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ORIGINAL ARTICLE

Evidence for the functional significance of diazotroph community structure in soil

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Microbial ecologists continue to seek a greater understanding of the factors that govern the ecological significance of microbial community structure. Changes in community structure have been shown to have functional significance for processes that are mediated by a narrow spectrum of organisms, such as nitrification and denitrification, but in some cases, functional redundancy in the community seems to buffer microbial ecosystem processes. The functional significance of microbial community structure is frequently obscured by environmental variation and is hard to detect in short-term experiments. We examine the functional significance of free-living diazotrophs in a replicated long-term tillage experiment in which extraneous variation is minimized and N-fixation rates can be related to soil characteristics and diazotroph community structure. Soil characteristics were found to be primarily impacted by tillage management, whereas N-fixation rates and diazotroph community structure were impacted by both biomass management practices and interactions between tillage and biomass management. The data suggest that the variation in diazotroph community structure has a greater impact on N-fixation rates than do soil characteristics at the site. N-fixation rates displayed a saturating response to increases in diazotroph community diversity. These results show that the changes in the community structure of free-living diazotrophs in soils can have ecological significance and suggest that this response is related to a change in community diversity.
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Introduction

Biological N fixation is the major natural process through which atmospheric N_2 is converted into forms that can be used by plants and animals, contributing 100–290 Tg N per year to the biosphere (Cleveland *et al.*, 1999). Although the majority of N fixation in terrestrial ecosystems is carried out by symbiotic bacteria in association with plants, free-living diazotrophs in soils have been shown to be important contributors to the N budgets of a number of ecosystems (Cleveland *et al.*, 1999). Progress in understanding the ecological significance of free-living diazotrophs has been limited, however, by the fact that many of these organisms are recalcitrant to laboratory cultivation. The *nifH* gene, which encodes a subunit of the nitrogenase enzyme, provides a useful marker that can be used to study the distribution and diversity of diazotrophs without the need for cultivation. Surveys of *nifH* diversity in soil commonly reveal sequence types that correspond to the diverse unidentified diazotrophs (Ueda *et al.*, 1995; Widmer *et al.*, 1999; Piceno and Lovell, 2000; Shaffer *et al.*, 2000; Poly *et al.*, 2001). Evidence indicates that these noncultivated diazotrophs, rather than their cultivated cousins, are the dominant N-fixing organisms in many soil systems (Poly *et al.*, 2001; Hamelin *et al.*, 2002; Tan *et al.*, 2003; Buckley *et al.*, 2007).

The ecological significance of free-living diazotrophs in terrestrial ecosystems can be difficult to constrain as estimates for N fixation by these organisms can vary widely, ranging from 0 to 60 kg Ha^{-1} per year (Cleveland *et al.*, 1999). Several environmental factors have been suggested to influence N fixation in soils including soil moisture, oxygen, pH, C quantity and quality, N availability and the availability of trace elements, such as Mo, Fe and V, Soil moisture, oxygen and pH have fairly straightforward effects on N-fixation rates. Increases in soil moisture (Brouzes *et al.*, 1969; Sindhu *et al.*, 1989) and reductions of oxygen tension (Brouzes *et al.*, 1969; O'Toole and Knowles, 1973; Kondo and Yasuda, 2003a) generally increase rates, whereas N fixation is not favored in soils of low pH (Roper and Smith, 1991; Limmer and Drake, 1996; Nelson and Mele, 2006). In contrast, the effects of C and N

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quantity and quality are less consistent. Increases in the availability of labile C generally stimulate N fixation (O'Toole and Knowles, 1973; Keeling *et al.*, 1998; Burgmann *et al.*, 2005; Kondo and Yasuda, 2003a, b), but in other cases have little or no effect (Brouzes *et al.*, 1969; Roper and Smith, 1991; Keeling *et al.*, 1998). Likewise, N availability can have either stimulatory (Azam *et al.*, 1988; Poly *et al.*, 2001) or inhibitory (Koteva *et al.*, 1992; Tan *et al.*, 2003) effects. It is reasonable to expect that the influences of these environmental parameters on N-fixation rates vary as a function of microbial community structure, but the functional significance of diazotrophic community composition remains poorly characterized.

Microbial community composition can have significant quantitative and qualitative impacts on important soil processes. Although soil processes that are widely distributed within the community, such as soil respiration, seem to occur largely independent of changes in community structure (Schimel, 1995), those that are mediated by a specialized subset of the community, such as denitrification (Cavigelli and Robertson, 2000), nitrification (Carney *et al.*, 2004; Hawkes *et al.*, 2005; Webster *et al.*, 2005), methane consumption (Gulledge *et al.*, 1997) and cellulose degradation (Wohl *et al.*, 2004) can be strongly impacted by the community level changes. Two recent studies have cast doubt on whether changes in diazotroph community structure have functional significance. Deslippe *et al.* (2005) were unable to find a significant association between *nifH* T-RFLP profiles and acetylene reduction assay (ARA)-based N-fixation rates in Arctic tundra in a short-term fertilization experiment. Likewise, Patra *et al.* (2006) were unable to find a significant association between overall *nifH* DGGE profiles and ARA-based N-fixation rates in a long-term grazing experiment, though 60% of the variance in N-fixation rates could be correlated to the changes in a subset of the DGGE bands observed.

Agricultural experiments provide an excellent opportunity to study the functional significance of microbial community structure in soil because of the relative simplicity and managed aspect of agricultural systems compared with natural ecosystems. Long-term agricultural experiments are particularly useful in this regard as microbial community structure can require long periods of time to equilibrate with respect to changes in management practice (Buckley and Schmidt, 2001, 2003). Thus, long-term treatments are required to investigate linkages between the composition and function of microbial communities in soil (Reed and Martiny, 2007). In this study, we examine the long-term (>30 years) effects of tillage and biomass management on N fixation, soil characteristics and diazotroph community structure to determine whether N-fixation rates are primarily determined by soil characteristics or whether the changes in community structure have functional significance. Diversity is evaluated as a function of its three main components—richness, evenness and genetic diversity. These three components of diversity, although frequently related, can vary somewhat independently (Magurran, 1988). Differences in community structure are evaluated as a function of both differences in the diversity and composition (that is, operational taxonomic unit (OTU) membership) of the communities being compared.

