Nitrogen

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Tree Species Influence Nitrate and Nitrous Oxide Production in Forested Riparian Soils

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Abstract: Abundance of soil microbial nitrogen (N) cycling genes responsible for nitrification, denitrification, and nitrous oxide reduction may vary with tree species and N inputs, and these variables may be used to predict or mediate nitrate (NO_3^-) and nitrous oxide (N_2O) from soil. Nitrification and denitrification rates have also been linked to tree mycorrhizal associations, as soil beneath species associated with arbuscular mycorrhiza (AM) shows greater nitrification rates than species forming ectomycorrhizal (ECM) associations. In this study, we integrated N microbial functional gene abundance in the soil influenced by six tree species in two sub-catchments receiving either high or low N inputs. The soils beneath the two ECM-associated tree species and the four AM-associated tree species were analyzed for inorganic N content and potential N_2O flux and microbial gene abundance (*nirK* and *nosZ*) was quantified using qPCR techniques. Other parameters measured include soil pH, moisture, and organic matter. We determined that tree species influence NO_3^- and N_2O production in riparian soils, particularly under high N enrichment. The soil beneath black cherry had the lowest pH, NO_3^- concentration, potential N_2O production, and OM, though this result did not occur in the low N catchment. The strongest predictors of soil NO_3^- and N_2O across the study sites were N enrichment and pH, respectively. These results provide a framework for species selection in managed riparian zones to minimize NO_3^- and N_2O production and improve riparian function.

Keywords: riparian soil; nitrate; nitrous oxide; tree species; West Run Watershed

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1. Introduction

Vegetated riparian zones, or the area of transition between land and water, are critical areas of management of nitrogenous pollutants, namely nitrate (NO_3^-) and nitrous oxide (N_2O gas; [1,2]). Owing to their bioremediation potential, establishing such zones is considered a best management practice (BMP) priority in agricultural, urban, and forested landscapes [3,4]. Plants, soils, and microbial communities in these zones can store, assimilate, immobilize, and/or reduce NO_3^- and N_2O, preventing them from entering aquatic systems and the atmosphere. However, these riparian management zones exhibit a wide variation in their capacity to store and/or transform N-containing compounds, differing as a function of N load, hydraulic gradient, soil texture, and hydrologic variables [5,6] and with differences in plant type (e.g., grass vs. trees), which are not consistent [7].

1.1. Nitrogen Pollutants

Soil nitrification and denitrification are microbially mediated steps in the N cycle, and both processes can create nitrogenous pollutants in the environment (Figure 1). One product of nitrification is NO_3^-, a highly mobile form of N, which may be easily transported to aquatic ecosystems and lead to eutrophication, toxic algal blooms and oxygen depletion,

and degraded habitability of waterways (e.g., dead zones in the Gulf of Mexico and the Chesapeake Bay, toxic algal blooms in the Great Lakes, the Ohio River, and the Florida coastline). Additionally, NO_3^- can infiltrate groundwater, affecting drinking water sources. Transport of NO_3^- from the soil profile may also deplete soil fertility by leaching soil nutrients such as calcium and magnesium and through increasing soil acidity via base cation depletion [8]. It is ultimately estimated that more than 100,000 miles of rivers and streams, approximately 1.01 million ha of lakes, reservoirs, and ponds, and more than 800 mi^2 of bays and estuaries in the United States have impaired water quality because of N and nutrient pollution [9].

Nitrification, denitrification, and dissimilatory nitrate reduction to ammonium (DNRA) are microbial processes that can produce N_2O, which accounts for approximately 10% of total greenhouse gas emissions [10]. Recently, there has been great interest in quantifying specific sources of N_2O as N_2O gas is the largest stratospheric ozone-depleting substance emitted through human activities [11,12] and has a 300-fold greater influence on global warming processes than carbon dioxide (CO_2; [13,14]). It is estimated that more than 65% of the global N_2O production comes from microbial nitrification and denitrification processes in soils, which have increased with greater N soil deposition as a result of anthropogenic influences, e.g., increasing use of agricultural fertilizers, intensive grazing on pastureland, and atmospheric deposition of N [15]. N_2O production may also be enhanced by greater soil carbon (C) content, as has been demonstrated in soils of no-till agriculture, in poorly aerated soil, and in riparian zones, where the soil C content is relatively greater [12,16–18]. The chemical quality of the C present may alter denitrification processes as well, as the addition of labile C substrates can decrease N_2O:N_2 product ratios, thus lowering the GHG emission potential [19]. Critically, N_2O can be either an intermediate or end product in the denitrification process as N_2O can be further reduced to inert N_2 gas if the denitrifying microbes possess the *nosZ* gene that encodes enzyme nitrous oxide reductase (Figure 1). However, ~33% of the microbial denitrifying population lacks this gene, resulting in a truncated denitrification process and emission of N_2O [12], which is likely common in acidified soils, as the assembly of the nitrous oxide reductase enzyme is disrupted in low-pH systems [20,21]. Hence, the microbial population may either be a sink (if N_2O is reduced to N_2) or a source of N_2O emissions from soil [22,23].

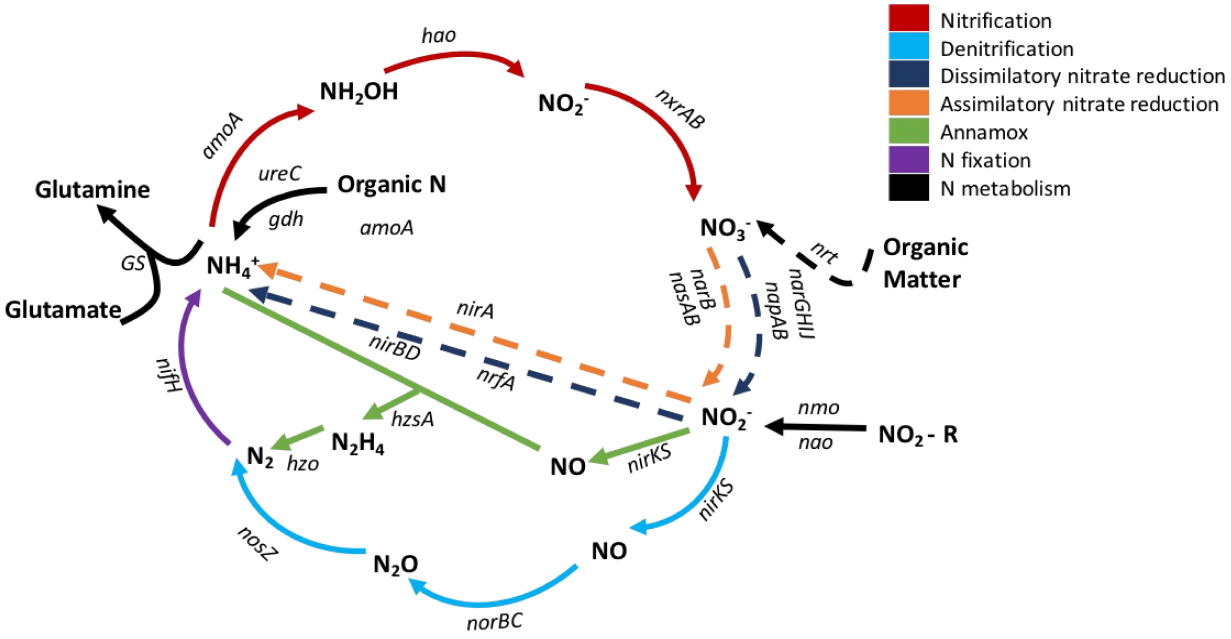

Figure 1. Products and processes of the nitrogen cycle. Functional genes associated with each step of the cycle are in italics. Image adapted from [24].

