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Genetic and Functional Variation in Denitrifier Populations along a Short-Term Restoration Chronosequence▽†

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Complete removal of plants and soil to exposed bedrock, in order to eradicate the Hole-in-the-Donut (HID) region of the Everglades National Park, FL, of exotic invasive plants, presented the opportunity to monitor the redevelopment of soil and the associated microbial communities along a short-term restoration chronosequence. Sampling plots were established for sites restored in 1989, 1997, 2000, 2001, and 2003. The goal of this study was to characterize the activity and diversity of denitrifying bacterial populations in developing HID soils in an effort to understand changes in nitrogen (N) cycling during short-term primary succession. Denitrifying enzyme activity (DEA) was detected in soils from all sites, indicating a potential for N loss via denitrification. However, no correlation between DEA and time since disturbance was observed. Diversity of bacterial denitrifiers in soils was characterized by sequence analysis of nitrite reductase genes (*nirK* and *nirS*) in DNA extracts from soils ranging in nitrate concentrations from 1.8 to 7.8 mg kg^{-1}. High levels of diversity were observed in both *nirK* and *nirS* clone libraries. Statistical analyses of clone libraries suggest a different response of *nirS*- and *nirK*-type denitrifiers to factors associated with soil redevelopment. *nirS* populations demonstrated a linear pattern of succession, with individual lineages represented at each site, while multiple levels of analysis suggest *nirK* populations respond in a grouped pattern. These findings suggest that *nirK* communities are more sensitive than *nirS* communities to environmental gradients in these soils.

The Hole-in-the-Donut (HID) is a 4,000-ha region within Everglades National Park, FL. The area originally consisted of oligotrophic sawgrass (*Cladium jamaicense* Crantz) prairies and short-hydroperiod pinelands but was converted to farmland during the early part of the twentieth century (4, 14). As preparation for the conversion to agriculture, intensive rock plowing pulverized underlying limestone bedrock and created coarse-textured, well-drained soil suitable for vegetable production (12). Farming in the HID ceased in 1976, and the abandoned farmland in this area was soon invaded by *Schinus terebinthifolius* Raddi (Brazilian pepper), an exotic shrub native to South America.

Everglades National Park initiated efforts to restore the HID in 1996 by complete removal of all plants and much of the soil down to bedrock. An individual plot within the HID is cleared at one time, such that plots representing a chronosequence after clearing are present at one time (4). Following clearing, individual plots are left undisturbed to allow natural establishment of microbial communities and colonization by native wetland plants. This staggered approach to clearing provides an excellent opportunity to study the redevelopment of soil, microbial communities, and ecosystem processes over a short-term chronosequence in this wetland.

A previous report on succession of methanogenic communities at this site indicated shifts within species and activities with time following restoration (33). In the current study, changes in denitrification rates and the composition of denitrifier populations were studied along the chronosequence as part of an effort to characterize potential changes in nitrogen cycling with time since disturbance.

Denitrification is the most significant loss mechanism of biologically preferred nitrogen from terrestrial ecosystems and the dominant anaerobic respiratory process based on nitrogen (18). Respiratory denitrification is distributed among a taxonomically diverse group of facultative anaerobic bacteria and a few archaea and fungi (31, 37, 39). High carbon inputs, water column-sediment surface exchange of reduced and oxidized forms of fixed nitrogen, and low oxygen partial pressures may be favorable conditions for the development of robust denitrifying communities in wetland soils (2, 22, 23). Denitrification in the Florida Everglades has been characterized in permanently flooded, nutrient-impacted, and oligotrophic regions (5, 42, 43) and in both dominant soil types of the ecosystem (marl and peat) (10). It is thought to be the most important nitrate loss mechanism in these regions (42); however, little information exists on the community composition of denitrifiers in these systems (21) or the development of denitrifying assemblages in a wetland undergoing restoration.

Nitrogen is most frequently the nutrient limiting primary productivity during primary succession (41). Thus, its retention within the ecosystem may be crucial to restoration success. Elucidation of the differences in composition and function of denitrifying communities at various stages of recovery will contribute to further interpretation of responses at the physiological and ecological scales. The overall goals of the research reported here were to evaluate potential relationships between restoration time and the phylogenetic composition and activities of denitrifiers in HID soils.

