Diazotroph community structure and abundance in wheat–fallow and wheat–pea crop rotations

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ABSTRACT

Biological input of nitrogen (N) from the atmosphere by free-living diazotrophs can help alleviate fertilizer use in agricultural systems. In this study, we investigated the effect of N fertilizer and winter pea (*Pisum sativum* L.) crop on the community structure and abundance of free-living diazotrophs in a two year study of dryland winter wheat (*Triticum aestivum* L.) no-till production system in Eastern Oregon, USA. Based on quantification of the *nifH* gene, diazotroph abundance was strongly influenced by plant species and the crop year in which the soil samples were collected. A greater amount of *nifH* copies was recovered in 2012 compared to 2011 either as copies per gram soil or normalized to the abundance of bacterial 16S rRNA genes. The quantity of genes was greater under pea than wheat in 2012 although no difference was observed in the preceding year. The *nifH* gene abundance was positively correlated to ammonium concentration in 2011 and bacterial abundance in 2012. Nitrogen application did not influence diazotroph abundance in the top 0–5 cm; however the abundance was reduced by application at the lower 5–10 cm depth under wheat crop. The diazotroph community structure appeared to be influenced more by N fertilization rather than plant species with the exception of wheat in 2012. Changes in the community structure over the two years were greater for fertilized than unfertilized soil. Collectively, these data suggest that year-to-year variability had a greater influence on diazotroph communities rather than specific parameters of plant species, fertilization, total N, total organic C, or soil pH. Multi-year studies are necessary to define the specific drivers of diazotroph abundance, community structure and function.

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1. Introduction

Nitrogen (N) is a critical plant nutrient and crop requirements range from 20 to 50 kg N ton^{-1} grain yield for cereals (Myers, 1988; Sylvester-Bradley and Kindred, 2009). Nitrogen fertilizer application promotes plant biomass and improves grain protein yields; however, it also imparts significant cost to the producer and could become an environmental challenge. Environmental concerns for N fertilization include increased greenhouse gas emissions (Dalal et al., 2003), soil acidification (Barak et al., 1997; Gollany et al., 2005), and groundwater contamination (Ambus et al., 2001; Gollany et al., 2004). Alternatively, biological nitrogen fixation (BNF) is an important source of N in some ecosystems (Cleveland et al., 1999) and may help meet some of the plant nutritional needs.

Diazotrophs, or N$_2$-fixers, contribute to plant available N by reducing atmospheric N$_2$ to ammonium in the soil. Diazotrophs are highly diverse and include members of α-, β-, γ-, and δ-*Proteobacteria*, Firmicutes, Cyanobacteria, and Archaea (Dixon and Kahn, 2004; Rösch et al., 2002), most of which are uncultivated. Although the diversity and distribution of non-symbiotic diazotrophs suggest that most soils have a capacity for BNF (Izquierdo and Nüsslein, 2006; Poly et al., 2001), the ecological impact of free-living diazotroph activity is disputable with estimates of activity varying widely from 0 to 60 kg N ha^{-1} yr^{-1} in cropland and natural ecosystems (Cleveland et al., 1999; Day et al., 1975; Gupta et al., 2006).

Diazotroph activity, abundance and community structure have been attributed to numerous factors related to microbial biomass (Hayden et al., 2010), sampling season (Mergel et al., 2001; Pereira e Silva et al., 2011) and soil physical and chemical properties including soil water content (Brouzes et al., 1969; Limmer and Drake, 1996; Nelson and Mele, 2006; Roper, 1985), soil texture (Pereira e Silva et al., 2011; Riffkin et al., 1999), soil aggregate size (Poly et al., 2001), soil pH (Limmer and Drake, 1996; Nelson and Mele, 2006), electrical conductivity (Hayden et al., 2010), oxygen (Brouzes et al.,

1969; Limmer and Drake, 1996, 1998), carbon (C) quality and quantity (Brouzes et al., 1969; Keeling et al., 1998; Limmer and Drake, 1996, 1998; Wakelin et al., 2010), and N availability (Hayden et al., 2010; Hsu and Buckley, 2009; Limmer and Drake, 1998; Nelson and Mele, 2006). Generally, some of the factors result in reproducible responses in N_2-fixation such as increased activity with decreased oxygen tension (Brouzes et al., 1969; Limmer and Drake, 1998) and increased soil water content (Brouzes et al., 1969; Roper, 1985), while others such as C (Brouzes et al., 1969; Limmer and Drake, 1996) and some soil physicochemical characteristics (Hsu and Buckley, 2009; Poly et al., 2001) produce inconsistent results. Although numerous factors may influence diazotroph populations, there is little knowledge regarding specific ecological drivers and soil properties do not appear to have similar effects on community structure and activity (Poly et al., 2001).

