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Soil amendments yield persisting effects on the microbial communities—a 7-year study

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ABSTRACT

Soil microbial communities are sensitive to carbon amendments and largely control the decomposition and accumulation of soil organic matter. In this study, we evaluated whether the type of carbon amendment applied to wheat-cropped or fallow soil imparted lasting effects on the microbial community with detectable differences in activity, population size, or community structure after a period of seven years post-amendment. The microbial communities from the top 10 cm of soil were analyzed for activity related to C-cycling (glucosidase, galactosidase), P-cycling (acid phosphatase), S-cycling (arylsulfatase), and N-cycling (β-glucosaminidase, arylamidase), in addition to fungal and bacterial abundance and structure. The amendments were applied at similar carbon rates for five years under annual wheat or continuous fallow and included cotton linters, sucrose, wheat residue, composted wheat residue, brassica residue, wood sawdust, alfalfa feed pellets, manure, biosolid and a no treatment control. Two crops, brassica and grass, were in the fallow treatments. The majority of the communities in the amended soils were not distinguishable from the no-treatment control. For amendments and crops that produced changes, significant differences in the population size and community structure were observable for fungi but not bacteria. Wood, sugar, and grass cropping produced the most pronounced effects on enzyme activity, fungal abundance and structure. Overall, the species of the planted crop had a significant effect on the soil enzyme activity and population size of fungi, with the greatest values under grass compared to wheat or brassica. The microbial communities were differentially affected by C source amendments in which the persistency of change and the aspect of the community affected (i.e. function, size, structure, kingdom) were dependent on amendment type.

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1. Introduction

The capacity of soil to store carbon (C) is largely influenced by the role of soil microbes in the formation and decomposition of organic matter (Paul, 2007; Six et al., 2006). The chemical composition (or quality) and quantity of detritus or C substrate has a strong impact on the rate and mode of decomposition. For example, litter with high lignin such as wood will decompose at much slower rates than cytoplasmic sugars and amino acid compounds. The type of substrate can also affect soil C and nitrogen (N) in which amendments of disparate composition produce different C/N ratios in soil when added as a surface amendment (Wuest and Gollany, 2013). Soil management strategies including fertilization, soil amendment, and selection of crop species will impact the rates of turnover in addition to the structure and function of the resident microbial communities (Acosta-Martínez et al., 2007; Acosta-Martínez and Tabatabai, 2001; Angers et al., 1993; Dodor and Tabatabai, 2002).

Extracellular soil enzymes have a key role in the stabilization and destabilization of organic matter and are important in the decay of insoluble plant materials such as cellulose and lignin. Soil management practices, environmental factors such as temperature and pH (Speir et al., 1980; Tabatabai, 1994), and the C and N status of the microbes (Allison and Vitousek, 2005; Geisseler and Horwath, 2009) affect the activity of enzymes in the soil. Enzyme synthesis is controlled under different regulatory mechanisms. Soil enzymes may be either constitutively expressed or responsive to the chemical environment, i.e. substrate availability may induce enzyme production (Geisseler and Horwath, 2009; Mobley and Hausinger, 1989; Suto and Tomita, 2001) or result in feedback inhibition. Enzyme activity can yield information regarding the substrate status of soil and may provide insight to the microbial community composition or abundance. As such, activities of several enzymes have been correlated to C and N mineralization

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rates (Allison and Vitousek, 2005; Ekenler and Tabatabai, 2002; Geisseler and Horwath, 2009), microbial respiration (Frankenberger and Dick, 1983) and microbial biomass of soils (Frankenberger and Dick, 1983; Taylor et al., 2002).

Microbial communities are dynamic and change rapidly in response to a disturbance such as an amendment. For example, changes in the community composition can be measured as quickly as 12 h post-addition (Cleveland et al., 2007). Most communities are sensitive to carbon amendments, meaning some aspect such as structure, composition, or function change in response to the addition. While some communities may remain unchanged after a perturbation (resistance), others may change and then revert back to the original composition (resilience) (Allison and Martiny, 2008). In a survey of 41 studies, the mean length of the studies showing sensitivity to disturbance (83% of studies) was far greater (4.9 ± 12.6 years) than studies showing resistance (0.15 ± 0.09 years) (Allison and Martiny, 2008). The difference in the response compared to the study duration suggests that a lag time may occur between C addition and community change, and that studies too short in length may miss key transformations. The resiliency of communities sensitive toward soil amendments is influenced by several factors including the type of carbon applied (Orwin et al., 2006), the dose (Saison et al., 2006), and the frequency of application (Saison et al., 2006). It is unclear how the microbial community resiliency is affected in farm production systems where the soil amendments may be infrequently applied or used as "pulse" applications and whether amendment type influences the response longevity.

A study on the effect different C source amendments on the soil C/N ratio in a wheat-fallow system revealed that the amendment composition had a large and lasting effect (3.5 yr) on the soil C/N ratio when application frequency and dose were constant (Wuest and Gollany, 2013). The study selected 9 different soil amendments with varying solubility, C structures, and nutrient and lignin contents to test the hypothesis that the amendment type, rather than specific dose, affects the soil organic carbon (SOC). The amendments included plant residues and compost, sugar, wood, cotton, manure and biosolid in addition to different living crops. Of the diverse amendments, wood produced a much higher C/N ratio than the other amendments including manure, compost and biosolid. The authors attributed the increased C/N ratio in the wood-amended soil to changes in the microbial community, presumably enhanced fungal populations (Wuest and Gollany, 2013). Although it is likely that many, if not all, of the amendments impacted the microbial communities to some degree, it is unknown whether the amendments produce lasting effects, particularly after the plots returned to cropping. Since the amendments were applied at similar doses based on C content and the same frequency (once a year for five years), it is possible to evaluate whether different carbon types influence microbial resiliency. The experiment initiated by Wuest and Gollany (2013) provided a unique opportunity to determine whether "pulse" amendments yield lasting effects on the microbial communities in a field setting rather than lab incubations. In the current study, we assessed the soil microbial communities of the amended plots of Wuest and Gollany (2013) which have been treated uniformly for the past 7 years post-amendment, with the final 2.5 years in crop rotation.

Community function was assessed based on the activity of extracellular enzymes functioning in C, N, phosphorous (P), and sulfur (S) cycling. Hydrolytic enzymes β-glucosidase, α-galactosidase, and β-glucosaminidase were used to query the C-cycling capacity of the soil. β-glucosidase is involved in cellulose degradation and produces glucose through the hydrolysis of the terminal, nonreducing ends (Deng and Popova, 2011). Similarly, α-galactosidase catalyzes the degradation of hemicellose and oligosaccharides by the hydrolysis of the terminal, nonreducing α-D-galactose residues (Adl, 2003; van den Brink and de Vries, 2011). The chitinase, *N*-acetyl-β-D-glucosaminidase, is involved in both C and N cycling through the release of the terminal *N*-acetyl-β-D-glucosamine residues from chitooligosaccharides (Ekenler and Tabatabai, 2002). Arylamidase catalyzes the release of the N-terminal amino acid from a peptide, amide or arylamide (Acosta-Martínez and Tabatabai, 2000a), and has been suggested as an index for net soil N-mineralization (Dodor and Tabatabai, 2002). Phosphatase produces plant available inorganic P through the hydrolysis of organic phosphomonoester (Tabatabai, 1994). Phosphatases are ubiquitous and pH sensitive, therefore the acid phosphatase was selected based on the pH range of the soil. Finally, arylsulfatase produces plant available sulfate by cleaving the O—S bond of ester sulfates, which is considered the dominant form of organic S in soil (Knauff et al., 2003; Scherer, 2009; Tabatabai, 1994). This subset of soil enzymes, in addition to assessment of the fungal and bacterial communities, was selected to determine whether the diverse composition of the amendments differentially impacted the microbial communities with persisting changes in function, structure, and/or abundance.

