APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2011, p. 911–919 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.01250-10

Diversity and Activity of Free-Living Nitrogen-Fixing Bacteria and Total Bacteria in Organic and Conventionally Managed Soils[▽]†

Caroline H. Orr,[1] Angela James,[1] Carlo Leifert,[2] Julia M. Cooper,[2] and Stephen P. Cummings[1]*

School of Applied Sciences, Northumbria University, Newcastle-upon-Tyne NE1 8ST, United Kingdom,[1] and Nafferton Ecological Farming Group, Newcastle University, Nafferton Farm, Stocksfield, Northumberland NE43 7XD, United Kingdom[2]

Received 27 May 2010/Accepted 24 November 2010

Agricultural soils are heterogeneous environments in which conditions affecting microbial growth and diversity fluctuate widely in space and time. In this study, the molecular ecology of the total bacterial and free-living nitrogen-fixing communities in soils from the Nafferton Factorial Systems Comparison (NFSC) study in northeast England were examined. The field experiment was factorial in design, with organic versus conventional crop rotation, crop protection, and fertility management factors. Soils were sampled on three dates (March, June, and September) in 2007. Total RNA was extracted from all soil samples and reverse transcribed. Denaturing gradient gel electrophoresis (DGGE) and quantitative PCR (qPCR) were used to analyze *nifH* and 16S rRNA genes in order to study free-living diazotrophs and the total bacterial community, respectively. Crop rotation was shown to have a significant effect on total bacterial diversity (and that of free-living N fixers) ($P \leq 0.001$). On all three dates, *nifH* activity was higher in the conventional crop rotation. In contrast, qPCR analysis of free-living N fixers indicated significantly higher levels of activity in conventionally fertilized plots in June ($P = 0.0324$) and in plots with organic crop protection in September ($P = 0.0143$). To our knowledge, the effects of organic and conventional farming systems on free-living diazotrophs have never been studied. An increased understanding of the impacts of management practices on free-living N fixers could allow modifications in soil management practices to optimize the activity of these organisms.

After water, nitrogen is most often the limiting factor for plant growth (45a). Crops such as wheat, rice, and maize need 20 to 40 kg soil N ha^{-1} over a period of 3 to 5 months to satisfy the N requirements for each tonne of grain produced (34). To meet such high demand, farmers must either apply inorganic synthetic N fertilizers to their land or rely on biological nitrogen fixation (BNF) and the input of recycled organic wastes, such as manure.

Adding nitrogen in the form of synthetic fertilizers can have negative environmental impacts since inorganic N, particularly nitrate; can be dispersed into surface and groundwater, leading to eutrophication (45a). In addition, the manufacture of N fertilizers relies on nonrenewable fossil fuels (the production of 1 kg N fertilizer requires 38,000 kJ of fossil energy) (39) and results in significant emissions of greenhouse gases (20). These environmental concerns, coupled with increasing fuel costs and a desire for improved sustainability have led some farmers to seek alternative N management strategies (34).

N cycling in natural ecosystems and traditional agricultural production relies on biological N fixation primarily by diazotrophic bacteria. Diazotrophs are highly diverse and are widely distributed across bacterial and archael taxa (13). Most (~80%) of biological nitrogen fixation (BNF) is carried out by diazotrophs in symbiosis with legumes (33). However, under specific conditions bacteria which are free-living in soil (e.g., cyanobacteria, *Pseudomonas*, *Azospirillum*, and *Azotobacter*) may fix significant amounts of nitrogen (0 to 60 kg N ha^{-1} $year^{-1}$) (5, 24). This may be particularly important in organically managed soils, which typically have a lower proportion of nitrogen in available forms (43).

The effects of crop management on diversity and function of the soil microbial community are equivocal. Many authors report an increase in total biomass and microbial activity when organic matter inputs are increased and chemical amendments are reduced (4, 7, 10). In contrast, Donnison et al. (15) found that a change in management had no effect on soil nutrient status, soil microbial biomass, and soil microbial activity. However, they did find that management practices significantly affected the soil microbial community structure and suggested that this was due to changes in plant composition and the form and quantity of fertilizer applied (29). Diazotrophic community structure and diversity have been shown to respond to changes in grazing, liming, the nature of the nitrogen added, and incorporations of crop residues (32, 46, 47). They are also especially sensitive to chemical inputs, such as pesticides (31).

The nitrogenase enzyme catalyzes the reduction of atmospheric dinitrogen to ammonia. This process is very energy expensive and is, therefore, tightly regulated (13). At neutral pH, low levels of fixed N and increased levels of C will allow more optimal conditions for free-living N fixation (12). These conditions are more likely to be found in organically managed soils as increased organic C is added in the form of manure and on average less readily available nitrogen is applied.

In this study, the diazotrophic population was monitored by PCR-denaturing gradient gel electrophoresis (DGGE) exploiting the *nifH* gene. The *nifH* gene is the most conserved gene in

* Corresponding author. Mailing address: School of Applied Sciences, Ellison Building, University of Northumbria, Newcastle-upon-Tyne NE1 8ST, United Kingdom. Phone: 44 191 227 3176. Fax: 44 191 227 3903. E-mail: stephen.cummings@unn.ac.uk.

† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 3 December 2010.

TABLE 1. CP protocols and FM used in the NFSC experiments for 2006 and 2007[a]

Crop, rotation type, and CP/FM protocol (yr)[a]	Management treatment(s)
Conventional rotation	
Winter barley (2006)	
ORG CP	Mechanical weeding (finger weeder)
CON CP	Pendimethalin[b] (2.5 liters/ha), isoproturon[b] (1.5 liter/ha), Duplosan[b] (1 liter/ha), Acanto[c] (0.4 liter/ha), Proline[c] (0.4 liter/ha), Corbel[c] (0.5 liter/ha), Fluroxypyr[c] (0.75 liter/ha), Amistar[c] (0.25 liter/ha), Bravo 500[c] (0.5 liter/ha), Cleancrop EPX[c] (0.4 liter/ha)
ORG FM	No amendment
CON FM	0:20:30 (64 kg P_2O_5/ha, 96 kg K_2O/ha), Nitram (170 kg N/ha)
Potatoes (2007)	
ORG CP	Mechanical weeding (ridging), copper-oxychloride[c] (23 kg/ha)
CON CP	Aldicarb[d] (33.5 kg/ha), linuron[b] (3.5 liters/ha), fluazinam[e] (1.5 liter/ha), mancozeb and metalaxyl-M[e] (4.7 kg/ha), oiquat[f] (2 liters/ha)
ORG FM	Composted cattle manure (equivalent to 180 kg N/ha)
CON FM	0:20:30 (134 kg P_2O_5/ha, 200 kg K_2O/ha), Nitram (180 kg N/ha)
Organic rotation	
Beans (2006)	
ORG CP	None
CON CP	Battalion[b] (2.8 liters/ha), Bravo 500[c] (1.5 liter/ha)
ORG FM	None
CON FM	0:20:30 (60 kg P_2O_5/ha, 90 kg K_2O/ha)
Potatoes (2007)	See conventional rotation for management treatments

