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Research Letter

Functional Predictions of Microbial Communities in Soil as Affected by Long-term Tillage Practices

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Core Ideas

- Microbial function is important but difficult to assess in soil.
- An omics-driven tool, PICRUSt, was used to characterize functions of soil microbial communities.
- No-tillage compared with plow tillage was functionally enriched for most nutrient cycles.
- Many other functions integral to soil health can be explored by the PICRUSt omics approach.

Abstract: Soil microbial communities affect the soil's biological, chemical, and physical properties, but there is still a knowledge gap regarding the long-term impact of tillage practices on soil microbial dynamics. Additionally, the accurate identification of belowground microbial functions is a topic of active interest. In this study, microbial community profiles and functions in soil from a 50-plus-year-old experiment in Ohio, representing one of the world's longest running comparisons of a plow-tillage system and a continuous no-tillage system, were compared. The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) algorithm was used to predict associated functional traits from 16S rRNA gene sequences. Analysis of the sequences revealed a large number of unidentified operational taxonomic units (67%), which is consistent with expectations of the soil ecosystem. Next, we investigated gene and enzyme predictions for nitrogen, sulfur, and methane metabolism and hydrocarbon degradation in soil. Results indicated that no-tillage was functionally enriched for most nutrient cycles. This study has allowed us to predict distinct functional profiles as a result of legacy land uses. It serves as an example of improved analysis of the functional differences in soil managed by long-term tillage versus no-till.

Soil microbiology is frequently limited by our ability to predict microbial ecosystem functions, especially for low diversity taxa. The exploration of microbial function is particularly important when considering the soil's nutrient cycles under legacy, or long-term, management systems. Agricultural ecosystems that are under long-term management studies are invaluable tools to understand how soil microbes affect various ecosystem services. One eventual goal of such studies is to provide predictive models of microbe-dependent ecosystem processes (Treseder et al., 2011; Krause et al., 2014).

Among different land management practices, tillage is known to alter many soil physicochemical properties, thereby influencing nutrient availability to microbes (Dick, 1984; Van Doren et al., 1984; Kumar et al., 2012). Varying results have been reported with regard to differences in microbial community composition between tillage treatments (Linn and Doran, 1984; Frey et al., 1999; van Capelle et al., 2012; Navarro-Noya et al., 2013; Sengupta and Dick, 2015). Ohio contains two of the longest, continuously maintained comparisons between plow (inversion) tillage and no-tillage in the world (Dick et al., 1991). At the time of sampling, this comparison had been in place for more than 50 yr. Thus, each plot has been replicated in time for the entire duration of the experiment (50-plus yr). Microbial community dynamics (i.e., diversity and functionality), as affected by tillage, are thus considered to be firmly established here because of legacy land use.

Increasingly, omics-based tools are being used to assess the phylogenetic composition of soil microbial communities. One such bioinformatics tool that allows prediction of functional profiles based on phylogenetic composition of communities is PICRUSt (Langille et al., 2013; Oh et al., 2016; Jiménez et al., 2014; Lopes et al., 2016). The objective of this study was to use this tool to

Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; NSTI, Nearest Sequenced Taxon Index; NT, no-till; OTU, operational taxonomic unit; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; PT, plow-till; QIIME, Quantitative Insights into Microbial Ecology.

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examine the predicted functional capabilities of the soil bacterial community under two different tillage systems, long-term plow tillage and long-term no-till.

Materials and Methods

Soil samples were collected from the Triplett–Van Doren long-term research site located near Wooster, OH. Treatment descriptions and the randomized experimental design are provided in Dick et al. (1991). The two treatments sampled for this study were plow-till (PT) and no-till (NT). The PT plots are characterized by spring moldboard (inversion) plowing to a depth of 20 to 25 cm, while the NT plots have had no tillage other than that accomplished by the planter. Other management practices, such as planting dates, liming, fertilizer and pesticide applications, are the same for both treatments.

Soil Sample Collection and Processing

Three subsamples (0–10 cm) were collected in June 2014 from three replicates of the continuous corn (*Zea mays* L.)–NT treatment and the continuous corn–PT treatment. This created a total of 18 subsamples (2 tillage treatments × 3 plot replicates × 3 cores per plot replicate). Subsamples from the NT and PT replicates were pooled together treatment-wise to make two composite samples. Composite soil samples have been used for other, similar studies (Acosta-Martínez et al., 2008). In our case, we have replication of tillage treatments over 50 yr to capture the legacy effects of these tillage practices. The composite samples were immediately prepared by passing the soil through a 2-mm sieve.