Materials and methods

Description of field site and sampling

The long-term tillage experiment was established in 1973 and is located at the William H Miner Institute in Chazy, Clinton County, New York (44°53.13′N, 73°28.40′W). All of the plots share a soil type, which is Raynham silt loam. The experimental site has a 2×2 factorial design, testing the effects of tillage and biomass management, and is arrayed in a randomized complete block with four replicate plots for each treatment. Maize has been grown continuously in all plots following standard agronomic practices for the region (26 kg Ha^{-1} per year fertilizer N). The treatments represent a gradient of soil disturbance with each of two treatments managed with annual tillage (T1 and T3; moldboard plowed and disked), and each of the two treatments managed as no-till systems (T2 and T4). These treatments are subdivided by biomass retention (T3 and T4) or removal (T1 and T2). In biomass removal plots, plant biomass was harvested for silage and only roots and stubble were retained in the field. In biomass retention plots, grain was harvested and all other biomass was retained in the field. In addition, a control site consisting of a never cultivated (NC; for >35 years) grassy field was also sampled. The NC site was immediately adjacent to the agricultural site, was on the same soil series and was managed by monthly mowing.

Soil samples were taken on 1 November 2005. A total of 20 soil cores (2.5 cm diameter and 5 cm depth) were taken across each replicate, and these cores were homogenized and sieved to 2 mm. Each replicate soil sample was split for the analysis of soil characteristics, N-fixation rates and diazotroph community structure. Soil characteristics were determined following standard methods with organic matter determined by loss on ignition, total C and N determined by mass spectrometry, and P, K, Mg, Ca, Fe, Al, Mn, Zn, Cu and NO_3 were measured following NH_4OAc extraction as described previously (Burt, 2004). Samples used for determining potential N-fixation rates were maintained at ambient temperatures, and were processed on the same day that they were sampled as described below. Samples used for analysis of diazotroph community structure were frozen on liquid nitrogen in the field, stored on dry ice for transport and archived at -80 °C.

Measurement of N-fixation rates

The ARA is the most common method for measuring N fixation and is based on the assumption that 3–4 mol acetylene are reduced to ethylene for every mole of N_2 fixed by nitrogenase enzyme (Stewart *et al.*, 1967; Jensen and Cox, 1983). However, conversion factors in the range of 0.022–22 have been reported for terrestrial habitats in which ARA has been standardized with $^{15}N_2$-based measurements (Spiff, 1973; Nohrstedt, 1983; Skujins *et al.*, 1987; Zechmeister-Boltenstern and Kinzel, 1990; Liengen, 1999). Thus, in this study, potential N-fixation rates were measured by $^{15}N_2$ incorporation into soil in relation to controls. This method has been widely applied (Brouzes *et al.*, 1969; O'Toole and Knowles, 1973; Nohrstedt, 1983; Skujins *et al.*, 1987; Montoya *et al.*, 1996) and has traditionally been recommended to verify N-fixation rates in cases where ARA is used (Stewart *et al.*, 1967). Briefly, 5 g soil was placed into 18×150 mm Balch tubes (Bellco Glass, Vineland, NJ, USA), and the headspace was replaced with synthetic air containing 20% O_2 and 80% $^{15}N_2$ (>98 atom % ^{15}N, Isotec, Miamisburg, OH, USA). Controls were processed in parallel and received unlabeled N_2 gas. Tubes were incubated horizontally at room temperature in the dark for 9 days. The atom % ^{15}N of soil samples was determined using a Finnigan MAT Delta Plus mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) plumbed to a Carlo Erba NC2500 elemental analyzer (CE Instruments, Wigan, UK) through a Conflo II open split interface for elemental and isotopic composition of solid samples (Thermo Electron Corporation). The net potential N-fixation rate was calculated from the difference of total ^{15}N in soils receiving $^{15}N_2$ relative to controls. It should be recognized that potential rates can differ from *in situ* rates because of bottle effects, but such potential rates are commonly useful for assessing relative differences in the activities of microbial communities.

Construction of nifH clone libraries

DNA was extracted from three subsamples of 0.33 g from each soil sample using the PowerSoil DNA Isolation Kit (MoBio Inc., Carlsbad, CA, USA) as per manufacturer's instructions and these samples were pooled. DNA concentrations were determined through the Pico-Green assay (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions. DNA from replicate samples was pooled with respect to treatment before the construction of *nifH* clone libraries. PCR of *nifH* genes was conducted with primers *nifH*-b1-112F (Burgmann *et al.*, 2004) and *nifH*623R (Steward *et al.*, 2004) in 50 μl volumes containing 70 ng of template DNA with each primer at a concentration of 0.25 μM, each dNTP at a concentration of 200 μM, 2.5 mM $MgCl_2$, 0.05% of BSA (New England Biolab, Ipswich, MA, USA), 2.5 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA), and $1 \times$ PCR buffer (supplied with Taq enzyme). Each PCR consisted of a 95 °C hold for 10 min followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 45 s at 72 °C, and a final extension for 15 min at 72 °C. Three PCR reactions were performed in parallel to the pooled DNA sample from each treatment, these PCR products were combined and gel purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA), and then cloned into pCR2.1-TOPO as per manufacturer's instructions (Invitrogen). DNA sequencing was performed using an Applied Biosystems Automated 3730 DNA Analyzer at Cornell University's Biotechnology Resource Center. The nucleotide sequences of the 349 *nifH* gene clones described in this study have been deposited in GenBank under accession numbers: FJ008168:FJ008514.

