Quantification of Nitrogen Reductase and Nitrite Reductase Genes in Soil of Thinned and Clear-Cut Douglas-Fir Stands by Using Real-Time PCR[†]

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The abundance of *nifH*, *nirS*, and *nirK* gene fragments involved in nitrogen (N) fixation and denitrification in thinned second-growth Douglas-fir (*Pseudotsuga menziesii* subsp. *menziesii* [Mirb.] Franco) forest soil was investigated by using quantitative real-time PCR. Prokaryotic N cycling is an important aspect of N availability in forest soil. The abundance of universal *nifH*, *Azotobacter* sp.-specific *nifH* (*nifH*-g1), *nirS*, and *nirK* gene fragments in unthinned control and 30, 90, and 100% thinning treatments were compared at two long-term research sites on Vancouver Island, Canada. The soil was analyzed for organic matter (OM), total carbon (C), total N, NH₄-N, NO₃-N, and phosphorus (P). The soil horizon accounted for the greatest variation in nutrient status, followed by the site location. The 30% thinning treatment was associated with significantly greater *nifH*-g1 abundance than the control treatment in one site; at the same site, *nirS* in the mineral soil horizon was significantly reduced by thinning. The abundance of *nirS* genes significantly correlated with the abundance of *nirK* genes. In addition, significant correlations were observed between *nifH*-g1 abundance and C and N in the organic horizon and between *nirS* and *nirK* and N in the mineral horizon. Overall, no clear influence of tree thinning on *nifH*, *nirS*, and *nirK* was observed. However, soil OM, C, and N were found to significantly influence N-cycling gene abundance.

Nitrogen (N) is a limiting nutrient in most Douglas-fir (*Pseudotsuga menziesii* subsp. *menziesii* [Mirb.] Franco) forest ecosystems. Understanding the links between forest management and forest ecosystem function, including the cycling of N, is of paramount importance to researchers and forest managers. Management practices such as thinning and clear-cutting can alter the soil microbial community, potentially altering the rate and amount of net N addition or loss to the forest floor. Clear-cutting alters the functional diversity of soil microorganisms and alters soil characteristics (temperature, pH, moisture, and nutrient status). Thinning and clear-cutting can increase nitrification, denitrification, and leaching of N in soil, all of which can reduce the available N (2, 13, 22, 41, 47). Clear-cutting in Douglas-fir forests can also remove associated gene pools of diazotrophic microorganisms (46). It is not yet well understood how clear-cutting or thinning affects the abundance of N-cycling microorganisms. We focus on two populations of N-cycling microorganisms: diazotrophs, which biologically fix N₂ gas to ammonia, and denitrifiers, which reduce N oxides and result in the release of N-containing gasses.

Fixation of N by diazotrophic microorganisms is the primary source of N addition to undisturbed, unfertilized forest soil ecosystems (9, 39). The diazotrophic community is most often studied *in situ* using the marker gene for nitrogenase reductase

(*nifH*); the diversity and abundance of diazotrophic microorganisms as determined by *nifH* characterization may be used as an indicator of overall soil ecological health. Diazotrophs can be symbiotic, associated (e.g., with a specific plant or fungal biomass), or free-living in the soil. Endophytic diazotrophs fix ~100 times more N than free-living strains (9). Free-living diazotrophs such as *Azotobacter vinelandii* and *A. chroococcum* may fix between 0 and 60 kg of N ha⁻¹ year⁻¹ (9) and, because of a relative dearth of endophytic interactions in coniferous forests, free-living diazotrophs can be an important source of N in these soils. Cultural studies have shown that free-living diazotrophs improve the establishment of mycorrhizae and conifer seedlings, with relative activity fluctuating according to season, site aspect, and moisture conditions (11). Fixed-N inputs act as a catalyst for interlinked N-cycling events, e.g., fungal decomposition of woody debris and organic material (28). Nitrogen fixation in temperate forest soil is directly related to the amounts of soil organic matter (17). However, it is unclear how *nifH* gene abundance relates to the amount of total carbon (C) and organic matter (OM) and N in forest soil. It is also unknown how common silvicultural practices (e.g., clear-cutting and thinning) affect diazotrophic abundance or how diazotrophic abundance may in turn affect cycling of soil nutrients.

The reduction of inorganic N oxides by denitrifying microorganisms can cause N loss from forest soil ecosystems, as well as the release of greenhouse gases into the atmosphere. The loss of N from temperate forest soil as N₂O has been reported as ranging from 0.2 to 7.0 kg ha⁻¹ year⁻¹, depending largely on soil nitrogen status, soil moisture, and temperature (57). Robertson and Tiedje (44) state that soil N loss in coniferous ecosystems due to denitrification is regulated by nitrification

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TABLE 1. Selected properties of field sites examined in this study

Site code	LOGS ^a site	Coordinates		Established	Date (yr)		Control tree density (stems ha ⁻¹)	Site index (m) ^b	Elevation (m) ^c	AAP ^d (mm)	Soil texture
		Latitude	Longitude		First treatment	Second treatment					
Site A	Shawnigan Lake	48°38'1.23"N	123°42'44.72"W	1948	1970	2003	2,162	29	335	1,174	Loam
Site B	Sayward Forest	50°2'24.20"N	125°33'25.50"W	1950	1969	1993	1,796	34	274	1,494	Silt loam

^a LOGS, levels of growing stock.^b Height of dominant trees at 50 years of age.^c That is, above sea level.^d AAP, average annual precipitation.

potential (e.g., nitrate levels) in the soil, and while not considered a major N loss component following clear-cutting, this loss is generally of the same magnitude as the N loss due to leaching. Denitrification is primarily studied using molecular approaches by monitoring several genes in the denitrification pathway: cytochrome *cd*₁-containing nitrite reductase (*nirS*), Cu-containing nitrite reductase (*nirK*), nitrous oxide reductase (*nosZ*), and membrane-bound nitrate reductase A (*narG*). The *nirS* and *nirK* genes were the denitrification genes used in the present study. Studies demonstrating (i) that the *nirS* gene is more diverse than *nirK* in soil and (ii) the domination of the *nirK* population by a relatively reduced number of clones have been published (42, 45). However, recent meta-analysis of studies involving *nirK* and *nirS* has shown that both communities tend to be phylogenetically clustered in undisturbed soils (23).

To compare the effects of silvicultural practices on the abundance of diazotrophs and denitrifiers, we used quantitative real-time PCR (qPCR) assays to quantify *nifH*, *nirS*, and *nirK* genes in soil. This method can be used to quantify target sequences in environmental samples. Several qPCR protocols for the analysis of functional gene abundance in soil have been developed for N-cycling genes, including *nifH*, ammonia monooxygenase (*amoA*), *nirK*, *nirS*, *nosZ*, and *narG* (21, 24, 31, 38, 43, 54, 55). The objectives of the present study were (i) to quantify *nifH*, *nirS*, and *nirK*; (ii) to compare the effects of thinning and clear-cutting in Douglas-fir stands on the abundance of total diazotrophs, free-living diazotrophs, and denitrifiers; and (iii) to elucidate the relationships between N-cycling genes and nutrient abundance in forest soils.

MATERIALS AND METHODS

Field site and soil sampling. Field sampling for the present study was conducted in long-term levels-of-growing-stock (LOGS) experimental installations located on Vancouver Island, British Columbia, Canada. The periodically thinned second-growth Douglas-fir plantation near Shawnigan Lake, British Columbia (Shawnigan Lake LOGS site; site A), and a nearby clear-cut plot were sampled on 25 January 2007 and 16 June 2008. The second-growth Douglas-fir plantation near Sayward, British Columbia (Sayward Forest LOGS site; site B), and a nearby clear-cut plot were sampled on 11 June 2008. Selected site characteristics are provided in Table 1. The clear-cut plot near site A (coordinates 48°37'56.37"N, 123°43'35.43"W; 330 m above sea level) was harvested in 2002, and the clear-cut plot near site B (coordinates 50°3'55.62"N, 125°32'56.77"W, 282 m above sea level) was harvested in 2003. Previous reports (1, 14) provide more detailed information about the sites used in the present study.

