

INTACT SOIL CORE EXPERIMENTS REVEAL THAT TEMPERATURE AND DEPTH INFLUENCE MICROBIAL  
COMMUNITY FUNCTION AND IMPACT THE FATE OF NITROGENOUS FERTILIZER AMENDMENTS

BY

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THESIS

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## Abstract

The application of nitrogenous fertilizer to agricultural soil has potentially large impacts on environmental quality, including large scale emission of the potent greenhouse gas nitrous oxide and runoff of nitrates. Through their metabolic activities, including nitrification, denitrification and dissimilatory nitrate reduction to ammonium (DNRA), soil microbial communities play an active role in determining the fate of nitrogenous species, thus thorough knowledge of microbial community structure and function is important to understanding ecosystem scale N-fluxes. While much work has been done to understand the role of microbes in soil N-cycling, rarely are such communities studied in ecological context of depth and temperature combined and we hypothesize that diurnal temperature variation, large in surface soils and more narrow at depth, will result in a differential response to N-input. To investigate both the fate of exogenous N-inputs and microbial community and functional response, intact soil cores (0-10 cm and 10-30 cm depth) taken from an agricultural field site in Havana, IL were imbibed with  $^{15}\text{N}$  labelled  $\text{NH}_4\text{Cl}$  and  $\text{KNO}_3$  to simulate fertilizer input. The unsaturated cores were then incubated under a summer diurnal temperature regime reflective of natural temperature profiles observed at specified depths (20-35°C shallow, 22-24°C deep). At nine time points over 21 days, replicate cores were destructively sampled for pH,  $\text{NO}_3^-$ ,  $\text{NH}_4^+$ , and  $^{15}\text{N}$  isotopic analyses. Stable isotope pool dilution was used to calculate gross transformation rates and microbial community structure and function were assessed by T-RFLP and qPCR, respectively. Comparison of 16S rRNA based T-RFLP total community profiles revealed a distinct temporal succession across soil depths, with shifts in community structure corresponding to changes in soil  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentration. While no significant difference in 16 rRNA based communities was evident between depths, a large temporal shift in nitrous oxide reductase gene (*nosZ*) based denitrifier community structure was evident after 7 days, resulting in denitrifier communities that were significantly distinct. Additionally, qPCR analysis of relevant functional gene transcripts reveals differential trends in gene expression between depths. These results

suggest that while diurnal temperature variation and depth may not significantly alter overall community structure, the functional response of community members to N-input is a function of temperature and these functional differences between soil communities has a direct impact on not only rates of transformation, but also for the timing and magnitude of N<sub>2</sub>O fluxes.

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## Introduction

The subject of anthropogenic climate change is well documented and well publicized. Most of the current conversation on this matter focuses on the effects of increased atmospheric loading of carbon dioxide (CO<sub>2</sub>). However, atmospheric nitrous oxide (N<sub>2</sub>O) concentrations have also been steadily on the rise for the last 250 years (Machida et al. 1995) and at no time in history have concentrations been higher than they are at present and atmospheric concentration of N<sub>2</sub>O continue to rise at an estimated rate of 0.2-0.3% per year. This is particularly alarming as N<sub>2</sub>O is a potent greenhouse gas (GHG), and though it accounts a significantly smaller portion (0.03%) of total yearly GHG emissions than CO<sub>2</sub>, it has a 300 times greater radiative capacity. Expressing this in The Intergovernmental Panel on Climate Change approved units of CO<sub>2</sub> equivalents, N<sub>2</sub>O accounts for 10% of global emissions (IPCC 2014). The increase in atmospheric N<sub>2</sub>O levels can be largely attributed to agricultural intensification and the use of nitrogen based fertilizers to achieve maximal crop yield with 65% of global N<sub>2</sub>O emissions coming from soil (Fields, 2004). The nitrogen cycle is arguably the best characterized of all elemental cycles, and anthropogenic manipulation via fertilizer use has largely altered the natural N budget. The application of nitrogenous fertilizer has skewed the cycle such that agricultural soils may act as a source rather than a sink for N<sub>2</sub>O.

Once applied to soil, the fate of N-fertilizer depends largely on the role of soil microorganisms (bacteria, fungi and archaea). Modern nitrogen fertilizer is often applied as Ammonium Nitrate which supplies inputs to all major microbial N metabolisms (Figure 1). The ammonium ion (NH<sub>4</sub><sup>+</sup>) is readily taken up by plants and is also relatively easily retained in soil due to its cationic nature however; NH<sub>4</sub><sup>+</sup> can also be readily oxidized to nitrite and nitrate by soil microorganisms through the process of nitrification. During the first step of nitrification the enzyme ammonia monooxygenase amoA, oxidizes ammonium to hydroxylamine (Hollocher, Tate, & Nicholas, 1981). A diverse collection of microorganism

are capable of this activity, both from domain bacteria (AOBs) and domain Archaea (AOAs). Recently it has been widely recognized that AOA are often the primary drivers of ammonia oxidation in a broad range of habitats (Francis et al., 2005; Wuchter et al., 2006), including soil (Gubry-Rangin, Nicol, & Prosser, 2010; Leininger et al., 2006). While providing an important link between reduced and oxidized N-compounds in the global nitrogen cycle, it has been shown that organisms capable of nitrification can often contribute significantly to the emission of  $\text{N}_2\text{O}$  from soils (Kool et al., 2011; Wrage et al., 2001).

In contrast to  $\text{NH}_4^+$ , the  $\text{NO}_3^-$  anion is more poorly retained in soil and can be lost due to leaching, leading to eutrophication of surface waters.  $\text{NO}_3^-$  is also subject to microbial metabolic process including denitrification, whereby  $\text{NO}_3^-$  is converted to gaseous products ( $\text{N}_2$  and/or  $\text{N}_2\text{O}$ ) and consequently lost from the system. In the final step in denitrification  $\text{N}_2\text{O}$  is reduced to  $\text{N}_2$  via the nitrous oxide reductase enzyme NosZ (Coyle, et al. 1985). Until recently, NosZ encoding genes were thought to be harbored solely by denitrifiers (typical *nosZ*) however, recent phylogenetic work has revealed a second, atypical NosZ clade, encompassed a wide variety of microorganisms, including those lacking other genes in the denitrification pathway (Sanford et al., 2012). This enzyme is not present in all denitrifying bacteria, however, and no fungal denitrifiers are known to reduce  $\text{N}_2\text{O}$  to  $\text{N}_2$  (Shoun et al. 1992) leading to incomplete denitrification and a large microbial population unable to effectively close the nitrogen cycle, resulting in a source of  $\text{N}_2\text{O}$  emissions. By contrast, the process of dissimilatory nitrate reduction to ammonia (DNRA) is capable of producing  $\text{NH}_4^+$  from  $\text{NO}_3^-$  through the catalytic activity of a dissimilatory nitrite reductase encoded by the *nrfA* gene (Einsle, 2011), thereby reducing GHG emissions and increasing N-retention. DNRA can thus be viewed as a favorable reductive pathway for  $\text{NO}_3^-$ . In the majority of agricultural systems, plant productivity and consequent C sequestration capacity is governed by soil N concentrations (Reich et al., 2006). N availability and C utilization in the soil system are intrinsically linked and N turnover in soil has implications not only for the global N cycle but also the biogeochemical cycling of C in terrestrial systems.

Denitrifiers and DNRA bacteria often coexist in terrestrial environments and compete for and utilize the same resources including organic material, electron donors and electron acceptors (Tiedje, 1988). It is therefore important to understand the relative contributions of both DNRA bacteria and denitrifiers to global nitrogen budgets as they play important roles in not only GHG emission but overall soil productivity. At present, the interactions between the two pathways and the environmental parameters favoring one pathway over the other remain relatively poorly understood. However, recent work suggests that certain environmental parameters such as C:N ratio, temperature and pH (Yoon et al., 2014) as well as nitrite (Yoon, Sanford, & Löffler, 2015) impact the reduction products of  $\text{NO}_3^-$  in pure culture, supporting the findings by Schmidt et al. (2011) that DNRA potential is related to  $\text{NO}_2^-$  concentrations, pH, sand content and bulk density in temperate arable soils. While it has been observed that in some soil ecosystems, particularly tropical regions, DNRA is the dominate  $\text{NO}_3^-$  reduction pathway (Silver et al. 2001; Templer et al. 2008), few studies to date have explored the importance of DNRA to total nitrate reduction in agricultural systems (Rütting et al. 2011). It was observed by Inselsbacher et al. (2010) that rates of DNRA relative to denitrification were negligible in two separate soil types. However it can be noted that based upon the findings of Schmidt et. al (2011), the environmental conditions may not have been conducive to DNRA, as both soils displayed a low sand content, and one soil was slightly acidic. The study also utilized sieved and homogenized soil, which has been shown to alter microbial community structure and respiration rates thus potentially impacting community functionality (Thomson et al., 2010), as such measured rates may not be truly reflective of *in situ* processes.

Much work has been focused on understanding the relationships between microbial community structure and their relationship to function (Balser & Firestone, 2005; Harter et al., 2014; Waldrop, Balser, & Firestone, 2000) as this is of particular importance if we desire to use community structure as a proxy for process.. However, microbial communities tend to be highly variable at the landscape, and

even field scale (Franklin & Mills, 2003) as even small changes in environmental conditions exert selective pressure. It has been observed that soil depth is an important parameter when considering the structural variability of microbial communities (Fierer et al., 2003; Griffiths et al., 2003). Jeffery et al. (2007) noted that significant variations in community structure can even be observed between the upper most centimeter of an arable soil and the soil below. With increasing depth, soils tend to become more oligotrophic and experience a more narrow range in diurnal temperature variation. While soil depth and temperature exert control over the structure and potentially function of indigenous microorganisms, these communities are rarely studied in the ecological context of depth and temperature combined.

In order to further our understanding of the community dynamics and function of microorganisms involved in N-cycling in an agricultural soil in response to anthropogenic N-loading, this study aims to simulate *in situ* field conditions by utilizing intact soil core incubations while maintaining the ecological relevance of both temperature and depth. It is hypothesized that differences will exist between surface and subsurface soils in both the structure of active soil communities as determined by terminal restriction length polymorphisms (T-RFLP) and function as assessed by analysis of relevant N-cycling gene expression and gross N-transformation rates.

## Materials and Methods

### *Site description and sample collection*

A total of 75 intact soil cores were taken from Central Illinois Irrigated Growers Association Research Plot, Havana, IL. The dominant soil series at the Havana site is Bloomfield (sandy, mixed, mesic Lamellic Hapludalfs) sand, and characteristics are fully describing in Table 1. All cores were taken at random to a depth of 30 cm from 3 m radius around a centroid in a section of the field under soy bean planting. Following core collection, individual cores were sectioned into shallow and deep sections (0-10 and 10-30 cm, respectively) on site utilizing a hand saw and transported at ambient temperature to the laboratory for analysis. Upon arrival at the laboratory, shallow and deep cores were placed into appropriate growth chambers under diurnal temperature regimes reflective of natural temperature profiles observed at each depth (20-35°C and 22-24°C for shallow and deep, respectively) and allowed to equilibrate for 6 days prior to the start of the experiment.

At the start of the experiment, cores were removed from their growth chambers and imbibed with a solution of 5 mM  $\text{KNO}_3$  and 10 mM  $\text{NH}_4\text{Cl}$ . Two sets of cores for each depth were created with the  $^{15}\text{N}$  label being either on the  $\text{NO}_3^-$  or  $\text{NH}_4^+$  (99 atom% excess). The solution was applied slowly from the bottom up via syringe fitted with plastic tubing securely attached to the bottom of the core to avoid disruption of the soil matrix and ensure uniform distribution of the label. Solution was pushed through the core until it visibly reached the top, and the syringe was then withdrawn to remove excess moisture to avoid induction of artificial anaerobiosis and maintain soil moisture near field conditions, after which the cores were fitted screened bottom cap to prevent soil loss but allow for drainage of excess fluids. Control cores received no fertilizer amendment but were imbibed with the same irrigation water used to make the nitrogenous solutions. Core mass was taken pre and post application. To avoid sample desiccation all cores were placed into racks and secondary bins lined with damp paper towel which was

remoistened as needed, approximately every other day before being placed into appropriate growth chambers (20-35°C for the shallow cores and 22-24°C for the deep cores) for the duration of the experiment. Cores were destructively sampled by in triplicate at 9 time points for experimental samples (0, 3, 10, 24, 48 and 72 hours and 7, 14 and 21 days) and 4 time points for controls (0, 48 hour, 14 and 21 days) for each treatment ( $^{15}\text{NO}_3$ ,  $^{15}\text{NH}_4$ , control) by transferring soil from the cores into Whirl Pak bags and briefly homogenizing. 24 hour before sampling, cores were placed into gas tight vessels constructed from PVC piping fitted with a rubber stopper gas sampling port.

### *Soil chemistry*

At each of the nine sampling time points soil  $\text{NO}_3^-$  and  $\text{NH}_4^+$  concentrations were determined using KCl extraction. 22.0 ( $\pm 0.5$ ) g of wet soil was placed into 100 mL of extra pure 2.0 M KCl and placed on a shaker for one hour at 200 rpm before being filtered through KCl washed Whatman filters. Liquid extracts were stored frozen until analysis utilizing a Lachat Flow Injection Analysis System. Soil pH was measured by placing 1.0 g (wet weight) soil into a glass vial containing 10 mL deionized water. Soil-water solution was allowed to equilibrate overnight before taking pH measurement using a pH probe.

