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Using metagenomics to reveal landscape scale patterns of denitrifiers in a montane forest ecosystem

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ABSTRACT

Denitrificationis an important process in the nitrogen cycle of many soil ecosystems, but the relationships between process rates and the genotype of denitrifying microorganisms are poorly understood. Genotyping may identify denitrifiers with less than the full complement of nitrogen-oxide reductases, which might be crucial for denitrification in nitrogen-limited environments, such as in montane forest landscapes. Therefore, a metagenomics survey was undertaken using soils from the Hubbard Brook Experimental Forest (HBEF) in New Hampshire, USA where steep elevation, vegetation, and soil gradients provide a complex landscape matrix to assess occurrence patterns of the genes involved in denitrification. DNA was extracted from soils taken from three soil horizons, at three elevations, in two watersheds. Metagenomic analysis of reads showed that the relative abundance of denitrification genes within a community did not differ across soil depths but did vary among elevation zones, with total denitrification reads in High Hardwood > Spruce Fir > Low Hardwood. Reads from *nirS* were extremely rare, which suggests that complete denitrification is uncommon across this forest landscape. The gene with the largest proportion of denitrification specific reads was the quinol-oxidizing nitric oxide reductase, *qnor*, which reduces toxic nitric oxide to nitrous oxide. The relative enrichment of specialized denitrification genes involved in intermediate reactions may indicate that environmental factors are selecting for a partial denitrification process, rather than complete denitrification. High Hardwood soils had the highest denitrification gene abundance and the greatest potential rate of denitrification, indicating that metagenomic information was consistent with the process measurements. Although little energy is generated from complete denitrification, due to the acidic soil conditions and low nitrate availability in HBEF soils, the denitrifier community appears to compensate by producing particular denitrification genes. In particular, *qnor* may help the community cope with toxic nitric oxide produced via chemodenitrification, making it a public good.

1. Introduction

Denitrification, which results in the conversion of nitrate (NO_3^-) to nitrite (NO_2^-) and then nitric oxide (NO), nitrous oxide (N_2O), and finally to dinitrogen (N_2) (Zumft, 1997), is an important reaction in the nitrogen (N) cycle of many soils. It is generally agreed that denitrification is a tightly coupled pathway, and thus measurements of denitrification can be inferred as providing information about the occurrence of all denitrification genes in the community. However, recent genomic studies have shown that partial denitrifiers, that is denitrifiers lacking the complete pathway, might be a common occurrence (Roco et al., 2017; Hallin et al., 2018). The high occurrence of partial denitrifiers implies that the notion of denitrification as a tightly coupled pathway for the denitrifier community to generate energy is inadequate, and thus environmental conditions are likely impacting the gene occurrence and preference for different steps in the pathway.

Upland forest ecosystems in the northeastern USA are an ideal location to study how the environment controls the denitrification rate and occurrence and denitrifier genotype. Denitrification is responsible for NO_3^- removal in these ecosystems (Houlton and Bai, 2009; Fang

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et al., 2015; Yu et al., 2016), even though the environment does not appear to be conducive to the denitrification process. Many forest soils are coarse, well-drained and aerated, which is at odds with the hypoxic oxygen (O_2) conditions favored by denitrifiers (Smith and Tiedje, 1979). In addition, soil NO_3^- levels are low due to tree growth and a gradual decline in atmospheric N deposition across the northeastern USA (reviewed in Groffman et al., 2018). Another possible confounding factor is acidic soil pH that is characteristic of many forest soils (cf., Johnson et al., 2014). Low pH inactivates the last step of denitrification, which could limit its selective benefit for denitrifiers and their occurrence in the community (Bergaust et al., 2010). On the other hand, NO_2^- in acidic soils is rapidly converted to toxic NO in an abiotic process termed chemodenitrification (Chalk and Smith, 1983; Lim et al., 2018), and the biological conversion of NO to non-toxic N_2O could be beneficial. Therefore, upland forest soils seem to require a denitrification community that is adapted for sporadic denitrification which may encourage the occurrence of partial denitrifiers.

The montane landscape where upland forests are found presents a complex environment to evaluate denitrification dynamics. For example, elevation and topography have important influences on soil characteristics, plant community composition, and nutrient cycling (Bohlen et al., 2001), and these factors are not necessarily independent of one another. Soil NO_3^- availability is often greater at higher than lower elevations because plant growth and N uptake decrease at higher elevations that are subjected to stresses from cold temperatures, high winds, and other environmental stressors that are more severe on hilltops (Bohlen et al., 2001). This pattern is complicated by hillslope hydrology, which depends on soil water flow paths downslope that can result in complex patterns of soil conditions in relation to elevation that are not obvious at the surface (Werner et al., 2011; Bailey et al., 2014). These watershed level differences result in denitrification rates being patchy in montane forested landscapes (Kulkarni et al., 2015, 2017; Morse et al., 2015). It is not clear if this patchiness arises only from changes in gene occurrence or if these changes in gene occurrence correlate with changes in genotype that indicate a more frequent reliance on denitrification (Hallin et al., 2018).

The work described here was undertaken to evaluate two hypotheses: 1) the denitrifier community in upland forests will show unique features as a consequence of adapting to environmental conditions seemingly inimical to the denitrification process, and 2) landscape scale differences are significant enough to drive localized changes in denitrification gene occurrence and/or genotype. A metagenomics approach was used to provide the genotypic resolution needed to assess levels of all critical genes (Daniel, 2005). The site chosen for this work was the Hubbard Brook Experimental Forest (HBEF) in New Hampshire, USA where long-term N budget studies (Yanai et al., 2013) have indicated a significant, unmeasured N sink that might be attributed to gaseous losses of denitrification products (Kulkarni et al., 2008) making it ideal for addressing hypothesis 1. Although denitrification occurs widely in the well-drained HBEF soils (Kulkarni et al., 2014) little is known about the controls on, or traits of the bacterial community responsible for the process. To address hypothesis 2, watersheds at the HBEF that represented a range of environmental and vegetation characteristics were sampled. Within these watershed three elevation zones were chosen based on known differences in soil NO_3^- levels and soil carbon content, suspected controllers of denitrification activity, and one watershed had received a calcium addition that raised soil pH, another suspected controller of denitrification activity (Fahey et al., 2015). The metagenomic survey was supplemented by measuring denitrification rates using a robotic system that allows quantification of multiple intermediates in the denitrification pathway (Molstad et al., 2007). This was done to better link gene occurrence with activity, because if partial denitrification is prominent, the reduction of NO_3^- could be bottlenecked at particular steps in the pathway if specific genes are less abundant or proteins are not active.