At both sites, sampling of the surface organic horizon (LFH; 0 to 2 cm) and the mineral horizon (Ae and Bh; 2 to 10 cm) took place in 0.081-ha treatment plots organized in a random complete-block design, including thinned (30 and 90%) and unthinned control plots (see Table 1 for the control tree density). Sampling in nearby clear-cut plots removed 0 to 10 cm of soil, primarily mineral soil with some organic material interspersed. The clear-cut plots generally contained a

cover of coarse woody debris overlaying a horizon of highly disturbed soil. Due to the removal of the majority of the organic horizon during the clear-cut process, soil collected in these plots was considered to be mineral soil. Soil samples were collected from the organic soil horizon and the mineral horizon separately, with nine ~200-ml samples collected in a 3-by-3 grid throughout each plot. The samples were pooled by plot, stones and large woody debris were removed, and the samples were homogenized. The samples were air dried in a laminar flow hood for 3 days. Oven drying was not used to prevent biochemical alterations to the soil organic matter and DNA. Three subsamples of 0.5 g were removed from the pooled soil from each plot for DNA extraction. The remainder was analyzed for particle size composition, organic matter (OM), exchangeable cations, effective cation exchange capacity (CEC), total C, total N, pH, ammonium (NH₄-N), nitrate (NO₃-N), and available phosphorus (P) by the British Columbia Ministry of Forest and Range Analytical Laboratory (Victoria, British Columbia, Canada). A summary of the soil nutrient values is provided in Table 2.

Soil DNA extraction. DNA was extracted from dried soil by using the MoBio UltraClean soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA) with modification to the manufacturer's protocol (30) to increase extraction yields and reduce organic PCR inhibitors (e.g., humic substances) commonly found in forest soil. Briefly, 0.125 g of the nuclease inhibitor aurintricarboxylic acid and 50 µl of 200 mM AlNH₄(SO₄)₂, a chemical flocculent used to remove coextracted humic substances, were added to each well prior to bead beating. DNA was stored at -20°C prior to PCR.

Bacterial cultures. Cultures of dinitrogen-fixing and denitrifier bacteria were used for standard curve construction for the universal *nifH* (*nifH*-universal), *Azotobacter* sp.-specific *nifH* (*nifH*-g1), and *nirK* genes, respectively. The standard curve for *nirS* quantification was constructed by using pre-extracted genomic DNA. *Azotobacter vinelandii* ATCC 12518 (American Type Culture Collection, Manassas, VA) was cultured on ATCC medium 14 (*Azotobacter* medium; H₂PO₄, 0.2 g liter⁻¹; K₂HPO₄, 0.8 g liter⁻¹; MgSO₄ · 7H₂O, 0.2 g liter⁻¹; CaSO₄ · 2H₂O, 0.1 g liter⁻¹; FeCl₃, 0.02 g liter⁻¹; Na₂MoO₄ · 2H₂O, 0.002 g liter⁻¹; yeast extract, 0.5 g liter⁻¹; sucrose, 20.0 g liter⁻¹; agar, 15.0 g liter⁻¹ [pH 7.2]) for 72 h at 26.0°C. *Azospirillum brasilense* ATCC 29729 and *Pseudomonas chlororaphis* ATCC 13985 were cultured on nutrient agar (Difco/BD Biosciences, Mississauga, Ontario, Canada). Bacterial inoculum was aseptically transferred to nutrient broth (Difco) in autoclaved 50-ml capped plastic tubes for 72 h at 30.0°C. DNA was extracted from broth cultures by using MoBio UltraClean microbial DNA isolation kits. *Pseudomonas aeruginosa* ATCC 47085D-5 genomic DNA was used for *nirS* standards. DNA was stored at -20°C prior to PCR.

Real-time PCR quantification of nitrogen-cycling genes. Quantitative real-time PCR used existing primer sets for *nifH*-universal (56), *nifH*-g1 (6), *nirS* (3), and *nirK* (8) gene amplifications (Table 3). The *nirK* primers used in the present study lack the degenerate nucleotide coding of the *nirS* primers. They were originally developed through the alignment of available *nirK* sequences, using *Pseudomonas chlororaphis* as a positive control (8). These primers are therefore not considered to be strain or genus specific (Reál Roy, personal communication). In the present study, the *nirK* and *nirS* primers are regarded as having roughly comparable levels of specificity. Reactions took place in a LightCycler 1.2 (Roche Applied Sciences, Indianapolis, IN). The primer sequences and characteristics are listed in Table 3. The *nifH*-universal qPCR used a nested protocol, whereas the *nifH*-g1, *nirS*, and *nirK* qPCRs used a single amplification step.

The first (non-qPCR) *nifH*-universal amplifications in the nested reaction took place under the following conditions: 1× reaction buffer (Bioline, Ltd., London, United Kingdom), 2.5 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 200 µM, each oligonucleotide primer at a concentration of 1 µM, and 0.2 µl (1 U) of Diamond *Taq* polymerase (Bioline). For the first PCR (25 µl), 5 µl of DNA sample containing 10 ng of DNA was used, and for the nested

TABLE 2. Summary of soil nutrients in the organic and mineral soil horizons of Douglas-fir stands and in nearby clear-cut plots in the Shawnigan Lake and Sayward Forest LOGS installations at each sampling date

Soil horizon	Site and date ^a	Mean content (SEM) of various soil nutrients ^c						
		OM ^b (%)	Total C (%)	Total N (%)	C/N ratio	NH ₄ -N (ppm)	NO ₃ -N (ppm)	Available P (ppm)
Organic	A2007	54.1 (5.9)*	29.8 (3.6)*	0.7 (0.1)*	41.2 (2.8)*	16.3 (5.0)*	0.4 (0.2)*	163.5 (45.6)*
	A2008	67.8 (10.0)**	39.1 (5.6)**	0.9 (0.1)*/**	45.1 (3.3)*	19.6 (2.3)*	0.4 (0.7)*	120.3 (35.2)*
	B2008	66.0 (10.9)**	38.3 (6.2)**	1.0 (0.2)**	40.8 (2.5)*	61.3 (37.8)**	0.1 (0.2)*	64.9 (22.3)**
Mineral	A2007	9.5 (1.1)*	4.5 (0.9)*	0.1 (0.1)*	38.9 (5.6)*	1.4 (0.6)*	0.1 (0.2)*	56.3 (17.5)*
	A2008	12.7 (2.18)**	6.7 (1.1)**	0.2 (0.1)*	38.4 (2.9)*	5.2 (0.9)**	<0.01 (-)**	61.2 (24.3)*
	B2008	12.2 (1.7)**	6.7 (1.5)**	0.2 (0.1)*	37.9 (4.0)*	11.8 (9.6)**	<0.01 (-)**	13.5 (7.7)**
Clear-cut	A2007	NA	NA	NA	NA	NA	NA	NA
	A2008	11.9 (1.0)*	6.0 (1.1)*	0.2 (0.1)*	37.1 (6.3)*	6.3 (2.4)*	<0.01 (-)*	19.7 (6.2)*
	B2008	11.8 (1.3)*	6.6 (0.5)*	0.2 (0.0)*	38.4 (3.5)*	8.0 (2.5)*	<0.01 (-)*	10.1 (4.1)**

^a A2007, Shawnigan Lake, January 2007; A2008, Shawnigan Lake, June 2008; B2008, Sayward Forest, June 2008.

^b Loss on ignition carbon.