Gravimetric water was determined for each sample by placing 5.0 g wet soil into an aluminum weigh boat and oven drying until constant mass was reached. Gaseous samples were taken for 24 hour gas flux analysis by removing approximately 15 mL headspace gas into evacuated gas tight vials. Trace gas concentrations was performed utilizing Shimadzu GC-2014 equipped with electron capture detector for  $\text{N}_2\text{O}$ , thermal conductivity detector for  $\text{CO}_2$ , and flame ionization detector for  $\text{CH}_4$ .

### *Isotopic Analysis*

Soil KCl extracts were prepared for isotopic analysis utilizing the diffusion method described by Davidson et al. (1991) and Hart et al. (1994). Briefly 40 mL, or enough to obtain approximated 100  $\mu\text{g}$  N KCl extract was added to a sample cup containing an acid washed glass bead and the pH of the solution

was raised via addition MgO to allow for the conversion of aqueous  $\text{NH}_4^+$  to  $\text{NH}_3$  gas. The diffused gas was trapped on an acidified paper disk suspended above the liquid. Samples were allowed to diffuse for 7 days and paper disks were placed into a desiccator for 24 hours. After removal of  $\text{NH}_4^+$ , Devarda's alloy was added to the solution to reduce  $\text{NO}_3^-$  to  $\text{NH}_4^+$  which was then diffused as previously described. Once dried, the paper disks were rolled into tin foils for analysis. The samples were measured for  $^{15}\text{N}$  atom percent using an Elementar Vario Cube (Hanau, Germany) elemental analyzer connected to an IsoPrime 100 isotope ratio mass spectrometer (Cheadle Hulme, UK). 8 atom% excess standards of varying concentrations were measured after every 8 samples. Measurements were corrected for instrumental drift by determination of the k-factor for each sample via interpolation of standard measurements.

#### *Rate Calculations*

Gross mineralization,  $\text{NH}_4^+$  consumption, nitrification and  $\text{NO}_3^-$  consumption were calculated using isotope pool dilution equations as described by Hart et al. (1994) and Kirkham & Bartholomew (1955):

$$m = \frac{M_0 - M_t}{\Delta t} - \frac{\text{Log}\left[\frac{H_o M_t}{H_t M_o}\right]}{\text{Log}\left[\frac{M_o}{M_t}\right]}$$

And

$$c = \frac{M_o - M_t}{\Delta t} \left[ \frac{\text{Log}(H_o H_t)}{\text{Log}(M_o / M_t)} \right]$$

Where  $M_o$  is equal to the initial  $^{14+15}\text{N}$  pool ( $\mu\text{gN gdw}^{-1}$ ),  $M_t$  is equal to post incubation  $^{14+15}\text{N}$  pool ( $\mu\text{gN gdw}^{-1}$ ),  $H_o$  is the initial  $^{15}\text{N}$  pool in atom percent excess (APE),  $H_t$  is the post incubation  $^{15}\text{N}$  pool (APE) and  $\Delta t$  is the time elapsed.  $m$  is the gross process (mineralization or nitrification) rate and  $c$  is the consumption rate.

Rates of DNRA were calculated according to Silver et al., (2001) using the following equations accounting for the rate of transport of  $^{15}\text{NH}_4$  into the  $^{15}\text{NH}_4$  pool, change of the  $^{15}\text{NO}_3$  pool over time and turnover of the  $^{15}\text{NH}_4$  pool:

$$^{15}\text{AC}_{\text{atm}} = ((\text{average } (^{15}\text{A}_{0\text{m}}, ^{15}\text{A}_{\text{fm}}) * \Delta t / M + ^{15}\text{A}_{\text{fm}}) / \text{A}_{\text{fm}}) * 100$$

Where  $^{15}\text{AC}_{\text{atm}}$  = Corrected final  $^{15}\text{NH}_4$  in atom %,  $^{15}\text{A}_{0\text{m}}$  =  $^{15}\text{NH}_4$  in  $\mu\text{g/g}$  at the initial sampling point ( $\text{NO}_3$  label experiment),  $^{15}\text{A}_{\text{fm}}$  =  $^{15}\text{NH}_4$  in  $\mu\text{g/g}$  at the final sampling point ( $\text{NO}_3$  label experiment),  $\Delta t$  = the time period of the experiment,  $M$  = the mean residence time of the  $\text{NH}_4$  pool, assumed to be 1 day,  $\text{A}_{\text{fm}}$  =  $\text{NH}_4$  in  $\mu\text{g/g}$  at the final sampling point ( $\text{NO}_3$  label experiment). And

$$\text{DNRA} = ((^{15}\text{AC}_{\text{atm}} - ^{15}\text{AC}_{\text{na}}) / 100 - ^{15}\text{A}_{0\text{atm}} / 100) * \text{A}_{\text{cm}} / \text{N}_{\text{catm}} * 100 / \Delta t$$

Where DNRA = Dissimilatory  $\text{NO}_3$  reduction to  $\text{NH}_4$  in  $\mu\text{g/g/d}$ ,  $^{15}\text{AC}_{\text{na}}$  =  $^{15}\text{NH}_4$  natural abundance in atom %,  $^{15}\text{A}_{0\text{atm}}$  =  $^{15}\text{NH}_4$  of the initial pool in atom % excess ( $\text{NO}_3$  label experiment),  $\text{A}_{\text{cm}}$  = average  $\text{NH}_4$  from initial and final sampling periods in  $\mu\text{g/g}$  ( $\text{NO}_3$  label experiment) and  $\text{N}_{\text{catm}}$  = average  $\text{NO}_3$  from the initial and final sampling periods in atom % excess ( $\text{NO}_3$  label experiment).

#### *Nucleic Acid Extraction*

Prior to nucleic acid extraction all glassware and solutions were rendered RNase free by 0.1% DEPC treatment. At each sampling time point 0.5 g (dry weight) soil was placed in 1.0 mL of RNA Later solution (Life Technologies). Preserved soils were stored at  $-20^\circ\text{C}$  until extraction. Based upon soil chemistry results 6 time points for each depth were chosen for nucleic acid extraction: 0, 24, 48, 72 hour and 7 and 21 day for shallow cores and 0, 24, 72 hour and 7, 14 and 21 day for deep cores. Prior to nucleic acid extraction, RNA Later preservative was removed via centrifugation at 2500 rpm for 5 minutes. Supernatant was removed and discarded. To ensure thorough removal any residual preservative, the soil was washed with 1.0 mL phosphate buffered saline. After rinsing, soil was

transferred to Lysing matrix tube E (MP Biomedicals) for nucleic acid extraction. To eliminate bias introduced by independent nucleic acid extraction DNA and RNA were coextracted utilizing a method modified from Griffiths et al. (2000) . Briefly 0.5 µL CTAB extraction buffer, 0.5 µL phenol:chloroform:isoamyl alcohol pH 8.0 and 10.0 µL β-mercaptoethanol (Töwe et al., 2011) were added to the extraction tube containing washed soil. Soil samples were disrupted for 2 minutes utilizing Disruptor Genie (Scientific industries) and nucleic acids extracted 2X with chloroform:isoamyl alcohol. All steps were performed on ice. One volume of 20% PEG-6000 in 2.0M NaCl with the addition of 1.0 µL 20 mg/mL RNA grade glycogen (Sigma Aldrich) was utilized for nucleic acid precipitation (-20°C for 2 hours). Nucleic acids were pelleted via centrifugation at 14000 rpm for 15 minutes at 4°C, washed with ice cold ethanol and resuspended in 100 µL of RNase free water. DNA portions were quantified using a Qubit 2.0 fluorometer. A 36 µL portion of the total extract was removed to generate the RNA fraction by treatment with 0.25 units of DNase 1 (Sigma) for 15 minutes at 37°C, followed by enzyme inactivation following manufacturer's protocols. Complete removal for DNA from the RNA fraction was verified via 16S rDNA PCR of the RNA fraction, with no PCR product detected via agarose gel electrophoresis and subsequent staining with ethidium bromide.

#### *Reverse transcription*

Reverse transcription as performed utilizing Life Technologies High Capacity Reverse Transcription Kit following manufacturer's protocols for randomly primed cDNA with the addition of 1.0 U/µL SUPERase• In™ RNase Inhibitor (Life Technologies) and 25 µg/mL T4 gene 32 protein (Roche) to improve reaction efficiency (Villalva et al., 2001). Gene specific cDNA was generated by substituting 0.5 µL of 10 µM primer (Table 2) for the supplied random hexamers. To decrease the incidence of mis-priming and generation of random cDNAs, bacterial and archaeal *amoA*, typical and atypical *nosZ* and *nrfA* primers were combined with RNA, incubated for 5 minutes at 65°C and immediately placed on ice

prior to the addition of the remaining reagents, after which the reaction was incubated for 2 hours at 46°C. Bacterial 16S cDNA was prepared without the additional high temperature incubation. cDNA was stored at -20°C until further analysis was completed.

#### *T-RFLP*

Bacterial 16S rDNA gene and 16S rRNA based community analysis was performed utilizing T-RFLP. Additionally, denitrifier community structure was monitored using typical *nosZ* gene and cDNA based T-RFLP. For each sample and target, independent PCR reactions were performed on 1:20 diluted DNA and undiluted cDNA. To each 50 µL reaction 1.0 µL template, 25 µg/mL T4 gene 32 protein (Roche), 0.5 mM each dNTP, 0.5 uM forward and reverse primer (Table 2), 1X supplied PCR Buffer, 0.025 U/µL TaKaRa Ex Taq DNA polymerase (Clontech) and 37.5 µL sterile water were added. 16S PCR amplification was performed with a 5 minute 94°C initial denaturation step followed by 25 cycles of 95°C for 90 seconds, 55°C for 90 seconds, 72°C for 90 seconds and a final extension step at 72°C for 5 minutes. Typical *nosZ* amplification was performed utilizing a touchdown cycling method: following initial denaturation at 94°C for 5 minutes, 10 cycles of 95°C for 30 seconds, 65°C for 90 seconds and 72°C for 120 seconds were performed with the annealing temperature decreasing 0.5° per cycle, after which 30 cycles of 95°C for 30 seconds, 57°C for 90 seconds and 72°C for 120 seconds were added with a final extension at 72°C for 5 minutes. Due to problems with the reverse transcription and PCR amplification resulting in a lack of temporal resolution, atypical *nosZ* T-RFLP was not performed. After verification of amplification via agarose gel electrophoresis, PCR products were cleaned using Qiagen PCR purification kit according to manufacturer's protocols. 5 µL of purified PCR product was then digested for 12 hours utilizing *HaeIII* (37°C) or *BstUI* (60°C) for 16S and *nosZ* respectively and supplied Cutsmart Buffer (New England Biolabs). Fragment analysis was performed at the Roy J. Carver Institute for Biotechnology via capillary electrophoresis with a 60 second injection time and ROX 1000 sizing standard.

### *Statistical Analysis*

T-RFLP profiles were processed on the basis of size in base pairs and peak height using GeneMapper v3.7 (Applied Biosystems). Peaks smaller than 50 bp and larger than 1000 bp in length were excluded from further analysis. Blank wells containing only water were used to determine background fluorescence, and a threshold height of 80 was set, below which the signal was indistinguishable from noise. For each sample, peak height was transformed to relative abundance by dividing the height of each peak by the sum height of all peaks in the sample. T-RFs with relative abundance less than 1.0% were excluded from further analysis. To determine the most active populations in each sample, the relative abundance of the cDNA generated T-RFs were scaled to the corresponding T-RFs from DNA fractions for the same sample. Further analysis was performed utilizing Primer v6.0 (Primer-E). To remove weighting of highly abundant T-RFs, all data were square root transformed prior to the generation of Bray-Curtis dissimilarity matrices which were analyzed utilizing 2 dimensional multidimensional scaling (MDS) plots. Relationships between community structure and time, depth and treatment were explored using ANOSIM analysis of dissimilarity, where  $R \geq 0.75$  delineates clearly distinct communities,  $R \geq 0.5$  represents communities that are overlapping, but still distinct and  $R \leq 0.25$  represents communities that are indistinguishable.

### *qPCR*

Bacterial 16S rDNA gene copies and bacterial and functional gene transcripts were quantified utilizing quantitative PCR. *nrfA* transcripts were undetectable throughout the course of the experiment, thus *nrfA* qPCR was performed on DNA, and solely gene copies will be further reported. All reactions were performed in triplicate utilizing SYBR green detection on Aligent Technologies' Mx3000P (formerly Stratagene) platform. With the exception of *nrfA* where primer concentration was increased to 0.8  $\mu\text{M}$ , all 25  $\mu\text{L}$  reactions contained 0.4  $\mu\text{M}$  forward and reverse primer (Table 2), 25  $\mu\text{g/mL}$  T4 gene 32 protein

and 1X TaKaRa SYBR Premix Ex Taq II (Tii RNaseH Plus) (Clontech). Cycling conditions for each target are provided in Table 3, with plate read after the extension time in each cycle and melt curve analysis performed following reaction completion. All targets were quantified via standard curve. Reactions displaying uncharacteristic melt curves, such as those indicative of primer dimers and inappropriate product were excluded from further analysis. To account for any variation in the efficiency of nucleic acid between samples, all gene copy and transcript copy numbers were normalized to ng DNA per reaction.