[a] Shown are results for crops under organic crop protection (ORG CP) or conventional crop protection (CON CP) and organic fertility management (ORG FM) or conventional fertility management (CON FM).
[b] Herbicide.
[c] Fungicide.
[d] Nematicide.
[e] Growth regulator.
[f] Dessicant.

the *nif* operon and encodes the Fe subunit of the nitrogenase enzyme (41). Due to the conserved nature of the *nifH* gene, it has been possible to identify primer sets that can be used for analysis of diazotrophs so that this community can be analyzed by a PCR-DGGE-based technique (5, 36, 42, 50).

In this study we have tested the hypothesis that the use of organic farming practices (crop rotation, fertility management [FM], and crop protection) enhances the diversity and activity of free-living N fixers and the total bacterial population. We also investigated the seasonal variability of the diversity (as measured by changes in DGGE expression fingerprints) and activity (as measured by changes in expression of genes measured by quantitative PCR [qPCR]) of free-living N fixers and total bacteria.

MATERIALS AND METHODS

Soil sampling. The soil used in this study was taken from the Nafferton Factorial Systems Comparison (NFSC) study, a field trial based at Nafferton Farm in the Tyne Valley, northeast England. The objective of the field trial is to study the effects of "low-input" and organic food production systems on crop productivity, sustainability, environmental impacts, and food quality and safety.

The NFSC was established in 2001 and consists of a series of four field experiments established within four replicate blocks: plots 1 to 4 in block 1, plots 5 to 8 in block 2, plots 9 to 12 in block 3, and plots 13 to 16 in block 4. The experiment is a split split-plot design with three factors. The main factor is crop rotation. An 8-year, conventional cereal intensive rotation is compared to an 8-year, diverse legume intensive organic crop rotation. Each main plot is split to compare two levels of crop protection: organic (ORG CP; according to Soil Association organic farming standards [45]) and conventional (CON CP; following British Farm Assured practice). Each crop protection subplot is further split into two fertility management sub-subplots: organic (ORG FM; using compost as a fertility amendment [applied 26 March]) and conventional (CON FM; using mineral NPK fertilizer as a fertility amendment [applied 12 and 25 April]). This design also allows the experiment to be analyzed within each level of crop rotation, as four separate production systems: fully organic (ORG), organic crop protection and conventional fertility management (ORG CP-CON FM), conventional crop protection and organic fertility management (CON CP-ORG FM), and fully conventional (CON).

Compost was applied to ORG FM plots on 26 March, and NPK and Nitram were applied to CON FM plots on 12 and 25 April, respectively. ORG CP plots received copper fungicide weekly between 20 June and 31 July. CON CP plots received pesticide on 25 April, herbicide on 2 May, and fungicide weekly between 20 June and 13 August. Full details of the organic and conventional fertility management and crop protection practices used in the potato crop and the preceding year are shown in Table 1. The soil used in this study is a uniform sandy loam (alluvial deposit) and was sampled from experiment 2 of the NFSC trial in year 4 of both crop rotations, when potatoes (cv. Santé) were grown. The previous crops (PCs) in the organic and conventional rotations were faba beans (cv. Fuego) and winter barley (cv. Pearl), respectively.

In order to allow for within-plot variability, five cores of soil (0 to 30 cm) were randomly sampled within a plot and immediately mixed to form one composite sample per plot, on 2 March (prior to planting), 11 June (potatoes in growth stage 30—elongation) and 24 September (after harvesting) 2007. There are 4 plots for each treatment, giving a total of 16 plots. Soils were sieved fresh (4 mm), and a portion (about 10 g) was frozen immediately and stored at −80°C before extraction of RNA. Another portion (about 500 g fresh) was stored at 4°C before measurements of soil basal respiration with the Sensomat measurement system (40). A further portion of fresh soil was frozen at −20°C until extraction for nitrate and ammonium-N (2 M KCl). Concentrations of NO_3^--N and NH_4^+-N in the KCl extracts were determined with a Brann-Luebbe autoanalyzer 3 and the hydrazine reduction method for nitrate and the salicylate method for ammonia. The September soil samples were used for pH analysis (1:1 in water). Representative samples from the plots were taken in November 2007, dried, and sieved (2 mm) before analysis for total C and N by Dumas combustion (LECO Corporation) and Mehlich-3 extractable macro- and micronutrients.

RNA extraction and PCR. RNA was extracted from 0.25 g of soil with the UltraClean microbial RNA isolation kit (MoBio) and reverse transcribed with the Superscript II reverse transcriptase kit (Invitrogen).

The *nifH* gene was amplified by a nested-PCR method adapted from Wartiainen et al. (48). The first reaction used primers PolF and PolR (36) to amplify a 360-bp fragment. In order to clamp the products for DGGE, a second round of PCR was needed using AQER-GC30 and PolFI primers (48). PCR and the

TABLE 2. PCR and qPCR primers used in this study

Primer	Sequence (5′→3′)	Reference
PolF	TGC GA(CT) CC(GC) AA(AG) GC (GCT) GAC TC	36
PolR	AT(GC) GCC ATC AT(CT) TC(AG) CCG GA	36
AQER-GC30	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCC GAC GAT GTA GAT (CT)TC CTG	48
PolFI	TGC GAI CC(GC) AAI GCI GAC TC	48
V3R	ATT ACC GCG GCT GCT GG	30
V3FC	CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG	30
Eub338	ACT CCT ACG GGA GGC AGC AG	26

qPCR primers are summarized in Table 2. The reaction mixture contained 1 μl of reverse-transcribed RNA, 0.5 μM each primer, 25 mM each deoxynucleoside triphosphate (dNTP), 50 mM $MgCl_2$ (25 mM when using AQER-GC30/PolFI), 5× *Taq* buffer, 5 U *Taq* polymerase (New England Biolabs) (2.5 U when using AQER-GC30/PolFI), and 0.1 mg bovine serum albumin (BSA) and made up to 50 μl with sterile water. PCR conditions were taken from Poly et al. (36). The annealing temperature for both rounds was 55°C. In order to amplify the total bacterial community, the V3FC and V3R primers were used as described by Baxter and Cummings (2).