1.2. Use of the Genes Associated with the N Cycle to Predict Process Rates

The abundance of functional microbial genes associated with N cycling in soil (Figure 1) can be a strong predictor of N cycling rates [25–28]. Although some uncertainty and decoupled gene abundance–process rate relationships are reported [29], other studies suggest that measures of functional gene abundance provide a powerful index to predict rates of biogeochemical processes, especially N process rates [20–23,25–30]. For example, across a vegetation gradient in Alaska, functional gene abundances were the variables most predictive of nitrification and denitrification rates, even when many other factors were considered (e.g., pH, organic matter content, N availability) [28]. That work showed that the potential nitrification rate was best explained by the abundance of the *amoA* bacterial gene, followed by ammonium content. Potential denitrification rates were best explained directly by the *nosZ* gene abundance and indirectly by *nir* and *nirS* gene abundance and nitrate content [28]. The ratio of *nirK* + *nirS:nosZ* gene abundance can also predict relative N_2O production [30,31].

1.3. Tree Species Influence on N Cycling

Vegetation-mediated variation in N cycling has been documented [32–40]. For example, soils beneath oaks, spruces, and American chestnuts (*Quercus*, *Picea* spp., *Castanea dentata*, respectively) have slower N mineralization rates and thus a lower availability of N relative to soils beneath maples and yellow poplars (*Acer* spp. and *Liriodendron tulipifera*) which have much faster N mineralization and greater inorganic N availability [38,40]. In a watershed comparison study, nitrification processes between soils influenced by Norway spruce or mixed hardwoods determined that net NO_3−N production was nine times higher in the hardwood-associated soil (mean = 183.51 mg N kg/28 d) than in the spruce-influenced soil (mean = 18.97 mg N kg/28 d) and, further, differences in the net NO_3−N production were attributed to differences in the quality of soil substrates that are available for microbial metabolism [38]. Moreover, a laboratory incubation study showed 5–8 times greater N_2O production potential in soils developed beneath grassland (1.36 mg N_2O kg/h) relative to soils beneath six tree species in central Siberia. Further, of the six tree species, the soil beneath larch and cedar had the greatest N_2O production potential (0.25 and 0.35 mg N_2O kg/h, respectively), above that beneath spruce, aspen, birch, and pine [41].

One of the primary influences that trees have on soil microbiomes is through inputs of organic matter, both above and below ground, which varies by tree species in quantity and chemical composition [36,42–44]. This variability in organic matter quantity and chemical composition is hypothesized to be the main driver for differential microbial and/or sorption processes by which nutrient bioavailability and/or storage are determined [45,46]. On a larger scale, forested watersheds dominated by different tree species may result in a differential export of NO_3^- to streams draining the watershed. For example, watersheds dominated by oaks or spruce have significantly lower stream NO_3^- concentrations and annual export of N relative to maple- or poplar-dominated watersheds [38,40,47]. However, little is known regarding the influence of tree species on N_2O emissions at the landscape scale [41].

A framework for predicting N availability (and potential for NO_3^- and N_2O production) associated with tree species has been developed based on the type of symbiotic mycorrhizal fungi associated with the tree [39]. Trees with ectomycorrhizal (ECM; e.g., *Quercus* spp.) associations generally have poorer quality litter (e.g., high C:N, high lignin:N), slower below-ground N cycling, and low net NO_3^- production when compared to trees with arbuscular mycorrhizal (AM; e.g., *Acer* spp.) associations [48–50]. Trees with AM associations commonly have a greater inorganic NO_3^- availability in the soil relative to ECM-associated trees [49] and, importantly, greater NO_3^- availability has been linked to greater N_2O production from the soil, e.g., the "Hole-in-Pipe" model [16,51,52]. It is plausible that riparian zone management practices within the above-described AM/ECM framework may inhibit nitrification and lead to positive environmental outcomes, as nitrification inhibition often leads to an associated decline in N_2O production [31]. For example,

mycorrhizal fungi can independently access forms of N, and the mycorrhizal community also influences the bacterial community structure in the soil [53]. This suggests a potential feedback mechanism tied to mycorrhizal fungi community structure and microbial N cycling to produce NO_3^- and N_2O gas. However, tree species-mediated influence on N cycling has not been consistent across the landscape and may be additionally related to the N load influencing the plant–soil system [47].

1.4. N Fertilization Influence on N Cycling

High N availability resulting from anthropogenic sources such as elevated atmospheric N deposition or agricultural application of fertilizers has altered soil fungal and bacterial communities and function; namely, decreased microbial respiration [54,55] and biomass [56] and altered archaeal [57], bacterial [57–59], and fungal [60–62] community composition. Decreased abundance and diversity of N cycling assemblages under elevated N deposition in northern USA hardwood forests have been reported, affecting assimilation, denitrification, and nitrification potential rates [63,64]. Regarding the N deposition gradient in Alaska, USA, Lilleskov et al. (2002) [65] reported a dramatic decline in the ECM fungal species richness associated with *Picea glauca* with increasing N availability and selection against ECM species specialized for N uptake under N-limited conditions. Avrahami et al. (2002) [66] showed that ammonium fertilization induced a shift in the soil-denitrifying community. Conversely, no change in the ammonia-oxidizing community in an incubation study ultimately resulted in an increased N_2O emission rate. The source or type of N fertilizer may also alter soil communities. For example, Enwall et al. (2005) [29] described that organic (cattle manure and sewage sludge) applications induced the greatest potential denitrification and respiration rates compared to inorganic ($Ca(NO_3)_2$ and $(NH_4)_2SO_4$) amendments. These changes in N cycling process rates occurred concomitantly with changes in the total soil N content and soil pH after 56 years of treatment. Similarly, alterations of the microbial community composition stemming from elevated atmospheric N deposition likely result from declines in plant allocation of C-based exudates below ground [62]. Although many studies focus on how anthropogenic N enrichment may affect microbial populations, impacts on specific microbial communities responsible for soil N cycling processes are poorly understood [63,64], especially as influenced by different tree species.