## Results

### *Soil Chemistry*

$\text{NH}_4^+$  declined slowly in deep cores from an average of 19.12  $\mu\text{g-N/g}$  with 50% loss occurring by day 7 and falling below detection at day 21 while  $\text{NO}_3^-$  increased from 11.54  $\mu\text{g-N/g}$  to 27.95  $\mu\text{g-N/g}$  by day 14 followed by little decline thereafter. Similar trends were observed in shallow cores though  $\text{NH}_4^+$  loss was more rapid, with 50% lost over the first 48 hours before falling below detection at after day 14 and maximum  $\text{NO}_3^-$  (35.12  $\mu\text{g-N/g}$ ) was observed at day 14, by day 21,  $\text{NO}_3^-$  concentrations had decline significantly ( $p < 0.5$ ) to concentrations close to starting values (Figure 2). Additionally soil pH declined from throughout the course of the experiment in both shallow and deep cores treated cores, transitioning from circumneutral to slightly acidic at day 14, with significant differences emerging in deep cores between treated (pH 6.48) and controls (7.63 pH) at day 21. No significant change in pH was observed over the 21 day incubation in untreated control cores (Figure 3).

### *Nitrous Oxide Flux*

$\text{N}_2\text{O}$  fluxes from shallow and deep cores were variable in both timing and magnitude. Shallow cores ( $n=3$ ) displayed low levels of  $\text{N}_2\text{O}$  emissions ( $\leq 0.39 \pm 0.03 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$ ) until day 3 when peak emissions of  $7.17 \pm 2.0 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$  were observed with decreasing fluxes thereafter until day 21 when a flux of  $0.08 \pm 0.01 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$  occurred (Figure 4). Deep core fluxes were negligible until day 3, when a flux of  $1.25 \pm 0.17 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$  was observed and a peak flux of  $7.41 \pm 2.64 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$  was noted at day 7. In contrast to shallow cores deep core  $\text{N}_2\text{O}$  flux, while decreasing from their day 7 high, remained elevated compared to earlier time points, though it can be noted that the magnitude of these fluxes were more variable between replicate treatments for both day 14 and day 21 ( $2.19 \pm 1.49$  and  $1.8 \pm 1.71 \text{ ngN- N}_2\text{O gdw}^{-1} \text{ day}^{-1}$ , respectively).

### *Gross transformation rates*

Due to the decline in ammonia concentrations beyond day 7, gross transformation rates could only be determined through 72 hours. A full list of transformation rates is providing in Table 4.

Consumption rates of  $\text{NH}_4^+$  exceeded  $\text{NO}_3^-$  consumption over all time intervals measured with the exception of 48 to 72 hours in shallow cores and 24 to 48 hours in deep cores. In shallow cores,  $\text{NH}_4^+$  consumption was nearly twice that of  $\text{NO}_3^-$  consumption over the first 3 hours ( $82.85$  vs  $48.60 \mu\text{gN gdw}^{-1}\text{day}^{-1}$ ) and 10 times higher ( $86.01$  vs  $7.79 \mu\text{gN gdw}^{-1}\text{day}^{-1}$ ) in deep cores, after which rates of  $\text{NH}_4^+$  consumption slowed to a minimum of  $19.16$  and  $11.72 \mu\text{gN gdw}^{-1}\text{day}^{-1}$  between 24 and 48 hours in shallow and deep cores respectively (Table 4). However, gross nitrification rates were less than the rate of  $\text{NH}_4^+$  consumption at all time intervals at both depths, and negative nitrification rates were recorded at two time points in both shallow and deep cores (3 to 10 and 24 to 48 hours shallow, 0 to 3 and 48 to 72 hours deep). In general, however, higher gross nitrification rates were observed in shallow cores. Rates of mineralization (conversion of organic to inorganic-N) were, with the exception of the first 3 hours, higher in shallow cores than deep cores, with values ranging from  $-2.44$  to  $1.099 \mu\text{gN gdw}^{-1}\text{day}^{-1}$  in shallow cores and  $-4.10$  to  $12.90 \mu\text{gN gdw}^{-1}\text{day}^{-1}$  in deep cores, though it can be noted that the highest mineralization rate in deep cores occurred from 0 to 3 hours and all rates were negative thereafter until 48 to 72 hours when the mineralization rate reached  $0.097 \mu\text{gN gdw}^{-1}\text{day}^{-1}$ . Positive DNRA rates were observed at all time points at both depths. Shallow core rates ranged from  $0.255 \mu\text{gN}$  to  $1.74 \text{gdw}^{-1}\text{day}^{-1}$ . Generally speaking, rates observed in deep cores ranging from  $0.124$  to  $1.27 \mu\text{gN gdw}^{-1}\text{day}^{-1}$  were approximately half of that observed in shallow cores, with the exception of 24 to 48 hours when the maximum rate was observed at both depths.

### *16S T-RFLP*

At all time points, no significant distinction existed between depths when bacterial communities were compared using s 16S rDNA gene based ( $R=0.184$ ) or 16S rRNA ( $R=0.1$ ) based T-RFLP (Figure 5). However, it was observed that rDNA gene based community structure was significantly distinct ( $R=0.626$ ) from communities based on rRNA transcripts (cDNA) (Figure 6). When investigating active communities based on the cDNA:DNA ratio of individual samples, both shallow and deep core active communities displayed a significant temporal succession where ANOSIM Global  $R=0.593$  for shallow and  $0.572$  for deep cores (Figure 7). Large shifts in active community structure were observed over the first 24 hours ( $R=0.926$  shallow,  $0.778$  deep) in response to N-input. Between 24 and 72 hour, no significant distinction is observed at either depth. However, communities again show a distinct shift between 72 hours and 7 days ( $R=0.667$  shallow  $0.583$  deep) where community structure is non-distinct from time 0 ( $R=-0.185$  shallow,  $0.148$ ). After day 7 trends in succession of the shallow and deep cores become divergent: shallow core active communities at day 7 and day 21 lack distinction ( $R=0.111$ ) while deep core active communities are significantly distinct between these two time points ( $R=1$ ). In both cases, active community structure at day 21 remains distinct from time 0 ( $R=0.63$  shallow,  $0.963$  deep).

#### *noZ T-RFLP*

At early time points (<7 days), no significant distinction in typical *nosZ* based active communities was observed at either depth. However, at day 7 shallow core active communities become significantly distinct (ANOSIM  $R=1$ ,  $0.1$ ) from all earlier time points (Figure 8). Communities at day 21 retain this distinction; however there is no significant distinction in community structure between days 7 and 21. While similar trends are observed in deep cores, the only significant difference between time points occurs between day 14, 0 hours and 72 hours (significance level=  $0.1$ ). In this case, the communities are not clearly distinct as they are in the shallow cores, but qualify as overlapping but still different. In the case of these later time points, PCR amplification of *nosZ* cDNA fractions resulted in the loss of

replication. Additionally, it is observed that while active *nosZ* based communities show no distinction between depth prior to day 7 ( $R \leq 0.25$ ), differences in community structure between depths begin to emerge at day 7 ( $R = 0.444$ ) with active denitrifier communities being completely distinct between depths ( $R = 1$ ) by day 21 (Figure 9).

#### *16S rDNA qPCR*

Average  $\pm$ SE ( $n=3$ ) 16S rDNA gene copy numbers at time zero were nearly identical for shallow and deep cores,  $8.71 \pm 0.12 \times 10^8$  and  $8.90 \pm 0.61 \times 10^8$  copies/ng DNA respectively. At 24 hours a drastic decrease in gene copy number was observed across both depths, though the magnitude of the decrease was much greater in deep cores than shallow, with a near order of magnitude decrease observed at depth (Figure 10). After the initial decline, 16S rDNA copy numbers began to rebound from the minima observed at 24 hours. Maximum copy numbers were observed at day 7 for shallow cores ( $9.28 \pm 0.46 \times 10^8$  copies/ng DNA) and day 14 for deep cores ( $9.28 \pm 0.152 \times 10^8$  copies/ng DNA). At day 21, copy numbers had decreased from their observed high to values similar to those observed at time 0,  $8.73 \pm 0.62 \times 10^8$  and  $8.85 \pm 0.17 \times 10^8$  copies/ng DNA for shallow and deep cores, respectively.

#### *nrfA qPCR*

Trends in *nrfA* gene copies were significantly different between shallow and deep cores, of all the genes and transcripts assessed in this study, only *nrfA* gene copies were higher in deep cores at all time points sampled (Figure 11). Deep core *nrfA* gene copies increased from an initial value of  $7.63 \pm 0.48 \times 10^6$  copies/ng DNA to a high of  $2.16 \pm 0.12 \times 10^7$  copies/ng DNA by day 7, with the most rapid rate of increase occurring over the first 24 hours. A slight decrease occurred between day 7 and 14 and *nrfA* gene copies remained elevated relative to time zero ( $1.53 \pm 0.06 \times 10^7$  copies/ng DNA) at day 21. Shallow core *nrfA* gene copies displayed a decreasing trend from starting values ( $1.86 \pm 0.17 \times 10^6$  copies/ng DNA) until reaching a minimum of  $3.01 \pm 0.16 \times 10^5$  copies/ng DNA at day 7. By day 21 *nrfA* gene copies had risen back to approximate starting values (Figure 11).

### *nosZ* qPCR

Both shallow and deep core typical *nosZ* transcript abundance increased within the first 24 hours of the experiment from  $2.35 \pm 0.39 \times 10^7$  to  $1.03 \pm 0.06 \times 10^8$  copies/ng DNA and  $4.13 \pm 2.13 \times 10^6$  to  $1.01 \pm 0.87 \times 10^8$  copies/ ng DNA for shallow and deep respectively. In shallow cores, the level of *nosZ* transcripts then remained relatively constant at all time points measured until day 21 when they increased to  $3.60 \pm 1.8 \times 10^8$  copies/ng DNA. In deep cores, however, a significant decrease in transcript abundance occurred at 72 hours, decreasing from their 24 hour high to  $3.93 \pm 1.22 \times 10^6$  copies/ng DNA. From 72 hours to 21 days, transcript levels displayed a slight upward trend, though copies/ng DNA remained on the same order of magnitude through day 14 before reaching  $3.74 \pm 1.79 \times 10^7$  copies/ng DNA at day 21 (Figure 12). Due to unreliable reverse transcription, atypical *nosZ* transcripts could not be quantified.

### *amoA* qPCR

Archaeal *amoA* (AOA) transcripts were significantly higher than bacterial *amoA* (AOB) in both shallow and deep cores at all time points by 3 to close to 4 orders of magnitude. AOA transcript levels in shallow cores remained static until over the first 24 hours, until increasing to a high of  $4.70 \pm 2.25 \times 10^8$  copies/ng DNA at 48 hours (Figure 13). After peaking at 48 hours, AOA transcript levels declined at 72 hours, though they remained elevated compared to time 0 and decreased to a minimum observed value of  $1.65 \pm 0.63 \times 10^7$  copies/ng DNA at day 7. An increase to  $1.41 \pm 0.68 \times 10^8$  copies/ng DNA was recorded at day 21. In contrast, deep core AOA transcripts decreased from  $6.82 \pm 1.80 \times 10^6$  to  $3.24 \pm 1.76$  copies/ng DNA over the first 24 hours before increasing to  $2.98 \pm 0.82 \times 10^7$  copies/ng DNA at 72 hours and remaining static until day 14. By day 21 AOA transcripts decreased to  $1.05 \pm 0.20 \times 10^7$  copies/ng DNA. On average, AOA transcript numbers were an order of magnitude lower in deep cores than shallow with the exception of day 7 when AOA transcripts were greater in deep cores (Figure 13).

AOB transcripts in shallow cores decreased over the first 24 hours from  $3.74 \pm 1.44 \times 10^4$  to a minimum observed value of  $1.46 \pm 0.64 \times 10^4$  copies/ng DNA (Figure 13), followed by an increase with maximum transcript levels reached at 48 hours ( $8.75 \pm 3.5 \times 10^4$  copies/ng DNA). Transcript levels declined slight until day 7, when values were very near levels observed at time zero. At day 21, however, transcript levels were again elevated when compared to time 0 ( $8.28 \pm 6.76 \times 10^4$  copies/ng DNA). In deep cores, no initial decrease from time zero values was observed, and transcript levels increased steadily over 72 hours from  $1.70 \pm 0.02 \times 10^4$  to  $8.45 \pm 4.23 \times 10^4$  copies/ng DNA before declining to  $1.74 \pm 0.13 \times 10^4$  copies/ng DNA at day 7 and apparently remaining constant until day 14. An increase in transcript abundance was observed at day 21 where a value of  $1.39 \pm 0.69 \times 10^5$  copies/ng was observed (Figure 13), though it can be noted that this value was quite variable between biologic replicates.

## Discussion

### *Active Community Structure*

The distinct temporal succession exhibited by active 16S rDNA:cDNA based communities is likely reflective of dynamical changes in soil chemistry, particularly the availability of nitrogenous species and changes in community structure in response to fertilizer amendment have been widely noted (Chu et al., 2007; O'donnell et al., 2001; Yevdokimov et al., 2008) The strong shift in active community structure observed in the first 24 hours after fertilizer amendment also corresponds to a decline in 16S rDNA gene copy number (Figure 10), particularly notable in deep cores. However, during this time there is an upregulation in both *nosZ* across both depths (Figures 14 and 15) and bacterial *amoA* transcripts in deep cores (Figure 15), thus community shifts may be driven by an increase in nitrifiers and denitrifiers at the expense of other community members. Owing to the increase in archaeal *amoA* transcripts at 48 hours, the drop in bacterial 16S rDNA gene numbers may also reflect an outgrowth of archaea, whose transcript abundance would likely show a temporal delay while the population increased in size. Interestingly, at day seven active 16S rDNA:cDNA based communities in both shallow and deep cores are indistinguishable from those at time zero (Figure 7) despite the strong divergence over the first 24 hour and it is also at this time point that active *nosZ* based denitrifier communities are significantly distinct from time 0 (Figure 8). These seemingly contrasting observations suggest that while significant changes can occur in a subset of an entire population, in this case denitrifiers, the structure of the active community as a whole can remain unaltered. Thus observations of total community may not be entirely reflective of structure of functional subsets within that community, and therefore speak little to the potential function of the entire community.