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TABLE 1. Biogeochemical parameters of HID soils as measured in November 2005[a]

Site	Soil depth (cm)	Moisture (%)	LOI (%)[b]	NH_4^+-N content (mg kg^{-1})	NO_3^--N content (mg kg^{-1})	Denitrification potential (mg N_2O-N/kg of soil/h)[c]
UND	10 (2–15)	43.8 (0.6)	16.71	9.9 (0.9)	6.8 (0.5)	0.51 (0.14)
R89	6 (1–17)	59.6 (2.4)	14.38	46.9 (1.9)	3.1 (0.5)	0.48 (0.14)
R97	5 (3–11)	58.8 (4.5)	14.19	49.5 (10.8)	1.8 (0.6)	1.01 (0.27)
R00	3 (1–4)	48.3 (1.4)	23.90	25.1 (7.7)	7.8 (2.8)	0.12 (0.03)
R01	3 (1–7)	41.6 (1.1)	23.58	15.2 (5.1)	6.5 (2.8)	1.15 (0.33)
R03	1 (0–3)	43.5 (10.1)	18.86	26.9 (9.2)	5.1 (2.8)	0.77 (0.11)

[a] Values in parentheses are standard deviations of the mean of three replicate samples.
[b] LOI is a surrogate for organic matter content.
[c] Potential denitrifying enzyme activity.

MATERIALS AND METHODS

Site characteristics, sampling, and biogeochemical characterization. Samples were collected in November 2005. Plots 20 by 20 m^2 were established in sites restored in 1989, 1997, 2000, 2001, and 2003 (R89, R97, R00, R01, and R03, respectively) and in an undisturbed site (UND). The range of elevation for the five plots was 0.5 to 0.6 m. Each plot was divided into 81 nodes, which were evaluated for soil depth, ground coverage, and elevation. Nine of the established nodes were chosen for sampling, based on the relative range of soil depth within each site. Soil samples were taken with a plastic coring device; however, due to nonuniform soil cover in recently restored sites, grab samples were collected at some nodes. Individual samples from each depth range were combined to make three composite samples per site that were used for biogeochemical and molecular analyses. Soil samples were kept on ice and transported to the laboratory within 72 h of collection, where they were manually mixed and large roots removed. Subsamples for DNA analysis were stored at −70°C until analysis. Biogeochemical analyses were conducted at the Wetland Biogeochemistry Laboratory (5, 43).

DEA and gas analysis. Laboratory denitrifying enzyme activity (DEA) was assessed in triplicate with samples collected in November 2005, using a slightly modified version of the method outlined by White and Reddy (42). Approximately 15 g of field-moist soil from each site was placed in triplicate 220-ml serum bottles, and anaerobic conditions were established by evacuation of the headspace to approximately −85 kPa and replaced with O_2-free N_2 gas. Approximately 15% of headspace gas was replaced with acetylene (C_2H_2) (1, 47). Eight milliliters of DEA potential solution (56 mg NO_3^--N $liter^{-1}$, 288 mg $C_6H_{12}O_6$ $liter^{-1}$, 100 mg $liter^{-1}$ chloramphenicol) was added to each bottle, creating a slight overpressure in the headspace. Samples were incubated in the dark at room temperature (24°C) and continually shaken, and headspace gas was sampled every 1 h for 4 h. Gas samples were analyzed for N_2O on a Shimadzu gas chromatograph (GC-14A; Shimadzu Scientific, Kyoto, Japan) fitted with a ^{63}Ni electron capture detector.

Nucleic acid extraction, PCR amplification, cloning, and sequencing. Nucleic acids were extracted from 0.25 g of soil with the Power Soil DNA isolation kit (MoBio, Carlsbad, CA) according to the manufacturer's instructions. To account for the spatial patchiness of soils and to more fully characterize diversity, bulk nucleic acid extracts from all soil samples (three) from within a site were combined prior to PCR amplifcation. PCR was conducted using primer sets and cycling conditions designed by Yan and colleagues (45); primers 583F and 909R amplify a 326-bp region of *nirK*, and 832F and 1606R amplify a 774-bp region of *nirS*.