2. Materials and methods

2.1. Study area and sampling

The study site was the Hubbard Brook Experimental Forest (HBEF), located in north-central New Hampshire, USA (43°56′ N, 71°45′ W). The site has been used for numerous forest research studies and is part of the Long Term Ecological Research (LTER) network (Fahey et al., 2015). Briefly, the climate is humid-continental with short, cool summers and long, cold winters. Soils are predominantly well-drained Spodosols (Haplorthods) and Inceptisols (Dystrochrepts) of sandy-loam texture formed from glacial till. The dominant vegetation is northern hardwood forest. The highest elevation plots contain a mixture of red spruce [*Picea rubens* Sargent], balsam fir [*Abies balsamea* (L.) Mill], and paper birch [*Betula papyrifera* var. *cordifolia* (Marsh.) Regel]. Sites at this elevation are referred to as Spruce Fir. The forests at lower elevations are dominated by a roughly equal mixture of sugar maple [*Acer saccharum* Marshall], yellow birch [*Betula alleghaniensis* Britton], and American beech [*Fagus grandifolia* Ehrh.] and are divided into High Hardwood zone at higher elevation and Low Hardwood at lower elevations. Other distinctive features of these elevation zones are described in Fahey et al. (2015). The HBEF is mostly second-growth forest developed following logging in the late 19th and early 20th centuries. Some areas were affected by a hurricane in 1938 as well as an intense ice storm in 1998 (Rhoads et al., 2002).

The study described here took place in two watersheds at the HBEF. One was the Bear Brook watershed, which is just west of the long-term reference watershed (WS6). Soil pH in the combined Oi/Oe layer in Bear Brook is on average 3.9. The other was watershed 1 (WS1), where in October 1999 (soon after leaf fall) 40.8 Mg of powdered and pelletized wollastonite ($CaSiO_3$) was evenly distributed by helicopter (Peters et al., 2004). The wollastonite application contained 1028 kg calcium (Ca) ha^{-1} and was designed to increase soil pH and exchangeable soil Ca and to decrease exchangeable aluminum in the soils. By 2012, soil pH in the Oi/Oe layer of WS1 averaged about 4.7 (Johnson et al., 2014). Elevations in both watersheds range from 488 to 747 m, and each watershed has an area of about 12 ha. Both watersheds are south facing, with slopes ranging from 5 to 25% (Fahey et al., 2005).

In July 2012, soil was collected from a subset of five replicate plots established by Fiorentino et al. (2003) in the Spruce Fir, High Hardwood, and Low Hardwood elevations in both watersheds. Two soil samples were taken from each of the 30 sampling plots (3 elevations · 5 replicate plots · 2 watersheds) and composited by distinct horizons: 1) organic Oi/Oe, 2) organic/mineral Oa/A, and 3) the top 10 cm of the B horizon. Once soils were collected, they were sieved (using sieve number 4) to remove large debris and create uniformity across the sample. After sieving the samples were split into two portions. One was stored at −20 °C between sampling and analysis (less than 1 week) for DNA. The other portion was kept at 4 °C until sent chilled to Norway (within a week of collection) where they were further stored at 4 °C in the dark until used for denitrification analyses (where stored for two weeks maximum). Soil moisture content was determined by drying at 60 °C for 48 h on fresh soil collected and sent to the lab chilled within a week of collection.

2.2. DNA extraction and shotgun sequencing of environmental metagenomes

DNA was extracted using the MoBio PowerSoil DNA Isolation Kit following the manufacturer's protocol. Within each starting soil sample, triplicate DNA extractions from the same soil sample were combined to account for inter-sample variability and to ensure enough quantity for sequencing. The experimental design resulted in 18 samples (2 watershed, 3 elevations per watershed, 3 soil depths site). Six additional samples were collected from the High Hardwood region in the Bear Brook watershed at two soil depths (Oi/Oe, B horizons) for purpose of

QA/QC. We could not use the larger sample size at all elevations, all soil depths, and in both watersheds as the cost for sequencing would have been prohibitive. Thus, we used one set of larger sample size to assess variations across sequencing metrics seen within a specific sample. This yielded 24 DNA samples in total that were sequenced at the JP Sulzberger Columbia Genome Center in New York, NY USA. Libraries for each metagenome were generated using the Illumina HiSeq next generation sequencing platform, yielding 2 x 100 bp paired end reads for each of the samples. On average each sample had 22,048,834 ± 1,009,491 reads. The raw reads were uploaded to the European Nucleotide Archive and their primary accession ID is PRJEB24179.

2.3. Shotgun metagenomic sequence processing and analysis

The denitrifier genes are given in Shapleigh (2013). Initial quality assurance/quality control (QA/QC) was carried out by the sequencing center, which included trimming sequencing adapters and bar codes from sequence reads. Additional QA/QC was performed using the CLC Genomics Workbench 4.0, which involved trimming low-quality reads and reads of < 80 bp. Some reads lost their pair after QA/OC analysis, but unpaired reads of sufficient quality were retained and included in later analysis where possible.

Custom datasets were used to identify reads assigned to the denitrification genes. Datasets consisted of a manually-curated set of full-length protein sequences derived from sequenced genomes in the IMG database (https://img.jgi.doe.gov/cgi-bin/m/main.cgi). These datasets were manually curated to contain diverse sequences while at the same time limiting multiple sequences from heavily sequenced species and are available at http://hdl.handle.net/1813/57594. For functional annotation, reads were aligned against the protein datasets using DIAMOND with an e-value cutoff of 1×10^{-3} (Buchfink et al., 2015). The DIAMOND output was converted to m8 blast format and analyzed in R. Reads were assigned as being from a particular gene if the aligned region was > 25 amino acids with an identity of > 60%. The method used for assigning *nosZ* reads to particular clades is described in Nadeau et al. (2019). Briefly, a functional analysis of reads was carried out using DIAMOND but with a database of *nosZ* of known clade assignment. Reads were assigned to a clade if the top three hits in the database were from the same clade. Otherwise a read was described as ambiguous. The method used to assign reads to particular taxonomic groups is described in Morse et al. (2018). Briefly, reads identified through DIAMOND analysis as coming from a particular gene were compared with a DNA sequence data set of that particular gene using a kmer-based approach.

Metagenome sample comparisons and clustering was carried out using Minhash ver. 1.1 (Ondov et al., 2016). The output was plotted using the Principal Coordinates Analysis (PCoA) function in the LabDSV: Ordination and Multivariate Analysis for Ecology package in R (R Core Team, 2017).

2.4. 16S read extraction and characterization

Reads from the 16S rRNA encoding region of bacterial genomes was extracted using a kmer-based approach. Bbduk (BBMap - Bushnell B. - sourceforge.net/projects/bbmap/) was used to extract reads by running it in filtering mode. The reference kmer dataset was compiled by Brian Bushnell from the Silva database (https://drive.google.com/file/d/0B3llHR93L14wS2NqRXpXakhFaEk/view?usp=sharing). Bbduk was used with a default kmer length of k = 31. Reads extracted using bbduk were clustered with vsearch with an id = 0.97. The clusters were then matched using vsearch to the Silva SSUref NR99 dataset to assign taxonomy. All further calculations were run in R.