Microbial DNA was extracted from 1 g of soil using MoBio's Ultra-CleanSoil DNA Isolation Kit (MO BIO Laboratories, Inc.) following the manufacturer's instructions. The V1 to V3 regions (~600 bp) of the bacterial 16S rRNA gene were targeted, and the amplicons were sequenced on a pyrosequencing platform using the 454 GS FLX Titanium system (454 Life Science, Roche) by ChunLab, Inc. Details of the primers, polymerase chain reaction conditions used for 16S rRNA gene amplification, and soil chemical characteristics were described in an earlier study (Sengupta and Dick, 2015). Sequence data for these samples were submitted to the Sequence Read Archive (SRA) under accession numbers SRR1610991 and SRR1610992.

Sequencing Data Analysis

Raw standard flowgram format (SFF) reads from the sequencing facility were denoised using the Denoiser (Reeder and Knight, 2010) program in Quantitative Insights into Microbial Ecology (QIIME) (Caporaso et al., 2010). Reads with lengths <200 bp and quality scores <25 were removed. Open reference operational taxonomic unit (OTU) picking was done using the QIIME (v. 1.8) pipeline against the Greengenes database (2013 version) (DeSantis et al., 2006) at 99% sequence similarity. Chimeric sequences were removed using Chimera Slayer (Haas et al., 2011) before construction of the OTU table. PICRUSt was then used to predict gene content (gene names and abundances for each OTU) from each sample's 16S rRNA sequence data, functionally annotate the data, and identify functional pathways predicted by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa and Goto, 2000).

The final output of this workflow was quantified in terms of predicted gene abundances per sample per OTU. To compare differences between the two treatments (NT and PT soil), we used a NT/PT ratio calculated by dividing the total predicted gene abundance for all contributing OTUs in the NT sample by the total predicted gene abundance for all contributing OTUs in the PT sample. A ratio >1 suggests the function predicted by the gene being analyzed is greater in NT compared with PT. A ratio <1 would imply the inverse. Welch's *t* test was used for significance testing between the functions predicted for NT and PT soil, with the threshold set at 0.05. It is, of course, possible that greater gene abundance need not translate to increased function under that treatment. The process of protein expression and function is complex and mediated by a number of factors, of which gene abundance is only one.

Results

Community Composition and Diversity

Of 7040 total sequences (after denoising), 4093 sequences were derived from the NT sample and 2947 from PT. At a sequence similarity of 99%, 506 OTUs (324 from NT and 259 from PT) were identified (Table 1).

At the phylum level, NT soil had greater diversity than PT, although exceptions were noted in the case of some classes of Proteobacteria, Gemmatimonadetes and Chlorobi. Bacterial diversity is generally higher under NT than PT (Adl et al., 2006; Lupwayi et al., 2012; van Capelle et al., 2012), but other studies have found no differences between tillage treatments (Jiang et al., 2011; Hartmann et al., 2014). Thus, there is still no clear consensus on the overall effect of tillage on bacterial diversity. However, our results come from a long-term study in which the effect of tillage is firmly established. They correspond with those studies showing an increase in bacterial diversity caused by NT.

The Nearest Sequenced Taxon Index (NSTI) is an indication of the phylogenetic distance between the OTUs in our samples and the reference genomes they are compared against for functional predictions. It is an indirect estimate of the confidence of PICRUSt predictions. For our soil samples, PICRUSt predicted an NSTI value of 0.17 for NT and 0.15 for PT, similar to those obtained for the soil datasets in Langille et al. (2013). These values do not indicate high availability of reference genomes for annotation, suggesting that there

Table 1. Statistics of sequence and operational taxonomic unit (OTU) distribution across samples.

Sample	Raw sequences†	Sequences with hits in GG‡	Number of OTUs	OTUs with hits in GG
No-till	4093 (2486)	2486	877	324
Plow-till	2947 (1528)	1528	441	259
Total	7040 (4014)	4014	1318	506

† Numbers in brackets indicate the sequences that passed all filters and were used for eventual analyses.

