O at each time step, a linear regression was performed to calculate the rate of N_2O as ppm per min. The linear regression was only performed on data sets where the values continued to increase. When data showed a decrease, only the values prior to that decrease were used for regression. In some cases, only 2 points could be used but in general a minimum of 3 points was utilized to calculate the value.

It is expected that treatment 13 (Fe, HA, G, Cu, NO3) should show lower yields as Cu and carbon are most available compared to other treatments. It is also expected that Treatment 10 (Fe, HA, G, NO3) would have one of the highest yields due to limited copper availability. If shown to be accurate, then the control of copper on denitrification is proven. The rate data allows an analysis of the production of N_2O but does not indicate the yield or total denitrification.

The current data shows that Treatment 8 (Fe, G, NO3) has the highest rate of N_2O production. In looking at comparing treatments with a glucose amendment versus a humic acid amendment, the rate of N_2O is lower in all cases with humic acid.
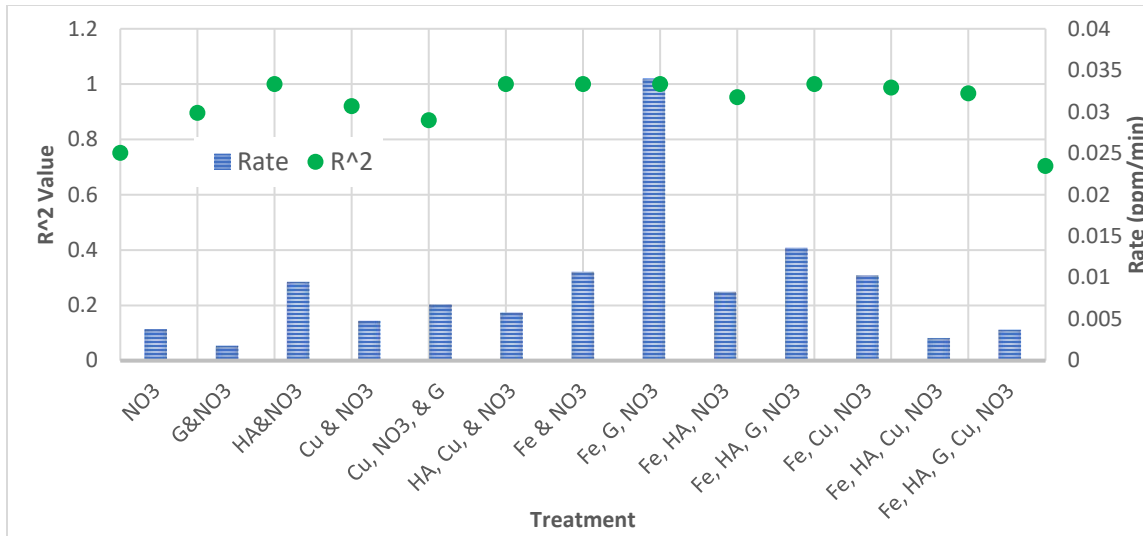
Figure 9 shows the rates of N₂O produced per minute based on the sampling shown in the above figures. The R^2 values indicate the fit of the linear regression performed to calculate these values.

Next, the flux of the slurries was calculated using the equation found in Jantalia et al., 2008.

