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# Linking microbial communities, functional genes and nitrogen-cycling processes in forest floors under four tree species

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**Abstract**

Tree species can influence rates of soil N transformations, but the question remains whether differences in N cycling rates are mirrored by the abundance of relevant functional genes. We studied whether the influence of tree species on soil N transformation processes and abundance of functional genes exist across two sites in British Columbia with different N availability. We used the $^{15}N$ pool-dilution method to estimate gross rates of ammonification and nitrification in forest floors of four conifers in a common garden experiment. The abundances of bacteria, fungi, nitrification (AOA *amoA*, AOB *amoA*) and denitrification (*nirS*, *nirK*) genes were determined by qPCR. Western red cedar (*Thuja plicata)* had the highest rates of gross ammonification and $NH_4^+$ consumption, followed by Sitka spruce (*Picea sitchensis),* hemlock (*Tsuga heterophylla),* and Douglas-fir (*Pseudotsuga menziesii)*; all species showed net nitrate immobilization. Western red cedar forest floors had the greatest abundance of bacterial *16S* genes and ammonia-oxidizing archaea *amoA* genes. This suggests that tree species foster different abundances of ammonification and denitrification functional groups. Differences in N transformation rates between the sites were related to site N status, as reflected in C:N ratios of the forest floor and microbial biomass, and were more closely tied to rates of N consumption rather than gross mineralization. Rates of most N transformation processes were related to microbial C:N ratio, indicating that the N status of microbes rather than their biomass or activity level determined the rates of N cycling. Ammonification rates were associated with forest floor and microbial biomass C:N ratio as well as bacterial and fungal abundances. Nitrification rates and denitrification gene abundance were associated with microbial biomass C:N ratios and AOA *amoA* gene abundance. The forest floor's genetic potential for denitrification was positively correlated with its nitrification potential as indicated by ammonia-oxidizer abundance. We conclude that tree species influenced forest floor N cycling and soil microbial gene abundances, and that functional genetics can be useful for exploring mechanistic links between tree species and nitrogen cycling processes.

**Keywords:** Ammonia oxidizers, nitrifiers, $^{15}N$ pool dilution, tree species effects, nitrogen cycling, forest floors

## 1. Introduction

Nitrogen (N) availability is often the primary limitation on ecosystem productivity in conifer forests (Vitousek et al., 1997, 2002; LeBauer and Treseder, 2008), and factors that regulate N availability have far-reaching consequences for ecosystem properties such as microbial activity, biogeochemical cycling, carbon (C) sequestration, aboveground biomass production and greenhouse gas (GHG) fluxes. Soil N transformation processes (e.g., mineralization of organic N to $NH_4^+$ and nitrification of $NH_4^+$ to $NO_3^-$) are largely mediated by interactions between functional communities of soil microorganisms and their environment (Bengtsson et al., 2003; Balser and Firestone, 2005; Högberg et al., 2013). The balance between gross and net rates of mineralization and nitrification can indicate turnover and immobilization rates of $NH_4^+$ and $NO_3^-$, respectively, which can be rapid in forest soil (Davidson et al., 1992; Stark and Hart, 1997). Rates of mineralization and nitrification have further implications for N loss through leaching of $NO_3^-$ and denitrification to $N_2O$ (Bengtsson et al., 2003; Szukics et al., 2010).

Quantification of marker genes for biogeochemical functions can be used to characterize N cycling processes and communities (Levy-Booth et al., 2014). Ammonia-oxidizing archaea (AOA) and bacteria (AOB) carry out the first, and potentially rate-limiting, step of nitrification. AOA and AOB abundance can be quantified using the ammonia-monooxygenase (*amoA*) gene (Rotthauwe et al., 1997; Francis et al., 2005; Leininger et al., 2006; Szukics et al., 2010). While AOB have long been thought to dominate ammonia-oxidation, the quantitative importance of AOA in soil has recently been recognized (Leininger et al., 2006; Petersen et al., 2012), and AOA are generally more abundant than AOB in acidic forest soil (Petersen et al., 2012). The relative contributions of AOA and AOB to gross and net nitrification in forests with different tree species remain to be seen. Denitrification is a biological pathway through which N returns to the atmosphere from soil or water by the reduction of nitrate to nitrous oxide (Henry et al., 2004) via the nitrite reductase enzyme genes *nirS* and *nirK*. Genes that encode enzymes involved in biogeochemical cycling can be used to compare measured N mineralization and nitrification rates with bacterial, fungal, nitrifier and denitrifier population sizes (inferred from gene abundances) and in so doing, link N-cycling pathways to functional microbial groups.

Tree species have been shown to influence chemical and biological properties of soil, particularly forest floor layers, and rates of processes therein. Tree species influence soils

directly via leaf litter inputs and formation of forest floors (Hobbie et al., 2006; Vesterdal et al., 2012), and via root litter inputs and alteration of soil structure. Tree species also influence rates of litter decomposition, nutrient release, C turnover and soil respiration through differences in foliar N, Ca, Mg and lignin concentrations (Hobbie et al., 2006; Vesterdal et al., 2012, 2013). Several studies have reported differences in rates of N-cycling processes in soils under different tree species (e.g., Ste-Marie and Paré, 1999; Malchair and Carnol, 2009; Christiansen et al., 2010), and distinct microbial communities have also been reported in soils and forest floors under different tree species (Leckie et al., 2005; Prescott and Grayston, 2014). However, despite decades of research on this question, categorization of species according to their influence of soil properties has proven elusive, as tree species effects have been inconsistent among studies, and even at different sites within a single study. For example, in a common garden experiment on Vancouver Island, the indirect influence of site factors (particularly slope position) on rates of net N mineralization and nitrification in the forest floors appeared to overwhelm the influence of tree species, with appreciable net rates detected only at the valley bottom sites where the forest floor C:N ratio was less than 35 (Prescott et al., 2000a). Likewise, Gurmesa et al. (2013) found an influence of broadleaved tree species on soil carbon only at relatively rich sites, which they attributed to the lack of earthworms at the infertile sites, regardless of tree species. These observations prompted Prescott and Vesterdal (2013) to propose that the expression of tree species influence on soils is context-dependent, and more likely to be detectable on rich or intermediate sites.

