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Soil microbial nitrogen-transforming genes influenced by tree species and fertilization in an Appalachian hardwood forest

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Soil microbial nitrogen-transforming genes influenced by tree species and fertilization in an Appalachian hardwood forest

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Thesis submitted
to the Eberly College of Arts and Science
at West Virginia University

In partial fulfillment of the requirements for the degree of
Master of Science in
Biology

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Abstract

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Emma Genevera Fox-Fogle

Many forest ecosystems have been receiving elevated nitrogen (N) deposition due to human activity. Increased ecosystem N threatens soil fertility, water and air quality, and modifies the soil microbially-mediated N cycle in complex ways. The current study investigates how the abundance of key functional N cycling genes within soil bacteria is altered by N deposition and further mediated by different dominant tree species at the Fernow Experimental Forest, WV. Soils were analyzed from two watersheds, where watershed 3 (WS 3) has been receiving experimental applications of N fertilizer that are 2x historic ambient N deposition and 5x the current ambient N deposition, relative to a reference watershed (WS 7). Abundance of four N cycling functional genes responsible for nitrate (NO_3^-) and nitrous oxide (N_2O) production (*amoA*, *nirK*, *nirS*, *nosZ*) were targeted for quantification using qPCR. We hypothesized that the abundance of these N cycling functional genes would be significantly different in soils influenced by elevated N deposition. It was expected that the abundance of genes would reflect the production of NO_3^- and N_2O from soils, measured as soil nitrate content and potential denitrification production from soil samples from beneath four individual tree species (tulip poplar, sweet birch, black cherry, and northern red oak). These tree species represent two mycorrhizal fungal associations (arbuscular and ectomycorrhizal); thus, we also tested whether functional gene abundance can be predicted by mycorrhizal association. It was expected that nitrification would be greatest in WS 3 and beneath arbuscular mycorrhizal trees because AM-associated trees sometimes have relatively more rapid litter decomposition leading to greater N losses than ECM associated tree species. Greater N_2O production was also expected in WS 3 due to the acidified soil altering the microbial capacity to reduce N_2O to N_2 gas. Soil beneath black cherry, an AM-associated species, contained significantly more NH_4^+ within WS 3. Fertilization has altered gene abundance in WS 3, leading to reduced *nirK* and *nosZ* abundance. Tree species did not influence gene abundance in either watershed, although AM-associated trees demonstrated reduced *nirK* abundance in WS 3. Soil beneath black cherry trees had reduced *nirK* and *nosZ* abundance as well as altered nitrification of NH_4^+ in WS 3, indicating that soils beneath black cherry trees may have altered N processing under fertilization. The reduction of functional gene abundance was not correlated with N transforming processes in either watershed. Gene abundance is not a strong predictor of N transforming rates in this study. Future research of the microbial community, paired with enzymatic activity, will enable better understanding of relationships between structure and function of the microbial community on N cycling processes.

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Introduction and Background

The soil microbiome is pivotal in the processes of nitrogen (N) cycling, as microbial-mediated transformations regulate biologically available N and emissions of atmospheric N (via N fixation, ammonification, nitrification, and denitrification). The global atmosphere is composed of 78% dinitrogen (N_2) gas, which is inaccessible to a majority of organisms due to the triple bond between the N atoms. N is historically the most common limiting nutrient for forest vegetation in the northern hemisphere (Vitousek, et al. 1997) and is essential for functioning ecosystems. However, up until the 1990s, areas in the northeastern U.S. had been receiving high amounts of N due to anthropogenic activities such as the burning of fossil fuels and agricultural fertilization (Driscoll, et al. 2001; Schwede & Lear, 2014). These activities increased global rates of N deposition to terrestrial ecosystems, which has been cited as a factor in a series of plant and soil responses (Thoms, et al. 2012; Gilliam, et al. 2016) such as; increased above ground biomass (Vitousek, et al. 1997), decreased root biomass (Nadelhoffer, 2000), increased nitrate (NO_3^-) leaching, and increased nitrous oxide (N_2O) emissions from soil (Aber et al., 1998) N deposition peaked in the northeastern U.S. at $15 \text{ kg N ha}^{-1} \text{ yr}^{-1}$ in 1979 (et al. 2021). The burning of fossil fuels releases greenhouse gases such as N_2O , nitrogen dioxide (NO_2), sulfur dioxide (SO_2), and sulfate (SO_4). West Virginia, USA has historically received high amounts of wet deposition N and sulfur (S) in the form of acid deposition, due the proximity of the coal burning powerplants of the Ohio River valley (Burnham, et al. 2017).

Acid deposition is the formation of sulfuric (H_2SO_4) and nitric (HNO_3) acids via SO_x and NO_x compounds emitted from the burning of fossil fuels. It was first reported to be an ecological issue in the 1960s (Grennfelt, et al. 2020), and is known to have impacts on forest health and productivity (DeHayes, et al. 1999). Forest health in the northeastern U.S. began declining in the late 1900s as calcium (Ca^{2+}) and other essential nutrients leached from the soil to water bodies such as streams, rivers, lakes, and the oceans (Adams, DeWalle, & Hom, 2006). These waterbodies also experienced intense acidification causing aquatic life to decline (Grennfelt, et al. 2020) and leading to federal regulations such as the Clean Air Act (EPA). Since the enactment of the Clean Air Act in 1970, and subsequent amendments in 1977 and 1990, the deposition of reactive N has decreased significantly in the eastern U.S. (Burns, et al. 2021; Mathias & Thomas, 2018). The 1990

amendments introduced regulatory programs for acid rain control, and while some recovery of impacted forests has occurred due to decreased emissions, it is a long-term process (Mathias & Thomas, 2018). A 2015 study indicated recovery in the form of decreasing plant available aluminum (Al^{3+}) and increasing soil pH in 13 of 27 forested sites across the northeastern U.S. and southeastern Canada (Lawrence, et al. 2015). NO_x emissions have decreased since the early 2000s due to the federal and state regulations put in place for NO_x emissions (Schwede & Lear, 2014) and SO_x emissions in the United States have decreased significantly over the three decades due to emission control policies (Aas, et al. 2019). However, atmospheric acid deposition is only one way by which ecosystems can receive excess amounts of N.

As biologically available N limited agricultural production in the 19th century, the industrial conversion process known as the Haber-Bosch process was engineered in the early 1900s. The use of the Haber-Bosch process has allowed high-yield agriculture to flourish, consequently introducing large amounts of reactive N into systems through the large-scale and widespread application of inorganic N fertilizers to agricultural land (Schlesinger & Bernhardt, 2013). Use of the Haber-Bosch process produces approximately 150 million tons of ammonia (NH_3) for fertilizer worldwide, through the pressurization of N_2 and H_2 gas (Kyriakou, et al. 2020). Atmospheric NH_3 deposition increased from the 1960's into the mid-2010s, from 33.1 Gg N year⁻¹ to 566.5 Gg N year⁻¹ (Xu, et al. 2018), largely due to agricultural fertilization and livestock production (Schwede & Lear, 2014). While NH_3 is not the focus of this study, it is a contributor to increased bioavailable environmental N.

Fertilization has improved our ability to produce food for a growing population. Unfortunately, excess N fertilization has created large-scale ecological impacts, including water quality issues related to the leaching of nitrate (NO_3^-) (Singh & Sekhon, 1979) and the volatilization of NH_3 and gaseous by-products of microbial conversion (particularly NO and N_2O), both of which can also harm air quality. NH_4^+ volatilization occurs when NH_4^+ is converted to NH_3 gas which is then released to the atmosphere, and largely occurs in moist, near neutral agricultural soils (Hamid & Mahler, 1994). NO and NO_2 are main contributors to smog and associated human health problems (EPA 2016). While industrial sources are a major contributor to NO_2 emissions (EPA 2016), fertilized soils also have a large potential for production of NO_2 (Oikawa, et al. 2015). Agricultural soils are also the major contributor to N_2O sources. as the need for higher crop yields increases

N₂O and NO₂ emissions are predicted to follow suit (Reay, et al. 2012). Elevated N deposition has contributed to altered biogeochemical cycling in many ecosystems, as N input affects the rates of microbially-driven N cycling processes such as nitrification and denitrification (Treseder, 2008; Wallenstein, et al. 2006).

