Title

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Abstract

We describe a novel approach for identifying microbial contributions to soil C-cycling dynamics using nucleic acid stable isotope probing coupled with next generation sequencing (SIP-NGS). In a series of parallel soil microcosms we amended soils with a complex mixture of model carbon (C) substrates and inorganic nutrients common to plant biomass, where a single C constituent is substituted for its ^{13}C-labeled equivalent. Using this approach we assessed incorportation of ^{13}C-xylose or ^{13}C-cellulose as proxies for labile soluble C and polymeric insoluble C utilization, respectively. Using CsCl gradient fractionation, incorporation of ^{13}C into DNA was measured over 30 days. The 16S rRNA gene sequences from CsCl gradient fractions were characterized by 454 pyrosequencing and classified into Operational Taxonomic Units (OTU). We describe specific patterns of C-assimilation by discrete OTUs as a function of substrate, time, and level of isotope incorporation. Incorporation of ^{13}C from xylose into OTUs was observed at days 1, 3, and 7, while notable incorporation of ^{13}C from cellulose was observed only after day 14. Of over 6,000 OTUs detected, a total of 43 and 35 unique OTUs significantly assimilated ^{13}C from xylose and cellulose, respectively. We did not observe consistent C utilization at the phylum level although both xylose and cellulose utilization were observed across 7 phyla each revealing a high diversity of bacteria able to utilize these substrates. OTUs that assimilate xylose and those that assimilate cellulose are largely mutually exclusive. Xylose assimilating OTUs are more abundant in the microbial community than cellulose assimilating OTUs, while cellulose OTUs demonstrate a greater substrate specificity than xylose OTUs. Furthermore, the increased depth provided by SIP-NGS allowed us to identify several novel cellulose utilizing bacteria.

monolayer | structure | x-ray reflectivity | molecular electronics

Abbreviations: SAM, self-assembled monolayer; OTS, octadecyltrichlorosilane

Introduction

We have only a rudimentary understanding of carbon flow through soil microbial communities. This deficiency is driven by the staggering complexity of soil microbial food webs and the opacity of these biological systems to current methods for describing microbial metabolism in the environment. Relating community composition to overall soil processes, such as nitrification and denitrification, which are mediated by defined functional groups has been a useful approach. However, carbon-cycling processes have proven more recalcitrant to study due to the wide range of organisms participating in these reactions and our inability to discern diagnostic functional genetic markers.

Excluding plant biomass, there are 2,300 Pg of carbon (C) stored in soils worldwide which accounts for ~80% of the global terrestrial C pool BATJES, 1996; Amundson, 2001. When organic C from plants reaches soil it is degraded by fungi, archaea, and bacteria. This C is rapidly returned to the atmosphere as CO_2 or remains in the soil as humic substances that can persist up to 2000 years Yanagita, 1990. The majority of plant biomass C in soil is respired and produces 10 times more CO_2 than anthropogenic emissions on an annual basis Chapin, 2002. Global changes in atmospheric CO_2, temperature, and ecosystem nitrogen inputs, are expected to impact primary production and C inputs to soils Groenigen *et al.*, 2006 but it remains difficult to predict the response of soil processes to anthropogenic change DAVIDSON *et al.*, 2006. Current climate change models concur on atmospheric and ocean C predictions but not terrestrial Friedlingstein *et al.*, 2006. These contrasting terrestrial ecosystem model predictions reflect how little is known about soil C cycling dynamics and it has been suggested that incosistencies in terrestial modeling could be improved by elucidating the relationship between dissolved organic carbon and microbial communities in soils Neff and Asner, 2001.

An estimated 80-90% of C cycling in soil is mediated by microorganisms Nannipieri *et al.*, 2003a; n.d. Understanding microbial processing of nutrients in soils presents a special challenge due to the hetergeneous nature of soil ecosystems and methods limitations. Soils are biologically, chemically, and physically complex which affects microbial community composition, diversity, and structure Nannipieri *et al.*, 2003a. Confounding factors such as physical protection/aggregation, moisture content, pH, temperature, frequency and type of land disturbance, soil history, mineralogy, N quality and availability, and litter quality have all been shown to affect the ability of the soil microbial community to access and metabolize C substrates Sollins *et al.*, 1996; Kalbitz *et al.*, 2000. Further, rates of metabolism are often measured without knowing the identity of the microbial species involved Nannipieri *et al.*, 2003b leaving the importance of community membership towards maintaining ecosystem functions unknown Nannipieri *et al.*, 2003b; Allison and Martiny, 2008; Schimel and Schaeffer, 2012. Litter bag experiments have shown that the community composition of soils can have quantitative and qualitative impacts on the breakdown of plant materials Schimel, 1995. Reciprocal exchange of litter type and microbial inocula under controlled environmental conditions reveals that differences in community composition can account for 85% of the variation in litter carbon mineralization Strickland *et al.*, 2009. In addition, assembled communities of cellulose degraders reveal that the composition of the community has significant impacts on the rate of cellulose degradation Wohl *et al.*, 2004.

