# The Effect of Nanofertilizers on the Soil Microbial Community

by

Kelsie Davis

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Graduate Supervisory Committee:

Ryan Penton, Chair
Fabio Suzart de Albuquerque
Yujin Park

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ABSTRACT

As the global population continues to increase, so does the need for agriculture resulting in increased fertilizer use. Nanofertilizers and biochar have been proposed as alternatives to fertilizers currently in use to reduce negative environmental impacts. In this study, the effects of various nanofertilizers and biochar on the soil microbial community were investigated. Soils treated with graphene nanoplatelet (GNP), graphene oxide (GO), reduced graphene oxide (rGO), graphite nano-additive (GNA) and biochar (BC) at concentrations of 5 mg/kg and 1000 mg/kg were sampled before and after a 28-day incubation period. Quantitative PCR assays were carried out against the following target genes: *16S rRNA, nirK, nirS, nifH, amoA* and *nosZ*. Overall, all treatments experienced a decrease in *16S rRNA* abundance after the incubation period with an average decrease of 48% however, all treatments were higher in abundance than the control. The abundances of nitrogen (N) cycling functional genes were evaluated in terms of relative abundance as a percentage of *16S rRNA*. There was an increase across all treatments in *nirK* relative abundance over time and when compared to the control. The most notable differences in abundance were in rGO (high) as well as BC. Both *nirS* and *nosZ* exhibited an increase over time but decreased compared to the control. A decrease in relative abundances of *nifH* in BC as well as GO (low) and rGO (high) was observed. Lastly, there was an increase in *amoA* relative abundance across all treatments after the incubation period. However, all treatments were significantly lower than the control. The increase of denitrifying genes (*nirK, nirS* and *nosZ*) and nitrifying genes (*amoA*) suggests the potential increase in denitrification which can result in increased N loss into the atmosphere and the potential decrease of nitrification resulting in reduced N loss into

waterways, respectively. At the time of writing, this study is one of the first to investigate and provide observations on the effects of nanofertilizers on *nifH*, which is responsible for N-fixation. The results presented here suggest that rGO and BC impart similar effects on the microbial community, whereas GNP had the most significant impact overall.

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# CHAPTER 1

## INTRODUCTION

As the global population continues to increase, with a quadrupling of the global population during the 20th century (Raliya et al, 2018), so too does the need for agriculture which, in turn, will result in an increase in fertilizer use. Estimates indicate that by the end of the 20th century, about 40% of the world's population relied on nitrogen (N) fertilizers for food production with 30-50% of crop yield increase due to the use of N fertilizer (Smil, 2001; Stewart et al., 2005). Additionally, the global consumption of N fertilizer has increased by ~600% from 1961 to 2000 (Scanlon et al., 2007). The wide availability of N fertilizers was made possible due to the introduction of the Haber-Bosch process which enabled agriculture to develop at a larger scale and served to sustain the world's growing population (Gruber & Galloway, 2008). It has been estimated that 80% of the N produced through the Haber-Bosch process was used in the manufacturing of agricultural fertilizers (Galloway et al., 2008). However, the application of traditional chemical fertilizers, such as ammonium nitrate, may lose effectiveness due to N loss through runoff and leaching as well as bacterial N transformations that can result in gaseous losses (Velthof et al, 2014). These losses result in a decrease in plant-available N. In addition, widespread overuse of chemical fertilizers has resulted in adverse effects on environmental health, such as the eutrophication of natural systems and global acidification (Gruber & Galloway, 2008; Diaz & Rosenberg, 2008). Additionally, fertilizer loss can result in the necessity of additional fertilizer applications, which comes at a great cost to both the grower and the environment (Zulfiqar et al, 2019). In one study, it was estimated that leaching was responsible for a loss of EUR 61.13 $ha^{-1}$

$yr^{-1}$ , or approximately USD 70.64 at the time of writing (Siksnane & Lagzdins, 2017). Nanofertilizers are now being investigated as an alternative to traditional fertilizers in order to address N losses in agricultural systems by slowing the release of N into the soil and delivering N directly to crops (Zulfiqar et al, 2019; Aacharya & Chhipa, 2020). However, little is known concerning the impact of these nanofertilizers on soil microbial communities and microbially-mediated N transformations. This study aims to further investigate these relationships.

# CHAPTER 2

# BACKGROUND

## Environmental effects of traditional N fertilizers

Although N applications occur locally, the high mobility of N allows its effects to spread much further than the initial application site (Vitousek et al., 1997). Traditional chemical fertilizers may be lost into the atmosphere as nitrous oxide ($N_2O$), ammonia ($NH_3$) and nitrogen gas ($N_2$) or transported to waterways as nitrite ($NO_2^-$) and/or nitrate ($NO_3^-$), thus contributing to increased pollution in the surrounding ecosystem (Raliya et al, 2018). Estimates suggest that approximately 40% of fertilizer N lost to the environment is lost through conversion to $N_2$ via denitrification (Galloway et al., 2004) with losses via denitrification estimated to be 15–30 kg N $ha^{-1}$ $yr^{-1}$ (Burkart and James, 1999; Hofstra and Bouwman, 2005; Seitzinger et al., 2006). Nitric oxide and $NH_3$ emissions have increased approximately five-fold since pre-industrial times (Galloway et al., 2004). Mosier (1998) reported that the atmospheric concentration of $N_2O$ has increased by 15% over the last 250 years. Nitrous oxide ($N_2O$) is especially important in the context of global climate change as it is ~270 times more reactive than $CO_2$ as a greenhouse gas (Ayoub, 1999). Gaseous losses of N can also occur through $NH_3$ volatilization, primarily when soil pH levels are increased (Raymond et al., 2016). Van der Weerden & Jarvis (1997) found that $NH_3$ losses were greatest from urea applications with 28.4-29.5% N being lost to the environment and that these losses were significantly greater than emissions from other treatments. Additionally, emissions are concentrated within the 3-6 weeks after fertilizer application and N losses are positively correlated with the amount of fertilizer applied, with larger amounts of fertilizer resulting in higher

emissions (Clayton et al., 1997; Mosier, 1998; Gu et al., 2009). One study showed that as N fertilizer rate increased, so did N losses (Huang et al., 2017). The same study reported that 315 kg $ha^{-1}$ of N fertilizer application resulted in a net loss of 6.90 kg $ha^{-1}$ , and this was also dependent on local conditions, with mountainous regions receiving more fertilizer as compared to plains (Huang et al., 2017).

One of the major environmental impacts due to N runoff is the eutrophication of freshwater and marine systems. When N is released into waterways, it becomes available for uptake by algae, thereby increasing their growth rates (Anderson et al., 2006). This ultimately results in dangerous algal blooms along rivers and coastlines downstream from agriculture production zones utilizing traditional N fertilizers. In 2009, China lost $1.6 \times 10^6$ t of N from crop production and the marine eutrophication potential for the same year was $5.3 \times 10^{10}$ PDF (potentially disappeared fraction of species) $m^3$ $yr^{-1}$ (Huang et al., 2017). The most soluble and mobile form of N is $NO_3^-$, which is easily leached by precipitation into groundwater, subsurface tile drainage, and streams (Goolsby & Battaglin, 2001). David et al. (1997) estimated N losses from $NO_3^-$ in a tile-drained mollisol cropland (maize and soybean) ranged from 20-50 kg N $ha^{-1}$ $yr^{-1}$. Nitrate is the main nutrient deposited from the Mississippi River into the Gulf of Mexico, contributing to the hypoxic conditions (oxygen < 2mg $L^{-1}$) and subsequent dead zones (Burkhart & James, 1999) that has resulted in devastating effects on wildlife, including fish and bird mortality as well as contaminated shellfish (Deeds et al., 2010, Van Deventer et al., 2012, Driggers et al., 2016, Fauquier et al., 2013). Atmospheric ammonia can also cause acidification of soils and waters, via ammonium sulfate oxidation to nitric and sulphuric

acid, which significantly changes the soil and water chemistry, disrupting the affected ecosystems (Van Breemen et al., 1982; Ayoub, 1999).

**Nanofertilizers**

Nanofertilizers can be described as nutrient fertilizers that are completely or partially comprised of nanostructured formulations that allow for more efficient plant nutrient uptake (Raliya et al, 2018). There are numerous types of graphene (GR) nanoparticles: Fullerenes, nanotubes, and graphene oxides (GO), all of which can be used for the basis of nanofertilizer production (Mittal et al., 2020). They are based on carbon (C) nanotubules or graphene sheets that have a high specific surface area (SSA) which can bind to N species (Zulfiqar et al, 2019; Aacharya & Chhipa, 2020). This results in a targeted, slower release of N for plant uptake and reduces the amount of N that will be lost due to leaching, increasing efficacy (Sangeetha et al., 2019). Nanofertilizers can also address environmental effects by reducing the overuse of fertilizers (Pitambara et al., 2019). Applying nanofertilizers to soils have yielded some positive effects, of which include the improvement of surface water quality near agricultural fields, better assimilation of N by plant roots, and increases in root activity marked by an increase in root exudates and the abundance of soil microbes (Teng, 2018). One group studied the effect of reduced graphene oxide on the microbial community by quantifying genes that are functional markers capable of identifying the presence of denitrifying (*nirK* & *nirS*) and nitrifying microbes. They found that with the addition of reduced graphene oxide (rGO), *nirK* and *amoA* decreased in relative abundance while *nirS* increased when compared to the control (Banach-Wiśniewska et al., 2021). Additional benefits of agricultural nanofertilizers include: increased plant production, higher plant nutrient

content, protection of natural resources, enhancement of soil biological health through increased microbial diversity, increased nutrient cycling, and reductions in traditional fertilizer demand (Raliya et al, 2018).

In a study investigating the growth of green peppers, treatment with slow-release nanofertilizers resulted in significant increases in both the height of the plant and the diameter of the stem when compared to a control treatment with no additives as well as a traditional chemical fertilizer treatment (Teng, 2018). In another study, sugar beets (*Beta vulgaris*) produced more leaves after application of a nanofertilizer when compared to the control (Jakiene, 2015). These increases in plant yield may be due to the ability of nanofertilizers to deliver nutrients directly to crops when compared to traditional chemical fertilizers (Lakzian, 2019). Overall, there are a large number of positive effects on plant growth when nanofertilizers are applied, which indicate that nanofertilizers can be beneficial alternatives to chemical fertilizers.