2.5. Denitrification rates and kinetics

Denitrification was measured *ex situ* in soil samples from the Oa/A and B horizons at all three elevations of Bear Brook watershed by anoxic batch incubation (Molstad et al., 2007). Five grams of fresh weight soil, each from five distinct field plots per elevation, were placed in two 120 ml serum bottles, equipped with a magnetic stirring bar and suspended in 40 ml of a 1 mM KNO_3 solution. To test for potential carbon (C)-limitation, one of the bottles received additional, pH-adjusted (pH = 7) glutamic acid to a final concentration of 1 mM. After crimp sealing with butyl septa, the bottles were made anoxic by six cycles of evacuation and He-filling while stirring the suspensions vigorously. The bottles were placed in a water bath adjusted to 15 °C and incubated for 200 h at 15 °C with continuous stirring at 400 rpm. Headspace concentrations of N_2, O_2, CO_2, CH_4, H_2, NO and N_2O were monitored by means of an automated GC set up described in detail by Molstad et al. (2016). In brief, the set up consists of a water bath, an automated sampling robot (CTC GC PAL), a gas chromatograph (Agilent GC -7890A) equipped with three detectors (ECD, TCD, FID), a Chemiluminescence NOx analyzer (Model 200A, Advanced Pollution Instrumentation, San Diego, USA) and a RCP-H_2 analyzer (Peak Laboratories, CA, USA). The set-up holds up to 30 constantly stirred 120 ml bottles and additional bottles for standards of CO_2, CH_4, H_2, N_2O in He and NO in N_2 (AGA). Air was used as standard for N_2 and O_2. Headspace samples (approx. 1 ml) were taken every 5 h via a needle mounted on the robotic arm of the autosampler and transferred to the measurement system by a peristaltic pump (Gilson). To maintain bottle pressure at ~1 atm, the pump was reversed after each sampling, pumping back a mixture of residual sample and pure He. All gas data were corrected for dilution and aqueous dissolution as described in Molstad et al. (2007). The incubation experiments were terminated after 200 h when NO_3^--N added to flasks was recovered as N_2. Periodically during the incubation period, two to three sample flasks of each soil were sacrificed to determine concentrations of NO_3^- and NO_2^-. To do so, a portion of the soil was crushed in a mortar and taken up with 0.9% NaCl solution. The turbid fluid was collected in Eppendorf tubes and centrifuged (10,000 rpm, 5 min) to obtain a clear solution, and NO_3^- and NO_2^- was quantified using standard colorimetric methods (Doane and Horwáth, 2003; Keeney and Nelson, 1982).

The high-resolution gas kinetics were used to estimate denitrification rates (sum of NO + N_2O + N_2 accumulation, expressed as μg N g $soil^{-1}$ h^{-1}) and gaseous product stoichiometries (N_2O, NO, N_2). Denitrification rates were calculated using the initial 60 h of incubation, however, product accumulation was monitored for 200 h. The longer time frame was not used due to concerns about growth and bottle effects. The efficiency of N_2O reductase (N_2OR) induction was estimated from the amount of N denitrified before $N_2O/(N_2O + N_2)$ ratios dropped below 1 (expressed as N denitrified before induction of N_2OR), suggesting a change in soil conditions whereupon significant gross reduction of N_2O was associated with alkalization of the slurry (Zhu et al., 2013).

2.6. Ancillary soil measurements

Soil pH was measured in the slurries before and after incubation by a pH-meter (Hach H170). After the incubation, the bottles were dried at 60 °C to determine soil dry weight. To further normalize measured process rates, the organic carbon (C_{org}) and organic N content of the soil samples was determined by an EA-IRMS (isotope ratio mass spectrometer coupled with an element analyzer, Thermo Finnigan MAT, Bremen, Germany) equipped with a thermal conductivity detector against EDTA as a standard. To simultaneously measure the abundance of $^{15/14}N$ ratio of the bulk soil as $\delta^{15}N$ against air, IAEA N1 ($\delta^{15}N = 4.7‰_{air\ N2}$) and IAEA N3 ($\delta^{15}N = 0.4‰_{air\ N2}$) standards were included. The precision of the analysis was < 0.2‰.

2.7. Environmental models

Log-linear models were used for differential analysis of read abundances across levels of experimental factors (elevation, soil depth, and watershed). The analyses were performed using R. For example, to compare the abundance of gene j at the different soil depths, the base log-linear model has the form

$$\log(\mu_{ijk}) = \beta_j + \beta_{jk} + \log(N_{ik})$$

where μ_{jk} is the expected read count for gene j in sample i at depth k. The $\log(N_{ik})$ term is an offset to account for the total read count, N_{ik}, in the sample. Standard errors of coefficient estimates were adjusted for over-dispersion between samples with respect to Poisson variation using the *quasipoisson* option. Since the read counts for a particular gene are a tiny fraction of the total for a given sample, the log-linear analysis is almost identical to that based on an over-dispersed binomial model. The base (one-factor) model is easily modified to allow for a multi-factor analysis to determine, for example, if differential abundances between soil depths are consistent at different elevations.

All figures showing data derived from read number comparisons were produced using the *emmeans* R package. They show confidence intervals for read "rates" derived from the log-linear model for the six denitrification genes, *nap*, *nar*, *nirK*, *cnorB*, *qnor* and *nosZ*, at three depths, Oi/Oe, Oa/A and Min, and three elevations, Spruce Fir, High Hardwood and Low Hardwood. The rates are the relative frequency of reads for each gene at each depth (or elevation) multiplied by the average of the total read counts for all samples. Confidence intervals are derived from the log-linear model fit by taking the exponential of the lower and upper endpoints of confidence intervals for the log of the expected rates. For each gene, pairwise comparisons between the three depths (or elevations) are made using Tukey's HSD method with a 5% significance level (Tukey, 1949). Note that it is possible for a pair of gene expression rates to be significantly different even if the corresponding 95% confidence intervals overlap. Community level comparisons based on 16S reads extracted from the metagenome were done using the same approach as for the denitrification reads. The relationship between denitrification gene read counts and soil parameters was also analyzed using a quasi-Poisson model with a log link. Values for the soil parameters (NO_3^-, NH_4^+, Soil Moisture Content, and Soil Carbon) are presented in Supplementary Table S1.

3. Results

3.1. Bacterial community composition

An assessment of differences in community composition was undertaken using the complete read set. A principal-coordinates analysis (PCoA) of this analysis indicated only minimal dissimilarities among the samples (Fig. 1). Samples from the High Hardwood sites were distinct from the Low Hardwood and Spruce Fir samples, which overlapped each other. Likewise, samples from the B horizon were distinct from the organic Oi/Oe and organic/mineral Oa/A samples, which overlapped each other.

Taxonomic assignment of the reads assigned to the 16s rRNA genes indicated that members of the Proteobacteria and Acidobacteria phyla were equally dominant, ranging from 30 to 40%, across all of the reads from each sample (Fig. 2). Members of the Actinobacteria were about 10% of the reads. Other phyla, such as the Verrucomicrobia, Planctomycetes, and Bacteroidetes made up about 5% of the total (Fig. S1). When grouped by elevation there were no significant differences in the occurrence of the top three phyla (Fig. 2b). However, significant differences were seen when grouped by soil depth (Fig. 2a). For example, the relative proportion of Actinobacteria and Proteobacteria were lower in the mineral soil ($P < 0.05$).

3.2. Genetic potential for denitrification

The prevalence of denitrification genes at the sites was initially analyzed by considering the complete denitrification pathway. To do this, reads assigned as coming from genes encoding the catalytic subunits of periplasmic NO_3^- reductase (*nap*), respiratory NO_3^- reductase (*nar*), copper containing NO_2^- reductase (*nirK*), cd_1-type NO_2^- reductase (*nirS*), cytochrome *c* oxidizing NO reductase (*cnor*), quinol oxidizing N_2O reductase (*qnor*) and nitrous oxide reductase (*nos*) were combined. The total number of normalized denitrification reads did not differ significantly between the two watersheds ($P = 0.7549$). In fact, a lack of difference between samples from the two watersheds held across all read-based comparisons and therefore no watershed comparisons are mentioned in the results. There also was no significant difference in the total number of normalized denitrification reads with soil depth (Fig. S2A). However, total normalized denitrification reads were significantly different ($P < 0.05$) when comparing elevations, with High Hardwood > Spruce Fir > Low Hardwood (Fig. S2B).