2. Materials and methods

2.1. Experimental design

Soil characteristics, annual precipitation averages, and previous management history of the research site located 15 km northeast of Pendleton, OR, USA is provided in Wuest and Gollany (2013). The plots were managed under no-till with crops planted in October and harvested late July according to regional practices. The experiment was initiated in 2002 in a split-plot design with two main factors of annual winter wheat (*Triticum aestivum* L.) or continuous fallow (referred to as main plots, or wheat or fallow main) and 12 subplots of 10 soil treatments and two different plant species (fallow only). The main plot locations were randomized within each of four block locations and were continuous (no rotation) throughout the experiment. The blocks were divided into randomly assigned subplots 9.29 m^2 (1.52 m × 6.10 m) with 0.15 m borders. Amendments were selected based on the common regional surface residues (wheat straw, brassica residue, alfalfa foliage) or amendments (manure and biosolids) in addition to other sources not typical of farming practices but high in concentration of different crop residue components (cotton linter is >85% cellulose [Han, 1998], sucrose is 100% sugar, wood is high in lignin). The 9 soil amendments were cotton (*Gossypium hirsutum* L.) linters, sucrose, wheat residue, composted wheat residue (referred to as compost), brassica residue (*Brassica napus* or *Brassica juncea*), wood sawdust (bark-free conifer species with 90% <1 mm size), alfalfa (*Medicago sativa* L.) feed pellets, cattle manure (un-aged, soil- and straw-free), dry biosolid from a municipal sewage treatment plant, and a no-amendment control (check). Additional amendment properties including C, N, and S contents are provided in Wuest and Gollany (2013). The amendments were added to the soil surface at targeted application rates of 250 g C m^{-2} at the end of summer 2002 just prior to planting the wheat main. The compost treatment received a lesser amount of C and N since the application rate was based on the pre-composted content of the wheat straw. The two different plant species (or crops), perennial grass tall fescue (*Festuca arundinacea* Schreb.) and winter brassica (*B. napus* or *B. juncea* L.), failed as intercrops in the wheat main and were therefore only present the fallow main. The grain drill used for planting and fertilization of the wheat was driven through the fallow plots for equal soil disturbance for the two main plots. The wheat main plots, excluding high N treatments of biosolid, manure and alfalfa, received fertilizer containing 58 g N m^{-2}, 9 g S m^{-2}, and 5 g P m^{-2} total over the five year period.

Brassica and grass plots received starter fertilizer for crop establishment. Grain was harvested from the wheat main plots and the straw and chaff were returned to the soil surface. Grass and brassica crops were clipped at the time of wheat harvest and the residue was weighed and returned to the plot surface.

The amendments were applied for 5 years. After the final crop of the wheat main was harvested in summer 2007, all plots remained fallow (the perennial grass was killed) and weed-free for approximately 3.5 years to monitor changes in SOC. The entire test area was placed back into crop rotation in the fall 2010 in which two wheat crops were sown followed by fallow. Soil samples were collected from the site in 2002 prior to the experiment, 2007 after the harvest of the wheat crop following the final amendment, in 2011 after the plots were placed back into rotation, and in 2013 at the end of the experiment. Soil C and N data for the 2002, 2007 and 2011 sampling events are published in Wuest and Gollany (2013) and the soil C, N, and P ratios for 2007, 2011 and 2013 are available in (Wuest and Reardon, 2016).

2.2. Sample collection

Soil samples were collected on July 1, 2013, approximately 7 years after the final amendment, using 2-cm diameter soil probes from the inner rows of the plots to avoid border effects. The top 10 cm of soil from 5 cores was composited, homogenized in the field and stored on ice. Upon arrival to the lab, 10–20 g of soil was transferred to a small plastic zippered bag and stored at −20 °C for DNA and nitrate analysis. The remaining soil was stored at 4 °C until analyzed for moisture, pH, and soil enzyme activity.

2.3. Soil analysis

Gravimetric soil water content was measured in soils dried at 105 °C. Nitrate-N was quantified in 1 M KCl extracts of frozen soil with an Astoria Analyzer (Astoria-Pacific International, Clackamas, OR, USA) using the sulfanilamide method (Mulvaney, 1996). Soil pH was measured in a 2:1 dilution of soil with 0.01 M calcium chloride.

2.4. Soil enzyme assays

Enzyme activities (β-glucosidase, β-galactosidase, β-glucosaminidase, acid phosphatase, and arylsulfatase) were quantified using *p*-nitrophenyl-labeled substrates in a microplate assay. The method was modified from previous protocols (Acosta-Martínez and Tabatabai, 2000a; Parham and Deng, 2000; Tabatabai, 1994) to accommodate smaller volumes. Substrates, concentrations and assay specific buffers are listed in Table 1. Substrate concentrations that differ from the original protocols were optimized in the microplate format. Visible roots and large soil aggregates were removed using sterile forceps. Approximately 1 g of moist soil was transferred to a sterile test tube containing 9 ml of assay-specific buffer. The tubes were vortexed for 10 s on max speed and 2 ml was transferred to a 12-well culture plate. The slurry was continually mixed using a micro stir bar during transfer to the microplate. The microplate was prepared for three technical replicates and one negative control for each sample, and a standard curve. Three of the sample wells contained 50 μl of substrate (no substrate was added for the negative control wells). Standards were measured in duplicate with wells containing 50 μl of 1X buffer and 200 μl of *p*-nitrophenol stock solutions ranging from 0 to 10 μg ml^{-1}. Soil slurry (200 μl) was added to each of the 4 test wells of the microplate. The plates were covered with a plastic lid and incubated at 37 °C for 1 h without shaking. The reaction was stopped and developed by the addition of 25 μl 0.5 M THAM buffer (Trizma base, pH 12) and 25 μl 0.6 M $CaCl_2$ to all wells of the microplate. The plate was sealed with tape, vortexed for 5 s and centrifuged for 2204 *g*. The tape was peeled back and substrate was added to the negative controls. The plates were resealed, vortexed briefly and centrifuged for 10 min at room temperature. The supernatant (200 μl) was transferred to a new microplate and read at 405 nm using a Molecular Devices VMax Microplate reader (Sunnyvale, CA, USA). Arylamidase activity was measured according to the methods of Acosta-Martínez and Tabatabai (2000a).