In this study, we sampled soils beneath six native deciduous trees in the West Run Watershed located in Morgantown, West Virginia, USA, 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 was compared to an adjacent reference sub-catchment that does not receive agriculture runoff (i.e., relatively high and low N inputs). We quantified the abundance of soil microbial functional genes related to denitrification (*nosZ*, *nirK*) in the soils sampled beneath the two ECM-associated trees and the four AM-associated trees. We also measured soil NH_4^+, NO_3^-, and potential N_2O production to investigate relationships between gene abundance and N cycling products. The objective was 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 would contribute to greater NH_4^+, NO_3^-, and N_2O concentrations that correspond to greater abundances of corresponding microbial functional genes in the N cycle.
2. Soil microbial N cycling gene abundance would differ significantly between tree species, specifically related to mycorrhizal association. We anticipated that the AM-dominant soils would likely contain greater abundances of *nirK* and NO_3^- relative to the ECM soils due to high quality, low C:N ratio as compared to the AM litter.
3. Soil influenced by tree species associated with AM fungi would contain a higher *nirK*:*nosZ* ratio, indicative of greater potential for N_2O production due to incomplete

nitrous oxide reduction by *nosZ* relative to trees associated with ECM fungi, especially in areas of high N.

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

2. Materials and Methods

2.1. Study Area

The West Run Watershed (23 km^2) 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 cover [67]. 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 (USDA Web Soil Survey, accessed May 2020); Morgantown, West Virginia (elevation 315 m), 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 [67]. The West Virginia University Animal Husbandry Farm is in the upper reaches of one sub-catchment that supports grazing livestock. Vegetation in the catchment is predominantly cove hardwoods of various ages, including sugar maple (*Acer saccharum*), red maple (*A. rubrum*), and tulip poplar (*Liriodendron tulipifera*), and some isolated plantings of conifers including Douglas fir (*Pseudotsuga menziesii*), Norway spruce (*Picea abies*), and bald cypress (*Taxodium distichum*).

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). 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. The 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 the subsamples were stored at either 4 °C or −20 °C. Soil pH was measured using a 0.01 *M* $CaCl_2$ solution in a 1:2 slurry (5 g soil:10 mL $CaCl_2$). The samples were shaken for 1 h and allowed to settle for 1 h prior to reading pH (Hanna Instruments). Gravimetric soil moisture content was quantified by drying field-moist soils for 24 h at 105 °C. Soil organic matter (SOM) was quantified via combustion in a muffle furnace at 500 °C for 12 h via loss-on-ignition. To determine soil NO_3^- and NH_4^+ concentrations, 10 g of soil were extracted with 40 mL of 2 *M* KCl, followed by colorimetric analysis in clear 96-well plates. The NO_3^- concentration was determined using a 2:1:1 solution of vanadium chloride (VCl_3), 2% sulfanilamide, and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride (NEDD). The NH_4^+ concentration was determined using 150 µL sodium salicylate and 150 µL sodium hydroxide bleach solution. The samples were incubated at room temperature (~20 °C) for 50 min for color development and analyzed in quadruplicate. Concentration (ppm) was determined via absorbance on a plate reader (Synergy HTX plate reader, Biotech, Winooski, Vermont) at 540 nm for NO_3^- and 650 nm for NH_4^+. Standard curves were generated

using the known standards for each replicate plate under the same conditions. All the calculations were corrected for soil moisture content.

Potential soil N_2O flux was measured using a dissolved N_2O microsensor (Unisense, Germany) [68,69]. Into 40 mL glass vials, 10 g field-moist soils and 35 mL nutrient solution containing KNO_3 and glucose (Kellogg Biological Station) [70–72] were added. Following the same protocol, a control for each sample was also analyzed, with 35 mL nutrient solution and no soil. The vials were incubated at 22 °C and the N_2O (mg/mL) concentrations were recorded after 90 s of equilibration at 18, 24, and 30 h utilizing the Unisense Microsensor by placing the microsensor needle through the rubber septum of each vial. All the N_2O concentrations were corrected for background values by subtracting control from the sample values, and all N_2O concentrations were normalized to the weight of the soil sample dried at 105 °C.

2.3. Genetic Analysis of nosZ and nirK in Soil Samples

Total genomic DNA was extracted from 0.25 g field-moist soil samples using a DNeasy Powersoil Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. The final DNA concentrations were measured with a Qubit fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using 197 µL buffer and 3 µL extraction using a dsDNA HS Assay kit and the standard protocols. An end-point polymerase chain reaction (PCR) was run on four microbial functional genes (Tables 1 and 2) involved in N processing using an AmpliTaq Gold Master Mix (Thermo Fisher) for *nosZ* and *nirK* using reference strains *Alcaligenes faecalis* and *Paracoccus denitrificans*, respectively, as templates ([73,74]; NRRL, USDA). The PCR was performed in a 25 µL reaction volume using 12.5 µL master mix (2× concentration) (Thermo Fisher), 9.5 µL dH_2O, 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 min; Stage 2: 95 °C for 1 min, 55 °C for 30 s, and 72 °C for 30 s for 35 cycles; and Stage 3: 72 °C for 7 min and 4 °C hold. We ran the PCR with amoA-1F and amoA-2R and reference strain *Pseudomonas fluorecens* to amplify *amoA* [75,76]. We also ran the PCR using A189 and A682 primers for *amoA* using *Pseudomonas fluorecens* as a template according to [77] and [78]. We also followed procedures outlined in [79] using GenAOAF/GenAOAR and amoA1F/GenAOB2R primer sets, *Pseudomonas fluorescens* as a template, and touchdown PCR (52–64 °C) with both AmpliTaq Gold and GoTaq Green Master Mixes. The standard PCR was performed for *nirS* using nirS4F/nirS5R and Cd3aF/nirSR3cd* primer sets using *Pseudomonas stutzeri* as a template and the AmpliTaq Gold Master Mix [60,80,81].

Table 1. Mean values of the measured parameters across the low- and high-N sub-catchments. The parameters marked by an asterisk indicate significantly different values by location at $\alpha = 0.05$.

	Low N	**High N**
Soil NO_3^- (mg N/kg soil) *	15.66 (3.02)	43.01 (5.02)
Soil NH_4^+ (mg N/kg soil) *	4.03 (0.50)	6.49 (0.84)
Soil pH_{CaCl2}	4.41 (0.16)	4.27 (0.13)
Soil OM_{LOI} (%)	13.19 (0.67)	15.59 (1.08)
Soil moisture (%) *	28.75 (1.47)	24.49 (1.14)
nirK (10^{12} copy number)	5.64 (1.48)	3.65 (1.20)
nosZ (10^9 copy number)	5.78 (1.34)	2.61 (0.53)
N_2O production (mg/kg/30 h)	0.51 (0.08)	0.37 (0.07)

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 a Wizard Gel Purification kit (Promega, Madison, WI, USA). Subsequent cloning was performed using a TA Cloning kit pCR2.1. TOPO plasmids were 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 a Zyppy Wash kit (Thermo Fisher), and the plasmid

DNA concentration (ng/μL) was confirmed using a 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).