A better understanding of the drivers or influencing factors on the diazotroph communities and BNF will help improve N use efficiency in cropping systems and may lead to reductions in fertilizer use. An indirect approach to assess the potential for BNF is the characterization of the diazotroph populations by molecular methods. Diazotroph communities are often characterized by the *nifH* gene which encodes the iron subunit of the nitrogenase enzyme (Hayden et al., 2010; Hsu and Buckley, 2009; Mao et al., 2011; Nelson and Mele, 2006; Orr et al., 2011; Poly et al., 2001; Rösch and Bothe, 2005). The *nifH* gene is encoded by all diazotrophs and mimics 16S rRNA gene phylogeny making it an ideal candidate for ecological studies of community structure and/or composition. We compared the abundance and community structure of diazotroph populations in a long-term no-till wheat–fallow and adjacent wheat–pea rotation to examine whether crop rotation and fertilization influence the microbial communities in the dryland Pacific Northwest (USA).

2. Methods

2.1. Site description and sample collection

The Pacific Northwest Columbia Plateau has a Mediterranean climate with cool, wet winters and hot, dry summers producing optimal conditions for dryland small grain production. Cropping practices are constrained in the region by limited annual precipitation and typically follow a two-year rotation with a fallow period of 12 or more months preceding winter wheat (W) which is planted in fall and harvested in July/August of the following year. The field site was located 15 km northeast of Pendleton, Oregon, USA (45° 72′ N, 118° 62′ W, elevation 458 m). Annual precipitation occurs mostly during the winter months and yearly averages were 447 mm, 571 mm, and 368 mm for crop years 2010, 2011, and 2012 (CY10, CY11, CY12). The soil is Walla Walla silt loam (coarse-silty, mixed, superactive, mesic Typic Haploxeroll) developed in loess overlying basalt (Wuest and Gollany, 2013). Treatments were arranged in a 2 by 2 factorial design with crop rotation as main plots and fertilization as subplots. The winter wheat–fallow rotation (W–F) main plot (NTA experiment) was established in 1982. The winter wheat-fallow rotation (NTB experiment) was initiated in 1997 and was converted to winter wheat–winter pea rotation (W–P) in 2010 by replacing the fallow period with Austrian pea. The NTB experiment is 7.62 m north of NTA and both are strictly no-till cropping systems. Nitrogen fertilizer was applied as urea at either 0 or 180 kg N ha^{-1} (160 lbs N ac^{-1}). Urea was banded at 10 cm depth during wheat planting and treatments referred to as "fertilized" pea and "fertilized" fallow received N only during the wheat phase of the rotation (Table 1). The CY11 and CY12 were the first pea harvests from each plot in the NTB experiment. In CY12, the winter wheat did not establish, therefore it was subsequently sprayed and re-planted to spring wheat on April 13, 2012. The naming convention for the plots lists first the crop under which the sample was taken, followed by the rotational crop and amount of the N fertilizer applied with wheat (e.g., W0-F for wheat of unfertilized W–F rotation and P-W180 for pea of W–P rotation in which wheat received 180 kg N ha^{-1}).

Soil sampling was initiated on June 28, 2011 and July 10, 2012 from the wheat and fallow plots of NTA and the pea plots of NTB and was completed within 2–3 days. In each plot, five soil cores were collected from the top 0–5 cm, 5–10 cm and 10–20 cm depths using a 1.85-cm diameter soil probe. Cores from the individual depths were pooled and homogenized by hand. Approximately 10–20 g of soil was transferred in the field into small zippered bags for DNA analysis and stored on ice until transfer to a −20 °C freezer upon arrival in the lab. The remaining soil was dried at 40 °C for chemical analyses. Three additional cores were retained for bulk density and soil water content analyses.

2.2. Soil chemical and physical properties

Soil water content and bulk density were calculated according to standard protocols. Dried soils were extracted for 30 min in 1 M potassium chloride prior to quantification of nitrate (NO_3–N) and

Table 1
Soil chemical properties under different crop rotations during wheat and fallow phase of wheat–fallow (W–F) rotation in the NTA experiment and pea phase of the wheat–pea (W–P) in the NTB experiment.

Properties		Wheat–fallow (NTA)[a]				Wheat–pea (NTB)		Pearson Coefficient[d]	
	Rotation	W–F		W–F		W–P			
	Crop, N	W0-F	W180-F	F-W0	F-W180	P-W0	P-W180	*nifH*	16S
Total Nitrogen (%)[b]									
2011		0.134[BC]	0.166[A]	0.133[C]	0.165[AB]	0.138[ABC]	0.150[ABC]	NS	NS
2012		0.122[C]	0.147[ABC]	0.128[C]	0.164[AB]	0.123[C]	0.138[ABC]	NS	NS
Total Organic Carbon (%)[c]									
2011		1.809[AB]	2.092[A]	1.691[AB]	1.978[AB]	1.815[AB]	1.826[AB]	NS	NS
2012		1.593[B]	1.861[AB]	1.706[AB]	2.087[A]	1.703[AB]	1.916[AB]	NS	+
pH									
2011		5.48[A]	5.08[B]	5.53[A]	5.50[A]	5.32[AB]	5.45[A]	NS	NS
2012		5.47[A]	5.32[AB]	5.52[A]	5.32[AB]	5.37[AB]	5.23[AB]	NS	NS

