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SOIL MICROBIOLOGY

Biosolids and Tillage Practices Influence Soil Bacterial Communities in Dryland Wheat

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Abstract

Class B biosolids are used in dryland wheat (*Triticum aestivum* L.) production in eastern Washington as a source of nutrients and to increase soil organic matter, but little is known about their effects on bacterial communities and potential for harboring human pathogens. Moreover, conservation tillage is promoted to reduce erosion and soil degradation. We explored the impacts of biosolids or synthetic fertilizer in combination with traditional (conventional) or conservation tillage on soil bacterial communities. Bacterial communities were characterized from fresh biosolids, biosolid aggregates embedded in soil, and soil after a second application of biosolids using high-throughput amplicon sequencing. Biosolid application significantly affected bacterial communities, even 4 years after their application. Bacteria in the families Clostridiaceae, Norcardiaceae, Anaerolinaceae, Dietziaceae, and Planococcaceae were more abundant in fresh biosolids, biosolid aggregates, and soils treated with biosolids than in synthetically fertilized soils. Taxa identified as *Turcibacter*, *Dietzia*, Clostridiaceae, and Anaerolineaceae were highly abundant in biosolid aggregates in the soil and likely originated from the biosolids. In contrast, Oxalobacteriaceae, Streptomyceteaceae, *Janthinobacterium*, *Pseudomonas*, *Kribbella*, and *Bacillus* were rare in the fresh biosolids, but relatively abundant in biosolid aggregates in the soil, and probably originated from the soil to colonize the substrate. However, tillage had relatively minor effects on bacterial communities, with only a small number of taxa differing in relative abundance between traditional and conventional tillage. Although biosolid-associated bacteria persisted in soil, potentially pathogenic taxa were extremely rare and no toxin genes for key groups (*Salmonella*, *Clostridium*) were detectable, suggesting that although fecal contamination was apparent via indicator taxa, pathogen populations had declined to low levels. Thus, biosolid amendments had profound effects on soil bacterial communities both by introducing gut- or digester-derived bacteria and by enriching potentially beneficial indigenous soil populations.

Keywords Biosolids · Soil · Microbiome · Pathogens · Tillage · Wheat · Dryland cropping systems

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Introduction

Soil loss due to wind erosion is a major problem worldwide. In the USA, billions of tons of soil are eroded by wind each year, with an estimated cost in the billions of dollars [1–4]. Tillage practices can be a major contributor to soil loss, especially in dryland cropping systems in the US Pacific Northwest (PNW) [5]. In this region, soils are composed mostly of silt and fine sand, have low organic matter content (< 1%), and are poorly aggregated. Further, high winds make soils in the low-precipitation zone (< 350 mm annual precipitation) highly susceptible to wind erosion and the release of particulates < 10 μm diameter (PM10) that adversely impact air quality [6]. Finally, due to the low precipitation, crop rotation is typically limited to winter wheat-summer fallow, where adequate soil moisture is available for only one crop every other year [7]. To combat soil loss due to

wind erosion and conserve soil moisture, regional growers are exploring numerous approaches to enhance the resilience of cropping systems, yet there is little information on how organic amendments impact soil bacterial communities [8, 9].

Tillage has been a crucial practice in agriculture for thousands of years to control weeds and prepare a seed bed [10]. However, soil disturbance during tillage is also a major contributor to soil loss [2, 10] because tillage breaks up soil aggregates, buries residue, and accelerates microbial activity and decomposition of crop residue [11]. Moreover, tillage can substantially modify the composition and diversity of soil microbial communities [12, 13]. In the dryland region of the PNW, tillage is typically conducted during the spring of the fallow year, then rod-weeded in late spring and summer to control weeds. This strategy creates a loose, dry surface soil mulch that breaks the capillary pores in the surface soil, thermally insulates the soil, and restricts evaporation of soil moisture during the hot, dry summer [14]. Although most growers still use tillage-based summer fallow, there is increasing interest in no-till summer fallow that conserves soil [9].

To circumvent the tradeoff between soil loss due to tillage and the conservation of moisture via disruption of soil capillaries, alternative methods to traditional disking are being promoted as best-management practices for summer fallow. Specifically, the use of an undercutter implement is supported by the UDSA Natural Resources Conservation Service via cost-sharing arrangements for growers purchasing undercutters [15]. The undercutter lifts without inverting the soil using wide V-shaped blades. This implement severs capillary continuity and preserves soil moisture and retains crop residue on the soil surface. Thus, it is likely that the ongoing transition from using the traditional tandem disk to the conservation undercutter implement will significantly affect soil microbial communities.

In addition to adapting conservation tillage practices to promote the sustainability of erosion-prone dryland cropping systems, the use of organic amendments is a means to stabilize soils, promote soil health, and reduce wind erosion [16]. In particular, use of processed biosolids as organic nutrients on agricultural fields allows for recycling of treated human waste while at the same time improving soil quality and plant productivity [17]. The biosolids are processed by anaerobically digesting the solid fraction of wastewater which stabilizes organic matter and reduces pathogen concentrations. The process generates a concentrated "biosolid" material that can be applied to fields [17]. Notably, biosolids are rich in organic C, N, and P, as well as many micronutrients [17, 18] and, in a 2-year winter wheat-summer fallow rotation, can serve as an adequate fertilizer replacement for two crops (i.e., one biosolid application every 4 years) [19]. Additionally, biosolid applications can increase soil organic matter, as well as aggregate size and stability [20, 21], thus potentially reducing soil erosion [22]. However, although digested, screened for high pathogen loads, and subject to field re-entry restrictions [23], most field-applied class B biosolids are not sterile and contain sewage-derived pathogens [24], including enteric bacteria such as *Clostridium* spp., *Escherichia coli*, and *Salmonella* [25]. Moreover, biosolids may also contain concentrations of heavy metals or pharmaceuticals [26]. As such, there are persistent concerns that field application of biosolids may modify soil communities in ways harmful to human health [25].

The impacts of biosolid amendments versus synthetic fertilizers on soil communities, including pathogen populations, may be highly dependent on tillage practices. For example, bacteria that are well-adapted to soil environments are expected to rapidly out-compete enteric bacteria introduced with biosolids and limit pathogen regrowth [27]. In other cases, if low populations of pathogens survive in soil, they may propagate if temperature and moisture conditions are conducive to rapid growth and populations are not suppressed by native microbes [28, 29]. As such, traditional heavy tillage, which break ups and buries organic material thereby exposing it to colonization of competitive soil microbial populations, may be needed to limit potential negative effects of biosolid applications. Moreover, burying field-applied biosolids with traditional tillage versus the low-disruption undercutter, may select for distinct soil bacterial populations. However, there is a paucity of information concerning tillage practices and biosolid amendments jointly influencing soil bacterial populations, including the prevalence of pathogenic taxa. High-throughput DNA sequencing (HTS) is a powerful tool for characterizing microbial communities in great detail [30] and may be especially useful for detecting a diverse suite of low-abundance populations of potential pathogens that are difficult to culture or viable-but-non-culturable (VBNC). As such, HTS may be useful for risk assessment or source tracking of field-applied biosolids [31].

In this work, we investigate the impacts of traditional (disk) tillage and conservation (undercutter) tillage on soil bacterial communities in a PNW dryland winter wheat-summer fallow cropping system managed with synthetic fertilizer or biosolids. Our objectives were to (1) determine the impacts of tillage and fertilizer treatments on soil bacterial community composition and diversity, (2) identify bacterial taxa introduced to soils with biosolids and their contribution to soil communities, (3) characterize bacterial consortia that colonize biosolid aggregates and compete with introduced populations, and (4) assess the potential for biosolids to introduce human pathogens to agricultural soils.

