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1 **Diazotroph Community Structure and Abundance in Wheat-Fallow and Wheat-Pea Crop**
2 **Rotations**

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4 **14 Abstract**

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6 15 Biological input of nitrogen (N) from the atmosphere by free-living diazotrophs can help
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9 16 alleviate fertilizer use in agricultural systems. In this study, we investigated the effect of N
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11 17 fertilizer and winter pea (*Pisum sativum* L.) crop on the community structure and abundance of
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14 18 free-living diazotrophs in a two year study of dryland winter wheat (*Triticum aestivum* L.) no-till
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16 19 production system in Eastern Oregon, USA. Based on quantification of the *nifH* gene, diazotroph
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19 20 abundance was strongly influenced by plant species and the crop year in which the soil samples
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21 21 were collected. A greater amount of *nifH* copies was recovered in 2012 compared to 2011 either
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24 22 as copies per gram soil or normalized to the abundance of bacterial 16S rRNA genes. The
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26 23 quantity of genes was greater under pea than wheat in 2012 although no difference was observed
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29 24 in the preceding year. The *nifH* gene abundance was positively correlated to ammonium
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31 25 concentration in 2011 and bacterial abundance in 2012. Nitrogen application did not influence
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34 26 diazotroph abundance in the top 0-5 cm; however the abundance was reduced by application at
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36 27 the lower 5-10 cm depth under wheat crop. The diazotroph community structure appeared to be
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38 28 influenced more by N fertilization rather than plant species with the exception of wheat in 2012.
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41 29 Changes in the community structure over the two years were greater for fertilized than
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43 30 unfertilized soil. Collectively, these data suggest that year-to-year variability had a greater
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46 31 influence on diazotroph communities rather than specific parameters of plant species,
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48 32 fertilization, total N, total organic C, or soil pH. Multi-year studies are necessary to define the
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51 33 specific drivers of diazotroph abundance, community structure and function.
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53 34
54 35 **Keywords:** Wheat, diazotroph, *nifH*, nitrogen-fixation, pea, Pacific Northwest
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37 **1 Introduction**

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

47 Diazotrophs, or N₂-fixers, contribute to plant available N by reducing atmospheric N₂ to
48 ammonium in the soil. Diazotrophs are highly diverse and include members of α -, β -, γ -, and δ -
49 *Proteobacteria*, Firmicutes, Cyanobacteria, and Archaea (Dixon and Kahn, 2004; Rösch et al.,
50 2002), most of which are uncultivated. Although the diversity and distribution of non-symbiotic
51 diazotrophs suggest that most soils have a capacity for BNF (Izquierdo and Nüsslein, 2006; Poly
52 et al., 2001), the ecological impact of free-living diazotroph activity is disputable with estimates
53 of activity varying widely from 0 to 60 kg N ha⁻¹ yr⁻¹ in cropland and natural ecosystems
54 (Cleveland et al., 1999; Day et al., 1975; Gupta et al., 2006).

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

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4 60 2001), soil pH (Limmer and Drake, 1996; Nelson and Mele, 2006), electrical conductivity
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6 61 (Hayden et al., 2010), oxygen (Brouzes et al., 1969; Limmer and Drake, 1996, 1998), carbon (C)
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9 62 quality and quantity (Brouzes et al., 1969; Keeling et al., 1998; Limmer and Drake, 1996, 1998;
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11 63 Wakelin et al., 2010), and N availability (Hayden et al., 2010; Hsu and Buckley, 2009; Limmer
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13 64 and Drake, 1998; Nelson and Mele, 2006). Generally, some of the factors result in reproducible
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15 65 responses in N₂-fixation such as increased activity with decreased oxygen tension (Brouzes et al.,
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17 66 1969; Limmer and Drake, 1998) and increased soil water content (Brouzes et al., 1969; Roper,
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19 67 1985), while others such as C (Brouzes et al., 1969; Limmer and Drake, 1996) and some soil
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21 68 physicochemical characteristics (Hsu and Buckley, 2009; Poly et al., 2001) produce inconsistent
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23 69 results. Although numerous factors may influence diazotroph populations, there is little
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25 70 knowledge regarding specific ecological drivers and soil properties do not appear to have similar
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27 71 effects on community structure and activity (Poly et al., 2001).
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33 72 A better understanding of the drivers or influencing factors on the diazotroph communities
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35 73 and BNF will help improve N use efficiency in cropping systems and may lead to reductions in
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37 74 fertilizer use. An indirect approach to assess the potential for BNF is the characterization of the
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39 75 diazotroph populations by molecular methods. Diazotroph communities are often characterized
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41 76 by the *nifH* gene which encodes the iron subunit of the nitrogenase enzyme (Hayden et al., 2010;
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43 77 Hsu and Buckley, 2009; Mao et al., 2011; Nelson and Mele, 2006; Orr et al., 2011; Poly et al.,
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45 78 2001; Rösch and Bothe, 2005). The *nifH* gene is encoded by all diazotrophs and mimics 16S
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47 79 rRNA gene phylogeny making it an ideal candidate for ecological studies of community
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49 80 structure and/or composition. We compared the abundance and community structure of
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51 81 diazotroph populations in a long-term no-till wheat-fallow and adjacent wheat-pea rotation to
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4 82 examine whether crop rotation and fertilization influence the microbial communities in the
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11 85 **2 Methods**