[a] Winter wheat–fallow (W–F) and winter wheat–winter pea (W–P) rotations were managed under no-till farming. All samples were collected in the summer during the crop listed first in the rotation (e.g. W–F sampled under wheat and F–W sampled under fallow). Nitrogen was applied as urea at either 0 or 180 kg N ha^{-1} rate to the plots during planting of the wheat phase of the rotation and is indicated after wheat (i.e., W0-F for unfertilized wheat and F-W180 for fallow crop previously fertilized under wheat).
[b] Values for soil properties followed by the same letter are not significantly different at $P < 0.05$ level over both years.
[c] Values are the same as total carbon since inorganic carbon measured was below detection.
[d] Pearson correlation coefficient of gene copy number and soil chemical properties; NS, not significant; ±, significant positive or negative correlation at $P < 0.10$.

ammonium (NH_4–N) using an Astoria Analyzer (Astoria-Pacific International, Clackamas, OR) equipped with a 305D digital detector, 303A analytical cartridge, 302D micropump, and 311XYZ sampler. Nitrate was reduced using an open tubular cadmium reactor and analyzed as nitrite using a sulfanilamide method (Mulvaney, 1996). Ammonia was quantified by the sodium salicylate method (Mulvaney, 1996). Soil pH was measured in a 2:1 dilution of soil with 0.01 M calcium chloride. Total C and N were determined by dry combustion using a combustion analyzer (Thermo Finnigan FlashEA 1112 Elemental Analyzer, Rodano, Italy).

2.3. DNA extraction

DNA was extracted from a volume of field moist soil equivalent to 0.5 g dry soil using the MoBio UltraClean DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA) according to the manufacturer's protocol with the following modifications: cells were lysed with a 10 s bead-beating step (BioSpec Products, Bartlesville, OK) and DNA was eluted with MD5 buffer heated to 80 °C then re-eluted with the eluant. DNA extracts were stored at −20 °C.

2.4. Gene quantification

The *nifH* gene was quantified from soil extracts using primers nifHF (5′-AAA GGY GGW ATC GGY AAR TCC ACC AC-3′) and nifHRb (5′-TGS GCY TTG TCY TCR CGG ATB GGC AT-3′) (Rösch and Bothe, 2005) and the bacteria quantified based on 16S rRNA gene sequence using the primers 341F (5′-CCT ACG GGA GGC AGC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Muyzer et al., 1993). Quantitative PCR (qPCR) reactions, prepared in 10 μL volumes, contained 0.8X Power SYBR Green PCR Master Mix (Life Technologies, Grand Island, NY), 0.1 μg μL^{-1} bovine serum albumin (BSA, Roche, Indianapolis, IN), 0.2 μM (*nifH*) or 0.1 μM (16S) primer and 1 μL of DNA template diluted 1:20 with H_2O. Template-free control reactions contained 1 μL of H_2O instead of DNA. Amplification was performed using an Applied Biosystems StepOnePlus instrument with the following conditions: denaturation for 10 min at 95 °C, 40 cycles of amplification for 15 s at 95 °C and 1 min at 60 °C, followed by a final melt curve of 15 s at 95 °C, 1 min at 60 °C with an increase of 0.3 °C to a final temp of 95 °C. All soil extracts were tested for PCR inhibition prior to analysis. Briefly, qPCR reactions were spiked with 3.6×10^5 copies of the pUC19 plasmid and the plasmid quantified using the M13F(-40) and M13R(-48) primers with a 60 °C annealing temperature. The C_T of the reactions containing diluted soil DNA extract was compared to the C_T of the plasmid-only controls. All of the soil extracts had C_T values varying less than 0.31 C_T of the plasmid-only control indicating a lack of PCR inhibition.

2.5. Community analysis

The community structure was analyzed by Terminal Restriction Fragment Length Polymorphism (T-RFLP) of the *nifH* gene. DNA was amplified from extracts pooled from the replicate plots. PCR reactions contained Phusion High-Fidelity PCR Master Mix with HF Buffer (New England Biolabs, Ipswich, MA), 0.1 μg μL^{-1} BSA and 0.4 μM nifHF and nifHRb primers in which the forward primer was labeled with the WellRED D3-PA fluor (Beckman Coulter, Indianapolis, IN). Amplification was carried out in quadruplicate 50 μL reactions with 1 μL of undiluted DNA or water under the following thermocycling conditions: denaturation for 1 min at 98 °C, 35 cycles of 10 s at 98 °C, 10 s at 64 °C and 15 s at 72 °C, followed by a final elongation for 5 min at 70 °C. Replicate reactions were pooled and gel purified from 1% low melt agarose using the PureLink™ Gel Extraction Kit (Life Technologies) with a 2 min elution step with 80 °C buffer. DNA was quantified by the Qubit dsDNA HS assay (Life

