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Tree species influence on microbial functional gene abundance and N cycling in riparian soil

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**Tree species influence on microbial functional gene abundance and N cycling in riparian
soil**

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Thesis submitted

To the Eberly College of Arts and Sciences
at West Virginia University

In partial fulfillment of the requirements for the degree of

Master of Science in

Biology

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Abstract

Tree species influence on microbial functional gene abundance and N cycling in riparian soil

Elizabeth A. Matejczyk

Little is known about how above-ground vegetation may differentially influence the below-ground microbial community structure, abundance, and function. Abundance of soil microbial N cycling genes responsible for nitrification (*amoA*), denitrification (*nirK* and *nirS*), and nitrous oxide reduction (*nosZ*) may vary with tree species and increasing N availability, and these variables may be used to predict production of NO_3^- and N_2O from soil. Variability of nitrification and denitrification rates have also been linked to tree mycorrhizal associations, with soil beneath tree species associated with arbuscular mycorrhizal (AM) exhibiting greater denitrification rates than tree species forming ectomycorrhizal- (ECM) associations. In this study, we integrate observed N microbial functional gene abundance in the soil as influenced by six different tree species in two sub-catchments receiving either high or low N inputs. Soils beneath two ECM-associated tree species (Northern red oak (*Quercus rubra*) and American beech (*Fagus grandifolia*)) and beneath four AM-associated tree species (sugar maple (*Acer saccharum*), tulip poplar (*Liriodendron tulipifera*), American sycamore (*Platanus occidentalis*), and black cherry (*Prunus serotina*)) were analyzed for inorganic N content, potential N_2O flux, and microbial gene abundance (*amoA*, *nirK*, *nirS*, and *nosZ*) was quantified using qPCR techniques. Other soil parameters monitored, including soil pH, moisture, and organic matter. These analyses were used to predict potential NO_3^- and N_2O production as influenced by tree species to provide a greater understanding of soil microbial community response to elevated N levels with tree species as a primary influence. We found that increasing N enrichment contributes to fluctuations in NH_4^+ and NO_3^- concentrations and subsequent soil acidification. pH was a driving influence on *nirK* and *nosZ* gene abundance, possibly due to reduced functionality. We also found greater concentrations of NH_4^+ beneath ECM associated tree species with implications of greater N-retention that may be helpful in the remediation of N polluted riparian buffer zones.

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1. Introduction

1.1 Sources and impacts of N pollutants

Nitrogen (N) is a vital nutrient for biological processes and historically was a limiting resource in most temperate terrestrial ecosystems (LeBauer and Treseder 2008). However, human population has increased dramatically over the last 200 years in part due to increasing combustion of fossil fuels and resulting in increased agricultural demand and industrial synthesis of N fertilizer using the Haber-Bosch process (Glass 2003). This has resulted in a large increase in global reactive N. Anthropogenic N inputs sourcing from fossil fuel burning and agricultural fertilization and runoff have increased ecosystem N deposition and bioavailable N with harmful consequences for ecosystem health and functioning. Manure is also a major source of N runoff in agricultural operations as it contains high levels of N due to the mineralization of organic N to ammonium (NH_4^+) that fuels the nitrification process to mobilize N (EPA). N fertilizer is widely available and used in agricultural operations with severe consequences for N leaching.

N enrichment has been shown to increase forest ecosystem productivity and (Tilman 1987; Bowman et al. 1993; Hooper and Johnson 1999; Gough et al. 2000; Oren et al. 2001; Thomas et al. 2010), but with prolonged N enrichment, shifts in flora biodiversity (Phoenix et al. 2012; Walter et al. 2016), soil pH, and fungal and microbial communities have been documented (Anderson and Joergensen 1997; Baath and Anderson 2003; Frey et al. 2004; Wallenstein et al. 2006; Hallin et al. 2009; Ramirez et al. 2010; Janssens et al. 2010; Averill et al. 2014; Freedman et al. 2013; Morrison et al. 2016; Carrara et al. 2018; Du et al. 2019). N enrichment has contributed to changes in carbon (C) allocation and subsequent fungal growth in soil (Egerton-Warburton et al. 2007).

Prolonged N enrichment eventually leads to decreasing soil pH wherein excess H^+ ions from the oxidation of NH_4^+ contributes to soil acidification (Wallace et al. 1994; Aber et al. 1998; Lu et al. 2009; Lu et al. 2014), in addition to the enhanced leaching of base cations from the soil profile. A shift in soil pH has been linked to a decrease in low N-adapted vegetation and an overall loss in plant biodiversity in heavily N disturbed areas (Bobbink et al. 2010), as the plant communities may become dominated by nitrophilic species (Gilliam et al. 2016).

N deposition from fertilizer increases the rate of microbially-mediated N cycling wherein nitrification and denitrification processes can produce potentially harmful pollutants such as nitrate

(NO_3^-) and nitrous oxide (N_2O gas) that may end up in aquatic and atmospheric systems and contribute to the degradation of water quality, the formation of aquatic dead zones, and ozone depletion (Erisman et al. 2013). In 2015, N pollution damage was estimated at \$157 billion in the US and remains a dominant threat to ecosystem and human health (Sobota et al. 2015). N pollutants have profound consequences for aquatic ecosystem health, commercial fisheries (Galloway et al. 2008; Ravishankara et al 2009; Giles et al. 2012; Ullah and Gladis 2006; Quan et al. 2016), and ultimately human livelihood and health (Rome et al. 2021). N fertilization contributes to eutrophication of waterways: high N inputs in streams cause algal blooms and subsequent decomposition, leading to anoxic conditions and fish kills with severe consequences for fisheries that support ecosystem health and fish supply for human consumption (Ullah and Gladis 2006; Galloway et al. 2008; Ravishankara et al 2009; Giles et al. 2012; Quan et al. 2016).

Excess N harms air quality through the soil microbial production of NO and N_2O gases. N_2O can be produced via the soil microbial processes of nitrification, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA), and soil emissions contribute approximately 70% of N_2O in the atmosphere (Conrad 1996). N_2O is a leading cause of stratospheric ozone depletion and is a harmful greenhouse gas, with a 300-fold greater influence on warming processes than carbon dioxide (CO_2), and accounts for approximately 10% of total greenhouse gas emissions (Bates et al. 2008; Richardson et al. 2009). Human health may also be directly influenced by gaseous N in the atmosphere via the burning of fossil fuels (NO is a precursor to smog which is a lung irritant), and N pollution of drinking water from agricultural leachate is linked to human disease and reproductive health complications (Richter et al. 2005; Liu et al. 2013).

Excess N may also perpetuate the “hole-in-pipe” model conceptualized by Davidson et al. (2000), relating NO_3^- and N_2O production with soil moisture in a leaky pipe. The hole-in-pipe model surmises that greater N availability in soil results in greater NO_3^- , NO, and N_2O products “leaking” from soil. This model also attributes other soil factors to the N products, such as soil moisture conditions, wherein dryer soils will primarily emit NO due to its high diffusivity. In wet soils, NO is quickly reduced to N_2O and escapes the soil (Davidson et al. 2000). In very wet soil conditions, NO is further reduced to N_2 gas and N_2 becomes the dominant N gas to “leak” from the soil. Thus, with increasing N fertilization, a greater leakage of N pollutants is expected, depending on soil conditions, due to greater flow through the metaphorical pipe (Firestone and Davidson 1989).

1.2 Importance of riparian zones in managing nitrogen pollutants

A riparian zone is an interface between terrestrial land and a body of water. It can provide habitat for wildlife, protection from flooding and erosion, and naturally filter pollutants to preserve water quality and ecosystem integrity (Lind et al. 2019). Implementation of functional riparian buffer zones in proximity to N pollutant sources may serve to mitigate and remediate the harmful effects of N pollution on ecosystem and human health (Lind et al. 2019). The EPA has estimated that approximately 101,000 miles of freshwater rivers and streams and 78% of U.S. coastal waters are impaired due to the influx of excess nutrients that create imbalances in the aquatic food chain (EPA Assessment and Total Maximum Daily Load Tracking and Implementation System as of 2012). Prolonged N enrichment in riparian buffer zones may exhibit “N saturation”, with decreased N retention and increased N leaching as explained by Aber et al. (1989). Riparian zone effectiveness of retaining N largely relies on width and it is recommended that when managing for pollutants, riparian zones should be at least 15-30 m in width (EPA), and wider zones are recommended in areas of steep slopes or coarse-textured soil. Riparian buffer zones are of special consideration in the remediation of N runoff sourcing from agricultural operations (Naiman et al. 2010; Franklin et al. 2019) because they have been documented to incorporate N runoff via vegetation assimilation and soil microbial denitrification and immobilization to transform and remove nitrate (NO_3^-) from surface runoff that prevent N leaching downstream and into the atmosphere, although efficiency of riparian buffer zones to remove N runoff may vary with vegetation composition and soil microbial and fungal interactions (Nelson et al. 1995; Hefting et al. 2006).