14 86 **2.1 Site description and sample collection**

16 87 The Pacific Northwest Columbia Plateau has a Mediterranean climate with cool, wet winters
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19 88 and hot, dry summers producing optimal conditions for dryland small grain production. Cropping
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21 89 practices are constrained in the region by limited annual precipitation and typically follow a two-
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24 90 year rotation with a fallow period of 12 or more months preceding winter wheat (W) which is
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26 91 planted in fall and harvested in July/August of the following year. The field site was located 15
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29 92 km northeast of Pendleton, Oregon, USA (45° 72' N, 118° 62' W, elevation 458 m). Annual
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31 93 precipitation occurs mostly during the winter months and yearly averages were 447 mm, 571
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34 94 mm, and 368 mm for crop years 2010, 2011, and 2012 (CY10, CY11, CY12). The soil is Walla
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36 95 Walla silt loam (coarse-silty, mixed, superactive, mesic Typic Haploxeroll) developed in loess
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39 96 overlying basalt (Wuest and Gollany, 2013). Treatments were arranged in a 2 by 2 factorial
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41 97 design with crop rotation as main plots and fertilization as subplots. The winter wheat-fallow
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43 98 rotation (W-F) main plot (NTA experiment) was established in 1982. The winter wheat-fallow
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46 99 rotation (NTB experiment) was initiated in 1997 and was converted to winter wheat-winter pea
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48 100 rotation (W-P) in 2010 by replacing the fallow period with Austrian pea. The NTB experiment is
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51 101 7.62 m north of NTA and both are strictly no-till cropping systems. Nitrogen fertilizer was
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53 102 applied as urea at either 0 or 180 kg N ha⁻¹ (160 lbs N ac⁻¹). Urea was banded at 10 cm depth
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56 103 during wheat planting and treatments referred to as “fertilized” pea and “fertilized” fallow
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58 104 received N only during the wheat phase of the rotation (Table 1). The CY11 and CY12 were the
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4 105 first pea harvests from each plot in the NTB experiment. In CY12, the winter wheat did not
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6 106 establish, therefore it was subsequently sprayed and re-planted to spring wheat on April 13,
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9 107 2012. The naming convention for the plots lists first the crop under which the sample was taken,
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11 108 followed by the rotational crop and amount of the N fertilizer applied with wheat (e.g., W0-F for
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14 109 wheat of unfertilized W-F rotation and P-W180 for pea of W-P rotation in which wheat received
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16 110 180 kg N ha⁻¹).

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19 111 Soil sampling was initiated on June 28, 2011 and July 10, 2012 from the wheat and fallow
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21 112 plots of NTA and the pea plots of NTB and was completed within 2-3 days. In each plot, five
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24 113 soil cores were collected from the top 0-5 cm, 5-10 cm and 10-20 cm depths using a 1.85-cm
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26 114 diameter soil probe. Cores from the individual depths were pooled and homogenized by hand.
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29 115 Approximately 10-20 g of soil was transferred in the field into small zippered bags for DNA
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31 116 analysis and stored on ice until transfer to a -20°C freezer upon arrival in the lab. The remaining
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34 117 soil was dried at 40°C for chemical analyses. Three additional cores were retained for bulk
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36 118 density and soil water content analyses.