**Biochar**

Biochar (BC) is a C-rich soil amendment produced through biomass pyrolysis. The high temperature and oxygen-free conditions mean that the biomass is not subject to complete combustion (Jha et al., 2010). It is the closest analogue to nanofertilizers. Biochar was discovered in fertile soils of the Amazon known as Terra Preta dating back ~2000 years and this area still remains fertile today (O'Neill et al., 2009). Different materials, typically plant litter, can be used as the base for BC including corn stover, yard waste, switchgrass, wood (as used in this study), maize, meadow grass, rice husk, etc. (Roberts et al., 2010; Břendová et al., 2012; Ghorbani et al., 2019). The addition of BC to soil has been reported to alter soil physical and chemical properties. These alterations

have led to nutrient retention as well as increased crop yields (Lehmann et al., 2006). Soil water-holding capacity was shown to increase when BC was applied at a concentration of 4t/ha (Karam D. S. et al., 2021). The ability for BC to increase the water-holding capacity in soils is due to the high amount of micro and meso pores, although the ability for the soil to hold water is also dependent on the soil type (Abukari, 2019; Karam D. S. et al., 2021). The structure of BC is highly porous, which increases soil surface area, and therefore allows water to penetrate the soil more effectively (Karam D. S. et al., 2021). In addition to increasing water-holding capacity, BC has also been found to increase cation exchange capacity (CEC). In one study, CEC increased by 20-30% in loamy sand and 9-13% in clay soil after the addition of BC (Ghorbani et al., 2019).

Additionally, the microbial communities within BC-treated soils were found to be more diverse when compared to the control (Yu et al., 2018). The area surrounding the roots, known as the rhizosphere, has been shown to harbor plant-microbe interactions. Plants recruit beneficial microbes from the surrounding soil into the rhizosphere through the production of root exudates (Sharma et al., 2021). Some beneficial microbes that interact with plants take part in the N cycle, making N more readily available to plants (Naik et al. 2019). One study found that the application of BC was able to double shoot biomass as well as increase root biomass by nine-fold when compared to the control (Zhaoxiang et al., 2020). When investigating the effects of BC on N-cycling, one study found that gross nitrification and total $N_2O$ emissions were not affected by BC application (Pereira et al., 2015). However, another study found that $N_2O$ emissions were reduced after the application of BC as well as a positive correlation between the higher rates of BC application and decrease in $N_2O$ production rates (Spokas et al., 2009).

**Nitrogen Cycling & Nitrogen Cycling Genes**

The N cycle is an incredibly complex and important process in agriculture and crop production. Nitrogen transformations are mediated by microbes that participate in distinct steps within the N cycle: Ammonification, nitrification, denitrification, dissimilatory nitrate reduction to ammonia (DNRA), anaerobic ammonium oxidation, and nitrite-nitrate interconversion (Stein & Klotz, 2016). Prior to modern agriculture and the application of chemical fertilizers, there existed a fine balance between the amount of $N_2$ being fixed from the atmosphere by microorganisms and the amount of $N_2$ being released back into the atmosphere by microbes (Fowler, 2013). However, the application of traditional chemical N fertilizers, particularly in agroecosystems under intensive cultivation, has resulted in a massive acceleration of the N cycle (Gruber & Galloway, 2008).

Nitrification is the process by which ammonium ($NH_4^+$) is oxidized to nitrite ($NO_2^-$) and $NO_2^-$ is further oxidized to nitrate ($NO_3^-$), whereas denitrification is the process by which $NO_2^-$ and $NO_3^-$ are reduced to nitrous oxide ($N_2O$), nitric oxide (NO) and dinitrogen ($N_2$), thereby removing N from the system and making it unavailable for plant uptake (Kreman et al., 2005). Coupled nitrification of ammonium and denitrification of nitrate occur in areas where both aerobic and anaerobic conditions are supported (Kreman et al., 2005; Conrad, 1996). Nitrogen fixation transforms $N_2$ into $NH_3$ making N available for plant uptake and occurs more in rhizosphere soils than bulk soils because of easily available root exudates (Bernhard, 2010; Dobereiner & Day, 1975). Dissimilatory nitrate reduction to ammonium (DNRA) is a shortcut in which nitrate is directly reduced into ammonium (Pandey et al., 2020). This pathway bypasses the usual

intermediates found in denitrification (Figure 1). Another pathway (not analyzed in this study) is anammox (anaerobic ammonium oxidation). This pathway oxidizes ammonium ($NH_4^+$) to $N_2$ by using $NO_2^-$ as an electron acceptor under anoxic conditions (Hu et al., 2011).

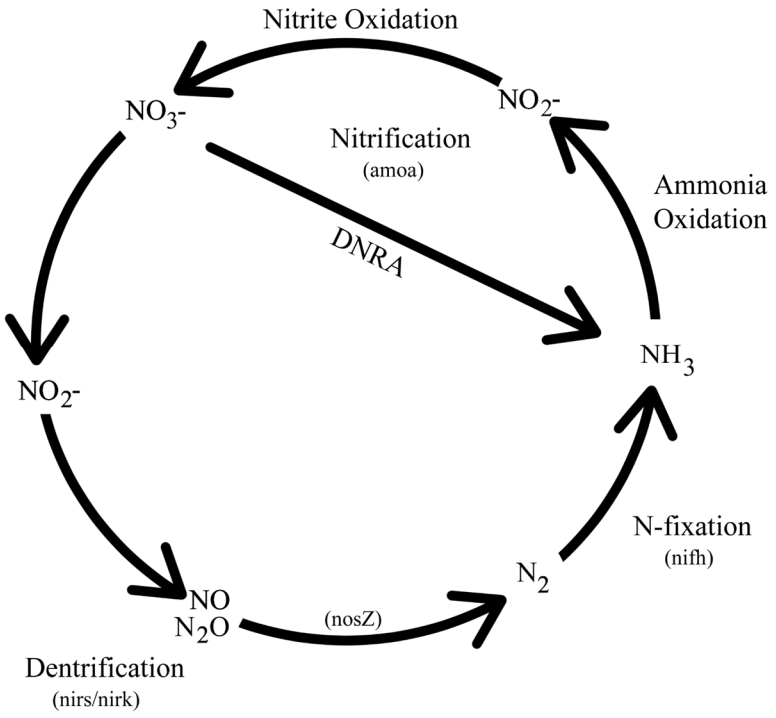


Figure 1. The nitrogen cycle. Pathways indicated by arrows. Listed in parentheses are the bacterial genes associated with each pathway. DNRA: Dissimilatory nitrate reduction to ammonium. Not included: anammox (anaerobic ammonium oxidation).

As microbes are responsible for carrying out these N transformations, the enzymes responsible for these processes are encoded by chromosomal or plasmid-borne genes. As such, there are multiple genes that can be used as markers for detecting the presence of microbes capable of carrying out these N cycling processes. For example, *nifH* is used to detect N-fixing organisms while *nirS* and *nirK* can be used to detect denitrifying organisms (Kuypers, 2018). The *nifH* gene encodes for the enzyme

nitrogenase, which can be found in all N-fixing organisms, meanwhile, *nirS* and *nirK* encode for two structurally different but functionally equal nitrate reductase enzymes (Poly et al., 2001; Hou et al., 2018). An additional gene, *nosZ*, encodes for the catalytic subunit of the nitrous oxide reductase enzyme which is responsible for carrying out the terminal step of denitrification: the reduction of $N_2O$ to $N_2$. (Henry et. al, 2006). The *amoA* gene encodes for the active site of the ammonia monooxygenase enzyme which can be used to detect microbes capable of nitrification (Sinigalliano et al, 1995). One study compared the effect of different organic fertilizers in conjunction with NPK fertilizers on the microbial community. They found that NPK fertilizer alone significantly increased the abundance of bacterial *amoA*, *nirK*, and *nosZ,* and had no significant effect on *nifH* and *nirS* genes, whereas wheat straw only increased *nirK* abundance and manures increased the abundance of all genes (Sun et al., 2015). Another study tested biochar (BC), bio-organic fertilizer, BC + bio-organic fertilizer, synthetic fertilizer, and BC + synthetic fertilizer and showed that *amoA* and *nosZ* increased with the application of bio-organic fertilizer, BC combined with bio-organic fertilizer, as well as BC combined with synthetic fertilizer (Zhaoxiang et al., 2020). They also found that *nirK* increased with all treatments except BC (Zhaoxiang et al., 2020). A comparison between biochar (BC) and urea applications found that *nirS* and *nirK* gene abundances decreased in BC treatment compared to urea treatment, however *nosZ* abundance resulted in the opposite effect of increasing with BC treatment compared to urea (Liao et al., 2020).

As the increase in agricultural production and the heavy use of N fertilizers continues, there is a need to find more sustainable solutions in order to keep up with the demand of a growing global population. The introduction of N fertilizers has positively

impacted the world's food supply by ramping up production but has also devastated natural ecosystems. Analyzing the effects of nanofertilizers and BC on the microbial community, more specifically those associated with the N cycle, can provide more insight on their effectiveness within the context of agriculture and their ability to mitigate environmental stresses like greenhouse gas emissions and eutrophication. This study hypothesizes (1) an increase in N-cycling genes and thus an increase in potential microbial N transformations with the addition of BC and nanofertilizers due to thier ability to decrease N leaching and release nutrients over time, respectively (Yu et al, 2019; Raliya et al, 2018) and (2) an increase in microbial abundance with the addition of nanofertilizers and BC, given that they are carbon-based substances and BC has previously been observed to increase microbial abundance (Ducey et al., 2013).