DGGE. DGGE was carried out using the D-Code system (Bio-Rad Laboratories) as described by Baxter and Cummings (2). Gels were electrophoresed at a current of 200 V for 6 h (*nifH*) or 200 V for 4.5 h (16S rRNA) at a constant temperature of 60°C. Bands were identified and relative intensities were calculated based on the percentageof intensity of each band in a lane. This was done with Quantity One software (Bio-Rad). Shannon's diversity index (H') was calculated by the formula $H' = -\sum p_i \ln(p_i)$, where p_i is the ratio of relative intensity of band i compared with the relative intensity of the lane.

qPCR. Reactions were set up using SYBR green (Thermo Fisher Scientific) according to Baxter and Cummings (1) with the Rotor-Gene RG 3000 (Corbett Research). All DNA was denatured at 95°C for 10 min prior to reaction setup. Reaction mixtures were heated to 95°C for 15 min to activate the SYBR green before completing 50 cycles of denaturation (95°C, 15 s), annealing (55/65°C [*nifH*/16S rRNA], 15 s), and extension (72°C, 15 s). PolF and PolR primers were used for *nifH* qPCR, and Eub338 and V3R were used for total bacteria qPCR. A standard curve was set up using 10-fold dilutions of pGEM-T Easy vector plasmid DNA containing either the *nifH* gene of *Rhizobium* sp. strain IRBG74 bacterium (isolated from root nodules of *Sesbania cannabina* by the International Rice Research Institute) (11) or the 16S rRNA gene of *Pseudomonas aeruginosa* NCTC10662. Each soil extraction, no-template control, and standard curve dilution was replicated three times. Average copy number was converted into copies of the gene per g of soil.

Standard deviation was determined (by the Rotor-Gene 6 software [Corbett Research]) on the replicate threshold cycle (C_T) scores. qPCR was repeated if the deviation was above 0.4. Samples were considered to be below reasonable limits of detection if the C_T score was above 30 (25). In the system used in this study, this would equate to results below 1.0×10^4 copies per g of soil being rejected. Although certain *nifH* copy numbers were low, none fell below this threshold. All no-template control results fell below this threshold (35.4 ± 2.8). The standard curve produced was linear ($r^2 = 0.98$), and the PCR efficiency was >0.9.

Statistical analysis. In all tests, significant effects/interactions were those with a P value of <0.05. All univariate data were analyzed using the linear mixed effects (lme) function in the nlme package of R (37). The combined data for all three dates were analyzed first, and where interaction terms were significant, further analyses were conducted at each level of the interacting factor. The hierarchical nature of the split split-plot design was reflected in the random error structures that were specified as block/date/precrop/crop protection. Where analysis at a given level of a factor was carried out, that factor was removed from the random error term. The normality of the residuals of all models was tested with QQ plots, and data were cube root transformed when necessary to meet the criteria of normal data distribution (2). Differences between main effects were tested by analysis of variance (ANOVA). Differences between the four crop management strategies within each level of crop rotation were tested with Tukey contrasts in the general linear hypothesis testing (glht) function of the multcomp package in R. A linear mixed effects model was used for the Tukey contrasts, containing a treatment main effect with four levels and with the random error term specified as block/crop protection.

Pearson's product-moment correlations were calculated using the cor.test function in R.

DGGE data were analyzed by detrended correspondence analysis (DCA) on relative intensities followed by direct ordination with Monte Carlo permutation testing. Direct ordination was either by canonical correspondence analysis (CCA) or redundancy discriminate analysis (RDA), depending on the length of the DCA axis (where an axis of >3.5 = CCA and an axis of <3.5 = RDA). CANOCO for Windows 4.5 and CANODRAW for Windows were used to carry out DCA, CCA, and RDA (28).

TABLE 3. Shannon diversity index values for 16S rRNA DGGE and *nifH* DGGE data sets and results of ANOVA carried out on these data

Crop management or statistical significance parameter	Shannon diversity index value for[b]:					
	nifH DGGE band data			16S rRNA DGGE band data		
	March	June	September	March	June	September
Precrop						
Barley	2.26	1.67	2.67	2.88	2.82	2.03
Beans	1.33	1.87	1.81	2.63	2.56	2.71
CP						
ORG	1.75	1.82	2.35	2.78	2.70	2.41
CON	1.84	1.72	2.14	2.73	2.69	2.33
FM						
ORG	1.66	1.78	2.22	2.71	2.69	2.34
CON	1.93	1.77	2.27	2.80	2.70	2.41
ANOVA P values[a]						
PC	**<0.0001**	0.154	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
CP	0.597	0.450	0.153	0.342	0.813	0.108
FM	0.121	0.916	0.695	0.057	0.796	0.154
CP × FM	0.381	0.537	0.546	0.584	0.407	0.514
FM × PC	0.381	0.731	0.599	0.391	0.191	0.268
CP × PC	0.558	**0.037**	0.859	0.696	0.441	0.539
FM × CP × PC	0.170	0.951	0.943	0.684	0.425	0.271

[a] PC, previous crop; FM, fertility management; CP, crop protection.
[b] Boldface is used for a significance (P) of <0.05.

RESULTS

Diversity and expression of *nifH*. (i) DGGE of *nifH*. DGGE gels are shown in Fig. S1 in the supplemental material. Analysis of the *nifH* DGGE Shannon's diversity index values for the whole data set (data not shown) indicated that sample date and crop rotation (precrop) significantly affected the *nifH* diversity (sample date, $P < 0.001$; precrop, $P < 0.001$; and sample date × precrop, $P < 0.001$). For these reasons, a separate analysis of variance (ANOVA) of the *nifH* DGGE Shannon's diversity index values was conducted at each date.

Soils in the conventional rotation with a previous crop (PC) of barley showed significantly higher *nifH* diversity than soils in the organic rotation with a previous crop of beans in March and September (the average H' values were 2.203 for the barley precrop and 1.674 for the beans precrop) (Table 3). Pearson's product-moment correlation found a significant negative correlation between nitrate and ammonium and *nifH* H' in March and a significant positive correlation between nitrate and ammonium and *nifH* H' in September (Table 4).