Methods

Field Experimental Design

Field plots were sampled at the Washington State University Dryland Research Station near Lind, Washington, USA (47°

00′ N, 118° 34′ W) in 2015 and 2016 as described in Schlatter et al. [8]. Briefly, this field site is in the low-precipitation zone (242 mm mean annual precipitation) and the crop rotation practiced by growers throughout this region is almost exclusively winter wheat-summer fallow. Soil is a Shano silt loam (coarse-silty, mixed, superactive, mesic, Xeric Haplocambids). Experimental treatments were established in a split-block design [32] with tillage (traditional vs. conservation) as the main-plot factor and nutrient sources (synthetic fertilizer vs. biosolids) as the subplot factor (Supplemental Fig. 1). Main and subplots were 76 × 8 m and 38 × 8 m, respectively. Each treatment combination was replicated four times. Two sets of plots were established so that both the wheat and the fallow portions of the rotation were present every year. Glyphosate [N-(phosphonomethyl) glycine] was applied in mid-March of the fallow year at a rate of 0.43 kg ae/ha to control weeds. Biosolid material (class B) was obtained from the King County Wastewater Treatment Division, Seattle, WA, and was applied with a manure spreader on 4 May 2015 (westside plots) and on 19 April 2016 (eastside plots) at a rate of 6508 kg/ha (dry weight) to meet the nutrient requirements of two wheat crops. Importantly, each plot had a history of biosolid use; the westside plots first received biosolids in 2011 and the eastside plots first received biosolid applications in 2012. Thus, biosolids were applied once every 4 years, whereas synthetic fertilizer (applied at a rate of 56 kg N plus 11 kg S/ha) was applied every 2 years. Traditional tillage involved the use of a tandem disk implement and conservation tillage the use of an undercutter implement. Tillage was conducted immediately after biosolid application in both 2015 and 2016. Liquid synthetic fertilizer was applied to the soil as aqua NH_3-N plus thiosol S in both the disk and undercutter treatments. The fertilizer was injected into the soil at a depth of 13 cm during primary spring tillage with the undercutter implement. In the disk treatment, fertilizer was applied to the soil surface then immediately incorporated into the soil (with the disk) to a depth of 13 cm. Plots were subsequently rod-weeded at a depth of 10 cm in 15 June in 2015 and in 3 June and 10 July in 2016. All field operations are described in Table 1.

Table 1 Field operations performed in the biosolids experiments

Year	Date	Field operations
Trial 1		
2011	18 April	Biosolids applied
2011	31 Aug	Winter wheat planted
2012	21 July	Winter wheat harvested
2013	3 Sept	Winter wheat planted
2014	22 July	Winter wheat harvested
2015	4 May	Apply biosolid/fertilizer/tillage treatments
	15 June	Rodweed
	18 June	Soil samples collected, air dried
	1 Oct	Biosolid aggregates removed from soil, DNA extracted from aggregates and soil
Trial 2		
2012	1 May	Biosolids applied
2012	5 Sept	Winter wheat planted
2013	24 July	Winter wheat harvested
2014	29 Aug	Winter wheat planted
2015	20 July	Winter wheat harvested
2016	13 April	Samples taken from biosolids pile
	19 April	Apply biosolid/fertilizer/tillage treatments
	3 June	Rodweed
	6 June	Soil samples collected and frozen
	1 Aug	Biosolid aggregates removed from soil, DNA extracted from aggregates and soil

Soil Sampling

The soil sampling information for the two sets of plots is presented in Table 1. In 2015, soil samples were collected to a depth of 3 cm on 18 June, 45 days after fertilizer treatments were applied. Soils were air dried and stored at room temperature (25 °C) for 6 months prior to DNA extraction. In 2016, samples were collected from the biosolid stockpile (biosolids were placed in a stockpile adjacent to field plots upon delivery to the Lind Station) and soil samples were collected from all plots in the same manner as 2015 prior to imposing treatments (13 April) and in 6 June 3 days after the first rod-weeding (48 days after applying biosolids) in 2016. Samples collected in 2016 were transported in a cooler to the laboratory and stored at − 20 °C until DNA extraction. Soil samples were collected from the plots using a flat-blade shovel while biosolid aggregates collected from plots were extracted from soil with a sterile tweezers, shaken free of loose soil, and treated as a distinct sample type. DNA was extracted from soil samples (~ 0.25 g) and biosolid aggregates (~ 0.1 g) using the Qiagen/MoBio Powersoil DNA extraction kit according to the manufacturers' instructions. Soil chemistry was evaluated for samples collected in June of both years for both nutrient and metal analysis as described in [33]. Briefly, concentrations of heavy metals (As, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Se, V, and Zn), macronutrients (C, N, P, K, Ca, Mg, and S), and micronutrients (Co, Fe, Mn, and V) were determined at the University of Idaho Analytical Sciences Laboratory. Moreover, the pH of biosolids was determined by the provider (King County, WA) in 2015 (pH = 8.80) and 2016 (pH = 8.99) and in soils under each treatment (pH for soils under Traditional-Synthetic = 5.83 and 5.70 in 2015 and 2016, respectively; pH for soils under Traditional-Biosolid = 5.94 and 5.72 in 2015 and 2016, respectively; pH for soils under Conservation-Synthetic = 5.80 and 5.65 for 2015 and 2016, respectively; and pH for soils under Conservation-Biosolid = 5.81 and 5.62 for 2015 and 2016, respectively. In total, we obtained $n = 16$ soil samples in 2015 (2 tillage treatments × 2 fertilizer amendments

× 4 replicates), $n = 32$ soil samples in 2016 (2 times [pre- and post-treatment] × 2 tillage treatments × 2 fertilizer amendments × 4 replicates), and $n = 20$ biosolid samples ($n = 4$ fresh biosolid in 2016, $n = 2$ tillage treatments × 2 years × 4 replicates field-collected biosolid aggregates).

Illumina Sequencing and Sequence Processing

Soil DNA was checked for quality on a nanodrop spectrophotometer and submitted to the University of Minnesota Genomics Center for amplification of the V1–V3 region of the bacterial 16S rRNA gene on the Illumina MiSeq platform (Version 3 chemistry, 2 × 300 paired-end reads) according to established protocols [34]. Briefly, an initial round of PCR using the gene-specific primers 27F (5′-AGAG TTTGATCMTGGCTCAG-3′) [34] and 534R (5′-ATTA CCGCGGCTGCTGG-3′) [34] with flow cell adapters was conducted with an initial denaturation of 95 °C for 5 min, 25 cycles of 98 °C for 20s, 55 °C for 15 s, and 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. After the first round of PCR, templates were diluted 1:100 and 5 μL of template used in a second indexing PCR to add barcodes and flowcell adapters, which included an initial denaturation at 95 °C for 5 min, 10 cycles of 98 °C for 20s, 55 °C for 15 s, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. KAPA HiFi DNA polymerase was used for each PCR (KAPA Biosystems, Wilmington, MA). Amplicons were pooled, size selected, denatured with NaOH, diluted to 8 pM in Illumina HT1 buffer, spiked with 20% PhiX, and heat denatured at 96 °C for 2 min prior to loading on a MiSeq machine. After sequencing, forward and reverse Illumina reads were paired using PEAR (v0.9.6) [35]. Barcodes and primer sequences were removed with cutadapt (v1.91) [36], and sequences with ambiguous bases or shorter than 350 bp were removed. Processed sequences were clustered following the UPARSE pipeline [37] using VSEARCH [38] for all steps with the exception of OTU clustering which used UPARSE (v8.1) [37]. Briefly, reads were quality filtered using a maximum expected error rate of 1, dereplicated, and singletons removed prior to OTU clustering at 97% similarity threshold using the cluster_otus command. Processed reads were then mapped to OTU clusters to generate an OTU abundance table. Taxonomy was assigned to OTU representative sequences (centroids) with the RDP Naïve Baysian Classifier [39] using the greengenes 13_8 reference database and an 80% confidence threshold. The OTUs were filtered to remove any OTUs that could not be classified to the bacterial Kingdom or those classified as mitochondria or chloroplasts using QIIME scripts (v1.9.1) [40]. The OTUs with a total sequence count of < 5 were removed to reduce poor quality OTUs and the OTU table was rarefied to 16,581 sequences per sample prior to analysis. Unrarefied OTU tables were retained for differential abundance analysis with DESeq2 (v1.12.4) [41]. One biosolid aggregate sample (conservation tillage in 2016) was discarded due to exceptionally low sequencing depth.