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40 120 **2.2 Soil chemical and physical properties**

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43 121 Soil water content and bulk density were calculated according to standard protocols. Dried
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45 122 soils were extracted for 30 min in 1 M potassium chloride prior to quantification of nitrate (NO₃-
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48 123 N) and ammonium (NH₄-N) using an Astoria Analyzer (Astoria-Pacific International,
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51 124 Clackamas, OR) equipped with a 305D digital detector, 303A analytical cartridge, 302D
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53 125 micropump, and 311XYZ sampler. Nitrate was reduced using an open tubular cadmium reactor
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55 126 and analyzed as nitrite using a sulfanilamide method (Mulvaney, 1996). Ammonia was
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58 127 quantified by the sodium salicylate method (Mulvaney, 1996). Soil pH was measured in a 2:1
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128 dilution of soil with 0.01 M calcium chloride. Total C and N were determined by dry combustion
129 using a combustion analyzer (Thermo Finnigan FlashEA 1112 Elemental Analyzer, Rodano,
130 Italy).

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132 **2.3 DNA extraction**

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

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139 **2.4 Gene quantification**

140 The *nifH* gene was quantified from soil extracts using primers nifHF (5'-AAA GGY GGW
141 ATC GGY AAR TCC ACC AC-3') and nifHRb (5'-TGS GCY TTG TCY TCR CGG ATB GGC
142 AT-3') (Rösch and Bothe, 2005) and the bacteria quantified based on 16S rRNA gene sequence
143 using the primers 341F (5'-CCT ACG GGA GGC AGC AG-3') and 518R (5'- ATT ACC GCG
144 GCT GCT GG-3') (Muyzer et al., 1993). Quantitative PCR (qPCR) reactions, prepared in 10 µL
145 volumes, contained 0.8X Power SYBR Green PCR MasterMix (Life Technologies, Grand
146 Island, NY), 0.1 µg µL⁻¹ bovine serum albumin (BSA, Roche, Indianapolis, IN), 0.2 µM (*nifH*)
147 or 0.1 µM (16S) primer and 1 µL of DNA template diluted 1:20 with H₂O. No template control
148 reactions contained 1 µL of H₂O instead of DNA. Amplification was performed using an
149 Applied Biosystems StepOnePlus instrument with the following conditions: denaturation for 10
150 min at 95°C, 40 cycles of amplification for 15 sec at 95°C and 1 min at 60°C, followed by a final

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151 melt curve of 15 sec at 95°C, 1 min at 60°C with an increase of 0.3°C to a final temp of 95°C.

152 All soil extracts were tested for PCR inhibition prior to analysis. Briefly, qPCR reactions were
153 spiked with 3.6×10^5 copies of the pUC19 plasmid and the plasmid quantified using the M13F(-
154 40) and M13R(-48) primers with a 60°C annealing temperature. The C_T of the reactions
155 containing diluted soil DNA extract was compared to the C_T of the plasmid-only controls. All of
156 the soil extracts had C_T values varying less than 0.31 C_T of the plasmid-only control indicating a
157 lack of PCR inhibition.

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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), $0.1 \mu\text{g } \mu\text{L}^{-1}$ BSA and $0.4 \mu\text{M}$ *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 $50 \mu\text{L}$ reactions with $1 \mu\text{L}$ of
undiluted DNA or water under the following thermocycling conditions: denaturation for 1 min at
 98°C , 35 cycles of 10 sec at 98°C , 10 sec at 64°C and 15 sec 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
 μL of DNA was digested in duplicate reactions with 10 units of *HaeIII* restriction enzyme for 6
hr 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 $40 \mu\text{L}$ of GenomeLab