1.3 Tree species and mycorrhizal influence on microbial community and N cycling

Above-ground vegetation influences below-ground soil N cycling processes and production of harmful NO_3^- and N_2O . Tree mycorrhizal associations contribute to species-specific intermediate N cycling and NO_3^- and N_2O production (Phillips et al. 2013). Soils beneath vegetation associated with arbuscular mycorrhizal fungi (AM) exhibit higher rates of nitrification and denitrification compared to soil beneath trees associated with ectomycorrhizal fungi (ECM) due to labile litter and greater access to inorganic N (Phillips et al. 2013; Averill et al. 2014). AM-associated plant species lack enzyme “priming” that ECM-associated species utilize to acquire N from soil organic matter (SOM); these environments tend to have higher inorganic N relative to organic N pools, contributing to higher rates of nitrification and subsequent N leaching from soil to waterways

similar to what the “hole-in-the-pipe” model predicts (Chapman et al. 2005; Kelly et al. 2010; Phillips et al. 2013; Levy-Booth et al. 2014; Phillips et al. 2014; Phillips et al. 2014; Peterjohn et al. 2015). Soils beneath AM-associated species such as maple (*Acer* spp.) and tulip poplar (*Liriodendron tulipifera*) exhibit greater nitrification and nitrate availability due to the capacity to access NH_4^+ pools, and with prolonged N enrichment, greater N leaching (Phillips et al. 2013; Midgeley and Phillips, 2016; Tatsumi et al, 2020). Soils beneath ECM-associated species such as oak (*Quercus* spp.) and beech (*Fagus* spp.) exhibit slow litter decomposition and N transformations, thus lowering N pollutant leaching potential (Gadgil and Gadgil 1971; Averill and Hawkes 2016; Fernandez and Kennedy, 2016).

1.4 Microbial functional genes

Soil microbial communities in ecosystems with N enrichment have the capacity to transform N compounds into less harmful forms depending on existence, abundance, and activation of microbial N cycling genes (Fenn et al. 1998; Liu et al. 2010; Phillipot et al. 2013; Fig. 2). Quantification of microbial functional genes responsible for the production of N-transforming enzymes may be useful to predict microbial production rates of NH_4^+ , NO_3^- and N_2O in soil. N mineralization and nitrification rates in soil have been linked to soil microbial functional gene abundance and are becoming an efficient way to predict these processes under elevated N conditions (Rotthauwe et al. 1997; Francis et al. 2005; Leininger et al. 2006; Szukics et al. 2010; Averill et al. 2014). More traditional measurements of N transformation in soil are time-consuming and complex, and in the case of measurement of denitrification products N_2O or N_2 , have long been prone to error related to gas capture and detection via the acetylene reduction method, along with the temporal and spatial variability of the process (Groffman et al. 2006; Butter-Bach Bahl et al. 2013). Thus, much work has been dedicated to the use of microbial genetic structure and function as a predictive tool in biogeochemical cycling.

Among these N-transforming functional genes is *amoA*, whose gene product is involved in the catalyzation of ammonia oxidation (Petersen et al. 2012; Levy-Booth et al. 2014). *amoA* is used by ammonia-oxidizing archaea and bacteria (AOA and AOB) to mediate the process of nitrification (conversion of NH_4^+ to NO_3^-). AOB *amoA* gene copies were reported to increase under NH_4^+ fertilization and were correlated to greater nitrification rates, suggesting *amoA* gene abundance is a useful proxy to predict nitrification rates in soil. Sterngren et al. (2015) showed that

nitrification corresponded to an increase in bacterial *amoA* copy numbers in a high N and amino acid treatment, and the highest nitrification rates were found in soils with high NH_4^+ content; this corresponded to an increase in bacterial *amoA* copy number (Sterngren et al 2015). Soil pH has been reported to be one of the most important influences in AOB and AOA community structure, especially when responding to N inputs, as soil pH can influence microbial diversity, abundance, and function (Stephen et al. 1998; Backman et al. 2003; Nugroho et al. 2007; Gubry-Rangin et al. 2011).

Denitrification potential has been predicted by using the N cycling genes *nirS* and *nirK*. *nirK* is Cu-containing nitrate reductase and *nirS* is cytochrome cd1-containing nitrate reductase. Although *nirS* and *nirK* are functionally identical, *nirS* is more diverse in soil and the two genes are thought to be mutually exclusive in that they rarely appear in the same organism (Zumft 1997; Levy-Booth 2014). *nirK* gene abundance may be used to predict NO emissions and *nirS* gene abundance has been correlated with N_2O emissions (Szukics et al. 2009). Levy-Booth and Winder (2010) found that *nirS* and *nirK* were correlated with organic matter content, total C, and total N in mineral horizon soil, and similar correlations were found with denitrification rates (Levy-Booth and Winder 2010). There is also evidence that pH is a strong influence on the functionality of *nirK* in that transcription errors can occur in low pH conditions (Brezinger et al. 2015).

Nitrous oxide reductase, *nosZ*, completes nitrous oxide reduction of N_2O gas into non-toxic N_2 gas and has become a powerful predictor of potential N_2 gas production. Acidic conditions may impact nitrous oxide reduction of *nosZ* by causing transcription failure in *nosZ* leading to lower abundances of *nosZ* in acidic soils and a greater production of N_2O (Liu et al. 2014). Thus, excess N enrichment may contribute to greater *nosZ* transcription failure and incomplete nitrous oxide reduction due to soil acidification caused by oxidation of N compounds (Liu et al. 2014). Morales et al. (2010) found that *nosZ* abundance was greater than *nirS* in forested areas and vice versa in agricultural areas. A higher *nirK+nirS:nosZ* ratio may indicate incomplete denitrification and N_2O reduction resulting in greater soil N_2O emissions (Petersen et al. 2012; Phillipot et. al 2013; Levy-Booth et al. 2014).

1.5 Objectives

In this study, we sampled soil beneath six native deciduous trees in the West Run Watershed located in Morgantown, West Virginia, USA (Fig. 1) to assess tree species influence on the abundance of soil microbial N cycling functional genes in two forested sub-catchments. One sub-catchment is influenced by agricultural runoff and is compared to an adjacent reference sub-catchment that does not receive agriculture runoff (i.e. relatively high and low N inputs). We aimed to quantify the abundance of key soil microbial N functional genes *amoA*, *nosZ*, *nirK*, and *nirS* in soils sampled beneath two ECM-associated tree species – Northern red oak (*Quercus rubra*) and American beech (*Fagus grandifolia*) – and four AM-associated tree species – sugar maple (*Acer saccharum*), tulip poplar (*Liriodendron tulipifera*), American sycamore (*Platanus occidentalis*), and black cherry (*Prunus serotina*). We also measured soil NH_4^+ , NO_3^- , and potential N_2O production to investigate relationships between gene abundance and N cycling products, to determine if gene abundance can be used as a proxy to predict NH_4^+ , NO_3^- , and N_2O concentrations from soil. We aimed to identify the influence of tree species and mycorrhizal association on the N cycling capacities of below-ground soil microbial communities under relatively high and low N levels.

We hypothesized that:

1. N enrichment will contribute to greater NH_4^+ , NO_3^- and N_2O concentrations that correspond to greater abundances of key microbial functional genes in the N cycle (*amoA*, *nirK*, *nirS*, and *nosZ*).
2. Soil microbial N cycling gene abundance will be significantly different between tree species, specifically related to mycorrhizal association. We expect that AM soils are likely to contain greater abundances of *amoA*, *nirK*, and *nirS* and thus elevated levels of NH_4^+ and NO_3^- relative to ECM soils due to greater access to ammonium pools in N enriched areas (Averill et al. 2014).
3. Soil influenced by tree species associated with AM fungi (*Acer saccharum*, *Liriodendron tulipifera*, *Platanus occidentalis*, and *Prunus serotina*) will contain a higher (*nirK*+*nirS*)/*nosZ* ratio, indicative of greater potential for nitrous oxide (N_2O) production due to incomplete nitrous oxide reduction by *nosZ*, relative to trees associated with ECM fungi (*Quercus rubra* and *Fagus grandifolia*), especially in areas of high N due to AM-

associated species being no longer limited by N and greater access to ammonia pools exacerbating N₂O production and soil acidification slowing transcription of *nosZ* that completes nitrous oxide reduction to N₂ gas.

A greater understanding of soil microbial communities and their associated N cycling capacities beneath specific tree species may help inform current ecosystem modeling strategies as well as support riparian buffer zone Best Management Practices (BMPs) to improve water and air quality in heavily disturbed and N-polluted ecosystems.