^c Values for each sampling date marked by different numbers of asterisks are significantly different at a minimum $P = 0.05$ following Tukey adjustment for multiple comparisons. NA, not applicable.

qPCR (25 μ l), 5 μ l of the first PCR product was used. After initial denaturation (6 min at 94°C), 30 amplification cycles were performed for 11 s at 94°C and 15 s at 92°C (denaturation), 8 s at 54°C, and 30 s at 56°C for the first reaction and for 8 s at 51°C and 30 s at 53°C for the nested reaction (annealing) and 25 s at 72°C (extension). The materials and parameters used for qPCR in the *nifH*-universal nested protocol included 5 $\times$ MasterPLUS SYBR green I master mix (Roche), 1 mM MgCl₂, each deoxynucleoside triphosphate at a concentration of 50 μ M, each oligonucleotide primer at a concentration of 0.75 μ M, and 5 μ l of the first nested PCR.

The *nifH*-g1 qPCR initial denaturation step was 6 min at 94°C, and 40 amplification cycles were performed for 11 s at 94°C and 15 s at 92°C (denaturation), 8 s at 58°C and 30 s at 60°C (annealing), and 25 s at 72°C (extension). The PCR conditions for *nirS* and *nirK* amplification were 40 cycles of 1 min at 94°C (denaturing), 1 min at 65°C (annealing), and 1 min at 72°C (extension), followed by a final extension of 3 min at 72°C. The qPCR solutions for *nifH*-g1, *nirS*, and *nirK* were identical to that provided for *nifH*-universal. Fluorescence was monitored during the extension step for all qPCRs.

Standard curve development. Known quantities of genomic DNA from pure cultures were amplified by using SYBR-Green qPCR to generate a standard curve for each gene target (see Fig. S1 in the supplemental material). Tenfold dilution series were used for each target, and the linear regression of DNA concentration and relative fluorescent units (RFU) at a threshold fluorescence value (C_T) were used to quantify gene targets in soil DNA extracts by comparing the RFU at the C_T to the standard curve. To determine the gene copy number used in standard curve generation, DNA extracted from bacterial pure cultures was assessed for quantity and quality by using a spectrophotometer to measure the absorbance at wavelengths of 260 and 280 nm. DNA was quantified as μ g ml⁻¹ and converted to gene copy number using published genome sizes and gene copy number per genome in a modified protocol following the approach of

Harrow et al. (20). Genome sizes of approximately 6.80, 4.70, 6.30, and 5.00 Mb were used for *Azospirillum brasilense* Sp7 (35), *Azotobacter vinelandii* (33), *Pseudomonas aeruginosa* PAO1 (48), and *P. chlororaphis* (18). The *A. vinelandii* genome is generally agreed to be between 4.50 and 4.70 Mb (15, 33, 34), although the U.S. Department of Energy Joint Genome Institute *A. vinelandii* AvOP genome sequencing project lists the genome size for this species as 5.34 Mb (<http://www.azotobacter.org>). This information has not yet been published in a peer-reviewed journal. We elected to use the most recent published values for the *A. vinelandii* genome size. The genome size was multiplied by the average molecular mass of a base pair (609.6 g mol⁻¹) to determine the molecular mass of a single genome copy (20), and the known mass of each bacterial genomic DNA extract was converted into the number of genome copies μ l⁻¹. From 1 to ~100 genome copies can be found in each cell depending on growth stage (33, 37). Due to the variability of *A. vinelandii* ploidy, we do not recommend quantifying *nifH* based on cell number using standards derived from the genomic DNA of pure cultures. Accordingly, comparisons in the present study are reported on the basis of gene copy number rather than cell number.

A. brasilense (ATCC 29729) was used to develop the *nifH*-universal standard curve, the equation for which was determined to be: $\log \text{ } nifH \text{ gene copies } \mu\text{l of soil (dry weight [dw])}^{-1} = -0.0831(C_T) + 3.005$ ($R^2 = 0.997$), where C_T is the cycle at which the critical threshold of fluorescence is reached during amplification. The critical threshold was 0.533 RFU for *nifH*-universal qPCR, the efficiency was 2.588 (129%), and the error was 0.0423. The detection limit was $\sim 10^4$ log *nifH* gene copies g of soil⁻¹. The *nifH*-g1 primer specificity was tested by using denaturing gradient gel electrophoresis (DGGE). Amplicons from soil-extracted DNA matched banding corresponding to *A. vinelandii* in DGGE (data not shown).

The *nifH*-g1 qPCR standard curve was determined to be: $\log \text{ } nifH\text{-g1 gene copies } \mu\text{l of soil (dw)}^{-1} = -0.0564(C_T) + 2.434$ ($R^2 = 0.997$). The critical

TABLE 3. Oligonucleotide primers used for PCR amplification of bacterial genes

Gene target	Primer, sequence (5'-3') ^a	Position(s) (nt)	Reference organism	Reference
Nitrogenase reductase (<i>nifH</i>)	ForA, GCI WTI TAY GGN AAR GGN GG ForB, GGI TGY GAY CCN AAV GCN GA Rev, GCR TAI ABN GCC ATC ATY TC	19–482, 112–482	<i>A. brasilense</i> ATCC 29729	56
<i>nifH</i> -universal	ForA, GGT TGT GAC CCG AAA GCT GA Rev, GCG TAC ATG GCC ATC ATC TC	112–482	<i>A. vinelandii</i> ATCC 12518	5
<i>nifH</i> -g1	nirS1F, CCT AYT GGC CGG CRC ART nirS3R, GCC GCC GTC RTG VAG GAA	763–1019	<i>P. chlororaphis</i> ATCC 47085	3
Nitrite reductase (<i>nirS</i>)	F560-589, GGG CAT GAA CGG CGC GCT CAT GGT GCT GCC R906-935, CGG GTT GGC GAA CTT GCC GGT GGT CCA GAC	560–935	<i>P. chlororaphis</i> ATCC 13985	8
Nitrite reductase (<i>nirK</i>)				

^a The degenerate nucleotide coding follows International Union of Pure and Applied Chemistry (IUPAC) conventions: B, G+T; M, A+C; N, A+C+G+T; R, A+G; S, G+C; Y, C+T; W, A+T.

threshold was 0.502 RFU for *nifH*-g1 qPCR, the efficiency was 1.834 (91.7%), and the error was 0.0220. The detection limit was $\sim 10^2$ log *nifH*-g1 gene copies g of soil⁻¹.

The *nirS* standard curve developed using *Pseudomonas aeruginosa* PAO1 genomic DNA was: log *nirS* gene copies μ l of soil (dw)⁻¹ = $-0.0819(C_T) + 2.594$ ($R^2 = 0.998$). The *nirS* standard curve amplification had an efficiency of 1.69 (84.5%), and the error was 0.0929. The critical threshold was 1.19 RFU, and the detection limit was $\sim 10^2$ log *nirS* gene copies g of soil⁻¹.

The *nirK* standard curve using *P. chlororaphis* genomic DNA was: log *nirK* gene copies μ l of soil (dw)⁻¹ = $-0.0592(C_T) + 2.529$ ($R^2 = 0.996$). For the *nirK* qPCR the critical threshold was 1.01 RFU, the efficiency was 1.970 (98.5%), and the error was 0.0418. The detection limit was $\sim 10^2$ log *nirK* gene copies g of soil⁻¹. Among the four gene targets, there was some variability in PCR efficiency, likely attributable to the presence of inhibitors in the soil-extracted DNA samples.

Experimental design and statistical analysis. The study sites (A and B) each contained 27 treatment plots organized in a completely random design. Our study used only three of the eight original treatments: control (0% thinning), long-term high thinning (90% thinning), and long-term low thinning (30% thinning). The plots used were subject to long-term thinning to maintain constant levels of tree retention for at least 50 years. Three replicate plots were sampled for each treatment with a total of nine samples removed per plot. The samples were pooled by plot, and three subsamples per plot were used in the final analysis to reduce artifacts resulting from microsite heterogeneity in each plot. The clear-cut plots were not part of the original experimental design of the sites and hence are included here for comparative purposes only and were not included in the statistical analysis.