PCR amplicons were ligated into pCRII-TOPO cloning vector and transformed into chemically competent *Escherichia coli* TOP10F′ cells according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). DNA sequences of the inserts were determined by the University of Florida Genome Sequencing Service Laboratory using internal vector-specific primers.

Phylogenetic and diversity analysis. Nucleotide sequences were manually aligned and translated into putative amino acids in Se-Al v.2.0, a11 (A. Rambaut, Se-Al: sequence alignment editor; http://tree.bio.ed.ac.uk/software/seal) and aligned with Clustal v.1.81 (35). Phylogenetic trees were produced for segments of NirK and NirS using Tejima and Nei corrected distance matrices in the TREECON software package (40). Bootstrap analysis (500 resamplings) was used to estimate reproducibility of phylogenies. Similarities of sequences obtained in this study were compared to those obtained from other studies using BLAST queries of the nucleotide database.

Community analyses were performed by generating operational taxonomic units (OTU) in DOTUR, using the furthest-neighbor algorithm and a 3% difference in nucleic acid sequences. Nonparametric estimates of richness and diversity were calculated using DOTUR (28), including Chao1, the Shannon index, and the Simpson index. Phylogenetic clusters were designated by visual inspection of the bootstrapped phylogenies. Sequence similarities for each cluster were determined in DOTUR.

Statistical analysis of phylogenetic data. To assess whether gross differences observed between denitrifier populations between sites represented statistically different communities, well-aligned subsets of each gene fragment were chosen for analysis using ∫-LIBSHUFF (27), with one million randomizations and a distance interval (D) of 0.01 (25), employing Jukes-Cantor corrected pairwise distance matrices generated with PAUP (34). Populations were considered significantly different with a P value below 0.0026 after a Bonferroni correction for multiple pairwise comparisons ($\alpha = 0.05, n = 20$). Libraries are distinct from one another if either of the comparisons (homologous [X] versus heterologous [Y] or Y versus X) is significant (30).

Analysis of molecular variance (AMOVA), pairwise comparisons of population-specific pairwise fixation indices (F_{ST}) (17), and determination of average pairwise sequence similarities were conducted with Arlequin (version 3.001, Genetics and Biometry Laboratory, University of Geneva; http://lgb.unige.ch/arlequin). All analyses were performed under default parameters, with the following exceptions: analyses were conducted at 90,000 iterations and distance-matrix-defined unique sequences. F_{ST} tests were employed as measures of genetic differentiation between all pairs of samples. Mantel tests (15, 16) were implemented in Arelquin and used to test correlations between population-specific F_{ST} values and geochemical parameters.

Parsimony tests (P-tests) were implemented in TreeClimber (29). ClustalX (version 1.83) was used to generate sequence alignments, constructed under default parameters. Trees were constructed by Bayesian analysis, as implemented in Mr. Bayes version 3.1 (11, 24) under default model parameters, with trees sampled every 1,000 generations. All Bayesian analyses were run for one million generations, of which 10% were discarded to account for initial divergence in log likelihood scores between chains. The resultant 900 trees were used for analysis in TreeClimber and compared to 1,000,000 randomly generated trees.

Statistical analysis of biogeochemical data. Environmental parameters were tested for significance across treatment groups (study sites) using one-way analysis of variance in JMP version 5.1 (SAS Institute) on both log-transformed and raw data. Pairwise comparisons of means were conducted in the same software using Tukey's honestly significant difference, which accounts for unequal variances among samples.

Nucleotide sequence accession numbers. The GenBank accession numbers for sequences determined in this study are DQ672387 to DQ672531 and EU442402 to EU442509 for *nirK* and *nirS*, respectively.