### *Transformation rates potential problems*

Negative gross transformation rates were calculated for all major N-transformations with the exception of DNRA. This is an unexpected result, and would imply that instead of the  $^{15}\text{N}$  product pools becoming diluted, as is the theory of staple isotope pool dilution, that product pools are becoming more enriched with time. Potential reasons for the anomalous results observed in this study include the necessity to destructively sample each core, meaning no time series measurement is a continuous record of measurements from one source, but instead reflect what is happening and has happened to that particular core. Thus inherent heterogeneities between cores may contribute to the observed variability in enrichment between replicates, and could result in an inaccurate estimate of initial pool sizes. Additionally, while every effort was made to ensure uniform distribution of our isotopically labeled solution throughout the entire soil core, it may be possible that in some cases applied the  $^{15}\text{N}$  labeled solution was not uniformly mixed with background N-pools. The importance of uniform label distribution is demonstrated by Davidson et al., (1991), whose sensitivity analysis revealed that the error in calculated transformation rates is exponentially related to the percent of microsites not receiving label.

In addition, problems with analytical solutions to N-transformation determination is their relative simplicity and underlying assumptions, including that once assimilated into biomass, labelled substrate is not remineralized (Kirkham & Bartholomew, 1955). Potential remineralization of assimilated label is often ignored over short time intervals owing to the observation by Bjarnason (1988) that remineralization of immobilized N does not occur for one week. It is likely that the addition of a high amount of mineral N in this study stimulated microbial activity, which may increase rates of remobilization and N-turnover between pools and it has been demonstrated that neglecting remineralization can lead to an underestimation of gross N-transformation rates (Tietema & Van Dam, 1996). To overcome these potential problems advanced numerical tracing models have been developed

(Müller et al, 2014; Müller et al., 2007; Rütting & Müller, 2007) and may provide greater insight into the phenomena observed here.

#### *nrfA* and DNRA

Despite problems with rate determination using isotope pool dilution, and even assuming that all rates are invalid, there is strong evidence that DNRA is the fact occurring in during this experiment. Raw  $^{15}\text{N-NH}_4^+$  data from the  $^{15}\text{N-NO}_3^+$  labeled core experiment show that the atom percent  $^{15}\text{N-NH}_4^+$  is increasing with time (Figure 17). It is then slightly surprising then that *nrfA* transcripts were undetectable at either depth over the course of the experiment, especially in shallow cores where calculated DNRA rates are higher (Table 4) and the  $\text{NH}_4^+$  pool is being enriched with  $^{15}\text{N}$  from a nitrate source. It is also interesting to note that not only are *nrfA* transcripts not detectable, but *nrfA* gene copy numbers are also decreasing over the first 48 hours. It has been observed that the fungi *Fusarium oxysporum* (Zhou et al., 2002) and *Aspergillus nidulans* (Takasaki et al., 2004) are capable of coupling dissimilatory nitrate reduction to ammonium to the oxidation of ethanol under anaerobic conditions utilizing alternate dissimilatory nitrite reductases not assessed in this study. Thus it is thus likely that nitrate ammonifying fungi are playing an active role in N-cycling in this system.

The increasing trend of *nrfA* gene copies in deep cores combined with undetectable *nrfA* transcripts is best explained by an increased abundance of organisms harboring a *nrfA* gene which are utilizing an alternate metabolic strategy to DNRA. *nrfA* genes are relatively ubiquitous environmentally (Mohan et al., 2004) and can be found in a variety of microorganisms spanning  $\delta$ ,  $\epsilon$ , and  $\gamma$ -proteobacteria (Simon, 2002). Notably, the existence of organisms harboring the genetic potential for both DNRA and denitrification has been demonstrated (Mania et al., 2014; Sanford et al., 2012). Given the energetic favorability of denitrification under carbon limiting conditions (Tiedje, 1982) as is often the case in agricultural soils due to large exogenous N-inputs, it can be expected that, under the

experimental conditions of low C:N ratio, denitrification would be the preferred metabolic strategy in both organisms capable of the two distinct  $\text{NO}_3^-$  reduction pathways and nitrate ammonifying and denitrifying populations in general. However, as noted by Strohm et al. (2007) growth yields and ATP generation are lower than thermodynamic calculations alone would suggest for denitrification, potentially due to inefficient energy conservation within the enzymatic machinery involved in denitrification. Such inefficiency may allow for the co-occurrence of DNRA and denitrification observed here. Due to the inability to detect *nrfA* transcripts and quantify rates of denitrification, a direct numerical comparison between the two processes is difficult, however it appears that denitrification is the dominant pathway for nitrate reduction.

#### *Distribution and temporal trends in functional gene transcripts*

The temporal trend evident in the expression of nitrogen cycling genes in this experiment reflects the dynamic relationship between metabolic activity, substrate availability and utilization, and environmental conditions. During the first 72 hours of the experiment, nitrification appears to be the dominant metabolic process as evidenced by rapidly declining  $\text{NH}_4^+$  concentrations and  $\text{NO}_3^-$  accumulation, however at 24 hours a drastic, transient increase in typical *nosZ* transcripts is evident in both shallow and deep cores, corresponding to a 5.45(Figure 14) and 24.5 (Figure 15) fold increase for shallow and deep cores respectively. It is likely that the activity of denitrifiers was stimulated by the addition of moisture to the soil cores. While efforts were taken to maintain field moisture conditions and the aerobic nature of the soil it is possible that anoxic microsites were created, thus promoting denitrification and associated transcripts. It has been reported that episodic increases in denitrification activity and  $\text{N}_2\text{O}$  production can occur after soil wetting events such as rain fall (Sexstone et al. 1988) and thaw events (Priemé & Christensen, 2001). As noted, this increase was highly transient, at the next time point (48 hours and 72 hours for shallow and deep respectively) relative typical *nosZ* expression

was <1 (Figures 14 and 15). It is worth noting that based on recent metagenomics work by Orellana et al., (2014) who demonstrated that that up to 70% gene sequences (DNA based) from the field site in Havana from which samples for this study were taken were classified as atypical. However, it remains to be determined to what extent atypical *nosZ* is actually expressed, as despite numerous efforts to reverse transcribe atypical *nosZ* transcripts from experimental samples, all attempts failed to generate a reliable and reproducible product.

Throughout the course of the experiment, archaeal *amoA* transcripts remained significantly elevated compared to bacterial, often by three orders of magnitude. This is in agreement with several studies in which AOA have been noted to numerically dominate their bacterial counterparts in a variety of ecosystems (Hugoni et al., 2013; Strauss et al., 2014; Wessén et al., 2010), and Leininger et al., (2006) demonstrated that AOA outnumber AOB in both unfertilized and fertilized agricultural soils. The numerical dominance of AOA may be related to their ability to grow mixotrophically or heterotrophically as observed by Jia & Conrad (2009) via stable isotope probing. Jia & Conrad, however, also noted that while numerically more abundant, AOB are more functionally relevant to overall nitrification with bacterial *amoA*, rather than archaeal being correlated to ammonia oxidation activity. Additionally, the upregulation of AOA in response to N-amendment observed here is in contrast to observations by Chen et al., (2013) who demonstrated that *amoA* gene copy number of AOB significantly increased in response to N-input while AOA did not, however, despite the increase in AOB gene copies in response to N-amendment, AOA gene copy number remained at least two orders of magnitude higher, even under the highest fertilizer amendment.

When comparing trends of AOA and AOB relative expression in shallow (Figure 14) and deep cores (Figure 15) it becomes evident that there is a preferential response of either AOA or AOB that is dependent upon depth. In shallow cores, both AOA and AOB are both significantly elevated relative to

time zero by 48 hours, though AOA display a 7.54 fold increase and AOB are only elevated 2.34 fold. In deep cores AOA upregulation begins relative to time zero as soon as 24 hours and at 72 hours both AOA and AOB display a near 5 fold increase. These data then explain the contrasting trends in AOA:AOB ratios between depths at early time points, implying that in shallow cores AOA dominantly respond to N-amendment, while in deep cores there appears to be a mixed response (Figure 16). While studies have attempted to constrain the apparent niche differentiation between AOA and AOB (Adair & Schwartz, 2008; Di et al., 2010; Prosser & Nicol, 2012; Zeglin et al., 2011) there is little consensus and it is likely that a combination of variables including the temperature differences between shallow and deep cores play a role in determination of AOA and AOB activity.

#### *Potential nitrous oxide sources*

Due to insufficient volume of gas recovery, isotopic analysis of produced  $\text{N}_2\text{O}$  could not be determined and therefore the process resulting in its generation cannot be confidently determined. However, differences in both the timing and magnitude of the fluxes were observed between the shallow and deep cores. In shallow cores, the largest flux of nitrous oxide was observed at 72 hours (gas collected between 48 and 72 hours). It is during this time period that transcripts of both bacterial and archaeal *amoA* are at their highest levels and concentrations of  $\text{NH}_4^+$  are in rapid decline. While AOB levels are comparable at 72 hours between shallow and deep cores, AOA transcript levels are notably higher in shallow cores. This may suggest that the larger flux of  $\text{N}_2\text{O}$  from shallow cores at this time is a result of increased ammonia oxidation by archaea.  $\text{N}_2\text{O}$  production by archaea has been observed (Kim et al., 2012; Santoro et al., 2011). While  $\text{N}_2\text{O}$  production via nitrification has been shown to be the dominant source of emissions in some ecosystems, including agricultural soils where it was demonstrated that up to 70% of the  $\text{N}_2\text{O}$  flux could be attributed to nitrification (Stevens et al., 1997). The observation that the magnitude of  $\text{N}_2\text{O}$  flux appears to be more strongly related to the expression of

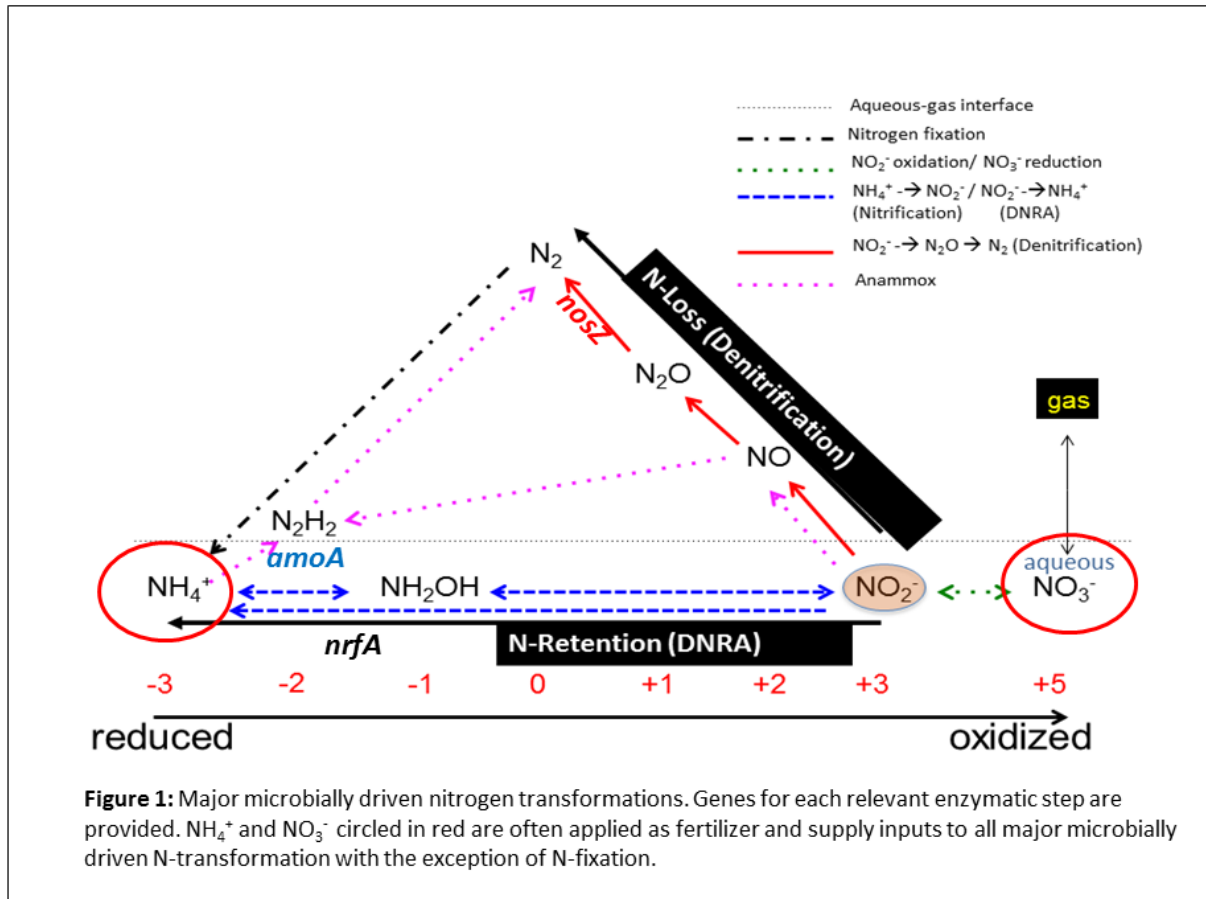
archaeal *amoA* supports the suggestion by Jung et al. (2014) that based both on their ability to produce  $\text{N}_2\text{O}$  and their numerical abundance, archaeal ammonia oxidizers may contribute largely to emissions from soils.