In the common garden experiment on Vancouver Island, previous studies of the forest floors discerned some distinct characteristics of the forest floors under four coniferous tree species, which have been summarized by Prescott and Vesterdal (2005). Western red cedar forest floors had the lowest rates of litter decomposition and net N mineralization, but the highest proportion of nitrate and the highest bacterial:fungal ratio of the four species. Western hemlock forest floors had low pH and low Ca concentrations, low bacteria:fungal ratios, and were dominated by $NH_4^+$ rather than nitrate. Sitka spruce forest floors had intermediate to high concentrations of N, P, Ca, and K, low bacterial:fungal ratios, and moderate rates of net N mineralization. Finally, Douglas-fir forest floors had intermediate pH and Ca, high bacterial:fungal ratios, high N concentrations and rates of net N mineralization and nitrification.

In this study, we revisit the common garden experiment on Vancouver Island and compare the forest floors that have developed under four tree species at one nutrient-rich, valley-bottom site and one nutrient-poor, mid-slope site. We examine differences in forest floors among tree species and the extent to which these differ according to the site. We measure rates of N mineralization and nitrification using $^{15}N$ pool-dilution and apply quantitative PCR of microbial genes involved in N-cycling processes to explore interactions among microbial communities (nitrifiers and denitrifiers) and rates of N-cycling processes.

## 2. Materials and Methods

### *2.1 Study location*

We sampled two sites, as case studies rather than replicates, from the EP571 common garden experiment on Vancouver Island: San Juan (48°35′N, 124°12′W) and Fairy Lake (48°35′N, 124°19′W), both located near Port Renfrew. The two sites were comparable in terms of elevation (65-85 and 75-85 m, respectively), and were within the Sub-montane Very Wet Maritime Coastal Western Hemlock variant (Prescott et al., 2000a). San Juan is a valley-bottom site with understory largely composed of *Rubus spectabilis* Pursh (salmonberry) and *Polystichum munitum* (Kaulf.) Presl. (swordfern), while Fairy Lake is a mid-slope site with understory dominated by *Gaultheria shallon* Pursh (salal) and *Vaccinium parvifolium* Smith (red huckleberry). Significantly higher N mineralization and nitrification rates and concentrations of P and K were measured in forest floors at the San Juan site compared with the Fairy Lake site (Prescott et al. 2000a), which were related to the differences in slope position and understory vegetation. We selected these two study sites because of their contrast in terms of N cycling, to enable us to test whether species effects on gross N cycling and functional genes would be consistent across contrasting sites.

Replicate plots of each species (western red cedar, western hemlock, Douglas-fir, and Sitka spruce) were planted in 1961, as a part of Experimental Project No. 571. The previous forest cover of western hemlock, western red cedar, amabilis fir, and Sitka spruce had been clear-cut and slash burned. The full experimental design contained 24 0.07-ha plots at each site, with 81 tree seedlings planted in three densities (2.7, 3.7, and 4.7 m); for the current study we sampled the two densest (2.7-m spacing) plots of each species. We used this pre-existing experimental design to determine if tree species differ in their dominant nutrient cycling characteristics

between N-rich and N-poor sites.

*2.2. Soil sampling and soil physical and chemical analyses*

We focused on the F-layer of the forest floor. This is the layer in which the greatest differences in soil microbial communities of these tree species have previously been found (Grayston and Prescott, 2005), and where soil fungi and fauna are expected to be most abundant and active (Kurbatova et al., 2009). It also ensured that we were comparing the influence of tree species on microbial communities at the same stage of decay, as recommended by Prescott and Grayston (2013).

Composite samples were collected from nine randomly selected 450 $cm^2$ samples of the F-layer in each plot in June of 2014. Brockett et al. (2012) showed that composite samples provide similar results to individually analyzed samples for plot-level values, albeit at a loss of information regarding spatial heterogeneity. Each composite forest floor sample was passed through a 2-mm mesh sieve and one 15-g subsample was immediately removed for the $^{15}N$ pool-dilution analysis. The remaining bulk sample was stored at -20° C for DNA extraction. For pH analysis, a 5-g field-moist subsample of forest floor was added to a small jar with 20 mL distilled water, shaken for 30 minutes then measured with a pH meter. A subsample of field-moist forest floor was also removed for C and N analysis. These samples were oven dried to 70°C for 48 h, ground using a mortar and pestle, and 5 mg weighed into tin capsules and analyzed for C and N by high-temperature flash combustion using a Vario EL Cube elemental analyzer (Elementar Americas Inc., Mount Laurel, NJ).