1.1 Nitrogen Pollutants and Global Change

In order to mitigate the impact of increased N in ecosystems, it is necessary to understand the ecosystem properties that influence N transformations and resultant N products (Backman & Klemetsson 2003; Hallin, et al. 2009; Kelly, et al. 2021). The N cycle is a complex biogeochemical cycle including biotic uptake, ammonification, nitrification, denitrification, and N fixation (Figure 1). Nitrogen compounds are common outputs from soils. One such pollutant, N₂O, is a gaseous form of N emitted by agricultural and anaerobic soils due to fertilization and oxygen depletion (Aamar, et al. 2020) via microbial denitrification and nitrification, with nitrification being the common pathway. N₂O is a major greenhouse gas and the dominant ozone-depleting substance produced by human activity (Ravishankara, et al. 2009), and soil is the main source of atmospheric N₂O, representing 56-70% of all global sources (Butterbach-Bahl, et al. 2013). Emissions of N₂O are particularly high in areas where N is available in excess, such as agricultural systems and areas of high N deposition such as the northeastern U.S. (Firestone & Davidson, 1989; Davidson & Verchot, 2000). However, current estimates of N₂O emissions from temperate forests vary significantly. Several European temperate forests report high annual N₂O emissions (Eickenscheidt, 2011; Eickenscheidt & Brumme, 2013). On the contrary, low N₂O emissions have been reported from eastern U.S. forests (Castro, et al. 1992; Peterjohn, et al. 1996). While N₂O accounts for only an estimated 10% of total greenhouse gas emissions, it has a 300-fold greater impact on global warming processes than carbon dioxide (CO₂) (Bates et al. 2008; Richardson et al. 2009). N₂O production in soil depends upon soil conditions, such as moisture, temperature, oxygen availability, available organic carbon and N, and C:N ratio of vegetative litter (Davidson, 1993; Pilegaard, 2013).

Another potential pollutant produced in the N cycle is NO₃⁻, which is formed via the oxidative microbial process of nitrification. It is a soluble, highly mobile anion repelled by soil surface negative charges, which are dominant in strongly weathered soil such as in the eastern U.S. Consequently, NO₃⁻ is prone to leaching from soil when water percolates through the soil profile

removing minerals causing acidification and downstream nutrient loading in water (Fenn, et al. 1998; Schlesinger & Bernhardt, 2013) and resulting in soil nutrient depletion (Aber, et al. 1995), soil acidification (Aber, et al. 1989), and water quality issues. Nutrient loading, due to excess N, may result in harmful water quality impacts, such as harmful algal blooms and eutrophication. Eutrophication occurs when nutrient loading of NO_3^- and phosphorus (P) increase the growth and biomass of algae. As the algae die and decomposes, the dissolved oxygen content in the aquatic system is depleted, potentially leading to fish kills and cyanobacteria blooms. Eutrophication is one of the most widespread water quality issues affecting river, streams, and lakes, leading to economic costs of approximately \$2.2 billion in U.S. freshwater systems alone (Smith, Tilman, & Nekola, 1999; Dodds, et al. 2009).

1.2 Ecological Impacts of Excess Nitrogen in Forests

The effects of excess N include eutrophication (discussed above), soil and water acidification, and shifts in species composition and biodiversity (Tsiknia, et al. 2015). Short term fertilization causes an increase in biomass and forest productivity due to alleviation of N limitations; however, when biological uptake capacity is reached productivity decline can ensue (Vitousek, et al. 1997). Long-term fertilization is associated with negative impacts due to exceeding the ecosystem N requirements (Aber, et al. 1989; Fenn, et al. 1998). Chronic N inputs from anthropogenic activities can lead to N levels in excess of biological demand, leading to what is commonly known as N saturation (Aber, et al. 1989; Fenn, et al. 1998; Lovett & Goodale, 2011). The N saturation theory as described by Aber et al. (1989), characterized the later-stage effects as forest decline, including NO_3^- leaching with associated base cation depletion, soil acidification, and increased nitrous oxide N_2O emissions. Forest decline due to N saturation is defined by visible tree death, the reduction of fine root biomass, reduction in mycorrhizal fungi, and reduction of bacterial biomass (Aber, et al. 1989; Fenn, et al. 1998; Treseder, 2008). Revised N saturation hypotheses indicate more of a continuum than distinct stages as ecosystems have differing starting points and N limitations (Aber, 1998; Lovett & Goodale, 2011). The reduction of fine root biomass is caused by a higher rate of fine root turnover and lesser nutrient allocation to production of fine roots (Nadelhoffer, 2000). Chronic N deposition can shift the associated soil microbial community structure, fungal abundance, and organic matter chemistry in forests (Wallenstein, et al. 2006; Frey, et al. 2014). Shifts in microbial community structure can influence the processes of nitrification and

denitrification (Petersen, et al. 2012), which in turn can further impact the production of N pollutants such as N_2O and NO_3^- . Symbiotic mycorrhizal fungi colonization of plant roots, both ectomycorrhizal (ECM) and arbuscular mycorrhizal (AM) fungi, may also decline with increased N, due to reduced carbon (C) allocation as N is no longer limiting for the host plant (Treseder, 2004; Hogberg, et al. 2010; Burnham, et al. 2017).

Soil acidification resulting from elevated NH_4^+ deposition can occur due to a buildup of hydrogen (H^+) cations, causing soil pH to decrease. Increases in H^+ occur when plants uptake N in the form of NH_4^+ , a process that leaves H^+ in the soil and leads to acidification (Schlesinger & Bernhardt, 2013), as well as through the oxidation process of nitrification. Large additions of NH_4^+ -based fertilizers can displace base cations that buffer the soil against acidification, causing them to leach out of the soil (Driscoll, et al. 2001). Soil acidification leads to changes in soil chemistry and microbial community composition and can impact vegetation. The loss of base cations such as Ca^{2+} and Mg^{2+} can cause nutrient limitations for vegetation leading to species composition shifts (Kogelman & Sharpe, 2006; Elias, Burger, & Adams 2009). In severe cases of soil acidification, aluminum (Al^{3+}) buffering can occur when there is minimal Ca^{2+} and Mg^{2+} left in the soil, causing vegetation damage (Schlesinger & Bernhardt, 2013).

1.3 Long-term Fertilization Studies

Long-term fertilization studies have provided large data sets on forest vegetation and soil response to increased N. Several long-term N fertilization experiments have focused on the response to chronic N additions over the course of decades - the USDA Forest Service Fernow Experimental Forest (FEF) (Adams, DeWalle & Hom, 2006) and the University of Maine Bear Brook Watersheds have paired watershed experiments that have been ongoing since 1989, although both are now in recovery phases of the study as fertilization has ceased after 30 years of fertilization (Venterea, et al. 2004). The long-term fertilization studies allow researchers to study the effects of N fertilization (Peterjohn, Adams & Gilliam 1996; Gilliam, et al. 2016; Gilliam, Adams and Peterjohn, 2020), and have documented ecosystem responses of decreased vegetative species diversity (Chapin III, et al. 2000) and shifts in the microbial community (Carrara, et al. 2018).

1.4 Plant-Microbe Interactions

Mutualistic plant-microbe interactions contribute directly to essential ecosystem processes. The soil microbial community plays an essential part in the processing and storage of N and is influenced by the dominant vegetation, due to differences in the chemical and physical make-up of the plant litter, as well as root physiology and mycorrhizal association (both AM or ECM fungi). Many studies have documented vegetation-mediated variation in N-cycling process rates (Melillo et al. 1982; Peterjohn et al. 2015; Ribbons et al. 2016). However, the influence of tree species on the soil microbial community may be altered by atmospheric deposition of N, or in areas receiving high amounts of N fertilizer (Treseder, 2008). Changes in dominant above-ground vegetation composition impact the associated soil microbiome in function, storage, and processing rates of N (Grayston & Prescott, 2005; Ribbons, et al. 2016).

The soil microbial community is influenced by tree species composition directly due to differences in leaf litter composition (Thoms, et al. 2012), as litter provides the initial nutrient content for decomposers and root exudates, and indirectly through fungal association (Phillips, Brzostek & Midgley 2013). Multiple studies (Chapman, et al. 2005; Phillips, Brzostek, & Midgley, 2013) have linked fungal association such as AM or ECM to a variety of feedback loops in nutrient cycling. AM-associated tree species are correlated with more rapid litter decomposition relative to ECM-associated species, due to a generally lower C:N ratio of the litter (Midgley & Phillips 2014). Additionally, ECM fungi have higher enzymatic capability for mining nutrients compared to AM fungi (Lucas & Casper, 2007), which allows the fungi to break down recalcitrant litter that is commonly associated with ECM species such as oaks, conifers, and birches (Cheeke, et al. 2016). Soil microbial processes such as nitrification differ among tree species, reflecting nutrient economy associated with the species. Trees with a more inorganic nutrient economy (commonly AM species) have greater NO_3^- losses due to the faster breakdown of the high quality, low C:N litter (Phillips, Brzostek & Midgley 2013). In turn, this may result in differing export of NO_3^- to streams through soil leaching (Peterjohn, et al. 2015), suggesting that watersheds dominated by slower N cycling trees such as ECM-associated oak species would have lower stream N exports relative to AM-dominated watersheds (Kelly, 2010; Phillips, Brzostek & Midgley, 2013; Midgley & Phillips 2014; Peterjohn, et al. 2015).