3.3. Analysis of occurrence of reads for specific genes

Reads coming from *nap*, *nar*, *nirK* and *qnor* were much more frequent than reads from *nirS*, *cnor* and *nosZ* (Fig. 3). The largest number of reads was assigned to *qnor*, accounting for 30–40% of total denitrification reads in all samples. When combined, the reads assigned to *nar* and *nap* made up about 15–30% of the total, while those assigned to *nirK* were between 10 and 20%. Reads assigned to *nos* were never more than 5% of the total denitrification reads. Across all samples, reads assigned to *nirS* were uncommon.

Among individual genes, *nar* reads showed no difference between samples from different soil depths (Fig. 3A), whereas *nap* reads were significantly higher ($P < 0.05$) in the Oi/Oe soil depth than the B horizon. When samples were grouped by elevation, relative abundance of reads from *nap* were higher in the High Hardwood than in either Spruce Fir or Low Hardwood. The Low Hardwood also had a lower proportion of *nar* reads than the High Hardwood site but, in contrast to *nap*, the Spruce Fir had about the same number as the High Hardwood site. Read numbers of *nirK*, *cnor, qnor* and *nosZ* did not change with soil depth (Fig. 3A). However, reads assigned to *nirK*, *cnor*, *qnor* and *nosZ* were all significantly higher ($P < 0.001$) in the High Hardwood plot than in the Low Hardwood site (Fig. 3B). Reads from *nirK* were also significantly higher in the High Hardwood than the Spruce Fir samples and accounted for nearly all the reads assigned to genes encodings dissimilatory nitrite reductases. The number of reads assigned as *nirK* from all 24 samples was > 6000 while only 31 reads in total (of the > 300 million total reads obtained) were assigned to *nirS*.

Further examination of *nosZ* assignments found that when reads assigned to clades I and clade II are clustered by depth, reads from each clade were about in equal proportion in the Mineral and Oi/Oe samples. In contrast, clade I reads were slightly more predominant than clade II reads in the Oa/A soils (Fig. S3A). When clustered by elevation clade I and clade II reads were in equal proportion in the High and Low Hardwood samples but clade I reads were of notably higher proportion in the Spruce Fir samples (Fig. S3B). Ambiguous reads, that is, reads assigned to both clades, were on average ~5% of the total reads assigned as *nosZ*.

3.4. Taxonomic assignment of denitrification genes

In general, Proteobacteria were the predominant source of reads assigned to denitrification genes, with the exception of *nar*. Therefore, taxonomic assignments were done at the Class level to achieve better resolution of differences between samples (Fig. 4, Fig. 5). With *nar*, Actinobacteria were the largest source of reads, with the remainder assigned to the various proteobacterial classes. When *nar* reads were clustered by soil depth, the number of reads assigned to Actinobacteria

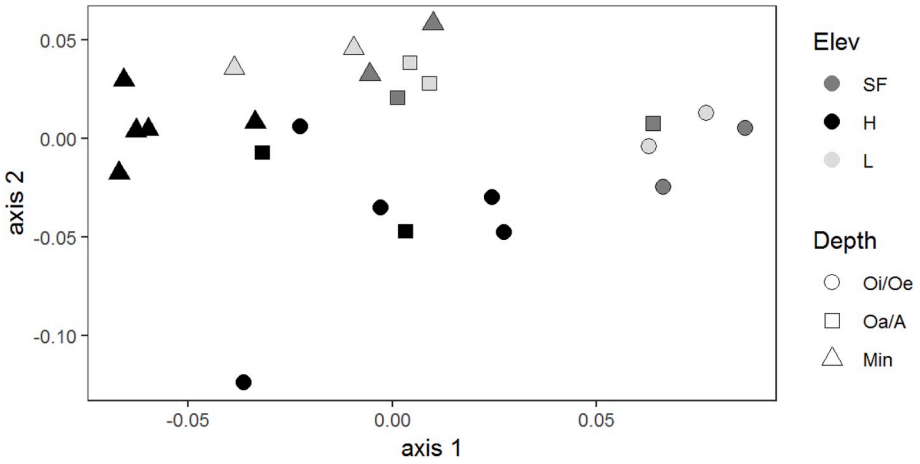

Fig. 1. Principal coordinate analysis (PCoA) plots derived from similarity comparisons of all sequenced reads. The shading of each symbol indicates elevation along the hillslope while symbol shape indicates depth in the soil column.

were similar across all samples (Fig. 4A). There was a decrease in actinobacterial reads with decreasing landscape elevation and this trend was also observed for the Proteobacteria (Fig. 4B). α- and β-Proteobacteria were the largest sources of reads assigned to *nap;* however, Acidobacteria were also a significant source making up about 15–20% (Fig. 5). The only reads in the denitrification gene set that were assigned to Acidobacteria were from *nap*. This is somewhat unexpected since 16S rRNA gene analysis showed that this group was as prevalent as the Proteobacteria (Fig. 2).

The α-Proteobacteria were the largest source of reads for both *nirK* and *qnor* (Fig. S4). The number of *nirK* reads assigned to particular classes did not change with soil depth but was significantly higher among α-Proteobacteria in the High Hardwood samples. The number of reads assigned as β- or γ-proteobacterial in origin showed similar patterns (Fig. S4). Similar trends across samples were noted for *qnor* except that δ-Proteobacteria were also predicted as a source of reads (Fig. S5). This group of bacteria was also assigned as a source of *nar* reads but not for any of the other genes. The number of *nosZ* reads from each site to which taxonomy could be assigned was small, only ~10–40 per sample making statistical comparisons of *nosZ* read taxonomic distribution problematic. However, in general, α-proteobacteria were the largest source of *nosZ* reads (Fig. S6). It is notable, however, that reads were also assigned to Flavobacteriia, Chitinophagia and Cytophagia. Bacteria that are members of these classes were not detected among reads from any of the other denitrification genes.

3.5. Denitrification rate and kinetics

During the incubation the initial gaseous product was N_2O (Fig. 6). However, prolonged incubation eventually lead to N_2 production. The rate of denitrification in the organic/mineral Oa/A soil had a median value of 0.96 μg N g soil^{-1} h^{-1}. The rate differed when grouped by elevation ($P = 0.002$), with High Hardwood > Spruce Fir > Low Hardwood (Fig. 7A). The addition of glutamate as a supplemental source of carbon did not change rates significantly in the organic/mineral Oa/A soil, but it caused an increase in the Low Hardwood soils.