‡ GG = Greengenes database.

remains a large portion of the soil microbiome that is yet to be sequenced and classified. Indeed, a closer look at the OTUs indicates that a substantial portion of them is unclassified, and this is reflected in the functional predictions as well. Thus, it is important to allow for an adequate margin of error in any such predictions made with incomplete reference databases.

To provide a frame of reference, the average NT/PT gene abundance ratio of four major soil microbial processes is displayed in Fig. 1. The genes with the highest predicted abundances and genes thought to be functionally critical are not the same. This suggests the most ubiquitous or abundant proteins may not be the most functionally relevant ones, and vice versa.

We next predicted the functional features of the bacterial community in both samples. Although any number of functional features can be investigated with the PICRUSt software, as examples we describe two nutrient cycling functions in brief—nitrogen metabolism and methane metabolism. A detailed description of all the components examined can be found in Hariharan (2015).

Nitrogen Metabolism

Forty-two genes from the KEGG database were predicted to be involved in nitrogen metabolism. Genes with highest predicted abundances are listed in Table 2, along with their associated proteins and related OTU information. Genes encoding the reversible reactions of ammonia to organic nitrogen had higher predicted abundances under NT, suggesting a larger metabolizable nitrogen pool size in NT. This would suggest that long-term NT soil, compared with PT soil, has a greater microbial capacity to mineralize and supply nitrogen to crops during the growing season.

Nitrification is catalyzed by the *amo* genes, all of which were predicted in very low abundance (four gene counts per OTU per sample). These genes were almost exclusively mapped to the Nitrosomonadaceae family and did not show any differences between the tillage treatments. The PT sample of the *amoC* gene did, however, indicate that *Bacillus* sp. and *Bradyrhizobium* sp. OTUs could also contribute to

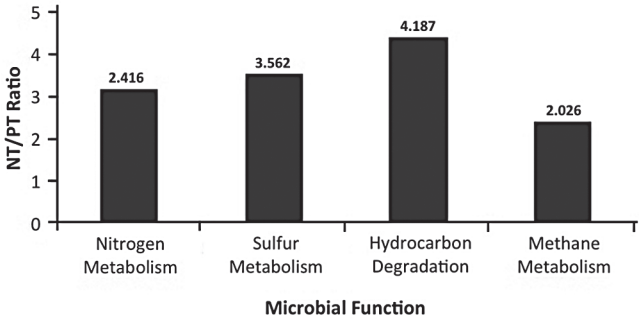

Fig. 1. Average no-till/plow-till (NT/PT) gene abundance ratios of four major soil microbial processes.

nitrifying activity. This link between the *amo* genes and ammonia-oxidizing bacteria like the Nitrosomonadaceae and *Bradyrhizobium* has been observed in previous findings (Rotthauwe et al., 1997; Purkhold et al., 2000; Francis et al., 2003). Many genes related to dissimilatory nitrate reduction (including the *nar* and *nir* genes) had NT/PT gene abundance ratios >1, suggesting that NT may exhibit greater potential for denitrification and production of N_2O than does PT.

Methane Metabolism

Carbon metabolism as a whole, including but not limited to the Wood–Ljungdahl pathway, Arnon–Buchanan cycle, Crassulacean acid metabolism (CAM) pathway, and Calvin cycle, was analyzed. PICRUSt predicted about 71 genes, of which the majority had NT/PT gene abundance ratios <1. Owing to the complexity and interlinked nature of carbon cycling, we focused on the cycling of methane, an important greenhouse gas that has a radiative forcing potential 23 times higher than that of carbon dioxide. Methanotrophic diversity in soils has also been previously studied (Sengupta and Dick, 2017).

Approximately 30 enzymes were connected with methane metabolism by soil microbes. Genes with the highest predicted abundances, along with their associated protein products and related OTU information are listed in Table 2. The NT/PT gene abundance ratio of 2.4 (Fig. 1) was lowest for

Table 2. Top five enzymes (by predicted gene abundances) for nitrogen and methane metabolism.