2. Methods

2.1 Study area

West Run Watershed (23 km²) in Monongalia County, West Virginia, United States is a tributary of the upper Monongahela River and is characterized by mixed land use, including farming, urban development, and forest (Kellner et al. 2018; Fig. 1). Our sampling area was located downstream of the West Virginia University Animal Husbandry Farm, in a wooded area of approximately 107 acres (known as the WVU Woodlot), west of the farm. The soil is classified as Clarksburg silt loam, fine-loamy, mixed, superactive, and mesic Oxyaquic Fragiudalfs, derived from siltstone, sandstone, and limestone on a 5% northeast facing slope (WSS; Fig.). Morgantown, West Virginia has an elevation of 1041 feet with a mild temperate climate characterized by warm summers (warmest monthly mean > 22°C) and cold winters (coldest monthly mean < 0°C) and an average annual precipitation of 106.2 cm (Peel et al. 2007; Kellner et al. 2018). The West Virginia University Animal Husbandry Farm is in the upper reaches of one sub-catchment that supports grazing livestock. Vegetation is predominantly cove hardwoods of various ages, including sugar maple, red maple, and tulip poplar, and some plantings of conifers including Douglas fir, Norway spruce, and bald cypress.

2.2 Soil collection, processing, and analysis

To investigate how tree species influence N transformations, A-horizon (0-10 cm) soil samples were collected in August 2020 beneath four individuals of six dominant native deciduous tree species in proximity to two stream drainages that flow into West Run Creek (n = 4 replicates; n = 6 tree species; n = 2 sub-catchments; N = 48; Fig. 1). One sub-catchment receives runoff from the University Farm and the other sub-catchment drains from a relatively undisturbed forested area

(these areas are denoted as high N and low N, respectively). Six native deciduous tree species were selected for this study, representing two ECM-associated and four AM-associated tree species. ECM-associated species were *Quercus rubra* (Northern red oak; REOA) and *Fagus grandifolia* (American beech; AMBE) and the AM-associated species were *Acer saccharum* (sugar maple; SUMA), *Liriodendron tulipifera* (tulip poplar; TUPO), *Platanus occidentalis* (American sycamore; AMSY), and *Prunus serotina* (black cherry, BLCH).

Field moist soils were sieved through a 2 mm mesh to remove rocks and large roots and subsamples and stored at either 4°C or -20°C. Soil pH was measured using a 0.01 M CaCl₂ solution in a 1:2 slurry (5 g soil:10 mL CaCl₂). Samples were shaken for 1 hour and allowed to settle for 1 hour prior to reading pH (Hanna Instruments). Gravimetric soil moisture content was quantified by drying field moist soils for 24 hours at 105°C. Soil organic matter (SOM) was quantified via combustion in a muffle furnace at 500°C for 12 hours and weight loss before and after was taken as SOM content (Bao et al. 2011). To determine soil NO₃⁻ and NH₄⁺ concentration, 10 g of soil was extracted with 40 ml of 2 M KCl, followed by colorimetric analysis in clear 96-well plates as outlined by the Deforest Lab at Ohio University (adapted from Miranda et al., 2001). NO₃⁻ concentration was determined by combining soil sample extractions with a 2:1:1 solution of vanadium chloride (VCl₃), 2% sulfanilamide, and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD). NH₄⁺ concentration was determined by combining sample extracts with 150 µl sodium salicylate and 150 µl sodium hydroxide bleach solution. Samples were incubated at room temperature (~20 °C) for 50 minutes for color development and analyzed in quadruplicate. Concentration (ppm) was determined by measuring absorbance on a plate reader (Synergy HTX plate reader, Biotech, Winooski, Vermont) at 540 nm for NO₃⁻ and 650 nm for NH₄⁺. Standard curves were generated using known standards for each replicate plate under the same conditions. All calculations were corrected for soil moisture content.

Potential soil N₂O flux was measured using a modified acetylene inhibition technique outlined in Estavillo et al. (2002) using a dissolved N₂O microsensor (Unisense, Germany, Estavillo et al. 2002; Meyer et al. 2008). Acetylene gas (C₂H₂, 15%) was synthesized by combining calcium carbide (CaC₂) with water. Using a needle and syringe, 8 ml of 15% acetylene gas was injected through a septum into the headspace of vials containing 10 g field moist soils and 35 ml nutrient solution containing KNO₃ and glucose (Kellogg Biological Station; Butterbach-Bahl et al. 2013; Liu et al.

2018; Kaden et al. 2020). A control for each sample was analyzed, following the same protocol, by replacing the vial headspace with O₂ instead of C₂H₂. N₂O (mg/ml) concentrations were recorded after 90 seconds equilibration at 18, 24, and 30 hours utilizing the Unisense Microsensor by placing the microsensor needle through the septum of each vial. All calculations were corrected for soil moisture content.

2.3 Genetic analysis of *nosZ*, *nirK*, *nirS*, and *amoA* in soil samples

Total genomic DNA was extracted from 0.25 g field moist soil samples using DNeasy Powersoil Kit (Qiagen) following manufacturer's protocol. Final DNA concentrations were measured with a Qubit fluorometer (ThermoFisher Scientific) using 197 µl buffer and 3 µl samples using the dsDNA HS Assay kit and standard protocols. End-point polymerase chain reaction (PCR) was run on four microbial functional genes (Tables 1 and 2) involved in N processing, using AmpliTaq Gold Master Mix (Thermofisher) for *nosZ* and *nirK* using reference strains *Alcaligenes faecalis* and *Paracoccus denitrificans*, respectively, as templates (Henry et al. 2006; Jung et al. 2013; NRRL, USDA). PCR was performed in a 25 µl reaction volume using 12.5 µl master mix (2x concentration) (Thermofisher), 9.5 µl dH₂O, 1 µl forward primer (10 µM), 1 µL reverse primer (10 µM), and reference strain template using the following conditions: Stage 1: 95°C for 1 minute, Stage 2: 95°C for 1 minute, 55°C for 30 seconds, and 72°C for 30 seconds for 35 cycles, and Stage 3: 72°C for 7 minutes and 4°C hold for 1 cycle. We ran PCR with *amoA*-1F and *amoA*-2R and reference strain *Pseudomonas fluorescens* to amplify *amoA* (Rottauwe et al. 1997; Levy-Booth et al. 2014). We also ran PCR using A189 and A682 primers for *amoA* using *Pseudomonas fluorescens* as template, according to Holmes 1995 and Jensen et al. 2000. We also followed procedures outlined in Meinhardt et al. (2015) using GenAOAF/GenAOAR and *amoA*1F/GenAOB2R primer sets, *Pseudomonas fluorescens* as template, and touchdown PCR (52°C-64°C) with both AmpliTaq Gold and GoTaq Green Master Mixes. Standard PCR was performed for *nirS* using *nirS*4F/*nirS*5R and Cd3aF/*nirS*R3cd* primer sets using *Pseudomonas stutzeri* as template and the AmpliTaq Gold Master Mix (Braker 1998; Michotey et al. 2000; Throback et al. 2004).

Gel electrophoresis was used to confirm DNA band size and reaction specificity for each PCR reaction. Bands corresponding to expected product sizes for *nosZ* and *nirK* were excised and purified using the Wizard Gel Purification kit (Promega). Subsequent cloning was performed using

TA Cloning kit pCR2.1 Topo plasmid transformed into *E. coli* competent cells, subsequently spread on agar plates using Super Optimal broth (SOC) media and incubated at 37 °C overnight. Plasmid DNA from individual colonies was extracted with Zyppy Wash kit (Thermofisher) and plasmid DNA concentration ($\text{ng}^{-1} \mu\text{l}$) was confirmed using the Qubit fluorometer as described above. All plasmid-transformed genes were sequenced by Sanger sequencing using M13 primers to confirm the identity of each insert (Genomics Core Lab at West Virginia University).

Standard curves for *nirK* and *nosZ* were determined using the cloned products via real-time quantitative PCR (qPCR), following the protocol outlined by Koos (2018). qPCR was run with a 10 μl final volume containing 2 μl PCR grade water, 0.5 μl each forward and reverse primers (total primer concentration = 500 nM), 5 μl of SYBR qPCR 2x master mix, and 1 μl template using the following conditions: Stage 1: 95 °C for 5 minutes 1x, Stage 2: 95 °C for 20 seconds and 60 °C for 45 seconds 35x, and Stage 3: 95 °C for 15 seconds for 1x. qPCR samples were run in triplicate. Using the same conditions, qPCR was then run for each soil sample DNA extraction and using the respective standard curve equations, gene copy number was calculated (Koos 2018). All calculations were corrected for soil moisture content so that gene copy numbers are standardized to g of dry soil.

2.4 Data analysis

Differences in soil pH, SOM, percent moisture content, NO_3^- concentration, NH_4^+ concentration, N_2O production, and *nirK* and *nosZ* gene copy numbers attributed to tree species or N level were analyzed using nonparametric tests due to a lack of normal distribution of all variables. When comparing two distinct groups (N-level), the Wilcoxon two-sample test was applied using a normal approximation. When more than two groups were compared (tree species comparison), a pairwise Wilcoxon two-sample test was applied, followed by Kruskal-Wallis test to compare the effect of tree species within a sub-catchment. For ease of interpretation, data are presented as calculated means and standard errors. Separate analyses were run for each sub-catchment. Statistical analyses were performed using RStudio version 1.4 using the following packages: *dplyr* for data organization, *ggplot2* and *ggpubr* to make figures, and *rstatix* for statistical analysis at $\alpha=0.05$ significance level. It is important to note that this study design is an example of pseudo-replication in that the sampling areas of high- and low N deposition are not replicated at the catchment scale.