*2.3 Gross rates of ammonification and nitrification*

The $^{15}N$ pool-dilution method (modified from Drury, 2008) was used to determine gross rates of ammonification and nitrification, with samples analyzed in triplicate. Six 15 g subsamples from each plot were passed through a 2-mm mesh sieve and transferred to 500 mL glass Mason jars and sealed with parafilm (n = 3 subsamples for both $^{15}NH_4^+$ and $^{15}NO_3^-$). The parafilm seal was punctured to enable gas exchange and maintain aerobic conditions. Samples were incubated in the dark at room temperature for 24 hours prior to initial $^{15}N$ treatments. These treatments consisted of either: 4 mL of $^{15}NH_4Cl$ solution (99 atom%; Cambridge Isotope Laboratories) or 4 mL of $K^{15}NO_3$ (99 atom%; Cambridge Isotope Laboratories) added to the

187 forest floor samples in each respective jar, which was an equivalent application rate of 12 μg N
188 $g^{-1}$ forest floor. Labeled N was injected into the samples in 1 mL intervals four times over one
189 minute, and gently homogenized to ensure isotopic labeled N was applied uniformly throughout
190 the forest floor sample, and the parafilm seal was replaced.
191 i) Immediately after $^{15}N$ addition and ii) 24 hours after $^{15}N$ addition, a 5-g forest floor
192 subsample was removed from each Mason jar, this subsample was added to 100 mL of 2.0 M
193 KCl, shaken for 1 hour, and filtered through glass fiber filter paper (Fisher Inc.). 10 mL of the
194 KCl extract were analyzed for $NH_4$-N and $NO_3$-N using a flow injection analyzer (Lachat;
195 Quickchem 8000) at the Environmental Engineering Department at the University of British
196 Columbia. The remainders of the extracts were used for microdiffusion of $^{15}NH_4$-N and $^{15}NO_3$-N
197 with the use of acid traps, and the sequential addition of MgO and Devarda's alloy according to
198 the protocol developed by the International Atomic Energy Agency (IAEA, 2001). Acid traps
199 were dried, packaged in tin cups, and sent to the University of Saskatchewan Isotope Laboratory
200 for stable isotope ratio analysis using a Costech ECS4010 elemental analyzer coupled to a Delta
201 V mass spectrometer with a Conflo IV interface. Net rates of ammonification were calculated as
202 the difference in $NH_4^+$ between the incubated samples and the initial soil extractions at the start
203 of the incubation. Net nitrification was calculated as the difference in $NO_3^-$ between the initial
204 and incubated samples. The gross rates of ammonification, nitrification and microbial
205 consumption were calculated following Hart et al. (1994).
206
207 *2.4 Microbial biomass determination*
208 Microbial biomass nitrogen (MBN) and microbial biomass carbon (MBC) were
209 determined using a modified chloroform-fumigation extraction (Brookes et al., 1985). Briefly,
210 100 mL of 2 M KCl was used in lieu of 40 mL of 0.5 M $K_2SO_4$ for extractions (Verchot et al.,
211 1999). 20 mL extracts were analyzed for total organic C (TOC) and total N (TN) on a Shimadzu
212 5000A TOC analyzer at the Analytical Services Laboratory of the University of Alberta. The
213 additional remaining extractant was used for acid diffusion traps, identical to the above protocol
214 for the $^{15}N$ pool-dilution method. To determine if microbial communities were immobilizing N
215 during the course of the incubation we assessed MBN and MBC in $^{15}N$-$NH_4$ and $^{15}N$-$NO_3$ added
216 samples at the end of the pool-dilution experiment, as well as from unfertilized soil samples.
217

*2.5 DNA isolation and quantitative PCR* DNA was extracted from 0.1 g of field-moist forest floor using the MoBio Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA). DNA quality and concentration was measured using a nanodrop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and electrophoresis in agarose gels (1% w/v in TAE), then stored at -20ºC prior to amplification.

Quantitative PCR (qPCR) was used to quantify gene copy numbers. Reactions were carried out with an Applied Biosystems® StepOnePlus™ real-time PCR system. Each 20 µl reaction contained 10 µl of SybrGreen (2x) PCR Master Mix (Life Technologies Corp., Carlsbad, CA, USA), 0.5 µl of each primer, 250 ng $\mu l^{-1}$ bovine serum albumin (BSA), and 1 µl of DNA template. Table 1 shows primer sequences for qPCR assays. PCR conditions for AOA *amoA*, AOB *amoA* were 10 min at 95ºC, followed by 40 cycles of 95ºC for 30 s, 30 s at 57ºC, and 72ºC for 1 min, and 10 s at 80ºC. Triplicate 10x standard curves ranged from $10^2$ to $10^7$ copy numbers of AOA and AOB *amoA* in linearized plasmids. PCR conditions for *nirK* and *nirS* were 10 min at 95 ºC and 40 cycles of 95 ºC for 1 min, 60 ºC for 1 min and 72 ºC for 1 min, with fluorescence quantified at extension (Levy-Booth and Winder, 2010). The standard curves for *nirS* and *nirK* used a triplicate 10-fold serial dilutions of $10^1$ to $10^7$ gene copies from *Pseudomonas aeruginosa* (ATCC 47085) and *Pseudomonas chlororaphis* (ATCC 13985) genomic DNA, respectively. Bacterial 16S rRNA PCR conditions were 5 min at 95ºC, followed by 40 cycles of 95ºC for 30 s, 30 s at 57ºC, and 72ºC for 1 min, and 10 s at 80ºC. Florescence quantification occurred during annealing. Triplicate standard curves were run using a 10x dilution of $10^2$ to $10^7$ amplified 16S rRNA in linearized plasmids. PCR conditions for fungal ITS were 10 min at 95ºC, followed by 40 cycles of 95ºC for 1 min, 30 s at 53ºC, 50 s at 72ºC and 10 s at 80ºC. Fluorescence was read at 80ºC to reduce the formation of non-target and primer self-complementation structures. Triplicate standard curves for fungal ITS quantification were constructed using 10x dilutions between $10^3$ to $10^9$ ITS copies amplified from soil and *Aspergillus citrisporus* genomic DNA. Standard curve ranges are indicative of lower and upper limits of detection, respectively. All qPCR analyses were run in duplicate.