1.5 Microbial Functional Genes

Microbial functional genes may be used as a predictive tool in biogeochemical cycling and enhance the understanding of environmental changes within an ecosystem (Braker, et al. 2000; Bernhard, et al. 2010). The quantification of functional gene abundance can be used as a measure of the microbial community containing the specific gene. N cycling specific functional genes have been suggested as an index of potential nitrification and denitrification rates (Venterea, et al. 2004), as the abundance of functional microbial genes associated with N cycling in soil is a strong predictor of N transformation rates (Philippot, et al. 2009; Petersen, et al. 2012; Ribbons, et al. 2016). For example, a study examining tree species effect on gene abundance and N cycling rates documented a significant positive correlation between *amoA* gene abundance and net nitrification (Ribbons, et al. 2016). Lab soil incubation studies have found that abundance of *nirK* is correlated to NO emissions, indicating *nirK* abundance is reflective of denitrification potential (Szukics, et al. 2009). The relative abundance of *nosZ* was shown to be a strong predictor of potential N₂O production, as lower abundance of *nosZ* correlated to a higher rate of N₂O production (Philippot et al. 2009). Thus, structure and function of soil microbial communities play a large role in the cycling of N, as the step-wise processes are highly controlled by metabolic capabilities of the microbial community but the relationship of N gene abundance and N process rates is largely unknown (Morales, Cosart, & Holben, 2010; Petersen, et al., 2012; Levy-Booth, Prescott, & Grayston, 2014; Rocca, et al., 2015, Ribbons, et al., 2016). This present study focuses on four key functional genes related to N transformation in soil, including *amoA*, *nirK*, *nirS*, and *nosZ* genes (Fig. 1), each of which is discussed briefly below.

Ammonia oxidation (amoA gene)

Chemolithotrophic ammonia oxidizers and nitrate oxidizers are required for the microbial process of nitrification (Levy-Booth, Prescott & Grayston 2014), which is the oxidation of ammonia to nitrite (NO₂⁻) and nitrate (NO₃⁻) (Fig. 1). The *amoA* gene encodes for ammonia monooxygenase, the main catalyst for ammonia oxidation, the first step in the nitrification process (Levy-Booth, Prescott & Grayston, 2014). Ammonia oxidation contributes to N₂O emissions through direct production and by contributing to denitrification that generates N₂O (Lehtovirta-Morley 2018). There are two categories of organisms that contain the *amoA* gene: ammonia oxidizing bacteria (AOB) and archaea (AOA). Functionality of AOB and AOA containing *amoA* genes are correlated with potential nitrification rate (Bernhard et al. 2007; Bernhard, et al. 2010). Environmental pH

and NH_3^+ concentration are key drivers of *amoA* abundance and diversity in ecosystems (Lehtovirta-Morley, 2018). Soil pH has been shown to affect the functionality and abundance of *amoA* bacterial species (Frijlink, et al. 1992). While nitrification still occurs in low pH soils, the bacterial community structure and function is altered compared to higher pH soils; Backman and Klemetsson (2003) demonstrated an increase in nitrification and nitrifying bacteria following liming of soils. In another study, bacterial transcript abundance of *amoA* increased with increasing pH in incubated microcosms (Nicol, et al. 2008). N fertilization can also alter the composition of the AOA and AOB communities (Hallin, et al. 2009; Wessen, et al. 2010) by increasing available NH_4^+ ; this allows for AOB to increase in abundance as AOB are typically limited by NH_4^+ availability (Backman & Klemetsson 2003).

Nitrate reductase (nirK/S genes)

Denitrification is the reduction of nitrate to nitrous oxide or dinitrogen; this is the process by which terrestrial N returns to atmospheric N (Fig. 1). The genes *nirK* and *nirS* catalyze the reaction of NO_2^- to N_2O (Levy-Booth, Prescott & Grayston 2014), and a change in the abundance of *nirK/S* can potentially drive NO_3^- and NO_2^- leaching from the environment (Barta, et al. 2010). The *nirK* and *nirS* genes are structurally different but functionally equivalent: *nirK* is copper (Cu) containing while *nirS* is cytochrome containing (Braker, et al. 2000; Prieme, Braker, & Tiedje, 2002). Due to the differences in structure, *nirK/S* are not found in the same organisms and thus have different biological niches (Braker et al. 2000; Jung, et al. 2011). Organisms containing either the *nirK* or *nirS* gene are common in soil and the *nirK/S* abundance is influenced by soil temperature (Jung, et al. 2011), soil organic matter (Petersen, et al. 2012), biologically available N additions (Ouyang, et al. 2018), and soil pH (Hallin, et al. 2009). For example, in the Hallin et al. (2009) study, *nirS* abundance was significantly lower in fertilized acidic (pH = 4.7) soils compared to the fertilized neutral pH (pH = 6.0) soils.

Nitrous oxide reductase (nosZ gene)

The reduction of N_2O to dinitrogen (N_2) is the final step in denitrification catalyzed by nitrous oxide reductase encoded by the *nosZ* gene (Scala & Kerkhof, 1998). *nosZ* abundance has been shown to increase significantly with N fertilization (Ouyang, et al. 2018). Low soil pH is known to decrease *nosZ* abundance (Liu, Frostegard, & Bakken, 2014; Amar, et al. 2020), which in turn increases N_2O emissions. Lab experiments on the model strain *Paracoccus denitrificans*

demonstrated a post-transcriptional error in *nosZ* impacting protein synthesis under low pH, leading to reduced functionality (Liu, Frostegard & Bakken 2014), suggesting that bacteria may not have the capacity to reduce N₂O to N₂ in highly acidic (pH < 5) soil, even if they possess the *nosZ* gene.

1.6 Research Objectives

Our research aims were to quantify soil microbial gene abundance related to N cycling as influenced by long-term forest fertilization and in association with specific tree species, as well as to determine the relationship between gene abundance and production of N₂O and NO₃⁻ in forest soil. We expected that the associated functional gene abundances would correspond to differences in N cycling resulting from long-term fertilization and acidification.

In the present study, soils sampled from two experimental forested watersheds in West Virginia. Watershed 3 (WS 3; fertilized) and Watershed 7 (WS 7; reference) in the Fernow Experimental Forest (FEF), were used to examine the abundance of the four key functional genes (*amoA*, *nirK*, *nirS*, and *nosZ*) in relation to N products measured in soil beneath four deciduous hardwood species. The tree species of interest were Northern red oak (*Quercus rubra*), sweet beech (*Betula lenta*) representing ECM-associated tree species; and tulip poplar (*Liriodendron tulipifera*), and black cherry (*Prunus serotina*), representing AM-associated tree species.

1.7 Hypotheses

Understanding how microbial functional genes contribute to the biogeochemical cycling is an important step in understanding ecosystem level processing of N and a critical research question for long-term fertilization sites. In many systems, differences in N cycling have been attributed to tree species (Lovett, et al. 2004; Midgley & Phillips 2014) and the soil microbial community (Philippot, et al. 2009; Hang, et al. 2015; Ribbons et al. 2016). Surprisingly, nitrification rates in WS 3 in mineral soils do not vary from the reference watershed, even after 30 years of ammonium sulfate fertilization (Gilliam, et al. 2016; Carrara, et al. 2018; Gilliam, Adams, & Peterjohn. 2020). To investigate the unexpected similarity in soil nitrification between WS 3 and WS 7, we measured N pools coupled with analyses of gene abundance in soil to test the following hypotheses:

1) Soil beneath trees species with higher nitrification rates (AM-associated species) will have a higher abundance of the N cycling functional genes *amoA*, *nirK*, *nirS*, and *nosZ*. Nitrification is the process of converting NH_4^+ to NO_3^- ; thus, trees with higher nitrification rates will have a larger pool of available N for microbial transformation, allowing for the step-wise process involving the associated genes to occur.

2) Increased soil acidification resulting from long-term fertilization will alter microbial gene abundance involved in the production of N_2O and NO_3^- . As soil becomes more acidic through elevated atmospheric deposition of N, and N becomes available in excess of biological demand, the product ratio of $\text{N}_2\text{O}:\text{N}_2$ trends upwards. The protein involved in the production of N_2O (*nosZ*) is sensitive to soil acidity (Liu, et al. 2010), which leads to the prediction that the fertilized WS 3 will have lower *nosZ* gene abundance due to acidification from N fertilization, resulting in a higher rate of soil N_2O production.

3) The abundance of the *amoA*, *nirS* and *nirK* functional genes will be positively correlated to the production of NO_3^- and N_2O in soils, while *nosZ* abundance will be negatively correlated.

Methods

2.1 Study Site

The study site is located at the USDA Forest Service Fernow Experimental Forest (FEF), a temperate mixed hardwood forest, located in north central West Virginia, USA (Fig. 2). Dominant tree species are black cherry, tulip poplar, sweet birch, and northern red oak (Gilliam, et al. 2016). Understory vegetation consists of common greenbrier (*Smilax rotundifolia*), Sassafras (*Sassafras albidum*), Woody nettle (*Laportea canadensis*) and several blackberry species (*Rubus* spp.) (Gilliam & Turrill, 1993; Gilliam, 2021). Climate of the study area is classified as humid continental with hot summers, cold winters, and abundant precipitation (Lockwood, 1985). Average annual precipitation is 1430 mm, with the greatest amount occurring in the spring and summer months (Gilliam et al. 2001). The FEF lies in the Allegheny Mountain section of the unglaciated Appalachian Plateau. The dominant soil series of the FEF is classified as the Calvin series, derived from sandstone and shale, leading to well-drained, medium-textured loams and silt loams (Peterjohn, Adams and Gilliam 1996). The Calvin series is a loamy-skeletal, mixed, active, mesic Typic Dystrudept (Web Soil Survey, USDA), with typically low base saturation, a thin,

poorly developed A horizon, and a weakly- to moderately-developed B horizon (USDA 2014). Soil pH in the FEF watersheds is relatively low due to the underlying geology of shale and sandstone, as well as the history of elevated N and S deposition; N deposition averaged about 10 kg ha⁻¹ yr⁻¹ (Gilliam et al. 2016).