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4 174 SLS with 0.25 μ l of GenomeLab DNA size standard 600 (Beckman Coulter). Digested DNA was
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7 175 separated using a CEQ 8800 Genetic Analyzer (Beckman Coulter) with the following protocol:
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9 176 capillary temperature of 50°C, denaturation for 120 sec at 90°C, injection for 15 sec at 2 kV, and
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12 177 separation for 90 min at 4.8 kV. Fragments were analyzed in the Fragment Analysis module of
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14 178 the CEQ software with a slope threshold of one, relative peak height threshold of 0.5%,
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16 179 confidence level of 95%, quartic model, time migration variable, peak calculation by height, and
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19 180 PA ver. 1 dye mobility calibration using calculated dye spectra. Peaks were manually edited,
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21 181 imported into T-REX online software (Culman et al., 2009), filtered with one standard deviation
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24 182 height, clustered at 1.0 bp, and averaged over the duplicate profiles. Only peaks present in both
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26 183 profiles were used for subsequent analyses. Cluster analysis was performed in PAST software
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29 184 (Hammer et al., 2001) using the paired group algorithm and Jaccard similarity index. The percent
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31 185 change in community structure was calculated from the Jaccard similarity indices obtained in
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33 186 PAST as $\text{change\%} = 100 - \text{similarity\%}$ (Pereira e Silva et al., 2011).
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38 188 **2.6 Calculations and statistical analyses**

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41 189 Gene abundance values are noted as copies per gram dry soil extracted. Percent *nifH*
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43 190 abundance was calculated by dividing the 16S rRNA gene copies g^{-1} soil by the *nifH* copies g^{-1}
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45 191 soil and multiplying by 100 for each individual plot. Differences in N-mineralization and gene
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48 192 abundance across both years were analyzed at a significance value of $P=0.05$ using PROC
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50 193 GLIMMIX procedure with a Tukey-Kramer adjustment for means separation and multiple
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53 194 comparisons (Gbur et al., 2012). Pearson correlation coefficients were calculated for gene copy
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55 195 number and soil physical properties by year using PROC CORR. All analyses were performed in
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58 196 SAS 9.2 (SAS Institute, Cary, N.C.).
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198 **3 Results**

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

211 Significant changes in the abundance of the *nifH* gene were measured over different depths in
212 the W-F rotation (Fig 2). The depth of sampling had a significant effect ($P<0.0001$) on the
213 abundance of the *nifH* gene. Nitrogen fertilization was considered a non-significant effect at the
214 $P=0.05$ level ($P=0.0514$) although comparison of the *nifH* gene abundance at 5-10 cm depth
215 between the two treatments revealed differences in the gene distribution. The *nifH* abundance
216 was similar in the top two depths for each treatment; however, *nifH* copies were significantly less
217 at the 5-10 cm depth in the fertilized wheat (W180-F) compared to unfertilized wheat (W0-F). In
218 both treatments, gene abundance was less in the lower 10-20 cm than the top 0-5 cm depths.

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219 Further experimentation was conducted at the top 0-5 cm sampling depth due to the greater
220 abundance of *nifH* genes in both treatments.

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

229 The increased abundance of the *nifH* gene in CY12 compared to CY11 was paralleled in the
230 overall bacterial abundance as quantified by the 16S rRNA gene (Fig 3b). Crop year imparted a
231 significant ($P<0.0001$) effect on the quantity of 16S genes g^{-1} soil. For CY11, bacterial
232 abundance was similar across all crops and fertilization treatments. However, differences in gene
233 abundance were apparent in CY12 for W0-F, which was less than in all but W180-F and F-
234 W180. When the *nifH* abundance was calculated as percent total bacteria, crop year was the
235 single significant ($P<0.0001$) effect and no differences were measured among any treatments
236 within a single year (Fig 3c). Overall, the average abundance of *nifH* for all treatments in a single
237 crop year was 4.7 times greater in CY12 than CY11. The increased *nifH* copy number was
238 positively correlated to bacterial abundance in CY12 ($r=0.87$, $P<0.0001$) but not in CY11 (Fig
239 3d). In CY11, a significant positive correlation was observed between *nifH* abundance and NH_4 -
240 N ($r=0.48$, $P=0.0173$) and a negative correlation between bacterial abundance and NO_3 -N ($r=-$
241 0.42 , $P=0.0385$). Regardless of year, no significant correlation was observed for either *nifH* or

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242 16S rRNA gene copy number with soil pH, % total N or % total organic C (TOC) at $P<0.05$;
243 however, a weak positive correlation ($r=0.38$, $P=0.0714$) was observed in CY12 for 16S gene
244 copy with %TOC (Table 1).