RESULTS AND DISCUSSION

Soil biogeochemical parameters along the restoration gradient. Measured values of nitrate, ammonium, and organic matter content (loss on ignition [LOI]) in HID soils ranged from 1.8 to 7.8 mg kg^{-1}, 15.2 to 49.5 mg kg^{-1}, and 14.2 to 23.9%, respectively (Table 1); values for these parameters did not differ significantly across sites. DEA ranged from 0.12 to 1.15 mg N_2O-N kg^{-1} h^{-1} (Table 1). There was no evident trend in DEA associated with restoration age or other mea-

TABLE 2. Relative abundance of *nirS* sequences from each study site within designated phylogenetic clusters

Cluster	Relative abundance of sequence from each *nirS* clone library (%)						Avg similarity (%)[a]	No. of sequences
	UND	R89	R97	R00	R01	R03		
I	32	40			10	7	97	44
II	11			13	30	14	92	11
III	7	20	20	25	15	21	97	19
IV	27	20	30	13	25	14	83	8
V	9		30	25	5	14	79	22
VI	14	20	20	25	15	29	72	13

[a] Based on pairwise comparison of deduced amino acid sequences within each cluster.

TABLE 3. Relative abundance of *nirK* sequences from each study site within designated phylogenetic clusters

Cluster	Relative abundance of sequence from *nirK* clone library (%)						Avg similarity (%)[a]	No. of sequences
	UND	R89	R97	R00	R01	R03		
A	14	32	20	40	21	26	95	41
B	7	4		13			96	7
C		7	4		17	9	96	9
D					13	17	96	7
E			8		4	4	98	4
F		7	36	3	8	9	98	16
G	14						96	4
H	18			3			95	6
I		4		7	8	4	94	6
J	7		4	3		4	88	5
K	4	11	4		17	4	85	10
L	11	25		13			96	14
Singletons	25	11	24	17	13	22		

[a] Based on pairwise comparison of deduced amino acid sequences within each cluster.

sured environmental parameters. Values determined in this study are within the range of those reported for oligotrophic surface soils in other regions of the Everglades using similar methods (42), as well as values reported for riparian (26) and agricultural soils (20). Although there was no clear trend associated with DEA and recovery stage in HID soils, statistical analysis of site effects on biogeochemical data indicated significant ($P < 0.0001$) within-site effects on DEA. This may indicate that, while there does not seem to be an obvious or homogenously controlling factor on DEA along the restoration gradient, activity may be more strongly controlled by different factors within each restoration site, such as soil structure and nutrient content, plant community composition (tissue quality), or other factors associated with soil redevelopment. Interestingly, relatively high DEA activities were observed in the two most recently restored sites, despite having less than 3 cm of soil and moisture contents similar to those of all other sites.

***nirS* and *nirK* phylogenies.** *nirS* denitrification genes were obtained from all study sites. These sequences grouped into six (I to VI) distinct phylogenetic clusters (see Fig. S1 in the supplemental material); the relative abundances of clones from each library are presented in Table 2. Of the 117 clones obtained from all study sites, 58% had the greatest similarity (ca. 85% of amino acids) to environmental clones obtained from a Michigan wetland (21); clusters I, II, and III were composed exclusively of these sequence types. It may be the case that these sequences represent wetland-adapted, *nirS*-type denitrifiers common to all HID soils. The remaining clones fell into clusters IV, V, and VI. These clusters were composed of loosely divergent lineages and shared the greatest similarity with uncultivated sequences obtained from groundwater (25), CO_2-enriched wetland soil (S. H. Lee, S. Y. Kim, and H. Kang, GenBank direct submission, 2005; http://www.ncbi.nlm.nih.gov/), activated sludge (T. Ohsaka, S. Yoshie, S. Tsuneda, A. Hirata, and Y. Inamori, GenBank direct submission, 2004; https://www.ncbi.nlm.nih.gov/), and cyanobacterial aggregates (38). Cluster VI sequences were the only sequences to have similarity to *nirS* of cultivated denitrifiers, sharing ca. 75% of amino acids with members of the betaproteobacterial genera *Thauera*, *Azoarcus*, and *Magnetospirillum*.

nirK sequences obtained from all study sites grouped into 12 distinct phylogenetic clusters (Table 3 and see Fig. S2 in the supplemental material). More so than *nirS*, a number of deeply divergent singletons were present in *nirK* clone libraries. Forty-two percent of the 158 *nirK* sequences had the greatest similarity (ca. 90% of amino acids) to environmental clones previously obtained from a wetland soil (21); these sequences composed clusters A through E (see Fig. S2 in the supplemental material). Sequences making up the remaining clusters (F through L) formed distinct, deeply branching clades and were most closely associated with uncultivated denitrifiers found in agricultural, forested upland, and peat soils (21, 36, 44). Several sequence clusters also had amino acid similarity to cultivated denitrifiers of the genera *Alcaligenes* (G, 82%), *Bradyrhizobium* (I, 93%), and *Ensifer* (J, 92%; L, 84%).