Salmonella and *Clostridium perfringens* Screening (PCR)

For the detection of *Clostridium perfringens*, PCR amplification of the *C. perfringens* alpha-toxin gene (*cpa* gene) was used as described previously [42]. *C. perfringens* produces as many as 17 exotoxins, four of which are considered the major toxins (alpha, beta, epsilon, and iota). Based on the presence of genes encoding these four toxins, *C. perfringens* is classified to five toxigenic types from A to E [43]. Because the *cpa* gene is conserved in all known types of *C. perfringens*, we selected *cpa* gene as target for detection of *C. perfringens* from all samples tested in this study. For amplification of *cpa* gene from *C. perfringens*, each PCR mixture consisted of 12.5 μL of DreamTaq PCR master mix (Thermo Fisher Scientific, CA, USA), 0.25 μM cpa1F primer (5′-GCTAATGTTACTGCCGTTGA-3′), 0.25 μM cpa1R primer (5′-CCTCTGATACATCGTGTAAA-3′) [42], 1 μL (~ 14 ng) of DNA and PCR-grade water to reach the final volume 25 μL. The amplification cycles consisted of 94 °C for 2 min, followed by 30 cycles of 1 min at 94 °C, 1 min at 53 °C, 1 min at 72 °C, and a final for 7 min at 72 °C. For the detection of *Salmonella*, PCR amplification of *invA* gene was used because this gene is well-conserved across *Salmonella* species [44]. For amplification of *invA* genes of *Salmonella*, each PCR mixture consisted of 12.5 μL of DreamTaq PCR master mix (Thermo Fisher Scientific, CA, USA), 0.04 μM *invA*_F primer (5′-GTGTCCTTTGGTATTAATCC-3′), 0.04 μM *invA*_R (5′-GTCTGAGCACTTCTTTAAG-3′) primer, 2 μL (~ 28 ng) of DNA, and PCR-grade water to reach the final volume 25 μL. The amplification cycles consisted of 95 °C for 5 min, followed by 39 cycles of 20 s at 95 °C, 20 s at 54 °C, 20 s at 72 °C, and a final for 5 min at 72 °C. PCR product size for *Salmonella* was 250 bp and for *Clostridium perfringens* was 324 bp. The detection limits of *C. perfringens* (*cpa* gene) and *Salmonella* (*invA*) PCRs were 330 bacterial cells and 1 bacterial cells, respectively. Positive controls (*Salmonella* and *C. perfringens* DNA) and negative control (water) were included during each PCR run. To rule out potential PCR inhibitors in gDNA samples obtained from soil samples, *C. perfringens* and *Salmonella* gDNA were spiked at a minimum detection limit in randomly selected soil samples. PCR amplicons were run into 1.5% agarose gel (Thermo Fisher Scientific, NJ, USA) along with 1 kb plus DNA ladder (Thermo Fisher Scientific, CA, USA).

Data Analysis

Variation in bacterial community structure was visualized using non-metric multidimensional scaling of Bray-Curtis

distances using the "metaMDS" function of the vegan package in R [45]. PERMANOVA was performed on Bray-Curtis distances with the "adonis" function to determine the significance of tillage and fertilizer treatments in each year and sampling date in 2016. Further, significant ($p < 0.05$) vectors of soil edaphic variables (June sampling) were fitted to the NMDS ordination using the "envfit" function, with 1000 permutations to determine significance. Considering only the June sampling time (post-treatment), Kruskal-Wallis tests were used to test for differences among abundant (> 1%) phyla between fertilizer and tillage treatments (synthetic fertilizer vs. biosolids, traditional vs. conservation tillage) followed by a Benjamini-Hochberg (BH) adjustment of p values to correct for false discovery. Further, significant effects of fertilizer, tillage, and year were evaluated on $\log_{10}(1 + x)$-transformed counts of abundant bacterial families using analysis of variance (ANOVA) with BH-adjustment of p values for false discovery. DESeq2 [41] was used to identify individual OTUs that differed significantly between fertilizer treatments within each tillage treatment and between tillage treatments with biosolids or fertilizer using the model: ~ year + tillage × fertilizer after filtering OTUs with a normalized count < 5 and found in fewer than three samples. The OTUs with a FDR-adjusted p value < 0.001, a base mean > 20, and a log2-fold difference > 2 were considered to be differentially abundant. Bacterial richness, Shannon diversity (H'), and Simpson's diversity ($1/D$) were calculated for each sample using vegan and differences in richness and diversity between treatments within each year were evaluated with ANOVA. Spearman correlations between soil edaphic characteristics and the relative abundances of abundant bacterial families (> 10% of sequences) were evaluated among June samples from both years. Differences in bacterial communities with a history of different fertilizer use (biosolid versus synthetic) were evaluated prior to a second round of treatments (April) using DESeq2 as described above. Further, shifts in communities under the fertilizer treatments from April (pre-treatment) to June (post-treatment) were also evaluated using DESeq2 with the model: ~ tillage + month × fertilizer. Finally, the bacterial communities of fresh- and field-collected biosolid aggregates were explored using NMDS and heatmaps of abundant OTUs found in biosolid aggregates.

Results

Bacterial 16S rRNA Gene Sequencing

In total, we obtained 2,277,482 sequences among 6957 OTUs from 48 soil samples (average of 47,447 ± 13,589 sequences per sample) and 879,059 sequences among 3358 OTUs from 19 biosolid aggregates (fresh and field-collected, average of 46,266 ± 12,299 sequences per sample) prior to rarefaction. After rarefaction, soil communities were dominated by Actinobacteria (40.4%), Proteobacteria (23.2%), Bacteroidetes (7.7%), Chloroflexi (6.7%), and Gemmatimonadetes (6.2%) (Fig. 1). Field-collected biosolid aggregates were dominated by Firmicutes (31.2%), Actinobacteria (26.4%), Proteobacteria (22.3%), Chloroflexi (9.2%), and Bacteroidetes (4.7%). In contrast, communities from fresh biosolid aggregates were composed of Bacteroidetes (22.2%), Firmicutes (18.6%), Proteobacteria (17.7%), and Chloroflexi (16.6%).

Impacts of Fertilizer and Tillage Treatments on Soil Bacteria

There was a significant effect of biosolids versus synthetic fertilizers on bacterial communities in 2016, but not among samples taken in 2015 (Fig. 2, Table 2), perhaps due to different sample storage conditions [46]. Contrasting communities from soils with synthetic fertilizer or biosolids, those with synthetic fertilizer had significantly greater abundances of Armatimonadetes (FDR $p = 0.009$), Chloroflexi (FDR $p = 0.025$), Verrucomicrobia (FDR $p = 0.01$), and those amended with biosolids had greater relative abundances of Firmicutes (FDR $p < 0.001$) and Proteobacteria (FDR $p = 0.004$). At the family level, numerous bacterial families differed in relative abundance between biosolid- and synthetically fertilized soils (Fig. 3). Notably, whereas some of the families enriched with biosolid amendments represent common soil- or plant-associated taxa (e.g., Streptomycetaceae, Oxalobacteraceae), others belonged to groups rarely found or in low abundances in soil environments (e.g., Dietziaceae, Aeroliniaceae, Turicibacteraceae, Planococcaceae) and are likely introduced with biosolids.

There were many specific OTUs that were significantly enriched in biosolid-amended versus synthetically fertilized soils under both traditional and conservation tillage, primarily belonging to the phyla Actinobacteria, Proteobacteria, and Firmicutes (Fig. 4). Some OTUs of note enriched under both tillage treatments included those related to *Turicibacter* (OTU38), SMB53 (family Clostridiaceae; OTU18), *Dietzia* (OTU67), and genus 02d06 (family Clostridiaceae; OTU107), which are likely gut derived. Other taxa enriched in biosolid-amended soil included taxa that are likely indigenous to the soil community, such as *Janthinobacterium* (OTU24), Streptomyces (OTUs 5, 23, and 3599), Oxalobacteraceae (OTUs 129 and 236), and *Rhodococcus* (OTU87). Only a few, low-abundance, OTUs (*Mycobacterium* OTU6077, Ellin5301 OTU174, and Cystobacteraceae OTU830) were less abundant in biosolid-amended versus synthetically fertilized soils.