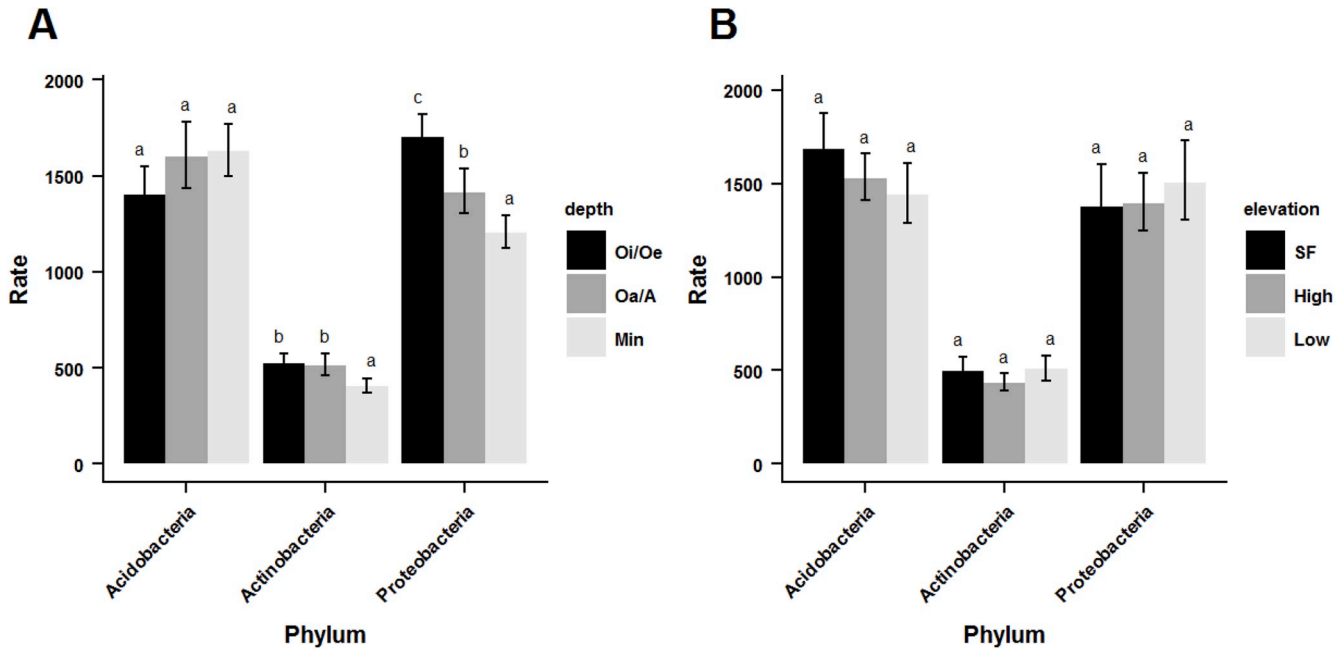

Fig. 2. Comparison of taxonomic assignments of 16S reads extracted from the metagenomes by A) depth or B) elevation. Only the top three occurring phyla are shown. Rates are the relative frequency of reads for each gene at each depth (or elevation) multiplied by the average of the total read counts for all samples. Error bars represent a 95% confidence interval and statistically significant groupings are indicated by different letters above the bars ($p < 0.05$).

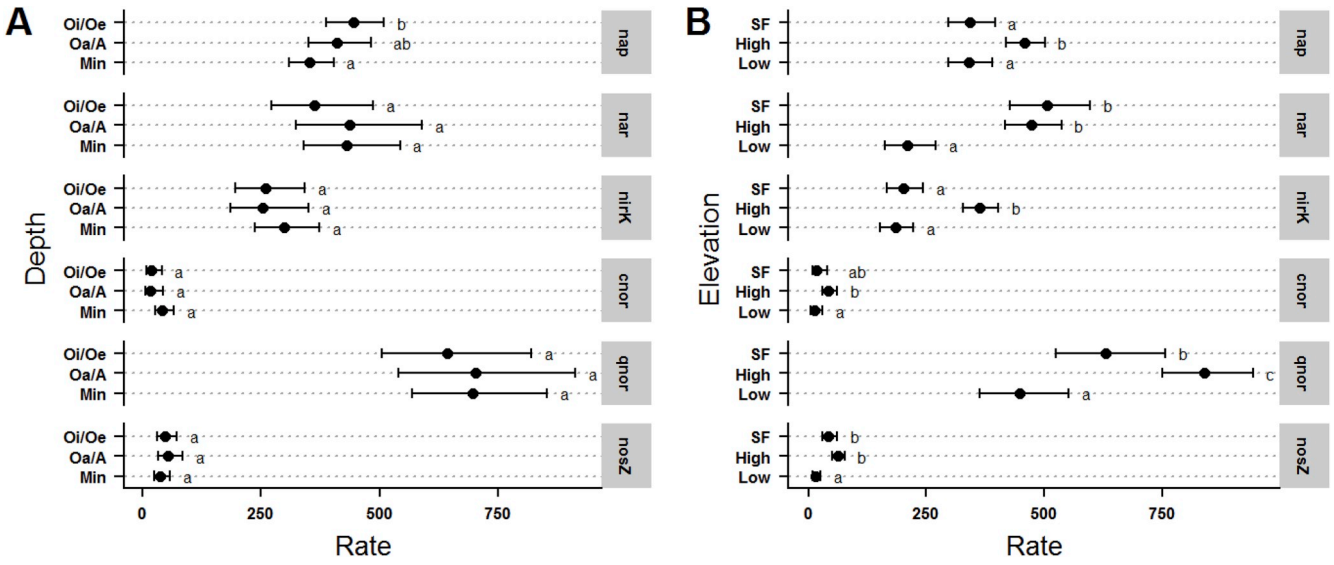

Fig. 3. Comparison of read numbers of particular genes associated with denitrification grouped by A) depth or B) elevation. Rates are the relative frequency of reads for each gene at each depth (or elevation) multiplied by the average of the total read counts for all samples. Error bars represent a 95% confidence interval and statistically significant groupings are indicated by different letters associated with the bars ($p < 0.05$).

Rates were significantly lower ($P < 0.001$) in the B horizon (median = 0.11 μg N g soil^{-1} h^{-1}) than in the organic/mineral Oa/A soils. Rates in the B horizon differed when grouped by elevation, with High Hardwood > Low Hardwood. As in the organic/mineral Oa/A soil, the addition of glutamate did not change rates significantly in the B horizon. Rates of denitrification in the organic/mineral Oa/A soils showed significant positive relationships with soil carbon ($R^2 = 0.41$, $P < 0.001$) and soil nitrogen ($R^2 = 0.46$, $P < 0.001$) (Fig. S7). Given that carbon fuels microbial activity, when rates were expressed per unit of soil carbon rather than per unit of soil values in the organic/mineral Oa/A soils showed a positive correlation with the $\delta^{15}N$ content of the soil. (Figs. S8A and S8B).