*2.6 Statistical analyses*

At each site one composite forest floor sample from the two replicate plots for each tree species was sampled (n=2). The influences of tree species and sites on forest floor chemistry,

249 microbial biomass C and N, process rates and gene abundances were evaluated using two-way
250 permutational analysis of variance (with 999 permutations). Permutational analysis of variance
251 was used to address the low sample sizes in this study (Andersen and Legendre, 1999) with tree
252 species and site as fixed effects. Microbial gene abundances were $\log_{10}$ transformed prior to two-
253 way permutational analysis of variance (with 999 permutations). We used multiple linear
254 regressions to determine which environmental variables were the best predictors of N
255 transformations on standardized data (*decostand* function in the *vegan* package in R (Oksanen et
256 al., 2013)). Rates of N transformations, including gross and net ammonification and nitrification,
257 were related to all potentially regulating parameters using multiple linear regressions, following
258 the removal of collinear variables. Model selection was based on stepwise variable selection with
259 Akaike's Information Criterion (AIC), whereby the lowest value indicates the model with the
260 highest explanatory power.
261 Redundancy analysis (RDA), a form of constrained ordination that determines how much
262 of the variation from one set of variables explains the variation in another set of variables, was
263 performed to test relationships between site and forest floor parameters and gene abundances
264 (*vegan* package in R ). Forward selection of explanatory variables for RDA was carried out using
265 the *packfor* function using the method recommended by Blanchet et al. (2008). The significance
266 of the RDA model and its individual terms were calculated using Monte-Carlo tests with 10000
267 permutations. All analyses were conducted with R v. 2.15.3 (R Core Team, 2013).
268
269 **3. Results**
270 *3.1 Forest floor pH, C and N*
271 There were no significant differences in pH, although Sitka spruce and western red cedar
272 had the highest average pH values (Table 2). Concentrations of total C and N also did not
273 significantly differ between forest floors under the four tree species or at the two sites (Table 3).
274 However, forest floor C:N ratio was significantly lower ($p = 0.02$) at the San Juan (nutrient-rich)
275 site (36.5 ± 1.1) than at Fairy Lake (nutrient-poor) (42.2 ± 1.5). C:N ratio also differed among
276 tree species, with hemlock forest floors having significantly higher C:N ratio (43.3 ± 2.3) than
277 Sitka spruce (36.4 ± 2.1; $p = 0.018$) and Douglas-fir (36.6 ± 1.2; $p = 0.015$). There were no
278 significant species-by-site interaction effects, indicating consistent tree species effects on C:N
279 ratios across sites.

*3.2 Microbial biomass C and N*

Microbial biomass C (MBC) differed among tree species across both sites and was significantly lower in forest floors at San Juan than Fairy Lake (Figure 1, Table 3). Significant species-by-site interaction indicated that influences of tree species on MBC differed between the two sites ($p = 0.03$). At Fairy Lake, Sitka spruce had lower MBC than other species whereas cedar had lower MBC than other tree species at San Juan (Figure 1). Microbial biomass N (MBN) did not differ significantly between sites ($p=0.89$) or among tree species across sites (Table 3, $p = 0.39$), but at San Juan, Douglas-fir had higher MBN than other species (Figure 1). Microbial C:N ratios did not differ significantly between sites or tree species, although there was a tendency for lower microbial C:N at San Juan (Figure 1, $p = 0.10$).

*3.3 Microbial gene abundance*

Bacteria *16S* rRNA gene abundance was significantly greater in forest floors from San Juan than from Fairy Lake (Table 3, $p = 0.01$), and differed significantly among tree species (Table 3, $p = 0.05$) with no species-by-site interaction. Bacterial genes were more abundant in forest floors of western red cedar at both sites, with hemlock or spruce having the lowest abundance depending on the site (Figure 2). Fungal *ITS* abundance did not differ between sites ($p = 0.55$) or tree species ($p = 0.89$). AOA *amoA* had the most pronounced site effect of any of the microbial genes quantified in this study, with San Juan having several orders of magnitude more AOA *amoA* genes than Fairy Lake ($p = 0.01$), but no species effects. AOB *amoA* genes were more abundant at San Juan than at Fairy Lake, but within the same order of magnitude ($p=0.10$). The abundance of the Cu-nitrite reductase *(nirK)* gene did not differ by site ($p = 0.19$), or tree species ($p = 0.13$), although hemlock tended to have the lowest gene abundance. In contrast, Cd-nitrite reductase (*nirS*) genes were significantly more abundant in forest floor at San Juan than at Fairy Lake ($p = 0.02$), but did not differ by tree species ($p = 0.27$).

*3.4 Gross and net N ammonification and nitrification rates*

Gross ammonification rates were significantly higher in forest floors at San Juan than at Fairy Lake (Figure 3a), with mean rates of 32.9 and 19.4 mg N $g^{-1}$ soil (dw) $day^{-1}$, respectively. A highly significant trees species effect on gross ammonification was observed with gross

ammonification under western red cedar being significantly greater than under Douglas-fir ($p$ = 0.03), hemlock ($p$ = 0.006) and Sitka spruce ($p$ = 0.03). Gross ammonium ($NH_4^+$) consumption was significantly higher in forest floors from San Juan than Fairy Lake ($p$ = 0.002), and was significantly greater in forest floors under cedar than the other species (Figure 3b). Net ammonification rates were mostly negative, indicating net immobilization of $NH_4^+$, with occasional positive mineralization occurring only in Sitka spruce at San Juan (Figure 3c). San Juan forest floors had higher (i.e. less negative) rates of net ammonification than Fairy Lake forest floors ($p$ = 0.01), and net ammonification rates differed among tree species ($p$ = 0.05).