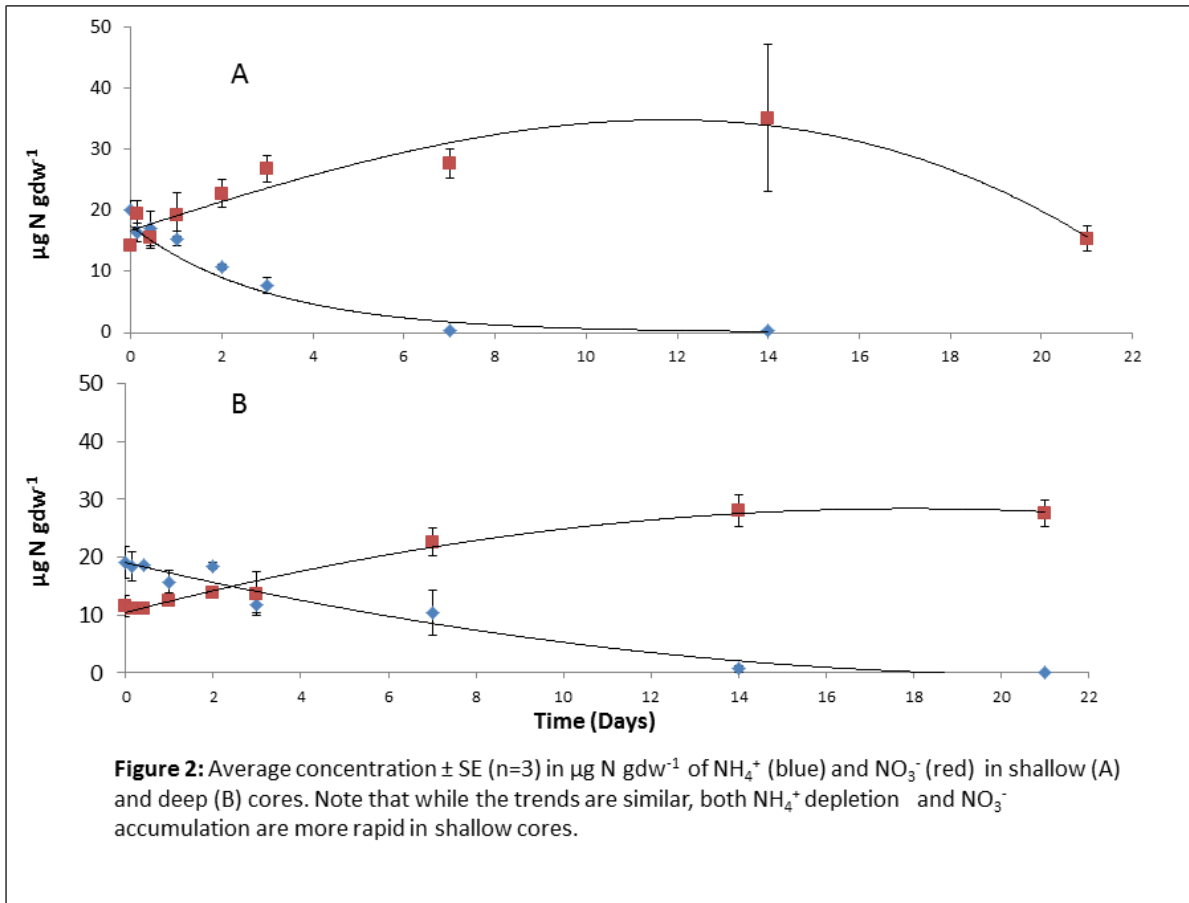
In contrast, the largest flux of nitrous oxide from deep cores occurs at day 7. At this time a large shift in the active typical *nosZ* community structure is observed at both depths, as well as a relative increase in *nosZ* transcripts, suggesting an increase in activity of denitrifiers. However, it is important to note that while active *nosZ* based communities show no distinction between depth prior to day 7 ( $R \leq 0.25$ ), differences in community structure between depths begin to emerge at day 7 ( $R = 0.444$ ) with active denitrifier communities being completely distinct ( $R = 1$ ) by day 21. Taken together, these data suggest that the dominant source of  $\text{N}_2\text{O}$  emissions in deep cores is a result of denitrification. Additionally, these observations imply that a temperature driven response to N-amendment may account for the divergence of denitrifier communities with time, with implications for the timing and magnitude of  $\text{N}_2\text{O}$  fluxes. Studies have shown that temperature has a direct effect on denitrifier community structure (Boulétreau et al., 2014; Braker et al., 2010) as well as the ratio of  $\text{N}_2\text{O}:\text{N}_2$  generated via denitrification (Avalakki et al., 1995). Holtan-Hartwig et al. (2002) demonstrated that in particular the *NosZ* enzyme was more affected by decreasing temperature than other N-species reductases. Thus it is likely that the decreased diurnal temperature variation experienced in deeper soil is a determinant factor when considering gross  $\text{N}_2\text{O}$  flux.

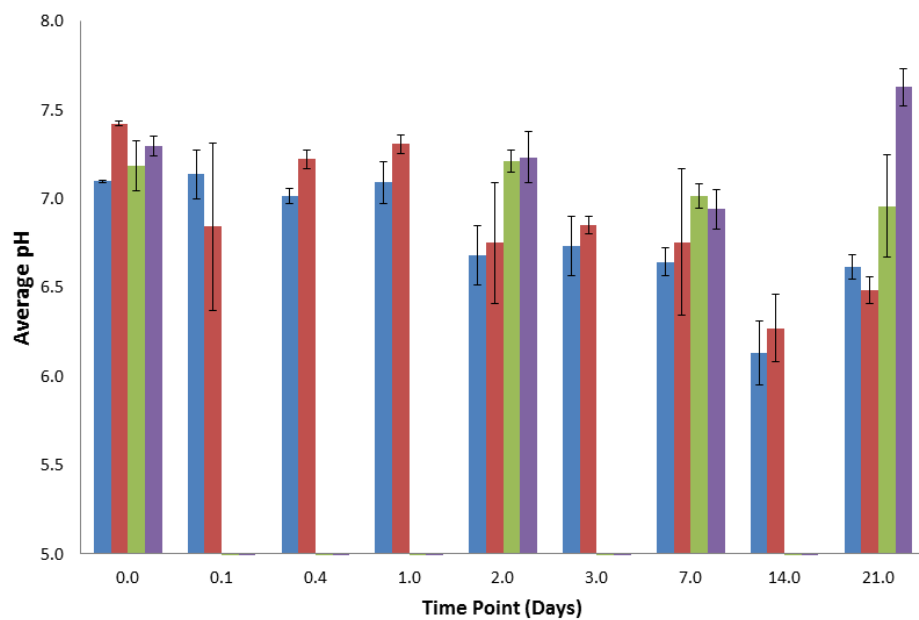
## Conclusions

This study provides insight into the complex dynamics of soil microbial communities both in terms of structure and in terms of function. While it was hypothesized that 16S rRNA based active community structure and function would vary between depths and temperature regime in response to N-amendment, it was shown that no significant differences in community structure resulted. However, active communities did display a distinct temporal succession in response to N-amendment and differential patterns emerged between depths in active denitrifier communities and overall functional gene expression, thus similarities in community structure may not be a predictive of functional similarity. These temperature and depth dependent functional differences directly impacted the fate of N-amendments, with particular implications for both the timing and magnitude of N<sub>2</sub>O fluxes.

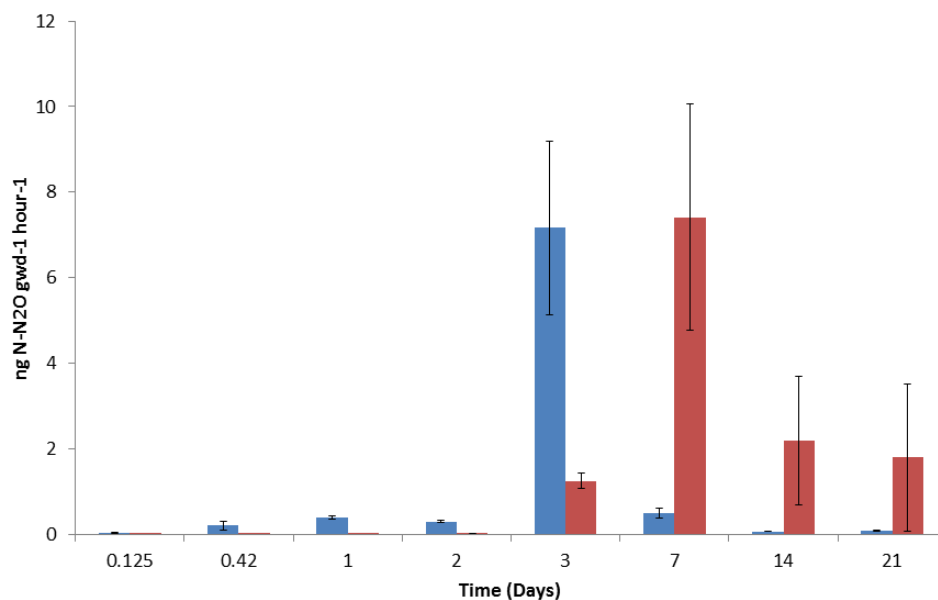
## Figures



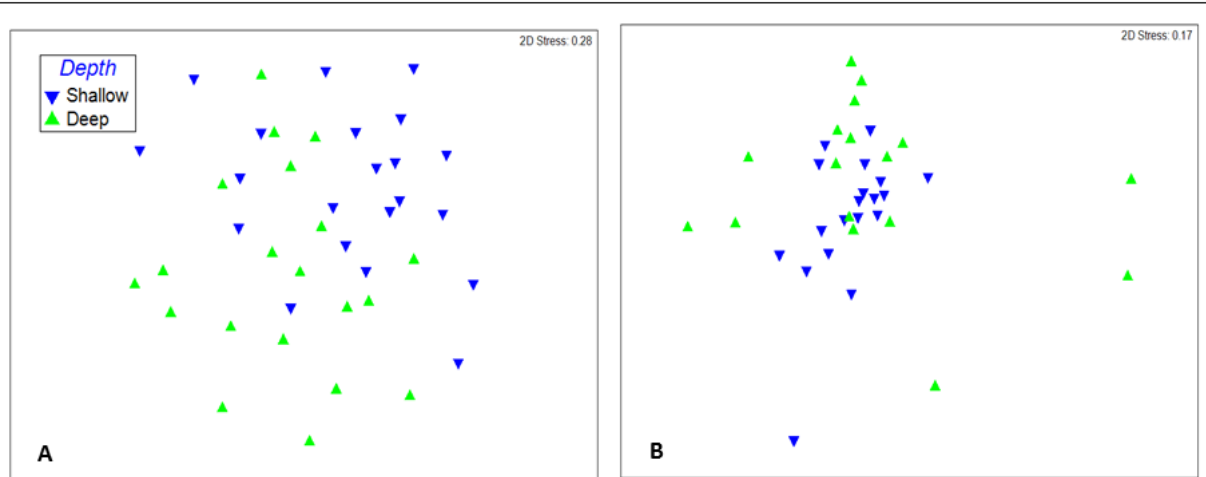




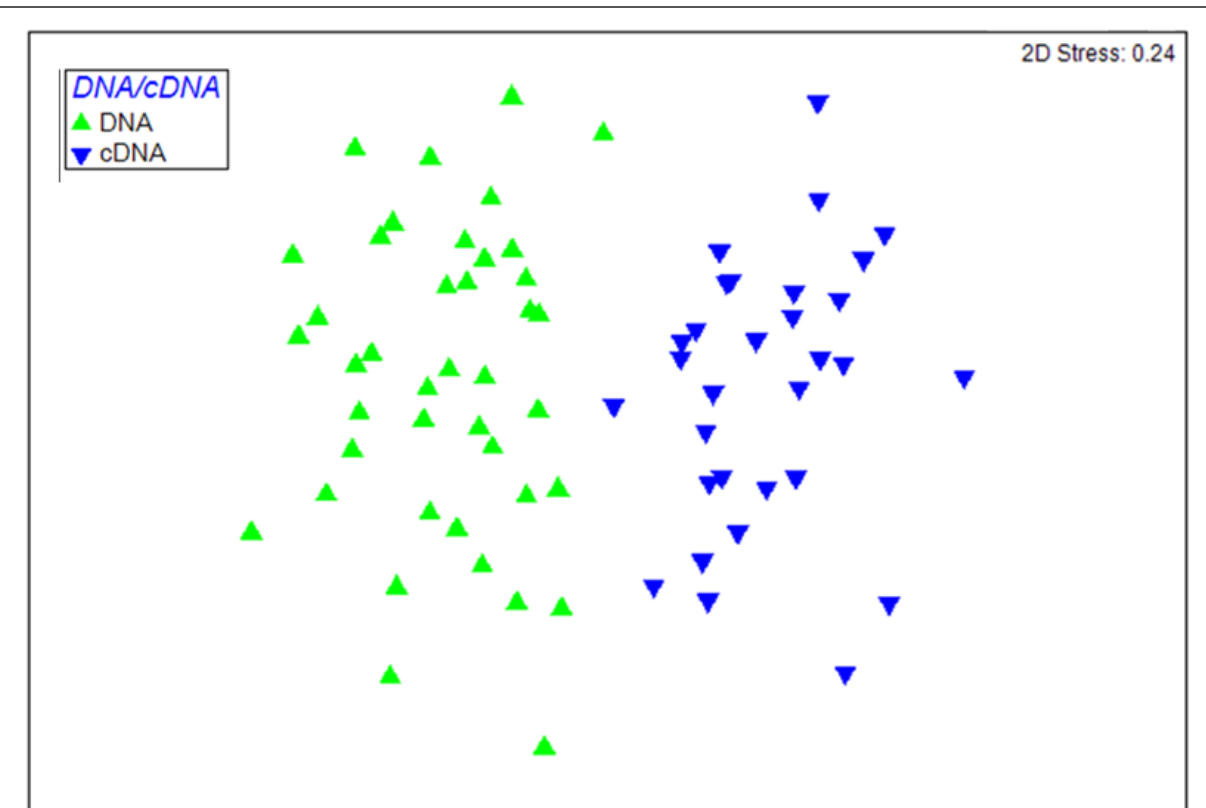
**Figure 3:** Average pH  $\pm$  SE (n=3) of shallow (blue), deep (red) experimental cores. Untreated controls were evaluated at time zero, 2, 7 and 21 days for shallow (green) and deep (purple) core segments.