Table 2. Stepwise regression model parameters included to predict soil NO_3^-, NH_4^+, and potential N_2O production. The overall model adjusted R^2 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 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 soil)	Tree species	2.19	0.075	0.444	
	nosZ gene abundance	3.93			
	Soil pH	5.78	0.021		
	Soil OM	6.44	0.015		
Potential N_2O (mg N/kg 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		

Standard curves for *nirK* and *nosZ* were determined using the cloned products via real-time quantitative PCR (qPCR) following the protocol outlined in [82]; a 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 2× master mix, and 1 μL template using the following conditions: Stage 1: 95 °C for 5 min, 1×; Stage 2: 95 °C for 20 s and 60 °C for 45 s, 35×; and Stage 3: 95 °C for 15 s for 1×. The qPCR samples were run in triplicate. Using the same conditions, a qPCR was then run for each soil sample DNA extraction, and using the respective standard curve equations, the gene copy number was calculated [82]. All the calculations were corrected for soil moisture content so that gene copy numbers were 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 the 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 the 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 at the $\alpha = 0.05$ significance level. In addition to the nonparametric statistical analysis we performed, we used SAS-JMP v. 11.0 (SAS Institute, Cary, NC, USA) to develop a predictive model using backward stepwise regression to determine important variables for predicting measured NO_3^-, NH_4^+, and N_2O concentrations compared to environmental variables including soil pH, moisture, SOM, *nirK* gene abundance, *nosZ* gene abundance, mycorrhizal associations, tree species, and location (relatively high or low N). The statistical model determines which soil variables best predict the NO_3^-, NH_4^+, and N_2O concentrations. The conditions selected for stepwise regression included the minimum BIC stopping rule with whole effects for inclusion of

categorical data, and the parameters were included at $\alpha = 0.10$. Mean response values by tree species were separated using Tukey's HSD at $\alpha = 0.05$.

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, the results may not be extrapolated to other locations [83].

3. Results

3.1. Nitrate and Ammonium

The NO_3^- concentration was significantly higher ($p < 0.001$) in the high-N catchment compared to the low-N catchment (43.01 and 15.67 mg N/kg soil, respectively; $p < 0.001$; Table 1). Within the high-N catchment, tree species influenced the NO_3^- concentration in soils, as soil NO_3^- beneath American beech (49.59 mg N/kg soil) was significantly lower than that beneath sugar maple (61.35 mg N/kg soil) and significantly higher than that beneath American sycamore (23.41 mg N/kg soil) ($p = 0.029$; Figure 2) and the NO_3^- concentration for American sycamore and black cherry (16.10 mg N/kg soil) was significantly lower than for northern red oak (63.72 mg N/kg soil) and sugar maple (61.35 mg N/kg soil) ($p = 0.029$; Figure 2). In the low-N catchment, the NO_3^- concentration was not significantly different between tree species ($p = 0.140$; Figure 2), nor did the NO_3^- concentration vary significantly between the ECM and AM fungal associations (0.444; Figure 3). In the high-N catchment, the NO_3^- concentration was significantly higher in the soils beneath the ECM-associated species relative to the AM species (56.65 and 36.19 mg N/kg soil, respectively; $p = 0.023$; Figure 3).

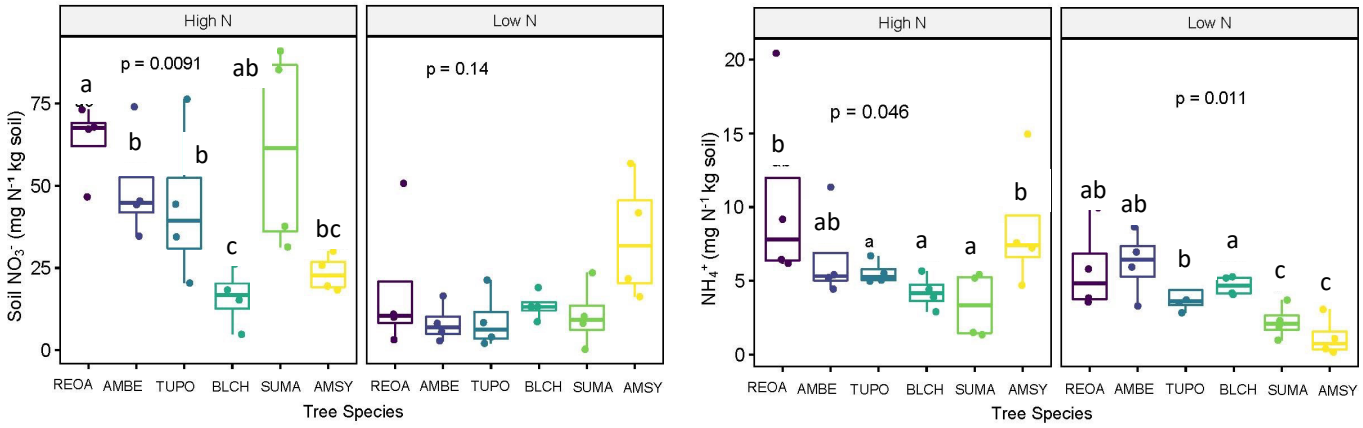

Figure 2. Soil nitrate and ammonium content in the high- and low-N catchments as influenced by tree species. The mean values marked with different letters indicate significant differences determined by Tukey's HSD mean separation at $\alpha = 0.05$.

The NH_4^+ concentration was also significantly greater in the soil from the high-N sub-catchment relative to the low-N catchment (6.49 and 4.03 mg N/kg soil, respectively; $p = 0.009$; Table 1). Tree species influenced soil NH_4^+ in both catchments. In the high-N catchment, the NH_4^+ concentration was significantly higher for northern red oak (10.56 mg N/kg soil) compared to black cherry (4.22 mg N/kg soil) and sugar maple (3.37 mg N/kg soil) ($p = 0.029$; Figure 2). In the low-N catchment, NH_4^+ was significantly lower in the soil beneath American sycamore (1.18 mg N/kg soil) compared to that beneath northern red oak (5.79 mg N/kg soil), American beech (6.21 mg N/kg soil), and black cherry (4.68 mg N/kg soil) and significantly lower for sugar maple (2.22 mg N/kg soil) compared to black cherry (4.68 mg N/kg soil) ($p = 0.029$; Figure 2). NH_4^+ was significantly higher in ECM compared to AM (6.00 and 3.04 mg N/kg soil, respectively) in the low-N catchment ($p = 0.01$; Figure 3), though NH_4^+ was not significantly different between the ECM- and AM-associated species in the high-N catchment ($p = 0.076$).