The suitability of the *nifH*-b1-112F and *nifH*623R primer sets for characterizing the diazotroph community present in our soils was validated empirically. A range of different primer sets were evaluated as described in Burgmann *et al.* (2004), and of the primer combinations tested the *nifH*-b1-112F and *nifH*623R provided the best amplification for our samples (data not shown). To confirm that the *nifH*-b1-112F and *nifH*623R primer sets provided a representative measurement of the diazotroph community for our samples, the *nifH* clone library generated with these primers was compared to a *nifH* clone library generated from the pooling DNA amplified in parallel using five different *nifH* PCR primer sets having a range of specificities as described previously (Burgmann *et al.*, 2004). All of these reactions used the reverse primer *nifH*623R and one of the following forward primers: *nifH*-b1-112F, *nifH*-a1-112F, *nifH*-c1-112F, *nifH*-f1-112F and *nifH*-g1-112F. The DNA template used for these PCR was from the NC soil; each reaction was performed in triplicate. The PCR products were then pooled across all primer sets, and this pooled PCR product was used to create a clone library as described above. A total of 70 *nifH* sequences from this library were determined and deposited in GenBank under accession numbers: FJ008515:FJ008582. The Chao1 estimator (see below) revealed a richness of 39 (95% confidence intervals: 29, 74) OTU in the mixed primer library, and 64 (95% confidence intervals: 44, 122) in the library constructed with primers *nifH*-b1-112F and *nifH*623R. The lack of a significant difference in diversity between these libraries suggests that the *nif*H-b1-112F and *nifH*623R primer sets provide a reasonable estimate of diazotroph community structure in soil from our site.

Phylogenetic analysis of nifH sequences

Phylogenetic analysis was performed using ARB (Strunk and Ludwig, 1997) and Phylip 3.64 (Felsenstein, 2005). Sequences were imported and aligned against an *nifH* database constructed from

Table 1 Soil and site characteristics of the long-term tillage experiment in Chazy, NY, as measured in November 2005

Treatment	Tillage	Biomass retention	N-fixed ($\mu g\,kg^{-1}\,d^{-1}$)	pH	Moisture (%)	Organic matter ($g\,kg^{-1}$)	DNA ($\mu g\,g^{-1}$)	Total C ($g\,kg^{-1}$)	Total N ($g\,kg^{-1}$)	NO_3 ($mg\,kg^{-1}$)
T1	Yes	No	382 ± 22	8.0 ± 0.1	20.4 ± 1.4	34.4 ± 3.6	6.4 ± 1.0	16.7 ± 1.8	1.3 ± 0.1	0.6 ± 0.1
T2	No	No	392 ± 55	7.6 ± 0.5	22.9 ± 1.5	50.0 ± 9.9	11.0 ± 1.9	23.3 ± 4.9	1.9 ± 0.4	9.2 ± 6.4
T3	Yes	Yes	351 ± 41	8.0 ± 0.03	21.0 ± 4.8	42.1 ± 9.3	9.0 ± 1.8	16.9 ± 2.1	1.3 ± 0.1	0.6 ± 0.1
T4	No	Yes	190 ± 11	7.7 ± 0.2	25.9 ± 3.6	57.8 ± 8.2	10.0 ± 0.5	27.6 ± 4.4	2.1 ± 0.2	2.7 ± 5.1
NC	No	Yes	321 ± 49	6.5 ± 0.3	26.4 ± 2.0	71.0 ± 4.2	15.4 ± 1.8	32.4 ± 2.4	2.7 ± 0.2	17.0 ± 6.8

Treatment	P ($mg\,kg^{-1}$)	K ($mg\,kg^{-1}$)	Mg ($mg\,kg^{-1}$)	Ca ($g\,kg^{-1}$)	Fe ($mg\,kg^{-1}$)	Al ($mg\,kg^{-1}$)	Mn ($mg\,kg^{-1}$)	Zn ($mg\,kg^{-1}$)	Cu ($mg\,kg^{-1}$)
T1	8.9 ± 1.0	42.0 ± 6.6	161.8 ± 29.1	4.46 ± 1.1	1.1 ± 0.7	6.2 ± 2.3	14.4 ± 5.2	0.9 ± 0.1	3.9 ± 2.2
T2	10.5 ± 1.2	38.3 ± 5.5	179.1 ± 42.1	3.60 ± 1.1	0.6 ± 0.3	4.9 ± 1.3	10.3 ± 1.0	0.9 ± 0.3	2.9 ± 0.3
T3	12.6 ± 3.8	54.1 ± 13.3	165.2 ± 16.2	3.88 ± 0.3	0.7 ± 0.8	5.7 ± 0.6	14.8 ± 2.2	1.1 ± 0.2	3.2 ± 0.7
T4	13.2 ± 3.3	72.4 ± 8.0	238.9 ± 31.6	3.39 ± 0.8	0.8 ± 0.4	3.6 ± 0.3	10.6 ± 1.3	1.1 ± 0.3	3.0 ± 0.7
NC	1.3 ± 0.7	64.5 ± 9.5	222.9 ± 24.1	2.34 ± 0.2	3.0 ± 1.9	13.0 ± 7.9	15.2 ± 1.5	1.6 ± 0.4	1.4 ± 0.9

sequences available in Genbank that were aligned against the Pfam Fer4_NifH amino-acid seed alignment (Finn *et al.*, 2006). Regions of ambiguous alignment were identified and excluded from subsequent phylogenetic analyses. Phylogenetic trees were generated by performing protein parsimony (Swofford, 1991), and maximum-likelihood analyses (Olsen *et al.*, 1994).

Analyses of diazotroph community composition

Diversity analyses were performed on aligned DNA sequences of 487 nucleotides in length. OTU classification was performed using DOTUR as described previously (Schloss and Handelsman, 2005). Protein-encoding genes from the strains of a given species generally have an average nucleotide identity, whicht is >93%–95% (Konstantinidis and Tiedje, 2005), and thus a conservative OTU cutoff of 93% similarity was used for diversity calculations. Community structure was evaluated as a function of changes in community diversity and composition as discussed previously (Schloss and Handelsman, 2008). Within community, diversity was evaluated both as richness, as determined by the Chao I estimator (Hughes *et al.*, 2001), and evenness, as calculated by the evenness component of the Shannon index (Pielou's J') (Magurran, 1988). Changes in OTU composition between communities were evaluated with Sorenson's index of similarity (Magurran, 1988), and with UniFrac analysis performed with unweighted data (which ignore the relative abundance of OTUs in the library). In addition, *nifH* clone libraries were compared using $\int$-Libshuff (Schloss *et al.*, 2004) and UniFrac analyses (Lozupone and Knight, 2005), which assess overall differences in community structure and are sensitive to the changes in richness, evenness, genetic diversity and the composition of communities as described previously (Schloss and Handelsman, 2008). Guide trees for UniFrac were generated through maximum-likelihood analysis as described above. Distance matrices generated with Unifrac were used to cluster communities using UPGMA and jackknife analysis was used to evaluate to the confidence of tree nodes. UniFrac was also used to perform principal coordinates analysis.