245 Diazotroph communities responded differently to crop and N treatments in the two crop
246 years (Fig. 4) and no single factor of influence was clear. The number of *nifH* phylotypes, or T-
247 RFLP peaks, detected in each treatment ranged from 28 to 83 with an average of 64 peaks in
248 CY11 and 57 peaks in CY12 (Table 2). Fertilized plots showed the greatest change in both
249 species richness and structure when compared over the two years. Fertilized wheat (W180-F) had
250 the largest increase in richness from CY11 to CY12 while both fertilized fallow (F-W180) and
251 pea (P-W180) showed the greatest decrease (Table 2). Communities collected from all
252 treatments showed significant changes in structure between the two CY, although both
253 unfertilized wheat (W0-F) and pea (P-W0) plots were least divergent with changes of 47% and
254 50%, respectively (Table 2). The community structures of fertilized fallow (F-W180) and
255 fertilized wheat (W180-F) were most divergent, with respective changes of 72% and 77%
256 between CY (Table 2). For comparison within CY, the microbial communities of F-W180 and P-
257 W180 in CY11 were most closely related and both treatments had the highest amount of $\text{NO}_3\text{-N}$
258 (Fig 4a, Fig 1a). However, the fertilized plots (F-W180, P-W180) also clustered with W0-F and
259 F-W0 which had markedly lower $\text{NO}_3\text{-N}$ and were of the same crop rotation. Similarly, in CY12
260 the most closely related communities were again recovered from F-W180 and P-W180 in which
261 the soil N of the pea plot was more in the form of $\text{NH}_4\text{-N}$ than $\text{NO}_3\text{-N}$ unlike the preceding CY
262 (Fig 4b, Fig 1a,b). Additionally, the wheat plots (W0-F, W180-F) of the W-F rotation of CY12
263 were most closely related to one another regardless of fertilization (Fig 4b).

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4 **265 4 Discussion**

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6 266 Diazotroph diversity, function and abundance have been associated with numerous factors
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9 267 such as microbial biomass, soil physicochemical characteristics, and management strategies
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11 268 (Hayden et al., 2010; Hsu and Buckley, 2009; Keeling et al., 1998; Limmer and Drake, 1996,
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13 269 1998; Nelson and Mele, 2006; Poly et al., 2001; Riffkin et al., 1999; Wakelin et al., 2010). In this
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15 270 study of a W-F and a newly established W-P rotation, the response of the diazotrophic
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17 271 communities varied between the two crop years in which the ecological drivers for abundance
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19 272 appeared to be related to microbial biomass in CY12 and soil chemistry in CY11. The quantity
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21 273 of *nifH* genes per g soil detected in the plots was consistent with other studies (Coelho et al.,
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23 274 2009; Mao et al., 2013; Orr et al., 2011; Pereira e Silva et al., 2011). Overall, the gene abundance
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25 275 in CY12 was much greater than in CY11 and was accompanied by a general increase in bacteria.
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27 276 Similarly, an association of microbial biomass and *nifH* abundance was reported for
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29 277 agriculturally managed and remnant sites in Australia (Hayden et al., 2010). The correlation
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31 278 observed in the current study suggests that the increase in *nifH* abundance is due to enhanced
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33 279 microbial biomass rather than specific guild enrichment.
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41 280 Plant species (Mao et al., 2011) and biomass retention (Hsu and Buckley, 2009) influence
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43 281 community structure and biomass retention has a positive effect on *nifH* gene abundance
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45 282 (Wakelin et al., 2007; Wakelin et al., 2010). In the current study, the crop rotations were
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47 283 conducted under no-till in which the biomass was retained. Wheat preceded both pea and fallow
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49 284 plots and, interestingly, the diazotroph community of the F-W180 was most similar to the P-
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51 285 W180 regardless of crop year. Like Poly et al. (2001) in which plant species was not a main
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53 286 factor of *nifH* diversity, the diazotroph communities appeared to be influenced more by N
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55 287 fertilizer application than crop. The variability in community structure between years was also
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288 greatly influenced by fertilization, such that the communities resident to fertilized plots diverged
289 more over the two year study than the unfertilized plots regardless of crop. Additionally, the
290 communities with similar fertilizer treatments were more closely related than those of similar
291 crop when comparisons were made within the same year. However, in the CY12 wheat plots, the
292 communities from fertilized (W180-F) and unfertilized (W0-F) treatments were most closely
293 related. It is important to note that the winter wheat plots in CY12 did not establish early in the
294 season and were therefore sprayed and re-planted to spring wheat. The replanting did not appear
295 to influence the diazotroph abundance since the microbial communities of both wheat and pea
296 plots had similar percent *nifH* gene compositions; however, it is not possible to discount that the
297 similarity in the community structure was an effect of either the spraying or replanting.