Statistical analysis of gene target abundance was performed by using a main-effects analysis of variance (ANOVA) (site $\times$ soil horizon $\times$ treatment) with $\alpha < 0.05$ using the SAS system for Linux, version 9.1 (SAS Institute, Cary, NC) with the "proc mixed" function. A Tukey test was used for all pairwise comparisons. The Shapiro-Wilks test was used to test normality assumptions of error distributions. Abundance of *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* targets conformed to assumptions for ANOVA. The residual errors were random and normally distributed around a zero mean. There were no outliers in the abundance of gene targets, as determined by the application of Cook's *D* statistical analysis of studentized residuals. For comparison of gene target abundance with soil nutrient values, linear regression analysis was performed. The average gene abundance was calculated for each treatment plot from the quantified subsamples and compared to the average nutrient value of the plot.

RESULTS

Soil nutrients. Soil nutrient status was affected by sample date and site location. Total C, OM, total N, and NO₃-N differed significantly between the January 2007 and June 2008 sampling dates at site A. NH₄-N and available P were significantly different between the site A and site B June 2008 sampling dates (Table 2). Tree-thinning levels did not significantly affect nutrient status for any nutrient at either site. Differences were similarly observed in both the organic and mineral soil horizons, although the mineral horizon had a significantly lower amount of all nutrients analyzed in the present study.

Quantification of nitrogen-cycling genes. The effect of thinning on the abundance of *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* genes in both the organic and the mineral soil horizons was tested using qPCR at the LOGS sites and nearby clear-cut plots at site A (January 2007 and June 2008) and Sayward Forest (June 2008 only) (Fig. 1). The abundance of *nifH*-universal was significantly affected by season and site location ($P < 0.001$) (Fig. 1a). The mean abundance ranged from 5.03 to 8.12 log gene copies g of soil⁻¹, corresponding to site B clear-cut on June 2008 and the organic horizon of the 30% thinning treatment at site A in January 2007, respectively. Soil horizon and thinning had no significant effects on *nifH*-universal abundance. Variability of the *nifH*-universal data set was generally higher than the other gene targets used in the present

study. The standard error ranged from 0.06 to 1.12 log gene copies g of soil⁻¹, corresponding to the organic horizon of the 30% thinning treatment at site B in June 2008 and the Shawngnan Lake clear-cut in June 2008, respectively.

The quantification of *nifH*-g1 revealed significant effects of sampling site and season ($P < 0.001$) and thinning ($P = 0.024$) and a significant site-horizon interaction ($P < 0.001$) (Fig. 1b). On average, site B (June 2008) had a greater abundance of *nifH*-g1 (3.19 log gene copies g of soil⁻¹) compared to site A in either January 2007 (2.83 log gene copies g of soil⁻¹) or June 2008 (2.75 log gene copies g of soil⁻¹). Although soil horizon was significantly different within each sampling season, it was not significantly different ($P = 0.054$) when the data were pooled for statistical analysis. The effects of thinning on *nifH*-g1 abundance were unclear, since only site B in June 2008 differed significantly due to thinning; the 30% thinning treatment had a significantly ($P = 0.033$) greater *nifH*-g1 abundance than the control treatment. The standard error of the mean (SEM) ranged from 0.06 to 0.40 log gene copies g of soil⁻¹ for the organic horizon of the control treatment at site A site in January 2007 and the mineral horizon at the 30% thinning treatment at site A in June 2008, respectively. The means *nifH*-g1 abundances ranged from 2.10 to 3.74 log gene copies g of soil⁻¹, which corresponds to the site A clear-cut in January 2007 and the mineral horizon of the 90% thinning treatment at site B in June 2008, respectively.

Site and horizon both significantly ($P < 0.001$) influenced *nirS* abundance (Fig. 1c). Significant site-by-treatment and horizon-by-treatment effects were observed, most notably in site B, where the control treatment had significantly greater *nirS* abundance than the 90% ($P = 0.013$) and 30% ($P = 0.026$) thinning treatments. In the site B mineral horizon, the mean $\pm$ SEM of the control treatment was 5.55 ± 0.23 log *nirS* gene copies g of soil⁻¹, whereas the abundance of *nirS* in the 30 and 90% thinned plots was 4.35 ± 0.15 and 4.56 ± 0.13 log *nirS* gene copies g of soil⁻¹, respectively. However, there were no clear trends that would indicate that tree thinning significantly reduces *nirS* levels in Douglas-fir forest soil.

Site and horizon had significant ($P < 0.001$) effects on *nirK* abundance (Fig. 1d). A clear pattern of *nirK* abundance unfolds in the organic horizon. Site B, which had a greater productivity and lower NO₃-N values, had significantly fewer *nirK* genes g of soil⁻¹ than the organic horizon of all treatments in the less productive site A. In the mineral horizon, however, no differences were noted between sites. A significant site-horizon interaction ($P < 0.001$) was also observed. The *nirK* assay had lower variability than either of the *nifH* quantifications. The mean *nirK* abundance ranged from 4.70 to 7.05 gene copies g of soil⁻¹ for the organic horizon in the control treatment plot at site B and the control treatment and the mineral horizon of the control treatment plot at site B. The SEM ranged from 0.08 to 0.62 log *nirK* gene copies g of soil⁻¹ for the mineral horizon of the control treatment at site B and the organic horizon of the control treatment at site B, respectively. The numbers of *nirS* and *nirK* gene copies were strongly correlated in both organic and mineral horizons of site A soil (Fig. 2): $nirK = 0.9228(nirS) + 1.6802$ ($R^2 = 0.938$, $P < 0.001$). No clear correlation was observed between the two genes in the site B soil: $nirK = 0.869(nirS) + 1.9783$ ($R^2 = 0.196$, $P = 0.075$).

FIG. 1. Real-time PCR quantification of *nifH* (universal) (a), *nifH-g1* (*A. vinelandii* specific) (b), *nirS* (c), and *nirK* (d) in Douglas-fir forest soil under thinning and clear-cut conditions. A2007, A2008, and B2008 refer to the Shawnigan Lake LOGS 2007 sampling, the Shawnigan Lake LOGS 2008 sampling, and the Sayward Forest LOGS 2008 sampling, respectively. The dashed lines separating the LOGS thinning treatments and the clear-cut treatments in the mineral horizon graphs denote that the clear-cut plot was located nearby the LOGS installation at both Shawnigan Lake and Sayward Forest but was not part of the original experimental design and thus not subjected to ANOVA ($n = 9$; ± 1 standard error of the mean).

FIG. 2. Linear regression plot of *nirS* and *nirK* gene abundance at the Shawnigan Lake and Sayward Forest LOGS sites in June 2008. The data points for each site represent the mean of three independent DNA extractions performed from pooled soil samples from each site. The data points for each site include both organic and mineral horizon soil samples.

Relationships between soil nutrients and nitrogen-cycling genes. Correlation-regression analysis was performed on the abundance of *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* gene targets and the amounts of organic and total C, as well as the organic and total N, in the soil for both the organic (Table 4) and mineral (Table 5) soil horizons. The abundance of *nifH*-

universal did not correlate significantly with any soil nutrient in the organic horizon. In the mineral horizon there were weak correlations of *nifH*-universal abundance with OM and total N when data were pooled by sampling point. The *nifH*-g1 target correlated significantly with OM, total C, and total N. For each soil nutrient, *nifH*-g1 abundance correlated at both sites and dates except OM, which did not correlate with *nifH*-g1 at site A (January 2007) sampling. Total C in particular had a strong ($R^2 > 0.5$; $P < 0.05$) relationship with *nifH*-g1 at both sites. The *nifH*-g1 target correlated strongly with OM at site B (June 2008), with total N at site A (June 2008), and with $\text{NH}_4\text{-N}$ at site A (January 2007). The *nirS* gene abundance correlated significantly with OM, total C, and total N in the mineral horizon of site A soil only. The abundance of *nirK* correlated strongly with OM, total C, and total N in the mineral horizon of both sites.