These data should be interpreted with that in mind and therefore, results may not be applicable to other locations.

3. Results

3.1 Nitrate and ammonium

NO_3^- concentration was significantly higher ($p=2.37\text{E-}5$) in the high N catchment (mean = 43.01 $\text{mg N}^{-1} \text{ kg soil}$) compared to the low N catchment (mean = 15.67 $\text{mg N}^{-1} \text{ kg soil}$) ($p=2.37\text{E-}5$; Fig. 3 a). Within the high N catchment, tree species influenced NO_3^- concentration in soils, as soil NO_3^- beneath American beech (mean = 49.59 $\text{mg N}^{-1} \text{ kg soil}$) was significantly lower than sugar maple (mean = 61.35 $\text{mg N}^{-1} \text{ kg soil}$) and significantly higher than American sycamore (mean = 23.41 $\text{mg N}^{-1} \text{ kg soil}$) ($p=0.029$; Fig. 7 a) and NO_3^- concentration in American sycamore (mean = 23.41 $\text{mg N}^{-1} \text{ kg soil}$) and black cherry (mean = 16.10 $\text{mg N}^{-1} \text{ kg soil}$) were significantly lower than northern red oak (mean = 63.72 $\text{mg N}^{-1} \text{ kg soil}$) and sugar maple (mean = 61.35 $\text{mg N}^{-1} \text{ kg soil}$) ($p=0.029$; Fig. 4 a). In the low N catchment, NO_3^- concentration was not significantly different between tree species ($p=0.14$; Fig. 7 a), nor did NO_3^- concentration vary significantly between ECM and AM fungal associations (0.444; Fig. 11 a). In the high N catchment, NO_3^- concentration was significantly higher in soil beneath ECM-associated species (mean = 56.65 $\text{mg N}^{-1} \text{ kg soil}$) relative to AM species (mean = 36.19 $\text{mg N}^{-1} \text{ kg soil}$) ($p=0.023$; Fig 12 a).

NH_4^+ concentration was also significantly greater in soil from the high N sub-catchment (mean = 6.49 $\text{mg N}^{-1} \text{ kg soil}$) relative to the low N catchment (mean = 4.03 $\text{mg N}^{-1} \text{ kg soil}$) ($p = 0.009$; Fig. 3 b). Tree species influenced soil NH_4^+ in both catchments. In the high N catchment, NH_4^+ concentration was significantly higher in northern red oak (mean = 10.56 $\text{mg N}^{-1} \text{ kg soil}$) compared to black cherry (mean = 4.22 $\text{mg N}^{-1} \text{ kg soil}$) and sugar maple (mean = 3.37 $\text{mg N}^{-1} \text{ kg soil}$) ($p=0.029$; Fig. 7 b). In the low N catchment, NH_4^+ was significantly lower in soil beneath American sycamore (mean = 1.18 $\text{mg N}^{-1} \text{ kg soil}$) compared to northern red oak (mean = 5.79 $\text{mg N}^{-1} \text{ kg soil}$), American beech (mean = 6.21 $\text{mg N}^{-1} \text{ kg soil}$), and black cherry (mean = 4.68 $\text{mg N}^{-1} \text{ kg soil}$) and significantly lower in sugar maple (mean = 2.22 $\text{mg N}^{-1} \text{ kg soil}$) compared to black cherry (mean = 4.68 $\text{mg N}^{-1} \text{ kg soil}$) ($p = 0.029$; Fig. 7 b). NH_4^+ was significantly higher in ECM (mean = 6.00 $\text{mg N}^{-1} \text{ kg soil}$) compared to AM (mean = 3.04 $\text{mg N}^{-1} \text{ kg soil}$) in the low N catchment in ($p=0.01$; Fig. 12 b), though NH_4^+ was not significantly different between ECM and AM associated species in the high N catchment ($p = 0.076$; Fig. 12 b).

3.2 Soil pH, soil organic matter, and soil moisture

Soil pH was not significantly different between high and low N catchments ($p = 0.613$; Fig. 4 a). Tree species influenced soil pH within the high N catchment and was lowest in soil beneath black cherry (mean = 3.89) and highest in sugar maple (mean = 4.86) ($p = 0.029$; Fig. 8 a). Soil pH was not significantly different between tree species in the low N catchment (Fig. 8 a). pH was not significantly different between ECM and AM fungal association in either the high or low N catchment ($p = 0.5$; $p = 0.759$, respectively).

Soil organic matter content was statistically similar between high and low catchments ($p=0.069$; Fig. 4 b). Within the high N catchment, organic matter was significantly higher in soil beneath northern red oak (mean = 18.66) compared to black cherry (mean = 9.89) ($p = 0.029$; Fig. 8 c). Organic matter content did not differ between tree species in the low N catchment ($p > 0.05$). Soil organic matter was not significantly different between ECM and AM fungal association in either the high or low N catchment ($p = 0.12$; $p = 0.928$, respectively).

Soil moisture content was also similar between high and low catchments ($p = 0.050$; Fig. 4 c). Within the low N catchment, soil moisture was significantly higher in soil beneath black cherry (mean = 0.35) compared to sugar maple (mean = 0.23) ($p = 0.029$; Fig. 8 b). Soil moisture did not differ between tree species in the high N catchment (Fig. 8 b). Soil moisture did not vary by ECM and AM fungal association in either the high or low N catchment ($p = 0.417$ for both catchments).

3.3 Potential N₂O production

Potential N₂O production after 30 hours incubation was similar between high and low N catchments ($p = 0.053$; Fig. 5). N₂O production was significantly lower in soil beneath black cherry (mean = 0.11) compared to American sycamore (mean = 0.66) and northern red oak (mean = 0.59) ($p = 0.029$; Fig. 9) in the high N catchment. Potential N₂O did not vary between tree species in the low N catchment (Fig. 9). Potential N₂O production did not vary between ECM and AM fungal association in either the high or low N catchment ($p = 0.350$; $p = 0.976$, respectively; Fig. 9 a and Fig. 9 b).

3.4 Target N cycling genes

nosZ gene abundance (copy number) was similar between the high and low N catchments ($p = 0.112$; Fig. 6 a). In the high N catchment, *nosZ* abundance did vary by tree species ($p = 0.029$), where *nosZ* was significantly higher in soil beneath American sycamore (mean = $4.96\text{E}+09 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) compared to black cherry (mean = $1.29\text{E}+09 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) ($p = 0.029$; Fig. 10 a). *nosZ* gene abundance did not vary between tree species in the low N catchment (Fig. 10 a). *nosZ* gene abundance was not significantly distinct between ECM and AM fungal association in either the high or low N catchment ($p = 0.192$; $p = 0.350$, respectively).

nirK gene abundance was also similar between the high and low N catchments ($p = 0.103$; Fig. 6 b). In the high N catchment, *nirK* abundance did vary by species ($p = 0.029$), where *nirK* was significantly lower in soil beneath American beech (mean = $7.93\text{E}+10 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) compared to American sycamore (mean = $6.27\text{E}+12 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) and sugar maple (mean = $9.10\text{E}+12 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) in the high N catchment ($p = 0.029$; Fig. 10 b). *nirK* abundance was not significantly different between tree species in the low N catchment (Fig. 10 b). *nirK* gene abundance was significantly higher in soil beneath AM-associated trees (mean = $4.97\text{E}+12 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) compared to ECM (mean = $1.02\text{E}+12 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) in the high catchment ($p = 0.011$; Fig. 13 a), although *nirK* abundance did not vary by fungal association the low N catchment ($p = 0.610$; Fig. 13 a).

The ratio of *nirK:nosZ* was not significantly different between high and low N catchments ($p=0.496$; Fig. 6 c). Within the high N catchment, *nirK:nosZ* did vary by tree species, where it was higher in soil beneath American sycamore (mean = $1008.52 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$), sugar maple (mean = $1677.09 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$), and black cherry (mean= $1078.05 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) compared to American beech (mean = $35.48 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) ($p = 0.029$; Fig. 10 c). *nirK:nosZ* did not differ between tree species in the low N catchment (Fig. 10 c). *nirK:nosZ* was significantly higher in soil beneath AM-associated trees (mean = $1098.06 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) compared to ECM (mean = $190.81 \text{ CN} * \text{ng DNA}^{-1} \text{ g soil}$) in the high N catchment, though the ratio did not vary by fungal association in the low N catchment ($p = 0.005$.; $p = 0.783$, respectively; Fig. 13 b).

Despite repeated attempts using different primer sets, PCR conditions, and polymerase formulations, we were unable to amplify usable targets within the *nirS* or *amoA* genes. As a result,

quantification of *nirS* and *amoA* gene abundances using qPCR was not achieved (Supplemental Figures 1-6).