Richness and diversity of *nirS* and *nirK* populations. Coverage values for *nirS* and *nirK* clone libraries for each site are presented in Table 4. Coverage for *nirS* libraries ranged from 45 to 90%: R01 and R03 libraries had the highest and lowest coverage values, respectively. *nirK* coverage values ranged from 45 to 100% and were highest for the UND library and lowest for the R01 library.

Both diversity indices presented in Table 4, as well as rarefaction analysis (see Fig. S3 in the supplemental material), suggest *nirS* to be more diverse than *nirK* in restoration sites. A greater overall diversity of *nirS* compared to *nirK* has been previously observed in other studies (3, 13), including wetland soils (21). Differences in diversity are more clearly pronounced by the Simpson values (Table 4), which are sample-size-independent estimates of diversity. Attempts to correlate measures of diversity with geochemical parameters or restoration age yielded no significance. This may be attributable to the narrow differences between diversity values of the indices presented in Table 4. However, the inability to correlate statistical measures of community composition with geochemical parameters may be due, in part, to the relatively poor understanding we have of factors controlling diversity of *nir* genotypes in the environment (25) or the fact that community structure is controlled by less quantifiable factors, such as spatial patchiness or nonequilibrium conditions common in disturbed ecosystems.

Population-based library compositions. $\int$-LIBSHUFF (27, 32) was employed to assess gross differences in denitrifier populations, as represented by our clone libraries, and a P-test was

TABLE 4. Values of *nirS* and *nirK* diversity and richness in HID soils, as estimated by Shannon diversity index, Simpson index, and Chao1 richness calculated using DOTUR[a]

Gene and site	No. of clones sequenced	No. of OTU	Shannon index	Diversity[b]	Richness[c]	No. of singletons	Coverage (%)
nirK							
UND	28	13	2.3 (1.9–2.6)	2.5	20 (13–54)	6	100[d]
R89	28	12	2.3 (1.8–2.5)	2.2	22 (14–66)	7	69
R97	25	11	2.0 (1.7–2.4)	1.9	21 (13–65)	7	86
R00	30	16	2.4 (1.9–2.8)	2.1	38 (21–102)	12	72
R01	24	12	2.3 (1.9–2.6)	2.4	19 (13–48)	7	45
R03	23	12	2.2 (1.9–2.6)	2.3	26 (15–79)	8	71
All	158						
nirS							
UND	21	8	1.8 (1.4–2.1)	1.8	11 (8–31)	4	48
R89	17	10	2.2 (1.8–2.5)	2.7	15 (11–39)	6	56
R97	15	16	2.7 (2.4–3.0)	3.7	32 (20–80)	12	67
R00	27	16	2.6 (2.2–2.9)	2.8	82 (38–212)	12	76
R01	15	12	2.4 (2.1–2.7)	3.1	16 (13–34)	7	90
R03	22	18	2.8 (2.5–3.1)	3.8	53 (27–144)	15	45
All	117						

[a] Estimates of OTU, Shannon index, diversity, and richness are all based on 3% differences in nucleic acid sequence alignments. Values in parentheses are 95% confidence intervals as calculated by DOTUR (28).
[b] Sample-size-independent estimate of diversity based on negative natural log transformation of Simpson's index values as calculated in DOTUR.
[c] Chao1 values, a nonparametric estimate of species richness.
[d] Coverage values for distance = 0.01, as calculated by ∫-LIBSHUFF (27).

used (17, 29) to test whether observed community structures were the result of random variation.