The intermediate reactants and products of potential denitrification showed patterns related to elevation. For instance, soils from the Low Hardwood zone accumulated significantly more NO_2^- than soils from the High Hardwood or Spruce Fir zones (Fig. 7A). In contrast, NO

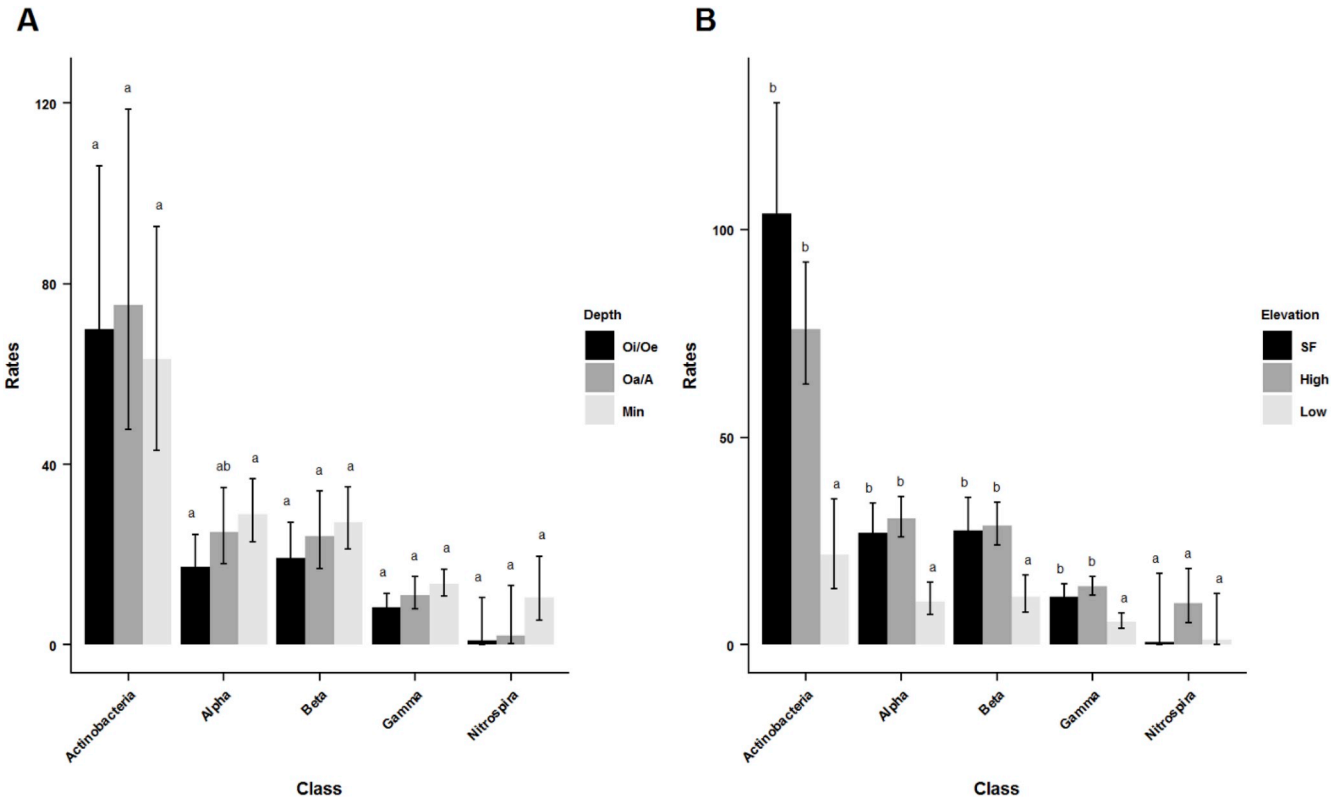

Fig. 4. Assignment of *nar* reads to particular bacterial classes grouped by A) depth or B) elevation. Only the top five classes are shown. Rates are the relative frequency of reads for each gene at each depth (or elevation) multiplied by the average of the total read counts for all samples. Error bars represent a 95% confidence interval and statistically significant groupings are indicated by different letters above the bars ($p < 0.05$).

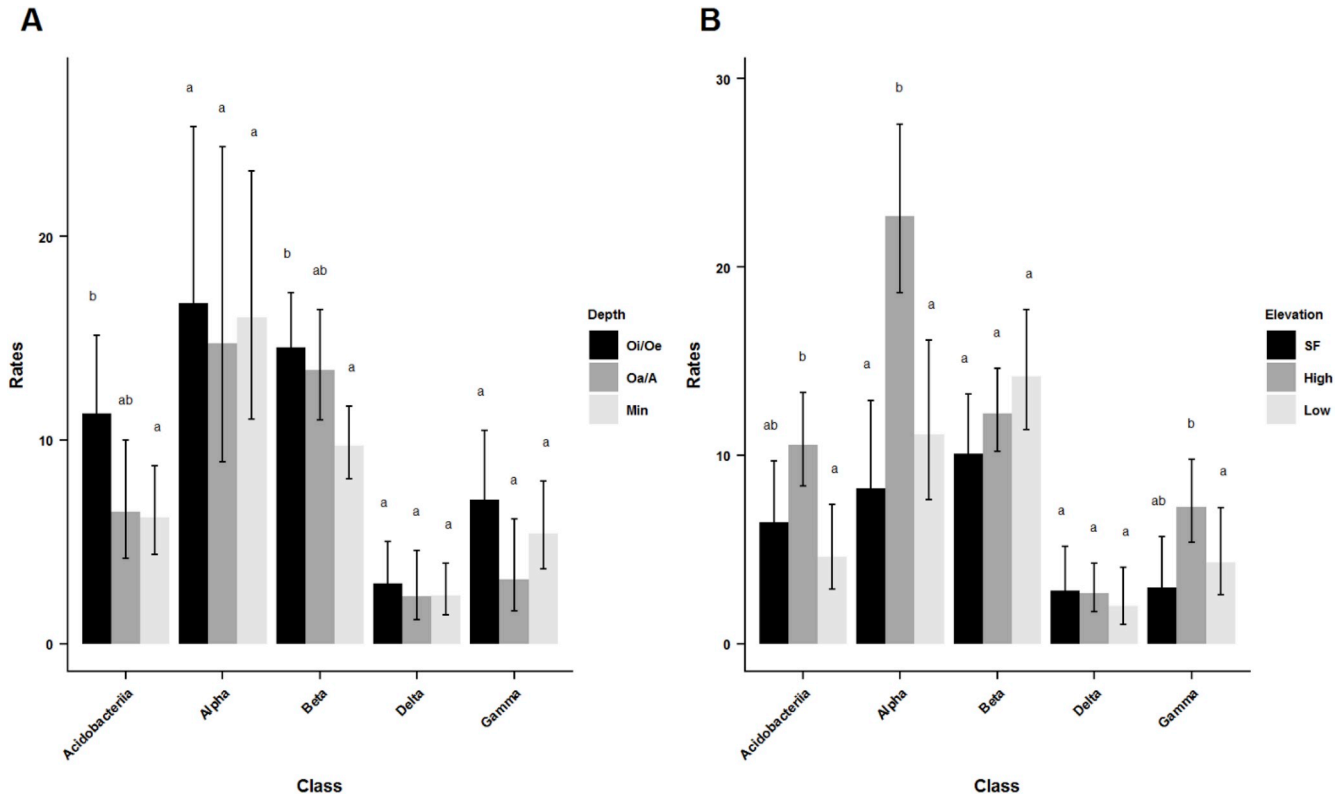

Fig. 5. Assignment of *nap* reads to particular bacterial classes. Only the top five classes are shown grouped by A) depth or B) elevation. Rates are the relative frequency of reads for each gene at each depth (or elevation) multiplied by the average of the total read counts for all samples. Error bars represent a 95% confidence interval and statistically significant groupings are indicated by different letters above the bars ($p < 0.05$).