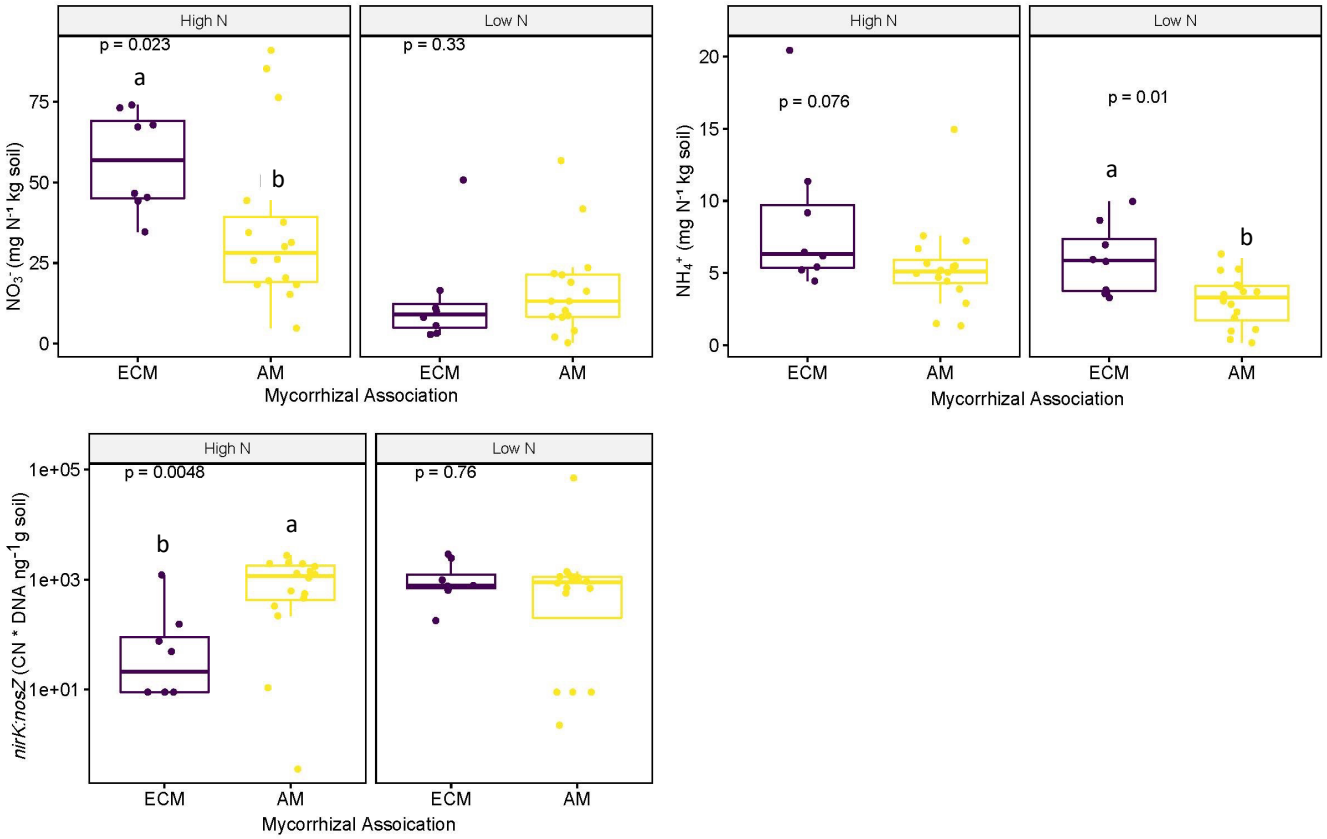

Figure 3. Soil nitrate and ammonium content and *nirK*:*nosZ* gene abundance ratio in the high- and low-N catchments as influenced by mycorrhizal fungal association (ECM or AM). The mean values marked with different letters indicate significant differences determined by Tukey's HSD mean separation at $\alpha = 0.05$.

3.2. Soil pH, Soil Organic Matter, and Soil Moisture

Soil pH was not significantly different (CI = 0.05) between the high- and low-N catchments ($p = 0.613$; Table 1). Tree species influenced soil pH within the high-N catchment and was lowest in the soil beneath black cherry (3.89) and highest for sugar maple (4.86) ($p = 0.029$; data not shown). Soil pH was not significantly different between tree species in the low-N catchment or between the ECM and AM fungal associations in either the high- or low-N catchment ($p = 0.50$ and $p = 0.759$, respectively).

The SOM content was similar between the high and low catchments ($p = 0.069$; Table 1). Within the high-N catchment, SOM was significantly higher in the soil beneath red oak relative to black cherry (18.66% and 9.89%, respectively; $p = 0.029$; data not shown). The SOM content did not differ between tree species in the low-N catchment ($p > 0.05$). SOM was not significantly different between the ECM and AM fungal associations in either the high- or low-N catchment ($p = 0.12$ and $p = 0.928$, respectively).

The soil moisture content was also similar between the high and low catchments ($p = 0.05$; Table 1). Within the low-N catchment, soil moisture was significantly higher in the soil beneath black cherry compared to sugar maple (0.35 and 0.23 g/g soil, respectively; $p = 0.029$). Soil moisture did not differ between tree species in the high-N catchment or between the ECM and AM fungal associations in either the high- or low-N catchment ($p = 0.417$ for both catchments).

3.3. Potential N_2O Production

The potential N_2O production after 30 h of incubation was similar between high- and low-N catchments ($p = 0.053$; Table 1). N_2O production was significantly lower in the soil beneath black cherry (0.11 mg N/kg soil) compared to American sycamore (0.66 mg N/kg soil) and northern red oak (0.59 mg N/kg soil) ($p = 0.029$; Figure 4) in the high-N catchment. Potential N_2O did not vary between tree species in the low-N catchment (Figure 4) or between the ECM and AM fungal associations in either the high- or low-N catchment ($p = 0.350$ and $p = 0.976$, respectively).

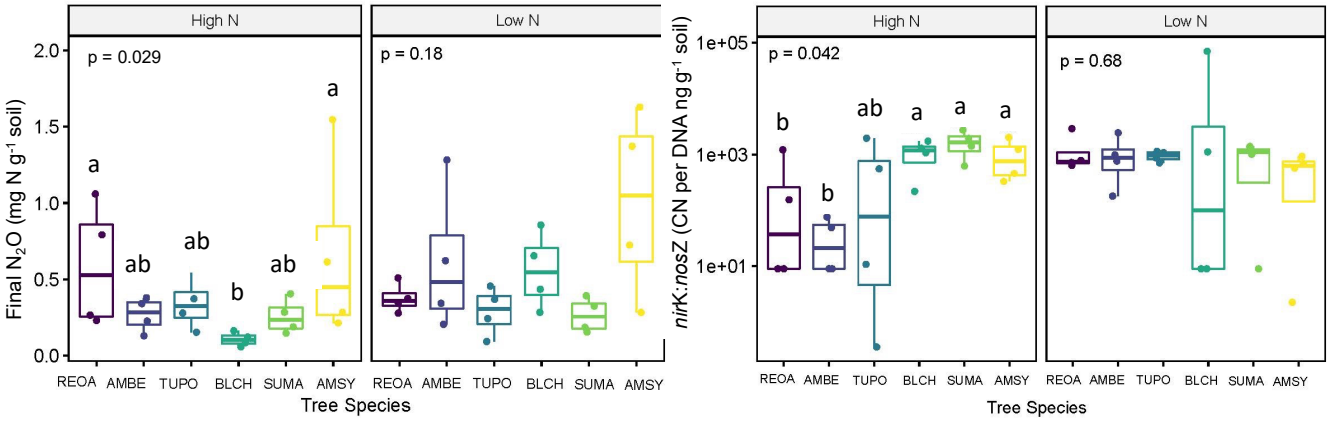

Figure 4. Soil nitrous oxide production and *nirK*:*nosZ* gene abundance ratio in the high- and low-N catchments as influenced by tree species. The mean values marked with different letters indicate significant differences determined by Tukey's HSD mean separation at $\alpha = 0.05$.