Technologies). Five microliters of DNA was digested in duplicate reactions with 10 units of HaeIII restriction enzyme for 6 h at 37 °C followed by a 10 min 80 °C enzyme inactivation step. Digests were desalted by ethanol precipitation with glycogen and the DNA pellet resuspended in 40 μL of GenomeLab SLS with 0.25 μL of GenomeLab DNA size standard 600 (Beckman Coulter). Digested DNA was separated using a CEQ 8800 Genetic Analyzer (Beckman Coulter) with the following protocol: capillary temperature of 50 °C, denaturation for 120 s at 90 °C, injection for 15 s at 2 kV, and separation for 90 min at 4.8 kV. Fragments were analyzed in the Fragment Analysis module of the CEQ software with a slope threshold of one, relative peak height threshold of 0.5%, confidence level of 95%, quartic model, time migration variable, peak calculation by height, and PA ver. 1 dye mobility calibration using calculated dye spectra. Peaks were manually edited, imported into T-REX online software (Culman et al., 2009), filtered with one standard deviation height, clustered at 1.0 bp, and averaged over the duplicate profiles. Only peaks present in both profiles were used for subsequent analyses. Cluster analysis was performed in PAST software (Hammer et al., 2001) using the paired group algorithm and Jaccard similarity index. The percent change in community structure was calculated from the Jaccard similarity indices obtained in PAST as change% = 100 − similarity% (Pereira e Silva et al., 2011).

2.6. Calculations and statistical analyses

Gene abundance values are noted as copies per gram dry soil extracted. Percent *nifH* abundance was calculated by dividing the *nifH* gene copies g^{-1} soil by the 16S rRNA gene copies g^{-1} soil and multiplying by 100 for each individual plot. Differences in N-mineralization and gene abundance across both years were analyzed at a significance value of $P < 0.05$ using PROC GLIMMIX procedure with a Tukey–Kramer adjustment for means separation and multiple comparisons (Gbur et al., 2012). Pearson correlation coefficients were calculated for gene copy number and soil physical properties by year using PROC CORR. All analyses were performed in SAS 9.2 (SAS Institute, Cary, N.C.).

3. Results

An *F*-test comparison across all treatments and both years revealed significant differences in nitrate concentration for fertilized treatments ($P = 0.0012$) and crop ($P < 0.0001$), in contrast to ammonium concentration which was significant across crop ($P < 0.0001$) and year ($P < 0.0001$) (Fig 1). In CY11, nitrate was greater in fallow (F-W0, F-W180) and pea (P-W0, P-W180) plots compared to wheat (W0-F, W180-F) regardless of fertilization albeit the increases were statistically significant only for plots fertilized during wheat (F-W180, P-W180) (Fig 1a). The fallow plots in CY12 had generally greater nitrate than either wheat or pea of the same crop year but values were statistically significant only for F-W180 compared to the W0-F and both pea plots (P-W0, P-W180). Unlike CY11, nitrate in CY12 was similar in wheat and pea plots regardless of fertilization (Fig 1a). N mineralization as evidenced by soil ammonium concentration was similar across all treatments in both years except for P-W180 in CY12 which had significantly greater ammonium than any other treatment excluding P-W0 (Fig 1b).

Significant changes in the abundance of the *nifH* gene were measured over different depths in the W–F rotation (Fig 2). The depth of sampling had a significant effect ($P < 0.0001$) on the abundance of the *nifH* gene. Nitrogen fertilization was considered a non-significant effect at the $P < 0.05$ level ($P = 0.0514$) although comparison of the *nifH* gene abundance at 5–10 cm depth between the two treatments revealed differences in the gene distribution.
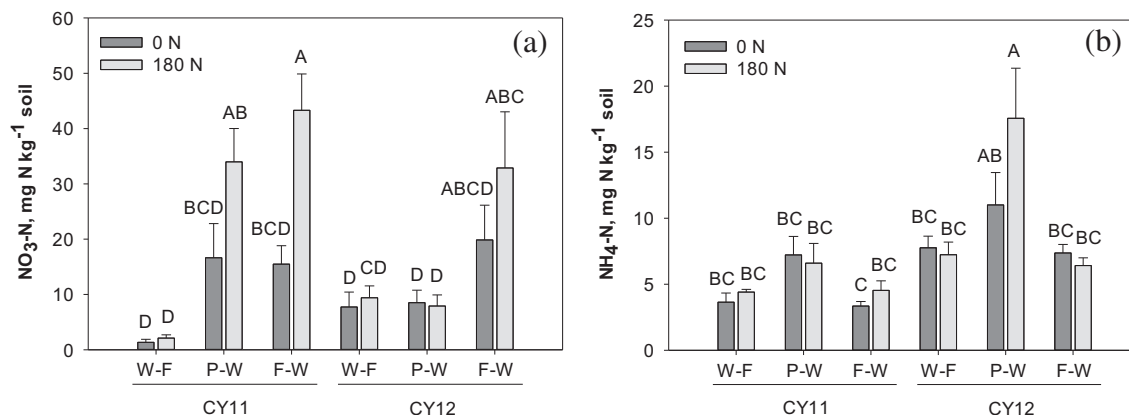
Fig. 1. Nitrate (a) and ammonium (b) concentration in the top 0–5 cm for each crop year (CY11, CY12). The crop listed first of the rotation is phase in which soils were sampled. The same letter above the data indicates that the data are not significantly different at $P < 0.05$ level ($n = 4$, Tukey post hoc test). Data is shown as mean ± standard error.