Table 5 summarizes results of CCA and RDA (for plots, see Fig. S3 in the supplemental material). Crop management effects were found to be significant in June in the organic rotation. Total carbon and nitrogen, available ammonium, and

TABLE 4. Changes to pH, available nitrate, and available ammonium across the field trial and Pearson's product-moment correlation analysis comparing data to *nifH* diversity and gene expression

Crop management or significance parameter	pH	Available NO_3^- (kg ha^{-1}) in[d]:			Available NH_4^+ (kg ha^{-1}) in[d]:		
		March	June[a]	September	March	June	September
Crop management[b]							
Precrop							
Barley	6.14 ± 0.05	5.81 ± 0.8	279.34 ± 43.2	27.08 ± 2.3	0.94 ± 0.2	7.58 ± 0.8	6.47 ± 0.5
Beans	6.23 ± 0.04	12.47 ± 0.7	234.42 ± 31.2	22.51 ± 1.7	5.95 ± 0.3	1.51 ± 0.3	0 ± 0
CP							
ORG	6.20 ± 0.05	8.68 ± 1.1	282.75 ± 40.5	30.07 ± 1.9	3.75 ± 0.1	4.85 ± 1.0	3.44 ± 1.0
CON	6.17 ± 0.04	9.60 ± 1.1	231.01 ± 34.3	19.52 ± 1.3	0.18 ± 0.9	4.24 ± 1.0	3.03 ± 0.8
FM							
ORG	6.26 ± 0.03	7.98 ± 1.2	125.04 ± 6.9	24.44 ± 1.3	3.71 ± 0.1	3.29 ± 0.6	3.68 ± 1.0
CON	6.11 ± 0.05	10.31 ± 0.9	388.72 ± 26.7	25.14 ± 2.7	3.18 ± 0.9	5.80 ± 1.2	2.79 ± 0.7
Statistical significance							
ANOVA *P* values							
PC	0.250	**<0.001**	**0.042**	**0.004**	**<0.001**	**<0.001**	**<0.001**
CP	0.603	0.327	**0.021**	**<0.001**	*0.051*	0.399	0.349
FM	**<0.001**	**0.018**	**<0.001**	0.632	*0.095*	**0.002**	**0.049**
ρ[c]							
Correlation with *nifH* DGGE *H′*	NS	−−	NS	+++	−−−	NS	+++
Correlation with *nifH* copy no.	+++	−−	NS	+++	−−−	+++	++

[a] Amounts of nitrate are often larger than amounts added to the soil due to mineralization of organic N to inorganic N by soil microorganisms.
[b] PC, previous crop; CP, crop protection; FM, fertility management.
[c] ρ, Pearson's product-moment correlation coefficient; NS, not significant; ++/−−, significant positive or negative correlation at $P < 0.05$; +++/−−−, significant positive or negative correlation at $P < 0.01$.
[d] Boldface is used for a significance (*P*) of <0.05, and italics are used for a significance of <0.1.

extractable phosphorus, as well as soil basal respiration, all significantly affect *nifH* diversity at some point over the sampling season, although the effects were not consistent across dates and between precrops.

(ii) qPCR of *nifH*. Analysis of the full set of data across sample dates indicated that there was a decrease in *nifH* copy number in June compared to March (average numbers of copies per g of soil, 5.70×10^5 in June versus 7.45×10^5 in March), followed by an increase in September (1.05×10^7 copies per g soil) which exceeded the March levels. There was a significant interaction between sample date and crop rotation (precrop effect) ($P = 0.0005$); therefore, the results of the ANOVA of the *nifH* qPCR are shown for each date in Table 6. On all sample dates, increased *nifH* copy number was seen in the conventional rotation (barley precrop) compared to the organic rotation (beans precrop). In the conventional rotation, the *nifH* copy number was 10 times higher in March and June than that in September. In June, increased *nifH* copy number was associated with conventional fertility management. In September, crop protection was a significant factor with increased *nifH* copy number when organic crop protection was used.

Pearson's product-moment correlation found a positive correlation between *nifH* copy number and pH, available ammonium in June and September, and available nitrate in September. There was a negative correlation between available nitrate and ammonium and *nifH* copy number in March (Table 4).

Total bacterial diversity and function. There are clearly differences, in the nitrogen-fixing community, between dates and between treatments. In order to ensure these factors are affecting the nitrogen-fixing community specifically and not the bacterial community as a whole, the 16S rRNA gene diversity and abundance were also analyzed.

DGGE of the 16S rRNA gene. DGGE gels showing diversity of total bacteria are shown in Fig. S2 in the supplemental material. As with the free-living nitrogen-fixing community, ANOVA results for the Shannon diversity index for the 16S rRNA gene indicated that date ($P < 0.001$) and date × crop rotation ($P < 0.001$) were significant factors (data not shown); therefore, each sample date was analyzed separately. This indicated that soils with a previous crop of barley (conventional rotation) showed significantly higher 16S rRNA diversity than soils following beans (organic rotation) in March and June; however, the situation was reversed in September (Table 3).

RDA and Monte Carlo permutation testing of the V3 DGGE profiles indicated that crop protection (June conventional rotation and September organic rotation) and fertility management (September conventional rotation) were significant drivers of bacterial community structure (Table 5). Total soil nitrogen and total soil carbon are also significant drivers of bacterial diversity in some cases. However, in all instances the differences in soil nitrogen and carbon are associated with different locations of the plots in the field (block effect) rather than the treatments themselves (see Fig. S4 in the supplemental material). The only significant driver which seems to be related to both crop management and soil diversity is pH in the

TABLE 5. Summary of CCA and RDA showing significant variables

Gene of interest	Previous crop	Sample date	Variable(s) tested	Significant variable(s) selected by forward selection[c]	Variance of DGGE data explained by the model (%)
nifH	Beans	March	FM		8.0
			CP		6.4
			Associated variables[a]	C	65.3
			Associated variables,[a] FM, CP[b]	C	76.3
		June	FM		6.1
			CP	CP	11.6
			Associated variables[a]	NH_4^+	48.2
			Associated variables,[a] FM, CP	CP, NH_4^+	65.7
		September	FM		7.2
			CP		7.4
			Associated variables[a]	P	36.9
			Associated variables,[a] FM, CP	P	48.3
	Barley	March	FM		7.5
			CP		5.8
			Associated variables[a]	N	51.0
			Associated variables,[a] FM, CP	N	61.9
		June	FM		9.2
			CP		6.4
			Associated variables[a]	SBR	53.7
			Associated variables,[a] FM, CP	SBR	68.1
		September	FM		7.0
			CP		5.2
			Associated variables[a]		40.9
			Associated variables,[a] FM, CP		51.7
16S rRNA	Beans	March	FM		6.6
			CP		5.7
			Associated variables[a]		41.8
			Associated variables,[a] FM, CP		55.0
		June	FM		6.9
			CP		7.7
			Associated variables[a]		46.6
			Associated variables,[a] FM, CP	N, pH	57.8
		September	FM		4.2
			CP	CP	11.4
			Associated variables[a]	N	30.7
			Associated variables,[a] FM, CP	CP, N	42.4
	Barley	March	FM		3.1
			CP		6.2
			Associated variables[a]		35.4
			Associated variables,[a] FM, CP		44.0
		June	FM		6.2
			CP	CP	14.1
			Associated variables[a]	C	42.0
			Associated variables,[a] FM, CP	CP, C	57.4
		September	FM	*FM*	11.3
			CP		4.7
			Associated variables[a]		31.3
			Associated variables,[a] FM, CP	*FM*	45.1

[a] These associated variables include available carbon (C) and nitrogen (N), pH, soil basal respiration (SBR), available phosphorus (P), ammonium (NH_4^+), and nitrate.