Rates of gross nitrification, nitrate consumption and net nitrification did not differ between species, but did differ between sites with slightly more N transformed at Fairy Lake than San Juan ($p$ < 0.01). At Fairy Lake, western red cedar had the highest rates of gross nitrification, which significantly differed from hemlock and Sitka spruce, but not from Douglas-fir. Douglas-fir and Sitka spruce had the highest and similar rates of nitrate consumption, followed by western red cedar, spruce, and hemlock at Fairy Lake. At San Juan, Sitka spruce had the highest rates of gross nitrification. Douglas-fir, western red cedar, and hemlock all had negligible rates of gross nitrification and nitrate consumption. Net nitrification rates were negative, indicating net nitrate immobilization in all plots except Sitka spruce at San Juan (Figure 3f).

*3.5 Relationships between N ammonification and nitrification rates and microbial parameters*

In general, we had greater explanatory power for ammonification rates than nitrification rates (Table 4). Gross ammonification was best explained by the total number of bacterial *16S* rRNA and fungal *ITS* genes. Gross $NH_4^+$ consumption was best explained by gross ammonification, pH, forest floor C:N ratio, and microbial biomass C:N ratio. Net ammonification was best explained by forest floor pH and C:N ratios and microbial biomass C:N ratios. Gross nitrification was best explained by microbial biomass C:N ratios and AOA *amoA* gene. Gross $NO_3^-$ consumption was best explained by net ammonification, gross nitrification, microbial biomass C:N ratios, bacterial 16S and AOA and AOB *amoA* genes. Net nitrification rates were best explained by net ammonification, microbial biomass C:N ratios, bacterial 16S, and AOA and AOB *amoA* genes. The sum of AOA and AOB gene abundances showed a strong, positive relationship with the sum of the abundance of nitrite reductase genes (*nirK* and *nirS*)

(Figure 4). Redundancy analysis showed clear separation of the two sites largely due to differences in forest floor C:N ratio and pH, but no clear grouping of microbial abundance patterns according to tree species (Figure 5). Fairy Lake was associated with high C:N ratio, and San Juan with higher microbial gene abundance for AOA and AOB *amoA, nirK, nirS,* and *16S.*

## 4. Discussion

The relationships between rates of production and consumption of N and microbial parameters uncovered in this study provide insights into the mechanisms underlying differences in N cycling and availability in forest floors. The prominence of forest floor C:N ratio in the relationships indicate that site N status exerts a dominant influence on N cycling. The proportion of mineralized N taken up by microbial biomass, rather than the gross mineralization rate, appeared to be the primary driver of N release, consistent with conclusions of Mooshammer et al. (2014). While gross ammonification was influenced by the abundance of bacteria and fungi (i.e. overall saprotrophic activity), consumption of the mineralized $NH_4^+$ depended on microbial demand for N, as indicated by microbial C:N ratio. This is consistent with forest floors at these sites having different rates of N mineralization and nitrification despite similar rates of litter decomposition and C mineralization (Prescott et al., 2000). The strong relationships between rates of most N transformation processes and microbial C:N ratio indicate that the N status of microbes, *i.e.* the degree to which N is available excess to their needs, rather than their biomass or activity level, determined the amount of $NH_4^+$ that remained available in the forest floor. High microbial N status (*i.e.* low microbial C:N ratio) led to a smaller proportion of the $NH_4^+$ being consumed by microbes, and the resulting 'excess' $NH_4^+$ stimulated nitrifying organisms (as indicated by the relationships between microbial biomass C:N ratios and *amoA* gene abundances). Nitrate production then creates conditions conducive to denitrifying organisms as indicated by the relationship between AOA and AOB gene abundances and abundance of nitrite reductase genes (*nirK* and *nirS*).

The manner in which these interrelationships between site conditions, soil microorganisms and N cycling processes play out at the two study sites is illustrated in Figure 6. Despite similar rates of decomposition and C mineralization between sites, the high C:N ratio in organic matter and microbes at Fairy Lake causes all of the mineralized $NH_4^+$ to be consumed by microbes and

plants. In contrast, at San Juan, the low C:N ratio of the organic matter causes more N to be mineralized per unit C mineralized, and only a portion of this is consumed by microbes and vegetation. The resulting accumulation of $NH_4^+$ stimulates ammonia-oxidizers (as evident in the higher microbial gene abundance for AOA and AOB *amoA)*, which liberate N in the form of nitrate. Some of this nitrate is consumed by microbes and plants, and some is used by denitrifiers, as evident in the detection of higher microbial gene abundance for *nirK,* and *nirS* in San Juan forest floors.

For many of the variables in this study, the influence of site N status on N cycling processes was greater than the influence of tree species. Observed gross ammonification rates support earlier findings of greater net N mineralization rates and higher bacterial:fungal ratio in forest floors at nutrient-rich, valley-bottom sites (including San Juan) than at nutrient-poor, mid-slope sites (including Fairy Lake) (Prescott et al., 2000a; Grayston and Prescott, 2005). Microbial gene abundances were also strongly affected by site, with forest floors from the San Juan site have a higher potential for nitrification and denitrification than those from Fairy Lake.

Forest floor C:N ratios were the primary feature of site influence on microbial biomass, gene abundance and N transformations. This is consistent with earlier findings that rates of N mineralization in forest floors along a site fertility gradient in Douglas-fir stands were most closely related to forest floor C:N ratios, with net mineralization being appreciable only at sites where the forest floor C:N ratio was less than 35. Similarly, Högberg et al. (2007) found forest floor C:N ratios to be as important as pH and base cations in predicting microbial community composition in forest floors in a boreal Fennoscandian forest ecosystem, while Chen and Högberg (2007) found negative correlations between fungal:bacteria ratios, forest floor C:N ratios and gross mineralization rates in forest floors. Bates et al. (2011) found soil C:N ratio to be the best predictor for archaeal relative abundances, with higher C:N ratios leading to higher archaeal relative abundances.