We assessed long-term fertilization effects at two forested watersheds within the FEF (Fig. 2). Watershed 3 (WS 3) is a 34-hectare watershed that had been receiving aerial application of additional N 35 kg N yr⁻¹ as ammonium sulfate ((NH₄)₂SO₄) fertilizer three times a year from 1989 until 2019 (Adams, Kochenderfer & Edwards, 2007). Watershed 7 (WS 7) is a 24-hectare watershed which has been allowed to naturally regenerate since 1969 after being clear-cut in sections from 1964 until 1967. Following harvest in WS 7, it was maintained as barren with herbicides until 1969. WS 7 serves as the vegetative reference watershed for WS 3 in numerous watershed studies (Adams, Kochenderfer & Edwards, 2007).

2.2 Field sampling

Four trees of four species (northern red oak, sweet birch, tulip poplar, and black cherry) were located and marked in both watersheds during the Fall of 2018 (see Fig. 2). Selected trees had a minimum diameter at breast height (DBH) of 16.25 cm, were visibly healthy, and of dominant canopy position. Tree species were selected to represent both dominant types of mycorrhizal association: Northern red oak and sweet birch are ECM-associated species, tulip poplar and black cherry are AM-associated species. Black cherry is the dominant species in WS 3 but not in WS 7, making it of special interest. Five soil samples were collected from the mineral A horizon (0-10 cm) under the drip line of each individual tree using a randomized collection approach (Peterjohn, et al. 2015). These subsamples were combined in the field to obtain one sample per tree (n = 2 watersheds; n = 4 tree species; n = 4 individual trees; N = 32 samples). All soil sampling took place over the course of one week in July 2020, corresponding to peak growth season. Sampling occurred after the last fertilization, but this is not a post- fertilization study as the watershed is not considered in the recovery stage at this time point. Moist samples were sieved with a 2 mm mesh sieve to remove roots and coarse debris and stored in a 4 °C cold room. Subsamples for DNA processing were stored at -20 °C until extraction.

2.3 Soil characteristics

To determine the effect of long-term fertilization on soil properties, soil physiochemical characteristics were analyzed that have been shown to affect plant and microbial function. Soil moisture content was determined gravimetrically, where 10 g of field-moist soil was dried at 105 °C for 24 h. Following drying, samples were re-weighed to determine dry mass. To determine soil organic matter (SOM) via loss on ignition (LOI), 10 g of field-moist soil was combusted at 500 °C for 12 h in a muffle furnace and allowed to cool. Following combustion and cooling, samples were reweighed, and weight adjusted for moisture content. Soil pH was measured using a 1:5 soil to 0.01 *M* CaCl₂ solution and CaCl₂ is used to mimic soil solution ionic composition. 5 g of air-dried soil were placed in 10 mL 0.01 *M* CaCl₂, shaken at room temperature for 30 minutes, and allowed to settle for 1 hour before reading solution by a pH electrode (GENERAL digital, Taiwan).

To determine inorganic N (NH₄⁺ and NO₃⁻) concentration in soils, samples were extracted using an extraction of 10 g field moist soil in 40 mL of 2 *M* KCl solution. Samples were shaken for 1 hour, allowed to settle, and supernatant was filtered through Whatman 2 filters. Quantification of NH₄⁺ and NO₃⁻ in filtered solutions was performed colorimetrically in 96-well plates on Synergy HTX plate reader (Biotek, Winooski, VT).

Extractable NH₄⁺ was determined using 2 *M* KCL extraction samples followed by 150 µL sodium salicylate and 150 µL sodium hydroxide bleach solution, absorbance values were read at 650 nm on the plate reader after a 50-minute incubation at room temperature (Deforest, 2011). All samples were run in quadruple and standard curves were generated for each plate. Sample weights were corrected for moisture content post analysis.

Extractable NO₃⁻ was determined using colorimetric reagents of 2:1:1 vanadium (VCl₃), 2% sulfanilamide and 0.1% NEDD solution; absorbance was read at 540 nm following incubation at 20 °C (Deforest 2011). All samples were run in quadruple and standard curves were generated for each plate. Sample weights were corrected for moisture content.

Potential N₂O-N emissions were measured using an N₂O microsensor (Unisense, Germany) using a modified acetylene (C₂H₂) inhibition technique (Liu, et al. 2018; Kaden, et al. 2020). C₂H₂ is known to inhibit the reduction of N₂O to N₂ (Estavillo, et al. 2002) and allows for the determination of the total denitrification rate (N₂O + N₂). 10 g of field moist soil was weighed out and added to a 50 ml vial with 35 ml of KNO₃ glucose nutrient solution (Liu, et al. 2018) and 8 ml C₂H₂ was injected to create a 15% environment of acetylene. A control was analyzed for each sample where

8 mL oxygen was added instead of C₂H₂. N₂O-N concentration readings were taken at 1 hr, 18 hr, 22 hr, and 36 hr by piercing the vial septum with the sensor needle. All readings were recorded after 60 s equilibration in the sample solution.

2.4 DNA extraction

DNA was extracted using 0.25 g field moist soil using the Qiagen PowerSoil DNA Extraction kit (Qiagen, Germany). DNA concentration (ng/μL) per sample was quantified using a Qubit fluorometer following the manufacturer's protocol using 197 μl buffer and 3 μl sample. DNA samples were stored at -20°C.

2.5 Functional gene analysis

We determined the effect of experimental N fertilization and the influence of tree species on bacterial gene abundance using quantitative polymerase chain reaction (qPCR) on the four key genes of interest (*amoA*, *nirK*, *nirS*, and *nosZ*). Table 1 summarizes the primers and reference strains used for each gene.

For genomic DNA isolation, *Pseudomonas fluorescens*, *Alcaligenes faecalis*, *Paracoccus denitrificans*, and *Pseudomonas stutzeri* were obtained through the USDA ARS culture collection and used as reference strains to obtain the gene segments with standard PCR (Table 1). Reference strains were grown in liquid media according to ARS specifications and genomic DNA was extracted using the ThermoFisher GeneJET Genomic DNA Extraction kit (ThermoFisher). Primers from the pure reference cultures and amplified using standard PCR using the following protocol. Standard curves for qPCR were generated from plasmid standards. Cloning of plasmid standards was done using Invitrogen TA Cloning kit pCR2.1 Topo.

Standard PCR was used to obtain amplified product for each gene of interest. 25 μl standard PCR reactions were run, using 12.5 μl Amplitaq Gold Master Mix (2x concentration), 1 μl Forward Primer (10 uM), 1 μl Reverse Primer (10 uM), 9.5 μl dH₂O, 1μl template DNA. The following amplification conditions were used: Stage 1: 95°C for 1 min, 1x cycle Stage 2: 95°C for 1 min, 55°C for 30 sec, 72°C for 30 sec, 35x cycles Stage 3: 72°C for 7 min, 4°C hold, 1x cycle (Biorad, USA). PCR reactions were run on a 1% agarose gel and visualized using SyberSafe (Promega, USA) to confirm the amplicon size. Bands were excised and purified using the Wizard clean up

kit (Promega, USA), then cloned using the Invitrogen TA Cloning kit PCR2.1. Individual colonies were picked and plasmid DNA purified using the Zippy DNA kit (ZYMO, USA). Plasmid DNA was sent to the WVU Genomics Core Lab for Sanger sequencing using M13 primers to verify insert sequence. Standard curves for qPCR were developed by serially diluting plasmid DNA to final concentrations between 1×10^1 to 1×10^8 copies μL^{-1} .

Touchdown PCR was attempted for *amoA* (50°C – 60°C) with primers *amoA* 1F mod and GenAOB R, and *nirS* (52°C – 62°C) with primers *nirS* 4F and *nirS* 5R, as standard PCR failed to amplify these gene targets. Touchdown PCR and standard PCR were also run using Promega Gotaq Green mastermix for *nirS* and *amoA* to determine if the polymerase was an issue. Touchdown PCR was not run for *nirK* or *nosZ* as amplification was successful using the standard approach.

For qPCR, a 10 μL final reaction volume was used, containing 2 μL of PCR grade water, 0.5 μL of forward and reverse primer (total primer concentration = 500 nM), 5 μL of SYBER qPCR 2X master mix, and 1 μL of template DNA. For all functional genes, real time amplification was performed using the following conditions: stage 1: 95 °C for 5 minutes 1x stage 2: 95 °C for 20 s and 60 °C for 45 s 35x stage 3: 95 °C for 15 s 1x. To assess the specificity of the PCR reaction a melt-curve was analyzed for each target gene (Fig. S1 – S2). Assays were run in 96-well plates with three replicates for each sample, positive and negative control. The number of gene copies (CN) was calculated by comparing measured values to genomic DNA of pure cultures with a known genome size and gene copy number.