# CHAPTER 3

# MATERIALS AND METHODS

## Obtaining soil samples

Soil samples were obtained from agricultural soil within the Maricopa Agricultural Center in Arizona which was categorized as a sandy loam (62% sand, 20% silt, and 18% clay) with a pH of 8.36 ± 0.02. The soil samples were then sieved through 2mm mesh and stored at 4°C before being treated. The following treatments (Table 1) were added as dry powders to 40g of dry soil in conjunction with conventional ammonium nitrate at a rate of 196 kg N $ha^{-1}$, each at a low concentration of 5 mg/kg soil and a high concentration of 1000 mg/kg soil: Graphene nanoplatelet (GNP), graphene oxide (GO), reduced graphene oxide (rGO), graphite nano-additive (GNA) and biochar (BC). Soils and treatments were mixed in a tumbler shaker for 20 minutes. Each treatment, and concentration, was replicated five times alongside five control replicates for a total of 55 samples. These samples were then incubated for a period of 28 days at 20°C in the dark in order to determine the effect of each treatment over a period of time. Each sample was sealed using a rubber septa and aluminum clamps to achieve an airtight seal. After the incubation period, soil samples were kept at -80°C until ready to be processed for DNA extraction.

Table 1. Nanofertilizer and biochar characteristics

| GNP materials | Description | Particle size | Preparation method | SSA ($m^2/g$) | Zeta potential (mV) in DI water at pH 8.4 | Zeta potential (mV) suspended in soil solution at pH 8.4 |
|---|---|---|---|---|---|---|
| GNP | 2-10 nm nanoscale particles of graphite | 2-10 nm thick, 2-7 µm diameter | Interlayer cleavage | 20-40 | -29.6 | -30.2 |
| rGO | graphene sheets made from chemical reduction of GO | ~1 nm thick, 0.5 -10 µm diameter | $N_2H_4$ Chemical Reduction | 180 | -31.9 | -22.6 |
| BC | pine wood chip biochar after screening for size | < 50 mesh (0.3 mm) | slow pyrolysis by kilns | ~ 400 | -30.1 | -28.8 |
| GNA | highly disordered graphite nanoparticles | < 20 nm | Electro-exfoliation of graphite | 18.5 | -42.7 | -30.2 |
| GO | NA | 1 -20 µm diameter | NA | NA | -37.1 | -24.6 |

SSA: specific surface area

**DNA extraction and quantification**

Microbial DNA was extracted from soil samples to be used as template DNA for qPCR assays against the following target genes: *16S rRNA, nirK, nirS, nifH, amoA* and *nosZ* (Table 2). Microbial DNA was extracted from 0.25g of soil using the DNeasy PowerSoil Kit (QIAGEN, Germany) following a modified protocol outlined by Yu et. el (2018). Extracted DNA was then quantified using the Qubit 3.0 Fluorometer using 2µl of the sample along with 198µl working solution which contained Qubit buffer and Qubit reagent (Invitrogen, Carlsbad, CA, USA). For each field replicate, 3 technical replicates were performed for a total of 39 assays. Quantitative PCR was performed using SYBR Green-based (Thermo Fisher, Waltham, MA, USA) chemistry on the QuantStudio 3

thermocycler (Thermo Fisher, Waltham, MA, USA). Each reaction well contained 1µl DNA template, 1µl forward primer, 1µl reverse primer, 7µl PCR-grade water and 10µl PowerUp SYBR Green Master Mix for a total of 20µl per well.

**Statistical analysis**

A standard curve was produced for each qPCR assay using the standard concentration as well as the CT (cycle threshold) value determined by qPCR. The line of best fit (□ = □□ + □) was used to determine the slope (□) of each standard curve. A series of equations were used to determine the number of copies $g^{-1}$ soil for each assay:

Log copies = (□□ − □)/□

Copies = $10^{(\square\square\square\ \square\square\square\square\square\square)}$

Copies $ng^{-1}$ DNA = □□□□□□/□□□□□□□□□ □□□□□□□□□□□□□□□

ng DNA $g^{-1}$ soil = (□□□□□□□□□ □□□□□□□□□□□□□□□) × (*100*) × (*1/0.25*)

Copies $g^{-1}$ soil = (□□□□□□ □□ $^{-1}$ □□□) × (□□ □□□ □ $^{-1}$ □□□□)

Statistical analysis was performed using RStudio version 4.0.3 (RStudio Team, Boston, MA, USA). A two-way analysis of variance (ANOVA) was conducted in order to determine the effect of different concentrations of each nanofertilizer on the relative abundance of each target gene. Tukey's honestly significant difference post hoc test ($p < 0.05$) was performed to detect any statistical differences within the data. These analyses were also carried out to determine any significant difference over time.

Table 2. qPCR Primers and Thermocycling Conditions

| Primers | Sequence (5'-3') | Base Pairs | Thermocycling conditions |
|---|---|---|---|
| ***16S rRNA***[1]**:**<br>Q341F_16S<br>Q797R_16S | CCT ACG GGA GGC AGC AG<br>GGA CTA CCA GGG TAT CTA ATC<br>*E. coli* K-12 | 466 | 95°C, 3 min, 1 cycle<br>95°C for 45 s, 60°C for 45 s, 72°C for 1 min, 40 cycles<br>72°C for 7 min, 1 cycle |
| ***nirK***[2]**:**<br>nirK876<br>nirK1040 | ATY GGC GGV CAY GGC GA<br>GCC TCG ATC AGR TTR TGG TT<br>*E. meliloti* (ATCC 51124D-5TM) | 165 | 95°C, 15 min, 1 cycle<br>95°C for 15 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s, 6 cycles<br>95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s, 40 cycles<br>95°C for 15 s, 60 to 95°C (+0.2°C per second), 1 cycle |
| ***nirS***[3]**:**<br>nirSCd3cd<br>nirSR3cd | AAC GYS AAG GAR ACS GG<br>GAS TTC GGR TGS GTC TTS AYG AA<br>*P. aeruginosa PAO1* | 425 | 95°C, 15 min, 1 cycle<br>95°C for 15 s, 63 to 58°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s, 6 cycles<br>95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s, 40 cycles<br>95°C for 15 s, 60 to 95°C (+0.2°C per second), 1 cycle |
| ***nosZ***[4]**:**<br>nosZ2F<br>nosZ2R´ | CGC RAC GGC AAS AAG GTS MSS GT<br>CAK RTG CAK SGC RTG GCA GAA<br>*P. stutzeri* (ATCC BAA-295) | 267 | 95°C, 15 min, 1 cycle<br>95°C for 15 s, 65 to 60°C for 30 s (-1°C by cycle), 72°C for 30 s, 80°C for 15 s, 6 cycles<br>95°C for 15 s, 60°C for 30 s, 72°C for 30 s, 80°C for 15 s, 40 cycles<br>95°C for 15 s, 60°C for 15 s, 95°C for 15 s, 1 cycle |
| ***nifH***[5] **:**<br>nifHF<br>nifHR | AAA GGY GGW ATC GGY AAR TCC ACC AC<br>TTG TTS GCS GCR TAC ATS GCC ATC AT<br>*E. meliloti* (ATCC 51124D-5TM) | 461 | 95°C, 15 min, 1 cycle<br>94°C for 45 s, 60°C for 45 s, 72°C for 45 s, 40 cycles<br>72°C for 5 min, 1 cycle |
| ***amoA***[6]**:**<br>AmoA1F<br>AmoA2R | GGG GTT TCT ACT GGT GGT<br>CCC CTC KGS AAA GCC TTC TTC<br>[K 5 G or T; S 5 G or C]<br>*N. europaea* C-31 (ATCC 25978) | 491 | 94 °C for 5 min, 1 cycle<br>93 °C for 35 s, 53 °C for 45 s, 72 °C for 1 min ,35 cycles<br>72 °C for 5 min, 1 cycle[7] |

[1] Lopez-Gutierrez J.C., Henry S., Hallet S., Martin-Laurent F., Catrou, G., Philippot L. (2004) Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *J Microbiol Methods* 57: 399-407.

[2] Henry S., Baudouin, E., López-Gutiérrez, J.C., Martin-Laurent, F., Brauman, A., Philippot, L. (2004) Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J Microbiol Methods* 59: 327-335. Corrigendum in *J Microbiol Methods* 61 (2): 289-290

[3] Throbäck N., Enwall K., Jarvis A., Hallin, S. (2004) Reassessing PCR primers targeting *nirS*, *nirK* and *nosZ* genes for community surveys of denitrifying bacteria with DGGE. *FEMS Microbiol Ecol* 49: 401-417.

[4] Henry S., Bru D., Stres B., Hallet S., Philippot, L. (2006) Quantitative detection of the *nosZ* gene, encoding nitrous oxide reductase, and comparison of the abundances of 16S rRNA, *narG, nirK*, and *nosZ* genes in soils. *Appl Environ Microbiol* 72: 5181-5189.

[5] Rösch, C., Mergel, A.,Bothe, H. (2002) Biodiversity of Denitrifying and Dinitrogen-Fixing Bacteria in an Acid Forest Soil. *Applied and Environmental Microbiology* 68 (8) 3818-3829

[6] Rotthauwe, J. H., Witzel, K. P., Liesack, W. (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Applied and Environmental Microbiology* 63 (12) 4704-4712.

[7] Dong, X., & Reddy, G., B. (2012) Ammonia-oxidizing bacterial community and nitrification rates in constructed wetlands treating swine wastewater. *Ecological Engineering* 40, 189-197

# CHAPTER 4

# RESULTS

In order to assess the effects of the soil amendments on the microbial community, the abundance of the *16S rRNA* gene, as well as key N cycling functional genes, were evaluated. The abundance of *16S rRNA* was used to determine changes in the abundance of the soil microbial community. Nitrogen cycling functional genes were used to gauge the ability of the microbial community to perform tasks such as nitrate reduction (*nirK* and *nirS*), N fixation (*nifH*), nitrous oxide reduction (*nosZ*), and nitrification (*amoA*). The abundance of each gene was analyzed for both day 0 soil samples and day 28 samples.