The *nifH* abundance was similar in the top two depths for each treatment; however, *nifH* copies were significantly less at the 5–10 cm depth in the fertilized wheat (W180-F) compared to unfertilized wheat (W0-F). In both treatments, gene abundance was less in the lower 10–20 cm than the top 0–5 cm depths. Further experimentation was conducted at the top 0–5 cm sampling depth due to the greater abundance of *nifH* genes in both treatments.

Crop and crop year significantly ($P = 0.0001$ and $P < 0.0001$, respectively) affected the abundance of the *nifH* gene in the top 0–5 cm over the two year study (Fig 3a). Apart from the W0-F plots, all CY11 plots had significantly less *nifH* gene copies than CY12 and neither crop nor fertilizer had significant effects in CY11 ($P = 0.6666$, $P = 0.8363$, respectively). In CY12, *nifH* abundance was greater under pea (P-W0, P-W180) compared to wheat (W0-F, W180-F) regardless of fertilization. The *nifH* copy number of the fallow plots (F-W0, F-W180) for CY12 was not statistically different from wheat (W0-F, W180-F) or pea (P-W0, P-W180) of the same year.

The increased abundance of the *nifH* gene in CY12 compared to CY11 was paralleled in the overall bacterial abundance as quantified by the 16S rRNA gene (Fig 3b). Crop year imparted a significant ($P < 0.0001$) effect on the quantity of 16S genes g^{-1} soil. For CY11, bacterial abundance was similar across all crops and

fertilization treatments. However, differences in gene abundance were apparent in CY12 for W0-F, which was less than in all but W180-F and F-W180. When the *nifH* abundance was calculated as percent total bacteria, crop year was the single significant ($P < 0.0001$) effect and no differences were measured among any treatments within a single year (Fig 3c). Overall, the average abundance of *nifH* for all treatments in a single crop year was 4.7 times greater in CY12 than CY11. The increased *nifH* copy number was positively correlated to bacterial abundance in CY12 ($r = 0.87$, $P < 0.0001$) but not in CY11 (Fig 3d). In CY11, a significant positive correlation was observed between *nifH* abundance and NH$_4$–N ($r = 0.48$, $P = 0.0173$) and a negative correlation between bacterial abundance and NO$_3$–N ($r = -0.42$, $P = 0.0385$). Regardless of year, no significant correlation was observed for either *nifH* or 16S rRNA gene copy number with soil pH, % total N or % total organic C (TOC) at $P < 0.05$; however, a weak positive correlation ($r = 0.38$, $P = 0.0714$) was observed in CY12 for 16S gene copy with %TOC (Table 1).