298 Previous studies of N application have produced inconsistent results on the soil or root-
299 associated diazotroph communities with some studies showing decreased diversity (Tan et al.,
300 2003), activity (Tan et al., 2003), and *nifH* abundance (Coelho et al., 2009) whereas others have
301 demonstrated no response (Ogilvie et al., 2008; Wakelin et al., 2007). The sensitivity of
302 diazotroph communities to fertilizer also appears to be a function of plant association since the
303 *nifH* abundance was decreased in response to high levels of fertilizer in the rhizosphere soils but
304 showed no response in bulk soil (Coelho et al., 2009). Similarly, in the current study fertilization
305 was inconsequential to the number of *nifH* genes recovered from the bulk soil at 0-5 cm for
306 either CY although it did reduce the abundance of genes at the lower 5-10 cm depth under wheat
307 cropping. Consistent with a study of an acidic forest soil (Mergel et al., 2001), the abundance of
308 *nifH* genes in the top 0-5 cm and 5-10 cm were similar in unfertilized plots and reduced at the 5-
309 10 cm depth when fertilizer was applied. Bacteria (Paul and Clark, 1989) and diazotrophs (Kloos

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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;

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333 Pereira e Silva et al., 2011). Numerous factors can contribute to differences in crop yield (e.g.,
334 soil and plant nutrient supply, general changes in climate, precipitation, disease, and crop
335 establishment). In fact, significant changes in diazotroph community structure between seasons
336 (April-November) have been reported in agricultural soil although the abundance was more
337 stable (Pereira e Silva et al., 2011). This study demonstrated significant changes in both
338 community structure and abundance over two CY and indicates that diazotroph community
339 structure rather than abundance is sensitive to N fertilizer application. Although fertilization,
340 crop and soil N influence diazotroph communities, the degree of impact can vary greatly
341 between years. Multi-year investigations are necessary to identify the factors influencing the
342 diazotroph populations and underlying year-to-year variability.

343

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350 opportunity provider and employer.

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473 **Figure legends**

474
475 **Table 1.** Soil chemical properties under different crop rotations during wheat and fallow phase
476 of wheat-fallow (W-F) rotation in the NTA experiment and pea phase of the wheat-pea (W-P) in
477 the NTB experiment.

478
479 **Table 2.** Jaccard similarity indices for different crop rotations during wheat and fallow phase of
480 wheat-fallow (W-F) rotation in the NTA experiment and pea phase of the wheat-pea (W-P) in the
481 NTB experiment. Values are noted as % similarity.

482
483 **Figure 1.** Nitrate (a) and ammonium (b) concentration in the top 0-5 cm for each crop year
484 (CY11, CY12). The crop listed first of the rotation is phase in which soils were sampled. The
485 same letter above the data indicate that the data are not significantly different at $P < 0.05$ level
486 ($n=4$, Tukey post hoc test). Bars indicate mean + standard error.

487
488 **Figure 2.** Abundance of *nifH* gene from different depths of soil sampled from wheat plots in
489 crop year 2011. Depths are 0-5 cm, 5-10 cm and 10-20 cm as indicated by the end depth. Bars
490 indicate mean + standard error and the same letter above the bars (all six) indicate no significant
491 difference at $P > 0.05$.