DISCUSSION

Quantification of *nifH* genes. The quantification of *nifH* was not based on cell number, as is typical of culture-based (e.g., most probable number) or visualization-based (e.g., fluorescence *in situ* hybridization) assays, or even some qPCR protocols (20, 38). Relative quantification is also used in many qPCR studies, although due to the lack of normalization it cannot be compared between datasets. We chose a gene-centric approach for two reasons: (i) the plasticity of the *A. vinelandii* genome precluded a cell-based assay for this target, and (ii) we wanted to maintain the ability to compare the assays conducted

TABLE 4. Regression analysis of *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* gene targets at sites A and B versus measurements of soil nutrient status in the organic leaf-litter soil horizon

Soil nutrient	Site and date ^a	<i>nifH</i> -universal			<i>nifH</i> -g1			<i>nirS</i>			<i>nirK</i>		
		XC ^b	R	P	XC	R	P ^c	XC	R	P	XC	R	P
OM (LOI ^d [%])	A2007	-0.02	0.40	0.29	-0.02	0.40	0.29						
	A2008	-0.04	0.30	0.44	-0.04	0.73	0.02	17	0.62	0.08	-0.02	0.62	0.08
	B2008	0.07	0.36	0.29	-0.05	0.79	0.01	20	0.37	0.32	0.01	0.22	0.56
	Total	-0.01	0.14	0.48	-0.05	0.77	<0.01	9.0	0.39	0.11	0.01	0.01	0.89
Total C (%)	A2007	0.01	0.10	0.79	-0.08	0.87	<0.01						
	A2008	-0.05	0.22	0.57	-0.07	0.80	0.01	9.1	0.60	0.09	-0.03	0.58	0.10
	B2008	0.06	0.30	0.44	-0.09	0.81	0.01	18.	0.66	0.05	0.02	0.17	0.65
	Total	-0.02	0.14	0.48	-0.08	0.84	<0.01	4.8	0.37	0.13	<0.01	0.01	0.97
Total N (%)	A2007	1.8	0.17	0.66	-3.33	0.74	0.02						
	A2008	-3.9	0.36	0.33	-3.24	0.74	0.02	0.21	0.69	0.04	-2.01	0.68	0.04
	B2008	0.27	0.20	0.60	-2.84	0.75	0.02	0.39	0.44	0.24	0.75	0.22	0.55
	Total	-0.45	0.10	0.68	-2.8	0.69	<0.01	0.04	0.10	0.66	-0.99	0.20	0.42
$\text{NH}_4\text{-N}$ (ppm)	A2007	0.04	0.30	0.47	-0.04	0.69	0.04						
	A2008	-0.16	0.32	0.42	-0.13	0.57	0.10	2.9	0.48	0.19	-0.04	0.28	0.46
	B2008	0.01	0.52	0.15	-0.01	0.24	0.54	93	0.51	0.16	0.01	0.46	0.21
	Total	0.01	0.20	0.30	-0.01	0.14	0.52	-26	0.35	0.16	-0.01	0.32	0.21
$\text{NO}_3\text{-N}$ (ppm)	A2007	0.33	0.20	0.63	-0.47	0.37	0.33						
	A2008	-0.34	0.26	0.49	0.04	0.10	0.88	0.01	0.10	0.99	0.21	0.45	0.23
	B2008	-0.94	0.32	0.41	-0.63	0.17	0.66	0.09	0.10	0.81	0.76	0.24	0.52
	Total	0.01	0.26	0.98	-0.07	0.01	0.81	0.25	0.22	0.39	0.54	0.37	0.13

^a A2007, Shawnigan Lake, January 2007; A2008, Shawnigan Lake, June 2008; B2008, Sayward Forest, June 2008.

^b XC, X-coefficient.

^c P values that are significant ($\alpha = 0.05$) are indicated in boldface.

^d LOI, loss-on-ignition carbon.

TABLE 5. Regression analysis of *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* gene targets at sites A and B versus measurements of soil nutrient status in the mineral silt loam soil horizon^a

Soil nutrient	Site and date ^b	<i>nifH</i> -universal			<i>nifH</i> -g1			<i>nirS</i>			<i>nirK</i>		
		XC ^c	R	P ^d	XC	R	P	XC	R	P	XC	R	P
OM (LOI ^e [%])	A2007	0.28	0.32	0.41	0.13	0.57	0.11	NA		NA	NA		NA
	A2008	-0.48	0.70	0.04	-0.04	0.17	0.69	2.8	0.28	0.45	-0.11	0.77	0.02
	B2008	-0.17	0.22	0.55	0.15	0.73	0.03	-2.1	0.80	0.01	-0.06	0.62	0.07
	Total	-0.35	0.56	<0.01	-0.01	0.10	0.97	-0.54	0.17	0.47	-0.09	0.71	<0.01
Total C (%)	A2007	-0.22	0.22	0.58	0.13	0.48	0.19	NA		NA	NA		NA
	A2008	-0.93	0.69	0.04	-0.02	0.10	0.93	0.66	0.14	0.73	-0.19	0.71	0.03
	B2008	-0.22	0.26	0.49	0.16	0.70	0.04	-2.1	0.87	<0.01	-0.07	0.68	0.05
	Total	-0.49	0.55	<0.01	0.03	0.10	0.61	-0.89	0.46	0.06	-0.12	0.63	<0.01
Total N (%)	A2007	-3.9	0.10	0.86	1.5	0.10	0.86	NA		NA	NA		NA
	A2008	-36	0.59	0.09	2.5	0.76	0.02	0.02	0.14	0.70	-8.8	0.73	0.03
	B2008	-8.2	0.28	0.46	0.16	0.71	0.04	-0.06	0.91	<0.01	-3.1	0.82	<0.01
	Total	-17	0.51	<0.01	1.5	0.14	0.45	-0.03	0.55	0.02	-4.5	0.69	<0.01
NH ₄ -N (ppm)	A2007	0.21	0.14	0.73	0.34	0.78	<0.01	NA		NA	NA		NA
	A2008	0.97	0.59	0.10	-0.19	0.32	0.41	1.6	0.39	0.30	0.02	0.10	0.86
	B2008	-0.03	0.20	0.59	0.01	0.28	0.45	-8.3	0.55	0.13	-0.01	0.62	0.08
	Total	-0.04	0.17	0.39	0.01	0.14	0.47	-7.3	0.64	<0.01	-0.01	0.26	0.30

^a Mineral soil nitrate levels were below detection limits. NA, not applicable.^b A2007, Shawigan Lake, January 2007; A2008, Shawigan Lake, June 2008; B2008, Sayward Forest, June 2008.^c XC, X-coefficient.^d P values that are significant ($\alpha = 0.05$) are indicated in boldface.^e LOI, loss-on-ignition carbon.

within the present study (e.g., *nifH*-universal versus *nifH*-g1) as well as assays used in other studies and datasets.

A nested protocol was used for quantification *nifH* using a universal primer set. Real-time PCR quantifies gene copies based on fluorescence during the exponential phase of amplification. To increase the sensitivity, a nested approach is used, whereby a larger gene target is first amplified in an initial conventional PCR, with a secondary PCR amplifying a short sequence within the initial amplicon. The shortcomings of this method may include increasing the likelihood of PCR bias by increasing the number of amplifications. However, nested real-time PCR has successfully been used previously to quantitatively detect *Mycobacterium tuberculosis* DNA (49, 50) and qualitatively detect *Aspergillus* DNA (19) in clinical samples, as well as *Helminthosporium solani* ITS DNA from soil (12).