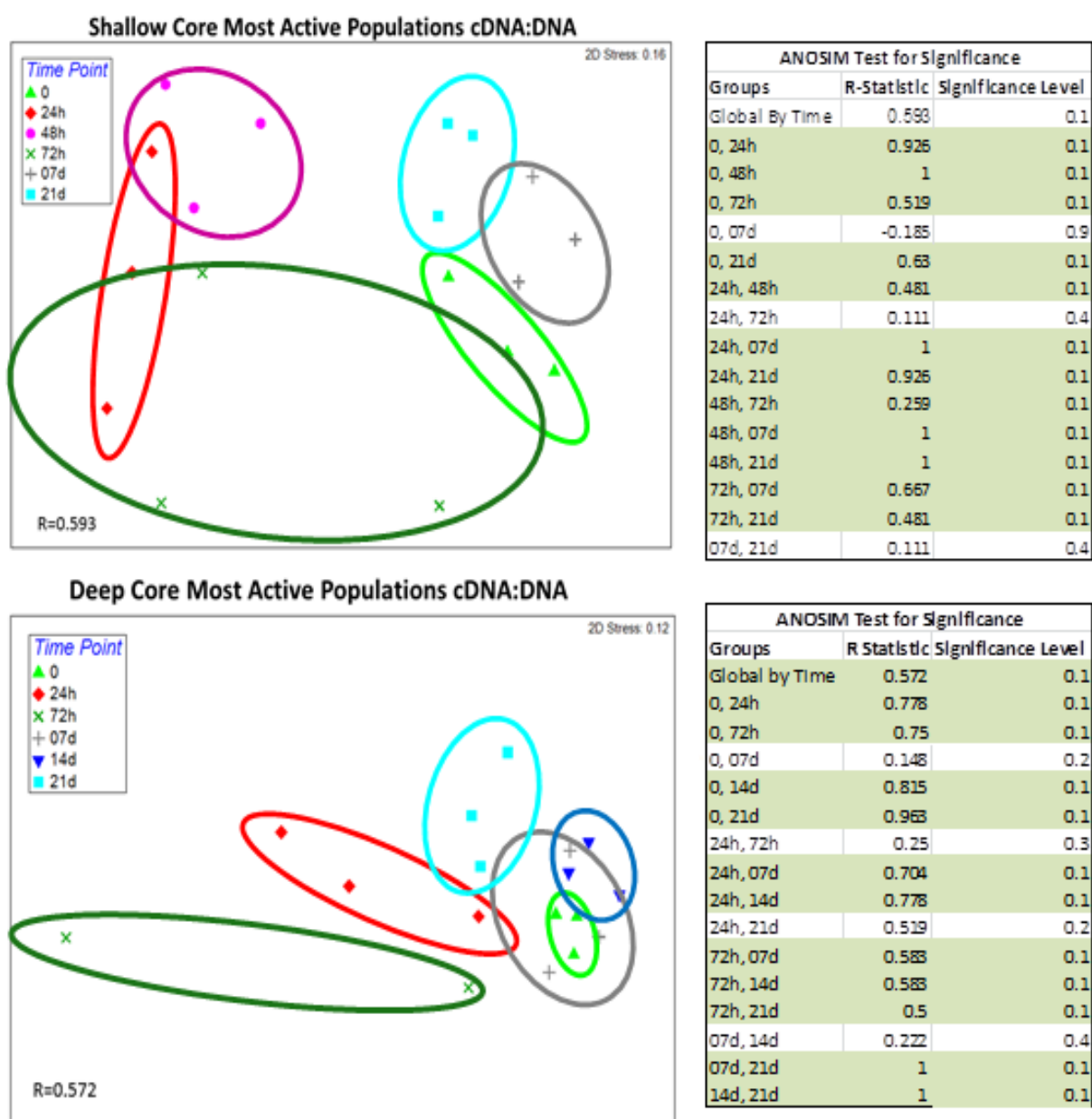
**Figure 4:** Average  $\pm$  SE (n=3) N<sub>2</sub>O flux in ng N-N<sub>2</sub>O gwd<sup>-1</sup> hour<sup>-1</sup> for shallow (blue) and deep (red) cores. Prior to 7 days, fluxes from deep cores were negligible. Note that there are distinct differences in the timing and magnitude of fluxes between shallow and deep cores, with more large fluxes observed from deep cores.



**Figure 5:** MDS scaling of 16S rDNA gene based (A) and 16S rRNA based (B) soil communities for shallow (blue) and deep (green) cores. No significant distinction exists between depth in either case ( $R = 0.184$  rDNA,  $0.1$  rRNA).

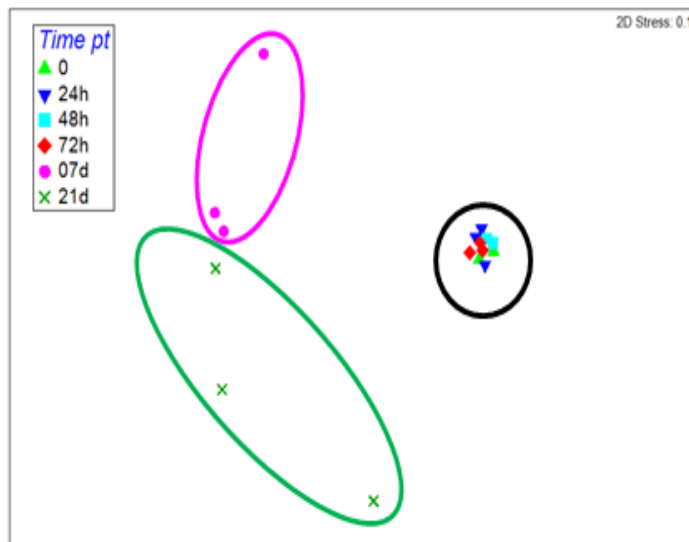


**Figure 6:** MDS scaling of communities derived from either DNA or cDNA for all time points and depth. ANOSIM analysis confirms that communities are significantly distinct ( $R = 0.626$ , confidence level  $0.1$ ) based on whether they are derived from DNA or cDNA.



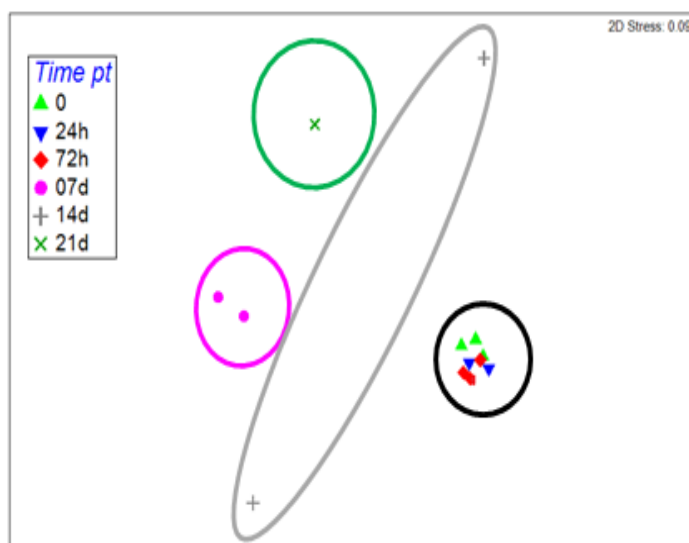
**Figure 7:** MDS scaling of 16S based most active populations for shallow (top) and deep (bottom) cores. Triplicate samples are shown for each time point and enclosed in colored circles.  $R \geq 0.5$  represents communities that are overlapping, but still distinct and  $R \leq 0.25$  represents communities that are indistinguishable, and significance levels  $\leq 0.1$  indicate statistical significance (green shading).

### Shallow Core Most Active Denitrifier Typical *nosZ* communities



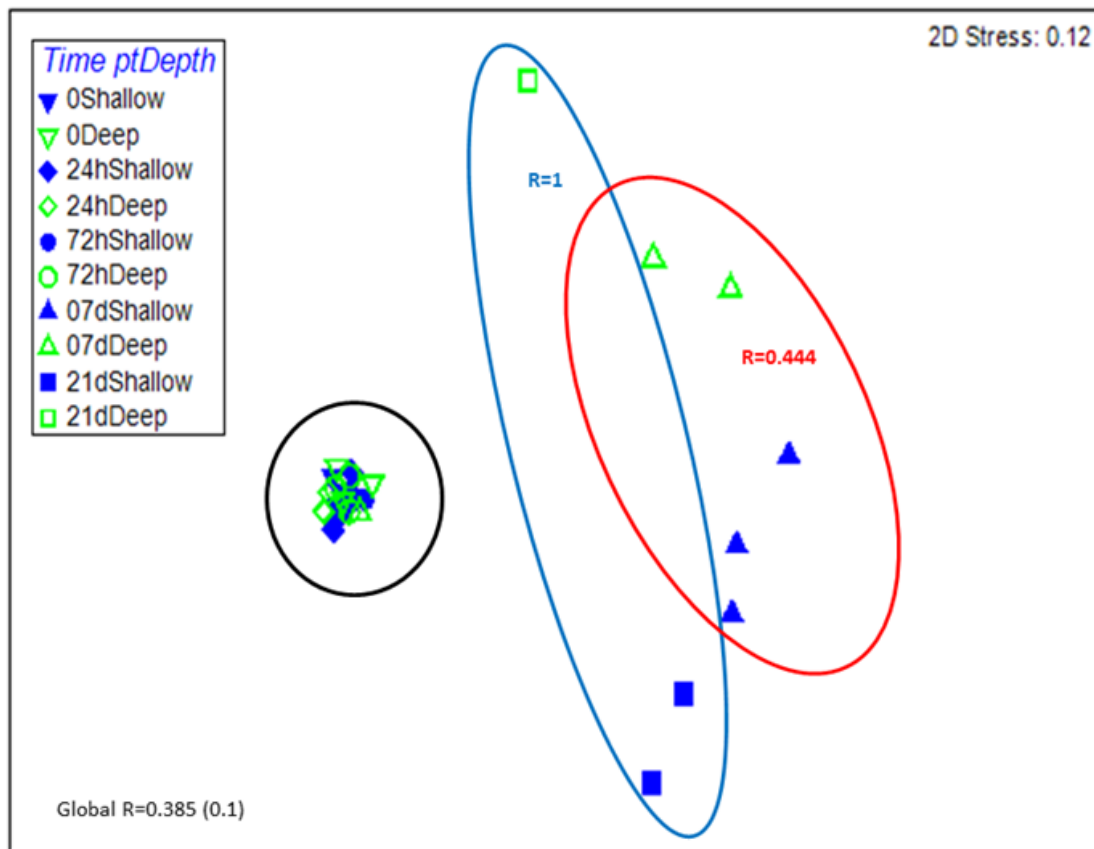
| Groups         | R Statistic | Significance Level |
|----------------|-------------|--------------------|
| Global by time | 0.378       | 0.2                |
| 0, 24h         | 0.25        | 0.3                |
| 0, 48h         | 1           | 0.33               |
| 0, 72h         | 0.25        | 0.4                |
| 0, 07d         | 1           | 0.1                |
| 0, 21d         | 0.167       | 0.3                |
| 24h, 48h       | -0.083      | 0.6                |
| 24h, 72h       | -0.185      | 0.9                |
| 24h, 07d       | 1           | 0.1                |
| 24h, 21d       | 0.444       | 0.2                |
| 48h, 72h       | 0.667       | 0.1                |
| 48h, 07d       | 1           | 0.1                |
| 48h, 21d       | 0.5         | 0.2                |
| 72h, 07d       | 0.926       | 0.1                |
| 72h, 21d       | 0.407       | 0.1                |
| 07d, 21d       | 0.111       | 0.4                |