2.6 Statistical Analysis

Differences in NO_3^- , NH_4^+ , N_2O , soil pH, *nirK* abundance, and *nosZ* abundance attributed to fertilization or tree species were analyzed using nonparametric tests. Non-parametric analysis was used due to the lack of normality for the distribution of data. Wilcoxon tests were run for watershed comparisons, pair-wise Wilcox tests were run for tree species comparisons within each watershed and a significance level of < 0.05 was used to indicate statistical significance. Simple correlation was performed using Pearson's correlation coefficient to examine linear relationships between variables.

All data was analyzed using R-studio v.1.4 with packages dplyr and tidyr for data organization, ggpubr for figure creation, and statix for statistical analysis. This watershed comparison study is an example of experimental pseudo-replication with an effective sample size of one. Differences in data are assumed to be a result of fertilization effects because 1) the soil descriptions in each watershed are predominantly the same and 2) previous analyses of soils in the watersheds indicate that changes primarily occurred after fertilization started in 1989 (Adams and Angradi 1996).

Results

3.1 Inorganic soil N

Soil inorganic N (NH_4^+ and NO_3^-) concentrations from the two watersheds are similar ($p > 0.05$; Fig. 3 b and c). Mean NH_4^+ concentrations were 9.45 (mg N kg^{-1}) in WS3 and 7.84 (mg N kg^{-1}) in WS7 (Table 2). Soil NH_4^+ concentrations under black cherry within WS 3 were significantly ($p = 0.03$) higher than tulip poplar and northern red oak (Fig. 4). Mean NO_3^- concentrations were not statistically distinct between watersheds (Table 2), WS 3 mean NO_3^- was 35.37 (mg N kg^{-1}) and 34.55 (mg N kg^{-1}) in WS 7. Soil NO_3^- concentrations within WS 7 were distinct by tree species, as soil beneath tulip poplar contained significantly more NO_3^- ($p = 0.02$) than soil NO_3^- concentrations beneath sweet birch (Fig. 5). Soils beneath sweet birch have a mean NO_3^- concentration of 16.94 (mg N kg^{-1}) and soils beneath tulip poplar have an average mean of 46.73 (mg N kg^{-1}) (Table 2) in WS 7. However, soil NO_3^- did not vary by tree species in WS 3. NO_3^- concentrations in soil beneath AM-associated trees in WS 7 was higher, though not significantly so ($p = 0.05$), compared to ECM-associated species in the same watershed (Fig. 6). No statistically significant difference in inorganic N between fungal association was observed in WS 3.

3.2 Soil pH, SOM, and Moisture

Soil pH varied significantly by watershed (Fig. 3 a), as mean soil pH was 3.98 in WS 3 and 4.35 in WS 7 ($p < 0.001$). There were no statistically significant differences in soil pH between tree species in either WS 3 or WS 7 (Table 2). Percent SOM did not significantly differ between watersheds or tree species within watershed (Table 2). Soil moisture also did not vary between watersheds or by tree species (Table 2).

3.3 Soil N_2O potential

Potential N₂O production increased throughout the 36-hour incubation period in all soils, with R values of 0.73 indicating a positively linear relationship of N₂O production over the 36-hour incubation (Fig. 7). Potential cumulative N₂O concentrations did not significantly differ between watersheds (Fig. 3 b). Average potential N₂O for WS 3 was 0.32 (mg N kg⁻¹). Within WS 3, tree species mildly ($P = 0.057$) influenced potential N₂O production, as soil beneath sweet birch trended higher than soils beneath red oak but were not statistically significant from sweet birch soil, producing an average of 0.47 (mg N kg⁻¹) and red oak soil produced 0.18 (mg N kg⁻¹) (Table 2). Tree species did not affect potential N₂O production in WS 7.

3.4 Gene Abundance

Soils in WS 7 had significantly ($p < 0.01$) greater abundance of *nosZ* than WS 3 (Fig. 9). WS 7 soil contained a mean of 1.14×10^{11} copies ng of DNA per gram of soil (CN·ng g⁻¹) and WS 3 soil contained 2.24×10^{11} CN·ng g⁻¹ (Table 2). *nosZ* abundance did not differ significantly among tree species (Table 2) or fungal association (Fig. 10) in either watershed.

WS 7 soil contained a significantly ($p < 0.01$) greater mean abundance of *nirK* relative to WS 3 2.28×10^{12} CN·ng g⁻¹ and 6.70×10^{10} CN·ng g⁻¹, respectively (Fig. 9). Within watersheds, *nirK* abundance did not differ significantly among tree species (Table 2). Soil beneath AM-associated species in WS 3, while not statistically significant ($p = 0.05$), had lower abundance of *nirK* (Fig. 10) relative to soil beneath ECM-associated species, though no differences were noted between fungal association in WS 7.

The ratio of *nirK:nosZ* (a higher ratio is reflective of greater N₂O production potential) was significantly higher ($p = 0.011$) in soil from WS 7 (Fig. 9) with an average of 6.41×10^5 of *nirK:nosZ* compared to WS 3 ratio of 131.80 (Table 2). There were no *nirK:nosZ* differences among tree species between individual watersheds (Table 2).

3.5 Relationships of Environmental Variable to Gene Abundance

Overall, *nirK* abundance exhibited a significant positive relationship with soil pH across both watersheds ($p < 0.01$), however, within the individual watersheds this relationship was not statistically significant (Fig. 11). *nosZ* abundance also exhibited a significant positive relationship

with soil pH across both watersheds ($p = 0.02$), however, within the individual watersheds this relationship was not observed (Fig. 11).

There was no correlation between gene abundance and soil NH_4^+ concentration (Fig. 12). In WS 3, N_2O production was negatively related to soil pH ($p = 0.03$), with lower N_2O production with increasing soil pH (Fig. 8). No correlation occurred between gene abundance and potential N_2O production across all samples (Fig. 13). Overall, no correlation was seen between gene abundance and soil NO_3^- concentration across watersheds (Fig. 14). There was also no correlation between *nirK:nosZ* at the watershed level in relation to N_2O production (Fig. 15).

Although many attempts and conditions were used, *amoA* and *nirS* were not successfully quantified and were thus removed from this study, as the standard PCR amplification was not successful (Table 3). Primers pairs AmoA189, AmoA682 and Cd3af, NirsR3cd amplified at 525 bp and 425 bp, respectively, but qPCR is not successfully run with fragment sizes above 300bp. Primer sets for *amoA*; AmoA1F, AmoA2R, GENAOB F, GENAOB R, and GEN AOB mod did not successfully amplify using a 55 °C annealing temperature, touchdown PCR using a gradient of 50°C to 60 °C, or use of Gotaq green mastermix. Primer set nirS4F and nirS5R did not successfully amplify at a 55 °C annealing temperature, touchdown PCR using a gradient of 52°C to 62 °C or use of Gotaq green mastermix. Electrophoresis gels for all PCR attempts can be found in the supplemental information (Fig. S3 – S8).

Discussion

4.1 Nitrogen dynamics

The processes of the N cycle are closely interlinked and have direct influence on each other (Fig 1). Due to the interconnectedness of the N cycle, an alteration at one step may impact the resultant end product and subsequent steps. Addition of NH_4^+ fertilizer impacts the subsequent N transformations due to an increased pool of N for downstream transformations. Surprisingly, no measurable differences were observed in the NH_4^+ , NO_3^- , or N_2O pools at the watershed level (Fig. 3).

It was expected that the fertilized watershed WS 3 would exhibit higher soil concentrations of NH_4^+ , NO_3^- , and N_2O compared to WS 7, the reference watershed.

Soil pH is significantly different between the watersheds with WS 3 being more acidic (Fig. 3 a). While WS 3 is highly acidic with an average pH of 3.88 it is important to keep in mind that soils in WS 7 are also considered acidic. Both watersheds functionally are described as acidic soils (pH < 5.0) in terms of biogeochemical processes (Deforest, et al. 2012; Shetty & Prakash, 2020). Acidic soils are known to impact soil properties (Ollivier, et al. 2011; Graham, et al. 2014), such as microbial processes and nutrient availability, and nutrient losses (Shetty & Prakash 2020). Acidity is produced when NH_3^+ containing materials such as N fertilizers are transformed to NO_3^- in the soil. WS 3 received consistently elevated amounts of N fertilizer each year, thus it was expected that WS 3 would have measurably higher NH_4^+ and NO_3^- .