Although tillage had a significant effect on bacterial community composition in 2015, there was no apparent difference between tillage treatments in 2016 (Fig. 2, Table 2). Moreover,

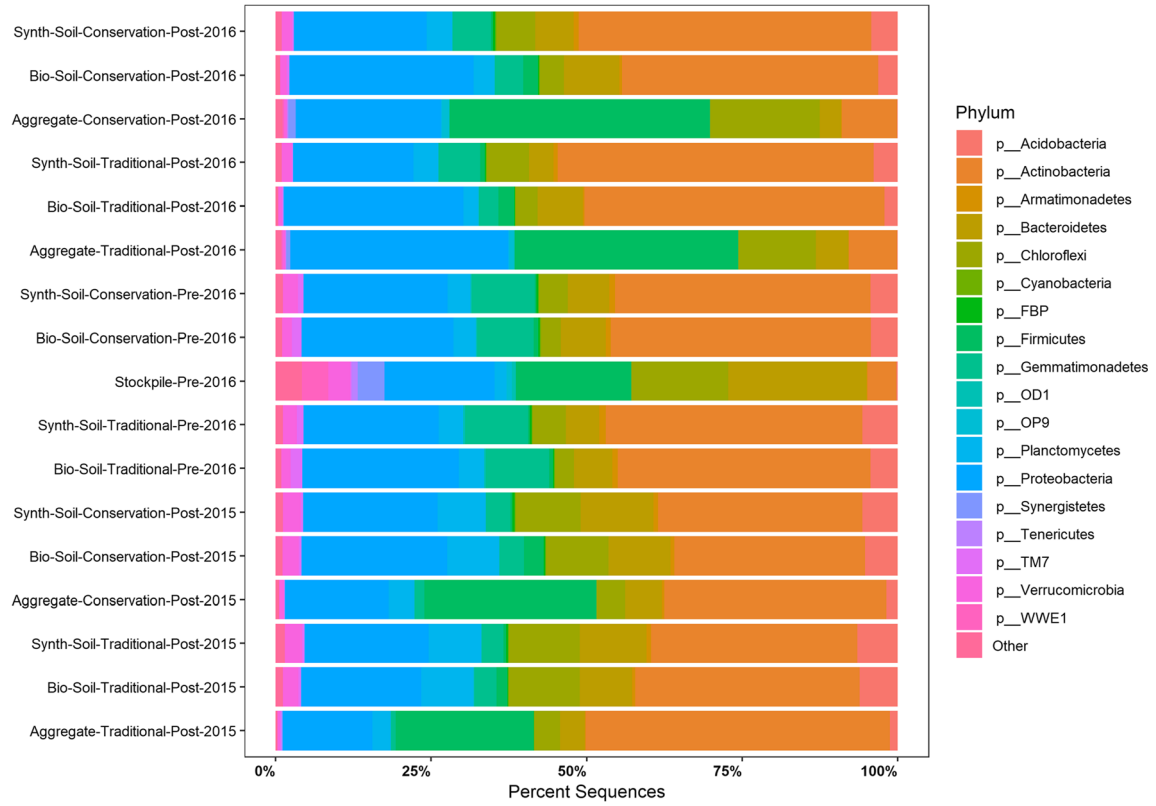

Fig. 1 Proportions of bacterial phyla among soil and biosolid aggregates under different tillage (conservation versus traditional) and fertilization (synthetic [synth] versus biosolid [bio]) regimes in April (pre-treatment) and June (post-treatment). Aggregate = biosolid aggregate embedded in and removed from soils

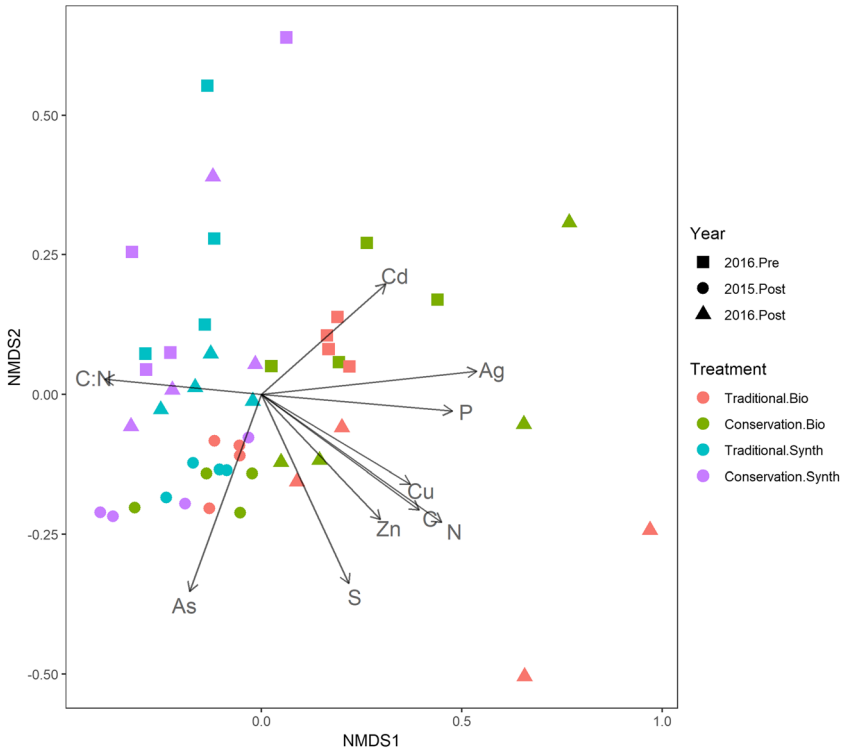

Fig. 2 Non-metric multidimensional scaling of bacterial communities from soil samples from different treatments at each time point. Arrows indicate significant ($p < 0.05$) vectors of soil chemical characteristics evaluated at the June sampling point. Bio = biosolids, Synth = synthetic fertilizer, Traditional = traditional tillage, Conservation = conservation tillage

Table 2 Impacts of fertilizer and tillage treatments on bacterial communities in each year evaluated by PERMANOVA

	F value	r^2	*p* value
2015			
Fertilizer	1.5	0.09	0.109
Tillage	2.35	0.14	0.011
Fertilizer × tillage	0.072	0.04	0.82
2016			
Month	4.87	0.11	0.001
Fertilizer	7.36	0.17	0.001
Tillage	1.26	0.029	0.19
Month × fertilizer	2.43	0.056	0.004
Month × tillage	1.13	0.026	0.273
Fertilizer × tillage	0.92	0.02	0.54
Month × fertilizer × tillage	1.04	0.024	0.37

there were no significant differences in the relative abundances of bacterial phyla (FDR $p > 0.2$) or families (FDR $p > 0.1$) between tillage treatments (Figs. 1 and 3). Concordantly, only a very small number of low-abundance OTUs were identified that differed significantly between tillage treatments in synthetically fertilized or biosolid-amended soils (Supplemental Fig. 2). Together, the minor differences in soil bacterial communities under different tillage treatments suggest that fertilization method, rather than tillage method, has a much larger influence on the bacterial community in soil in this system.

Impact of Fertilization and Tillage on Soil Bacterial Richness and Diversity

Soil bacterial diversity, but not OTU richness, was significantly affected by fertilizer and tillage treatments (Table 3). Specifically, there were no differences in OTU richness of soil communities between fertilizer or tillage treatments in 2015 (ANOVA; fertilizer $F = 2.02$, $p = 0.21$ and tillage $F = 0.59$, $p = 0.50$), in April 2016 (ANOVA; fertilizer $F = 0.21$, $p = 0.67$ and tillage $F = 0.29$, $p = 0.63$), or in June 2016 (ANOVA; fertilizer $F = 3.08$, $p = 0.13$ and tillage $F = 0.05$, $p = 0.84$). However, there was a significant effect of fertilizer treatments on Shannon and Simpson's diversity indices at each time point (Shannon H': 2015 $F = 6.08$, $p = 0.049$; April 2016 $F = 8.64$, $p = 0.026$; June 2016 $F = 0.633$, $p = 0.046$; Simpson's $1/D$: 2015 $F = 14.55$, $p = 0.009$; April 2016 $F = 30.86$, $p = 0.001$; June 2016 $F = 6.14$, $p = 0.048$.). In general, biosolid-amended soils had lower richness and diversity than synthetically fertilized soils (Table 3). However, individual contrasts among treatments were significant only for Simpson's diversity in April 2016, where soils previously amended with biosolids under traditional tillage