3.4. Target N Cycling Genes

Abundance of the *nosZ* gene (copy number) was similar between the high- and low-N catchments ($p = 0.112$; Table 1). 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 (4.96E+09 CN per ng DNA/g soil) compared to black cherry (1.29E+09 CN per ng DNA/g soil) ($p = 0.029$). Abundance of the *nosZ* gene did not vary between tree species in the low-N catchment or between the ECM and AM fungal associations in the high- or low-N catchments ($p = 0.192$; $p = 0.350$, respectively).

Abundance of the *nirK* gene was also similar between the high- and low-N catchments ($p = 0.103$; Table 1). In the high-N catchment, *nirK* abundance did vary by species ($p = 0.029$), where *nirK* was significantly lower in the soil beneath American beech (7.93E+10 CN per ng DNA/g soil) compared to American sycamore (6.27E+12 CN per ng DNA/g soil) and sugar maple (9.10E+12 CN per ng DNA/g soil) in the high-N catchment ($p = 0.029$). Abundance of *nirK* was not significantly different between tree species in the low-N catchment. Abundance of the *nirK* gene was significantly higher in the soil beneath the AM-associated trees compared to the ECM ones (4.97E+12 and 1.02E+12 CN per ng DNA/g soil, respectively) in the high catchment ($p = 0.011$). However, *nirK* abundance did not vary by the fungal association in the low-N catchment ($p = 0.610$).

The ratio of *nirK*:*nosZ* was not significantly different between the high- and low-N catchments ($p = 0.496$; Table 1). Within the high-N catchment, *nirK*:*nosZ* varied by tree species, where it was higher in the soil beneath American sycamore (1008.52 CN per ng DNA/g soil), sugar maple (1677.09 CN per ng DNA/g soil), and black cherry (1078.05 CN per ng DNA/g soil) compared to American beech (35.48 CN per ng DNA/g soil) ($p = 0.029$; Figure 3). The *nirK*:*nosZ* ratio did not differ between tree species in the low-N catchment (Figure 3). The *nirK*:*nosZ* ratio was significantly higher in the soil beneath the AM-associated trees (1098.06 CN per ng DNA/g soil) compared to the ECM ones (190.81 CN per ng DNA/g soil) in the high-N catchment, though the ratio did not vary by fungal association in the low-N catchment ($p = 0.005$ and $p = 0.783$, respectively; Figure 3).

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 the *nirS* and *amoA* gene abundances using qPCR was not achieved.

3.5. Relationships between Gene Abundance and Soil Parameters

Soil pH was positively correlated to the *nosZ* and *nirK* copy number ($p < 0.001$ and $p = 0.001$, respectively; Figure 5) across all the samples. Within the catchments, the *nosZ* copy number and soil pH were positively correlated in both the high- and low-N catchments ($p = 0.042$ and $p < 0.001$, respectively). Soil pH and *nirK* gene abundance were also positively correlated in the high-N catchment ($p < 0.001$), but this relationship did not occur in soil from the low-N catchment ($p = 0.250$).

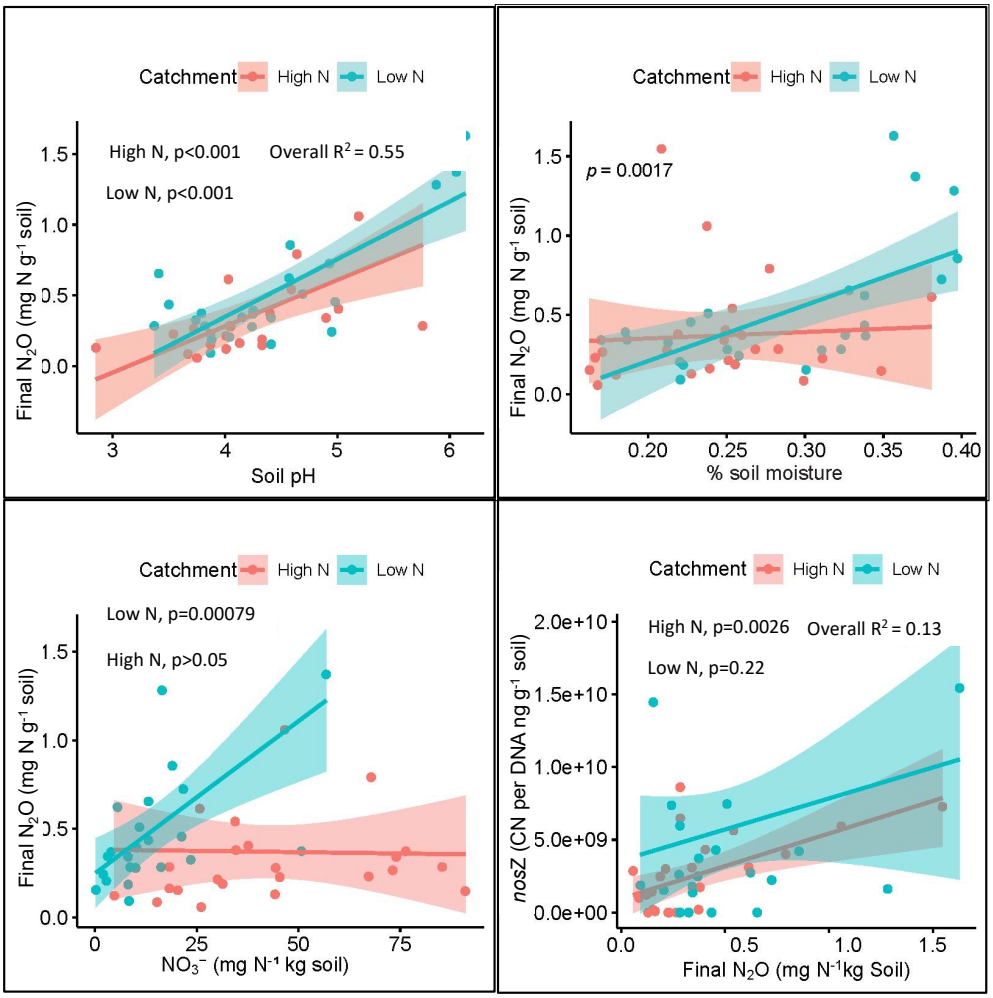

Figure 5. Correlative relationships between nitrous oxide production and soil pH, soil moisture, soil nitrate content, and *nosZ* gene abundance in the high- and low-N catchments.