Diazotroph communities responded differently to crop and N treatments in the two crop years (Fig. 4) and no single factor of influence was clear. The number of *nifH* phylotypes, or T-RFLP peaks, detected in each treatment ranged from 28 to 83 with an average of 64 peaks in CY11 and 57 peaks in CY12 (Table 2). Fertilized plots showed the greatest change in both species richness and structure when compared over the two years. Fertilized wheat (W180-F) had the largest increase in richness from CY11 to CY12 while both fertilized fallow (F-W180) and pea (P-W180) showed the greatest decrease (Table 2). Communities collected from all treatments showed significant changes in structure between the two CY, although both unfertilized wheat (W0-F) and pea (P-W0) plots were least divergent with changes of 47% and 50%, respectively (Table 2). The community structures of fertilized fallow (F-W180) and fertilized wheat (W180-F) were most divergent, with respective changes of 72% and 77% between CY (Table 2). For comparison within CY, the microbial communities of F-W180 and P-W180 in CY11 were most closely related and both treatments had the highest amount of NO$_3$–N (Figs. 4a and 1a). However, the fertilized plots (F-W180, P-W180) also clustered with W0-F and F-W0 which had markedly lower NO$_3$–N and were of the same crop rotation. Similarly, in CY12 the most closely related communities were again recovered from F-W180 and P-W180 in which the soil N of the pea plot was more in the form of NH$_4$–N than NO$_3$–N unlike the preceding CY (Figs. 4b and 1a,b). Additionally, the wheat plots (W0-F, W180-F) of the W–F rotation of CY12 were most closely related to one another regardless of fertilization (Fig 4b).
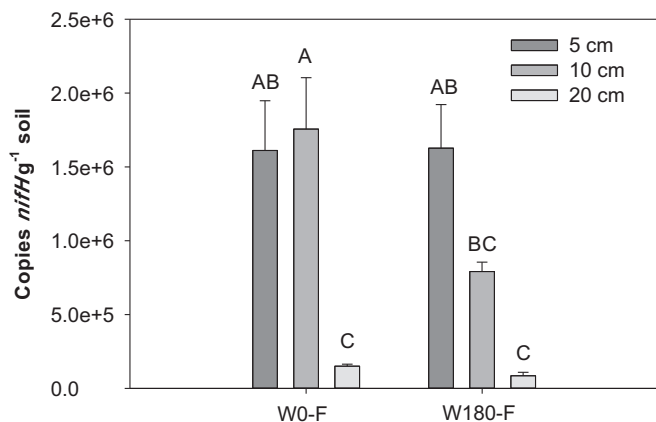

Fig. 2. Abundance of *nifH* gene from different depths of soil sampled from wheat plots in crop year 2011. Depths are 0–5 cm, 5–10 cm and 10–20 cm as indicated by the end depth. Data is shown as mean ± standard error and the same letter above the bars (all six) indicate no significant difference at $P < 0.05$.
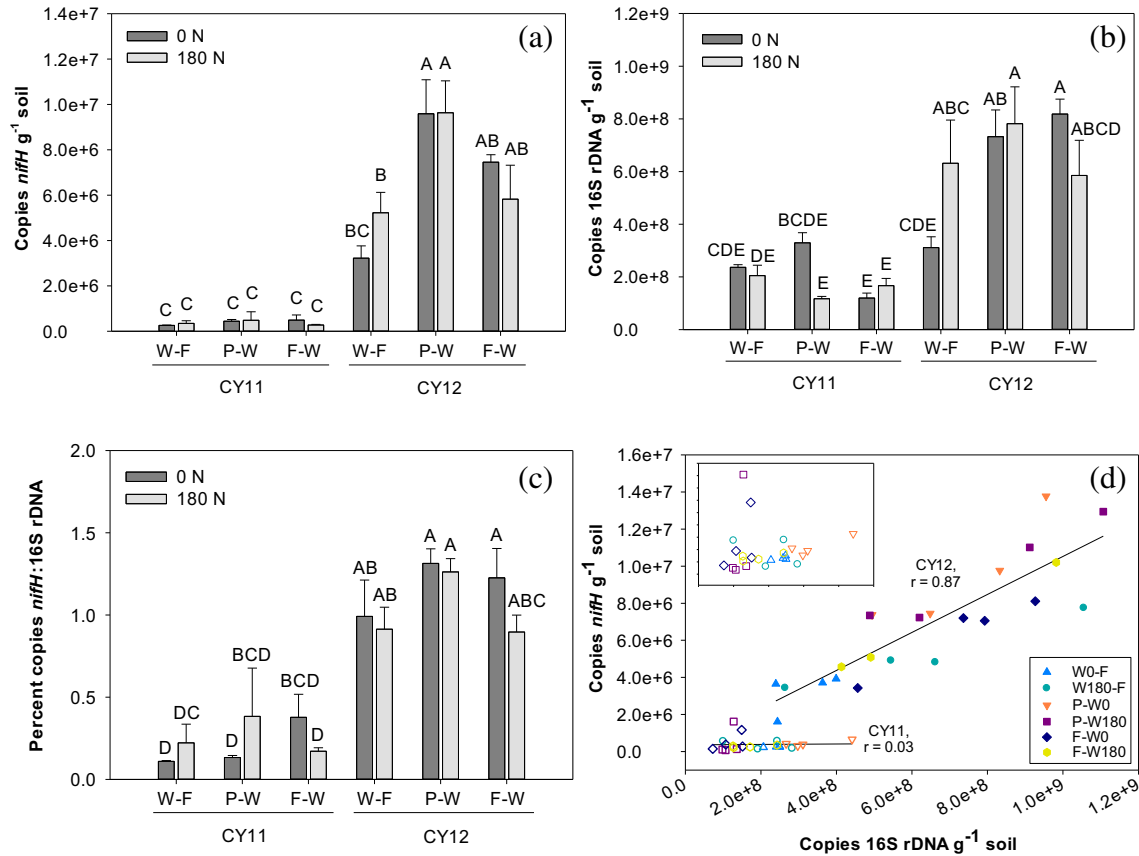
Fig. 3. Abundance of diazotrophs and total bacteria in the top 5 cm of soil as measures of *nifH* gene (diazotroph) quantity (a), bacterial 16S gene quantity (b), relative percent *nifH* (c), and correlation of *nifH*:16S copy number (d). For graphs a–c, data is shown as the mean ± standard error and data with the same letter are not significantly different at $P < 0.05$. In plot d, the open symbols are from crop year 2011, the closed symbols are crop year 2012 and the regression lines are shown.