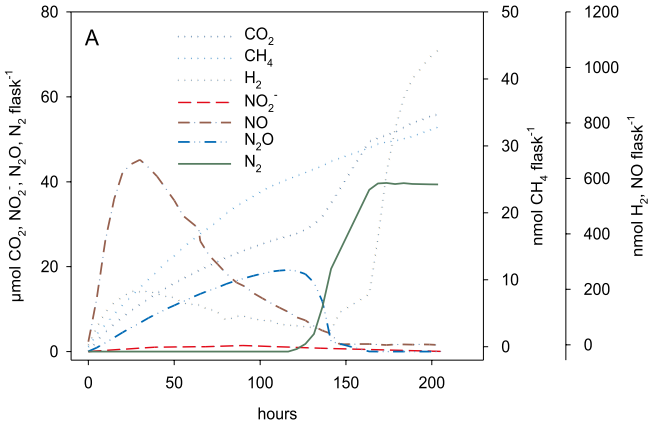

Fig. 6. A typical example of product accumulation during the incubation to assess denitrification rates, together with CO_2, CH_4 and H_2 dynamics, for a 200-hr incubation.

accumulation was smallest in Low Hardwood soils and largest in the High Hardwood soils (Fig. 7C). In general, NO release was moderate, resulting in nM NO concentrations in the slurries. The denitrification product ratio ($N_2O/(N_2 + N_2O)$) was highly variable but showed a trend with soils from the Spruce Fir producing more N before N_2O reduction than soils from High Hardwoods and Low Hardwoods (Fig. 7D).

Although denitrification in the B horizon exhibited low rates, there were a few notable patterns. Most notably, the B horizon accumulated on average more NO_2^- (3–6 μmol NO_2^- g^{-1} soil) than soils from the organic/mineral Oa/A (0–1 μmol NO_2^- g^{-1} soil; Fig. 7B). Nitrite accumulation did not lead to more NO accumulation in the B horizon, however. Glutamate addition did not markedly change measured parameters.

3.6. Influence of environmental conditions

Relationships between abundances of reads from denitrification genes and the likely explanatory environmental variables (NO_3^-, NH_4^+, soil moisture content, soil pH and soil carbon) were determined using a log-linear model. Read numbers for *nap*, *nirK* and *nosZ* were positively correlated only with NO_3^- ($P < 0.001$). The number of *qnor* reads also showed significant positive relationships with NO_3^- ($P < 0.001$) and with soil carbon ($p < 0.05$). However, when NO_3^- was removed from the variable set carbon was no longer significant, but when carbon was removed NO_3^- still showed a significant effect ($P < 0.0001$). This suggests that the model prediction for carbon was spurious. In contrast, *nar* read numbers showed no significant relationships with any of the soil parameters. Models were not run for *cnor* or *nirS* due to low read numbers.

4. Discussion

The metagenomics survey described here provides strong evidence that the generally acidic, low N input environment in the northeast USA biases the denitrifier genotype to be enriched in particular genes and depauperate in others. This pattern supports the hypothesis that environmental conditions act on individual genes in the pathway and favor the occurrence of partial denitrifiers. As well, landscape positions at HBEF can change the occurence of denitrification genes in the denitrifier community. In particular, when samples were clustered by elevation zone, total denitrification read occurrence was highest in the High Hardwood zone and lowest in the Low Hardwood zone. This pattern suggests that conditions allowing denitrification are more persistent in the High Hardwood zone than at the other two elevations, with the Low Hardwood zone having the least favorable conditions. Similarly, concentrations of NO_3^- in soil solution and stream water also are consistently higher in the High Hardwood zone than in either the

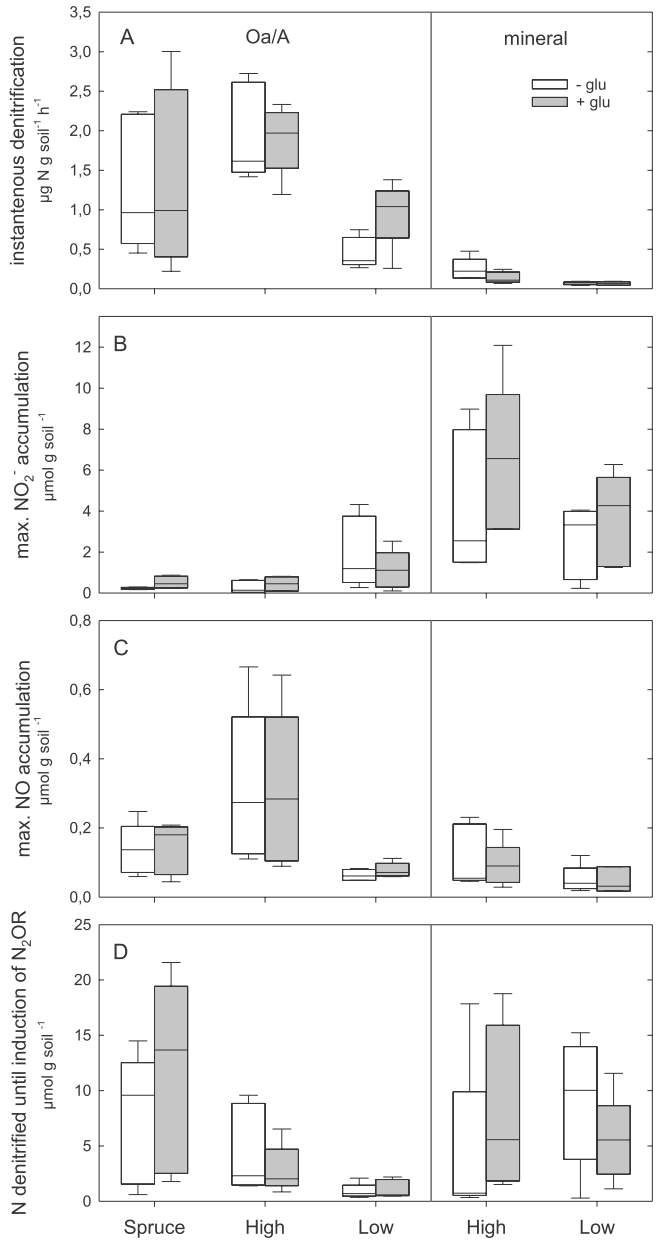

Fig. 7. Box-Whisker plots of denitrification rates (A), maximum NO_2^- accumulation (B), maximum NO (C) accumulation, and denitrification progress until apparent induction of N_2O reductase activity (D) during incubation of Oa/A (left panel) and mineral soils (right panel) from different elevations of HBEF WS6. Data is shown with and without the addition of glutamate (glu) as a carbon source.