$$f = \frac{\Delta G}{\Delta t} * \frac{V}{A} * \frac{m}{V_m}$$

Where f = gas flux, N₂O (mg N-N₂O m^{-2} day^{-1})
ΔG = concentration of N₂O during sampling
Δt = time period that chamber remained closed
V and A = volume and soil surface area covered by chamber, respectively
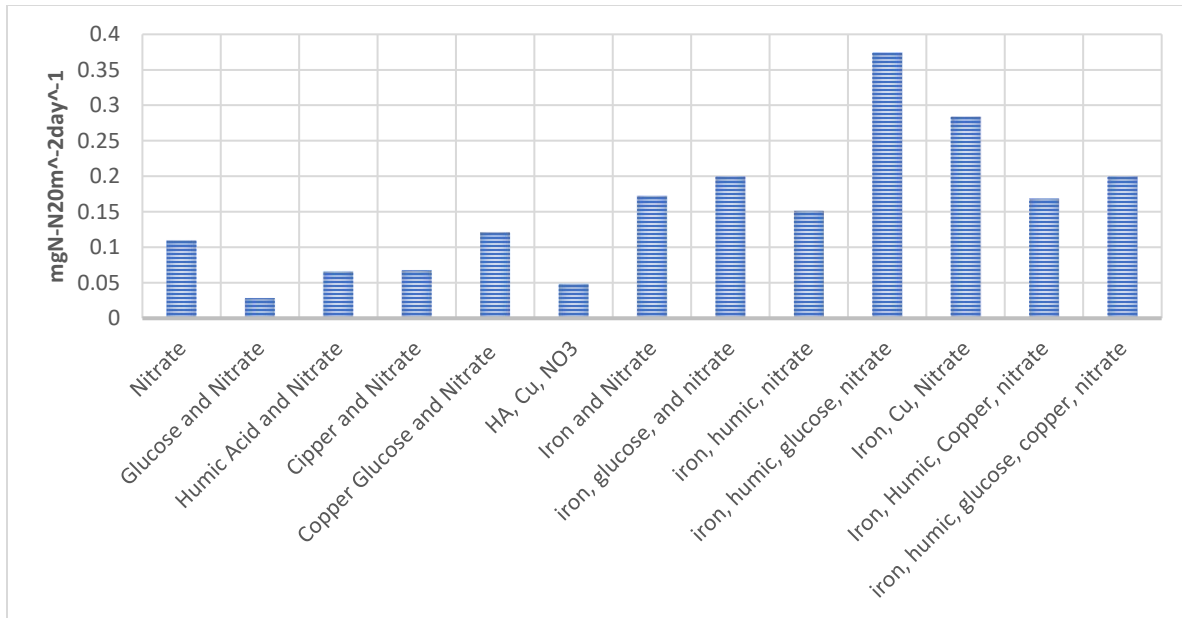m = molecular weight of N₂O
V_m = molecular volume of N₂O

Figure 10 shows the flux of N₂O for each treatment

Yield and Total Denitrification

Based on these yield calculations, it is expected that if carbon and copper are acting as a control on denitrification that in scenarios where Cu and C are available the yield will be lower and total denitrification will favor N_2. In Figure 10, the treatment with the highest total denitrification was treatment 10. Treatment 10(Fe, HA, G, NO3) had no additional Cu in it and therefore it was expected that while denitrification would still occur due to the plentiful carbon and nitrate source that the lack of Cu may encourage more incomplete denitrification.
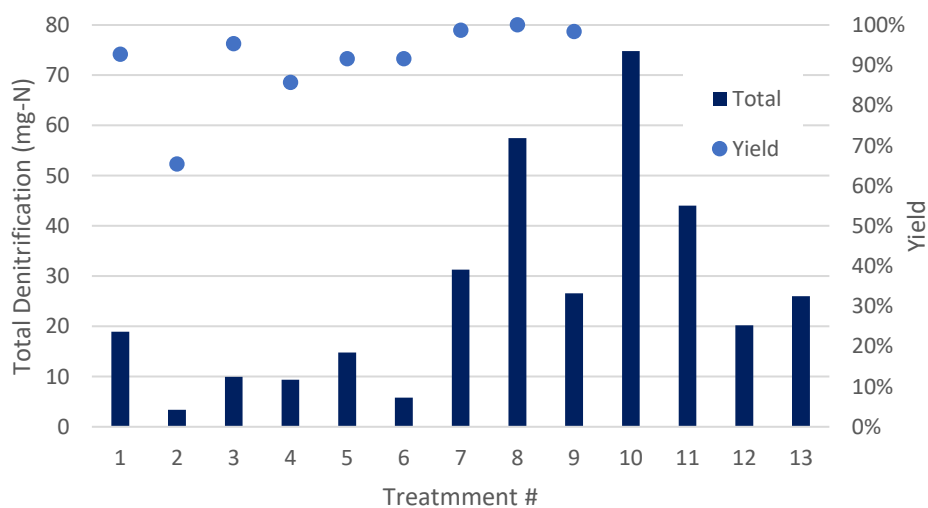

Figure 11. The total denitrification (bar graph) and yield (scatter plot) are shown for each treatment where Treatment 10 shows the highest total.

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