The results of ∫-LIBSHUFF analysis of *nirS* clone libraries indicate a level of shared similarity between sites most closely related in time since disturbance (Table 5). This pattern may be explained by the presence of a single lineage common to all sites: for example, the closely related group of sequences that make up cluster IV (Table 5) or a succession of shared lineages between the most closely related sites. An analysis of community covariance with phylogeny (P-test) confirms that community structures from each site are significantly different ($P < 0.02$), and removal of any site from the analysis did not lead to loss of significance. Prior studies that removed distinct groups from P-test analysis were able to discern groups responsible for differentiation (17, 29). Combined, these results support the idea that all restoration sites harbor groups of *nirS*-type denitrifiers not present in clone libraries for other restoration sites, despite that fact that there is an underlying level of shared lineages between restoration sites, though factors controlling a response of this nature are currently unclear.

∫-LIBSHUFF analysis of *nirK* libraries indicates a different response from that of *nirS*. A clear linear response was not evident: instead, communities native to R03 and R01 appear to be drawn from the same population. R00 maintained an independent population, while R97 and R89 shared a community, establishing three response groups. The reason for this response pattern is less clear upon investigation of the phylogenetic analysis (see Fig. S2 in the supplemental material). A P-test including all populations indicated significant differences ($P = 0.032$), and pairwise comparisons for all sites were also significant ($P < 0.02$). Thus, while early stage and late-stage response groups share an underlying community, indi-

TABLE 5. Population similarity *P* values for comparison of *nirK* and *nirS* clone libraries determined using the Cramer-von Mises test statistic, implemented in ∫-LIBSHUFF[a]

Gene (*n*)	Site for homologous library (*X*)	*P* value for comparison of heterologous library (*Y*) with *X*[b]				
		R89	R97	R00	R01	R03
nirK (158)	R89		0.036	**0.000**	**0.000**	**0.000**
	R97	0.400		**0.000**	**0.000**	**0.000**
	R00	**0.000**	**0.000**		**0.000**	**0.000**
	R01	**0.000**	**0.000**	0.040		0.957
	R03	**0.000**	**0.000**	**0.000**	0.304	
nirS (117)	R89		0.421	**0.000**	**0.000**	**0.000**
	R97	0.125		0.040	**0.000**	**0.000**
	R00	**0.000**	0.210		0.056	**0.000**
	R01	**0.000**	**0.000**	0.269		**0.000**
	R03	**0.000**	**0.000**	**0.000**	**0.000**	

[a] See reference 27.
[b] Boldface values indicate significant *P* values ($P < 0.0026$) after Bonferroni correction for multiple pairwise comparisons. Libraries are distinct from one another if either of the comparisons (*X* versus *Y* and *Y* versus *X*) is significant (30).

TABLE 6. Fixation indices, average pairwise differences, nucleotide diversity, and unique sequences of *nirK* clone libraries as calculated by Arlequin[a]

Site	F_{ST}	Avg pairwise difference ($\theta[\pi]$)	Nucleotide diversity[b]	No. of unique OTU
R89	0.030	89 (44)	0.25 (0.12)*	23
R97	0.033	83 (37)	0.24 (0.11)*	21
R00	0.042	66 (29)	0.19 (0.01)†	28
R01	0.043	64 (27)	0.18 (0.01)†	17
R03	0.045	62 (28)	0.17 (0.09)†	18

[a] See reference 8.
[b] Values sharing the same symbol are not statistically different, based on pairwise Student's *t* test ($P \leq 0.05$).

vidual sites comprising the groups harbor unique lineages, possibly selected for by the disturbance recovery stage. Finally, in contrast to the ∫-LIBSHUFF analysis, a P-test comparison of libraries pooled into "late-stage" recovery (R89 and R97) to "early stage" recovery (R00, R01, and R03) yielded the greatest level of significance of all comparisons ($P = 0.012$), suggesting a two-group pattern of succession.