Statistical analyses

Statistical tests were performed using StatView v5.0.1 (SAS Institute Inc., Cary, NC, USA). Analysis of variance models included one-way analysis of variance to evaluate the differences between control (NC) and agricultural sites, and 2×2 factorial analysis of variance to evaluate the main effects of tillage and biomass retention among the agricultural sites. In addition, stepwise regression was used to evaluate linear relationships between soil variables and N-fixation rates. Stepwise regression was performed using forward and backward procedures and collinear variables were removed, so that both models produced identical results.

Results

Impacts of tillage and biomass treatments on soil characteristics

Tillage and biomass management were both found to have significant impacts on soil characteristics (Tables 1 and 2). The effect of three decades of continuous tillage was clearly evident at the site as 12 of the 19 soil characteristics (do not include N fixation) examined varied significantly with respect to tillage and one variable, extractable soil K, demonstrated an interaction between the effects of tillage and biomass management that was significant (Table 2). In contrast, the impact of biomass retention was more modest, and the only effects observed were increases in soil P and K content in fields where biomass was retained (Tables 1 and 2). Ignoring management type, the agricultural treatments differed from NC control sites in all soil characteristics measured except extractable K, Mg and

Table 2 Results from ANOVA examining effects of tillage and biomass on soil characteristics presented in table 1 (and excluding the NC sites)

	N-fixed	*pH*	*Moisture*	*OM*	*DNA*	*C*	*N*	*NO_3*	*C/N*	*C/P*	*N/P*
Tillage											
Mean Square	2.3×10^{-8}	0.559	0.028	988	91.9	297	1.96	114.9	0.22	9928	1508389
F-value	2.38	9.6	5.7	13.3	22.4	21.8	32.7	6.95	0.301	5.367	4.7
P-value	0.143	0.008	0.031	0.003	<0.001	<0.001	<0.001	0.022	0.587	0.036	0.048
Biomass											
Mean Square	5.4×10^{-8}	0.008	0.004	242	31.8	20.93	0.09	42.8	0.035	287509	2217
F-value	7.23	0.078	0.577	1.901	3.81	0.629	0.465	2.59	0.049	0.704	0.923
P-value	0.018	0.784	0.46	0.19	0.071	0.441	0.506	0.134	0.828	0.415	0.353
Tillage × biomass											
Mean Square	3.0×10^{-8}	0.02	0.003	0.006	0.271	16.61	0.04	41.9	0.615	194206	431
F-value	6.8	0.31	0.526	9.59E-05	0.132	1.303	0.676	2.53	0.792	0.581	0.222
P-value	0.030	0.588	0.481	0.992	7.723	0.276	0.427	0.137	0.391	0.646	0.646

	P	*K*	*Mg*	*Ca*	*Fe*	*Al*	*Mn*	*Zn*	*Cu*
Tillage									
Mean Square	4.55	214	8271	1818	0.108	12.21	68.0	0.001	1.24
F-value	0.518	2.72	8.52	2.42	0.312	6.78	7.83	0.009	0.87
P-value	0.439	0.125	0.013	0.146	0.587	0.023	0.016	0.926	0.369
Biomass									
Mean Square	39.46	2129	3987	636	0.054	3.060	0.519	0.20	0.365
F-value	6.27	27.05	4.12	0.847	0.155	1.698	0.060	4.27	0.257
P-value	0.025	<0.001	0.065	0.375	0.701	0.220	0.811	0.061	0.621
Tillage × biomass									
Mean Square	0.992	484.2	3181	134	0.422	0.679	0.008	0.000	0.567
F-value	0.144	6.15	3.28	0.179	1.217	0.377	0.001	0.008	0.400
P-value	0.711	0.029	0.095	0.680	0.292	0.551	0.976	0.932	0.539

Abbreviation: ANOVA, analysis of variance.

Mn (Table 1). Soil moisture ($F_{1,18} = 9.23$, $P = 0.008$), organic matter ($F_{1,18} = 17.44$, $P = 0.0006$), DNA ($F_{1,18} = 10.263$, $P = 0.0049$), total C ($F_{1,18} = 14.553$, $P = 0.013$), total N ($F_{1,18} = 21.6$, $P = 0.0002$), nitrate ($F_{1,18} = 23.23$, $P = 0.0002$), Fe ($F_{1,18} = 18.13$, $P = 0.0005$), Al ($F_{1,18} = 16.02$, $P = 0.0008$) and Zn ($F_{1,18} = 19.49$, $P = 0.0003$) were all higher in NC than in agricultural sites (Table 1). In contrast, pH ($F_{1,18} = 61.299$, $P < 0.0001$), P ($F_{1,18} = 44.39$, $P < 0.0001$), Ca ($F_{1,18} = 10.94$, $P = 0.004$) and Cu ($F_{1,18} = 9.28$, $P = 0.007$) were lower in NC than in agricultural sites (Table 1).

Impacts of treatment and soil characteristics on N-fixation rates

Analysis of N-fixation rates in soil revealed that there was an interaction between the effects of tillage and biomass management and the result was significant (Table 2). *Post hoc* tests revealed that this effect was largely driven by the rate of N fixation in the no-till sites with biomass retention (T4), which was significantly lower than that observed in all other sites (Fisher's PLSD, $P < 0.05$). Despite this interaction effect, the main effect of biomass retention was still observed to cause a reduction in soil N fixation and the result was significant (Table 2).

Relationships between soil characteristics and N-fixation rates were explored by both regression and stepwise multiple regression. Soil K was found to be negatively correlated with N-fixation rates and was the only single variable shown to explain significant variation in N-fixation rates ($R^2 = 0.680$, $P < 0.001$). Stepwise regression using all independent soil variables identified P and K as the main variables associated with N-fixation rates ($R^2 = 0.747$; $F_{2,18} = 23.60$, $P < 0.0001$), but of these, only K was found to have a significant standardized regression coefficient (-0.826, $P < 0.0001$). If K was removed from the stepwise regression procedure, then a model containing the variables P, Ca and Mg was still found to explain significant variation in N-fixation rates ($R^2 = 0.575$; $F_{3,19} = 7.22$, $P = 0.003$), and the standardized regression coefficients of these variables (P: -0.599, $P = 0.004$; Mg: -0.544, $P = 0.004$; Ca: 0.453, $P = 0.023$) were significant.