492
493 **Figure 3.** Abundance of diazotrophs and total bacteria in the top 5 cm of soil as measures of
494 *nifH* gene (diazotroph) quantity (a), bacterial 16S gene quantity (b), relative percent *nifH* (c), and
495 correlation of *nifH*:16S copy number (d). For graphs a-c, bars indicate mean + standard error and

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496 data with the same letter are not significantly different at $P=0.05$. In plot d, the open symbols are
497 from crop year 2011, the closed symbols are crop year 2012 and the regression lines are shown.

498
499 **Figure 4.** Effect of N and crop on the diazotrophs community structure as characterized by
500 cluster analysis of the T-RFLP data for the *nifH* gene for crop years 2011 (a) and 2012 (b). Crop
501 and fertilization (0 and 180 kg N ha⁻¹) are noted. The Jaccard similarity coefficient is shown on
502 the Y axis and the scale varies between the two crop years. Bootstrap values are indicated at the
503 nodes (based on 10,000 bootstrap replicates).

504

Table 1

Properties	Rotation Crop, N	Wheat-Fallow (NTA) ^a				Wheat-Pea (NTB)		Pearson Coefficient ^d	
		W-F		W-F		W-P		<i>nifH</i>	16S
		W0-F	W180-F	F-W0	F-W180	P-W0	P-W180		
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 a different letter are 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 $P < 0.10$.

Table 2

Properties	Rotation Crop, N	Wheat-Fallow (NTA) ^a				Wheat-Pea (NTB)	
		W-F		W-F		W-P	
		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							
	2011-2012	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 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).

Figure 1

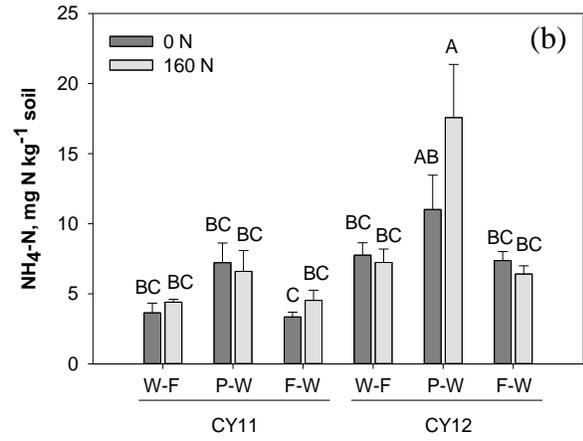
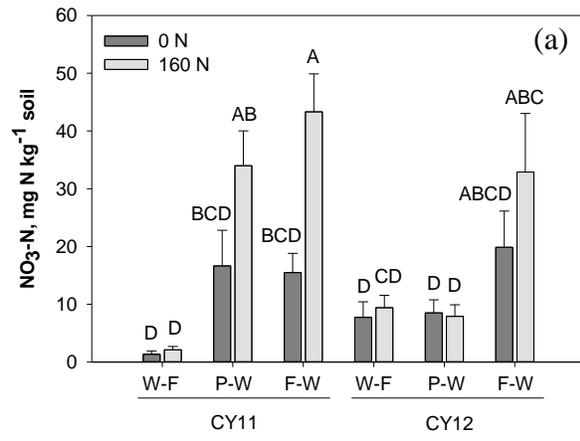