2.5. DNA extraction and quantitative PCR

DNA was extracted from field moist soil equivalent to 0.25 g dry soil using the PowerLyzer PowerSoil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) with a 10 min vortex lysis step according to the manufacturer's protocol. DNA was eluted with 80 °C buffer and re-eluted with the eluant. Bacteria and fungi were quantified as a measure of the abundance of the 16S rRNA gene and internal transcribed spacer (ITS) region, respectively. Bacterial DNA was amplified using the primers 338F (5′-ACT CCT ACG GGA GGC AGC AG-3′) and 518R (5′-ATT ACC GCG GCT GCT GG-3′) (Øvreås et al., 1997) and fungal DNA using primers NSI1 (5′-GAT TGA ATG GCT TAG TGA GG-3′) (Vilgalys and Hester, 1990) and 5.8 S (5′-CGC TGC GTT CTT CAT CG-3′) (Martin and Rygiewicz, 2005). Quantification was carried out in 10 μl reactions with 0.8× PowerSYBR Green Master Mix (Life Technologies, Grand Island, NY, USA), 0.1 μg μl^{-1} BSA (Roche, Indianapolis, IN, USA), 0.1 μM bacteria or 0.4 μM fungi-specific primers, and 1 μl of soil DNA diluted 1:20 in H_2O. Thermocycling was performed using a StepOnePlus Real-Time PCR System (Life Technologies) with the following conditions: denaturation 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 s^{-1} to a final temp of 95 °C. Soil DNA extracts were tested for the presence of inhibitors according to published protocol (Reardon et al., 2014). None of the diluted extracts showed signs of inhibition as all of the sample's C_q values were less than 0.5 of the plasmid-only control.

2.6. Terminal-Restriction Fragment Length Polymorphism analysis

Community structure was analyzed by Terminal Restriction Fragment Length Polymorphism of the bacterial 16 S rRNA gene

Table 1
Enzyme assay buffers and substrates.

Enzyme	EC[a]	Substrate (final conc., mM)[b]	Buffer[c]	Reference
Acid Phosphatase	3.1.3.2	*p*-NP-phosphate (50)	MUB, pH 6.5	Tabatabai (1994)
Galactosidase	3.2.1.21	*p*-NP-α-D-galactopyranoside (50)	MUB, pH 6.0	Tabatabai (1994)
Glucosidase	3.2.1.21	*p*-NP-β-D-glucopyranoside (10)	MUB, pH 6.0	Tabatabai (1994)
Arylamidase	3.4.11.2	L-Leucine β-naphthylamide (8)	0.1 M THAM, pH 8.0	Acosta-Martínez and Tabatabai (2000a,b)
β-Glucosaminidase	3.2.1.30	*p*-NP-*N*-acetyl-β-D-glucosaminide (10)	0.1 M Acetate, pH 5.5	Parham and Deng (2000)
Arylsulfatase	3.1.6.1	Potassium *p*-NP-sulfate (10)	0.5 M Acetate, pH 5.8	Tabatabai (1994)

[a] Enzyme Commission Number.
[b] *p*-NP stands for *p*-nitrophenyl.
[c] MUB, modified universal buffer (Parham and Deng, 2000); THAM, tris(hydroxymethyl) aminomethane.

and fungal ITS region. DNA was amplified in duplicate 12.5 μl PCR reactions containing final concentrations of 1× PCR Master Mix (Promega, Madison, WI, USA), 0.1 μg μl^{-1} BSA (Roche), and 0.2 μM WellRED-labeled forward (Sigma–Aldrich, St. Louis, MO, USA) and unlabeled reverse primers. Bacterial DNA was amplified with WellRED D4-labeled 8F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 518R. Fungal DNA was amplified using WellRED D3-labeled ITS1-F (5′-CTT GGT CAT TTA GAG GAA GTA A-3′) (Gardes and Bruns, 1993) and ITS4 primers (5′-TCC TCC GCT TAT TGA TAT GC-3′) (Mitchell et al., 1994). Thermocycling was performed for 2 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C, with a final extension for 7 min at 72 °C. Replicate PCR products were pooled and visualized by gel electrophoresis to verify amplification of correctly sized bands. PCR product (5 μL) was digested in duplicate 20 μl reactions with 10 U MspI (bacteria) or 10 U each of HhaI/HaeIII (fungi) for 6 hrs at 37 °C and the enzymes inactivated at 80 °C for 10 min. Digests were precipitated with glycogen and EtOH and the pellets resuspended in 20 μl SLS (Beckman Coulter, Indianapolis, IN, USA). Equal volumes of bacterial and fungal digests were added to duplicate wells of a 96 well plate containing 0.25 μl of DNA Size Standard 600 (Beckman Coulter) and SLS (final volume of 20 μl). Fragments were 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. Data were imported to T-REX software (Culman et al., 2009), and the peaks were clustered by 1.0 bp, the peak areas filtered by one standard deviation, and the profiles relativized within samples. Profiles were averaged over replicates for ordination and diversity indices.

2.7. Calculations and statistical analyses

Gene abundance and enzyme activity were normalized to grams dry soil extracted or activity per gram dry soil, respectively. Means separation was performed with the Tukey–Kramer adjustment using the generalized linear mixed model (GLIMMIX) procedure of SAS 9.4 (SAS Institute, Cary, NC) with a significance value of $P < 0.05$. The means separation of pH values was performed on [H^+] concentration whereas the arithmetic means and standard deviations of pH values were plotted for visualization of the data. Pearson's correlation coefficients were determined in SAS 9.4. Differences in the total soil enzyme activity were calculated by means separation of the sum of each enzyme activity. Intercropping of brassica and grass in the wheat main failed therefore all comparisons of main effects were conducted without the crop treatments (i.e. brassica and grass crop) unless otherwise noted. In several analyses, the values for the grass crop were greater than the other treatments, therefore means separation of the treatments were analyzed with and without grass and brassica crops. T-RFLP data were analyzed in PAST 3.06 software (Hammer et al., 2001). One-way analysis of similarity (ANOSIM) was used to determine a significant effect of treatment on the replicate T-RFLP profiles using the Bray–Curtis similarity indices at a significance value of $P < 0.05$. Separation of the communities was based on the ANOSIM R statistic using the scale of Ramette (2007) in which $R > 0.75$ are well separated, $R > 0.5$ are separated but overlapping, and $R > 0.25$ are barely separable, and $R = 0$ are not separable. Diversity indices and non-metric multidimensional scaling (NMDS) ordination of the Bray–Curtis similarity index were performed using the averages of the treatment T-RFLP profiles.

3. Results

Significant differences in crop and treatment were observed for NO_3-N and soil pH. The wheat main had significantly more NO_3-N in the 0–10 cm than the fallow main ($P < 0.0001$, Fig. 1). The soil NO_3-N in the biosolid treatment was significantly greater than all other treatments in both the wheat and the fallow main when crops were included. When crops were omitted from the analysis of the fallow main, sugar was not statistically different from biosolid or the other treatments. Although the pH of the two main plots were not different ($P = 0.9419$), a treatment effect was observed ($P < 0.0001$). Manure and brassica residue increased the soil pH from the check in both the wheat and fallow main although the increase was significant only in the wheat main (Fig. 2). Biosolid reduced the soil pH from the check under fallow but not wheat. Nitrate values were strongly and negatively correlated to pH in the fallow main regardless of whether the grass and brassica crop were included in the analysis ($r = -0.4383$, $P = 0.0018$ with crops included and $r = -0.4928$, $P = 0.0012$ without crops) (Fig. 2). No correlation was observed between nitrate and pH in the wheat main ($P = 0.8030$).