Overall, all treatments experienced a decrease in *16S rRNA* abundance after the 28 day incubation period with the total abundance ranging from $1.54 \times 10^8$ to $5.06 \times 10^8$ copies $g^{-1}$ soil at day 0 to $6.44 \times 10^7$ to $3.33 \times 10^8$ copies $g^{-1}$ at day 28 (Figure 2). The control soils showed an average decrease of 48% over time. The treatments with the greatest decrease over time were GO, GNA, and rGO with decreases greater than 60% at both high and low concentrations. Both GO (low) and rGO (high) were found to be statistically significant. All treatments resulted in an increase of *nirK* after the incubation period. Abundances at day 0 ranged from $3.75 \times 10^4$ to $1.35 \times 10^5$ copies $g^{-1}$ while at day 28 abundances ranged from $3.08 \times 10^4$ to $4.87 \times 10^5$ copies $g^{-1}$. The control was the only one to show a decrease over time and when compared to the control all treatments resulted in an increase. Three treatments were found to be significantly different: rGO (high) and BC. The abundances of *nirS* ranged from $6.78 \times 10^5$ to $2.06 \times 10^6$ copies $g^{-1}$ at the start of the incubation period and $1.07 \times 10^6$ to $3.16 \times 10^6$ copies $g^{-1}$ by the end of the incubation period. The most notable differences were seen in both concentrations of GNP

amendment with more than twice as many copies/g soil after the 28 days. The high concentration of GNP was found to be statistically significant. On day 0, *nosZ* abundances ranged from $6.21 \times 10^5$ to $2.12 \times 10^6$ copies $g^{-1}$ and had a similar range, $6.68 \times 10^5$ to $2.97 \times 10^6$ copies $g^{-1}$, on day 28. No significant differences were observed between treatments and the control or over time with regard to *nosZ* abundance. All treatments resulted in a decrease of *nifH* abundance over time ranging from $5.46 \times 10^5$ to $1.25 \times 10^6$ copies $g^{-1}$ at day 0 and $1.81 \times 10^5$ to $9.23 \times 10^5$ copies $g^{-1}$ at day 28. Both concentrations of BC as well as GO (low) were found to be statistically significant. Lastly, *amoA* abundances at day 0 ranged from $5.22 \times 10^4$ to $5.42 \times 10^5$ copies $g^{-1}$ and $5.06 \times 10^4$ to $4.22 \times 10^5$ copies $g^{-1}$ at day 28. The abundance did not change significantly over time with the exception of both concentrations of GNA and GNP (high) which were significantly higher after the incubation period. However, of these three treatments only GNA (high) and GNP (high) were found to be statistically significant over time, while BC (low) was found to be statistically significant compared to the control.

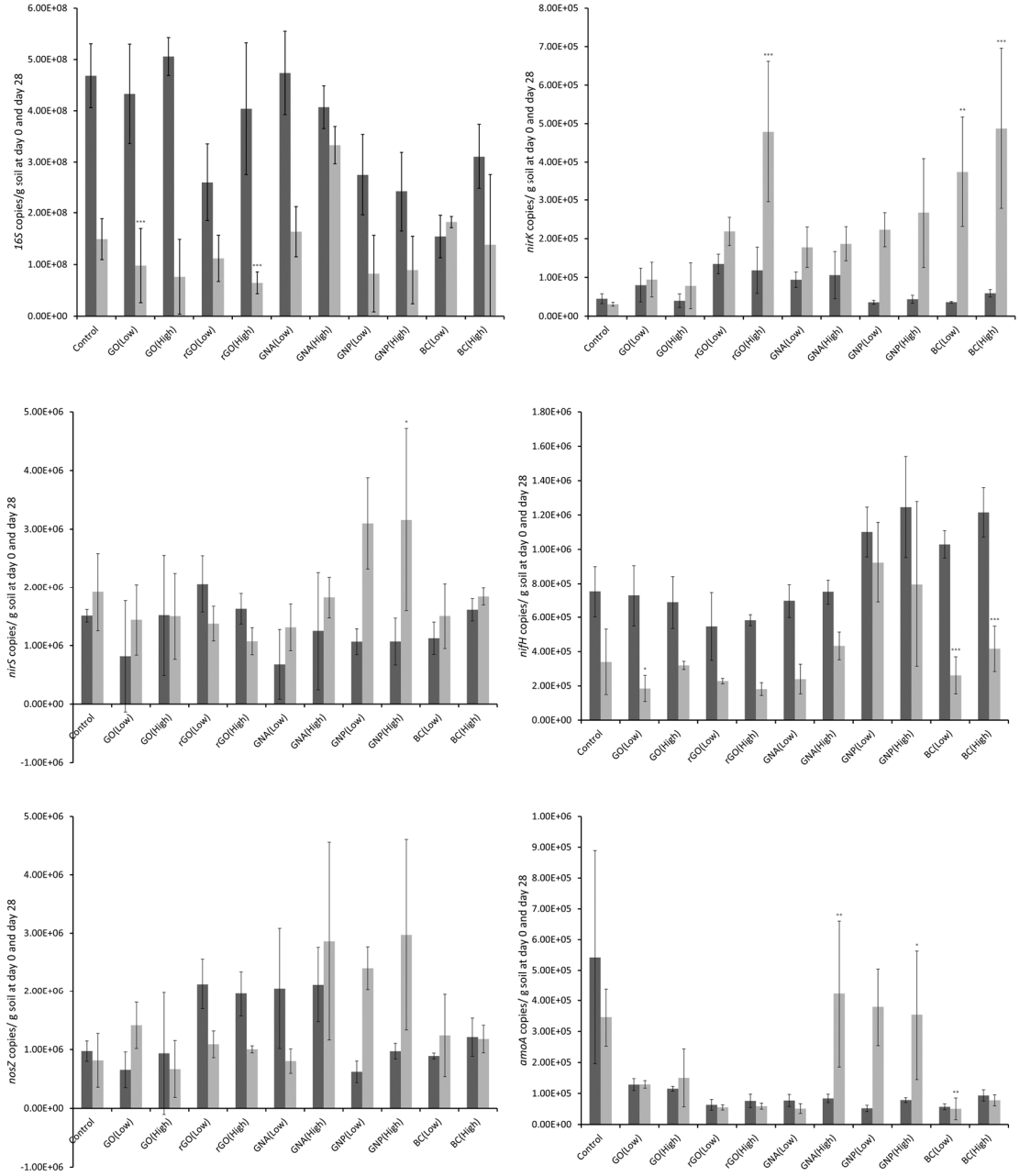


Figure 2. Abundances of *16S rRNA* as well as N cycling functional genes in copies/ g soil. Black bars and grey bars represent day 0 and day 28, respectively. Error bars show ± standard deviations. Asterisks indicate significant differences at *p<0.05, **p<0.01 and, ***p<0.001 compared to the control.

The N cycling functional genes were then evaluated in terms of relative abundance as a percentage of *16S rRNA* to determine what percent of the community is occupied by each functional group (Figure 3). On day 0, *nirK* ranged in relative abundance from 0.0049%-0.0434%. On day 28 it ranged from 0.022%-0.205%. Overall, there appears to be an increase in the *nirK* relative abundance over time. The most notable differences are in rGO (high) as well as BC (high) and BC (low). All treatments experienced an increase in *nirK* when compared to the control after the 28 day incubation period. For the *nirS* gene, the relative abundance ranged from 0.0922%-1.1814% at day zero and 0.488%-1.288% on day 28. All treatments exhibited an increase of *nirS* over time, and all treatments showed a decrease when compared to the control at both sampling times. Similarly, *nosZ* ranged from 0.0895%-0.7291% before the incubation period and 0.252%-0.714% after the incubation period. Again, all treatments experienced an increase over time, but all treatments were decreased compared to the control before and after the incubation period. Before the incubation period, *nifH* ranged from 0.0837%-0.2232%. After the incubation period *nifH* ranged from 0.080%-0.316%. A decrease in relative abundance in both concentrations of BC as well as GO (low) and rGO (high) was observed. Lastly, on day 0, *amoA* ranged from 0.0089%-0.0177% and 0.274%-0.017% on day 28. Overall there was an increase in *amoA* across all treatments after the incubation period. However, all treatments were significantly lower than the control.

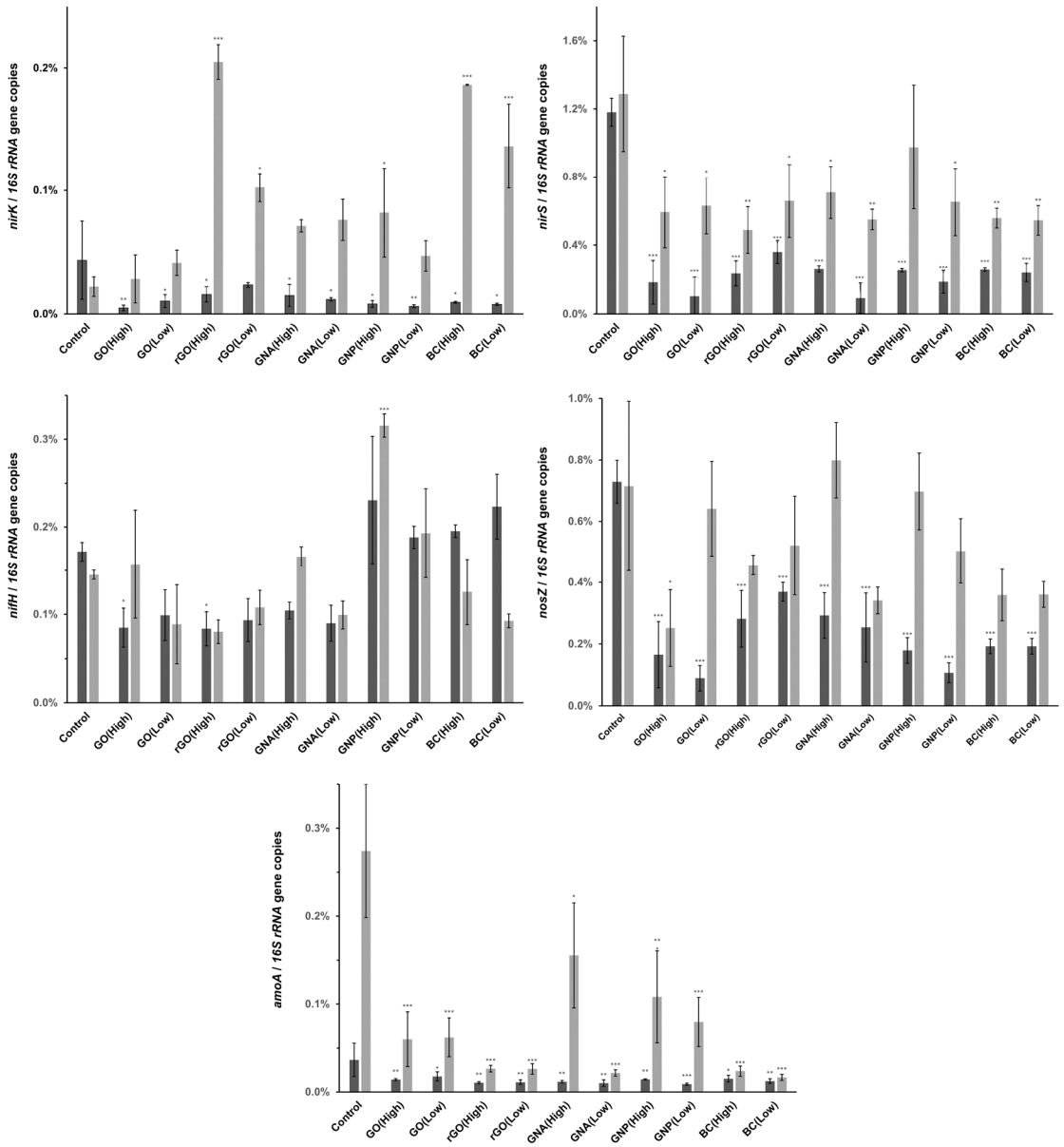


Figure 3. Relative abundances of N cycling functional genes as a proportion of *16S rRNA* gene abundances. Black bars and grey bars represent day 0 and day 28, respectively. Error bars show ± standard deviations. Asterisks indicate significant differences at *p<0.05, **p<0.01 and, ***p<0.001 compared to the control.