Impacts on the diversity of the diazotroph community

The Chao1 estimator was used to assess the richness of diazotrophic communities as revealed through the analysis of *nifH* clone libraries. The accumulation curves indicate that the diazotroph community

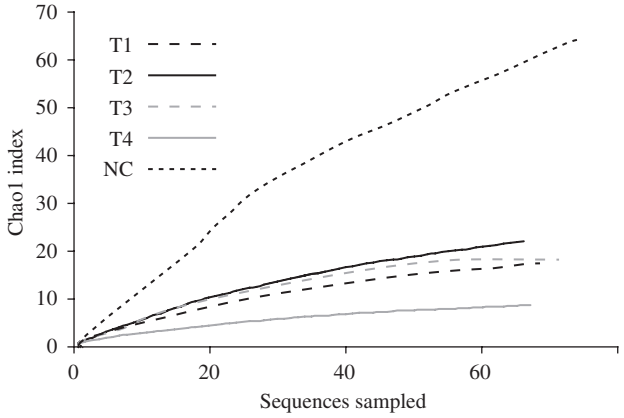


Figure 1 Chao I richness estimates for *nifH* clone libraries with OTUs defined by a 93% DNA similarity cutoff. Confidence intervals are provided in Table 3. OTUs, operational taxonomic units.

was well sampled in agricultural sites but may still be under sampled in the NC sites (Figure 1). Analysis of *nifH* clone libraries from the five treatments revealed that the no-till plots with biomass retention (T4) had the lowest richness, and the NC sites had the highest richness of all sites examined, and these results were significant (Table 3). When pooled by management type within the agricultural sites, differences in the richness of *nifH* with respect to tillage (till: 47 ± 19; no-till: 30 ± 6) or biomass management (retention: 26 ± 4; removal: 31 ± 4) were not significant (s.d. of the mean is reported). In contrast, evenness varied considerably across the sites (Figure 2) with greater dominance observed in biomass retention plots than in plots where biomass was removed or in the control plots (Figure 2).

Impacts on the structure of the diazotroph community
Diazotroph community structure differed across the treatments as a function of both biomass and tillage management. Results from $\int$-Libshuff analysis of the *nifH* libraries indicated that the community structure did not differ significantly between plots in which biomass was retained (T3 and T4), but there were significant differences in community composition between all other pairwise combinations of treatments ($P<0.05$, after Bonferroni correction). UniFrac analysis of *nifH* clone libraries also supports the conclusion that biomass retention has an impact on the composition of the diazotroph community and the result is significant (Figure 3a). When *nifH* sequences were pooled with respect to management type both the effect of biomass retention and tillage were found to have significant impacts on diazotroph community composition ($\int$-Libshuff, $P<0.05$, after Bonferroni correction). To evaluate the effect that OTU relative abundance has on these analyses, UniFrac analysis was also performed with (Figure 3a) and without (Figure 3b) respect to OTU

Table 3 Estimates of *nifH* richness for all sites. OTUs defined by a 93% similarity cutoff

	T1	*T2*	*T3*	*T4*	*NC*
Total sequences	69	66	72	67	75
Observed OTU	16	17	15	8	35
Chao 1 (mean ± s.d.)	18 ± 2	21 ± 4	18 ± 3	8 ± 1	64 ± 18
Chao 1 upper 95% CI	28.0	39.3	32.6	12.1	121.7
Chao 1 lower 95% CI	16.2	17.8	15.5	8.0	44.4

Abbreviations: CI, confidence interval; OUT, operational taxonomic unit.

abundance in the library. In the unweighted analysis, it was possible to discern the effects of tillage on the composition of the diazotoph community (Figure 3b). Likewise, Sorenson's index of similarity, which evaluates the presence or absence of OTUs without respect to abundance, indicated greater similarity between the diazotrophic communities in tilled fields (T1 and T3: 0.92) relative to either no-till fields (T2 and T4: 0.52), plots that shared biomass management (T1 and T2: 0.62; T3 and T4: 0.048), or any comparison of the agricultural and control plots (0.15 ± 0.05, $n=4$ comparisons). These results are broadly consistent with an interaction of tillage and biomass management on diazotroph community composition in which biomass retention results in an increase in the dominance of certain OTUs (that is, reduced evenness) in T3 and T4, whereas tillage results in an increase in the number of shared OTUs between T1 and T3.

The impact of treatment effects on the abundance of particular OTUs in *nifH* clone libraries was investigated. The abundance of a set of four OTUs that comprise a monophyletic grouping within a cluster of *nifH* genes most commonly associated with *Alphaproteobacteria* (Figure 4, shaded box) was found to be greater in plots with biomass retention (93 of 139 sequences) than in plots where biomass was removed (55 of 135 sequences) and the result is significant (Fisher's exact test, $P=0.0001$). The majority of these sequences (82%) fall into a single dominant OTU (Figure 4), and when these data are removed, the effect of biomass is still significant (Fisher's exact test, $P=0.001$). In contrast, the abundance of this group in clone libraries does not vary significantly with respect to tillage (Fisher's exact test, $P>0.05$). As a result, we can conclude that this single group of *nifH* sequences has become enriched as a result of biomass retention and is largely responsible for the differences observed in the evenness of the diazotrophic community.