Total soil enzyme activity was similar ($P = 0.0838$) between the main plots (Fig. 3). Treatment differences were observed only in the fallow main regardless of whether crops were included ($P < 0.0001$) or omitted ($P = 0.0435$). The sum enzyme activity measured in the grass crop was greater than the other fallow main treatments although the differences were not significant from alfalfa, manure or wheat compost (Fig. 3). When the enzymes were considered separately in the fallow main, significant differences were observed for acid phosphatase, galactosidase, and arylamidase activity albeit treatment differences of galactosidase were dependent on inclusion of the crop treatments (Table 2). The treatment differences in the fallow main were similar for all comparisons when crops were excluded except that arylamidase activity was different between biosolid and brassica residue. Apart from arylsulfatase, enzyme activity was generally greater in the grass crop compared to the other treatments. Additionally, grass was greater than both wheat (wheat main check) and brassica crop for all enzymes when crops were considered separately, although the data were not always significant (Table 3). Arylamidase was the

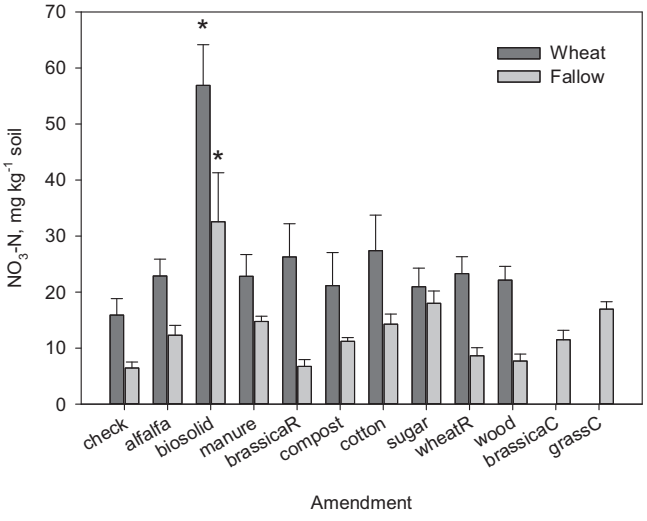

Fig. 1. Soil NO_3-N values from top 0–10 cm depth for treatments in both wheat and fallow main plots. Capital letters in the treatment name indicate whether the amendment was a plant residue (R) or living crop (C). Asterisks indicate significance at $P = 0.05$ for either the wheat main or fallow main when crops were included. When crops were excluded in the analysis of the fallow main, biosolid was statistically different from the other amendments excluding sugar.

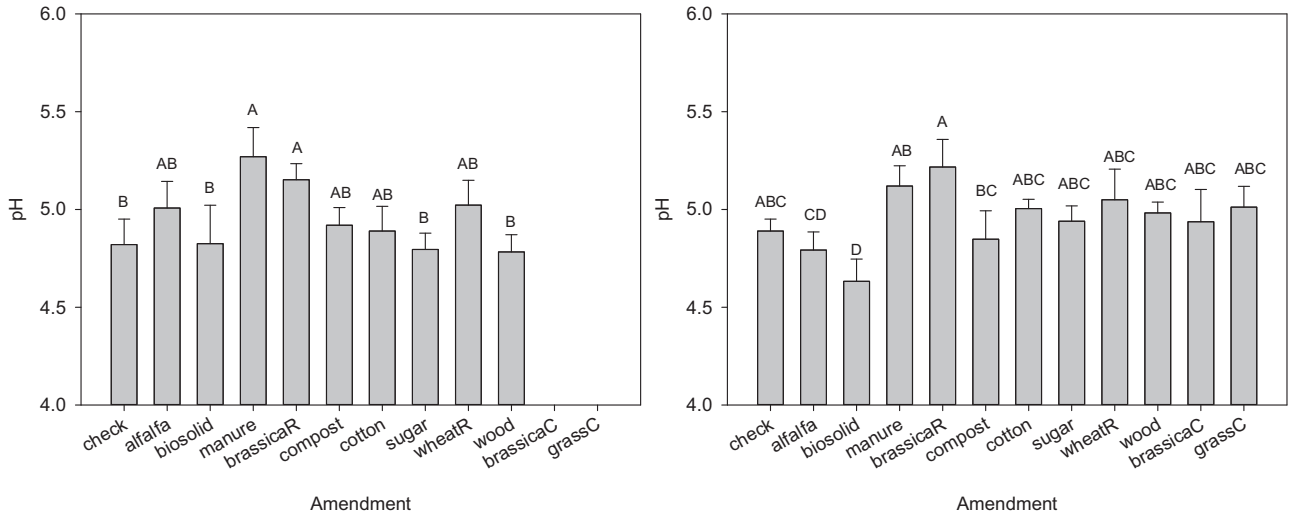

Fig. 2. Soil pH values for the different treatments applied in either the wheat (left) or fallow (right) main plots. Bars indicate the arithmetic mean and standard deviation of the pH values. Shared letters above the bars indicate the pH value based on $[H^+]$ are not significantly different at $P = 0.05$. Crops were included in the analysis of the fallow main and omission of the crops in the data analysis does not change the significance of the other treatments.

only enzyme with treatment differences in the wheat main in which wheat residue was greater than sugar and wood (Table 2). Arylamidase activity was strongly correlated to pH ($P < 0.0001$) with correlation coefficients of $r = 0.6905$ for wheat, $r = 0.5745$ for fallow main with crop and $r = 0.5832$ for fallow main without crop. No correlations were observed between pH and any other enzyme.

Measurable differences between the main plots were observed in the abundance of the fungal ($P = 0.0126$) but not bacterial ($P = 0.5692$) populations when brassica and grass crops were omitted (Fig. 4). Fungi were more abundant in the wheat compared to fallow main plots. Treatment differences were highly significant for fungi in both main plots regardless of the inclusion of crops (P values <0.005). For the wheat main, fungi were greatest in the wood treatment although the difference was significant only for comparisons with check, manure, brassica residue, cotton, and wheat residue (Fig. 4). In the fallow main, the abundance of fungi in the grass crop was significantly greater than any other treatment. Fungi was significantly greater in sugar than the check regardless of whether crops were included. For bacteria, treatment effects were weakly significant in the fallow main ($P = 0.0549$) when crops included ($P = 0.0860$ when crops excluded) but not in the wheat main ($P = 0.4068$). Crop species had a significant effect on the fungal but not bacterial population density (Table 3). Fungi were greater under grass cropping than either wheat or brassica.

The bacterial abundance correlated to the fungi ($r = 0.5348$, $P < 0.0001$) across both mains and all treatments excluding crops. No significant correlation was observed between bacterial or fungal abundance to pH ($P = 0.5122$ and $P = 0.0949$, respectively). Fungal abundance was correlated to β-glucosaminidase activity in the fallow but not wheat main (Table 2). Bacteria showed a strong correlation to arylamidase activity in the fallow main regardless of whether crop was included. In the wheat main, bacteria were weakly and negatively correlated to acid phosphatase, α-galactosidase, and arylsulfatase; however, in the fallow main the correlations between bacterial abundance and acid phosphatase and α-galactosidase were positive and dependent on the inclusion of crops (Table 2).