There was a negative relationship between the NH_4^+ concentration and the *nosZ* copy number across all the samples ($p = 0.001$). The low-N catchment showed a greater *nosZ* copy number with lower levels of the NH_4^+ concentration compared to those in the high-N catchment ($p < 0.001$; $p = 0.230$). A significant positive correlation was noted between *nosZ* abundance and potential N_2O production across all the samples ($p = 0.011$). The high-N catchment displayed a significant positive relationship with the *nosZ* copy number and N_2O ($p = 0.003$; Figure 5). The *nosZ* copy number and N_2O did not display a significant relationship in the low-N catchment ($p = 0.220$; Figure 5). No significant relationship was found in *nosZ* and NO_3^- in the high- or low-N catchments.

The soil NH_4^+ concentration was negatively correlated to *nirK* abundance across all the samples ($p = 0.003$), largely driven by a strong, significant negative relationship in the low-N catchment ($p = 0.003$). Abundance of *nirK* was not related to increased potential N_2O production among all the samples ($p = 0.089$), though within the catchments, a significant positive relationship between *nirK* and N_2O was noted in the high-N catchment ($p = 0.045$). The soil NO_3^- concentration was unrelated to *nirK* gene abundance across all samples.

The *nirK*:*nosZ* ratio was not related to NO_3^-, NH_4^+, or N_2O in either the high- or low-N catchment. Within the high-N catchment, *nirK*:*nosZ* did exhibit a significant positive relationship with soil pH ($p = 0.002$) and a significant negative relationship with the soil NH_4^+ content ($p = 0.033$). These relationships were not present in the low-N catchment.

Within the low-N catchment, soil moisture and potential N_2O exhibited a strong significant positive relationship ($p < 0.001$; Figure 5), although this relationship was not present in the high-N catchment.

Soil pH was positively correlated to potential N_2O production and had a significant positive relationship across all the samples ($p < 0.001$; Figure 5) and from samples within both the low- and high-N catchments ($p < 0.001$ for both catchments). Soil pH was negatively correlated to the soil NH_4^+ content across all the samples ($p = 0.004$), and in the low-N catchment, a significant negative relationship between the NH_4^+ concentration and pH occurred ($p = 0.022$), and a significant positive relationship between soil pH and NO_3^- was noted in the low-N catchment samples ($p = 0.033$). Potential N_2O production and the soil NO_3^- content were not related across all the samples, but a significant positive relationship was noted in the low-N catchment ($p < 0.001$; Figure 5). No relationship occurred between potential N_2O and the soil NH_4^+ content among or within the high- and low-N catchments ($p = 0.1$).

From the stepwise regression analysis including all the predictive variables, we found that the strongest predictor of soil NO_3^- in our data was location (high N/low N), followed by soil moisture ($p < 0.001$ and $= 0.009$, respectively; Table 2). Tree species was also an important factor for soil NO_3^- ($p = 0.033$); overall, the greatest soil NO_3^- concentration was noted in the soil beneath oak, and the lowest NO_3^- was found beneath cherry (Table 3). The strongest predictor of soil NH_4^+ was the SOM content, followed by soil pH (Table 2; $p = 0.015$ and 0.021, respectively). Abundance of the *nosZ* gene and tree species were also important in predicting soil NH_4^+. The greatest soil NH_4^+ was, again, found in the soil beneath oak, the lowest—beneath cherry (Table 3). The strongest predictor of potential N_2O production was soil pH (increasing pH resulted in a greater N_2O), followed by the SOM content (Table 2; $p < 0.001$ 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 the soils beneath sycamore and the least N_2O was produced in the soil beneath sugar maple (Table 3).

Table 3. Mean response of the measured soil NO_3^-, NH_4^+, and potential N_2O production by tree species across both catchments as significant parameters in predictive regression models. Significant differences in the mean response for each variable are noted by different letters (Tukey's HSD $\alpha < 0.05$).

Predicted Dependent Variable	Tree Species	Mean Response
Soil NO_3^- (mg N/kg soil)	Sycamore (AMSY)	28.77 ^{ab}
	Cherry (BLCH)	14.79 ^{b}
	Oak (REOA)	41.21 ^{a}
	Beech (AMBE)	28.91 ^{ab}
	Poplar (TUPO)	26.41 ^{ab}
	Sugar maple (SUMA)	35.94 ^{ab}

Table 3. *Cont.*

Predicted Dependent Variable	Tree Species	Mean Response
Soil NH_4^+ (mg N/kg soil)	Sycamore (AMSY)	4.90 ^{ab}
	Cherry (BLCH)	4.45 ^{b}
	Oak (REOA)	8.17 ^{a}
	Beech (AMBE)	6.40 ^{ab}
	Poplar (TUPO)	4.83 ^{ab}
	Sugar maple (SUMA)	2.79 ^{ab}
Potential N_2O (mg N/kg soil/30 h)	Sycamore (AMSY)	0.83 ^{a}
	Cherry (BLCH)	0.33 ^{ab}
	Oak (REOA)	0.48 ^{ab}
	Beech (AMBE)	0.44 ^{ab}
	Poplar (TUPO)	0.31 ^{ab}
	Sugar maple (SUMA)	0.26 ^{b}

4. Discussion

N fertilization and deposition contribute to pollution of ecosystems, pose threats to water and air quality [84,85], contribute to global climate change [10], and are of special consideration in ecosystem management and stream remediation efforts [86]. Our objective was to investigate relationships between the abundance of microbial functional genes critical to key N transformations and soil N process rates in soils beneath six tree species in stream catchments containing relatively high and low N inputs. Given the differences in N inputs between our high- and low-N sampling locations, differences in N cycling were expected. Soil NO_3^- and NH_4^+ content were greater in the high-N catchment (Table 1), likely resulting from additional N influx from fertilizer and manure runoff from agricultural operations in the upper reaches of the sub-catchment. This is consistent with previous studies in the West Run Watershed. Martin et al. (2021) [87] found a positive association with stream NO_3^- concentrations in the West Run Watershed areas impacted by agriculture [67,87].

Although we found no significant differences in soil pH, soil organic matter content, 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 the high- and low-N catchments between tree species and mycorrhizal association. Furthermore, these differences were not consistent between the catchments. We predicted that the soil microbial functional gene abundance would be significantly different between tree species, specifically related to mycorrhizal association, in that the soil beneath the AM-associated tree species (*L. tulipifera*, *P. serotina*, *A. saccharum*, *P. occidentalis*) would contain a greater abundance of *nirK* compared to *nosZ* relative to the ECM-associated tree species (*Q. rubra* and *F. grandifolia*). The NO_3^- concentration was significantly higher beneath the ECM-associated tree species in the high-N catchment (Figure 3) and NH_4^+ was significantly higher beneath the ECM species in the low-N catchment (Figure 3). We expected that the NO_3^- and NH_4^+ concentrations would be higher beneath the 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 [39].