### Deep Core Most Active Denitrifier Typical *nosZ* communities

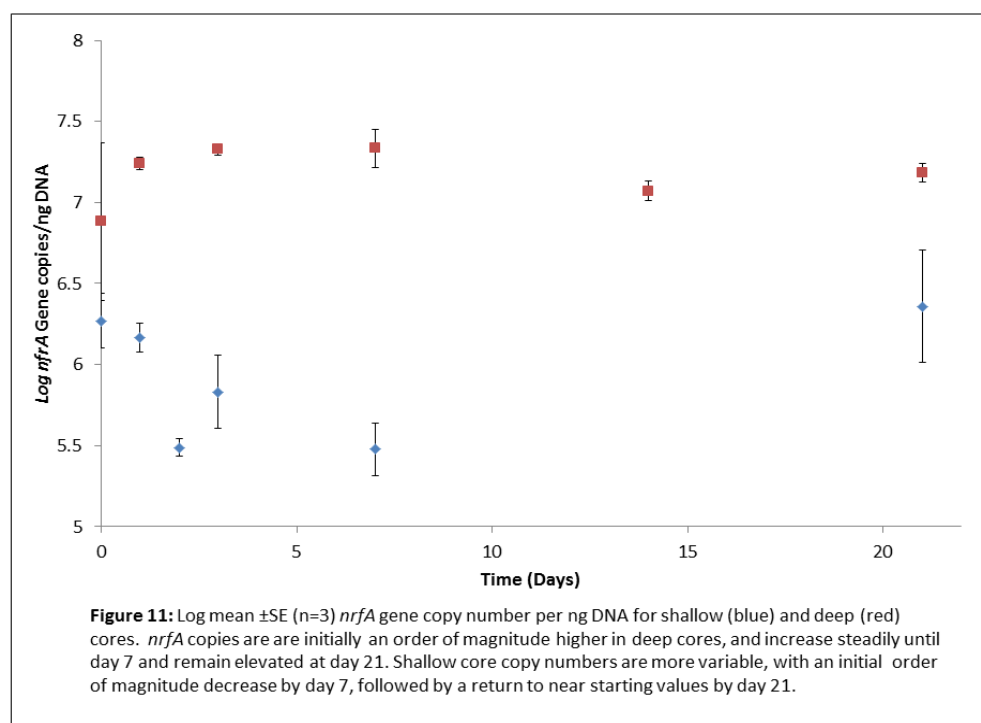
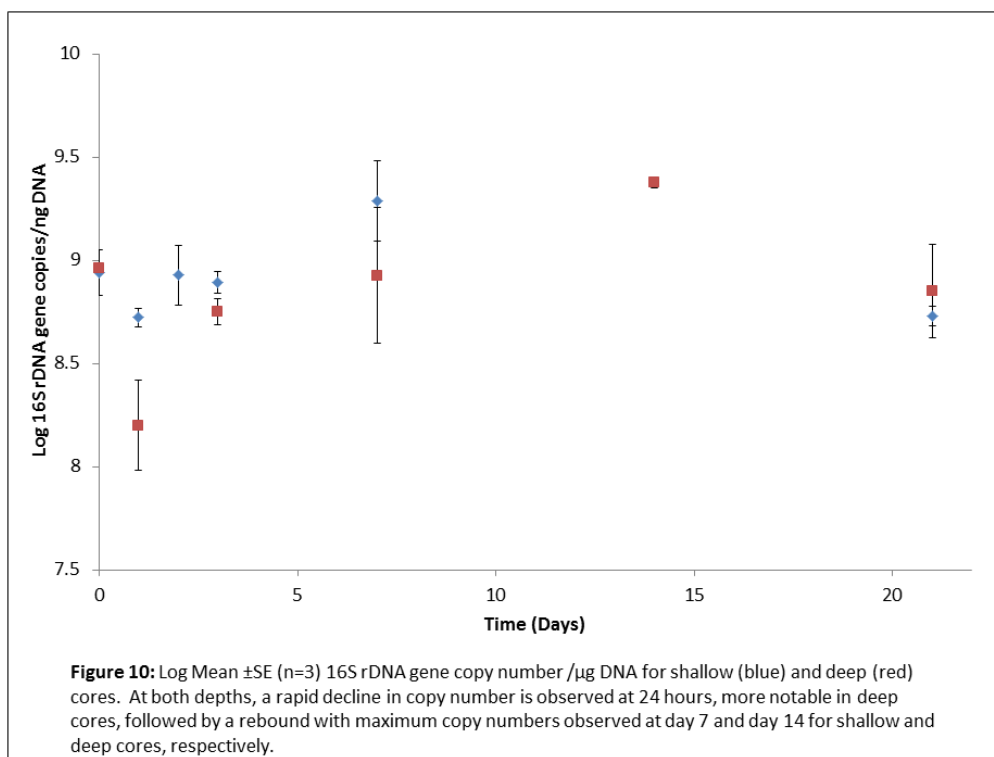


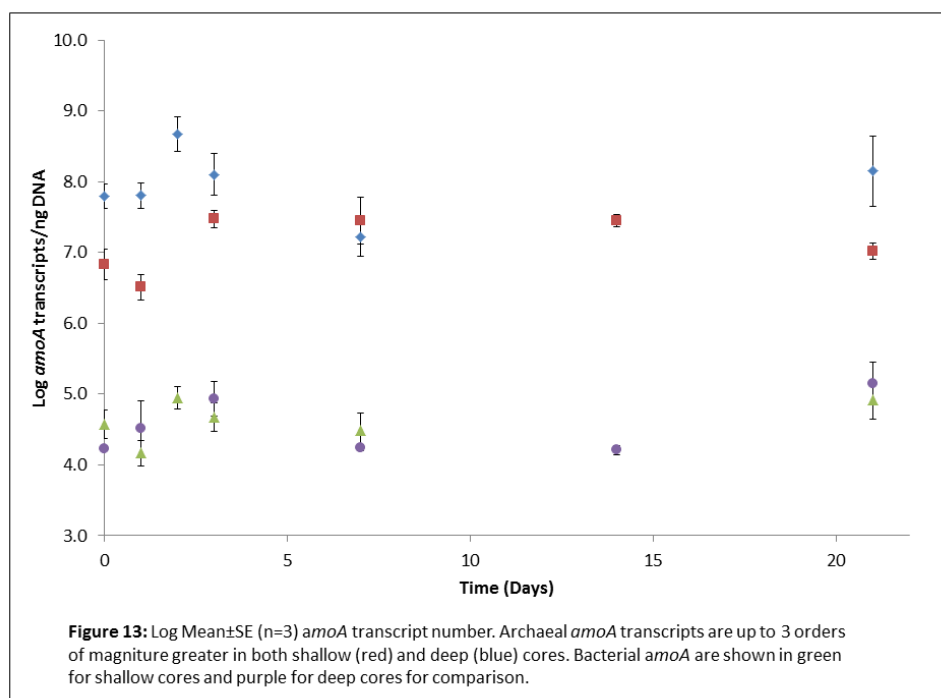
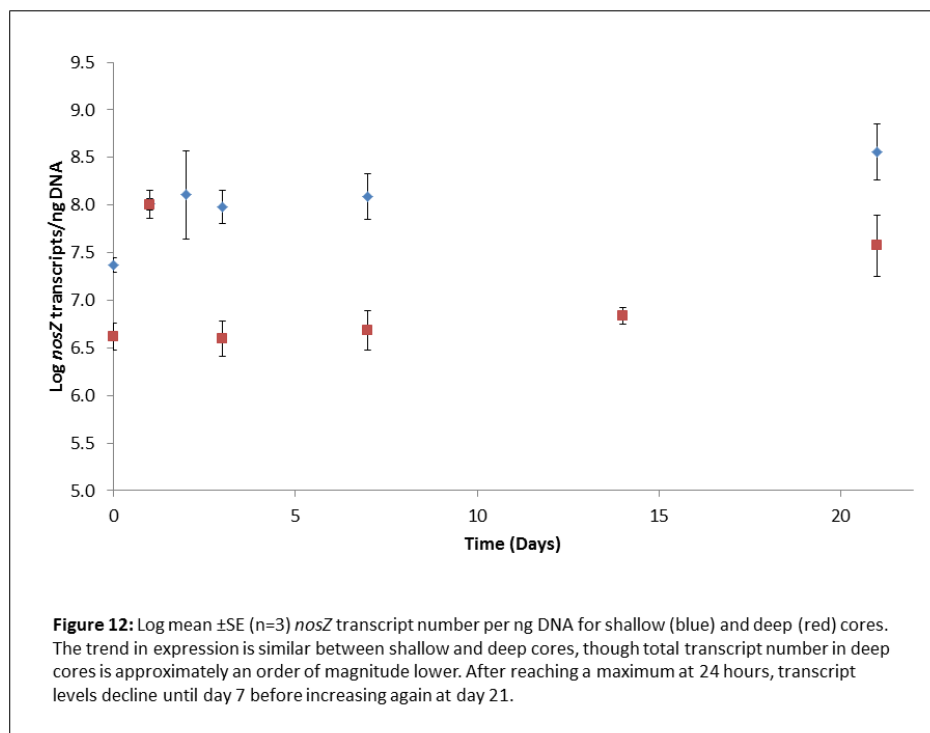
| Groups         | R Statistic | Significance Level |
|----------------|-------------|--------------------|
| Global by Time | 0.382       | 0.6                |
| 0, 24h         | -0.083      | 0.5                |
| 0, 72h         | 0.37        | 0.2                |
| 0, 07d         | 0.481       | 0.2                |
| 0, 14d         | 0.5         | 0.1                |
| 0, 21d         | 1           | 0.25               |
| 24h, 72h       | 0.083       | 0.6                |
| 24h, 07d       | 0.417       | 0.2                |
| 24h, 14d       | 0           | 1                  |
| 24h, 21d       | 1           | 0.33               |
| 72h, 07d       | 0.259       | 0.3                |
| 72h, 14d       | 0.5         | 0.1                |
| 72h, 21d       | 1           | 0.25               |
| 07d, 14d       | 0.167       | 0.3                |
| 07d, 21d       | 0.556       | 0.5                |
| 14d, 21d       | -1          | 1                  |

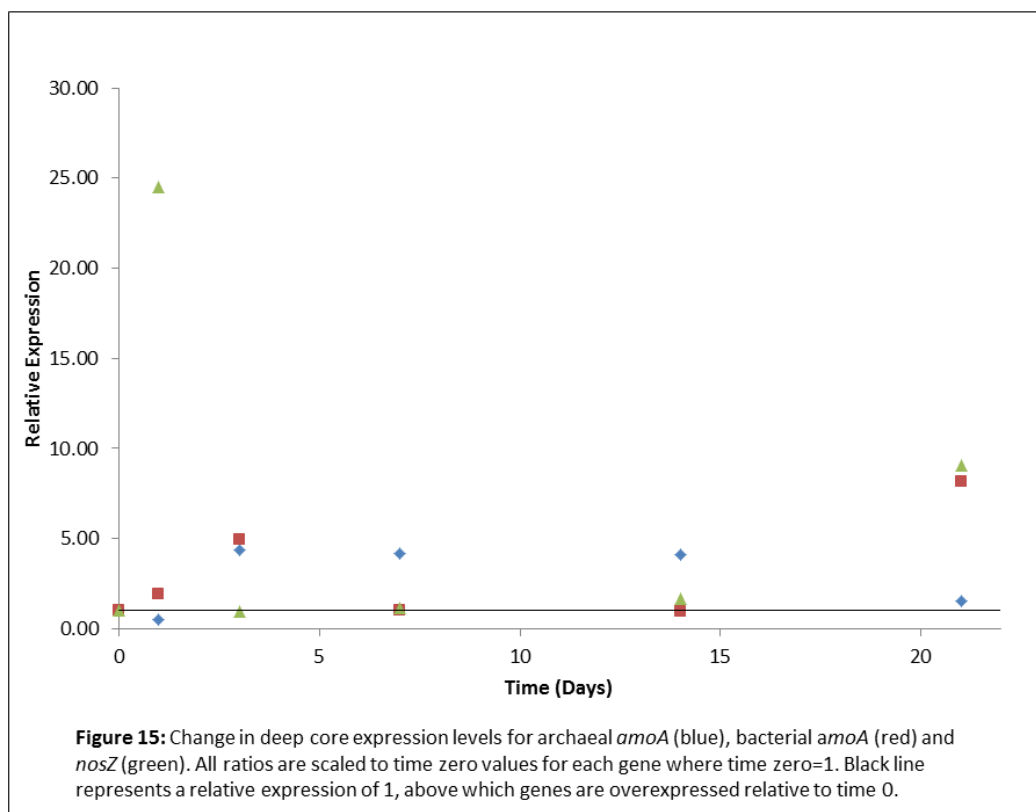
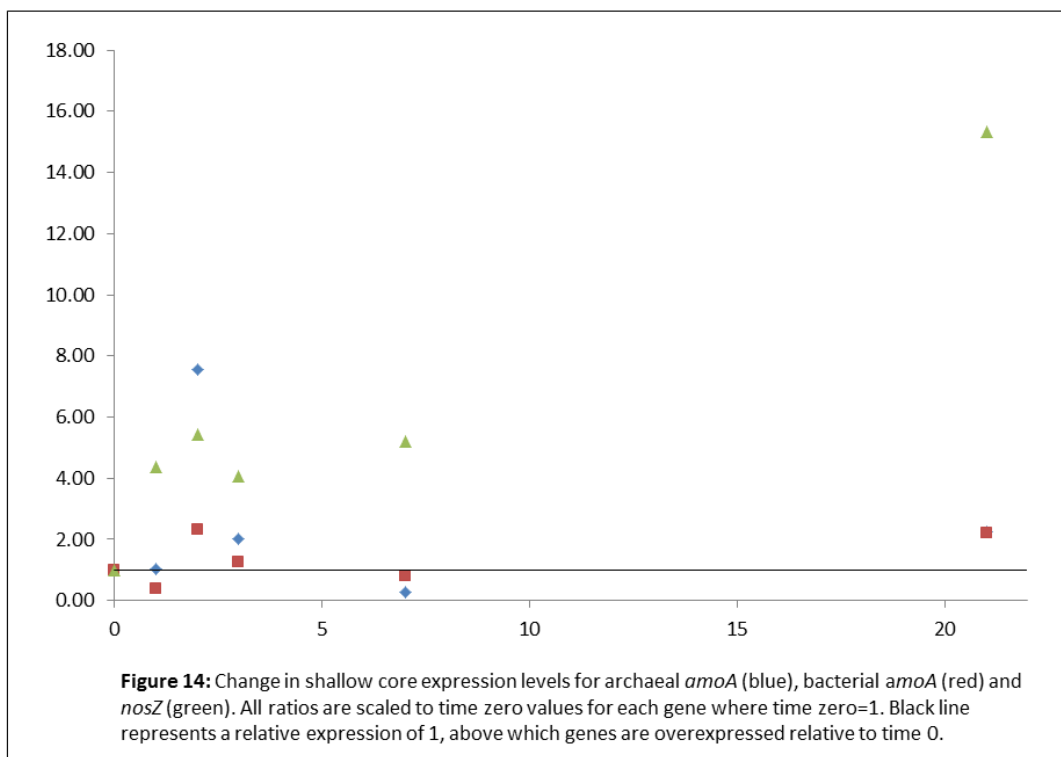
**Figure 8:** MDS scaling Most active typical *nosZ* based communities for shallow (top) and deep (bottom) cores. ANOSIM  $R \geq 0.75$  delineates clearly distinct communities,  $R \geq 0.5$  represents communities that are overlapping, but still distinct and  $R \leq 0.25$  represents communities that are indistinguishable., and significance levels  $\leq 0.1$  indicate statistical significance (green shading).