Similar numbers of distinct phylotypes or T-RFs were detected in the wheat and fallow mains, which ranged from 170 to 198 for bacteria and 158–205 for fungi. Crop species had a significant effect on the fungal community structure as the R statistic in the crop comparison indicated a high degree of separation (Table 4). Also,

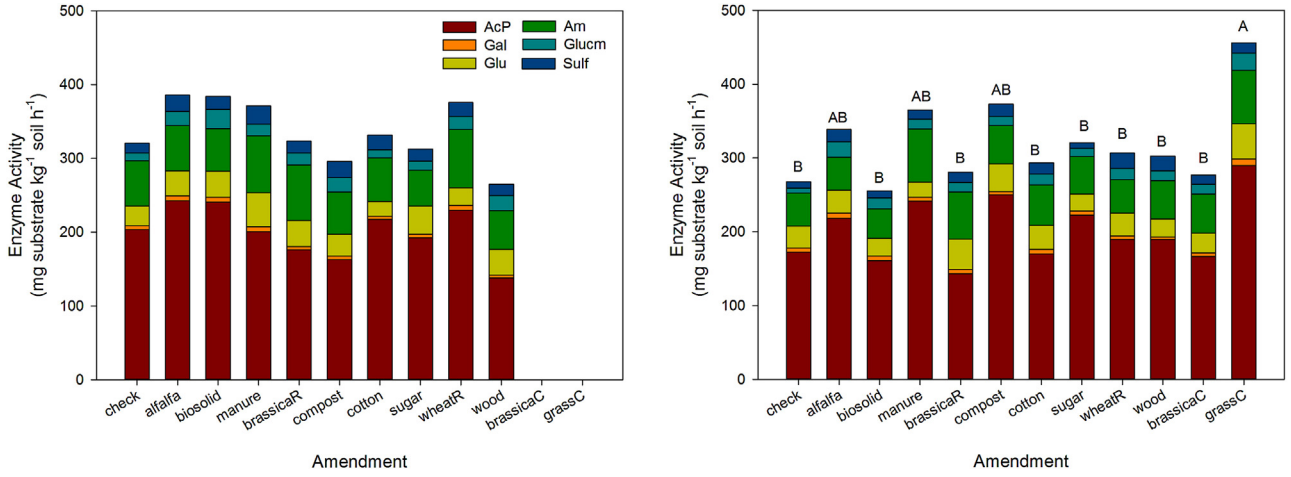

Fig. 3. Total soil enzyme activity of the different treatments in the wheat (left) or fallow (right) main plots. Shared letters above the bars indicate the sum of each enzyme activity is not significantly different at $P = 0.05$. The enzyme abbreviations in the legend are: AcP, acid phosphatase; Gal, α-galactosidase; Glu, β-glucosidase; Am, arylamidase; Glucm, β-glucosaminidase; and Sulf, arylsulfatase.

Table 2
Soil enzyme activity of plots amended with different carbon sources under either wheat or fallow. Activity is shown as the mean ± standard error (n = 4). Data followed by the same capital letter in each column are not significantly different at $P = 0.05$. Significance levels are shown for fallow plots when grass and brassica were included (+C) or excluded (−C). Activity is indicated as mg *p*-nitrophenol kg^{-1} soil h^{-1} for all enzymes except arylamidase which is mg β-naphthylamine kg^{-1} soil h^{-1}.

Enzyme	Acid phosphatase		Galactosidase		Glucosidase		Arylamidase		β-Glucosaminidase		Arylsulfatase	
Amendment[a]	Wheat	Fallow	Wheat	Fallow	Wheat	Fallow	Wheat	Fallow	Wheat	Fallow	Wheat	Fallow
check	204 ± 34	172 ± 26^{BC}	5 ± 0.4	6 ± 1^{AB}	27 ± 8	30 ± 9	61 ± 8^{ABC}	45 ± 2^{B}	10 ± 3	7 ± 1	13 ± 3	8.5 ± 2
alfalfa	242 ± 21	218 ± 3^{ABC}	7 ± 1	7 ± 1^{AB}	34 ± 6	31 ± 15	61 ± 4^{ABC}	45 ± 5^{B}	19 ± 4	21 ± 7	22 ± 4	17 ± 4
biosolid	241 ± 15	161 ± 29^{BC}	7 ± 1	6 ± 1^{AB}	35 ± 10	24 ± 3	58 ± 6^{ABC}	40 ± 7^{B}	26 ± 6	15 ± 4	18 ± 3	10 ± 3
manure	200 ± 22	242 ± 21^{ABC}	7 ± 2	5 ± 1^{AB}	46 ± 24	21 ± 3	78 ± 7^{AB}	72 ± 8^{A}	15 ± 8	13 ± 4	25 ± 6	12 ± 3
brassicaR	176 ± 26	144 ± 19^{C}	4 ± 0.3	6 ± 1^{AB}	35 ± 9	41 ± 13	76 ± 4^{AB}	64 ± 4^{AB}	16 ± 3	12 ± 4	16 ± 4	14 ± 4
compost	163 ± 26	250 ± 29^{AB}	5 ± 0.2	4 ± 1^{B}	30 ± 4	37 ± 14	57 ± 2^{ABC}	52 ± 5^{AB}	20 ± 5	12 ± 5	22 ± 5	16 ± 2
cotton	217 ± 36	170 ± 43^{BC}	4 ± 0.3	6 ± 1^{AB}	20 ± 3	32 ± 10	59 ± 10^{ABC}	55 ± 4^{AB}	11 ± 3	14 ± 2	20 ± 6	15 ± 3
sugar	192 ± 24	222 ± 36^{ABC}	5 ± 1	6 ± 1^{AB}	38 ± 8	23 ± 4	48 ± 4^{C}	51 ± 8^{AB}	13 ± 4	11 ± 5	16 ± 4	8 ± 3
wheatR	230 ± 40	190 ± 32^{ABC}	7 ± 2	5 ± 1^{B}	24 ± 6	31 ± 9	79 ± 9^{A}	45 ± 7^{B}	18 ± 2	15 ± 4	19 ± 4	21 ± 5
wood	138 ± 20	190 ± 35^{ABC}	4 ± 0.3	4 ± 0.3^{B}	35 ± 13	24 ± 4	52 ± 3^{BC}	52 ± 4^{AB}	21 ± 6	13 ± 1	15 ± 3	20 ± 3
brassicaC	NA	167 ± 20^{BC}	NA	5 ± 1^{AB}	NA	27 ± 7	NA	53 ± 10^{AB}	NA	13 ± 5	NA	13 ± 3
grassC	NA	290 ± 30^{A}	NA	9 ± 1^{A}	NA	48 ± 7	NA	73 ± 7^{A}	NA	23 ± 7	NA	14 ± 5
P (+C)		0.0003		0.0041		0.1615		0.0007		0.1464		0.1101
P (−C)[b]	0.0792	0.0229	0.1857	0.1396	0.7688	0.5675	0.0025	0.0007	0.1959	0.3821	0.2758	0.0665
P (Main)	0.7118		0.9876		0.4120		<0.0001		0.0514		0.0052	
Pearson correlations[c]												
Bacteria												
(+C)		0.31*		0.34*				0.59***				
(-C)	−0.36*		−0.34*					0.49**			−0.39*	
Fungi												
(+C)										0.41**		
(-C)										0.33*		

Significance is indicated at $P > 0.05$ (*), $P > 0.01$ (**), and $P > 0.001$(***) levels.
[a] Capital letters indicate plant residue (R) or living crop (C).
[b] *P* value for treatment significance of each main when brassicaC and grassC are included (+C) or excluded (−C) from the analysis. Letters are based on (+C) analysis. Main effects, shown as *P* (Main), excluded brassicaC and grassC from the analysis.
[c] Pearson correlations and significance.

inclusion of crop to the fallow main increased the amount of separation in the fungal populations. The fungal communities were more separable than the bacterial communities in all comparisons.