4. Discussion

Diazotroph diversity, function and abundance have been associated with numerous factors such as microbial biomass, soil physicochemical characteristics, and management strategies (Hayden et al., 2010; Hsu and Buckley, 2009; Keeling et al., 1998; Limmer and Drake, 1996, 1998; Nelson and Mele, 2006; Poly et al., 2001; Riffkin et al., 1999; Wakelin et al., 2010). In this study

of a W–F and a newly established W–P rotation, the response of the diazotrophic communities varied between the two crop years in which the ecological drivers for abundance appeared to be related to microbial biomass in CY12 and soil chemistry in CY11. The quantity of *nifH* genes per g soil detected in the plots was consistent with other studies (Coelho et al., 2009; Mao et al., 2013; Orr et al., 2011; Pereira e Silva et al., 2011). Overall, the gene abundance in CY12 was much greater than in CY11 and was accompanied by a
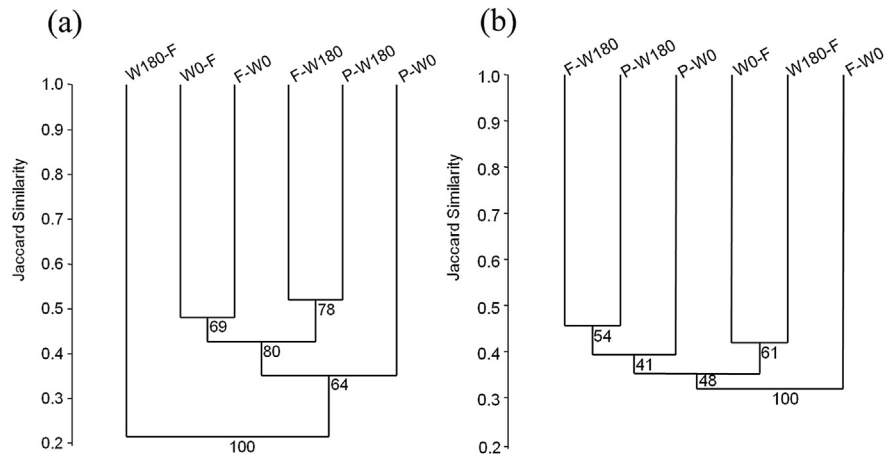

Fig. 4. Effect of N and crop on the diazotroph community structure as characterized by cluster analysis of the T-RFLP data for the *nifH* gene for crop years 2011 (a) and 2012 (b). Crop and fertilization (0 and 180 kg N ha⁻¹) are noted. The Jaccard similarity coefficient is shown on the Y axis. Bootstrap values are indicated at the nodes (based on 10,000 bootstrap replicates).

Table 2
Jaccard similarity indices for different crop rotations during wheat and fallow phase of wheat–fallow (W–F) rotation in the NTA experiment and pea phase of the wheat–pea (W–P) in the NTB experiment.

Properties		Wheat–fallow (NTA)[a]				Wheat–pea (NTB)	
	Rotation	W–F		W–F		W–P	
	Crop, N	W0-F	W180-F	F-W0	F-W180	P-W0	P-W180
Richness (phylotypes)[b]							
2011		77	28	83	76	48	70
2012		82	67	61	43	51	40
Percent change in structure[c]		47	77	57	72	50	64

[a] Winter wheat–fallow (W–F) and winter wheat–winter pea (W–P) rotations were managed under no-till farming. All samples were collected in the summer during the crop listed first in the rotation (e.g. W–F sampled under wheat and F–W sampled under fallow). Nitrogen was applied as urea at either 0 or 180 kg N ha^{-1} rate to the plots during planting of the wheat phase of the rotation and is indicated after wheat (i.e., W0-F for unfertilized wheat and F-W180 for fallow crop previously fertilized under wheat).
[b] Number of peaks present in both duplicate T-RFLP profiles from pooled DNA extracts from the replicate plots.
[c] Calculated from Jaccard similarity index of the two years as change% = 100 − similarity%.

general increase in bacteria. Similarly, an association of microbial biomass and *nifH* abundance was reported for agriculturally managed and remnant sites in Australia (Hayden et al., 2010). The correlation observed in the current study suggests that the increase in *nifH* abundance is due to enhanced microbial biomass rather than specific guild enrichment.