In this study, no significant differences in inorganic N contents were observed at the watershed scale in the mineral A horizon (Table 2). Soil analyses from 2015 in these same watersheds demonstrated a significantly greater amount of NH_4^+ and NO_3^- in WS 3, contrary to our findings (Gilliam, Adams and Peterjohn 2020), which could be due to more intensive sampling at multiple time points. Carrara et al. (2018) found that nitrification in the organic (O) horizon in WS 3 was 51% higher, but detected no difference in N transformation rates in the mineral soil between WS 3 and WS 7, which is consistent with our results. O horizon soil and mineral soil have differing chemical and biological properties such as organic matter content, root densities, and microbial compositions (Brzostek and Finzi 2011). In the current study, no differences in SOM between watersheds were observed (Table 2), which is also supported by results from Gilliam et al. (2020), showing a similar OM content in soil from WS 3 and WS 7.

At the tree species level, soils beneath black cherry contained significantly more NH_4^+ within WS 3 (Fig. 4). Black cherry, an AM-associated species, is a NO_3^- -demanding species and does not utilize NH_4^+ as efficiently as other co-occurring species (Royo, et al. 2021), which could result in residual NH_4^+ from fertilization accumulating in the soil. Soils beneath sweet birch produced significantly less NO_3^- than tulip poplar within WS 7 (Fig. 5). Sweet birch is an ECM-associated species while tulip poplar is an AM-associated species. ECM species typically have less soil NO_3^- loss compared to AM species due to the development of a more organic nutrient economy (Phillips, Brzostek and Midgley 2013).

Soils on plots dominated by AM-tree species have been shown to have higher NO_3^- concentration than ECM-dominated plots (Midgley and Phillips 2014). AM-associated tree species produce litter

that is more easily decomposed compared to ECM associated species; this leads to larger pools of inorganic N such as NO_3^- in the soil (Cheeke, et al. 2016). Black cherry and tulip poplar are AM-associated species which are known to have a relatively faster nitrification rate than ECM-associated species (Phillips, Brzostek & Midgley 2013); this would lead to a larger pool of bioavailable N in the soil. This effect is observed within WS 7, as soil beneath AM-associated species have a significantly higher concentration of NO_3^- , relative to ECM-associated species (Fig. 6). This effect was not observed in WS 3, which supports the idea that fertilization decreases the effect of fungal association (Midgley & Phillips, 2014) and fertilization may result in a homogenization of N cycling and other biogeochemical processes (Wu, et al. 2019). Fungal biomass can also decrease in response to N fertilization (Treseder, 2004; Zhang, Chen & Ruan, 2018), as N limitation is alleviated through fertilization, the need for the host species to allocate nutrients belowground to associated fungi decreases (Treseder, 2008), causing a decrease in fungal biomass and limiting the metabolic influence of AM and ECM fungi on N transformations (Wallenstein, et al. 2006).

It was surprising to observe similar rates of N_2O produced in both watersheds (Fig. 7 a and b), as WS 3 is significantly more acidic which has been shown to be an important factor in increased N_2O production (Firestone & Davidson, 1989). This could be due to the selected methodology of N_2O production as a nutrient solution was used to stimulate microbial respiration for potential N_2O , which may not reflect field conditions. In a 1998 study, field measures of N_2O in WS 3 exhibited significant spatial variability with certain soil cores having 60% less N_2O from a plot lower in the watershed (Peterjohn, et al. 1998). In the current study, N_2O emissions were positively correlated with soil NO_3^- concentration across both watersheds, indicating increasing NO_3^- increases N_2O production (Fig. 8 c). This is explained by Firestone and Davidson's (1989) "hole in the pipe" theory, which suggests that increasing amounts of NO_3^- in the soil directly results in elevated N_2O emissions.

4.2 Fertilization Influence on Gene Abundance

Though there are few differences in measured soil properties between the watersheds, we documented a significant reduction in both *nirK* and *nosZ* gene abundance in the fertilized watershed, WS 3 (Fig. 9). *nosZ* gene abundance was significantly lower in WS 3 relative to WS 7 (Fig. 9 b), but *nosZ* abundance was not correlated to potential denitrification rates (Fig. 13 a). This

finding supports our hypothesis that increased soil acidification would alter nitrification associated gene abundance leading to WS 3 having decreased *nosZ* abundance. Several studies (Dandie, et al. 2008; Attard, et al. 2010) have also shown that *nosZ* abundance was not significantly associated with potential denitrification rates. However, to the contrary, several studies previously linked *nosZ* abundance to potential denitrification rates (Hallin, et al. 2009; Petersen, et al. 2012). The functionality of N₂O reductase encoded by *nosZ* has been shown to be inhibited at low pH (pH < 6.1), whether by a mutation causing silencing of the gene or an assembly issue within the bacterial cell, the cause of impaired *nosZ* at low pH is poorly understood (Liu, Frostegard, & Bakken 2014). While significant differences in gene abundance were observed between the watersheds, cumulative N₂O production did not differ significantly (Fig. 3 d). The abundance of *nosZ* and *nirK* functional genes were not correlated to the production of NO₃⁻ (Fig. 14) or to potential N₂O production (Fig. 13) in soil within our study.

In multiple studies, the total *nirS* and *nirK* abundance is used as an indicator of the total denitrifier population present (Philippot, et al. 2009; Hallin, et al. 2009), as *nirS* is functionally identical to *nirK* but structurally different. Organisms that contain *nirS* do not generally also contain *nirK* (Levy-Booth, Prescott & Grayston, 2014; Ribbons), thus the total *nirK/S* abundance is used for an estimate of nitrate reductase in multiple studies. Although *nirK* is significantly lower in WS 3 (Fig. 9 c), this does not necessarily mean that nitrate reductase is lacking in WS 3 soils, it is plausible that *nirS* is the dominant denitrifying gene and we were not able to produce satisfactory results to quantify *nirS* abundance. Thus, *nirS* abundance may be an important indicator of process rates in these forest soils and could better account for N₂O emissions observed between the watersheds (Fig. 9), though we were not able to quantify *nirS* in the present study.

The ratio of *nirK:nosZ* has previously been used as an indicator of endpoint N₂O production (Philippot, 2002), though the ratio of *nirK:nosZ* was not correlated to potential N₂O production in this study (Fig. 15). The watersheds demonstrated significant difference in *nirK:nosZ* (Fig. 9 c) but no significant difference in potential N₂O production (Fig. 3 d), indicating the ratio of *nirK:nosZ* was not indicative of N₂O production here. Previous studies (Hallin, et al. 2009; Philippot, et al. 2011; Qin, et al. 2020) have also not detected a significant correlation between the proportion of denitrifying genes and N₂O production.

Contrary to our hypothesis, concentrations of NH_4^+ (Fig. 12), N_2O (Fig. 13) and NO_3^- (Fig. 14) were not correlated to any measured gene abundance. The enzymes produced by organisms containing these genes mediate N transformations (Morales, Cosart, & Holben, 2010), which makes it surprising that no significant relationship was observed at the watershed level. However, measures of *amoA* gene abundance may be better related to NH_4^+ and NO_3^- , as it encodes for the enzyme production responsible for the oxidation of ammonium, though we were unable to successfully quantify *amoA* in our study.

The ability to use gene abundance as an indicator of potential N cycling rates is still unresolved, as (Graham, et al. 2014), as gene presence does not necessarily indicate community functionality (Levy-Booth, Prescott & Grayston, 2014). DNA based analysis such as qPCR does not differentiate between dormant and active DNA sources (Levy-Booth, Prescott & Grayston, 2014). Available primers for functional genes are unlikely to capture the full diversity of the target genes in environmental samples for which they are designed, as sequences are derived from singular organisms (Green, et al. 2010; Levy-Booth, Prescott & Grayston, 2014). It has previously been shown (Liu, Frostegard, & Bakken 2014) that the assembly of functional N_2O reductase is altered in acidic soil, but not necessarily the relative abundance of the *nosZ* gene. Liu (2014) explored N_2O reductase functionality in soils ranging from pH of 4.0 to 8.0 and reported soils with pH < 6.1 were unable to produce functional N_2O reductase in a laboratory setting. It is hypothesized that organisms containing *nosZ* are inhibited by low pH, which decreases their abundance and capability to reduce N_2O (Liu, Frostegard & Bakken 2014). Shifts in specific gene composition are not necessarily directly correlated to shifts in measurable functions such as N transformations, as the active bacterial community may differ from the total community (Freedman, et al. 2015; Moore, et al. 2020).

4.3 Tree Species Influence on Gene Abundance

Few significant differences occurred in measured soil parameters or gene abundance as a function of tree species in either watershed in this study. Gene abundance did not differ significantly among tree species in either watershed (Table 2). This contrasts with the study by Kelly, et al. (2021), where tree species did significantly alter the gene abundance of multiple key genes in the N cycle. Although black cherry had a higher *nirK* and *nosZ* abundance than American chestnut, no significant difference between soil beneath red oak and black cherry *nirK* and *nosZ* abundance was

found. This could indicate that soils beneath black cherry and red oak are more similar in soil properties influencing N transformations than soil beneath chestnut.