Ordination of the Bray–Curtis indices revealed that a few of the treatments had persisting effects on the community structure although some were dependent on the plot main (Fig. 5). Sugar had a pronounced effect on the fungal community structure under both wheat and fallow with significant treatment-based R statistics of >0.85, and in most cases $R = 1$ ($P < 0.05$). The wood treatment in the wheat main was barely separable from wood of the fallow main ($R = 0.38$) yet well-separated from all other treatments ($R > 0.91$, $P < 0.026$) (data not shown). The bacterial communities of the biosolid treatment in the wheat main and brassica crop of the fallow were outliers in the ordination plot but the R statistics were not significant from the main checks. Separation was observed for the biosolid plot and other treatments of the wheat main. The R statistic of the bacterial community structure of the biosolid treatment was significant yet barely separable ($R = 0.30$ to $R = 0.40$) from brassica residue, manure, cotton, wheat residue, and grass (data not shown).

4. Discussion

Soil amendments imparted persistent effects on soil microbiology, diversity, and/or abundance lasting seven years after the final treatment, but the effects were dependent on the amendment type and varied in the aspect of the community that was affected. The current study included several factors: continuous wheat or fallow main plots, 9 different amendments at the same carbon rate, and two alternative crops (grass and brassica). Apart from wood, sugar, manure and alternative crop treatments, the majority of plots were indistinguishable from the check on the basis of enzyme activity, microbial abundance and/or community structure.

Although the lack of short-term samples precludes our ability to rule out that the communities were resistant to the amendments, it seems more likely that the communities were indeed sensitive and resilient. Changes in the microbial abundance and activity in response to compost have be observed as quickly as four days after amendment in a microcosm experiment by Saison et al. (2006). In the study, the communities responded in a dose-dependent manner in which the changes induced by a low-level compost treatment (0.5% w/w, equivalent to a surface addition of

Table 3
Soil enzyme activity and microbial abundance under different crops. Values indicate the mean ± standard error ($n = 4$). Soil enzyme data is from Table 3 but includes statistical analyses specific to cropped plots. Data followed by the same capital letter in each column are not significantly different at $P = 0.05$. Activity is indicated as mg *p*-nitrophenol kg^{-1} soil h^{-1} for all enzymes except amidase which is mg β-naphthylamine kg^{-1} soil h^{-1}. Microbial abundances are indicated as 10^7 gene copies g^{-1} dry soil.

Crop	Acid phosphatase	Galactosidase	Glucosidase	Arylamidase	β-Glucosaminidase	Arylsulfatase	Bacterial abundance[b]	Fungal abundance[b]
wheatC[a]	204 ± 34^{AB}	5 ± 0.4^{B}	27 ± 8^{B}	61 ± 8	10 ± 3^{B}	13 ± 3	49 ± 12.5	11.9 ± 2.7^{B}
brassicaC	166 ± 20^{B}	5 ± 1^{B}	27 ± 7^{B}	53 ± 10	13 ± 5^{AB}	13 ± 3	54.9 ± 12.1	12.3 ± 2.8^{B}
grassC	290 ± 30^{A}	9 ± 1^{A}	48 ± 7^{A}	73 ± 7	23 ± 7^{A}	14 ± 5	94.8 ± 25.8	28.5 ± 4.7^{A}
P value	0.0344	0.0049	<0.0001	0.3278	0.0297	0.9369	0.0722	0.0042

[a] Wheat crop (wheatC) refers to the wheat main check plot and brassica crop (brassicaC) and grass crop (grassC) were in the fallow main.
[b] Bacterial and fungal abundances are based on quantification of the 16S rRNA gene and ITS region, respectively.