Enzyme	Function	Gene	NT/PT†	Associated OTUs‡
Nitrogen metabolism				
Carbonic anhydrase	Cyanate degradation	*cynT, can*	2.54	54, 41
Glutamine synthetase	Ammonia degradation	*glnA*	2.22	61, 44
Glutamate synthase small chain	Glutamate formation	*gltD*	2.18	59, 43
Glutamate synthase large chain	Glutamate formation	*gltB*	2.43	59, 47
Nitronate monooxygenase	Nitrite synthesis	*ncd2, npd*	2.29	44, 31
Methane metabolism				
Formate dehydrogenase major subunit	CO_2 formation	*fdoG, fdfH*	2.42	60, 39
Formate dehydrogenase iron-sulfur subunit	CO_2 formation	*fdoH*	2.64	43, 29
Formate dehydrogenase alpha subunit	CO_2 formation	*fdhA1*	2.99	5, 2
Formylmethanofuran dehydrogenase subunit E	Formate synthesis	*fwdE, fmdE*	2.899	27, 17
Formate dehydrogenase subunit gamma	CO_2 formation	*fdoI*	1.627	22, 16

† NT/PT = no-till/plow-till gene abundance ratio.

‡ OTU = operational taxonomic unit. First number is number of OTUs observed for that enzyme in the no-till sample; second number is number of OTUs observed in the plow-till sample.

methane metabolism relative to the other functional categories we studied. This may not necessarily be reflective of the methane metabolizing potential of either soil but is possibly due to the rarity of methanogenic microbes in the top stratum of soil (i.e., the 0- to 10-cm soil layer). In fact, Jacinthe et al. (2014) found that PT soils were a source of methane whereas long-term NT soils were a sink.

The *pmo* set of genes (closely related to the *amo* genes) were predicted to be the major contributors to methane catabolism, followed by cytochrome c-associated methanol dehydrogenase enzymes (*mdh1, mdh2*). An interesting finding was that the methanol dehydrogenase-encoding genes were predicted for the PT sample alone at very low abundances (in *Hyphomicrobium* sp.). This is a rare instance wherein genes encoding an enzyme were predicted exclusively for the PT sample.

Discussion

This study evaluates *in silico* the genes contributing to nutrient cycling and other processes in the context of legacy (approximately 50 yr) soil tillage practices. Our results suggest NT soils harbor more diverse soil bacterial communities (quantified at the phylum level) with higher predicted gene content, although exceptions were noted in the case of some classes of Proteobacteria, Gemmatimonadetes, and Chlorobi. Bacterial diversity is generally reported to be higher under NT than PT (Lupwayi et al., 2012; van Capelle et al., 2012; Adl et al., 2006). Thus, there is still no clear consensus on the overall effect of tillage on bacterial diversity. However, our results come from a long-term study in which the effect of tillage is firmly established, and they correspond with studies showing an increase in bacterial diversity caused by NT.

Long-term land management practices shape the composition and function of soil microbial communities, and continuously maintained NT seems to be associated with better nitrogen and methane metabolizing capabilities, based on the 16S rRNA gene sequences and predicted metagenome analyses performed. The unique description of the bacterial players in the long-term tillage plots also suggests maintaining NT practice for long periods of time may provide diverse benefits to agroecosystems.

Myriad biological and physicochemical factors control specific biological functions in terrestrial ecosystems, especially related to nutrient cycling. Quantifying such functions in the laboratory setting is difficult due to issues in culturing of many soil microbes as well as the extensive number of functions that would need to be tested. Enzyme assays and measurements of soil nutrient concentrations done at the time of sampling could improve functional interpretations. However, an *in silico* analysis is extremely useful for predicting the putative roles of all observed OTUs in a soil sample and formulating hypotheses that could then be functionally validated. Although these studies are not meant to replace bench experiments and functional validation, using such tools can help develop hypotheses for large-scale microbial ecology studies. In the larger context of functional pathways, in silico analyses could also pinpoint key rate-limiting steps as well as critical microorganisms performing unique functions in the soil. Predictive functional tools could also be of use in the identification of candidate microorganisms for commercially important genes.

Conclusions

The temporal stability offered by the long-term no-tillage plots used in this study provides unique ecological significance to our results. Linking PICRUSt, as a prediction tool, to 454 pyrosequencing data revealed the utility of this approach to predict functional differences as affected by land management, in this case, long-term tillage practices. The functional categories studied here represent only a small portion of soil microbial processes. There are a multitude of other functions that are integral to the maintenance of soil health that can be studied. As more and better tools and technologies are developed to open the proverbial microbial "black box," we should revise our understanding of microbial contributions to biogeochemical processes.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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