[b] These associated variables, including FM and CP, show the total effect of treatments and variables.

[c] All variables shown are significant at a P value of <0.05 unless shown in italics ($P < 0.1$).

TABLE 6. ANOVA of qPCR results

Crop management factor or significance parameter[a]	Avg no. of copies of *nifH*/g of soil[b]			Avg no. of copies of 16S rRNA/g of soil[b]		
	March	June	September	March	June	September
Precrop						
Barley	$1.3 \times 10^6 \pm 7.9 \times 10^5$ A	$1.1 \times 10^6 \pm 3.5 \times 10^5$ A	$2.1 \times 10^7 \pm 6.1 \times 10^6$ B	$6.2 \times 10^7 \pm 1.7 \times 10^7$ C	$5.2 \times 10^7 \pm 1.1 \times 10^7$ C	$4.8 \times 10^7 \pm 1.0 \times 10^7$ C
Beans	$1.5 \times 10^5 \pm 8.1 \times 10^4$ D	$3.0 \times 10^4 \pm 6.6 \times 10^3$ D	$6.6 \times 10^4 \pm 1.7 \times 10^4$ D	$2.7 \times 10^7 \pm 5.2 \times 10^6$ E	$5.3 \times 10^7 \pm 1.5 \times 10^7$ E	$4.4 \times 10^7 \pm 1.1 \times 10^7$ E
CP						
ORG	$3.3 \times 10^5 \pm 1.0 \times 10^5$ F	$4.3 \times 10^5 \pm 1.9 \times 10^5$ F	$1.5 \times 10^7 \pm 6.4 \times 10^6$ G	$5.1 \times 10^7 \pm 1.6 \times 10^7$ H	$3.9 \times 10^7 \pm 8.9 \times 10^6$ H	$3.9 \times 10^7 \pm 5.8 \times 10^6$ H
CON	$1.2 \times 10^6 \pm 8.1 \times 10^5$ I	$7.2 \times 10^5 \pm 3.5 \times 10^5$ I	$5.9 \times 10^6 \pm 2.7 \times 10^6$ I	$3.8 \times 10^7 \pm 1.0 \times 10^7$ J	$6.6 \times 10^7 \pm 1.6 \times 10^7$ J	$5.4 \times 10^7 \pm 1.4 \times 10^7$ J
FM						
ORG	$3.1 \times 10^5 \pm 9.8 \times 10^4$ K	$2.6 \times 10^5 \pm 1.1 \times 10^5$ K	$1.2 \times 10^7 \pm 6.2 \times 10^6$ K	$5.5 \times 10^7 \pm 1.7 \times 10^7$ L	$5.7 \times 10^7 \pm 1.5 \times 10^7$ L	$5.0 \times 10^7 \pm 1.3 \times 10^7$ L
CON	$1.2 \times 10^6 \pm 8.1 \times 10^5$ M	$8.9 \times 10^5 \pm 3.7 \times 10^5$ M	$8.9 \times 10^6 \pm 3.5 \times 10^6$ N	$3.4 \times 10^7 \pm 7.8 \times 10^6$ O	$4.8 \times 10^7 \pm 1.1 \times 10^7$ O	$4.3 \times 10^7 \pm 7.6 \times 10^6$ O
ANOVA *P* values						
PC	**0.012**	**0.006**	**<0.001**	**0.003**	0.897	0.366
CP	0.426	0.876	**0.014**	0.195	**0.027**	*0.079*
FM	0.163	**0.032**	0.447	**0.039**	0.524	0.982
CP × FM	0.539	0.734	0.718	0.372	0.608	0.728
FM × PC	0.194	0.340	0.530	0.488	0.297	0.406
CP × PC	0.103	0.156	0.266	0.285	0.314	0.700
FM × CP × PC	*0.096*	0.736	0.270	0.881	0.610	0.508

[a] PC, previous crop; CP, crop protection; FM, fertility management.

[b] The values shown for management factors are means ± standard errors. Values followed by the same letter in the same row at each level of management factor are not significantly different (Tukey's honestly significant difference; $P > 0.05$). Boldface is used for a significance (P) of <0.05, and italics are used for a significance of <0.1.

organic rotation in June. Here an increase in pH is associated with organic fertility management.

qPCR of 16S rRNA gene. The aim of the qPCR analysis was to compare differences between plots rather than absolute quantification. There was a significant interaction between sample date and crop protection when the 16S bacterial populations were quantified using qPCR ($P = 0.017$). For this reason, a separate analysis was conducted at each date (Table 6). In contrast with the DGGE results for the 16S rRNA gene, the previous crop was the only significant factor in March. On the same date, a history of organic fertilization also resulted in higher numbers of the 16S rRNA gene copy. In June, crop protection affected 16S rRNA gene copy numbers, with higher numbers where conventional crop protection was used. This positive effect of conventional crop protection continued into September, although it was no longer significant ($P = 0.079$).

Pearson's product-moment correlation showed that there is no link between increased expression of the 16S gene and increased expression of the *nifH* gene (data not shown).

DISCUSSION

This study allowed a detailed analysis of the effects of key components of organic and conventional farming systems on soil bacterial and free-living N-fixing bacterial population structure and gene expression. Expression of *nifH* and 16S rRNA genes was compared using Pearson's product-moment correlation, and no link was found between the levels of expression of both genes. This suggests that factors which affect the free-living N fixers do not necessarily affect the community as a whole. Most molecular studies looking at the *nifH* gene use DNA rather than RNA. The numbers of copies of *nifH* per g of soil seem low in some instances and suggest *nifH* is not always transcribed. This was also observed in pine forest soil by Izumi et al. (21), who found although diverse populations of nitrogen-fixing organisms were found using DNA, *nifH* could often not be amplified from RNA samples.