Variance within *nirK* clone libraries. To further investigate the response pattern of *nirK*-type denitrifers along the restoration chronosequence, AMOVA was implemented (8); AMOVA has been previously applied for differentiation of microbial communities characterized by sequence analysis of functional genes (6, 19, 46). Only *nirK* libraries were chosen for this level of analysis due to the observed difference in response to recovery (compared to *nirS*), the generally greater coverage of expected diversity for *nirK* libraries (Table 4 and see Fig. S3 in the supplemental material), and the more conserved nature of *nirK* in comparison to *nirS*, which commonly has larger regions of insertion or deletion. AMOVA results indicate that 98% of variation observed in *nirK* populations is attributable to within-site factors, meaning that the diversity of sequences within sites is the greatest factor contributing to total variance. While small, the remaining 2% of variance between populations differed significantly from the pooled population ($P = 0.013$), in support of P-test results and further evidence that unique lineages of *nirK*-containing denitrifiers at each site are responsible for statistical differences between communities.

F_{ST} values for each site declined with time since disturbance (Table 6); these values are measures of genetic diversity within a population compared to the total (pooled) population. The general decline in values with time since disturbance suggests that populations of *nirK*-containing denitrifiers become more reflective of total observed diversity in HID soils as recovery progresses. F_{ST} values between sites nearer in time since disturbance are closely related. Values for R89 and R97 range from 0.030 to 0.033, and values for R00, R01, and R03 range from 0.045 to 0.042. This trend is also evident in pairwise sequence similarity ($\theta[\pi]$) and statistically supported by nucleotide diversity estimates (Table 6). A similar, yet statistically insignificant, pattern is also evident in the geochemical data. The organic matter, nitrate, and ammonium contents are more similar among soils in later stages of recovery than between later and earlier stages (Table 1).

Overall, two levels of a statistical analysis indicate a bimodal response of *nirK*-type denitrifier communities to succession, while ∫-LIBSHUFF suggested a three-group response. Firm conclusions about which of the observed response patterns is most accurate must be made with caution, as the clone libraries employed in this study do not fully cover the expected richness of *nirS* and *nirK* communities. However, the unifying result of all analyses is that *nirK*-type denitrifiers respond to succession in a different manner from *nirS*-type denitrifiers, which suggests that *nirK* communities are more sensitive to environmental gradients or that they exhibit greater habitat selectivity (25, 36, 44).

A Mantel test (7, 16) was used to examine the correlation between pairwise differences in *nirK* population-specific F_{ST} values between sites to matrices of pairwise differences in geochemical parameters. Such analyses have been used previously to test whether observed differences in functional gene diversity correlated with geochemical variables between sampling sites (9). In the context of this study, the test was employed to determine correlations between observed differences in *nirK* diversity between sites and measured environmental parameters along the chronosequence and to ultimately gain an understanding of environmental factors most likely controlling the observed differences in *nirK*-type denitrifier populations in HID soils. Pairwise F_{ST} matrices (see Table S1 in the supplemental material) were tested for correlation with environmental factors most likely controlling denitrifier activity: organic matter (loss on ignition), moisture content, and soil oxygen demand. Differences in *nirK* F_{ST} values between sites were strongly correlated with differences in soil moisture content ($r = 0.895$, $P = 0.017$) and marginally correlated with differences in organic matter content ($r = 0.61$, $P = 0.05$). The results suggest that soil moisture plays a strong role in *nirK* population diversity within each site; this correlation becomes intuitive when one considers the influence variations in soil moisture can have on oxygen availability and, to some extent, redox potential in soils—two factors likely to influence the distribution and diversity of denitrifying bacterial communities in soils.

Conclusions. While geochemical data for sites of various stages of recovery since complete soil removal suggest loose trends associated with time since disturbance, several lines of evidence indicate the existence of significantly different populations of denitrifying bacterial communities at each of the study sites. *nirS* clone libraries suggest an approximately linear response with time since disturbance, while *nirK* sequences appear to be characterized by two or three independent response groups. In either case, this suggests that diversity of functionally redundant enzymes results from adaptation to particular environments. The factors governing community diversity are not entirely clear. However, the most obvious variable is recovery stage, the gradual accumulation of nutrients, soil, and associated moisture, and the maturing of plant communities. Furthermore, results of AMOVA indicate population diversity within sites declines with time since disturbance, which may indicate a gradual decrease in species recruitment as conditions within each site converge toward stability. These results highlight the sensitivity of denitrifying bacterial communities to environmental conditions and provide insight into microbial community dynamics in response to ecosystem recovery.

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