Our findings may reflect slower 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 likely process inorganic N in the low-N catchment so that it does not accumulate in the soil as it would with N enrichment [39]. Elevated NH_4^+ in the soil beneath ECM-associated tree species in the low-N catchment (Figure 3) may be attributable to the same mechanism, in that ECM-associated tree species typically promote soil with less NO_3^- leaching and low inorganic N availability [39]. 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 (Figure 2). Similarly, in the high-N catchment, the NH_4^+ concentration was significantly higher in the soil beneath ECM-associated northern red oak compared to black cherry and sugar maple (Figure 2). A metagenomic study which compared the abundance of genes associated with the key N pathways as influenced by three tree species indicated that the soil microbiome below ECM-associated American chestnut contained the lowest abundances of the *amoA* functional gene, which encodes enzymes involved in nitrification, and of the functional genes *nirK*, *norB*, and *nosZ*, which encode enzymes involved in the denitrification pathway. In the dissimilatory nitrate reduction to ammonium pathway, chestnut soils contained the lowest gene abundance of nitrate reductase genes *narJ* and *napA* as well as nitrite reductase genes *nrfA* and *nrfH* [88]. The measured process rates from incubated soil and litter reflected the low functional gene abundances in the chestnut soils, as N mineralization was lowest in the chestnut soil (7.84 mg N/kg) relative to that of AM-associated cherry (11.51) and ECM-associated oak soils (12.02). Indeed, functional gene abundances were significantly correlated to N mineralization rates and inorganic N availability in that study.

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, the soil beneath black cherry had the lowest pH, NO_3^- concentration, potential N_2O production, and OM in the high-N catchment (Figures 2 and 3). 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 [89]. 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 [90,91].

Abundance of the *nirK* gene in the high-N catchment was significantly higher in the soil beneath sugar maple compared to those beneath American beech and American sycamore. This may be related to faster N mineralization and nitrification relative to neighboring tree species [40]. Likewise, the high *nirK*:*nosZ* ratio for black cherry, sugar maple, and American sycamore compared to American beech in this catchment may be attributed to the influence of the mycorrhizal fungal association. This is consistent with the hypothesis that AM soils would 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 a greater access to NH_4^+ pools in the high-N catchment [49]. In the low-N catchment, *nirK*, *nosZ*, and *nirK*:*nosZ* did not differ between tree species, perhaps due to the similar pH across the catchment [92].

4.1. Relationships of Environmental Variables to Gene Abundance

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 by oxidizing NH_4^+ compounds [93]. Although soil pH was not distinct between the high- and low-N catchments (Table 1), N enrichment likely contributed to species-specific changes in soil pH that appear to influence other variables in each catchment. Significantly positive correlations with soil pH were displayed by *nirK*, *nosZ*, *nirK*:*nosZ* copy number, and potential N_2O production (e.g., Figure 5). On the other hand, NH_4^+ displayed a significant negative correlation with soil pH, likely due to ammonification causing soil acidification and potentially contributing to altered functionality of *nirK* and *nosZ* [94,95]. Several studies have found altered transcription processes encoding *nirK* and *nosZ* due to low pH in pure culture and soil [21,92,96]. Liu et al. (2014) [21] documented N_2O reductase inhibition (*nosZ*) at pH < 6.1. This reduction in functionality of *nirK* and *nosZ* may contribute to differences in N cycle rates. Reduced *nirK* functionality results in lower nitrate reductase, and the reduction of *nosZ* functionality has been linked to incomplete reduction of N_2O to N_2 gas, especially in high N conditions with lowered pH [92]. Soil pH

and N_2O also displayed a significant positive relationship in our results, possibly due to greater functionality of *nirK* in higher-pH soils (Figure 5).

Inconsistent with our hypothesis, N_2O production increased with increasing *nosZ* abundance (Figure 5), 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 beneath AM-associated tree species due to high N conditions and a greater access to NH_4^+ pools. N_2O and *nirK* did exhibit a positive relationship, 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 any alterations in functionality of nitrous oxide reduction by *nosZ* [16,52,92].

Lower cumulative N_2O production is further related to the soil moisture content, as evidenced by the significant positive relationship between relative soil moisture and the final N_2O concentration (Figure 5). This is supported by the hole-in-pipe mechanism of production where wetter soil contributes to more N_2O [16,52]. In this study, potential N_2O production was not related to either NO_3^- or NH_4^+ in either high- or low-N catchment, although N_2O and NO_3^- showed a slight positive relationship 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*. The *nirK*:*nosZ* ratio was unrelated to N_2O production among the samples or within the catchments.

4.2. Limitations and Future Directions

A potential limitation of this study is the sample size. We collected and processed 48 soil samples in total, 24 from the high-N catchment and 24 from the low-N catchment. Within the catchments, we sampled beneath 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 a higher analytical power to discern more subtle effects. Additionally, understory vegetation may have contributed to the variance in our data; other studies have shown that understory plants influence forest N cycling [89,97]. It is difficult to separate the influence of understory vegetation, soil conditions, and tree species because they are closely associated in a field setting.

Additionally, had our attempts in *nirS* and *amoA* quantification been successful, we may have identified stronger links with the NO_3^- and NH_4^+ concentrations and implications of nitrification and denitrification rates [15,76,98]. A more comprehensive soil metagenomic analysis may lead to a more comprehensive understanding of the N-related genes in our study [99].

4.3. Limitations of Quantifying Gene Abundance

Quantitative PCR measures the presence of a gene, but it does not measure the function or turnover. Thus, the copy numbers of *nosZ* and *nirK* measured here may include genes that are not currently active [100]. With gene abundance measures, we may be underestimating or overestimating their influence on NO_3^- and N_2O production. This may explain the lack of congruence between gene abundance values and the NH_4^+, NO_3^-, or N_2O concentration [100]. 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.

5. Conclusions

We determined that tree species influence NO_3^- and N_2O production in riparian soils, particularly under high N enrichment. Soil acidification, likely from elevated NH_4^+, is related to decreasing *nirK* and *nosZ* gene abundance and potentially to reduced functionality of these genes. However, a weak relationship occurred between gene abundance and N process rates. The mycorrhizal association was not a consistent predictor of NO_3^- or N_2O production, with AM-associated black cherry driving lower than expected AM-associated NO_3^- and N_2O. ECM-associated tree species appear to be retaining more soil 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 BMPs to improve N retention in riparian buffer zones. The strongest predictors of soil NO_3^- and N_2O across study sites were N enrichment and pH, respectively. Quantitative PCR is useful for quantifying the presence of genes but 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.

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