N fixation and diazotroph community structure
N fixation rates were observed to vary as a function of the diversity and composition of the diazotrophic community. N-fixation rates displayed a unimodal

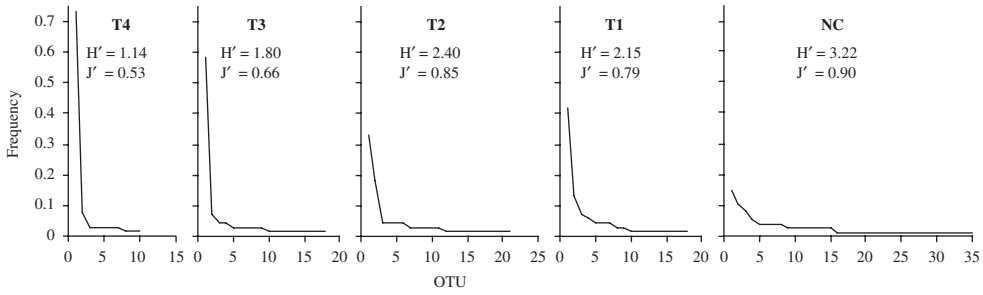


Figure 2 Frequency distribution for OTUs observed in *nifH* clone libraries. The label for each panel provides the treatment identifier, the Shannon index of diversity (H') and the evenness component of the Shannon index (Pielou's J') for each library. OTUs, operational taxonomic units.

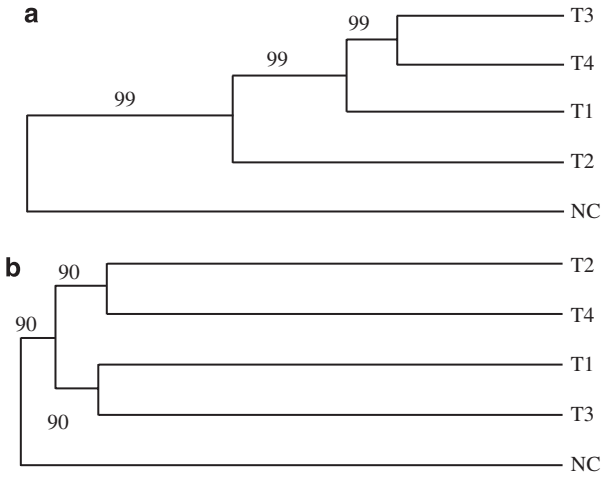


Figure 3 Dendograms from UniFrac analyses of *nifH* clone libraries. Numbers indicate the frequency with which nodes were supported by jackknife analysis. Analyses were performed with respect to the abundance of each OTU (weighted data, **a**), and by ignoring OTU abundance (unweighted data, **b**). OTU, operational taxonomic unit.

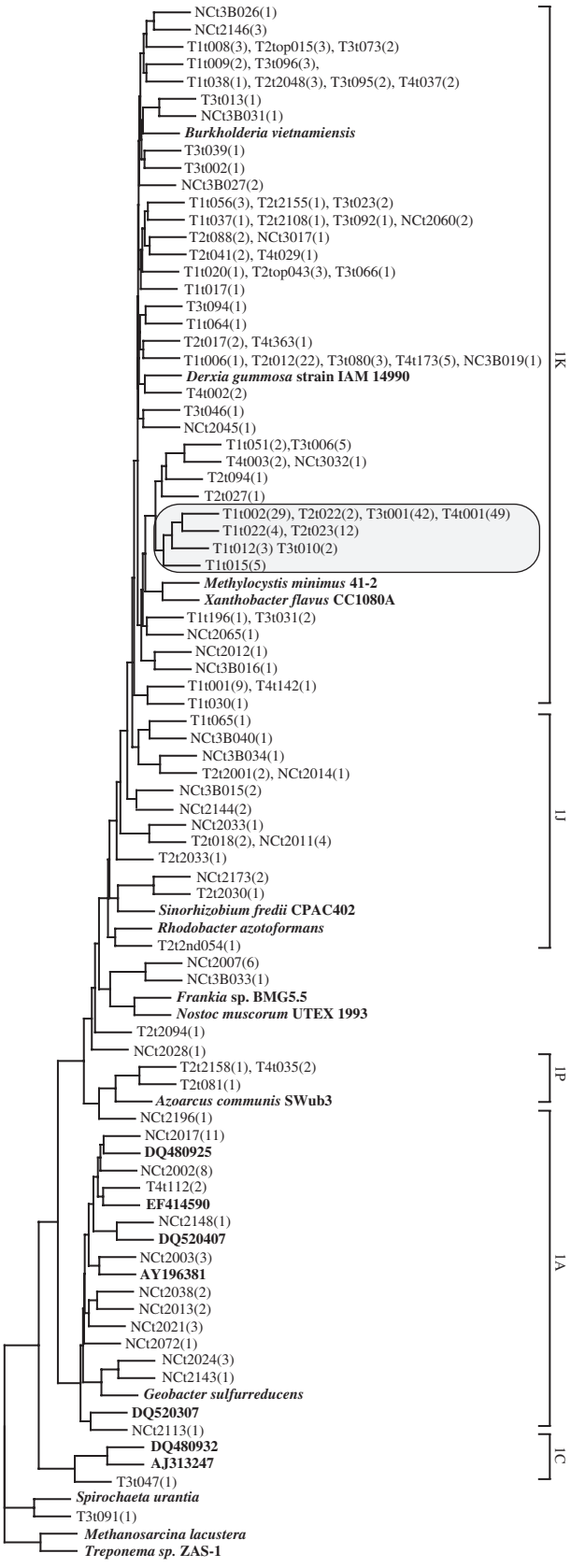
distribution with respect to both evenness (Figure 5a) and, to a lesser degree, the richness (Figure 5b) of the diazotophic community, and the results were significant. The effect of the lowest diversity sample (T4) on this analysis was examined, and when these datare removed, the relationship between N fixation and evenness remains significant ($R^2 = 0.999$, $P < 0.001$), whereas the relationship to richness is no longer significant. A similar relationship between N-fixation rates and diazotrophic community composition was detected as a result of principal component analysis of community similarity conducted using UniFrac analysis (Figure 5c). Change in a principal component representing 30% of variation in community membership was found to correlate with differences in N-fixation rate (Figure 5c). This analysis was conducted with unweighted data, and is therefore not influenced by the changes in evenness or abundance caused by the dominant OTU found on the agricultural site (as described above).