Tree species effects were smaller than site effects, and were more pronounced for $NH_4^+$ transformations than for $NO_3^-$ transformations. Western red cedar had consistently high abundance of *16S* and AOA *amoA* at both sites, and higher rates of gross ammonification and $NH_4^+$ consumption, which made it the most ecologically extreme of the four tree species. This is consistent with previous studies highlighting the different N transformation pattern and microbial community structure in western red cedar forest floors (Turner and Franz, 1985; Prescott et al.,

2000a; Prescott and Grayston, 2005). Douglas-fir and Sitka spruce had similar abundances of all targeted genes, which did not significantly differ from each other. Some tree species effects appeared to depend on site N status. For example, Sitka spruce and western hemlock differed in rates of net ammonification, but only at the nutrient-poor site, Fairy Lake. The data from this study support both direct effects of tree species on soil N cycling and soil microbial communities, but also highlight the context-dependency of tree species effects (Prescott and Vesterdal 2013).

The positive correlation between gross ammonification rate and abundance of bacterial 16*S* rRNA genes suggests an important role of bacteria in ammonification, which aligns with current thinking about N-cycling processes (Laverman et al., 2001, Kowalchuk and Stephen, 2001, Wallenstein et al., 2006). The significantly positive correlations between the abundance of AOA *amoA* genes and rates of both gross and net nitrification indicate that nitrification in these forest floors is modulated primarily by archaeal ammonia-oxidation, with AOB playing a negligible role. Although archaea are considered to contribute little to soil microbial biomass (Gattinger et al., 2002; Bardgett and Griffiths, 1997), archaea in forest soils can have a functional role in N cycling akin to a keystone species (Prosser and Nicol, 2008; Verhamme et al., 2011). AOA are generally more abundant than AOB in acidic forest soils (Petersen et al., 2012) and AOA can have higher ammonia-oxidation rates relative to AOB under similar N availabilities (Wertz et al., 2012). The strong, positive relationship between the abundance of nitrite reductase genes (*nirK, nirS*) and the sum of AOA and AOB indicates that the genetic potential for denitrification in these forest floors was strongly influenced by ammonia oxidizer abundance. We measured gene abundance rather than directly assessing activity associated with specific genes; in other studies, gene abundances have shown a high degree of correlation with substrate concentrations and process rates (McGill et al., 2006; Wertz et al., 2009; Penton et al., 2013; Levy-Booth et al., 2014). Recent studies have characterized the comammox *Nitrosospira* species which contain enzymes that catalyze complete nitrification (van Kessel et al., 2015 and Daims et al., 2015), but these organisms were not considered in this study.

Bacterial *16S* and *nirK* varied amongst tree species, which suggests that tree species foster different abundances of denitrifying bacteria, in addition to the elevated AOA *amoA* in western red cedar plots. AOA *amoA* can oxidize ammonia via an alternate pathway that requires less oxygen than the bacterial (AOB *amoA*) channel, which enables ammonia oxidation in anoxic

soils (Schleper and Nicol, 2010; Levy-Booth et al., 2014). Similarly, organisms that contain the gene *nirS* often do not contain *nirK*, which suggests that tree species-specific soil microbial communities are associated with specific denitrifying bacteria (Levy-Booth et al., 2014). Consistent with previous studies of forest floor microbial communities associated with these tree species (Grayston and Prescott, 2005; Turner and Franz, 1985), we found cedar forest floors to be more bacteria-dominated (*16S*) while forest floors of all four tree species were similar in fungal abundance (fungal *ITS*).

The net N transformation rates presented here were derived from the 24-hour gross-N incubations, not a standard 28-day incubation for rate of net N mineralization such as in the previous study of forest floor N dynamics in the common garden experiment (Prescott et al., 2000a). The short-term incubation study found tree species differences in N ammonification rates, but these were overshadowed by site effects. This is consistent with results of the longer-term incubations (Prescott et al., 2000a), although the values should not be directly compared, and more studies are needed to confirm the patterns. Nevertheless, this study demonstrates that a) differences between tree species and sites are discernible with these methods, and b) insights into the linkages between forest floor physico-chemical parameters, microbial gene abundance and biogeochemical cycling can be gained using these methods.

## 5. Conclusions

Quantification of key microbial marker genes involved in biogeochemical transformations were used to explore mechanistic links between site factors, tree species and N cycling processes. Rates of N transformation and microbial gene abundances were higher at the San Juan site, which had higher forest floor C:N ratios, higher microbial gene abundances related to nitrification and denitrification, and higher gross N transformation rates. Differences between the sites were related to site N status, as reflected in C:N ratios of the forest floor, and were more closely tied to rates of N consumption rather than gross mineralization. The relative contributions of AOA and AOB to gross and net nitrification in forests were mainly influenced by site N status. Tree species influenced gross and net ammonification and $NH_4^+$ consumption. Western red cedar forest floors were the most distinct of the four tree species, with highest rates of $NH_4^+$ N transformation, and the most distinctive forest floor microbial communities in terms of *16S*

and *nirK* gene abundances. The coupling of techniques for assessing ecosystem process rates with molecular techniques, such as functional gene abundances, can provide a greater mechanistic understanding of links between tree species and N transformation processes, as demonstrated in this study.