We compared a broad-ranging primer set in a nested PCR for *nifH*-universal amplification (56) with a taxon-specific *nifH* protocol (6). The abundance of the *nifH*-g1 target was considerably less than the total *nifH*-universal abundance, accounting for up to 10% of the total *nifH* community. Some caution is warranted in interpreting this estimate. Although a chemical flocculent was used to counter the inhibitory effects of humic soil substances, high efficiency noted for the *nifH*-universal amplification could reflect the presence of residual inhibitors that would in turn bias the *nifH*-universal measurement. As previously shown (6), the *nifH*-universal primer set amplified genes of *Rhizobium phaseoli*, *Sinorhizobium meliloti*, *R. tropici*, *Clostridium pasteurianum*, *A. vinelandii*, *Pseudomonas stutzeri*, *R. leguminosarum*, *Paenibacillus azotofixans*, and *Nostoc muscorum*. It did not amplify *nifH* genes of *Frankia* sp., *Azoarcus communis*, or *A. brasilense*. The latter result is contradicted by our phylogenetic analysis after *nifH*-universal PCR-DGGE, where the majority of amplified targets were highly homolo-

gous to *A. brasilense nifH* sequences (unpublished data). The *nifH*-g1 primer set was shown to weakly amplify *P. azotofixans* and *Frankia* genes in addition to preferential amplification of *A. vinelandii* and *A. chroococcum* genes (6). Despite the potential for minor amplification of nontarget *nifH* sequences, the *nifH*-g1 melting curves matched *A. vinelandii*-positive controls (unpublished data).

We hypothesized a decrease in microbial functional groups from soil in clear-cut plots due to the disturbance and removal of the organic LFH horizon, and we expected that the retention of a functional leaf litter horizon in thinned plots would preserve functional gene abundance in Douglas-fir forest soil. Symbiotic N₂-fixing bacteria are active primarily in the organic leaf litter soil horizon. Acetylene reduction assays have shown that nitrogen fixation in a Scots pine forest ranged from 0.007 ± 0.002 to 0.342 ± 0.042 nmol of C₂H₄ g (dw)⁻¹ h⁻¹ in the A₀ horizon, depending on the season, moisture, organic material, and soil depth (17). Mineral horizon acetylene reduction ranged from 0.009 ± 0.004 to 0.002 ± 0.001 nmol of C₂H₄ g (dw)⁻¹ h⁻¹ (17). In the present study there were no significant differences in *nifH* gene copies between soil horizons (Fig. 1a and b). However, correlations between soil nutrient values and *nifH*-g1 gene copy number were stronger in the organic horizon, indicating that the asymbiotic N₂-fixing bacteria monitored here greatly influenced their habitat in this niche. In N-limited natural ecological systems these relationships are important for ecological functioning and thus require a greater degree of understanding. In addition, qPCR does not differentiate between active, dormant, and dead cells, nor does it provide information on the activity levels of N₂-fixing bacteria or *nifH* gene expression. Bürgmann et al. developed a protocol for monitoring *nifH*-g1 expression in *A. vinelandii* inoculated liquid and soil culture (5). These authors showed

that expression can be correlated with nitrogenase activity, while neither is indicative of cell density of N_2 -fixing bacteria. The application of this protocol to monitor *in situ* N_2 -fixing populations will greatly assist the elucidation of the effects of environmental disturbance on diazotrophic activity.

The loss of diazotrophic communities may limit the available N in forest soil. Granhall (16) hypothesized that the removal of organic material during clear-cutting would have adverse effects on the sustainability of diazotrophic populations due to the links between soil organic material and diazotrophic activity. Clear-cutting removes *nifH* gene pools (46). In the present study we have shown that although clear-cutting can reduce *nifH* gene abundance (Fig. 1), it does not completely remove the gene from the soil ecosystem. Thus, the removal of the organic horizon during harvesting and the subsequent loss of diazotrophic activity are thought to be of greatest consequence in the loss of ecosystem functioning in harvested plots.

Quantification of *nirS* and *nirK* genes. The *nirS* (3) and *nirK* (8) protocols were based on the *P. aeruginosa* (ATCC 47025) and *P. chlororaphis* (ATCC 13985) nucleotide positions, respectively. Quantitative and melt-curve analysis using qPCR revealed a lack of nontarget positive fluorescence and no detectable primer self-complementation. We found that the *nirS* and *nirK* primers performed robust and specific amplification of target DNA in thinning and clear-cut plots.

Site was a major influence for the abundance of *nirS* and *nirK* genes; their ratio correlated in a linear fashion at the drier Shawngnan Lake site but did not correlate well at the wetter Sayward Forest site. This finding parallels the meta-analysis of Jones and Hallin (23), who hypothesized that *nirK* and *nirS* communities may respond differently to environmental gradients. Unlike *nirK*, there were significantly fewer *nirS* gene copies in the thinned treatments at site B. Site clearly affected the abundance of *nirK* in the organic horizon of thinned plots and in soil in clear-cut plots (Fig. 1d). Clear-cutting can affect denitrifier abundance due to alterations in nutrient status, soil temperature, oxygen content, soil moisture, and pH (53). Site B had a higher productivity than site A and yet had significantly fewer *nirK* gene copies g of soil⁻¹. This can partially be explained by the significantly lower amount of nitrate available in the soil at site B (Table 2). The lack of differences between sites in the mineral soil horizon is likely due to the lack of significant differences in concentrations of organic and total C, total N, ammonium, and nitrate.

Correlations between *nirS* and *nirK* abundance and soil nutrients in the thinning plots further elucidate the interactions between gene copy number and soil nutrient status. The *nirS* and *nirK* genes both catalyze the reduction of nitrite to nitric oxide. These genes do not appear together in the same strain (10) and do not demonstrate any functional differences. Although there is no obvious relationship between these genes, they exhibited a close linear relationship at site A in both soil horizons (Fig. 2). Again, some caution is warranted when interpreting the slope of this trend, because the lower efficiency noted for amplification of *nirS* may indicate the presence of residual inhibitors that could bias that measurement. Above a threshold of available substrate, denitrifying bacteria can facultatively switch to nitrite reduction in the absence of oxygen. These results indicate that organisms containing *nirS* and *nirK*

react in a similar manner to differences in the available nitrite levels in the soil analyzed in the present study.

Relationships between soil nutrients and nitrogen-cycling genes. A previous study of agricultural soils has shown a relationship between soil chemistry and the abundance of *nifH*, *nirS*, and *nirK* fragments (36). In the present study, the majority of relationships between soil nutrients and nitrogen-cycling genes tested did not produce significant correlations (Tables 4 and 5). However, two important patterns emerged following regression analysis in our study that provide insight into the relationships between nitrogen-cycling functional gene abundance and soil nutrient status: (i) *nifH*-g1 primarily correlated with soil C and N in the organic horizon, and (ii) *nirK* primarily correlated significantly with soil C and N in the mineral horizon. The abundance of the *nifH*-g1 gene and the *nirK* gene showed high levels of correlation with soil nutrient values. Correlations between *nifH* gene abundance and soil nutrient concentrations were primarily found in the organic soil horizon. This was expected, since N_2 fixation primarily occurs in this horizon in forest soil.

In previous studies, the effects of C and N quality and quantity on nitrogen fixation have been inconsistent. In general, the availability of labile C stimulates N fixation (7, 26, 27), although not always (25). Nitrogen fixation can also be inhibited (51) or stimulated (40) by available N in the soil. We found that *nifH*-g1 gene abundance increased when OM, total C, and total N declined. As OM increased from 46 to 83%, *nifH*-g1 gene copies g of soil⁻¹ decreased from log 3.39 to log 2.13. Similar relationships were observed with total C and total N. We hypothesize that the fixation of N_2 made available organic N that facilitated the breakdown of soil organic material by fungal and bacterial communities. N_2 fixation is linked to the activity of decay fungi, specifically white-rot fungi, and may act as a catalyst for interlinking ecological events such as litter breakdown (16, 52). Further study is required to test these hypotheses.

The *nirS* and *nirK* qPCR protocol used in the present study also shows potential for relating ecological functioning to functional gene abundance. Rates of denitrification are highly correlated with soil OM (4). The *nirS* gene was significantly correlated with OM, total C, and total N in the mineral horizon soil samples from site B. Correlations between *nirK* abundance and OM, total C, and total N were detected in the mineral horizon at both sites. The strong relationships between *nirS* and *nirK* and soil nutrients in the mineral horizon were unexpected, since denitrification predominates in the organic soil horizon (29), and it was therefore hypothesized that the nutrient values in the organic horizon would be coupled to *nirK* abundance with higher levels of correlation than in the mineral horizon. Denitrification rates in forest soil are influenced greatly by several environmental factors, including temperature and water content (32). The nature of the relationships between *nirS* and *nirK* gene abundance and soil temperature and moisture are currently unknown, although these factors may have greater influence at the upper soil horizons, leading to the differences observed in the present study.