Discussion

Treatment effects on diazotroph diversity

The experimental site had been established for more than 30 years as a randomized complete block to control for variation in the landscape. The treatments are relatively small in scale with the whole site occupying less than 1 Ha and all plots having the same soil type and being planted to the same crop for the entire history of the experiment. Thus, extraneous variation is minimized in this experiment relative to many studies that focus on the functional significance of soil communities. In addition, the diversity of the diazotrophic community in soil is typically far lower than that of the overall community, with estimates of *nifH* richness generally between 11–99 $OTU_{0.03}\,g^{-1}$ soil (Bothe *et al.*, 2002; Izquierdo and Nusslein, 2006; Roesch *et al.*, 2008). Regardless, the response of the diazotrophic community to the effects of tillage and biomass management that we observed was complex and differed with respect to the richness, evenness and the composition of the community.

Biomass retention caused a dramatic change in the evenness of the diazotroph community (Figure 2), and this result was likely responsible for the results obtained by both $\int$-Libshuff and weighted UniFrac analyses (Figure 3a), which indicated a significant effect of biomass management on diazotroph community structure. This change in evenness was primarily driven by the enrichment of a single group of dominant *nifH* sequences (Figures 4) in response to biomass retention. However, $\int$-Libshuff indicated that the tillage also had a significant impact on community structure. The effect of tillage became more apparent, when the effects of changes in evenness were removed by performing analyses that ignore OTU abundance (Figure 3b). Taken together, these results indicate an interaction between the effects of tillage and biomass retention on diazotroph community structure. The interaction seems to result from the impact of biomass management on evenness (caused by the enrichment of a dominant OTU in response to biomass retention) and the impact of tillage on community composition (caused by the



homogenization of community composition in response to tillage of the soil). The richness results also support an interaction effect as data pooled with respect to either tillage or biomass did not reveal significant differences with respect to richness, whereas richness was significantly reduced in no-till biomass retention (T4) plots relative to either tilled biomass retention (T3) plots or no-tilled biomass removal (T2) plots (Table 3).

A range of treatment effects can impact the structure of the diazotrophic community in soils including changes in plant community composition (Tan *et al.*, 2003; Patra *et al.*, 2006), burning (Yeager *et al.*, 2005), pH (Nelson and Mele, 2006), soil N (Bothe *et al.*, 2002; Mergel *et al.*, 2001; Tan *et al.*, 2003) and biomass retention (Nelson and Mele, 2006; Wakelin *et al.*, 2007). In this study, plant community composition was held constant across agricultural sites, but differed considerably between those and the control (NC) sites. The effects of pH on diazotroph community structure have largely been documented to be important at pH values below 5.3 (Limmer and Drake, 1996; Nelson and Mele, 2006), but have not been observed to have effects in the pH range of 6.5–8.0 that characterized the soil from our site. The effects of soil N on diazotroph community structure tend to be somewhat complex. Maximal *nifH* abundance has been observed to coincide with peak values for nitrate and total N (Bothe *et al.*, 2002; Mergel *et al.*, 2001), but fertilization does not necessarily result in an increase in *nifH* abundance (Wakelin *et al.*, 2007). In addition, the changes in diazotroph community structure show no relationship to N availability in upland soils (Shaffer *et al.*, 2000; Poly *et al.*, 2001) or salt marsh soils (Piceno and Lovell, 2000), but do in alpine soils (Zhang *et al.*, 2006) and in paddy soils (Tan *et al.*, 2003).

Impacts of treatment and soil characteristics on N-fixation rates

Given the inconsistent response of free-living diazotrophs to N availability in soil as described above, it is perhaps not surprising that we did not find a relationship between N-fixation rates and soil N or nitrate. In contrast, the presence of high C/N crop biomass clearly has the potential to stimulate N fixation in soils, and biomass retention can impact N fixation through the effects on both soil moisture

Figure 4 Maximum parsimony tree of representative *nifH* sequences described in this study. The tree was constructed from translated data using 109 amino-acid positions. Each unique OTU is represented once in the tree. Branch labels contain multiple sequence names to indicate when an OTU occurs in more than one site, and a representative sequence from each site is provided. The number in parenthesis following sequence names indicates the number of sequences observed for each OTU in each site. The names of reference sequences not determined in this study are in bold. The shaded box encompasses the dominant group of OTUs as discussed in the text. OUT, operational taxonomic unit.

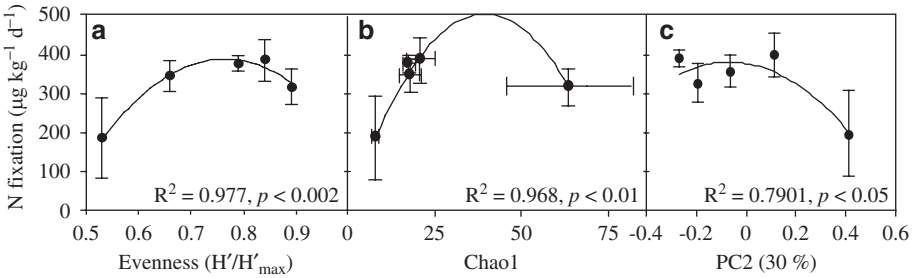


Figure 5 Relationship between N-fixation rate and diazotroph diversity as estimated from *nifH* clone libraries and defined by evenness, richness and community composition. Community evenness (**a**) is defined by the evenness component of the Shannon index (Pielou's $J' = H'/H'_{max}$); Community richness (**b**) is defined by the Chao1 estimator; and community composition (**c**) was assessed by Unifrac principle coordinate analysis of unweighted data from *nifH* libraries (and is thus a measure of composition unaffected by the differences in evenness). The principle coordinate (PC) depicted describes 30% of the variation in *nifH* library composition. Regression was performed with a second order polynomial function consistent with the observation of a unimodal response. Error bars indicate s.d. of the mean.

and temperature (Roper, 1983). Biomass retention or addition can increase N-fixation rates in cereal crops, including wheat, rice and maize (Roper, 1983; Roper *et al.*, 1989, 1994; Gupta *et al.*, 2006), increase *nifH* abundance in soil (Wakelin *et al.*, 2007) and change diazotroph community structure (Nelson and Mele, 2006). In addition, the effect of biomass retention can interact with the effect of tillage, because crop residues have different effects when left on the soil surface than they do when incorporated into the soil by tilling (Roper *et al.*, 1989). The estimates of N fixation in agricultural fields where biomass is retained, range from 0.03–1.6 kg N Ha^{-1} d^{-1}, with an estimate that optimal conditions can provide a rate of 0.75 kg N Ha^{-1} d^{-1} (Gupta *et al.*, 2006). The average N-fixation rate observed for the agricultural plots at our site was 0.33 ± 0.13 kg Ha^{-1} d^{-1}.