Soils beneath black cherry trees had a higher amount of NH_4^+ suggesting an altered nitrification rate. While soils beneath black cherry did not differ significantly in gene abundance, *nirK* in soils beneath black cherry in WS 3 was highly suppressed (Fig. 9 a), which could indicate *nirS* as the dominant gene for nitrite reductase as NO_3^- did not differ in WS3 among tree species (Fig. 5). Ribbons, et al. (2018) reported no significant differences in *nirK* abundance in soils beneath a suite of broadleaf species, but a significantly lower abundance of *nirK* in spruce soils relative to hardwood. Preliminary data (not shown) collected from an additional watershed that was planted to Norway spruce (WS 6) within the FEF is contradictory, as the spruce soils contained a significantly greater abundance of *nirK* and *nosZ* genes compared to both WS 3 and WS 7 hardwood soils measured here.

Kelly, et al. (2021) reported that tree species may mediate abundance of certain functional genes, with black cherry soils containing a relatively high *nirK* abundance compared to other tree species. This finding is contrary to our results, wherein black cherry soils had lower *nirK* abundance in both watersheds and *nirK* abundance in WS 3 soils beneath black cherry trees was very low and rarely detectable (Table 2). Other studies (Ribbons, et al. 2016) have indicated that *nirK* may be less species-dependent than *nirS*.

Within WS 3, *nirK* abundance was significantly lower in soils beneath AM-associated species relative to ECM species (Fig. 10). This is attributable to the strong influence of black cherry on *nirK*, as it was nearly non-detectable beneath black cherry trees. Relative *nosZ* abundance did not differ by species or fungal association within the watersheds (Table 2). The *nirK:nosZ* ratio did not significantly differ by tree species (Table 2) which is supported by the Kelly et al. (2021) findings in that similar *nirK:nosZ* ratios were observed across tree species. A higher ratio of *nirK:nosZ* has been indicated to be a potential indicator of higher N_2O production, as N_2O production was statistically similar across species in the individual watersheds this is reflective of the similar *nirK:nosZ* ratios among species. As discussed previously, the *nirK:nosZ* ratio was higher in WS 3 but N_2O production did not differ indicating *nirK:nosZ* is not indicative of N_2O production.

There was no significant difference in gene abundance of *nirK*, *nosZ*, or *nirK:nosZ* associated with tree species in this present study, thus our hypothesis that gene abundance would be influenced by tree species was not supported. Although, *nirK* abundance is lower in soils beneath AM-associated species in WS 3 (Fig 10), there were no significant differences between the NH_4^+ , N_2O , or NO_3^- pools in WS 3 associated with fungal association. It is possible that the diversity of neighboring trees and understory vegetation may have masked any species level influences on the soil microbial community. Several studies (e.g., Yang, et al. 2018) have shown that understory vegetation can strongly affect the soil microbial biomass and extracellular enzyme activities, which may make differentiation of the influence by dominant tree species difficult. Tree species influence on soil microbial genetic composition and metabolic function is still poorly understood (Ribbons, et al. 2018; Dukunde, et al. 2019; Kelly, et al. 2021).

4.4 Acidification Influence on Gene Abundance

The positive correlation of soil pH with gene abundance (Fig.11) supports our hypothesis that WS 3 would exhibit decreased *nosZ* abundance in comparison to WS 7. We identified a significant difference in functional gene abundance at the watershed scale (Table 2). Abundance of *nirK* and *nosZ* was significantly lower under N fertilization in WS 3 (Fig. 9). As previously discussed, N fertilization leads to soil acidification through base cation leaching and creation of H^+ protons. Soil pH has been shown to be a major driver of bacterial biodiversity as acidic environments influence species tolerant of high acidity (low pH) (Palmer and Horn 2012) and inhibit bacterial growth (Liu, Frostegard, & Bakken 2014) due to nutrient limitations. Soil acidity is a particularly strong driver of microbial diversity as homogeneity is an effect of low pH (Deforest, et al. 2012; Zhang, Chen & Ruan, 2018; Shetty & Prakash, 2020).

Our data show that both *nirK* and *nosZ* relative abundance are significantly lower in WS 3 (Fig. 9), and this finding is correlated to lower soil pH (Fig. 11); as soil pH increases, *nirK* and *nosZ* abundances increases. This is congruent with findings from previous studies (e.g. Liu, Frostegard, & Bakken 2014; Xiao, et al. 2019; Amar, et al. 2020) that demonstrate that acidic soils reduce functionality and abundance of *nirK* and *nosZ* genes. Reductions in *nirK* abundance in WS 3 may be due to acidic soils influencing abundance of *nirS*, as organisms containing *nirS* are more acid tolerant and *nirK* is not found in organisms containing *nirS* (Palmer & Horn, 2012). Due to the relatively acidic nature of soil from both watersheds, the microbial community functionality may

be similar even though the relative abundances are distinct, resulting in similar the N transformation rates measured here (Fig 12-15) (Liu, Frostegard, & Bakken 2014).

4.5 Limitations of the study and future directions

Our study design attempted to capture spatial variability by creating homogenized, composite samples from 5 points under each tree sampled. Due to the relatively small sample size ($n = 32$), however, the full microbial community and N transformations may not have been sufficiently examined. As previously stated, understory variability may have a masking effect on tree species influence on N transformations and gene abundance. Decoupling tree species impact and understory impact is difficult in field-based experiments with diverse understories. In order to fully address the question of tree species impact on gene abundance, a greenhouse-based study or experimental vegetation plantings could isolate the specific influence of tree species (and associated fungal effects). Monodominant plot-based field studies have been able to isolate fungal associated effects (Brzostek, et al. 2015; Midgley & Sims 2020).

We were unable to generate amplicons necessary to quantify *amoA* and *nirS*; qPCR is limited by amplicon size and large fragments ($bp > 150$) reduce the accuracy and specificity of the reaction (Smith & Osborn 2009). Thus, we were not able to fully capture the abundance of key genes linked to denitrification and nitrification within the watersheds or for soils underneath specific species. qPCR may not capture all genes associated with N_2O reductase and nitrite reductase. For example, *nosZ* is known to have a canonical type and a divergent type, and the commonly used *nosZ* primer sets are not usually capturing the full *nosZ* diversity (Sanford., et al. 2012; Li., et al. 2017), as the primer sets used for *nosZ* were designed to capture canonical *nosZ*. Full metagenomic sequencing of these soils may help in quantification of the functional genes of interest.

Additionally, inclusion of measures of enzyme activities to link to gene abundance would allow for a broader picture as well as aid in support of using gene abundance as a proxy for microbial N transformations, as, no relationship between N transformations and gene abundance was observed.

Conclusions

Our results show that the 30 years of forest fertilization with $(NH_4)_2SO_4$ has altered soil microbial gene abundance, as fertilized soils exhibit decreased *nirS* and *nosZ* gene abundance. This shift in gene abundance is correlated with lower soil pH. While there may be other drivers of gene

abundance, acidity appears to be the main driver in this study. Measured N pools were not observed to be correlated to gene abundance in this study. Tree species influences on the soil parameters suggest that black cherry is a unique driver of N cycling. Gene abundance of *nirK* and *nosZ* were not observed to be impacted by tree species in this study. Soils beneath AM-associated species in WS 3 demonstrated reduced *nirK* abundance, indicating mycorrhizal association may alter microbial genetic composition. N management is important for ecosystem health, as the N cycle is primarily microbially driven, it is pertinent to understand the driving forces behind N pollutant production through bacterial metabolic transformations encoded by N cycling genes. While the presence of functional genes does not necessarily indicate an active community, it can provide insights into the complexity of biogeochemical cycles such as the N cycle.

Tables

Table 1. Genes with reference strains, primers, and references

Gene	Primer	Reference Strain	Amplicon size	Sequence (5'-3')	Ref
<i>amoA</i>	amoA-1F	<i>Pseudomonas fluorescens</i>	491	GGGG TTTCTACTGGTGGT	Rottauwe et al 1997, Booth 2014
	amaA-2R			CCCCTCKGSAAAGCCTTCTTC	
	A189		525	GGNGACTGGGACTTCTGG	Holmes 1995, Jensen et al 2000
	A682			GAASGCNGAGAAAGASGC	
	amoA 1F mod		120	CTG GGG TTT CTA CTG GTG GTC	Meinhart 2015
<i>nirK</i>	GenAOB R			GCA GTG ATC ATC CAG TTG CG	
	nirK876 nirK1040	<i>Alcaligenes faecalis</i>	147	ATYGGCGVCA YGGCGA GCCTCGATCAGRTTGTGGTT	Henry et al 2006
<i>nirS</i>	Cd3aF	<i>Pseudomonas stutzeri</i>	425	GT(C/G) AAC GT(C/G) AAG GA(A/G) AC(C/G) GG	Michotey et al. 2000; Throck et al 2004
	nirSR3cd*			GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A	
	nirS 4F		192	TTC(A/G)TCAAGAC(C/G)CA(C/T)CCGAA	Braker 1998
	nirS 5R			CTTGTTG(A/T)ACTCG(C/G)(C/G)CTGCAC	
<i>nosZ</i>	nosZ 2F	<i>Paracoccus denitrificans</i> ,	267	CGCRACGGCAASAAGGTSMSST	Jung et al. 2013; Henry et al. 2006
	nosZ 2R			CAKRTGCAKSGCRTGGCAGAA	