One fundamental objective of the present study was to assess tools for researchers and forest managers to better understand the effects of thinning and clear-cutting on the abundance N_2 fixers and denitrifiers and to elucidate the interactions between

soil nutrient status and N-cycling microorganisms. To do this, we applied qPCR protocols for *nifH*-universal, *nifH*-g1, *nirS*, and *nirK* quantification from previously designed PCR primer sets and developed standard curves for their use. We showed that retention of as little as 10% of the tree volume of an unthinned control stand would not significantly reduce *nifH*, *nirS*, and *nirK* abundance in most cases. However, reductions in N-cycling genes were observed in the clear-cut plots of both sites, and nutrient levels were greatly reduced due to the removal of the organic horizon from these soils. Strong correlations between *nifH* and soil C and N in the organic horizon, as well as between *nirS*, *nirK*, and soil C and N in the mineral horizon demonstrate the functional relationship between gene abundance and soil nutrient status. The retention of the soil organic leaf-litter horizon, where the majority of nutrient-cycling events take place in forest soil ecosystems, contributes greatly to the preservation of a functioning soil ecosystem.

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REFERENCES

- Beddows, D. 2002. Levels-of-growing-stock cooperative study in Douglas-fir: report no. 16—Sayward Forest and Shawnigan Lake. Information report BC-X-393. Canadian Forest Service, Pacific Forestry Centre, Victoria, British Columbia, Canada.
- Bottomley, P. J., A. E. Taylor, S. A. Boyle, S. K. McMahon, J. J. Rich, D. Cromack, Jr., and D. D. Myrold. 2004. Responses of nitrification and ammonia-oxidizing bacteria to reciprocal transfers of soil between adjacent coniferous forest and meadow vegetation in the Cascade Mountains of Oregon. *Microb. Ecol.* **48**:500–508.
- Braker, G., A. Fesefeldt, and K. P. Witzel. 1998. Development of PCR primer systems for amplification of nitrite reductase genes (*nirK* and *nirS*) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol.* **64**:3769–3775.
- Burford, J. R., and J. M. Bremner. 1975. Relationships between the denitrification capacities of soils and total, water-soluble, and readily decomposable soil organic matter. *Soil Biol. Biochem.* **7**:389–394.
- Bürgmann, H., F. Widmer, W. V. Sigler, and J. Zeyer. 2003. mRNA extraction and reverse transcription-PCR protocol for detection of *nifH* gene expression by *Azotobacter vinelandii* in soil. *Appl. Environ. Microbiol.* **69**:1028–1035.
- Bürgmann, H., F. Widmer, W. Von Sigler, and J. Zeyer. 2004. New molecular screening tools for analysis of free-living diazotrophs in soil. *Appl. Environ. Microbiol.* **70**:240–247.
- Bürgmann, H., S. Meier, M. Bunge, F. Widmer, and J. Zeyer. 2005. Effects of model root exudates on structure and activity of a soil diazotroph community. *Environ. Microbiol.* **7**:1711–1724.
- Chénier, M. R., D. Beaumier, R. Roy, B. T. Driscoll, J. R. Lawrence, and C. W. Greer. 2003. Impact of seasonal variations and nutrient inputs on nitrogen cycling and degradation of hexadecane by replicated river biofilms. *Appl. Environ. Microbiol.* **69**:5170–5177.
- Cleveland, C. C., A. R. Townsend, D. S. Schimel, H. Fisher, R. W. Howarth, L. O. Hedin, S. S. Perakis, E. F. Latty, J. C. Von Fischer, A. Elseroad, and M. F. Wasson. 1999. Global patterns of terrestrial biological nitrogen (N_2) fixation in natural ecosystems. *Global Biogeochem. Cycle* **13**:623–645.
- Coyne, M. S., A. Arunakumari, A. Averill, and J. M. Tiedje. 1989. Immunological identification and distribution of dissimilatory heme *cd*₁ and non-heme copper nitrite reductases in denitrifying bacteria. *Appl. Environ. Microbiol.* **55**:2924–2931.
- Cracknell, P. C., and J. D. Lousier. 1988. Improving forest fertilization decision-making in British Columbia, p. 293. In J. D. Lousier, H. Brix, R. Brockley, R. Carter, and V. G. Marshall (ed.), *Improving forest fertilization decision-making in British Columbia: Proceedings of a Forest Fertilization Workshop*, March 1988. Research Branch, Ministry of Forests, Victoria, British Columbia, Canada.
- Cullen, D. W., A. K. Lees, I. K. Toth, and J. M. Duncan. 2001. Conventional PCR and real-time quantitative PCR detection of *Helminthosporium solani* in soil and on potato tubers. *Eur. J. Plant Pathol.* **107**:387–398.
- DeLuca, T. H., and K. L. Zouhar. 2000. Effects of selection harvest and prescribed fire on the soil nitrogen status of ponderosa pine forests. *Forest Ecol. Manag.* **138**:263–271.
- Diggle, P. K. 1972. Levels of growing stock cooperative study in Douglas fir in British Columbia: report no. 3. Information report BC-X-66. Environment Canada, Victoria, British Columbia, Canada.
- Fogel, G. B., C. R. Collins, J. Li, and C. F. Brunk. 1999. Prokaryotic genome size and SSU rDNA copy number: estimation of microbial relative abundance from a mixed population. *Microb. Ecol.* **38**:93–113.
- Granhall, U. 1981. Biological nitrogen fixation in relation to environmental factors and functioning of natural ecosystems, p. 131–144. In P. Clark and T. Rosswall (ed.), *Terrestrial nitrogen cycles*. Ecological Bulletin, Stockholm, Sweden.
- Granhall, U., and T. Lindburg. 1978. Nitrogen fixation in some coniferous forest ecosystems, p. 178–192. In U. Granhall (ed.), *Environmental role of nitrogen-fixing blue-green algae and symbiotic bacteria*. Ecological Bulletin, Stockholm, Sweden.
- Grothues, D., and B. Tümmeler. 1991. New approaches in genome analysis by pulsed-field gel electrophoresis: application to the analysis of *Pseudomonas* species. *Mol. Microbiol.* **5**:2763–2776.
- Halliday, C., Q. W. Wu, G. James, and T. Sorrell. 2005. Development of a nested qualitative real-time PCR assay to detect *Aspergillus* species DNA in clinical specimens. *J. Clin. Microbiol.* **43**:5366–5368.
- Harrow, S. A., V. Ravindran, R. C. Butler, J. W. Marshall, and G. W. Tannock. 2007. Real-time quantitative PCR measurement of ileal *Lactobacillus salivarius* populations from broiler chickens to determine the influence of farming practices. *Appl. Environ. Microbiol.* **73**:7123–7127.
- Henry, S., E. Baudoin, J. C. López-Gutiérrez, F. Martin-Laurent, A. Brauman, and L. Philippot. 2004. Quantification of denitrifying bacteria in soils by *nirK* gene targeted real-time PCR. *J. Microbiol. Methods* **59**:327–335.
- Holmes, W. E., and D. R. Zak. 1999. Soil microbial control of nitrogen loss following clear-cut harvest in northern hardwood ecosystems. *Ecol. Appl.* **9**:202–215.
- Jones, C. M., and S. Hallin. 2010. Ecological and evolutionary factors underlying global and local assembly of denitrifier communities. *ISME J.* **4**:633–641.
- Kandeler, E., K. Deiglmayr, D. Tscherko, D. Bru, and L. Philippot. 2006. Abundance of *narG*, *nirS*, *nirK*, and *nosZ* genes of denitrifying bacteria during primary successions of a glacier foreland. *Appl. Environ. Microbiol.* **72**:5957–5962.
- Keeling, A. A., J. A. Cook, and A. Wilcox. 1998. Effects of carbohydrate application on diazotroph populations and nitrogen availability in grass swards established in garden waste compost. *Bioresour. Technol.* **66**:89–97.
- Kondo, M., and M. Yasuda. 2003. Effects of temperature, water regime, light, and soil properties on N_2 - 15 fixation associated with decomposition of organic matter in paddy soils. *Jpn. Agric. Res. Q.* **37**:113–119.
- Kondo, M., and M. Yasuda. 2003. Seasonal changes in N_2 fixation activity and N enrichment in paddy soils as affected by soil management in the northern area of Japan. *Jpn. Agric. Res. Q.* **37**:105–111.
- Larson, M. G., M. F. Jurgensen, and A. E. Harvey. 1978. N_2 -fixation associated with wood decayed by some common fungi in western Montana. *Can. J. For. Res.* **8**:341–345.
- Laverman, A. M., H. R. Zoomer, D. Engelbrecht, M. P. Berg, M. van Straalen, H. W. van Verseveld, and H. A. Verhoef. 2000. Soil horizon-specific variability in net nitrification and denitrification in an acid coniferous forest. *Biol. Fertil. Soils* **32**:427–434.
- Lerat, S., L. S. England, M. L. Vincent, K. P. Pauls, C. J. Swanton, J. N. Klironomos, and J. T. Trevors. 2005. Real-time polymerase chain reaction quantification of the transgenes for Roundup Ready corn and Roundup Ready soybean in soil samples. *J. Agric. Food Chem.* **53**:1337–1342.
- López-Gutiérrez, J. C., S. Henry, S. Hallet, F. Martin-Laurent, G. Catroux, and L. Philippot. 2004. Quantification of a novel group of nitrate-reducing bacteria in the environment by real-time PCR. *J. Microbiol. Methods* **57**:399–407.
- Maag, M., and F. P. Vinther. 1996. Nitrous oxide emission by nitrification and denitrification in different soil types and at different soil moisture contents and temperatures. *Appl. Soil Ecol.* **4**:5–14.
- Maldonado, F., J. Jiménez, and J. Casadesús. 1994. Changes in ploidy during the *Azotobacter vinelandii* growth cycle. *J. Bacteriol.* **176**:3911–3919.
- Manna, A. C., and H. K. Das. 1997. The *Azotobacter vinelandii* chromosome. *J. Genet.* **76**:55–60.
- Martin-Didonet, C. C. G., L. S. Chubatsu, E. E. Souza, M. Kleina, F. G. M. Rego, L. U. Rigo, M. G. Yates, and F. G. Pedrosa. 2000. Genome structure of the genus *Azospirillum*. *J. Bacteriol.* **182**:4113–4116.
- Morales, S. E., T. Cosart, and W. E. Holben. 2010. Bacterial gene abundances as indicators of greenhouse gas emission in soils. *ISME J.* **4**:799–808.
- Nagpal, P., S. Jafri, M. A. Reddy, and H. K. Das. 1989. Multiple chromosomes of *Azotobacter vinelandii*. *J. Bacteriol.* **171**:3133–3138.
- Okano, Y., K. R. Hristova, C. M. Leutenegger, L. E. Jackson, R. F. Denison, B. Gebreyesus, D. Lebauer, and K. M. Scow. 2004. Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl. Environ. Microbiol.* **70**:1008–1016.