# CHAPTER 5
# DISCUSSION

This study observed and analyzed the relationship between various soil amendments (nanofertilizers and biochar) and the soil microbial community. These soil amendments are now being investigated as alternatives to current fertilization practices which have numerous negative impacts on the environment. It is important to carry out thorough testing of nanofertilizers and biochar (BC) in order to determine if their use in agriculture is feasible as an alternative to current fertilization practices. The addition of nanofertilizers should result in (1) an increase in N-cycling genes (and increase in potential microbial N transformations) due to the slow-release capabilities of nanoparticles and the ability for BC to decrease leaching therefore making N more readily available (Raliya et al, 2018; Yu et al, 2019), as well as (2) an increase in overall microbial abundance with the addition of both nanofertilizers and BC since they are carbon-based substances and BC has previously been observed to increase microbial abundance (Ducey et al., 2013).

The abundance of the bacterial community, determined by the number of *16S rRNA* gene copies, decreased across all treatments, including the control, after the 28 day incubation period. This may be due to C limitation given that no additional C was introduced into the soil samples at any point throughout the incubation period after the addition of the carbon-based treatments and that the collected soil already had a low-carbon content of 1.76%. In another study in which the soil had a carbon content between 1.39% to 3.60%, it was found that the total microbial abundance was between $1.8 \times 10^8$ and $1.2 \times 10^{10}$, which is consistent with what was found in the present study ($1.54 \times 10^8$

to $5.06 \times 10^8$ copies g-1 soil at day 0 and $6.44 \times 10^7$ to $3.33 \times 10^8$ copies g-1 at day 28 (Figure 2)) (Ligi et al., 2014). However, there was no direct evidence to confirm this and further testing would need to be performed at a later date. In a maize-tomato (*Zea mays* L.–*Lycopersicum esculentum* L.) cropland, three cropping systems were studied: a conventional system (annual synthetic fertilizer application), a low-input system (synthetic fertilizer and cover crops applied in alternate years) and an organic system (annual composted manure and cover crop). They found that *16S rRNA* genes averaged across the cropping systems increased from $1.06 \times 10^8$ copies $g^{-1}$ soil at $T_0$ (day 0) to $3.34 \times 10^8$ and $7.70 \times 10^8$ copies $g^{-1}$ soil at $T_i$ (8 months) and $T_h$ (11 months), respectively (Kong et al., 2010). This increase may be explained by the presence of plants in the system which could have fostered plant-microbe interactions. In an artificial soil incubation, Vogel et al. (2014) found that *16S rRNA* abundance increased after an incubation period of 561 days and after the addition of manure, ranging from $6 \times 10^9$ to $2 \times 10^{10}$ copies per gram of soil. They sampled the soils again after 839 days and found *16S rRNA* abundance had significantly increased since day 561, although no specific abundances were provided. These results are not consistent with the results presented in the present study, which found *16S rRNA* abundance to decrease over time.

Our results showed that *nirK* abundance increased over the course of the incubation period. Abundances at day 0 ranged from $3.75 \times 10^4$ copies $g^{-1}$ soil to $1.35 \times 10^5$ copies $g^{-1}$ soil while at day 28 abundances ranged from $3.08 \times 10^4$ copies $g^{-1}$ soil to $4.87 \times 10^5$ copies $g^{-1}$ soil. These results indicate that there is the potential for an increase in nitrate reduction over time after the addition of these nanofertilizers. Other studies have found varying results in *nirK* abundance with the addition of inorganic as well as

organic fertilizers. Ligi et al. (2014) reported a higher abundance, $8.9 \times 10^7$ to $5.6 \times 10^8$ copies/g, than what is reported here. Their soil characteristics consisted of a pH ranging from 5.60 to 7.16, lower than the pH of the present study (8.36). In a sand to silty sand with a pH of 5.56 to 7.53, *nirK* abundances were reported as ranging from $7.4 \times 10^2$ to $1.4 \times 10^3$ copies $ng^{-1}$ DNA (Kandeler et al., 2006). Acidic soils (pH 3.53 to 5.45) were observed to have *nirK* abundances ranging from $2.2 \times 10^4$ to $1.6 \times 10^6$ copies $g^{-1}$ soil (Lindsay et al., 2010). Two recent studies have shown that the application of BC decreased the abundance of *nirK*, which is in direct contrast to our results that found there to be an increase in *nirK* abundance after the application of BC (Zhaoxiang et al., 2020; Liao et al., 2020). Organic fertilizers such as bio-organic fertilizer, manure, and wheat straw increased the abundance of *nirK* (Sun et al., 2015; Zhaoxiang et al., 2020). Another study analyzed the effects of rGO on the soil microbial community. It found that rGO decreased the relative abundance of *nirK* which is consistent with the results that were found at the beginning of the 28 day incubation period (Banach-Wiśniewska et al., 2021). However, after the 28 day incubation period in our study, the relative abundance of *nirK* was found to increase. The differences in these results could be due to differences in soil type and pH as acidic soils reported lesser abundances than those reported here.

Treatment with biochar and the various nanofertilizers resulted in mixed results with regards to the abundances of *nirS*. The rGO treatment resulted in a decrease of *nirS* abundance over time at both rGO concentrations. Previously, *nirS* abundance was found to increase with the addition of rGO (Banach-Wiśniewska et al., 2021). The observed higher *nirS* abundance with rGO addition only occurred upon the initial addition of rGO, not over time. Similar to *nirK*, the abundance of *nirS* was found to increase with the

addition of manure and decrease with the addition of BC (Sun et al., 2015; Zhaoxiang et al., 2020). Our BC results are consistent with this finding, as we found that *nirS* abundance decreased when compared to the control soil. Although, over time, we found the abundance did increase slightly in BC amended samples. Other studies have reported abundances of *nirS* in varying soil types with and without soil amendments. In a slightly acidic soil, *nirS* abundance was reported as $6.8 \times 10^7$ to $3.4 \times 10^9$ copies $g^{-1}$, which is greater than what is reported in the present study ranging from $6.78 \times 10^5$ to $2.06 \times 10^6$ copies $g^{-1}$ at the start of the incubation period and $1.07 \times 10^6$ to $3.16 \times 10^6$ copies $g^{-1}$ (Ligi et al., 2014). In another soil with a similar pH, the abundance of *nirS* was reported in copies $ng^{-1}$ DNA: $2.5 \times 10^2$ to $6.4 \times 10^3$ (Kandeler et al., 2006). Again, the differences observed here are most likely due to the differences in soil type and characteristics such as soil pH and texture as the studies presented utilized soils with a lower pH than the present. An additional study which investigated a sandy loam (pH 6.0) was not able to amplify *nirS* suggesting that the gene was either not present in the soil samples or too low to detect (Sharma et al. 2005). However, they utilized a primer pair (nirS1F-nirS6R) that differed from the one used in this study (nirSCd3cd-nirSR3cd), which may be a cause as to why *nirS* was not able to amplify. Sharma et al. (2005) also stated that many others had difficulty amplifying *nirS* utilizing the same primer pair (Avrahami et al., 2002; Priemé et al., 2002). Jung et al., (2011) discovered that *nirS* outnumbered *nirK* in acidic soils (pH 4.6-5.5) after an incubation period of 30 days, regardless of treatments tested, which is consistent with the referenced studies. This behavior might be explained by the negative charge on both the nanofertilizers and the biochar. This negative charge may bind to positively-charged copper ($Cu^{2+}$), which makes it unavailable for copper-

dependent nitrate reductase, thereby limiting the growth of the *nirK* population and reducing the abundance of *nirK*. More nitrite was also available for *nirK* and *nirS* transformation to pave the way for denitrification intermediates: nitrous and nitric oxides.

All soil amendments presented in this study resulted in a decrease of *nifH* over time. At the time of writing, no studies were found which detail the effects of nanoparticles on *nifH*. Other researchers have studied the effects of synthetic and organic fertilizers on soil microbial communities and have reported mixed results. Sun et al. (2015) reported that there was no significant effect of NPK fertilizer on the abundance of *nifH* whereas organic fertilizers such as manure were found to increase *nifH* abundance. Inorganic fertilizers (such as NPK) do not contain carbon, whereas organic fertilizers (such as manure) do contain carbon, which may explain the effects of inorganic and organic fertilizers on *nifH* abundance. Microbes containing *nifH* are heterotrophs, meaning that they need an organic carbon source for energy. It is possible that the lack of carbon in inorganic fertilizers is what contributes to the negligible or negative effects they have on *nifH* abundance. This also ties into the possible carbon limitation affecting the overall microbial abundance. In an acidic soil (pH 3.53 to 5.45), *nifH* resulted in the lowest abundance of all the functional genes observed (*nirk, nifH*, and *amoA*) with a reported abundance of $5.0 \times 10^2$ to $2.1 \times 10^4$ copies $g^{-1}$ soil (Lindsay et al., 2010). In the present study (pH 8.6), results indicated that among these three genes (*nirk, nifH*, and *amoA*), *nifH* had the highest abundance ranging from $5.46 \times 10^5$ copies $g^{-1}$ to $1.25 \times 10^6$ copies $g^{-1}$ at day 0 and $1.81 \times 10^5$ to $9.23 \times 10^5$ at day 28. In another acidic soil (pH 4.6–5.5), the abundance of *nifH* was found to be between $4.0 \times 10^4$ and $1.4 \times 10^5$ copies $g^{-1}$ (Jung et al., 2011). This suggests that *nifH*-containing bacteria favor soils with a pH

closer to neutral rather than acidic soils and, based on the referenced studies, there is a trend in increasing *nifH* abundance as pH reaches neutral. Further analyzing the effects of nanofertilizers on *nifH* communities is imperative for understanding how they alter N fixation in soils as N fixation is one mechanism that makes N available for plant uptake.