We observed that the biomass retention had a significant impact on N fixation and that there was an interaction between the effects of biomass and tillage (Table 2). As maize residues have a high C/N ratio (30:1–50:1) the *a priori* expectation would be a positive correlation between biomass inputs and N-fixation rates; however, the reverse was observed (Table 1). This unexpected result suggests that the addition of homogeneous residues has different impacts on soil processes (and possibly on soil communities) over long periods of time than it does in short-term experiments (such as those described above). Although total soil C, N, NO_3, C/N, pH, moisture and organic matter content might all be expected to influence N-fixation rates, these variables were not impacted significantly by biomass management (Table 2), and did not explain significant variation in N-fixation rates as assessed either by regression or stepwise regression. Biomass retention did cause a significant increase in extractable cP and K (Table 2), and variation in these parameters and Mg were found by multiple regression to be negatively correlated with N-fixation rates. As P, K, Ca and Mg are in order, the four most abundant trace elements in plant biomass following C and N, it seems likely that the correlations observed between these variables and N-fixation rates are caused indirectly by their correlation with plant biomass inputs (the different response of extractable Ca is likely explained by the interaction between pH and Ca mineralogy). As biomass retention was observed to decrease N fixation, we would expect soil variables that track biomass addition, such as P and K, to correlate with N fixation. Changes in these soil variables, however, are unlikely to be driving changes in N fixation, as increases in K, P and Mg were correlated with decreases in N fixation. Limitation of P and K can inhibit N-fixation rates (Alahari and Apte, 1998; Moisander *et al.*, 2003) indirectly by limiting growth, but there is no mechanism known by which the modest increases in K, P and Mg could inhibit N fixation. Thus, changes in soil characteristics caused by biomass retention cannot mechanistically explain the reductions in N-fixation rate that we observed.

Evidence that diazotroph diversity impacts N-fixation rate

Changes in diazotroph community structure mirrored those of N-fixation rates in that they were both primarily impacted by biomass management and also by the interaction of tillage and biomass. Evidence was found that the changes in community structure as assessed by the evenness (Figure 5a), and to a lesser extent the richness (Figure 5b) and composition (Figure 5c) of the diazotroph community, were correlated with the changes in N-fixation rates. These results show that changes in diazotroph community structure at the site have functional significance and suggest that N-fixation rates vary as a function of the diversity of the diazotroph community. The ecological significance of community diversity has been investigated widely in plant communities and to a lesser degree in microbial systems. The general focus of many of these studies is the influence of diversity on productivity. The most common observation at local and regional

scales is a unimodal or hump-shaped relationship between plant community diversity and productivity (for reviews see (Symstad *et al.*, 2003; Hooper *et al.*, 2005)). This observation fits with the general expectation of a saturating response of ecosystem properties to increasing diversity (Hooper *et al.*, 2005). Thus, the observation of a unimodal relationship between N fixation and diazotroph diversity (Figure 5a and b) fits existing theory pertaining to the potential significance of diversity on ecosystem properties. It can, however, be difficult to disentangle the ecological significance of changes in the diversity and the composition of communities. The effect of biomass retention on the evenness and richness of the diazotroph community is clearly related to the changes in the abundance of the dominant OTU at the site. The distribution and activity of highly dominant species have previously been shown to exert large impacts on ecosystem function in plant communities (Symstad *et al.*, 2003). These impacts can be caused either directly by the activity of the dominant species, or indirectly through the impacts of dominant species on the diversity or activity of the rest of the community (Symstad *et al.*, 2003). It should be noted, however, that a relationship between community composition and N-fixation rate was also observed when OTU abundance (and hence evenness) was ignored (Figure 5c), demonstrating that the relationship between community structure and N-fixation rates is not solely a function of the distribution of any one OTU at the site.

Problems associated with inferring the ecological significance of community composition from environmental treatments have been reviewed previously (Reed and Martiny, 2007). Two common problems encountered are that experimental treatments are not maintained for sufficient time to allow for community composition to reach equilibrium, and that the effects of community composition on a given ecological process cannot be resolved from those of the treatment itself. The current experiment has been maintained for more than 30 years, and so the former concern does not seem immediately relevant, but the latter requires some consideration. Biomass and tillage treatments would primarily impact N-fixation rates through their impacts on soil characteristics; however, variation in the soil characteristics observed at the site did not satisfactorily explain N-fixation rates. The quality of biomass inputs can impact N-fixation rates (Vitousek and Hobbie, 2000), but all C inputs derived from this experiment were from maize, and only varied in quantity between treatments. One potentially confounding variable in this study is the effect of soil physical structure on the community. Aggregate stability has been observed to vary as a function of both tillage and biomass at our site (Harold van Es, personal communication), and soil aggregate size can have a profound impact on both N-fixation rates and on diazotrophic community composition (Poly *et al.*, 2001; Chotte *et al.*, 2002). However, changes in community structure and N-fixation rates are likely to be intimately related within soil aggregates and difficult to disentangle. As a result, it seems clear that the changes in community structure have functional significance at the site, but it is not clear whether this response is mechanistically explained by a change in community diversity itself or from treatment-induced changes in soil structure and their resulting impacts on community structure and function.

Future perspectives

A final consideration is the degree to which N-fixation rates measured at a single time are ecologically significant or the degree to which N-fixation rates or diversity may vary temporally at the site. Research is currently ongoing to address this issue as N-fixation rates, soil characteristics and diazotroph community structure in the sites have been monitored over a period of 2 years. Preliminary evidence suggests that when averaged over time, N fixation continues to be depressed in plots where biomass is retained relative to where it is removed (data not shown). It is expected that measurement over time of changes in the abundance and composition of the diazotroph community in relation to N-fixation rates will make it possible to better constrain the functional significance of community composition. We have shown that the changes in rates of N fixation are related to the changes in diazotroph community structure and cannot be satisfactorily explained solely by the soil variables of the site. These results show that the changes in the community structure of free-living diazotrophs in soils can have ecological significance and suggest that this response is related to a change in community diversity.

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