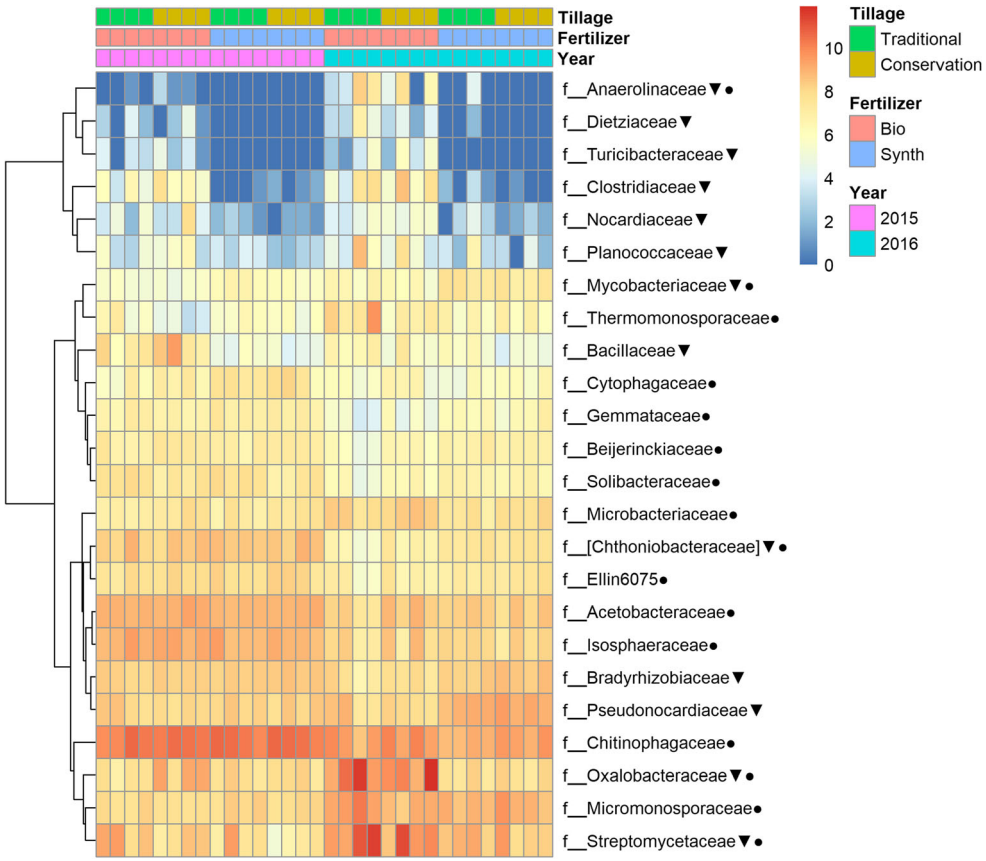

Fig. 3 Heatmap of bacterial families that differed in abundance between fertilizer (circle) or tillage (triangle) treatments. Heatmap colors are based on log2(1 + *x*)-transformed sequence counts. Bio = biosolids, Synth = synthetic fertilizer, Traditional = traditional tillage, Conservation = conservation tillage. Soils in this comparison were all from the June sampling in 2015 and 2016

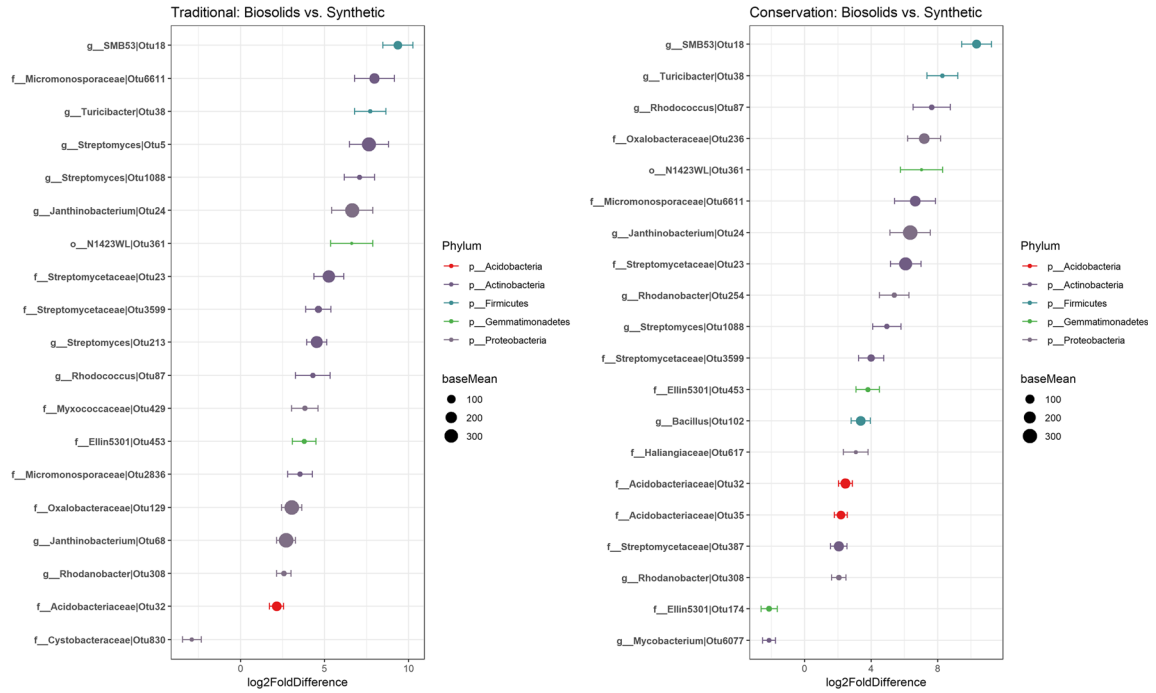

Fig. 4 Plots of OTUs that were differentially abundant between biosolid- and synthetically fertilized soils managed with traditional tillage (left) or conservation tillage (right). Points represent individual OTUs colored by the phylum to which they belong and scaled by their base mean. The lowest level of taxonomy that could be confidently assigned is indicated on the *y*-axis

had significantly lower bacterial diversity than other treatments, and in the conservation tillage in the June 2016 sample (Table 3). In contrast, the significance of tillage treatments for soil bacterial diversity was inconsistent and significant only for Simpson's diversity in April 2016 ($F = 52.58$, $p = 0.005$), though not in other years ($F < 9.7$, $p > 0.05$ for each time). Thus, bacterial diversity tended to be lower after biosolid amendments versus synthetic fertilizers, even several years after the previous biosolid application.

Relationships Between Community Composition and Diversity with Soil Chemistry

Soil chemical parameters were significantly related to variation in bacterial communities (Fig. 2). Specifically, C, N, P, S, and trace metals tended to be associated with bacterial communities from biosolid-amended soils. In contrast, the C/N ratio was associated with bacterial communities from synthetically fertilized soils. Further, the relative abundances of bacterial families were significantly correlated with soil chemical characteristics (Fig. 5). In particular, the families Oxalobacteraceae, Micrococcaceae, Microbacteriaceae, and Streptomycetaceae were positively correlated with C and N, whereas other families, such as Pseudonocardiaceae and Micromonosporaceae, were negatively correlated with concentrations of various trace metals (Fig. 5). Thus, differences in bacterial community structure between fertilization treatments were likely due, at least in part, to their effects on soil chemistry, especially C and N.

Temporal Patterns (April–June 2016)

There were significant shifts in soil bacterial communities from April to June in 2016 for both biosolid-amended and synthetically fertilized plots (Fig. 2, Table 2). Interestingly, there was a significant difference in community composition between fertilizer treatments in April of 2016, prior to subsequent amendment (Fig. 2, Table 2), suggesting a residual effect of biosolids from the previous application 4 years earlier. Specifically, contrasting soil bacterial communities from different fertilizer treatments at the April sampling time, biosolid-amended soils had higher relative abundances of many taxa, such as *Streptomyces*, *Micromonospora*, *Kribbella*, *Rhodanobacter*, SMB53, and *Turicibacter* (Fig. 6). In biosolid-amended plots, the individual taxa that increased in relative abundance from April to June included many Proteobacteria (*Pseudomonas*, Oxalobacteraceae, *Janthinobacterium*) and Actinomyces (*Streptomyces*) (Fig. 6). In contrast, many fewer taxa increased in abundance over this same time period in synthetically fertilized plots (Fig. 6). Thus, the taxa that proliferate in biosolid-amended plots are likely soil taxa feeding on biosolid material, whereas bacterial populations in the relatively carbon-poor soils receiving synthetic fertilizers are less likely to increase in abundance over the season.