Much like *nirS,* the abundance of *nosZ* varied by treatment. The present study found that there was an increase in *nosZ* abundance with the application of the low concentration of BC after the 28 day incubation period. Another study revealed that BC, BC + biofertilizer, and BC + synthetic fertilizer also resulted in an increase in *nosZ* abundance, which indicates that BC can magnify the effects of other locally applied soil amendments (Zhaoxiang et al., 2020). On day 0, *nosZ* abundances ranged from $6.21 \times 10^5$ to $2.12 \times 10^6$ and had a similar range, $6.68 \times 10^5$ copies $g^{-1}$ soil to $2.97 \times 10^6$ copies $g^{-1}$ soil, on day 28 which is consistent with results from Ligi et al. (2014) who reported *nosZ* gene abundance ranged from $4.9 \times 10^6$ to $7.0 \times 10^7$ copies $g^{-1}$ . Kandeler et al. (2006) reported $1.2 \times 10^3$ to $5.5 \times 10^3$ *nosZ* copies $ng^{-1}$ of DNA. Kong et al. (2010) found that copies of *nosZ* were greater in conventional and low-input cropping systems, over time, when compared to an organic cropping system and attributed this result to the effects of C availability on the denitrifier community. This supports some of the findings in this study in which, *nosZ* abundance increased over time with certain treatments (like GNP). The GNP treatment had the greatest impact on *nosZ* abundance, significantly increasing it. This increase may be explained by the structure of GNP which was described as having a low specific surface area (SSA) of 20-40 $m^2$ $g^{-1}$.

When compared to the control, *amoA* abundance decreased with the addition of BC which is not consistent with the results of Zhaoxing et al. (2020) who found *amoA*

abundance increased after the addition of BC. Our study also found that *amoA* abundance decreased with the addition of rGO, which is consistent with the findings of Banach-Wiśniewska et al. (2021) that also reported a decrease in *amoA* after the addition of rGO. The present study found that *amoA* abundances at day 0 ranged from $5.22 \times 10^4$ copies $g^{-1}$ to $5.42 \times 10^5$ copies $g^{-1}$ and $5.06 \times 10^4$ copies $g^{-1}$ to $4.22 \times 10^5$ copies $g^{-1}$ at day 28. In a soil with a much lower pH range (3.53 to 5.45), *amoA* abundances ranged from 0 to $5.1 \times 10^5$ copies $g^{-1}$ (Lindsay et al., 2010). Kong et al. (2010) reported that *amoA* gene abundance was six times greater in an organic cropping system at the beginning of the study and two times greater after 8 months when compared to the conventional cropping system and the low-input cropping system which is consistent with our results that *amoA* abundance decreased over time. Ammonium nitrate was used to supplement the treatments which should have provided ammonium for microbial transformation. Nitrifying bacteria are autotrophs, so there would be negligible effects from the carbon-based treatments in terms of C-limitations. The decrease in *amoA* abundance would also mean that there is a decrease in nitrate and nitrite production, meaning that there is the potential for a decrease in N losses via leaching. As mentioned previously, nitrate is the most mobile form of nitrogen and is easily lost into waterways.

Overall, there is no clear answer regarding the effects of nanofertilizers and biochar on the soil microbial community. Many studies have observed both increases and decreases in the abundance of N-cycling functional genes. The difference in observations is mainly due to the soil type as well as soil characteristics such as pH, CEC, and C content. In the referenced studies, pH was identified as having the largest effect in N-cycling functional gene abundances while C content was identified as having the largest

effect on overall microbial abundance (*16S rRNA*). Additionally, biochar varies greatly in carbon base (e.g. wood vs. rice husk) and method of preparation, which in turn alters the overall characteristics of the biochar.

The results of this study revealed that GNP had the largest impact on the abundance of some N-cycling genes, namely *nirS, nosZ*, and *amoA*. The abundance of all of these genes greatly increased over time. The increase in abundance may be attributed to the structure of GNP which had a particle size of 2-10 nm thick and a diameter of 2-7 μm. The specific surface area (SSA) of GNP was 20-40 $m^2$ $g^{-1}$ which is the second lowest SSA of the treatments tested. A comparable treatment was GNA, although it had a different preparation method and was described to be highly disordered. These reasons may be why GNA did not have a greater effect on the soil microbial community. As for rGO, it had the largest effect on overall microbial abundance with a decrease in *16S rRNA* abundance greater than 60%. The rGO treatment also resulted in an increase in *nirK* abundance as well as a decrease in abundance of all other N-cycling genes. Reduced graphene oxide was the treatment that had the greatest impact on the increase of *nirK* abundance. Biochar also had a large impact on *nirK* abundance. The results of the present study revealed that *nirK* increased in abundance after the addition of BC while others reported an decrease in *nirK* abundance. It was also found that BC significantly decreased the abundance of *nifH* and no other studies could be found to support this finding. As for the other N-cycling genes, BC had very little effects on their abundance. It appears that rGO and BC had similar effects across N-cycling genes which may be due to their similar characteristics. As mentioned previously, BC is the closest analogue to nanofertilizers, and although these treatments vary in particle size, their zeta potential in both soil and

water is very similar. In addition, rGO was found to have the largest SSA among the nanofertilizers (180 $m^2$ $g^{-1}$) compared to BC with a much higher SSA (~400 $m^2$ $g^{-1}$). Given the results presented in this study, it appears that rGO and BC impart similar effects on the microbial community, whereas GNP had the most significant impact overall.

The study presented here is novel in its approach. The study of nanofertilizers is a relatively new avenue of research and little has been published in terms of determining their effects on the soil microbial community. Current studies emphasize the microbial community as a whole via testing of *16S rRNA* rather than focusing on other functional genes. In order to gain more insight into the relationship between the soil microbial community and nanofertilizer amendments, more research and testing must be conducted. The methodology outlined in this study should be carried out on a variety of soils, particularly those that are commonly used for agricultural applications in order to compile more specific data. In addition to controlled lab tests, there should be field tests to account for the uncontrolled field conditions that are more likely to occur in real-world situations. Having this data would be incredibly beneficial in real-world applications if this technology is to be seriously considered for applications in agriculture. Furthermore, to determine the toxicity of these materials on the soil microbial communities, community sequencing should be conducted. Community sequencing could provide insight into how the community composition changes with the addition of these substances. It would also prove beneficial to test these nanofertilizers with plant subjects rather than bare soil samples. By incorporating plants, further scrutiny can be applied toward plant-microbe interactions and how those relationships change with the addition

of these materials. However, if bare soil studies were to continue, it is recommended using soil cores, rather than gathering and mixing topsoil. By utilizing soil cores, the soil microbial community is able to stay intact for a more accurate representation of results.

REFERENCES


Abukari, A. (2019). Influence of Rice Husk Biochar on Water Holding Capacity of Soil in The Savannah Ecological Zone of Ghana. *Turkish Journal of Agriculture-Food Science and Technology*, 7(6), 888-891 DOI: 10.24925/turjaf.v7i6.888-891.2488

Aacharya, R., Chhipa, H. (2020) 15 - Nanocarbon fertilizers: Implications of carbon nanomaterials in sustainable agriculture production. *Micro and Nano Technologies, Carbon Nanomaterials for Agri-Food and Environmental Applications,* 297-321 DOI: 10.1016/B978-0-12-819786-8.00015-3

Anderson, D. M., Glibert, P. M., & Burkholder, J. M. (2002). Harmful algal blooms and eutrophication: nutrient sources, composition, and consequences. *Estuaries*, 25(4), 704-726 DOI: 10.1007/BF02804901

Avrahami, S., Conrad, R., & Braker, G. (2002). Effect of soil ammonium concentration on N2O release and on the community structure of ammonia oxidizers and denitrifiers. *Applied and environmental microbiology*, *68*(11), 5685-5692 DOI: 10.1128/AEM.68.11.5685-5692.2002

Ayoub, A.T. (1999). Fertilizers and the environment. *Nutrient Cycling in Agroecosystems* 55, 117–121 DOI: 10.1023/A:1009808118692

Banach-Wiśniewska, A., Tomaszewski, M., Hellal, M. S., & Ziembińska-Buczyńska, A. (2021). Effect of biomass immobilization and reduced graphene oxide on the microbial community changes and nitrogen removal at low temperatures. *Scientific Reports,* 11(1), 1-12 DOI: 10.1038/s41598-020-80747-7

Bernhard, A. (2010) The Nitrogen Cycle: Processes, Players, and Human Impact. *Nature Education Knowledge* 3(10):25

Břendová, K., Tlustoš, P., Száková, J., & Habart, J. (2012). Biochar properties from different materials of plant origin. *European Chemical Bulletin*, *1*(12), 535-539.