Plant species (Mao et al., 2011) and biomass retention (Hsu and Buckley, 2009) influence community structure and biomass retention has a positive effect on *nifH* gene abundance (Wakelin et al., 2007, 2010). In the current study, the crop rotations were conducted under no-till in which the biomass was retained. Wheat preceded both pea and fallow plots and, interestingly, the diazotroph community of the F-W180 was most similar to the P-W180 regardless of crop year. Like Poly et al. (2001) in which plant species was not a main factor of *nifH* diversity, the diazotroph communities appeared to be influenced more by N fertilizer application than crop. The variability in community structure between years was also greatly influenced by fertilization, such that the communities resident to fertilized plots diverged more over the two year study than the unfertilized plots regardless of crop. Additionally, the communities with similar fertilizer treatments were more closely related than those of similar crop when comparisons were made within the same year. However, in the CY12 wheat plots, the communities from fertilized (W180-F) and unfertilized (W0-F) treatments were most closely related. It is important to note that the winter wheat plots in CY12 did not establish early in the season and were therefore sprayed and re-planted to spring wheat. The replanting did not appear to influence the diazotroph abundance since the microbial communities of both wheat and pea plots had similar percent *nifH* gene compositions; however, it is not possible to discount that the similarity in the community structure was an effect of either the spraying or replanting.

Previous studies of N application have produced inconsistent results on the soil or root-associated diazotroph communities with some studies showing decreased diversity (Tan et al., 2003), activity (Tan et al., 2003), and *nifH* abundance (Coelho et al., 2009) whereas others have demonstrated no response (Ogilvie et al., 2008; Wakelin et al., 2007). The sensitivity of diazotroph communities to fertilizer also appears to be a function of plant association since the *nifH* abundance was decreased in response to high levels of fertilizer in the rhizosphere soils but showed no response in bulk soil (Coelho et al., 2009). Similarly, in the current study fertilization was inconsequential to the number of *nifH* genes recovered from the bulk soil at 0–5 cm for either CY although it did reduce the abundance of genes at the lower 5–10 cm depth under wheat cropping. Consistent with a study of an acidic forest soil (Mergel et al., 2001), the abundance of *nifH* genes in the top 0–5 cm and

5–10 cm were similar in unfertilized plots and reduced at the 5–10 cm depth when fertilizer was applied. Bacteria (Paul and Clark, 1989) and diazotrophs (Kloos et al., 1998; Mergel et al., 2001; Rösch et al., 2002) are most abundant in the top 5 cm of soil therefore comparisons between fertilizer treatments and crop rotation were made at this depth.

Year-to-year variability was observed in several facets of the microbial communities from bacterial and diazotroph abundance to community structure and nitrification/mineralization. In addition to increased *nifH* abundance, the bacteria were also overall significantly more abundant in soils collected in CY12 compared to CY11. The differences in quantity cannot be attributed to DNA extraction efficiency since the disparity in abundance was also reflected when the two genes were normalized as percent copies *nifH*:16S rDNA. Interestingly, correlations in gene quantity and N were observed in CY11 but not CY12. Ammonium has previously been reported as a main predictor of *nifH* gene abundance (Pereira e Silva et al., 2011). In CY11, *nifH* gene had a weak positive correlation to ammonium, and the bacteria were negatively correlated to nitrate even though neither correlated to total nitrogen and no significant difference was observed in the abundance of either gene among the treatments.

A lack of consensus exists regarding the exact drivers for diazotroph activity, abundance and diversity. It has been suggested that based on the distribution of diazotrophs most soils should have a capacity for nitrogen fixation (Bürgmann et al., 2003; Izquierdo and Nüsslein, 2006; Poly et al., 2001); however, the *nifH* gene cannot be detected in all soils (Hayden et al., 2010). In this study, no factors reproducibly influenced diazotroph abundance and community structure in the two sequential crop years. Several studies that have identified different soil physicochemical properties or management strategies that influence diazotroph communities were not replicated over year (Hayden et al., 2010; Hsu and Buckley, 2009; Limmer and Drake, 1996; Nelson and Mele, 2006; Poly et al., 2001; Riffkin et al., 1999; Roper, 1985; Wakelin et al., 2010) even though populations are known to fluctuate with both season and year (Mergel et al., 2001; Pereira e Silva et al., 2011). Numerous factors can contribute to differences in crop yield (e.g., soil and plant nutrient supply, general changes in climate, precipitation, disease, and crop establishment). In fact, significant changes in diazotroph community structure between seasons (April–November) have been reported in agricultural soil although the abundance was more stable (Pereira e Silva et al., 2011). This study demonstrated significant changes in both community structure and abundance over two CY and indicates that diazotroph community structure rather than abundance is sensitive to N fertilizer application. Although fertilization, crop and soil N influence diazotroph

communities, the degree of impact can vary greatly between years. Multi-year investigations are necessary to identify the factors influencing the diazotroph populations and underlying year-to-year variability.

Acknowledgments

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