16S rRNA copy numbers are also are lower than would be expected in agricultural soil and are more similar to numbers observed in forest soil (49). This is possibly due to the efficiency of RNA extraction/reverse transcription. The purpose of qPCR in this experiment is to compare between sample plots not absolute quantification.

The dominant management factor affecting microbial population structure and function in this study was crop rotation. Rotation (identified by the previous crop in this study) had a strong effect on both total bacterial and free-living N-fixing bacterial population structure (measured by DGGE profiles) and activity (measured by gene copy numbers).

Most research into the effect of crop species on the soil's microbial community has been carried out on rhizosphere soils. Any changes to the microbial community are attributed to changes in organic root exudates affecting microbial activity in a species-specific manner (18, 51). In this study, these changes were detected in the bulk soil and were apparent even though the crop was the same in both rotations in the sampling year. The different crop species grown in each rotation in the previous 3 years had resulted in fundamental changes to the structure and activity of both the free-living N-fixing bacteria and the broader bacterial community.

A considerably more active and diverse diazotroph community was seen in soils previously under barley (conventional rotation). Even on the final sample date, differences in the composition of the *nifH* community (between organic and conventional rotation) were evident (Procrustes rotation of the PCA axes; $P = 0.003$) (data not shown). These findings support those of Larkin (27), who suggested that plant effects (i.e., crop rotation) are the most important drivers of soil microbial community characteristics within a given site and soil type. Crop rotational effects on populations of free-living N-fixing bacteria were also reported by Chunleuchanon et al. (6), who found that when rice was grown in rotation there was higher diversity of nitrogen-fixing cyanobacteria than when it was grown in monoculture. However, to our knowledge, this is the first study which documents increased *nifH* expression and diversity of a free-living diazotroph following a rotation containing nonlegumes compared with a rotation containing legumes.

It could be hypothesized that the dramatic effect of crop rotation on the free-living nitrogen-fixing community in our study is due to the fundamental differences between the two rotations. Faba beans can derive 90% of their N from N_2 fixation (19); therefore, beans do not have as high an N demand as barley (22). For this reason, even under conventional fertility management, no N fertilizer is applied to the beans in the NFSC experiments. Cereal crops such as barley efficiently utilize available N in the soil, depleting mineral N during crop growth. The low N levels in soils under cereal crops may make the soil a more suitable environment for free-living diazotrophs, resulting in the increased numbers seen in the soil in the conventional rotation. Indeed, even in March of the following year (2007), there was still more available nitrate and ammonium (Table 4) in the soil under potatoes following a crop of beans in the previous year than there was in soil under potatoes following barley ($P = 0.0895$). This suggests that higher levels of mineral N throughout the season in the soil after a legume precrop may be suppressing the activity of free-living N-fixing bacteria.

Organic farming practices rely on the addition of organic material to the soil, and it was expected that organic fertility management would promote more gene expression and diversity in the soil microbial community (44). However, in this study fertility management affected microbial populations to a lesser extent than crop rotation, influencing the activity of the *nifH* gene on one date (June 2007) and the activity of the 16S rRNA gene on one date (March 2007).

However, some fertility-related factors did affect the nitrogen-fixing community according to Pearson's product-moment correlation (Table 4), although the factors affected the community differently at different sampling dates. Levels of N in bulk soils may not affect the *nifH* community consistently. Most studies that have reported this effect have been conducted on free-living N-fixing bacteria in the rhizoplane or rhizosphere soils. Coelho et al. (8) found higher levels of nitrogen fertilizer decreased N fixation in rhizosphere soils but found it had no effect in bulk soil.

In the NFSC experiments, the different fertility management (FM) regimens do not just involve application of different forms of nitrogen: the conventionally managed plots receive superphosphate and potassium chloride, whereas the organic plots receive only compost (which contains various amounts of P and K as well as other macro- and micronutrients) (Table 1). Reed et al. (38) found that the addition of phosphorus to soil more than doubles nitrogen fixation, due to the energy requirements of nitrogen fixation. While it is possible that the positive effect of conventional fertility management (see the number of copies of the *nifH* gene for June 2007) has nothing to do with the nitrogen applied to the field but more to do with the increased availability of phosphorus in this treatment, this is not supported by CCA/RDA analysis (Table 5).

Organic and conventional crop protection practices were also shown to have an impact on the bacterial community. Conventional crop protection of potatoes and the preceding cereal crops involves the use of a variety of synthetic pesticides (Table 1). These chemicals can have a marked effect on the bacterial community structure and function as some microorganisms may be suppressed and some will proliferate in the vacant ecological niches (23). Organic farming practices have been criticized for relying on copper products to control disease: for example, copper oxychloride is used for control of fungal diseases in the NFSC experiments. Studies of copper oxychloride have found that it only significantly affects bacterial communities in concentrations over 100 mg/kg (16). Annual rates of application total 6 kg Cu ha^{-1}, or approximately 3 mg Cu kg^{-1} soil in the NFSC experiments, suggesting that Cu levels in the system are well below safe limits for bacterial communities. In September, there was increased expression of *nifH* after organic crop protection. This could be a result of the cumulative effect of crop protection over the season (and the previous 3 years), possibly inhibiting the free-living N-fixing community where conventional crop protection was used. Studies into the environmental impacts of pesticides have shown that they can significantly affect the bacterial community as a whole and that diazotrophs could be particularly affected. For example, the fungicide mancozeb was found to exert an inhibitory effect on aerobic dinitrogen fixers in soil (14).

Strong seasonal effects and interactions between the sample date and crop management factors were detected. Temperature is one of the most important environmental factors affecting the soil bacterial community (35). The optimum temperature for nitrogen fixers' growth and activity is between 10 and 25°C (this is the temperature in the field between June and September) (3, 17). The temperature in the field on the March sampling date was approximately 4.5°C. It seems likely that the activity of the free-living N-fixing population was suppressed by temperature at this time. The average numbers of copies of the *nifH* gene on the March sampling date were 7.45×10^5 g^{-1} soil, and even on the June sampling date, copy number had not yet recovered (5.7×10^5 copies g^{-1} soil); however, by September the population had increased 14-fold to 1.1×10^7 copies g^{-1} soil. Eckford et al. (17) suggested that the free-living diazotrophs may only be active seasonally *in situ*. Interestingly, the populations of the total metabolically active bacteria were not affected by seasonal variations in temperature, with populations only ranging between 4.5×10^7 and 5.2×10^7 copies g^{-1} soil on the three sampling dates.