Burkart, M. R., & James, D. E. (1999). *Agricultural nitrogen contributions to hypoxia in the Gulf of Mexico* (Vol. 28, No. 3, pp. 850-859). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America DOI: 10.2134/jeq1999.00472425002800030016x

Clayton, H., McTaggart, I. P., Parker, J., Swan, L., & Smith, K. A. (1997). Nitrous oxide emissions from fertilised grassland: A 2-year study of the effects of N fertiliser form and environmental conditions. *Biology and fertility of soils*, 25(3), 252-260 DOI: 10.1007/s003740050311

Conrad, R. (1996). Soil microorganisms as controllers of atmospheric trace gases (H2, CO, CH4, OCS, N2O, and NO). *Microbiological reviews*, 60(4), 609-640 DOI: 10.1128/mr.60.4.609-640.1996

David, M. B., Gentry, L. E., Kovacic, D. A., & Smith, K. M. (1997). *Nitrogen balance in and export from an agricultural watershed* (Vol. 26, No. 4, pp. 1038-1048). American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America DOI: 10.2134/jeq1997.00472425002600040015x

Deeds, J. R., Wiles, K., VI, G. B. H., White, K. D., & Abraham, A. (2010). First US report of shellfish harvesting closures due to confirmed okadaic acid in Texas Gulf coast oysters. *Toxicon*, *55*(6), 1138-1146 DOI: 10.1016/j.toxicon.2010.01.003

Diaz, R. J., & Rosenberg, R. (2008). Spreading dead zones and consequences for marine ecosystems. *science*, *321*(5891), 926-929 DOI: 10.1126/science.1156401

Dobereiner, J. O. H. A. N. N. A., & Day, J. M. (1975). Nitrogen fixation in the rhizosphere of tropical grasses. *Nitrogen fixation by free-living microorganisms*, 39-56.

Driggers III, W. B., Campbell, M. D., Debose, A. J., Hannan, K. M., Hendon, M. D., Martin, T. L., & Nichols, C. C. (2016). Environmental conditions and catch rates of predatory fishes associated with a mass mortality on the West Florida Shelf. *Estuarine, Coastal and Shelf Science*, *168*, 40-49 DOI: 10.1016/j.ecss.2015.11.009

Ducey, T. F., Ippolito, J. A., Cantrell, K. B., Novak, J. M., & Lentz, R. D. (2013). Addition of activated switchgrass biochar to an aridic subsoil increases microbial nitrogen cycling gene abundances. *Applied soil ecology*, *65*, 65-72.

Fauquier, D. A., Flewelling, L. J., Maucher, J. M., Keller, M., Kinsel, M. J., Johnson, C. K., ... & Landsberg, J. H. (2013). Brevetoxicosis in seabirds naturally exposed to Karenia brevis blooms along the central west coast of Florida. *Journal of wildlife diseases*, *49*(2), 246-260 DOI: 10.7589/2011-09-270

Fowler, D., Coyle, M., Skiba, U., Sutton, M.A., Cape, J.N., Reis, S., Sheppard, L.J., Jenkins, A., Grizzetti, B., Galloway, J.N., Vitousek, P., Leach, A., Bouwman, A.F., Butterbach-Bahl, K., Dentener, F., Stevenson, D., Amann, M., Voss, M. (2013). The global nitrogen cycle in the twenty-first century. *Phil Trans R Soc,* 368 DOI: 10.1098/rstb.2013.0164

Galloway, J. N., Dentener, F. J., Capone, D. G., Boyer, E. W., Howarth, R. W., Seitzinger, S. P., ... & Vöosmarty, C. J. (2004). Nitrogen cycles: past, present, and future. *Biogeochemistry*, 70(2), 153-226 DOI: 10.1007/s10533-004-0370-0

Galloway, J. N., Townsend, A. R., Erisman, J. W., Bekunda, M., Cai, Z., Freney, J. R., ... & Sutton, M. A. (2008). Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. *Science*, 320(5878), 889-892 DOI: 10.1126/science.1136674

Ghorbani, M., Asadi, H., & Abrishamkesh, S. (2019). Effects of rice husk biochar on selected soil properties and nitrate leaching in loamy sand and clay soil. *International soil and water conservation research*, 7(3), 258-265 DOI: 10.1016/j.iswcr.2019.05.005

Goolsby, D. A., & Battaglin, W. A. (2001). Long□term changes in concentrations and flux of nitrogen in the Mississippi River Basin, USA. *Hydrological Processes*, *15*(7), 1209-1226 DOI: 10.1002/hyp.210

Gruber N., & Galloway J. N., (2008) An Earth-system perspective of the global nitrogen cycle. *Nature*, 451, 293-296. DOI: 10.1038/nature06592

Gu, C., Maggi, F., Riley, W. J., Hornberger, G. M., Xu, T., Oldenburg, C. M., Spycher, N., Miller, N. L., Venterea, R. T., and Steefel, C. (2009), Aqueous and gaseous nitrogen losses induced by fertilizer application, *J. Geophys. Res*., 114, G01006, DOI:10.1029/2008JG000788

Henry, S., Bru, D., Stres, B., Hallet, S., Philippot, L. (2006). Quantitative Detection of the nosZ Gene, Encoding Nitrous Oxide Reductase, and Comparison of the Abundances of 16S rRNA, narG , nirK , and nosZ Genes in Soils. *Applied and Environmental Microbiology*, 72(8), 181–5189 DOI: 10.1128/AEM.00231-06

Hofstra, N., & Bouwman, A. F. (2005). Denitrification in agricultural soils: summarizing published data and estimating global annual rates. *Nutrient Cycling in Agroecosystems*, *72*(3), 267-278 DOI: 10.1007/s10705-005-3109-y

Hou, S., Ai, C., Zhou, W., Liang, G., He, P. (2018). Structure and assembly cues for rhizospheric nirK- and nirS-type denitrifier communities in long-term fertilized soils. *Soil Biology and Biochemistry,* 119, 32-40 DOI: 10.1016/j.soilbio.2018.01.007

Hu, B. L., Shen, L. D., Xu, X. Y., & Zheng, P. (2011). Anaerobic ammonium oxidation (anammox) in different natural ecosystems. *Biochemical Society Transactions*, *39*(6), 1811-1816 DOI: 10.1042/BST20110711

Huang, J., Xu, C. C., Ridoutt, B. G., Wang, X. C., & Ren, P. A. (2017). Nitrogen and phosphorus losses and eutrophication potential associated with fertilizer application to cropland in China. *Journal of Cleaner Production*, *159*, 171-179 DOI: 10.1016/j.jclepro.2017.05.008

Jakienė, E., Spruogis, V., Romaneckas, K., Dautartė, A., & Avižienytė, D. (2015). The bio-organic nano fertilizer improves sugar beet photosynthesis process and productivity. *Zemdirbyste-agriculture,* 102, 141-146. DOI: 10.13080/z-a.2015.102.018

Jha, P., Biswas, A., Lakaria, B., & Rao, A. (2010). Biochar in agriculture – prospects and related implications. *Current Science*, 99(9), 1218-1225

Jung, J., Yeom, J., Kim, J., Han, J., Lim, H. S., Park, H., ... & Park, W. (2011). Change in gene abundance in the nitrogen biogeochemical cycle with temperature and nitrogen addition in Antarctic soils. *Research in microbiology*, *162*(10), 1018-1026 DOI: 10.1016/j.resmic.2011.07.007

Kandeler, E., Deiglmayr, K., Tscherko, D., Bru, D., & Philippot, L. (2006). Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. *Applied and environmental microbiology*, 72(9), 5957-5962 DOI: 10.1128/AEM.00439-06

Karam D. S., Nagabovanalli P., Rajoo K. S., Ishak C. F., Abdu A., Rosli Z., Muharam F. M., Zulperi D. (2021). An overview on the preparation of rice husk biochar, factors affecting its properties, and its agriculture application, *Journal of the Saudi Society of Agricultural Sciences* DOI: 10.1016/j.jssas.2021.07.005

Karimi, E., & Fard, E. M. (2017). Nanomaterial effects on soil microorganisms. In *Nanoscience and plant–soil systems* (pp. 137-200). Springer, Cham DOI: 10.1007/978-3-319-46835-8_5

Kong, A. Y., Hristova, K., Scow, K. M., & Six, J. (2010). Impacts of different N management regimes on nitrifier and denitrifier communities and N cycling in soil microenvironments. *Soil Biology and Biochemistry*, *42*(9), 1523-1533 DOI: 10.1016/j.soilbio.2010.05.021

Kreman, A., Bear, J., Shavit, U., Shaviv, A. (2005). Model Demonstrating the Potential for Coupled Nitrification Denitrification in Soil Aggregates. *Environmental Science & Technology,* 39 (11), 4180-4188 DOI: 10.1021/es048304z

Kuypers, M., Marchant, H. & Kartal, B. (2018). The microbial nitrogen-cycling network. *Nat Rev Microbiol.* 16, 263–276 DOI: 10.1038/nrmicro.2018.9

Lakzian A., Bayat M., Gadzhikurbanov A., & Zargar M., (2019). The role of nanotechnology for improving crop production. *RUDN Journal of Agronomy and Animal Industries*, 14(4), 297-305. DOI: 10.22363/2312-797X-2019-14-4-297-305

Lehmann, J., Gaunt, J., Rondon, M. (2006). Bio-char sequestration in terrestrial ecosystems – a review. *Mitigation and Adaptation Strategies for Global Change*, 11, 403-427 DOI: 10.1007/s11027-005-9006-5

Liao, J., Liu, X., Hu, A., Song, H., Chen, X., & Zhang, Z. (2020). Effects of biochar-based controlled release nitrogen fertilizer on nitrogen-use efficiency of oilseed rape (Brassica napus L.). *Scientific reports*, *10*(1), 1-14 DOI: 10.1038/s41598-020-67528-y

Ligi, T., Truu, M., Truu, J., Nõlvak, H., Kaasik, A., Mitsch, W. J., & Mander, Ü. (2014). Effects of soil chemical characteristics and water regime on denitrification genes (nirS, nirK, and nosZ) abundances in a created riverine wetland complex. *Ecological Engineering*, 72, 47-55 DOI: 10.1016/j.ecoleng.2013.07.015

Lindsay, E. A., Colloff, M. J., Gibb, N. L., & Wakelin, S. A. (2010). The abundance of microbial functional genes in grassy woodlands is influenced more by soil nutrient enrichment than by recent weed invasion or livestock exclusion. *Applied and Environmental Microbiology*, *76*(16), 5547-5555 DOI: 10.1128/AEM.03054-09

Mittal, S. K., Goyal, D., Chauhan, A., Dang, R. K. (2020). Graphene nanoparticles: The super material of the future. *Materials Today: Proceedings*, 28(3), 1290-1294. DOI: 10.1016/j.matpr.2020.04.260

Mosier, A. R. (1998). Soil processes and global change. *Biology and fertility of soils*, 27(3), 221-229 DOI: 10.1007/s003740050424