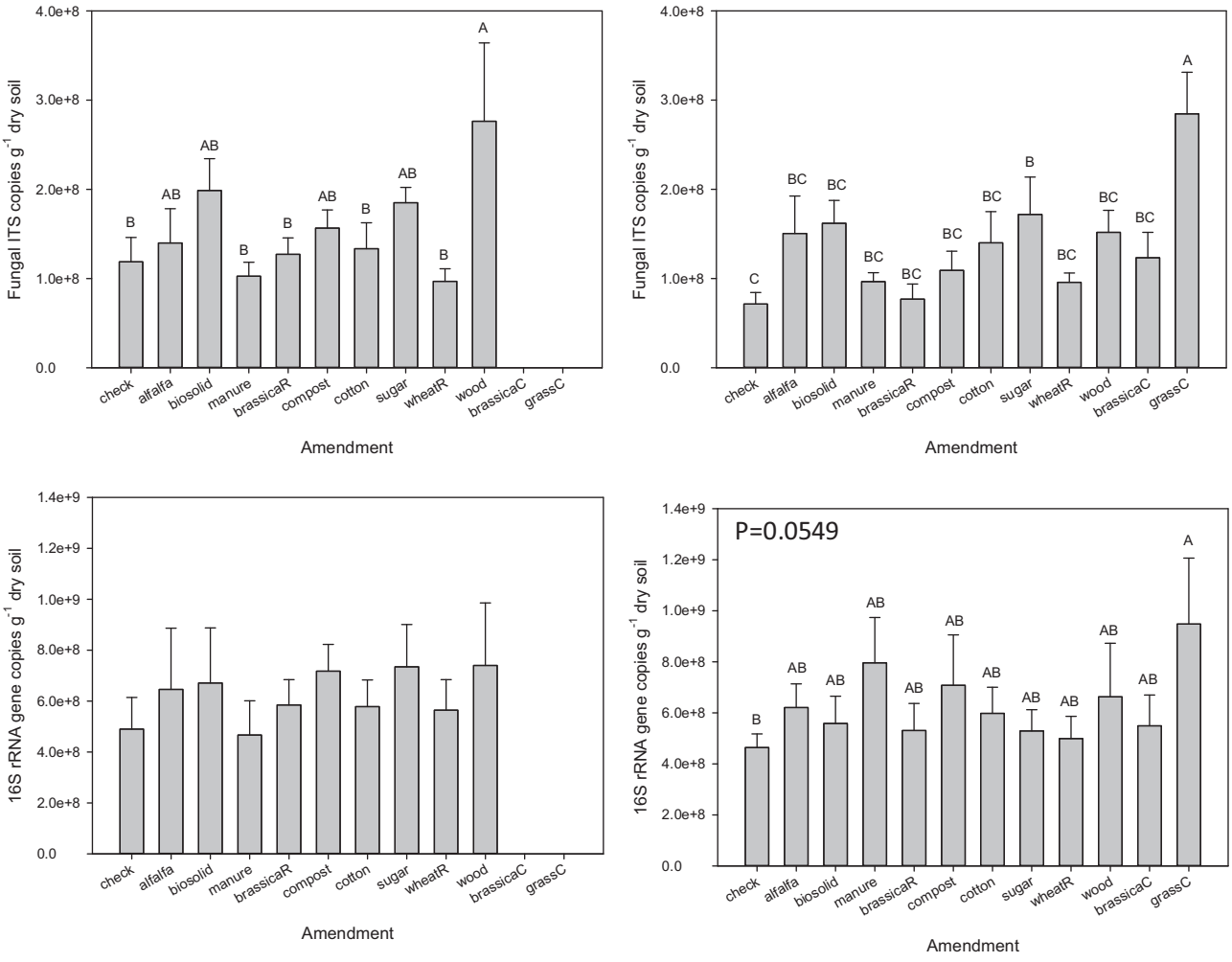

Fig. 4. Fungal (top) and bacterial (bottom) abundance per gram soil in the wheat (left) and fallow (right) mains with different amendments or crops. The same letters above the bars indicates values were not significantly different at $P < 0.05$ for fungi and $P < 0.1$ for bacteria.

approximately 62 g C m^{-2}) disappeared within the 6 month study, but the changes induced by high level compost addition (5% w/w, equivalent to a surface addition of approximately 620 g C m^{-2}) persisted the length of the study (Saison et al., 2006). The amount of compost used in the high and low amendments by Saison et al. (2006) brackets the rate of C (250 g m^{-2} yr^{-1}) in the amendments applied annually for five consecutive years of the current study, although the amount of C in the compost amendment varied as the rate was based on pre-compost C content. In consideration of the relatively short 6 month experiment length of Saison et al. (2006), it is probable that the communities in the current study changed in response to the amendments and, for the most part, recovered to a similar pre-amendment state.

Table 4
ANOSIM of Bray–Curtis diversity indices of bacterial and fungal communities (1 = distinct or separable).

Global R statistic[a]		
Comparison[b]	Fungi	Bacteria
Wheat + fallow	0.14**	0.04**
Wheat	0.50**	0.01
Fallow (−C)	0.40**	0.07
(+C)	0.51**	0.07*
Crop[c]	0.86**	0.02

[a] Significance values indicated as *$P > 0.05$, **$P > 0.01$.
[b] Comparisons for the fallow main with brassica and grass crop included (+C) and excluded (−C).
[c] Crop comparison included grass (fallow main), brascrop (fallow main), and wheat (check, wheat main).

Lasting effects on one or more aspects of the community structure, size or activity were observed for wood, sugar, manure amendments, and crops. A main plot effect, either continuous wheat or fallow, was apparent in the community function—treatment effects in wheat were observed only for arylamidase activity whereas effects under fallow were observed for acid phosphatase, α-galactosidase and arylamidase. Wood, applied at a total amount of 1247 g C m^{-2} over the amendment period, induced long-term changes in the fungal abundance and community structure in wheat but not fallow. Surface addition of a mixture of wood chips and sawdust at a similar amount of 950 g C m^{-2} over 2 years to ex-arable land increased the fungal abundance and fungal/bacterial ratio indicating that wood was an available C source for fungi (Eschen et al., 2007). Stimulation of the fungal population by wood is unsurprising as wood is comprised of 18-30% dry weight of lignin in which decomposition is primarily attributed to fungi (2003). The response of fungi to wood in the wheat but not fallow main is likely not a factor of the crop biomass since addition of wheat residue in the fallow did not affect the fungal abundance or community composition. Rather, the response of fungi may be related to nutrient availability through root deposits or application of nitrogen and sulfur fertilizer during seeding of wheat. Disagreement exists on the effect of N fertilization on decomposition rates. A meta-analysis of studies

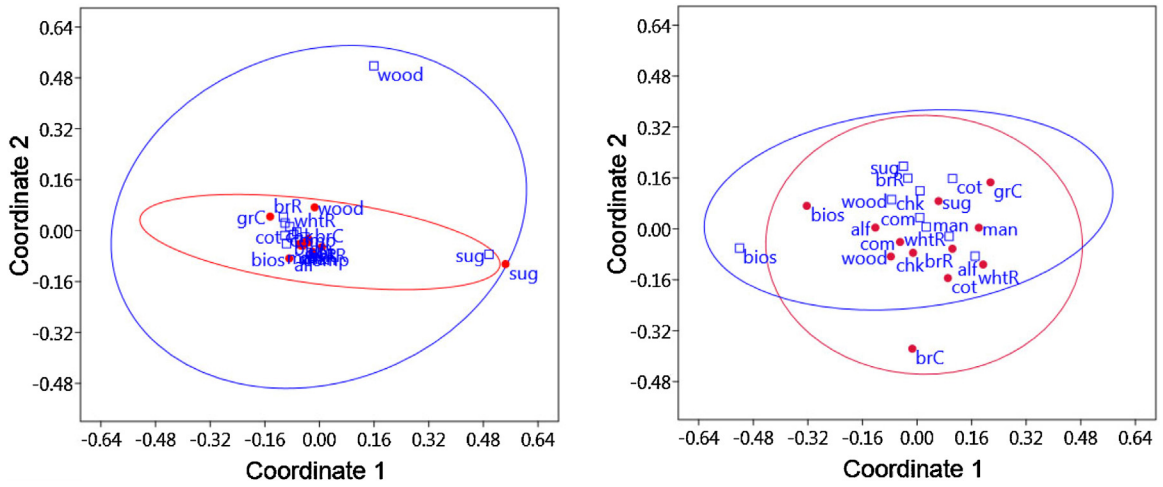

Fig. 5. Non-metric multi-dimensional scaling (NMDS) of the Bray–Curtis diversity indices of fungal (left) and bacterial (right) T-RFLP profiles in wheat and fallow main plots. Open blue squares are communities from the wheat main and red filled circles are communities from the fallow main. The ellipses indicate the 95% concentration region. Abbreviations are: chk, check; alf, alfalfa; bios, biosolid; man, manure; brR, brassica residue; com, compost; cot, cotton; sug, sugar; whtR, wheat residue; wood, wood; brC, brassica crop; and grC, grass crop.

indicated that N fertilization inhibited decomposition of high-lignin litters (Knorr et al., 2005); however, decomposition rates of wood by specific fungal groups were increased by addition of N at low levels (Allison et al., 2009).