Table 3 Richness and diversity of bacterial OTUs from soils under different treatments at each time point. Different letters indicate significant differences (Tukey HSD < 0.05) within each time point

OTU richness	Traditional		Conservation	
Year (Month)	Synthetic	Biosolids	Synthetic	Biosolids
2015 (June)	2188 ± 184 a	2039 ± 182 a	2233 ± 305 a	2142 ± 184 a
2016 (April)	1736 ± 467 a	1683 ± 50 a	1753 ± 529 a	1617 ± 266 a
2016 (June)	2013 ± 184 a	1436 ± 397 a	1886 ± 418 a	1510 ± 544 a
Shannon (*H'*)	Traditional		Conservation	
Year (month)	Synthetic	Biosolids	Synthetic	Biosolids
2015 (June)	6.69 ± 0.12 a	6.58 ± 0.12 a	6.71 ± 0.18 a	6.58 ± 0.08 a
2016 (April)	6.55 ± 0.14 a	6.27 ± 0.07 a	6.57 ± 0.18 a	6.32 ± 0.19 a
2016 (June)	6.6 ± 0.1 a	5.77 ± 0.65a	6.55 ± 0.23 a	6.1 ± 0.5 a
Simpsons (1/*D*)	Traditional		Conservation	
Year (Month)	Synthetic	Biosolids	Synthetic	Biosolids
2015 (June)	336 ± 41 a	301 ± 37 a	330 ± 46 ab	263 ± 41 b
2016 (April)	293 ± 20 a	184 ± 34 b	296 ± 17 a	227 ± 47 ab
2016 (June)	279 ± 29 a	121 ± 99 a	272 ± 56 a	76 ± 108 a

Bacterial Communities of Biosolid Aggregates

Both fresh biosolid aggregates (not yet applied to the field) and those that were embedded in and recovered from soil in June harbored diverse bacterial communities, with an average of 554 and 574 OTUs in fresh and field-recovered aggregates, respectively. Although the composition of the bacterial colonists of biosolids shifted from fresh to field-recovered aggregates (Fig. 7), many of the taxa that dominated the fresh biosolid community were also found in field-collected aggregates (e.g., OTUs related to T78 [Chloroflexi], SB-1 [Bacteroidetes], SMB53 [Firmicutes], and Planococcaceae [Firmicutes]), indicating that these persist after field application (Fig. 8). However, other taxa not initially present in fresh biosolid aggregates, such as *Streptomyces*, *Pseudomonas*, *Arthrobacter*, *Rumellibacillus*, and *Kribbella*, were also found

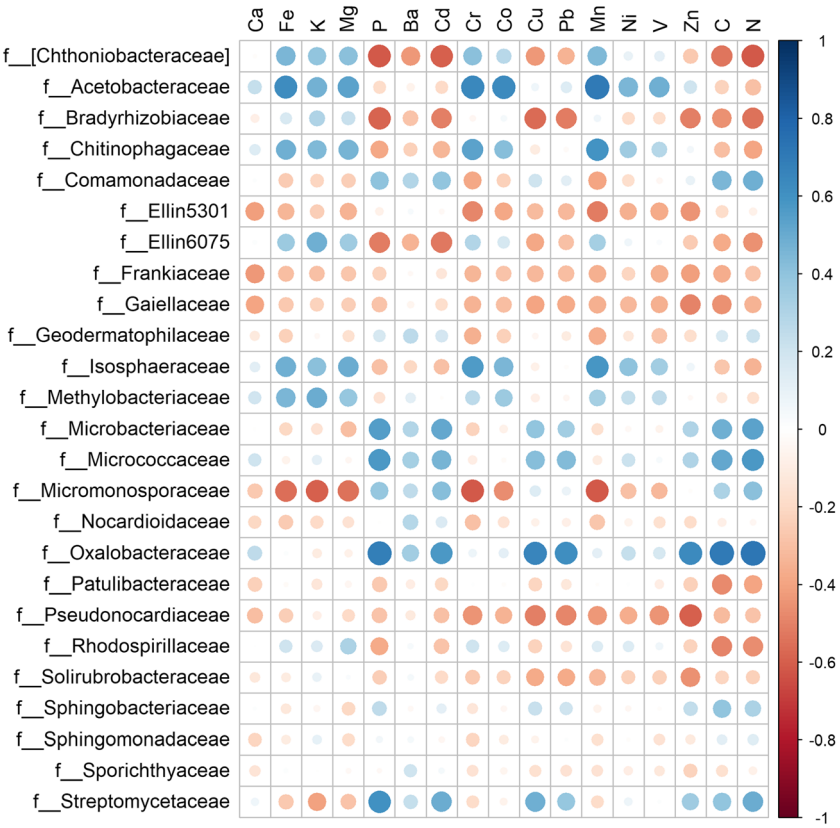

Fig. 5 Correlation heatmap (Spearman) of abundant bacterial families with soil characteristics. Positive correlations are represented in blue, where stronger correlations have larger circles. Correlations that did not meet a significance threshold of $p < 0.05$ are left empty

in high abundance in field-collected aggregates, especially those under traditional tillage, suggesting that these are soil taxa that colonize and grow on biosolid material and compete with the introduced populations.

Pathogenic Potential of Bacterial Communities from Biosolids

Although some of the taxa enriched in biosolid-amended soils were likely gut derived, *Salmonella* and *C. perfringens*-specific genes were not detected by the conventional PCR used in this study. It is important to note the PCR screening was performed to detect *C. perfringens* as being the most important species causing several forms of enteric diseases, including food poisoning and fatal enterotoxemia. However, it is possible that other clostridial species could be present in these samples.

Discussion

Much of the literature on biosolids focuses on the benefits to soil and use as a replacement for synthetic fertilizers [47, 48]. This includes benefits such as improving soil structure, increasing organic matter and soil fertility [17]. In terms of microbes, most studies have focused on pathogenic bacteria that may be present in the biosolids, such as Clostridia [49–52], *Salmonella* [53], *E. coli* [27], and enterococci [54]. As expected, we found very low abundances of Enterobacteriaceae and other enteric bacteria. However, all samples were PCR negative when screened for the presence of the gut-derived pathogens *Salmonella* and *Clostridium perfringens*. Few studies have used molecular techniques to study the detailed bacterial communities in the soil as influenced by biosolids. Zerzghi et al. [55] used a clone library (350 clones) with the 16S rRNA gene to explore bacterial diversity 20 years after high rates of Class B biosolids were applied and found only minor differences in soil communities. The use of high-throughput sequencing in our study provides a new detailed insight into the influence of biosolids on soil bacteria.