Management factors may also have played a role in the seasonal variations in *nifH* activity. Soil mineral N also varies seasonally with levels, being generally highest in June, after fertilizers have been applied but before the crop's root system has developed sufficiently to take up the available N. Coelho et

al. (8, 9) found that 30% more free-living diazotrophs could be isolated from soil in the presence of low levels of nitrogen fertilizer compared with high levels of nitrogen fertilizer. In our study, June soil samples were very high in mineral N, even in the organic fertility management treatments (~200 kg mineral N ha^{-1} for ORG FM and ~400 kg mineral N ha^{-1} for CON FM in June 2007), and exceeded the amounts added to the soil, suggesting mineralization of organic N is occurring in the soil. The relatively high soil available N levels in June could, therefore, have suppressed the activity of the free-living N-fixing bacteria. By September, levels of mineral N in the potato soils were approximately 30 kg N ha^{-1}, regardless of the fertility treatment.

Conclusions. The effect of crop rotation was consistent. The increase in nitrogen uptake by the barley crop is likely to create conditions more favorable to free-living nitrogen fixation. Although the increased amounts of nitrate and ammonia found in the soil following the bean crop are only apparent in March, the free-living diazotrophs are more likely established in the soil following barley and the community appears more diverse and abundant throughout the growing season.

The results show that the management regimen clearly affects both the total bacterial community and the free-living diazotroph community. However, the communities are not always affected in the same way and the effects are often subtle/short-lived. When looking at the total bacterial community in this study, significant differences were found as a result of changing management. However, although this gives a greater understanding of the structure of the community, it does not necessarily tell us anything about function. The effect of fertility management and crop protection on free-living N fixation was not consistent. However, organic crop protection and conventional fertility management often had positive effects on *nifH* diversity and activity. This study supports the work of Bossio et al. (4) and suggests that although management does affect community structure/activity, it could be secondary to other factors such as time of sampling and previous crop. Current work aims to make these findings more robust by studying further sample years.

ACKNOWLEDGMENTS

This work was supported by Yorkshire Agricultural Society, Nafferton Ecological Farming Group, and the Northumbria University Research Development Fund.

REFERENCES

1. **Baxter, J., and S. P. Cummings.** 2008. The degradation of the herbicide bromoxynil and its impact on bacterial diversity in a top soil. J. Appl. Microbiol. **104:**1605–1616.
2. **Baxter, J., and S. P. Cummings.** 2006. The impact of bioaugmentation on metal cyanide degradation and soil bacteria community structure. Biodegradation **17:**207–217.
3. **Beauchamp, C. J., G. Lévesque, D. Prévost, and F.-P. Chalifour.** 2006. Isolation of free-living dinitrogen-fixing bacteria and their activity in compost containing de-inking paper sludge. Bioresour. Technol. **97:**1002–1011.
4. **Bossio, D. A., K. M. Scow, N. Gunapala, and K. J. Graham.** 1998. Determinants of soil microbial communities: effects of agricultural management, season, and soil type on phospholipid fatty acid profiles. Microb. Ecol. **36:**1–12.
5. **Burgmann, H., F. Widmer, W. Von Sigler, and J. Zeyer.** 2004. New molecular screening tools for analysis of free-living diazotrophs in soil. Appl. Environ. Microbiol. **70:**240–247.
6. **Chunleuchanon, S., A. Sooksawang, N. Teaumroong, and N. Boonkerd.** 2003. Diversity of nitrogen-fixing cyanobacteria under various ecosystems of Thailand: population dynamics as affected by environmental factors. World J. Microbiol. Biotechnol. **19:**167–173.
7. **Clough, Y., A. Kruess, and T. Tscharntke.** 2007. Organic versus conventional arable farming systems: functional grouping helps understand staphylinid response. Agric. Ecosyst. Environ. **118:**285–290.
8. **Coelho, M. R. R., et al.** 2008. Diversity of *nifH* gene pools in the rhizosphere of two cultivars of sorghum (*Sorghum bicolor*) treated with contrasting levels of nitrogen fertilizer. FEMS Microbiol. Lett. **279:**15–22.
9. **Coelho, M. R. R., et al.** 2009. Molecular detection and quantification of *nifH* gene sequences in the rhizosphere of sorghum (*Sorghum bicolor*) sown with two levels of nitrogen fertilizer. Agric. Ecosyst. Environ., Appl. Soil Ecol. **42:**48–53.
10. **Colvan, S., J. Syers, and A. O' Donnell.** 2001. Effect of long-term fertiliser use on acid and alkaline phosphomonoesterase and phosphodiesterase activities in managed grassland. Biol. Fertil. Soils **34:**258–263.
11. **Cummings, S. P., et al.** 2009. Nodulation of *Sesbania* species by *Rhizobium* (*Agrobacterium*) strain IRBG74 and other rhizobia. Environ. Microbiol. **11:**2510–2525.
12. **DeLuca, T. H., L. E. Drinkwater, B. A. Wiefling, and D. M. DeNicola.** 1996. Free-living nitrogen-fixing bacteria in temperate cropping systems: influence of nitrogen source. Biol. Fertil. Soils **23:**140–144.
13. **Dixon, R., and D. Kahn.** 2004. Genetic regulation of biological nitrogen fixation. Nat. Rev. Microbiol. **2:**621–631.
14. **Doneche, B., G. Seguin, and P. Ribereau-Gayon.** 1983. Mancozeb effect on soil microorganisms and its degradation in soils. Soil Sci. **135:**361–366.
15. **Donnison, L. M., G. S. Griffith, J. Hedger, P. J. Hobbs, and R. D. Bardgett.** 2000. Management influences on soil microbial communities and their function in botanically diverse haymeadows of northern England and Wales. Soil Biol. Biochem. **32:**253–263.
16. **Du Plessis, K. R., et al.** 2005. Response of the microbial community to copper oxychloride in acidic sandy loam soil. J. Appl. Microbiol. **98:**901–909.
17. **Eckford, R., F. D. Cook, D. Saul, J. Aislabie, and J. Foght.** 2002. Free-living heterotrophic nitrogen-fixing bacteria isolated from fuel-contaminated Antarctic soils. Appl. Environ. Microbiol. **68:**5181–5185.
18. **Funnell-Harris, D., J. Pedersen, and D. Marx.** 2008. Effect of sorghum seedlings, and previous crop, on soil fluorescent Pseudomonas spp. Plant Soil **311:**173–187.
19. **Hauggaard-Nielsen, H., S. Mundus, and E. Jensen.** 2009. Nitrogen dynamics following grain legumes and subsequent catch crops and the effects on succeeding cereal crops. Nutr. Cycl. Agroecosyst. **84:**281–291.
20. **IPCC.** 2006. Agriculture, forestry and other land use. 2006 IPCC guidelines for national greenhouse gas inventories, vol. 4. Intergovernmental Panel on Climate Change, Geneva, Switzerland.
21. **Izumi, H., I. C. Anderson, I. J. Alexander, K. Killham, and E. R. B. Moore.** 2006. Diversity and expression of nitrogenase genes (*nifH*) from ectomycorrhizas of Corsican pine (Pinus nigra). Environ. Microbiol. **8:**2224–2230.
22. **Jensen, E. S., H. Hauggaard-Nielsen, J. Kinane, M. K. Andersen, and B. Jørnsgaard.** 2005. Intercropping—the practical application of diversity, competition and facilitation in arable and organic cropping systems researching sustainable systems. Proceedings of the First Scientific Conference of the International Society of Organic Agriculture Research (ISOFAR). International Society of Organic Agriculture Research, Bonn, Germany.
23. **Johnsen, K., C. Jacobsen, V. Torsvik, and J. Sørensen.** 2001. Pesticide effects on bacterial diversity in agricultural soils—a review. Biol. Fertil. Soils **33:**443–453.
24. **Kahindi, J. H. P., et al.** 1997. Agricultural intensification, soil biodiversity and ecosystem function in the tropics: the role of nitrogen-fixing bacteria. Appl. Soil Ecol. **6:**55–76.
25. **Karlen, Y., A. McNair, S. Perseguers, C. Mazza, and N. Mermod.** 2007. Statistical significance of quantitative PCR. BMC Bioinformatics **8:**131.
26. **Lane, D.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* M. Stackebrandt and E. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, London, United Kingdom.
27. **Larkin, R. P., and C. W. Honeycutt.** 2006. Effects of different 3-year cropping systems on soil microbial communities and *Rhizoctonia* diseases of potato. Phytopathology **96:**68–79.
28. **Lindström, E. S., and A.-K. Bergström.** 2005. Community composition of bacterioplankton and cell transport in lakes in two different drainage areas. Aquat. Sci. **67:**210–219.
29. **Mäder, P., et al.** 2002. Soil fertility and biodiversity in organic farming. Science **296:**1694–1697.
30. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
31. **Omar, S. A., and M. H. Abd-Alla.** 1992. Effect of pesticides on growth, respiration and nitrogenase activity of *Azotobacter* and *Azospirillum*. World J. Microbiol. Biotechnol. **8:**326–328.
32. **Patra, A. K., et al.** 2006. Effects of management regime and plant species on the enzyme activity and genetic structure of N-fixing, denitrifying and nitrifying bacterial communities in grassland soils. Environ. Microbiol. **8:**1005–1016.
33. **Peoples, M. B., D. F. Herridge, and J. K. Ladha.** 1995. Biological nitrogen