Naik, K., Mishra, S., Srichandan, H., Singh, P. K., Sarangi, P. K. (2019). Plant growth promoting microbes: Potential link to sustainable agriculture and environment, *Biocatalysis and Agricultural Biotechnology*, 21 DOI: 10.1016/j.bcab.2019.101326

O'neill, B., Grossman, J., Tsai, M., Gomes, J. E., Lehmann, J., Peterson, J., ... & Thies, J. E. (2009). Bacterial community composition in Brazilian Anthrosols and adjacent soils characterized using culturing and molecular identification. *Microbial ecology*, *58*(1), 23-35 DOI: 0.1007/s00248-009-9515-y

Pandey, C. B., Kumar, U., Kaviraj, M., Minick, K. J., Mishra, A. K., & Singh, J. S. (2020). DNRA: A short-circuit in biological N-cycling to conserve nitrogen in terrestrial ecosystems. *Science of the Total Environment*, *738*, 139710 DOI: 10.1016/j.scitotenv.2020.139710

Pereira, E. I. P., Suddick, E. C., Mukome, F. N., Parikh, S. J., Scow, K., & Six, J. (2015). Biochar alters nitrogen transformations but has minimal effects on nitrous oxide

emissions in an organically managed lettuce mesocosm. *Biology and Fertility of Soils*, *51*(5), 573-582 DOI: 10.1007/s00374-015-1004-5

Pitambara, Archana, Shukla Y.M. (2019) Nanofertilizers: A Recent Approach in Crop Production. In: Panpatte D., Jhala Y. (eds) Nanotechnology for Agriculture: Crop Production & Protection. Springer, Singapore. DOI: 10.1007/978-981-32-9374-8_2

Poly F., Ranjard L., Nazaret S., Gourbière F., Monrozier L. J., (2001). Comparison of nifH Gene Pools in Soils and Soil Microenvironments with Contrasting Properties. *Applied and Environmental Microbiology,* 67(5), 2255-2262. DOI: 10.1128/AEM.67.5.2255-2262.2001

Priemé, A., Braker, G., & Tiedje, J. M. (2002). Diversity of nitrite reductase (nirK and nirS) gene fragments in forested upland and wetland soils. *Applied and Environmental Microbiology*, *68*(4), 1893-1900.

Raliya, R., Saharan, V., Dimkpa, C., & Biswas, P. (2018). Nanofertilizer for Precision and Sustainable Agriculture: Current State and Future Perspectives. *Journal of Agricultural and Food Chemistry.* 66 (26), 6487-6503 DOI: 10.1021/acs.jafc.7b02178

Raymond, J. E., Fox, T. R., Strahm, B. D., & Zerpa, J. (2016). Ammonia volatilization following nitrogen fertilization with enhanced efficiency fertilizers and urea in loblolly pine (Pinus taeda L.) plantations of the southern United States. *Forest Ecology and Management*, *376*, 247-255 DOI: 10.1016/j.foreco.2016.06.015

Roberts, K. G., Gloy, B. A., Joseph, S., Scott, N. R., & Lehmann, J. (2010). Life cycle assessment of biochar systems: estimating the energetic, economic, and climate change potential. *Environmental science & technology*, *44*(2), 827-833 DOI: doi.org/10.1021/es902266r

Sangeetha J., Mundaragi A., Thangadurai D., Maxim S.S., Pandhari R.M., Alabhai J.M. (2019) Nanobiotechnology for Agricultural Productivity, Food Security and Environmental Sustainability. In: Panpatte D., Jhala Y. (eds) Nanotechnology for Agriculture: Crop Production & Protection. Springer, Singapore. DOI: 10.1007/978-981-32-9374-8_1

Scanlon, B. R., Jolly, I., Sophocleous, M., & Zhang, L. (2007). Global impacts of conversions from natural to agricultural ecosystems on water resources: Quantity versus quality. *Water resources research*, 43(3) DOI: 10.1029/2006WR005486

Seitzinger, S., Harrison, J. A., Böhlke, J. K., Bouwman, A. F., Lowrance, R., Peterson, B., ... & Drecht, G. V. (2006). Denitrification across landscapes and waterscapes:

a synthesis. *Ecological applications*, *16*(6), 2064-2090 DOI: 10.1890/1051-0761(2006)016[2064:DALAWA]2.0.CO;2

Sharma, P., Kumar, T., Yadav M., Gill, S. S., Chauhan N. S. (2021). Plant-microbe interactions for the sustainable agriculture and food security. *Plant Gene*, 28 DOI: 10.1016/j.plgene.2021.100325

Sharma, S., Aneja, M. K., Mayer, J., Munch, J. C., & Schloter, M. (2005). Diversity of transcripts of nitrite reductase genes (nirK and nirS) in rhizospheres of grain legumes. *Applied and Environmental Microbiology*, 71(4), 2001-2007 DOI: 10.1128/AEM.71.4.2001-2007.2005

Siksnane, I., & Lagzdins, A. (2017). Assessment of economic losses associated with nitrogen leaching in agricultural land in Latvia. In *Rural development 2017: bioeconomy challenges: proceedings of the 8th international scientific conference, 23-24 November, 2017, Aleksandras Stulginskis University, 2017, p. 423-427* DOI: 10.15544/RD.2017.204

Sinigalliano, C. D., Kuhn, D. N., Jones, R. D. (1995). Amplification of the amoA gene from diverse species of ammonium-oxidizing bacteria and from an indigenous bacterial population from seawater. *Applied and Environmental Microbiology,*61 (7) 2702-2706 DOI: 10.1128/aem.61.7.2702-2706.1995

Smil, V. (2001). Enriching the earth: Fritz Haber, Carl Bosch, and the transformation of world food production. *MIT press.*

Spokas, K. A., Koskinen, W. C., Baker, J. M., & Reicosky, D. C. (2009). Impacts of woodchip biochar additions on greenhouse gas production and sorption/degradation of two herbicides in a Minnesota soil. *Chemosphere*, *77*(4), 574-581.

Stein L.Y., Klotz M.G. (2016). The nitrogen cycle. *Current Biology,* 26(3), R94-R98. DOI: 10.1016/j.cub.2015.12.021

Stewart, W. M., Dibb, D. W., Johnston, A. E., & Smyth, T. J. (2005). The contribution of commercial fertilizer nutrients to food production. *Agronomy journal,* 97(1), 1-6 DOI: 10.2134/agronj2005.0001

Sun, R., Guo, X., Wang, D., & Chu, H. (2015). Effects of long-term application of chemical and organic fertilizers on the abundance of microbial communities involved in the nitrogen cycle. *Applied Soil Ecology*, 95, 171-178 DOI: 10.1016/j.apsoil.2015.06.010

Teng, Q., Zhang, D., Niu, X., & Jiang, C. (2018). Influences of application of slow-release Nano-fertilizer on green pepper growth, soil nutrients and enzyme activity.

*IOP Conf. Ser.: Earth Environ. Sci*. 208 DOI: 10.1088/1755-1315/208/1/012014/meta

Van Breemen, N., Burrough, P. A., Velthorst, E. V., Van Dobben, H. F., de Wit, T., Ridder, T. D., & Reijnders, H. F. R. (1982). Soil acidification from atmospheric ammonium sulphate in forest canopy throughfall. *Nature*, *299*(5883), 548-550 DOI: 10.1038/299548a0

Van der Weerden, T. J., & Jarvis, S. C. (1997). Ammonia emission factors for N fertilizers applied to two contrasting grassland soils. *Environmental Pollution*, 95(2), 205-211 DOI: 10.1016/S0269-7491(96)00099-1

Van Deventer, M., Atwood, K., Vargo, G. A., Flewelling, L. J., Landsberg, J. H., Naar, J. P., & Stanek, D. (2012). Karenia brevis red tides and brevetoxin-contaminated fish: a high risk factor for Florida's scavenging shorebirds? DOI: 10.1515/bot.2011.122

Velthof, G.L., Lesschen, J.P., Webb, J., Pietrzak, S., Miatkowski, Z., Pinto, M., Kros, J., Oenema,O. (2014). The impact of the Nitrates Directive on nitrogen emissions from agriculture in the EU-27 during 2000–2008. *Science of The Total Environment*, 468-469, 1225-1233 DOI: 10.1016/j.scitotenv.2013.04.058

Vitousek, P. M., Aber, J. D., Howarth, R. W., Likens, G. E., Matson, P. A., Schindler, D. W., Schlesinger, W. H., Tilman, W. G. (1997). Human alteration of the global nitrogen cycle: sources and consequences. *Ecological Applications*. 7(3), 737-750 DOI: 10.1890/1051-0761(1997)007[0737:HAOTGN]2.0.CO;2

Vogel, C., Babin, D., Pronk, G. J., Heister, K., Smalla, K., & Kögel-Knabner, I. (2014). Establishment of macro-aggregates and organic matter turnover by microbial communities in long-term incubated artificial soils. *Soil Biology and Biochemistry*, *79*, 57-67 DOI: 10.1016/j.soilbio.2014.07.012

Yu, J., Deem, L. M., Crow, S. E., Deenik, J. L., Penton, C. R. (2018). Biochar application influences microbial assemblage complexity and composition due to soil and bioenergy crop type interactions. *Soil Biology and Biochemistry*, 117, 97-107 DOI: 10.1016/j.soilbio.2017.11.017

Yu, J., Deem, L.M., Crow, S.E., Deenik, J., Penton, C.R., (2019). Comparative metagenomics reveals enhanced nutrient cycling potential after 2 years of biochar amendment in a tropical oxisol. *Appl. Environ. Microbiol.* 85, e02957-18. DOI: 10.1128/AEM.02957-18

Zhaoxiang, W., Huihu, L., Qiaoli, L., Changyan, Y., & Faxin, Y. (2020). Application of bio-organic fertilizer, not biochar, in degraded red soil improves soil nutrients and plant growth. *Rhizosphere*, 16, 100264 DOI: 10.1016/j.rhisph.2020.100264

Zulfiqar, F., Navarro, M., Ashraf, M., Akram, N.A., & Munné-Bosch, S. (2019). Nanofertilizer use for sustainable agriculture: Advantages and limitations. *Plant Science*, 289 DOI: 10.1016/j.plantsci.2019.110270