3.5 Relationships between gene abundance and soil parameters

Soil pH was positively correlated to *nosZ* and *nirK* copy number ($p < 0.001$, Fig. 17 a; $p = 0.0014$, Fig. 16 b, respectively) across all samples. Within catchments, *nosZ* copy number and soil pH were positively correlated in both the high and low N catchments ($p = 0.042$; $p < 0.001$, respectively, Fig.). Soil pH and *nirK* gene abundance were also positively correlated in the high N catchment ($p < 0.001$, Fig. 16 b), but this relationship did not occur in soil from the low N catchment ($p = 0.25$; Fig. 16 b).

There was a negative relationship between NH_4^+ concentration and *nosZ* copy number across all samples ($p = 0.0013$; Fig. 15 a). The low N catchment showed greater *nosZ* copy number with lower levels of NH_4^+ concentration compared to that in the high N catchment ($p = 0.00062$; $p = 0.23$; Fig. 15 a). A significant positive correlation was noted between *nosZ* abundance and potential N_2O production across all samples ($p = 0.011$; Fig. 20 a). The high N catchment displayed a significant positive relationship with *nosZ* copy number and N_2O ($p = 0.0026$; Fig. 15 a). *nosZ* copy number and N_2O did not display a significant relationship in the low N catchment ($p = 0.22$; Fig. 20 a). No significant relationship was found in *nosZ* and NO_3^- in high or low N catchments ($p = 0.97$; $p = 0.18$).

Soil NH_4^+ concentration was negatively correlated to *nirK* abundance across all samples ($p = 0.0029$; Fig. 15 b), largely driven by a strong significant negative relationship in the low N catchment ($p = 0.0026$; Fig. 15 b). *nirK* abundance was related to increased potential N_2O production among all samples ($p = 0.089$; Fig. 20 b), and within catchments, a significant positive relationship between *nirK* and N_2O was noted in the high N catchment ($p = 0.045$; Fig. 20 b). Soil NO_3^- concentration was not related to *nirK* gene abundance across all samples.

nirK:nosZ was not related to NO_3^- , NH_4^+ , or N_2O in either the high and low N catchments. Within the high N catchment, *nirK:nosZ* did exhibit a significant positive relationship with soil pH ($p = 0.0021$; Fig. 17 b) and a significant negative relationship with soil NH_4^+ content ($p = 0.033$; Fig. 16 a). These relationships were not present in the low N catchment.

Within the low N catchment, soil moisture and potential N₂O exhibited a strong significant positive relationship ($p = 0.00088$; Fig. 19 b), although this relationship was not present in the high N catchment.

Soil pH was negatively correlated to soil NH₄⁺ content across all samples ($p = 0.0037$; Fig. 18 a) and in the low N catchment, a significant negative relationship between NH₄⁺ concentration and pH occurred ($p = 0.022$; Fig. 18 a) and a significant positive relationship between soil pH and NO₃⁻ was noted in the low N catchment samples ($p = 0.033$; Fig. 18 b). Soil pH was positively correlated to potential N₂O production had a significant positive relationship across all samples ($p = 5.7\text{e-}9$; Fig. 19 a) and from samples within both low and high N catchments ($p = 5.70\text{E-}9$; $p = 4.10\text{E-}6$, respectively; Fig. 19 a).

Potential N₂O production and soil NO₃⁻ content were not related across all samples, ($p = 0.59$), but a significant positive relationship was noted in the low N catchment ($p = 0.00079$; Fig. 21 a). No relationship occurred between potential N₂O and soil NH₄⁺ content occurred among or within high and low N catchments ($p = 0.1$, Fig. 21 b).

4. Discussion

N fertilization and deposition contributes to pollution of ecosystems and poses threats to water and air quality (Sabota et al. 2015; Rome et al. 2021). Excessive N in the environment contributes to global climate change and is of special consideration in ecosystem management and stream remediation efforts (Suddick et al. 2013). Our objective was to investigate relationships between abundance of microbial functional genes critical to key N transformations and soil N process rates in soil beneath six tree species in stream catchments containing relatively high and low N inputs. We used West Run Watershed in Morgantown, West Virginia as our study site; this watershed has previously exhibited an influence of land use intensification and evidence of reduced stream microbial biodiversity (Dang et al. 2021). Given the differences in N inputs between our high and low N sampling locations, differences in N cycling were expected. Our target genes have been linked to nitrification and denitrification potentials in soil by previous studies, and we anticipated our study would provide insight into eutrophication and the complex N cycling dynamics of forest ecosystems (Levy-Booth et al. 2014).

Soil NO_3^- and NH_4^+ content was greater in the high N catchment (Figs. 3 a and 3 b, respectively), perhaps resulting from additional N influx from fertilizer runoff in the form of $(\text{NH}_4)_2\text{SO}_4$ and NH_3 in animal excrement (NRCS), from agricultural operations in the upper reaches of the sub-catchment, and NH_4^+ is subsequently nitrified to NO_3^- . This is consistent with previous studies in West Run Watershed. Martin et al. (2021) found a positive association with stream NO_3^- concentrations in West Run Watershed areas impacted by agriculture (Kellner et al. 2018; Martin et al. 2021).

Although we found no significant differences in soil pH, percent soil organic matter content, percent soil moisture, cumulative N_2O concentration, *nosZ* and *nirK* gene abundance, or *nirK:nosZ* gene abundance between the two sub-catchments, there were considerable differences within high and low N catchments between tree species and mycorrhizal association. Furthermore, these differences were not consistent between the catchments. We predicted that soil microbial functional gene abundance would be significantly different between tree species, specifically related to mycorrhizal association, in that soil beneath AM-associated tree species (*L. tulipifera*, *P. serotina*, *A. saccharum*, *P. occidentalis*) would contain a greater abundance of *nirK* compared to *nosZ* relative to ECM-associated tree species (*Q. rubra* and *F. grandifolia*). NO_3^- concentration was significantly higher beneath ECM-associated tree species in the high N catchment (Fig 12 a) and NH_4^+ was significantly higher beneath ECM species in the low N catchment (Fig 12 b). We expected that NO_3^- and NH_4^+ concentrations would to be higher beneath AM-associated tree species in the high N catchment due to fast decomposition of high-quality litter pools and rapid N mineralization from organic to inorganic N forms (Phillips et al. 2013).

Our findings may be a reflection of slow inorganic N processing by ECM-associated tree species with N enrichment that results in higher retention of inorganic N relative to AM-associated tree species. ECM-associated tree species are likely processing inorganic N in the low N catchment so that it does not accumulate in the soil as it would with N enrichment (Phillips et al. 2013). Elevated NH_4^+ in soil beneath ECM-associated tree species in the low N catchment (Fig. 12 b) may be attributable to the same mechanism, in that ECM-associated tree species typically promote soil with less NO_3^- leaching with low inorganic N availability (Phillips et al. 2013). This is partially supported here by the significantly higher NH_4^+ concentration in the low N catchment beneath American beech, an ECM-associated species, compared to black cherry, sugar maple, and

American sycamore (Fig. 7 b). Similarly, in the high N catchment NH_4^+ concentration was significantly higher in soil beneath ECM-associated northern red oak compared to black cherry and sugar maple (Fig. 7 b). These differences can be attributed to slower mineralization and decomposition beneath ECM-associated tree species. Soil beneath ECM-associated tree species exhibits relatively greater proportions of organic N compared to soil beneath AM-associated species, which generally have a greater proportion of inorganic forms of N, potentially resulting in greater N leaching in the form of NO_3^- (Malchair and Carnol 2009; Phillips et al. 2013).

In the high N catchment, the measured parameters of soil pH, OM, NO_3^- , NH_4^+ , potential N_2O production, *nosZ* and *nirK* gene abundance, *nirK:nosZ*, exhibited significant differences as a function of tree species. Perhaps most notably, soil beneath black cherry had the lowest pH, NO_3^- concentration, potential N_2O production, and OM in the high N catchment (Figs. 8 a, 7 a, 9, and 8 c, respectively). This effect was not observed in the low N catchment. Black cherry is an AM-associated tree species and is considered an N-demanding species (Royo et al. 2021). It has been associated with greater soil N mineralization rates resulting in greater available NO_3^- and NO_3^- losses relative to ECM-associated tree species (Schwaner and Kelly 2019; Royo et al. 2021).

nirK gene abundance in the high N catchment was significantly higher in soil beneath sugar maple compared to American beech and American sycamore (Fig. 10 b). This may be related to faster N mineralization and nitrification relative to neighboring tree species (Peterjohn et al. 2015). Likewise, the high *nirK:nosZ* ratio in black cherry, sugar maple, and American sycamore compared to American beech in this catchment (Fig. 10 c) may be attributed to the influence of mycorrhizal fungal association. This is consistent with the hypothesis that AM soils will likely contain greater abundances of *nirK* and *nirK:nosZ*, and thus elevated levels of NH_4^+ and NO_3^- relative to ECM soils, due to greater access to NH_4^+ pools in the high N catchment (Averill et al. 2014). *nirK*, *nosZ*, and *nirK:nosZ* did not differ in the low N catchment between tree species (Figs. 10 b, 10 a, and 10 c, respectively), perhaps due to the similar pH across the low N catchment (Brezinger et al. 2015).