Figure 2

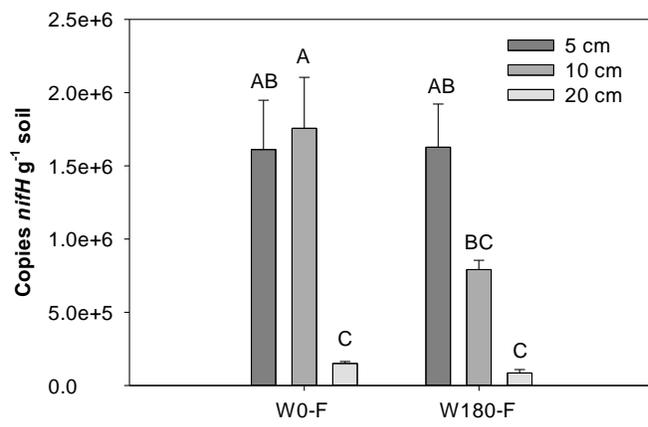


Figure 3

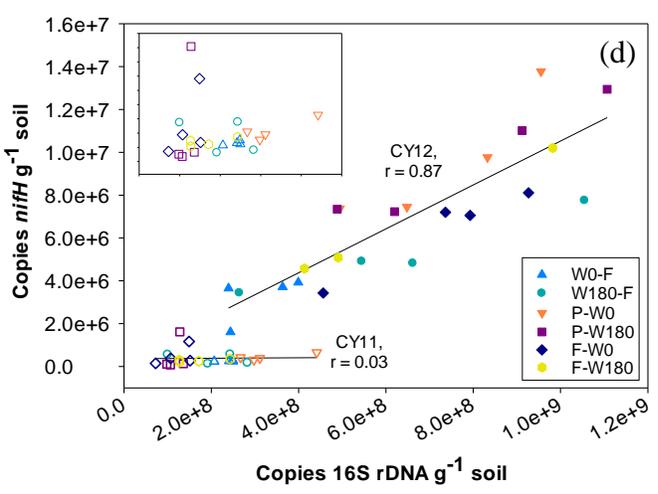
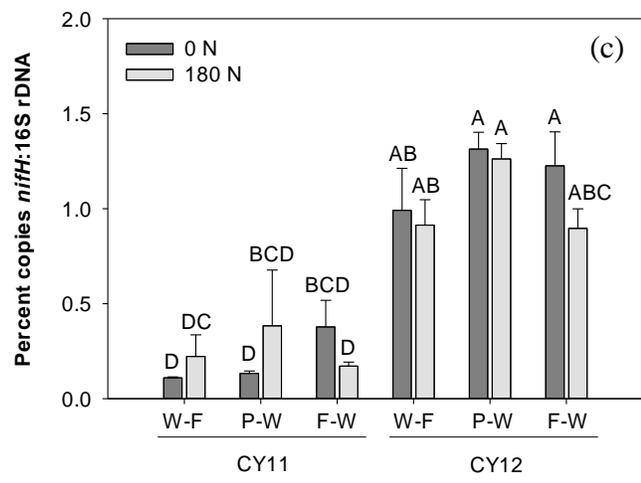
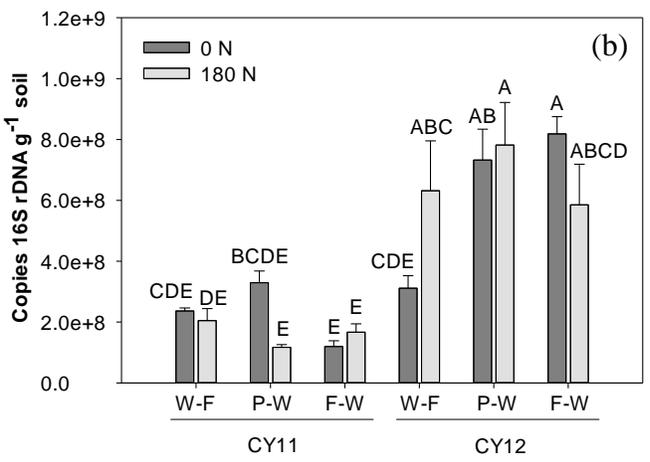
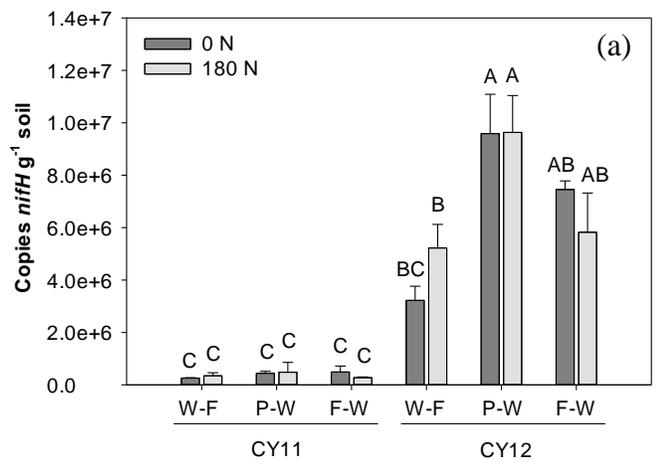


Figure 4