Table 2. Mean soil pH percent soil organic matter (SOM), percent moisture, NO_3^- , NH_4^+ , cumulative potential N_2O , *nirK*, *nosZ*, and *nirK:nosZ* values from each watershed and beneath the four tree species. Values indicate mean ($\pm$ standard error). Values with different letters indicate statistically significant difference according to Wilcoxon pairwise means separation ($p < 0.05$)

WS	Soil pH	%OM	NO_3^- (mgN/kg)	NH_4^+ (mgN/kg)	Cumulative N_2O (mgN/kg Soil/hr)	<i>nosZ</i> (CN-ng DNA g ⁻¹ soil)	<i>nirK</i> (CN-ng DNA g ⁻¹ soil)	<i>nirK:nosZ</i> Z (CN-ng DNA g ⁻¹ soil)	Species	Soil pH	%OM	NO_3^- (mgN/kg)	NH_4^+ (mgN/kg)	Cumulative N_2O (mgN/kg Soil)	<i>nosZ</i> (CN-ng DNA/g soil)	<i>nirK</i> (CN-ng DNA/kg Soil)	<i>nirK:nosZ</i> (CN-ng DNA g ⁻¹ soil)
3	3.88 ^a (0.21)	1.8 (0.55)	35.37 (18.85)	9.54 (4.06)	0.32 (0.16)	2.264E+11 ^a (9.05E+11)	6.70E+10 ^a (1.63E+11)	131.80 ^a (4.97E+02)	PRSE	3.79 (0.18)	2.24 (0.64)	22.87 (7.52)	14.59 ^a (2.86)	0.33 (0.12)	3.61E+07 (7.15E+07)	2.41E-01 (6.06E-02)	0.64 (0.75)
									BELE	3.86 (0.25)	1.73 (0.68)	41.5 (29.04)	7.19 ^{ab} (3.94)	0.47 (0.20)	9.05E+10 (1.81E+12)	1.43E+11 (2.86E+11)	1.01 (0.57)
									QURU	3.94 (0.29)	1.73 (0.51)	37.75 (16.94)	9.23 ^b (1.99)	0.18 (0.10)	3.82E+08 (7.63E+07)	3.82E+10 (7.65E+10)	26.03 (49.48)
									LITU	3.93 (0.11)	1.52 (0.21)	39.37 (39.13)	7.14 ^b (2.41)	0.27 (0.018)	4.34E+07 (8.67E+07)	8.65E+10 (1.73E+11)	499.51 (996.43)
7	4.35 ^b (0.35)	1.88 (0.66)	34.55 (20.20)	7.84 (3.17)	0.37 (0.22)	1.415E+11 ^b (3.24E+11)	2.28E+12 ^b (4.98E+12)	6.41E+05 ^b (2.56E+07)	PRSE	4.47 (0.24)	1.92 (0.90)	36.25 ^a (17.37)	9.45 (3.71)	0.31 (0.11)	6.25E+08 (1.24E+09)	1.30E+10 (2.06E+10)	1.71E+03 (2.37E+03)
									BELE	4.12 (0.43)	1.88 (0.42)	16.94 ^b (2.94)	8.23 (2.8)	0.38 (0.30)	2.52E+11 (4.99E+11)	2.45E+12 (4.60E+12)	3.72E+03 (6.23E+02)
									QURU	4.36 (0.41)	1.77 (0.36)	38.26 ^a (29.75)	6.29 (2.08)	0.24 (0.13)	2.28E+11 (4.39E+11)	5.21E+12 (8.86E+12)	2.92E+01 (4.03E+01)
									LITU	4.46 (0.31)	1.95 (1.05)	46.73 ^{ab} (14.55)	7.4 (4.15)	0.55 (0.25)	8.45E+09 (1.69E+11)	1.47E+12 (2.30E+12)	2.56E+07 (5.12E+07)

Table 3. Primer sets for four functional genes with annealing temp and whether amplification was successful with use of standard PCR.

*Indicate touchdown PCR was run

Gene	Primer set	Annealing	Amplification
<i>amoA</i>	amoA-1F	54	-
	amoA-2R		
	A189	54*	+
	A682		
	GenAOB F	57*	-
	GenAOB R		
	GenAOB 1F	54*	-
	mod		
<i>nirK</i>	nirK876	57	+
	nirK1040		
<i>nirS</i>	Cd3af	51*	-
	nirSR3cd*		
	nirS 4f		-
	nirS5R		
<i>nosZ</i>	nosZ 2F	52	+
	nosZ 2R		

Figures

Figure 1. The nitrogen cycle with all steps and products indicated with associated functional gene in red.

Figure 2. A map of WS3 and WS7 in the FEF. Watershed 3 is dark green and watershed 7 is light green, AM trees are orange points and ECM trees are green.

Figure 3. Soil property differences between watersheds. Panel a is showing soil pH between watersheds, WS3 has significantly lower pH than WS7. Panels b-d show N concentrations in different forms which did not vary significantly between watersheds. Boxplots with different lower-case letters indicate significantly different means according to Wilcoxon two sample test ($p < 0.05$).

Figure 4. NH_4^+ concentrations correlated to tree species split by watershed. For each species, boxplots with different letters within each watershed are significantly different according to pairwise Wilcoxon test ($p < 0.05$).

Figure 5. Soil NO_3^- concentrations in WS 3 and WS 7. For each species, boxplots with different letters within each watershed are significantly different according to pairwise Wilcoxon two sample test ($p < 0.05$).

NO₃ by Fungal Association

Figure 6. Soil NO₃⁻ concentrations in WS 3 and WS 7 by fungal association. For each watershed, boxplots with different letters within each watershed are significantly different according to pairwise Wilcoxon two sample test ($p < 0.05$).

Figure 7. Potential N_2O production over the 36-hour incubation time with standard error: (a) Potential N_2O production by watershed. (b) Potential N_2O production over a 36-hour incubation for all samples. (c) Potential N_2O production in the high N watershed over the incubation period. (d) Potential N_2O production in the reference watershed over the incubation period. R values indicates a relationship with N_2O production over time. Colors delineate tree species.

Figure 8. The relationship of cumulative potential N_2O to soil pH and NO_3^- in (a) relationship between soil pH and potential end point N_2O ; (b) relationship of end point N_2O to soil pH for all samples demonstrating no significant trend; (c) relationship of NO_3^- to potential N_2O production in WS 3 (red) and WS 7 (blue); and (d) relationship of NO_3^- to potential N_2O production for all samples.

Figure 9. Relative abundance of *nirK*, *nosZ* and *nirK:nosZ* between watersheds. Boxplots with different lower-case letters indicate significantly different means according to Wilcoxon two sample test ($p < 0.05$).

Figure 10. Relative abundance of *nirK* and *nosZ* by fungal association. Boxplots with different lower-case letters are significantly different according to Wilcoxon two sample test ($p < 0.05$).

Figure 11. Relationship between gene abundance and soil pH. (a) *nosZ* abundance in relation to soil pH. (b) *nirK* abundance in relation to soil pH. R and p-values are denoted for each panel for all data across watersheds.

Figure 12. Relationship between gene abundance and NH_4^+ . A. *nosZ* abundance in relation to NH_4^+ . B. *nirK* abundance in relation to NH_4^+ . R and p-values are denoted for each panel for all data across watersheds

Figure 13. Relationship between gene abundance and N_2O . (a) *nosZ* abundance in relation to N_2O . (b) *nirK* abundance in relation to N_2O . R and p-values are denoted for each panel for all data across watersheds.

Figure 14. Relationship between gene abundance and NO_3^- . (a) nosZ abundance in relation to NO_3^- . (b) nirK abundance in relation to NO_3^- . R and p-values are denoted for each panel for all data across watersheds

Figure 15. Ratio of *nirK* to *nosZ* in relation to cumulative potential N₂O by watershed and overall ratio of *nirK* to *nosZ* in relation to cumulative potential N₂O. R and p-values are denoted for each panel for all data across watersheds

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Supplemental Information

Figure S1. *nosZ* melt curve for standard curve creation using plasmid DNA from *paracoccus denitrificans* with primers nosZ2F and nosZ2R.

Figure S2. *nirK* melt curve for standard curve creation using plasmid DNA from *Alcaligenes faecalis* with primers nirK876 and nirK1040.

Figure S3. *nirK* 876 and 1040 and *nosZ*2F and *nosZ*2R primer set using standard PCR showing amplification at indicated bp.

Figure S4. NosZ2F primer set, nirK876 primer set, amoA1F primer set, nirScd3af primer set run with Promega gotaq green mastermix and amplitaq gold mastermix on the Biorad thermocycler.

Figure S5. nirScd3af primer set and amoA189 and amoA682 primer set with nirK as the positive control.

Figure S6. Touchdown PCR run with AmoA1F mod and GENAOB R primer set

Figure S7. amoA1F mod and GEN AOB R primer set, nirScd3af and nisSR3cd primer set.

Figure S8. Touchdown PCR run with nirS 4F 5R primer set