4.1 Relationships of environmental variables to gene abundance

We quantified *nosZ* and *nirK* microbial N cycling functional genes using qPCR and measured soil NH_4^+ , NO_3^- , N_2O , pH, soil organic matter content, and soil moisture content. We hypothesized that N enrichment would correspond to greater concentrations of NH_4^+ , NO_3^- , and N_2O and greater

abundances of microbial N cycling functional genes *nosZ* and *nirK*. We also assumed that N enrichment would contribute to lower soil pH via oxidation of NH_4^+ compounds (Grennfelt and Hultberg 1986).

Although soil pH was not distinct between the high and low N catchments (Fig. 4 a), N enrichment contributed to species-specific changes in soil pH that appear to have an influence on other measurements within each catchment (Fig. 8 a). *nirK*, *nosZ*, *nirK:nosZ* copy number, and potential N_2O production all displayed significantly positive correlations with soil pH (Figs. 16 b, 16 a, 17 b, 19 a, respectively). On the other hand, NH_4^+ displayed a significant negative correlation with soil pH (Fig. 18 a), likely due to ammonification causing soil acidification and potentially contributing to altered functionality of *nirK* and *nosZ* (Hartman et al. 2008; Lauber et al. 2009; Ramirez et al.; Morales et al. 2010). Various studies have found altered transcription processes encoding *nirK* and *nosZ* due to low pH in pure culture and soil (Bergaust et al. 2010; Liu et al. 2010; Liu et al. 2014; Brezinger et al. 2015). Liu et al. (2014) found N_2O reductase inhibition at $\text{pH} < 6.1$. This reduction in functionality of *nirK* and *nosZ* may contribute to differences in N cycle rates downstream. A loss in *nirK* functionality is a loss in nitrate reductase and a loss in *nosZ* functionality has been linked to incomplete reduction of N_2O to N_2 gas, especially in high N conditions with lowered pH (Brezinger et al. 2015). Soil pH and N_2O also displayed a significant positive relationship, possibly due to greater functionality of *nirK* in low N (high pH) samples (Fig 19 a).

Inconsistent with our hypothesis, N_2O production increased with increasing *nosZ* abundance (Fig. 20 a), especially in high N areas. We predicted that increasing *nirK* and *nirK:nosZ* would contribute to greater N_2O production due to the incomplete reduction of N_2O to N_2 by *nosZ* especially among AM-associated tree species due in high N conditions and greater access to NH_4^+ pools. *nirK* and N_2O did exhibit a positive relationship (Fig. 20 b), supporting our hypothesis. The positive relationship between N_2O and *nosZ* may be related to the influence of soil pH and soil moisture in the denitrification process outweighing the functionality of nitrous oxide reduction by *nosZ* (Firestone and Davidson 1989; Davidson et al. 2000; Brezinger et al. 2015).

Our data support the possibility that increasing NH_4^+ leads to a decrease in soil pH and inhibition of *nosZ* and *nirK* function. Low pH was generally associated with high NH_4^+ concentrations in both high and low N catchments (Fig. 18 a). This is consistent with NH_4^+ contributing to

acidification of soil via excess H^+ ions (Wallace et al. 1994; Aber et al. 1998; Lu et al. 2009; Lu et al. 2014). Additionally, loss of *nirK* functionality at low pH likely contributes to decreased N_2O production in low pH samples, since the *nirK* product catalyzes reduction of NO_3^- to NO and N_2O (Szukics et al. 2009; Morales et al. 2010; Rasche et al. 2011; Bothe et al. 2000; Richardson et al. 2009; Morales et al. 2010). If *nirK* and N_2O production do not align, it could be because *nirS* is filling the role of nitrate reductase. Unfortunately, we were unsuccessful in quantifying *nirS* in this study, but it is thought to be functionally equivalent to *nirK* and could be filling the same function (Zumft 1997).

Lower cumulative N_2O production is further related to soil moisture content, as evident in the significant positive relationship between percent soil moisture and final N_2O concentration (Fig. 19 b). This is supported by the hole-in-pipe mechanism that soil moisture is related to N_2O production in that wetter soil contributes to more N_2O (Firestone and Davidson 1989; Davidson et al. 2000; Fig. 19 b), pH can also contribute to N_2O concentration by influencing *nirK* functionality (Brezinger et al. 2015). In this study, potential N_2O production was not related to either NO_3^- or NH_4^+ in either high and low N catchments, although N_2O and NO_3^- show a slight positive trend in the low N catchment. This relationship between potential N_2O production and soil NO_3^- may be a result of NO_3^- being further reduced to N_2O by abundant *nirK*. *nirK:nosZ* did not have a significant relationship to N_2O production among samples or within catchments.

4.2 Limitations and future directions

Perhaps the most obvious limitation of this study is sample size. We collected and processed 48 soil samples total, 24 from the high N catchment and 24 from the low N catchment. Within catchments, we sampled four individuals from each of the six tree species. Given the number of considerable outliers and large variance in some of the measured parameters, a larger sample size would have provided higher analytical power to discern more subtle effects.

Additionally, understory vegetation may have contributed to the high variance in our data; other studies have shown that understory plants play a significant role in forest N cycling (Gilliam et al. 2016). Gilliam et al. (2016) found shifts in herbaceous layer community composition with N enrichment in an Appalachian forest (Gilliam et al. 2016). They found initial increases in biomass and eventually decreases in N-efficient plant species potentially influencing N cycling dynamics. It is difficult to separate the influence of understory vegetation, soil conditions, and tree species

because each of these are intimately meshed in a field setting. Perhaps studying a monoculture of a single tree species or implementing a greenhouse experiment would eliminate the influence of understory vegetation and provide the capacity to determine the singular influence of tree species on soil and microbial processes.

Pseudo-replication may contribute to statistical weakness in our study, in that we did not replicate catchments – instead, we compared one high N catchment with one low N catchment – and within catchments we sampled an uneven number of ECM and AM associated species.

Additionally, had *nirS* and *amoA* quantification been successful, we may have seen considerable significant links with NO_3^- and NH_4^+ concentrations and implications of nitrification and denitrification rates (Zumft et al. 1997; Levy-Booth et al. 2014; Sterngren et al. 2015). A more comprehensive soil meta-genomic analysis might lead to more informative results for the quantification of *nirS* and *amoA* (Pester et al. 2011).

4.3 Limitations of quantifying gene abundance

qPCR measures the presence of a gene, but it does not measure function or turnover. Thus, the copy numbers of *nosZ* and *nirK* measured here may include genes that are not currently active (Wertz et al. 2019). With gene abundance measures, we may be underestimating or overestimating their influence on the production of NO_3^- and N_2O . This may explain the lack of congruence between gene abundance values and NH_4^+ , NO_3^- , or N_2O concentration (Wertz et al. 2019). In future studies, it may be useful to consider measures of gene expression, protein levels (or turnover), enzymatic processes, or additional N functional genes and related pathways.

4.4 Conclusions

We determined that increasing N enrichment contributes to NH_4^+ and NO_3^- fluctuations, and that subsequent soil acidification from NH_4^+ contributes to decreasing *nirK* and *nosZ* gene abundance and potentially to reduced functionality of these genes. Tree species influence is less conclusive, possibly due to our relatively low sample size and the potential masking influence of the understory vegetation. Ultimately, we found important connections between the measured parameters, but further study is needed to determine the accuracy of using N cycling functional genes in predicting N pathway processes. qPCR is useful to quantify the presence of genes, although not necessarily the extent of their function, and when combined with other types of genetic analysis may be useful

in predicting N cycling that may be considered when implementing N pollution remediation in riparian buffer zones and forested ecosystems. ECM associated tree species appear to be retaining more inorganic N, as reflected by greater abundances of NH_4^+ beneath American beech and northern red oak trees and these species should be of consideration when implementing BMP's to improve N retention in riparian buffer zones.

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6. Figures and Tables

Figure 1: West Run Watershed in Morgantown, West Virginia. West Run Creek is a tributary of the Monongahela River. West Run Creek receives runoff from sources of forest, agriculture, and urban development areas.

Figure 2. Target microbial functional genes associated with nitrogen cycling.

Table 1: Genes with primers and reference strains used in this study.