Spruce Fir or Low Hardwood zones (Palmer et al., 2004), suggesting that concentrations of available NO_3^- determine denitrification read abundance. However, our current understanding of denitrification suggests that NO_3^- alone is insufficient to explain the gene trends and available O_2 should also be an important factor. At HBEF, the hydropedological features based on soil pits at the sampled sites (Bailey, 2019) do not provide evidence of persistent and widespread anoxia, and no such differences were noted between the sites with higher (High Hardwood) and lower (Spruce Fir and Low Hardwood) denitrification gene occurrence, particularly at the depths of sampling used in this study. Perhaps episodic hydrological conditions favoring denitrification are of sufficient duration to contribute to the patterns observed, but more detailed study of soil hydrology would be needed to test such a hypothesis

When total denitrification reads were grouped by soil depth, there was no significant difference in normalized read abundance between the three depths (Fig. S3). Since lower soil depths tend to have lower O_2 concentration (Werner et al., 2011), it might be expected there would be greater denitrification read occurrence at greater depth. However, the relationship between soil depth and duration of saturation at HBEF is complicated. Although well-drained Spodosols predominate, Bailey et al. (2014) described a complex catena series from ridgetops through upper to lower slopes in an adjacent watershed (W3) that accounted for lateral flow paths and consequent saturated areas and perched water tables at mid elevations. Nevertheless, based on soil pits that were excavated in the study plots (Bailey, 2019), none of the soils exhibited features associated with anoxia above the B horizon/C horizon interface (> 50 cm) which is well below the depth of sampling. Thus, it is not surprising that denitrification gene occurrence was unrelated to soil depth.

A similar absence of trends in total denitrification read abundance occurred when reads were grouped by watershed. This finding indicates that the addition of the wollastonite and consequent incerease increase in soil pH levels from 3.9 to 4.7 in the Oi/Oe soil, and to a lesser extent of 0.2 pH units in the A soil, did not impact denitrifier genotypegenotype. It is possible that the increase in pH was not large enough. Also, it is possible that read abundance does not depend on soil pH, but rather denitrification rates are affected by acidic pH at the enzyme level, that is, acidity affects enzyme structure and function rather than the denitrification gene abundance (Conthe et al., 2019).

Examination at the individual gene level showed roughly similar trends to those measured for total denitrification abundance. For most genes the highest proportion of reads occurred in the High Hardwood zone and there was little change in their proportion in the community when samples were clustered by soil depth in the soil column (Fig. 3). Proteobacteria were the major source of most of the denitrification-related reads, as expected (Jones et al., 2008), particularly reads from genes encoding proteins reducing NO_2^- and more reduced N-oxides. In the analysis of 16S reads, the reads from Acidobacteria were equal to those from proteobacteria. Denitrification genes are only rarely found in the Acidobacteria (Kielak et al., 2016; Eichorst et al., 2018), explaining their absence from the taxonomic assignment of denitrification reads, with the exception of *nap* (Fig. 5).

In all the HBEF samples reads from *qnor* occurred at higher levels than the other denitrification genes (Fig. 3). This is unexpected since previous analysis of the occurrence of denitrification genes in sequenced genomes has found that *nar* occurs at much higher frequencies than the other genes encoding active-site containing proteins required for denitrification (Roco et al., 2017; Hallin et al., 2018). This is likely due to the fact that dissimilatory nitrate reduction can be coupled to ammonia production as well as denitrification. Moreover, characterization of soil isolates has found strains with just the capacity to reduce NO_3^- to NO_2^- are common in soils, suggesting its high prevalence in soil communities (Lycus et al., 2017; Roco et al., 2017). The relatively high occurrence of *qnor* in the HBEF soils likely is a result of the fact that at low pH values, NO_2^- will become protonated leading to the rapid production of NO (Lim et al., 2018). The protein product of *qnor* will convert the toxic NO to the relatively more inert N_2O, making it beneficial in acidic soils.

It is important to note that since NO is toxic, due to its high affinity for the enzymatic metal centers found in most organisms (Rubbo et al., 1996), it is a broad-spectrum antimicrobial agent. By providing the enzyme that reduces the levels of a freely diffusible molecule like NO, denitrifiers with an abundance of *qnor* not only protect themselves, but other members of the local community. This means that *qnor* fits the definition of a public good (Morris, 2015). If *qnor* were not present most other organisms in the community would likely be inhibited and grow more slowly. However, those organisms with *qnor* bear a cost. Although cheaters would be expected to occur, they are likely kept in balance by the widespread nature of NO generation, making it difficult for

communities depauperate in *qnor* to attain high numbers (Wakano et al., 2009; Morris, 2015).

Another notable trend in the occurrence of denitrification reads at HBEF is the near absence of *nirS*. Previous work has shown that *nirS* is more commonly associated with organisms that have the full suite of denitrification genes (Graf et al., 2014). Given that *nirS* requires additional enzymes for assembly and function, the energetic overhead associated with its utilization might preclude its usefulness at HBEF where the conditions compatible with denitrification are infrequent. The rare occurrence of *nirS* is not due to an issue with the metagenomics approach since an analysis done using the same analytic approaches and databases as in the work described here found significant levels of *nirS* (Nadeau et al., 2019).

While read numbers reflect a community in which denitrification *sensu stricto* is not a common phenotype, this does not mean the community there is incapable of reducing nitrogen oxides to gaseous nitrogen oxides. Soil gathered from all the sites sampled for metagenomic analysis was capable of reducing NO_3^- to gaseous end products, with the greatest rates in the High Hardwood zone followed by Spruce Fir and then Low Hardwood zones (Fig. 7). This trend matches the distribution of denitrification reads in soils from these sites (Fig. 3B). As expected, N_2O was the main product during the majority of the incubation period reflecting the sensitivity of NosZ to low pH (Fig. 6) (Bergaust et al., 2010). Dinitrogen was eventually produced. However, this required extended incubation times where it is possible bottle-effects resulted in localized increase in pH values. Nitric oxide concentrations remained in the nM range in all the samples. These low levels have been observed in denitrifiers incubated under steady state conditions in whole cells and supports the role of nitric oxide reductase in controlling NO levels in HBEF soils (Hassan et al., 2016). The accumulation of NO_2^- in the mineral soils is puzzling since there was no particular trend in read occurrence that would suggest the potential for less Nir activity here relative to other depths.

One notable trend in the rate measurements reported here is the positive relationship between denitrification rates and soil carbon (Fig. S7A). This trend is driven largely by the much greater rates in the organic/mineral Oa/A horizon than in the B horizon. This most likely reflects much less microbial biomass in mineral soils than in the organic-rich forest floor (Bohlen et al., 2001). For example, comparing rates in the organic/mineral Oa/A horizon to rates in the B horizon (Fig. S9) reveals a relationship with a slope 0.12 for rates expressed on mass of soil basis (Fig. S9A), whereas the slope increases to only 0.31 when the rates are normalized for soil carbon (Fig. S9B). Thus, even when potential denitrification is normalized to account for differences in amounts of soil carbon in the forest floor versus the mineral soil, potential denitrification in the B horizon is only 32% of that in the organic/mineral Oa/A horizon. Denitrifiers are likely less abundant in the B horizon than in the forest floor, which is plausible given the fact that organic matter in the forest floor has a much larger specific surface area and provides more adequate energy to fuel microbial growth. Moreover, because glutamate added as a carbon source and electron donor did not induce greater rates, except in the Oa/A horizon in the Low Hardwood zone, the results suggest that available carbon per se does not limit denitrification in these acidic forest soils.

The metagenomic survey described here showed that conditions selected for particular genes, such as *qnor,* but disfavored others like *nirS*. Moreover, trends of potential denitrification rates generally matched trends in denitrification read occurrence in the samples. These results demonstrate that metagenomics can be used to better resolve variation in the occurrence of denitrification at the landscape scale. This work also suggests that the study of denitrification provides a unique opportunity to investigate how environmental conditions directly drives gene selection in processes providing critical ecosystem services.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2019.107585.

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