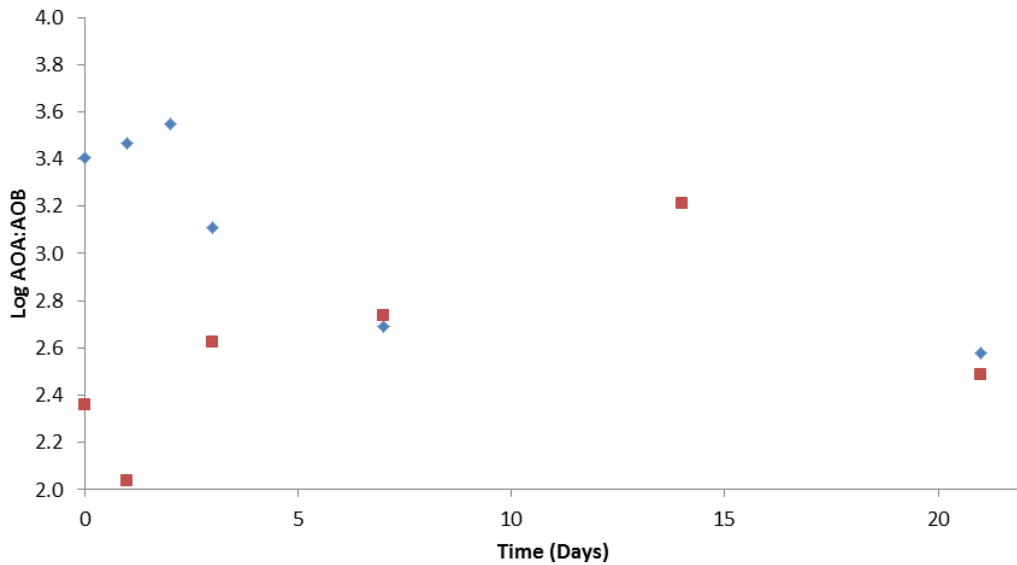


**Figure 9:** MDS scaling of active *nosZ* based communities by depth and time point for shallow (blue) and deep (green) cores. Early time points ( $\leq 7$  days) display no distinction with depth (black circle) however, at day 7 (red circle) communities begin to diverge and by day 21 (blue circle), communities are clearly distinct ( $R=1$ ).

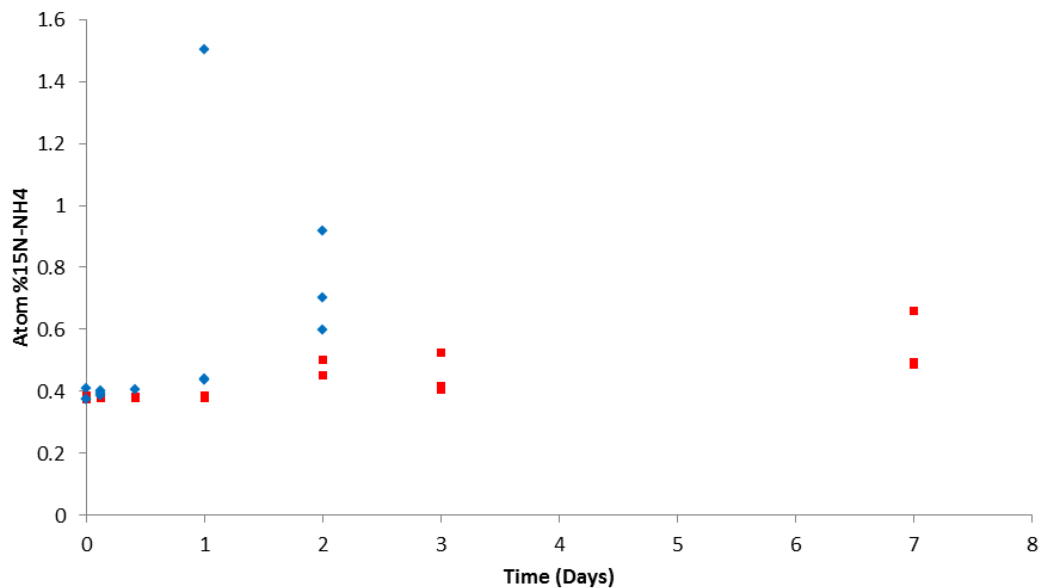








**Figure 16:** Log Ratio of archaeal *amoA* transcripts to bacterial *amoA* for shallow (blue) and deep (red) cores. While this ratio is initially much higher in shallow cores than deep, due the overall decrease in the ratio in shallow cores and increase in deep cores ratio, by day 21 the ratios are similar between depths.



**Figure 17:** Measured atom percent  $^{15}\text{N-NH}_4^+$  from  $^{15}\text{N-NO}_3^-$  labeled shallow (blue) and deep (red) cores. In the absence of transport from the from the  $^{15}\text{N-NO}_3^-$  pool, atom percentages would be expected to be at natural abundance (0.364) thus the increasing values suggest dissimilatory nitrate reduction to ammonium.

## Tables

**Table 1: Soil Characteristics**

| Soil Parameter                             | 0-10 cm | 10-30 cm |
|--------------------------------------------|---------|----------|
| Total Organic Matter (%)                   | 1       | 0.5      |
| Available P (ppm-P)                        | 78      | 65       |
| K (ppm)                                    | 72      | 40       |
| Mg (ppm)                                   | 91      | 65       |
| Ca (ppm)                                   | 662     | 418      |
| pH (A&L)                                   | 7.1     | 7.3      |
| NO <sub>3</sub> <sup>-</sup> -N (ppm)      | 3       | 2        |
| NH <sub>4</sub> <sup>+</sup> -N (ppm)      | 6       | 6        |
| Total Kjeldahl N (%)                       | 0.069   | 0.023    |
| Extractable Fe (ppm)                       | 139     | 167      |
| CEC (meq/100g)                             | 4.3     | 2.7      |
| Percent Base Saturation                    |         |          |
| %K                                         | 4.3     | 3.8      |
| %Mg                                        | 17.8    | 19.8     |
| %Ca                                        | 77.8    | 76.4     |
| %H                                         | 0       | 0        |
| Single Site Composite Parameters (0-30 cm) |         |          |
| Bulk Density (g/cm <sup>3</sup> )          |         | 1.51     |
| Sand (%)                                   |         | 93       |
| Silt (%)                                   |         | 0        |
| Clay (%)                                   |         | 7        |
| Soil Textural Class                        |         | Sand     |
| Water Holding Capacity (%)                 |         |          |
| at 1/3 Bar                                 |         | 3.86     |
| at 15 Bar                                  |         | 1.95     |

**Table 2: List of Primers Utilized**

| Gene Target           | Primer                  | Sequence                          | Reference               |
|-----------------------|-------------------------|-----------------------------------|-------------------------|
| <b>Reverse</b>        |                         |                                   |                         |
| <b>Transcription</b>  |                         |                                   |                         |
| Bacterial 16S rRNA    | 1492R                   | 5'-TAC GGY TAC CTT GTT ACG ACT T  |                         |
| Archaeal <i>amoA</i>  | crenamoA616r            | 5'-GCC ATC CAT CTG TAT GTC CA     |                         |
| Bacterial <i>amoA</i> | amoA2R                  | 5'-CCC CTC KGS AAA GCC TTC TTC    |                         |
| typical <i>nosZ</i>   | NosZRDnit2              | 5'-DRT GCA KNG CRT GGC AGA A      |                         |
| Atypical <i>nosZ</i>  | NosZRAdeh2              | 5'-RTG SAR BGC SSW GCA RAA G      |                         |
| <i>nrfA</i>           | nrfAR1                  | 5'-TWN GGC ATR CAR TC             |                         |
| <b>T-RFLP</b>         |                         |                                   |                         |
| Bacterial 16S rRNA    | FAM F27                 | 5'-AGA GTT TGA TCM TGG CTC AG     |                         |
|                       | 1492R                   | 5'-TAC GGY TAC CTT GTT ACG ACT T  |                         |
| typical <i>nosZ</i>   | FAM NosZ661FDenit       | 5'-MGC YTG KGG SVW GAC YAA        | Throbäck et al., 2004   |
|                       | NosZRDnit2              | 5'-DRT GCA KNG CRT GGC AGA        |                         |
| atypical <i>nosZ</i>  | FAM NosZ661FAdeh        | 5'-SGG CTA YGG CTW YGA YGA        |                         |
|                       | NosZRAdeh2              | 5'-RTG SAR BGC SSW GCA RAA G      |                         |
| <b>qPCR</b>           |                         |                                   |                         |
| Bacterial 16S         | S-D-Bact-0341-b-S-17    | 5'-CCT ACG GGN GGC WGC AG         | Klindworth et al., 2013 |
|                       | S-D-Bact-0515-a-A-19MOD | 5'-TTC CCG CGG CTG CTG G          |                         |
| Archaeal <i>amoA</i>  | amoA19F                 | 5'-ATG GTC TGG CTW AGA CG         | Meinhardt et al., 2015  |
|                       | amo277Rmod              | 5'-CRA YGA AGT CRT AHG GRT ADC C  |                         |
| Bacterial <i>amoA</i> | amoA1F                  | 5'-GGG GTT TCT ACT GGT GGT        | Rotthauwe et al., 1997  |
|                       | amoA2R                  | 5'-CCC CTC KGS AAA GCC TTC TTC    |                         |
| Typical <i>nosZ</i>   | nosZ1F                  | 5'-WCS YTG TTC MTC GAC AGC CAG    | Henry et al., 2006      |
|                       | nosZ1R                  | 5'-ATG TCG ATC ARC TGV KCR TTY TC |                         |
| <i>nrfA</i>           | nrfAF2aw                | 5'-CAR TGY CAY GTB GAR TA         | Welsh et al., 2014      |
|                       | nrfAR1                  | 5'-TWN GGC ATR CAR TC             |                         |

**Table 3: qPCR Cycling Conditions**

| Gene                  | Initial Denaturation | Denaturation     | Annealing        | Extension        | Cycle Number |
|-----------------------|----------------------|------------------|------------------|------------------|--------------|
| 16S                   | 95°C, 30 seconds     | 95°C, 30 seconds | 60°C, 30 seconds | 72°C, 20 seconds | 35           |
| Archaeal <i>amoA</i>  | 95°C, 30 seconds     | 95°C, 15 seconds | 55°C, 40 seconds | 72°C, 20 seconds | 40           |
| Bacterial <i>amoA</i> | 95°C, 30 seconds     | 95°C, 20 seconds | 60°C, 30 seconds | 72°C, 45 seconds | 40           |
| Typical <i>nosZ</i>   | 95°C, 30 seconds     | 95°C, 30 seconds | 55°C, 40 seconds | 72°C, 40 seconds | 40           |
| <i>nrfA</i>           | 95°C, 30 seconds     | 95°C, 15 seconds | 53°C, 30 seconds | 72°C, 30 seconds | 40           |

**Table 4: Gross Transformation Rates**

|         | Time Interval | Nitrification | $\text{NO}_3^-$ Consumption | Mineralization | $\text{NH}_4^+$ Consumption | DNRA  |
|---------|---------------|---------------|-----------------------------|----------------|-----------------------------|-------|
| Shallow | 0 to 3        | 20.465        | 48.596                      | -2.435         | 82.858                      | 0.255 |
|         | 3 to 10       | -9.541        | 44.737                      | 0.436          | 46.323                      | 0.390 |
|         | 10 to 24      | 11.677        | 18.925                      | 0.115          | 26.994                      | 0.926 |
|         | 24 to 48      | -1.251        | 15.450                      | 0.456          | 19.163                      | 1.742 |
|         | 48 to 72      | 2.720         | 23.701                      | 1.099          | 18.214                      | 1.471 |
| Deep    | 0 to 3        | -1.810        | 7.791                       | 12.898         | 86.019                      | 0.124 |
|         | 3 to 10       | 1.816         | 5.554                       | -4.104         | 44.733                      | 0.208 |
|         | 10 to 24      | 0.254         | -0.750                      | -0.030         | 29.894                      | 0.401 |
|         | 24 to 48      | 4.620         | 15.951                      | -0.064         | 11.716                      | 1.269 |
|         | 48 to 72      | -1.335        | 7.513                       | 0.097          | 21.293                      | 0.511 |

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