Regardless of the tillage treatment, biosolid amendments induced significant shifts in community composition and diversity. Although differences in community composition between synthetic and biosolid fertilizer treatments were not statistically significant in 2015, communities from both years amended with biosolids were enriched in Clostridiaceae, Nocardiaceae, Planococcaceae, Turicibacteriaceae, Dietziaceae, and Anaerolinaceae, suggesting that biosolids consistently boost populations of these bacterial lineages. Notably, many of these bacterial groups are not typically abundant soil inhabitants but are often among the most abundant groups found in the human gut or anaerobic digesters. The anaerobic family Clostridiaceae is well-known as a gut inhabitant [56] and common in sewage sludge and biosolids

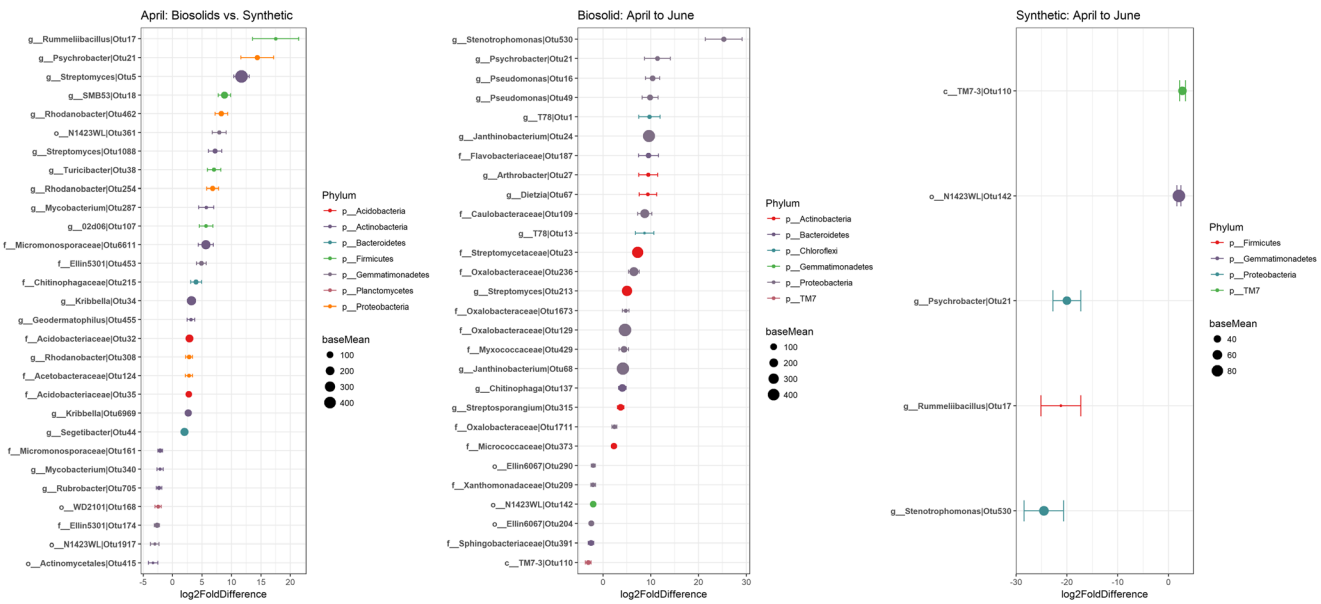

Fig. 6 Plots of OTUs that were differentially abundant between biosolid- and synthetically fertilized soils in April 2016, prior to a second round of treatments (left plot), between April and June in 2016 after a second biosolid amendment (center), and between April and June in 2016 after a second synthetic fertilizer amendment (right). Points represent individual OTUs colored by the phylum to which they belong and scaled by their base mean. The lowest level of taxonomy that could be confidently assigned is indicated on the *y*-axis. In far-left plot, taxa to the right of zero are more abundant in biosolid treatment. In the center plot, taxa to the right of zero are more abundant in April than June in the biosolid treatments (i.e., declined over time). In the far-right plot, taxa to the left of zero are more abundant in June than April in the synthetic fertilizer treatments (i.e., increased over time)

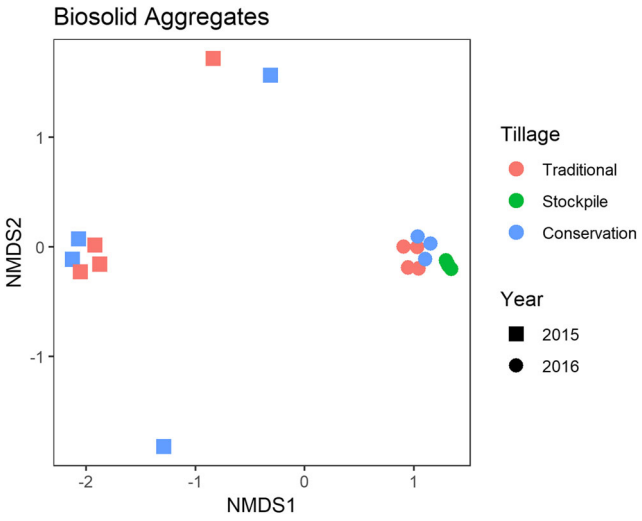

Fig. 7 Non-metric multidimensional scaling of bacterial communities from fresh (stockpile) and field-collected biosolid aggregates from traditional and conservation tillage treatments. Samples from the fresh stockpile were unavailable in 2015

[57]. Anaerolinaceae in the phylum Chloroflexi was only established in 2006 [58] and has been found in anaerobic digesters [59] and methane biogas digesters [60]. *Turcibacter*, an anaerobic bacterium in the phylum Firmicutes, first described in 2002 [61], has been found in the gut microbiome of rats, cattle, pigs, and humans [62–65]; thus, it is not surprising to find members of this group in biosolids. *Dietzia*, an actinobacterium, is commonly found in soil and has been investigated in bioremediation [66, 67]. Some *Dietzia* species are human pathogens [68], but there are very few papers exploring their role in the gut microbiome, for example in dairy [69]. Nocardiaceae is another family in Actinobacteria and *Nocardia* are widely found in sewage sludge where they break down hydrophilic compounds, are involved in foam formation, and can interfere with the processing of the sludge [70–72]. The family Planococcaceae in the order Bacilliales has been found in guts of dairy cattle [73], human colostrum [74], wheat roots [75], soils [76], swine manure pits, and pre-anaerobic phase of digestors [77]. At the OTU level, there were several groups that could not be assigned to a genus, but which were more common on biosolids. These include 02d06 (Clostridiaceae) SMB53

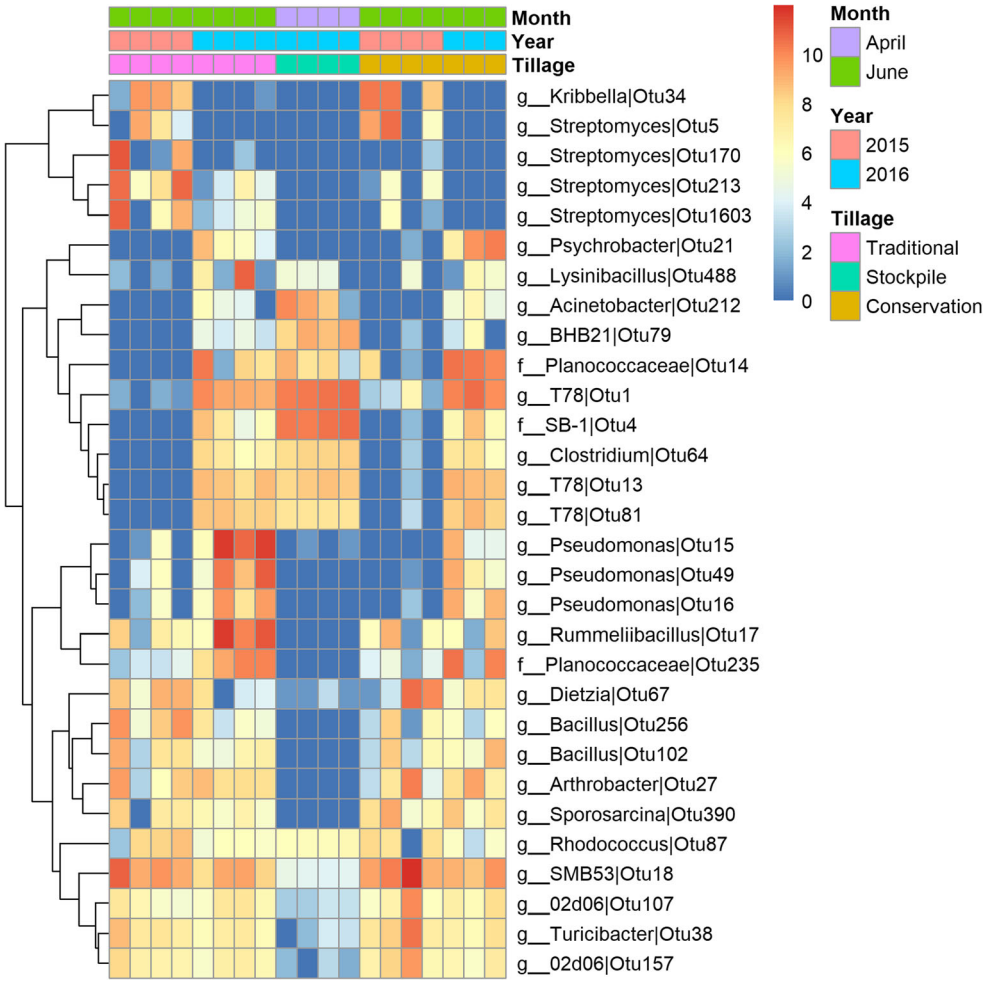

Fig. 8 Abundant OTUs (> 0.5%) found in biosolid aggregates from fresh biosolids pile (stockpile) (April 2016) and field-collected aggregates (June) in different years and tillage treatments (traditional and conservation)

(Clostridiaceae), T78 (Anaeroliniaceae) and SB-1 (Bacteroidales).