Gene	Primer	Reference Strain	Amplicon size	Sequence (5'-3')	Reference
<i>amoA</i>	amoA-1F	<i>Pseudomonas fluorescens</i>	491	GGGG TTTCTACTGGTGGT	Rottauwe et al. 1997; Levy-Booth 2014
	amoA-2R			CCCCTCKGSAAGCCTTCTTC	Rottauwe et al. 1997; Levy-Booth 2014
	A189		525	GGNGACTGGGACTTCTGG	Holmes 1995; Jensen et al. 2000
	A682			GAASGCNGAGAGAASGC	Holmes 1995; Jensen et al. 2000
	GenAOB 1F GenAOB 2R		120	CTG GGG TTT CTA CTG GTG GTC	Meinhardt et al. 2015
<i>nirK</i>	GenAOB 1F mod				
	nirK876	<i>Alcaligenes faecalis</i>	147	ATYGGCGGVCAVGGCGA	Henry et al. 2006
	nirK1040			GCCTCGATCAGRTTRTGTT	
<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; Throback et al. 2004
	nirSR3cd*			GA(C/G) TTC GG(A/G) TG(C/G) GTC TTG A	Michotey et al. 2000; Throback et al. 2004
	nirS 4F		192	TTC(A/G)TCAAGAC(C/G)CA(C/T)CCGA A	Braker 1998
				CTTGTTG(A/T)ACTCG(C/G)(C/G)CTGCA C	
<i>nosZ</i>	nirS 5R nosZ 2F nosZ 2R	<i>Paracoccus denitrificans</i>	267	CGCRACGGCAASAAGGTSMSST CAKRTGCAKSGCRTCAGAGAA	Jung et al. 2013; Henry et al. 2006

Table 2. Gene primer sets annealing conditions and amplification results. *touchdown PCR was performed

Gene	Primers	Annealing Temp	Amplification
<i>amoA</i>	amoA-1F amoA-2R	54	-
	A189 A682	54*	+
	GenAOB 1F GenAOB 2R	57*	-
	GenAOB 1F mod	54*	-
<i>nirS</i>	Cd3aF nirSR3cd*	51*	+
	nirS 4F nirS 5R		-
<i>nirK</i>	nirK876* nirK1040*	57	+
<i>nosZ</i>	nosZ 2F* nosZ 2R*	52	+

Table 3. High N catchment summary statistics.

Treatment	Soil pH	% OM	% Moisture	NO ₃ ⁻ (mg N ⁻¹ kg soil)	NH ₄ ⁺ (mg N ⁻¹ kg soil)	N ₂ O at 30 Hr (mg N ⁻¹ kg soil)	nosZ (CN ⁺ ng DNA ⁻¹ g soil)	nirK (CN ⁺ ng DNA ⁻¹ g soil)	nirK:nosZ (CN ⁺ ng DNA ⁻¹ g soil)
High N									
	4.27	15.59	0.25	43.01	6.49	0.37	2.61E+7	3.65E+12	795.65
	(0.66)	(5.3)	(0.06)	(24.57)	(4.13)	(0.34)	(2.62E+09)	(5.89E+12)	(830.04)
					</				

Table 4. Low N catchment summary statistics.

Treatment	Soil pH	% OM	% Moisture	NO ₃ ⁻ (mg N ⁻¹ kg soil)	NH ₄ ⁺ (mg N ⁻¹ kg soil)	N ₂ O at 30 Hr (mg N ⁻¹ kg soil)	nosZ (CN ⁺ ng DNA ⁻¹ g soil)	nirK (CN ⁺ ng DNA ⁻¹ g soil)	nirK:nosZ (CN ⁺ ng DNA ⁻¹ g soil)	Species	Soil pH	% OM	% Moisture	NO ₃ ⁻ (mg N ⁻¹ kg soil)	NH ₄ ⁺ (mg N ⁻¹ kg soil)	N ₂ O at 30 Hr (mg N ⁻¹ kg soil)	nosZ (CN ⁺ ng DNA ⁻¹ g soil)	nirK (CN ⁺ ng DNA ⁻¹ g soil)	nirK:nosZ (CN ⁺ ng DNA ⁻¹ g soil)
Low N																			
	4.41 (0.78)	13.19 (3.28)	0.29 (0.07)	15.67 (14.81)	4.03 (2.45)	0.52 (0.40)	5.78E+09 (6.55E+09)	5.64E+12 (7.26E+12)	3778.97 (14255.95)	REOA	4.22 (0.37)	11.40 (1.60)	0.27 ^{ab} (0.06)	18.70 (21.64)	5.79 ^{ac} (2.95)	0.38 (0.1)	3.79E+09 (2.63E+09)	3.46E+12 (1.56E+12)	1257.06 (1095.16)
										AMBE	4.73 (0.80)	13.88 (1.09)	0.28 ^{ab} (0.10)	8.23 (5.91)	6.21 ^a (2.24)	0.61 (0.48)	1.92E+09 (5.62E+08)	1.96E+12 (1.51E+12)	1091.47 (963.38)
										TUPO	4.51 (0.55)	10.78 (3.73)	0.26 ^{ab} (0.05)	8.92 (8.68)	4.10 ^{abc} (1.53)	0.29 (0.16)	4.00E+09 (2.47E+09)	3.92E+12 (3.03E+12)	944.36 (198.43)
										BLCH	3.72 (0.58)	15.43 (3.32)	0.35 ^a (0.03)	13.48 (4.26)	4.68 ^c (0.64)	0.56 (0.25)	1.05E+09 (2.10E+09)	1.17E+12 (2.33E+12)	17939.89 (35131.11)
										SUMA	4.07 (0.31)	11.20 (1.98)	0.23 ^b (0.05)	10.53 (9.68)	2.22 ^{ab} (1.13)	0.26 (0.11)	1.33E+10 (9.14E+09)	1.60E+13 (1.19E+13)	895.09 (610.83)
										AMSY	5.24 (1.09)	16.47 (3.30)	0.34 ^{ab} (0.06)	34.12 (18.68)	1.18 ^b (1.32)	1.00 (0.61)	1.07E+10 (7.87E+09)	1.07E+10 (7.87E+09)	545.96 (391.84)

Figure 3 a. Soil NO_3^- concentration was significantly higher in the high N catchment compared to the low N catchment ($p=2.37\text{E-}5$).

Figure 3 b. Soil NH_4^+ concentration was significantly higher in the high N catchment compared to the low N catchment ($p=0.009$).

Figure 4 a: Soil pH was similar between high and low N catchments ($p=0.61$).

Figure 4 b. Percent soil organic matter was similar between high and low catchments ($p=0.069$).

Figure 4 c. Percent soil moisture was similar between high and low catchments (p=0.050).

Figure 5. N₂O was similar between high and low N catchments (p=0.053)

Figure 6 a. *nosZ* copy number was similar between high and low N catchments (p=0.112).

Figure 6 b. *nirK* copy number was similar between high and low N catchments (p=0.103).

Figure 6 c. *nirK:nosZ* was similar between high and low N catchments (p=0.5).

Figure 7 a. Soil NO_3^- content varied by tree species in the high N catchment. Within the high N catchment, NO_3^- concentration in American beech was significantly lower than sugar maple and significantly higher than American sycamore and American sycamore and black cherry were significantly lower than northern red oak and sugar maple ($p=0.029$). NO_3^- concentration was not significantly different between tree species in the low N catchment ($p=0.14$).

Figure 7 b. Soil NH_4^+ content varied by tree species in the high N catchment and was significantly higher in northern red oak compared to black cherry and sugar maple ($p=0.029$). Within the low N catchment, soil NH_4^+ was significantly lower in soil beneath American sycamore compared to northern red oak, American beech, and black cherry and significantly lower in sugar maple compared to black cherry ($p=0.029$).

Figure 8 a. Soil pH varied by tree species in the high N catchment and was significantly higher in soil beneath sugar maple compared to black cherry (post-hoc $p=0.029$). Soil pH was not significantly different between tree species in the low N catchment ($p=0.15$).

Figure 8 b. Soil moisture content varied by tree species in the low N catchment and was significantly higher in soil beneath black cherry compared to sugar maple (post-hoc $p=0.029$). Soil moisture did not differ between tree species in the high N catchment.

Figure 8 c. Soil organic matter content varied by tree species in the high N catchment and was significantly lower in black cherry compared to northern red oak ($p=0.029$). Organic matter content did not differ between tree species in the low N catchment ($p=0.069$).

Figure 9. Potential N₂O production after 30 hours of incubation was different within high N catchment between tree species. Final N₂O concentration was significantly lower in soil beneath black cherry compared to American sycamore and northern red oak (post-hoc $p=0.029$) in the high N catchment. Final N₂O concentration was not distinct between tree species in the low N catchment ($p=0.18$).

Figure 10 a. *nosZ* copy number was significantly higher in American sycamore compared to black cherry in the high N catchment ($p=0.029$). *nosZ* copy number was not significantly different between tree species in the low N catchment ($p=0.098$).

Figure 10 b. *nirK* copy number was significantly lower in American beech compared to American sycamore and sugar maple in the high N catchment ($p=0.029$). *nirK* copy number was not significantly different between tree species in the low N catchment.

Figure 10 c. The ratio of *nirK:nosZ* varied by tree species in the high N catchment and was higher in soil beneath black cherry, sugar maple, and American sycamore compared to American beech (post-hoc, $p=0.029$). *nirK:nosZ* copy number did not differ between tree species in the low N

Figure 11. (a) NO_3^- concentration was similar between soil beneath ECM and AM fungal association (0.444). (b) NH_4^+ concentration was significantly higher beneath ECM-associated trees relative to AM fungal association (0.002). (c) *nirK* copy number was not significantly different between ECM and AM fungal association ($p=0.073$). (d) *nirK:nosZ* was not significantly different between ECM and AM fungal association ($p=0.155$).