39. Philippot, L., and J. C. Germon. 2005. Contribution of bacteria to initial input and cycling of nitrogen in soils, p. 159–176. *In* F. Buscot and A. Varma (ed.), *Microorganisms in soils: roles in genesis and functions*. Springer, Berlin, Germany.
40. Poly, F., L. Ranjard, S. Nazaret, F. Gourbiere, and L. J. Monrozier. 2001. Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. *Appl. Environ. Microbiol.* **67**:2255–2262.
41. Prescott, C. E. 2002. The influence of the forest canopy on nutrient cycling. *Tree Physiol.* **22**:1193–1200.
42. Priemé, A., G. Braker, and J. M. Tiedje. 2002. Diversity of nitrite reductase (*nirK* and *nirS*) gene fragments in forested upland and wetland soils. *Appl. Environ. Microbiol.* **68**:1893–1900.
43. Qiu, X. Y., R. A. Hurt, L. Y. Wu, C. H. Chen, J. M. Tiedje, and J. Z. Zhou. 2004. Detection and quantification of copper-denitrifying bacteria by quantitative competitive PCR. *J. Microbiol. Methods* **59**:199–210.
44. Robertson, G. P., and J. M. Tiedje. 1987. Nitrous oxide sources in aerobic soils: nitrification, denitrification and other biological processes. *Soil Biol. Biochem.* **19**:187–193.
45. Rösch, C., A. Mergel, and B. Hermann. 2002. Biodiversity of denitrifying and dinitrogen-fixing bacteria in an acid forest soil. *Appl. Environ. Microbiol.* **68**:3818–3829.
46. Shaffer, B. T., F. Widmer, L. A. Porteous, and R. J. Seidler. 2000. Temporal and spatial distribution of *nifH* gene of N₂-fixing bacteria in forests and clearcuts in Western Oregon. *Microb. Ecol.* **39**:12–21.
47. Smith, N. R., B. E. Kishchuk, and W. W. Mohn. 2008. Effects of wildfire and harvest disturbances on forest soil bacterial communities. *Appl. Environ. Microbiol.* **74**:216–224.
48. Stover, C. K., X. Q. Pham, A. L. Erwin, S. D. Mizoguchi, P. Warrenner, M. J. Hickey, F. S. Brinkman, W. O. Hufnagle, D. J. Kowalik, M. Lagrou, R. L. Garber, L. Goltry, E. Tolentino, S. Westbrook-Wadman, Y. Yuan, L. L. Brody, S. N. Coulter, K. R. Folger, A. Kas, K. Larbig, R. Lim, K. Smith, D. Spencer, G. K. Wong, Z. Wu, I. T. Paulsen, J. Reizer, M. H. Saier, R. E. Hancock, S. Lory, and M. V. Olson. 2000. Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**:959–964.
49. Takahashi, T., and T. Nakayama. 2006. Novel technique of quantitative nested real-time PCR assay for *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* **44**:1029–1039.
50. Takahashi, T., M. Tamura, Y. Asami, E. Kitamura, K. Saito, T. Suzuki, S. N. Takahashi, K. Matsumoto, S. Sawada, E. Yokoyama, and T. Takasu. 2008. Novel wide-range quantitative nested real-time PCR assay for *Mycobacterium tuberculosis* DNA: development and methodology. *J. Clin. Microbiol.* **46**:1708–1715.
51. Tan, X. Y., T. Hurek, and B. Reinhold-Hurek. 2003. Effect of N-fertilization, plant genotype and environmental conditions on *nifH* gene pools in roots of rice. *Environ. Microbiol.* **5**:1009–1015.
52. Thompson, M. V., and P. M. Vitousek. 2006. Asymbiotic nitrogen fixation and litter decomposition on a long soil-age gradient in Hawaiian montane rain forest. *Biotropica* **29**:134–144.
53. Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179–244. *In* A. J. B. Zehnder (ed.), *Biology of anaerobic microorganisms*. John Wiley & Sons, Inc., New York, NY.
54. Wakelin, S. A., M. J. Colloff, P. R. Harvey, P. Marschner, A. L. Gregg, and S. L. Rogers. 2006. The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundance under irrigated maize. *FEMS Microbiol. Ecol.* **59**:661–670.
55. Wallenstein, M. D., and R. J. Vitousek. 2005. Quantitative analyses of nitrogen cycling genes in soils. *Pedobiologia* **49**:665–667.
56. Widmer, F., B. T. Shaffer, L. A. Porteous, and R. J. Seidler. 1999. Analysis of *nifH* gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. *Appl. Environ. Microbiol.* **65**:374–380.
57. Wolf, I., and R. Brumme. 2002. Contribution of nitrification and denitrification sources for seasonal N₂O emissions in an acid German forest soil. *Soil Biol. Biochem.* **34**:741–744.