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**Figure legends**

**Figure 1.** Microbial biomass C, N and C:N ratio in forest floors of four tree species at the two sites (blue bars=Fairy Lake; green bars=San Juan; DF=Douglas-fir; SS=Sitka spruce; WH=western hemlock; WRC=western red cedar).

**Figure 2.** Mean (± SE) gene abundance (log of gene copies/g soil) of total ammonia oxidizers (AOA *amoA* and AOB *amoA)* and total denitrifiers *(nirK* and *nirS,* bacteria (*16S),* and fungi (*ITS)* in the forest floors of four tree species at the two sites. Blue bars=Fairy Lake; green bars=San Juan; DF=Douglas-fir; SS=Sitka spruce; WH=western hemlock; WRC=western red cedar.

**Figure 3.** Rates of nitrogen transformations in the forest floors of four tree species at the two sites; (mean ± SE). Blue bars=Fairy Lake; green bars=San Juan; DF=Douglas-fir; SS=Sitka spruce; WH=western hemlock; WRC=western red cedar.

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**Figure 4.** Regression of the sum of denitrification (*nirK* and *nirS*) and ammonia-oxidation (AOA and AOB *amoA*) gene abundance by site ($p > 0.05$, *; $p > 0.01$, **, $p > 0.001$, ***). Sites are differentiated in the coordinate plot by colour (blue=Fairy Lake; green=San Juan) and tree species are differentiated by symbols (circle=hemlock; triangle=Douglas-fir; diamond=spruce; square=cedar).

**Figure 5.** Canonical redundancy analysis (RDA) and variation partitioning to determine the factors contributing to the abundance of functional gene abundance at Fairy Lake and San Juan sites. RDA was used to ordinate gene abundance measurements for total bacteria (16S), AOA (*amoA*), AOB (*amoA*) and nitrite reducers (*nirK* and *nirS*) against forest floor physico-chemical factors (pH, total C, total N and C:N ratio). Sites are differentiated in the coordinate plot by colour (orange, Fairy Lake; green, San Juan) and tree species are differentiated by symbols (circle=hemlock; triangle=Douglas-fir; diamond=spruce; square=cedar).

**Figure 6.** Conceptual model of N cycling processes at the two study sites with differing N status – Fairy Lake and San Juan.

679 **Table 1.** Group-specific primers for qPCR gene quantification assays.

| *Primer Target* | *Primer Name* | *Primer Sequence (5'-3')* | *Reference* |
|---|---|---|---|
| All Bacteria (16S rRNA) | 519F<br>907R | CAG CMG CCG CGG TAA NWC<br>CCG TCA ATT CMT TTR AGTT | Baker et al. (2003)<br>Muyzer et al. (1995) |
| All Fungi (ITS) | ITS-1F<br>5.8s | TCC GTA GGT GAA CCT GCG G<br>CGC TGC GTT CTT CAT CG | Gardes and Bruns (1993)<br>Vilgalys and Hester (1990) |
| AOA[a] *(amoA)* | *amoA*-23F<br>*amoA*- 616R | ATG GTC TGG CTW AGA CG<br>GCC ATC CAT CTG TAT GTC CA | Francis et al. (2005) |
| *AOB*[b] *(amoA)* | *amoA*-1F<br>*amoA*-2R | GGG GTT TCT ACT GGT GGT<br>CCC CTC KGS AAA GCC TTC TTC | Rotthauwe et al. (1997) |
| Cd-nitrite reductase (*nirS*) | *nirS*-1F<br>*nirS*-3R | CCT AYT GGC CGG CRC ART<br>GCC GCC GTC RTG VAG GAA | Braker et al. (1998) |
| Cu-nitrite reductase (*nirK*) | *nirK-1F*<br>*nirK-1R* | GGG CAT GAA CGG CGC GCT CAT GGT G<br>CGG GTT GGC GAA CTT GCC GGT GGT C | Braker et al. (1998) |

680 [a]Ammonia Oxidizing Archaea, [b]Ammonia Oxidizing Bacteria

**Table 2.** Forest floor chemistry: pH, total soil C (mg/g), N (mg/g), and C:N ratios of the four tree species at the two sites. Mean ± SE.

| Site | Tree species | pH | Total C (mg $g^{-1}$) | Total N (mg $g^{-1}$) | C:N ratio |
|---|---|---|---|---|---|
| San Juan | WRC | 4.01 ± 0.18 | 436 ± 31 | 110 ± 0 | 38.5 ± 3.1 |
| | DF | 3.95 ± 0.02 | 466 ± 9 | 135 ± 1 | 34.6 ± 0.9 |
| | WH | 3.98 ± 0.21 | 449 ± 17 | 114 ± 1 | 39.4 ± 1.0 |
| | SS | 4.63 ± 0.45 | 369 ± 85 | 112 ± 26 | 33.6 ± 0.9 |
| Fairy Lake | WRC | 4.31 ± 0.30 | 485 ± 8 | 11.0 ± 0.1 | 44.0 ± 0.6 |
| | DF | 4.05 ± 0.01 | 430 ± 52 | 11.1 ± 1.1 | 38.5 ± 0.9 |
| | WH | 3.75 ± 0.03 | 498 ± 4 | 10.5 ± 0.2 | 47.2 ± 0.7 |
| | SS | 4.29 ± 0.05 | 460 ± 18 | 11.8 ± 0.5 | 39.2 ± 3.1 |