fixation: an efficient source of nitrogen for sustainable agricultural production? Plant Soil **174:**3–28.
34. **Peoples, M. B., and E. T. Craswell.** 1992. Biological nitrogen fixation: investments, expectations and actual contributions to agriculture. Plant Soil **141:**13–39.
35. **Pettersson, M., and E. Bååth.** 2003. Temperature-dependent changes in the soil bacterial community in limed and unlimed soil. FEMS Microbiol. Ecol. **45:**13–21.
36. **Poly, F., L. J. Monrozier, and R. Bally.** 2001. Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. Res. Microbiol. **152:**95–103.
37. **R Development Core Team.** 2006. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
38. **Reed, S. C., et al.** 2007. Phosphorus fertilization stimulates nitrogen fixation and increases inorganic nitrogen concentrations in a restored prairie. Appl. Soil Ecol. **36:**238–242.
39. **Refsgaard, K., N. Halberg, and E. S. Kristensen.** 1998. Energy utilization in crop and dairy production in organic and conventional livestock production systems. Agric. Syst. **57:**599–630.
40. **Robertz, M., S. Eckl, T. Muckenheim, and L. Webb.** 1999. A cost-effective laboratory method for determining microbial soil respiration. Schriftenr. Ver Wasser Boden Lufthyg. **51:**48–53.
41. **Roeselers, G., L. J. Stal, M. C. M. van Loosdrecht, and G. Muyzer.** 2007. Development of a PCR for the detection and identification of cyanobacterial *nifD* genes. J. Microbiol. Methods **70:**550–556.
42. **Rosado, A. S., G. F. Duarte, L. Seldin, and J. D. Van Elsas.** 1998. Genetic diversity of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analyzed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. Appl. Environ. Microbiol. **64:**2770–2779.
43. **Rosen, C. J., and D. L. Allan.** 2007. Exploring the benefits of organic nutrient sources for crop production and soil quality. HortTechnology **17:**422–430.
44. **Shannon, D., A. M. Sen, and D. B. Johnson.** 2002. A comparative study of the microbiology of soils managed under organic and conventional regimes. Soil Use Manag. **18:**274–283.
45. **Soil Association.** 2005. Soil Association organic standards. Soil Association, Bristol, United Kingdom.
45a. **Vitousek, P. M., et al.** 2000. Human alteration of the global nitrogen cycle: causes and consequences. Issues Ecol. **1:**1–16.
46. **Wakelin, S. A., et al.** 2009. Pasture management clearly affects soil microbial community structure and N-cycling bacteria. Pedobiologia (Jena) **52:**237–251.
47. **Wakelin, S. A., et al.** 2007. The effects of stubble retention and nitrogen application on soil microbial community structure and functional gene abundance under irrigated maize. FEMS Microbiol. Ecol. **59:**661–670.
48. **Wartiainen, I., T. Eriksson, W. Zheng, and U. Rasmussen.** 2008. Variation in the active diazotrophic community in rice paddy-*nifH* PCR-DGGE analysis of rhizosphere and bulk soil. Appl. Soil Ecol. **39:**65–75.
49. **Whitman, W. B., D. C. Coleman, and W. J. Wiebe.** 1998. Prokaryotes: the unseen majority. Proc. Natl. Acad. Sci. U. S. A. **95:**6578–6583.
50. **Widmer, F., B. T. Shaffer, L. A. Porteous, and R. J. Seidler.** 1999. Analysis of *nifH* gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain range. Appl. Environ. Microbiol. **65:**374–380.
51. **Wieland, G., R. Neumann, and H. Backhaus.** 2001. Variation of microbial communities in soil, rhizosphere, and rhizoplane in response to crop species, soil type, and crop development. Appl. Environ. Microbiol. **67:**5849–5854.