Sugar in the form of sucrose imparted a large, seven-year effect on the fungal community size and structure. Sucrose decomposes rapidly in the soil (Engelking et al., 2007a) and has been used in several studies as an easily available C substrate (Engelking et al., 2007a; Eschen et al., 2007) or plant exudate analog (Badri and Vivanco, 2009; Grayston et al., 1998; Griffiths et al., 1998). Variable responses of microbial populations to sucrose amendment have been reported. In a greenhouse incubation study, the fungal and bacterial abundance did not differ between soil amended three times a week for four weeks compared to a no-amendment control (Grayston et al., 1998). In contrast, sucrose and inorganic N amendment to soil increased the bacterial and fungal biomass but resulted in a lower fungal C/bacterial C ratio after 33 days (Engelking et al., 2007b). When soil was heated to remove organic matter prior to amendment with sucrose, inorganic N and microbial inoculum, the microbial community shifted toward fungi as demonstrated by a linear increase in the fungal C/bacterial C ratio from 5 to 67 days incubation (Engelking et al., 2008). The response of fungi to sucrose amendment also appears to depend on dose, as increasing concentrations of readily assimilable substrates (sugars, carboxylic and amino acids) resulted in increasing effects on the fungal/bacterial ratio and degree of microbial community divergence (Griffiths et al., 1998).

Of the high N amendments only manure enhanced arylamidase activity, an enzyme involved in the mineralization of N from organic matter in soil (Acosta-Martínez and Tabatabai, 2000a). Arylamidase is sensitive to soil pH with greater activity at near-neutral to slightly basic pH (Acosta-Martínez and Tabatabai, 2000a; Acosta-Martínez and Tabatabai, 2000b). Arylamidase activity was strongly correlated to soil pH which is in agreement with results from a limed agricultural study (Acosta-Martínez and Tabatabai, 2000b). The increased activity in the manure amendment is likely a factor of soil pH as both manure and brassica residue amendments had the greatest soil pH values and generally the greatest arylamidase activities. Arylamidase activity was also strongly correlated to the bacterial abundance, which was slightly elevated in the manure plots of the fallow main. The correlation of arylamidase and bacterial abundance, which has also been demonstrated on spruce litter (Müller et al., 2004), suggests that bacteria are major contributors to the measured activity. Romaní et al. (2006) revealed that the biomass specific leucine-aminopeptidase activity (activity per amount microbial C) was greater in bacteria than fungi when inoculated separately on *Phragmites* leaves. Also, arylamidase activity was more widespread among the bacteria isolated from the phyllosphere of spruce in contrast to fungi. Eighty percent of the bacteria tested positive compared to only one-third of the fungi (Müller et al., 2004).

Both biosolid and manure had little or no detectable effect on the size and structure of the microbial communities although other reports indicate that the microbial communities often respond to the amendments in a dose-dependent manner (Leita et al., 1999; Peacock et al., 2001; Saison et al., 2006; Speir et al., 2004; Sullivan et al., 2006). Increased basal respiration and microbial biomass C have been measured in response to composted biosolid amendment two years after the final treatment (Speir et al., 2004) when applied at a higher but not lower rate of yearly application. Additionally, the microbial biomass in soil amended with municipal refuse compost increased with increasing rates of application applied for 12 years (Leita et al., 1999). The amendment rate of the current study (0.25 kg C m^{-2} yr^{-1} or 1.25 kg m^{-2} C total application) is far less than the amounts employed by the above studies with ranges of approximately 1.23–6.86 kg C m^{-2} yr^{-1} (total application of 4.94–27.44 kg C m^{-2} over 4 years) (Speir et al., 2004) and 0.68–2.04 kg C m^{-2} yr^{-1} (total of 8.16–24.5 kg m^{-2} over 12 years) (Leita et al., 1999). The lack of distinguishability of the biosolid and manure amendments from the no treatment control may be due to either the relatively low amendment dosage compared to other studies or the long sampling period which may have missed early effects. While not a part of this study, it would be interesting to determine how much the application rate affects the resiliency of the communities in long term studies.

Although biosolid did not have a strong influence on the soil biology, it did impart a significant effect on the chemical properties of the soil. Biosolids acidified the soil in fallow but not under wheat cropping. Several studies have shown that pH can have a large impact on the bacterial community composition (Rousk et al., 2010; and therein), but the effects on the fungal community may be modest (Rousk et al., 2010). Although the pH was significantly lower in the biosolid treatment of the fallow main, the bacterial composition of the wheat main was more separable suggesting that factors other than pH were influential to the bacterial communities.

The most notable factor in the biosolid amendment was the soil NO_3-N content. Higher concentrations of oxidized N (nitrate and nitrite) in biosolid-amended rather than synthetic fertilizer-treated soils has been previously reported and attributed to increased rates of nitrification and abundance of ammonia oxidizers (Kelly et al., 2011). In fact, the effects of biosolid on the microbial communities could be detected six years after a single amendment event (Barbarick et al., 2004). The metabolically active biomass (as indicated by substrate-induce respiration), CO_2-respiration, and N mineralization were greater in incubations of grassland and shrubland soils that had received biosolid six years prior compared to soils that received none. The greater rates of N mineralization were attributed to labile N still available in the soil (Barbarick et al., 2004).

The sum community activity demonstrated that crop had a significant and persisting effect on nutrient cycling capacity, in agreement with other studies (Acosta-Martínez et al., 2007; Kramer et al., 2013). Comparison of different crops, cropping intensities, grass and native pasture revealed that microbial biomass and enzyme activities increased under greater cropping intensities with the greatest activities in grass and pasture (Acosta-Martínez et al., 2007). The enhanced microbial communities in pasture and grass compared to the agricultural soils were attributed to differences in ground cover, root exudates and biomass, and tillage (Acosta-Martínez et al., 2007; Acosta-Martinez et al., 2004). Similarly, compared to the other treatments grass enhanced the microbial abundance and enzyme activity except for sulfatase, although not all measurements were significant. Sulfatase activity was not responsive to the amendment treatments in this study but activity was greater in the wheat compared to the fallow main. No crop effect was observed for sulfatase activity; however, a plant species effect has been noted with greater rhizosphere activities of grass (*Lolium perenne*) > wheat > *B. napus* (Knauff et al., 2003). Grass also enhanced the fungal abundance compared to wheat or brassica crop. This is in contrast to a report in which fungi and bacteria were significantly greater in the rhizosphere of wheat than ryegrass (Grayston et al., 1998). Although rhizosphere communities can be highly specific to plant species, plant effects in bulk soil, as analyzed in this study, may be less apparent (Kowalchuk et al., 2002).

In summary, detectable differences in soil enzyme activity, fungal community structure and abundance were observable seven years post-amendment and post-cropping treatment, with the greatest differences observable in the wood and sugar amendments and grass cropping. The bacterial communities were not distinguishable between the check and the treatments, indicating a higher level of resiliency or less sensitivity to C inputs for bacteria compared to fungal communities. Crops and C amendments affected different components of the microbial communities: crop had a greater effect than amendment on community function whereas amendment had a greater influence on fungal diversity and both crop and amendment were influential in fungal population size. The conservative statistical approach for mean separation discerned only the strongest or most probable differences in the community aspects. Although we are unable to demonstrate exactly how much the communities diverged from the check in response to the different amendments, it is clear that the amendment composition influenced the longevity of measurable and discernable changes in the fungal and bacterial communities. Seven years of uniform fallow and wheat cropping did not obliterate the influence of treatments as simple as sugar, or differences between perennial grass roots and wheat roots. The most durable soil differences were related to fungal abundance, fungal structure, and enzyme activity.

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