WRC=western red cedar; DF=Douglas-fir; WH=western hemlock; SS=Sitka spruce

**Table 3.** F-statistics following permutation ANOVA testing of tree species (western red cedar, Douglas-fir, western hemlock, Sitka spruce, df=3), site (Fairy Lake, San Juan, df=1), and interaction (T x S, df=3) effects on forest floor chemistry (pH, total C, total N, C:N ratio), microbial biomass (C, N and C:N ratios), N transformations (gross and net nitrogen ammonification and nitrification, and $NH_4^+$ and $NO_3^-$ consumption) and microbial gene abundances.

| *Forest floor chemistry* | Tree (T) | | Site (S) | | Tree x Site (T x S) | |
|---|---|---|---|---|---|---|
| | *F* | *p* | *F* | *p* | *F* | *p* |
| pH | 2.93 | 0.10 | 0.08 | 0.79 | 0.90 | 0.48 |
| C | 0.89 | 0.49 | 2.03 | 0.20 | 0.97 | 0.43 |
| N | 0.58 | 0.64 | 0.87 | 0.40 | 0.71 | 0.59 |
| C:N | 8.49 | 0.01 | 23.77 | <0.01 | 0.45 | 0.71 |
| *Microbial biomass* | | | | | | |
| MBC | 6.77 | 0.02 | 33.16 | <0.01 | 5.25 | 0.03 |
| MBN | 2.26 | 0.18 | 0.02 | 0.90 | 1.11 | 0.39 |
| MBC:N | 1.62 | 0.28 | 3.90 | 0.10 | 1.67 | 0.29 |
| *N transformation rates* | | | | | | |
| Gross ammonification | 18.43 | <0.01 | 64.57 | <0.01 | 1.08 | 0.40 |
| Ammonium consumption | 6.75 | 0.01 | 7.31 | 0.03 | 0.50 | 0.73 |
| Net ammonification | 4.43 | 0.05 | 18.27 | 0.01 | 0.73 | 0.58 |
| Gross nitrification | 0.74 | 0.58 | 20.81 | <0.01 | 1.95 | 0.18 |
| Nitrate consumption | 1.19 | 0.38 | 132.72 | <0.01 | 1.68 | 0.25 |
| Net nitrification | 1.41 | 0.34 | 67.09 | <0.01 | 5.84 | 0.02 |
| *Microbial gene abundances* | | | | | | |
| Bacteria *16S* | 3.88 | 0.05 | 10.20 | 0.01 | 1.24 | 0.32 |
| Fungal *ITS* | 0.20 | 0.90 | 0.38 | 0.55 | 0.99 | 0.45 |
| AOA *amoA* | 0.76 | 0.51 | 18.62 | 0.01 | 0.02 | 1.00 |
| AOB *amoA* | 1.06 | 0.46 | 4.02 | 0.07 | 1.42 | 0.33 |
| *nirK* | 2.73 | 0.13 | 1.94 | 0.20 | 0.97 | 0.45 |
| *nirS* | 1.47 | 0.27 | 8.54 | 0.02 | 1.73 | 0.22 |

C=carbon; N=nitrogen; MB=microbial biomass

**Table 4.** Multiple linear regressions of gross and net N ammonification and nitrification rates, and consumption rates with adjusted $R^2$ values, with best models selected using the lowest AIC values.

| *Gross ammonification* = bacterial *16S* gene copies - fungal *ITS* gene copies | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| bacteria *16S* gene copies | 1.057 | 6.253 | 2.96E-05 |
| fungal *ITS* gene copies | -0.537 | -3.179 | 0.007 |
| F-statistics: 19.68 on 2 and 13 degrees of freedom; Adj. R2: 0.71; p-value: 0.0001168 | | | |

| *Gross NH4+ consumption* = gross ammonification + C:N ratio + MBC:N ratio | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| Gross ammonification | 1.055 | 13.084 | 1.84E-08 |
| Forest Floor C:N ratio | 0.243 | 2.68 | 0.020 |
| Microbial Biomass C:N ratio | 0.262 | 3.178 | 0.008 |
| F-statistics: 61.5 on 3 and 12 degrees of freedom; Adj. R2: 0.92; p-value: <0.001 | | | |

| *Net ammonification* = - C:N ratio - MBC:N ratio | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| C:N ratio | -0.526 | 0.1538 | 0.005 |
| Microbial biomass C:N ratio | -0.486 | 0.1538 | 0.008 |
| F-statistics: 21.73 on 2 and 13 degrees of freedom; Adj. R2: 0.73; p-value: <0.001 | | | |

| *Gross nitrification* = MB C:N ratio - *amoA* AOA gene copies | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| Microbial biomass C:N ratio | 0.3401 | 1.77 | 0.100 |
| *amoA* AOA gene copies | -0.5998 | -3.179 | 0.008 |
| F-statistics: 7.265 on 2 and 13 degrees of freedom; Adj. R2: 0.46; p-value: 0.008 | | | |

| *Gross NO3- consumption* = gross nitrification | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| gross nitrification | 0.7552 | 0.1752 | <0.001 |
| F-statistics: 18.58 on 1 and 14 degrees of freedom; Adj. R2: 0.54; p-value: <0.001 | | | |

| *Net nitrification* = *amoA* AOB gene copies | | | |
|---|---|---|---|
| Variable | Coefficient | t value | p-value |
| *amoA* AOB gene copies | 0.4775 | 2.034 | 0.061 |
| F-statistics: 4.135 on 1 and 14 degrees of freedom; Adj. R2: 0.1729; p-value: 0.061 | | | |

Ammonification rates: mg N $kg^{-1}$ soil (dw) $d^{-1}$; nitrification rates: mg N $g^{-1}$ soil (dw) $d^{-1}$; microbial C and N, mg N $g^{-1}$ soil (dw); Bacterial *16S,* Fungal *ITS,* AOA and AOB *amoA*: genes $g^{-1}$ soil (dw).