Under both traditional and conservation tillage, significant populations of some biosolid-derived bacteria persisted after field application. Notably, due to their long-lived, hardy spores, *Clostridium* species (e.g., *C. perfringens*, *C. bifermentans*) have been used as indicators of fecal contamination after biosolid amendments and used for source tracking bioaerosols [78, 79]. However, this group may not be especially useful as a pathogen indicator, since it survives for prolonged periods where other pathogens are more sensitive to heat and moisture stress [80]. In either case, the consistent enrichment of *Clostridium* species in biosolid-amended soil suggests that this group is a hallmark of biosolid use and may be useful in source tracking fecal contamination of bioaerosols after high-wind events [78, 79]. Importantly, although biosolid-associated bacteria persisted in soil, potentially pathogenic taxa were extremely rare and no toxin genes for key groups (*Salmonella*, *Clostridium*) were detectable, suggesting that although fecal contamination was detectable via indicator taxa, pathogen populations had likely declined to low levels. We cannot rule out the possibility of the presence of very small amount of *Salmonella* and *Clostridium perfringens*, which may be below the detection limit of the PCR assays.

In addition to human gut- or digester-derived bacteria, populations of many soil-inhabiting bacteria also proliferated after biosolid use. In particular, members of *Streptomyces*, Oxalobacteraceae, and *Janthinobacteria* were consistently enriched in biosolid-amended soil, and *Streptomyces*, *Kribbella*, and *Pseudomonas* species were abundant colonists of field-collected biosolid aggregates. These groups, especially *Streptomyces* and *Pseudomonas*, are generally regarded as plant-beneficial soil bacteria involved in suppression of plant disease [81], P-solubilization [82, 83], and induction of plant resistance [84, 85]. Thus, beyond supplying plant nutrients, biosolid amendments may improve soil health by supporting the growth of soil microbes that limit plant disease or improve plant nutrition [86, 87]. Moreover, because many of the taxa that proliferated in biosolid aggregates can grow rapidly and are among the most potent producers of antibiotic compounds (e.g., *Streptomyces*, *Pseudomonas*) [88–90], these are likely the populations that compete with bacteria introduced with biosolids, limit the regrowth of potential human pathogens, and contribute to the rapid decline of pathogen populations [53]. Recent work on fungal communities after biosolid applications also found that fast-growing, resource competitive fungal taxa (e.g., *Fusarium*, *Ulocladium*, *Mortierella*) were enriched [8], suggesting that competition and/or niche partitioning among these fungal and bacterial groups are key determinants of microbial dynamics after biosolid amendments [91]. However, more research is needed on the potential impacts of biosolid amendments on plant-beneficial soil bacteria and their role in the suppression of regrowth of introduced pathogens in soil.

Temporal changes in soil bacterial communities were much more pronounced after biosolid amendment than after synthetic fertilization of soils. This, along with the significant relationships between biosolid-amended communities and soil C and N, suggests that biosolids select for bacterial populations are proliferating on, or are primed by, the introduced organic material. For example, positive relationships between soil C and N with relative abundances of Streptomycetaceae, Oxalobacteraceae, Micrococcaceae, and Microbacteriaceae suggest that greater resources after biosolid amendments select for these groups, or that synthetic fertilizer use suppresses their growth over the season. Thus, in contrast to synthetic fertilizers, biosolids are likely to promote microbial growth and activity via the introduction of organic C and N at periods (April to June) when it is most likely that temperature and moisture conditions will be growth-conducive. The impacts of biosolid amendments on soil microbial communities, as has been demonstrated in other work [8, 92, 93], were apparent 4 years after the previous biosolid application, suggesting that soil communities undergo relatively long-term changes in composition after biosolid use. These "legacy effects" were characterized by higher abundances of many of the same bacterial taxa that appeared to grow on the biosolid aggregates, as well as the more recalcitrant members (Clostridiaceae OTUs) introduced with biosolids. For example, even prior to the second application of biosolids (April, 2016), many of these groups (e.g., Streptomycetaceae, Clostridiacaeae, *Kribbella*) were more abundant than in plots previously fertilized with synthetic N and S. Notably, whereas many of the taxa that characterized the legacy effect of biosolids can form hardy, desiccation-resistant spores [51, 94, 95], other taxa that increased consistently after biosolid amendment over the season and do not have the ability to produce spores (e.g., Oxalobacteraceae, Janthinobacteriaceae, Flavobacteriaceae), do not appear to survive for prolonged periods. Thus, biosolids appear to promote many taxa in the short-term, with longer term impacts on populations of spore-forming groups. The long-term effects of biosolids are likely to be related to both spore-forming capacities, as well as the continued decomposition of biosolid material.

The adoption of conservation tillage and use of organic fertilizers, such as biosolids, are crucial strategies for reducing soil erosion, improving plant health, and enhancing the sustainability of wheat production in the dryland areas of eastern Washington [7, 13]. However, despite the potential benefits of biosolid application, there is little information on how biosolids interact with tillage practices to impact soil microbial communities, and concerns regarding the potential of biosolid application to introduce human pathogens to agricultural systems remain. In this work, traditional tillage with a disk implement versus conservation tillage with an undercutter

implement had little impact on the composition or diversity of soil bacterial communities. Although tillage is often found to modify soil communities in other systems [96, 97], the small influence of tillage in this study is consistent with recent work that described only minor differences in bacterial communities between no-till and traditional tilled fields [13]. Moreover, the minor effects of tillage in this work parallels the findings of a companion study where fungal communities also did not differ significantly between tillage practices [6, 8]. The weak effects of tillage practices on soil bacterial communities in this system may be due to the relatively short timeframe of tillage treatments, where the undercutter was used for only 4 years. Longer periods of conservation tillage (or no-tillage) that modify soil structure, moisture, or organic matter may be required to detect significant shifts in soil bacterial communities. Additionally, environmental constraints on microbial growth, especially temperature and moisture, may limit the response of bacterial communities in this system to differences in tillage. For example, in the PNW, most precipitation occurs during the winter months, when temperatures are low, and whereas summers are hot and dry, thus providing a relatively short window during which bacterial populations near the soil surface may be active.

In summary, we found few significant differences between soil bacterial communities under with traditional tillage compared to conservation tillage. In contrast, biosolid amendments had large and lasting effects on soil bacterial communities relative to synthetic fertilizers, both by introducing gut- or digester-derived bacteria and enriching indigenous soil populations. Although biosolid amendments did not appear to boost populations of pathogens and are thus not likely to pose a risk to human health, non-pathogenic *Clostridium* species may provide a useful indicator for source tracking. Moreover, the soil bacteria that proliferated after biosolid amendments included many plant-beneficial taxa. Further work characterizing the impacts of biosolids on plant-associated taxa and their plant-beneficial phenotypes will offer deeper insight into how field-applied biosolids contribute to enhanced plant productivity without posing a risk to human health.

Conclusions

Biosolid applications significantly shifted the soil bacterial community, compared to synthetic fertilizer and these legacy effects lasted for more than 4 years. Biosolids not only introduced gut-associated bacteria to the soil but also selected for soil-derived saprotrophs which colonized the biosolids. Traditional (tandem disk) vs. conservation (undercutter) tillage had little effect on bacterial communities. Human pathogens (*Clostridium perfringens* and *Salmonella*) were not detectable in soils after biosolid application.

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