Figure 12 (a) NO_3^- concentration was significantly higher in ECM compared to AM ($p=0.023$) in the high N catchment. NO_3^- concentration was not significantly different between ECM and AM associated species in the low N catchment ($p=0.33$). (b). NH_4^+ was significantly higher in ECM compared to AM in the low N catchment in ($p=0.01$). NH_4^+ was not significantly different between ECM and AM associated species in the high N catchment ($p=0.076$).

Figure 13 (a) *nirK* copy number was significantly higher in AM compared to ECM in the high catchment ($p=0.011$) but did not vary by fungal association in the low N catchment ($p=0.610$). (b) The ratio of *nirK:nosZ* was significantly higher in soil beneath AM compared to ECM in the high N catchment and not significantly different in the low N catchment ($p=0.005$; $p=0.783$).

Figure 14. N₂O concentration measured at 1, 18, 24, and 30 hours. N₂O concentration was not significantly different between the high and low N catchment (top) but showed significant differences between black cherry and American sycamore and northern red oak in the high N catchment after 30 hours (bottom; $p=0.029$).

Figure 15 a: The low N catchment showed greater *nosZ* copy number with lower levels of NH₄⁺ concentration compared to that in the high N catchment (left; p=0.00062; p=0.23). There was a negative relationship between NH₄⁺ concentration and *nosZ* copy number across all samples (right; p=0.0013).

Figure 15 b: *nirK* copy number and NH₄⁺ concentration showed a significant negative relationship in the low N catchment (left; p=0.0026) with a significant negative trend across all samples (right; p=0.0029)

Figure 16 a: Within the high N catchment, *nirK:nosZ* copy number had a significant negative relationship with NH₄⁺ (p=0.033).

Figure 16 b: Soil pH and *nirK* copy number showed a significant positive relationship in the high N catchment (p<0.001) and no significant relationship in the low N catchment (left; p=0.25). Soil pH showed a significant positive relationship with *nirK* copy number (p=0.0014) across all samples (right).

Figure 17 a: *nosZ* copy number and soil pH had a significant positive relationship within high and low N catchments (left; $p=0.042$ and $p<0.001$, respectively). Soil pH had a significant positive relationship with *nosZ* and *nirK* copy number ($p=0.00045$) across all sample (right).

Figure 17 b. Within the high N catchment, *nirK:nosZ* had a significant positive relationship with pH ($p=0.0021$).

Figure 18 a: Soil pH and NH_4^+ concentration had a significant negative relationship in the low N catchment (left; $p=0.022$) and across all samples (right; $p=0.0037$).

Figure 18 b: Soil pH and NO_3^- concentration showed a significant positive relationship in the low N catchment (left; $p=0.033$) and exhibited a positive trend and insignificant differences across all samples (right; $p=0.17$).

Figure 19 a. Soil pH and N₂O final concentration at 30 hours had a significant positive relationship within high and low N catchments (left; $p=5.70\text{E}-9$; $p=4.10\text{E}-6$, respectively) and across all samples (right; $p=5.7\text{e}-9$).

Figure 19 b. Within the low N catchment, percent soil moisture and final N₂O have a strong significant positive relationship (left; $p=0.00088$). Percent soil moisture and N₂O show a significant positive relationship across all samples (right; $p=0.0017$).

Figure 20 a. *nosZ* and N₂O displayed a significant positive relationship at 30 hours in the high N catchment compared to that in the low N catchment (left; $p=0.0026$; $p=0.22$, respectively). *nosZ* and N₂O showed a significant positive relationship across all samples (right; $p=0.011$).

Figure 20 b. *nirK* copy number and N₂O final concentration did not display significant relationships in the high or the low N catchment (left). *nirK* copy number and N₂O final concentration showed a slight positive trend among all samples (right; $p=0.089$).

Figure 21 a. NO_3^- and final N_2O concentration showed a significant positive relationship in the low N catchment (left; $p = 0.00079$) and no significant relationship across all data (right; $p = 0.59$).

Figure 21 b. NH_4^+ and final N_2O concentration showed no significant relationships within the high or the low N catchment (left) or across all data (right; $p = 0.1$).

7. Supplemental Material

S1. *nirK* and *nosZ* PCR amplification.

S2. *nirS* cd3af primer set and *amoA* 189 and 682 primer set with *nirK* positive control.

S3. *amoA* 1F mod and GEN AOB R and *nirS* cd3af and R3cd primer sets.

S4. *nirS* 4F 5R primer set using touchdown PCR.

S5. *nosZ* 2F, *nirK* 876, *amoA* 1F, *nirS* cd3af primer sets run with Promega GoTaq Green and Amplitaq Gold.

S6. *amoA* 1F mod and GEN AOB R primer set run with touchdown PCR.

S7. Melt curves for *nosZ* (top) and *nirK* (bottom)

S8. qPCR standard curves for *nirK* (top) and *nosZ* (bottom).

S9. *nirK* and *nosZ* plasmid transformations with insert length.

8. Data Analysis Addendum

In addition to the non-parametric statistical analysis we performed, we used SAS-JMP v. 11.0 (SAS Institute, Cary NC) to develop a predictive model using backward stepwise regression to determine important variables for predicting measured NO_3^- , NH_4^+ , and N_2O concentration compared to environmental variables including soil pH, moisture, SOM, *nirK* gene abundance, *nosZ* gene abundance, mycorrhizal associations, tree species, and location (relative high or low N). The statistical model determines which soil variables are the best predictors for NO_3^- , NH_4^+ , and N_2O concentrations. Conditions selected for stepwise regression included the minimum BIC stopping rule with whole effects for inclusion of categorical data and parameters were included at $\alpha = 0.10$. Mean response values by tree species were separated using Tukey's HSD at $\alpha = 0.05$.

We found that the strongest predictor of soil NO_3^- in our data was location (high N/low N), followed by soil moisture (Table 5; $p < 0.001$ and $= 0.009$, respectively) and tree species was also an important influence on soil NO_3^- ($p = 0.033$). The greatest soil NO_3^- occurred in soil beneath oak and the lowest NO_3^- occurred beneath cherry (Table 6).

The strongest predictor of soil NH_4^+ was SOM content, followed by soil pH (Table 5; $p = 0.015$ and 0.021 , respectively). *nosZ* gene abundance and tree species were also important in predicting soil NH_4^+ . The greatest soil NH_4^+ occurred again in soil beneath oak and the lowest NH_4^+ occurred beneath cherry (Table 6).

The strongest predictor of potential N_2O production was soil pH, followed by SOM content (Table 5; $p < 0.001$ and $= 0.016$, respectively). Additional significant predictors of N_2O were location ($p = 0.036$) and tree species ($p = 0.015$). The greatest potential N_2O production occurred in soil beneath sycamore and the least N_2O was produced in soil beneath sugar maple (Table 6).

Table 5. Stepwise regression model parameters included to predict soil NO₃⁻, NH₄⁺, and potential N₂O production. Overall model adjusted R² and model p-values are presented inclusive of the variables listed for each dependent variable.

Predicted dependent variable	Explanatory independent variable	F Ratio	Parameter P-Value	Model Adjusted R ²	Model P-Value
Soil NO ₃ ⁻ (mg N kg ⁻¹ soil)	Tree species	2.72	0.033	0.442	<0.001
	Location	33.89	<0.001		
	Soil moisture	7.37	0.009		
Soil NH ₄ ⁺ (mg N kg ⁻¹ soil)	Tree species	2.19	0.075	0.444	
	<i>nosZ</i> gene abundance	3.93			
	Soil pH	5.78	0.021		
	Soil OM	6.44	0.015		
Potential N ₂ O (mg N kg ⁻¹ soil)	Tree species	3.31	0.015	0.686	<0.001
	Location	4.73	0.036		
	Soil pH	63.77	<0.001		
	Soil OM	6.33	0.016		

Table 6. Mean response of measured soil NO_3^- , NH_4^+ and potential N_2O production by tree species as significant parameters in the predictive regression models. Significant differences in mean response for each variable are noted by different letters (Tukey's HSD $p < 0.05$).

Predicted Dependent Variable	Tree Species	Mean Response
Soil NO_3^- (mg N kg^{-1} soil)	Sycamore	28.77 ^{ab}
	Cherry	14.79 ^b
	Oak	41.21 ^a
	Beech	28.91 ^{ab}
	Poplar	26.41 ^{ab}
	Sugar maple	35.94 ^{ab}
Soil NH_4^+ (mg N kg^{-1} soil)	Sycamore	4.90 ^{ab}
	Cherry	4.45 ^b
	Oak	8.17 ^a
	Beech	6.40 ^{ab}
	Poplar	4.83 ^{ab}
	Sugar maple	2.79 ^{ab}
Potential N_2O (mg N kg^{-1} soil)	Sycamore	0.83 ^a
	Cherry	0.33 ^{ab}
	Oak	0.48 ^{ab}
	Beech	0.44 ^{ab}
	Poplar	0.31 ^{ab}
	Sugar maple	0.26 ^b