An important step in understanding soil C cycling dynamics is to identify individual contributions of discrete microorganisms and to investigate the relationship between genetic diversity, community structure, and function O'Donnell *et al.*, 2002. The vast majority of microorganisms continue to resist cultivation in the laboratory, and even when cultivation is achieved, the traits expressed by

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a microorganism in culture may not be representative of those expressed when in its natural habitat. Stable-isotope probing (SIP) provides a unique opportunity to link microbial identity to activity and has been utilized to expand our knowledge of a myriad of important biogeochemical processes Chen and Murrell, 2010. The most successful applications of this technique have identified organisms which mediate processes performed by a narrow set of functional guilds such as methanogens Lu, 2005. The technique has been less applicable to the study of soil C cycling because of limitations in resolving power as a result of simultaneous labeling of many different organisms in the community. Additionally, molecular applications such as TRFLP, DGGE, and cloning that are frequently used in conjunction with SIP provide insufficient resolution of taxon identity and depth of coverage. We have developed an approach that employs a complex mixture of substrates added to soil at a low concentration relative to soil organic matter pools along with massively parallel DNA sequencing. This greatly expands the ability of nucleic acid SIP to explore complex patterns of C-cycling in microbial communities with increased resolution.

A temporal cascade occurs in natural microbial communities during the plant biomass degradation in which labile C degradation preceeds polymeric C Hu and Bruggen, 1997; Rui *et al.*, 2009. The aim of this study is to track the temporal dynamics of C assimilation through discrete individuals of the soil microbial community to provide greater insight into soil C-cycling. Our experimental approach employs the addition of a soil organic matter (SOM) simulant (a complex mixture of model carbon sources and inorganic nutrients common to plant biomass), where a single C constituent is substituted for its ^{13}C-labeled equivalent, to soil. Parallel incubations of soils amended with this complex C mixture allows us to test how different C substrates cascade through discrete taxa within the soil microbial community. In this study we use ^{13}C-xylose and ^{13}C-cellulose as a proxy for labile and polymeric C, respectively. Using a novel approach we couple nucleic acid stable isotope probing with next generation sequencing (SIP-NGS) to elucidating soil microbial community members responsible for specific C transformations. Amplicon sequencing of 16S rRNA gene fragments from many gradient fractions and multiple gradients make it possible to track C assimilation by hundreds of different taxa. Ultimately we identify discrete microorganisms responsible for the cycling of specific C substrates.

Results and Discussion

In this study, we couple nucleic acid SIP with next generation sequencing (SIP-NGS) to observe C use dynamics by the soil microbial community. A series of parallel soil microcosms all amended with a C substrate mixture were incubated for 30 days. The substrate mixture was identical for each bottle except in one series of bottles the cellulose was ^{13}C-labeled in another the xylose was ^{13}C-labeld and in the last no sustrattes were labeled. The C substrate mixture was designed to approximate freshly degrading plant biomass. Xylose or cellulose carried the isotopic label so we could examine C assimilation dynamics for labile, soluble C versus insoluble, polymeric C. 5.3 mg total mass of C substrate mixture per gram soil (including 0.42 mg xylose-C and 0.88 mg cellulose-C g soil^{-1}) was added to each microcosm representing 18% of the total soil C. Microcosms were harvested at several time points during the incubation period and ^{13}C assimilation was observed by sequencing 16S rRNA gene amplicons from bulk soil DNA and CsCl gradient fractions. Assimilation of ^{13}-C from Xylose degradation peaked immediately, while cellulose ^{13}C assimilation peaked after two weeks of incubation (Figure 1).

^{13}C from cellulose assimilated by canonical cellulose-degrading and uncharacterized microbial lineages in many phyla including Chloroflexi and Verrucomicrobia. Only 2 and 5 OTUs were found to have incorporated ^{13}C from labeled cellulose at days 3 and 7, respectively. At days 14 and 30, however, 42 and 39 OTUs were found to incorporate ^{13}C from cellulose into biomass. An average 16% of the ^{13}C-cellulose added was respired within the first 7 days, 38% by day 14, and 60% by day 30. A *Cellvibrio* and *Sandaracinaceae* OTU assimilated ^{13}C from cellulose at day 3. Day 7 responders included the same *Cellvibrio* responder as day 3, a *Verrucomicrobia* OTU and three *Chloroflexi* OTUs. 50% of Day 14 responders belong to Proteobacteria (66% Alpha-, 19% Gamma-, and 14% Beta-) followed by 17% *Planctomycetes*, 14% *Verrucomicrobia*, 10% *Chloroflexi*, 7% *Actinobacteria* and 2% *Cyanobacteria*. *Bacteroidetes* OTUs begin to incoporate ^{13}C from cellulose at day 30 (13% of day 30 responders). Other day 30 responding phyla include *Proteobacteria* (30% of day 30 responders; 42% Alpha-, 42% Delta, 8% Gamma-, and 8% Beta-), *Planctomycetes* (20%), *Verrucomicrobia* (20%), *Chloroflexi* (13%) and *Cyanobacteria* (3%). *Proteobacteria*, *Verrucomicrobia*, and *Chloroflexi* had relatively high numbers of responders with heavy response across multiple time points (Figure 2).

Proteobacteria represent 46% of all cellulose responding OTUs identified. *Cellvibrio* accounted for 3% of all Proteobacterial responding OTUs detected. *Cellvibrio* was one of the first identified cellulose degrading bacteria and was originally described by Winogradsky in 1929 who named it for its cellulose degrading abilities (Boone, 2001). All ^{13}C-cellulose responding *Proteobacteria* share high sequence identity with 16S rRNA genes from sequenced type straints (Table XX) except for OTU.442 (best type strain match 92% sequence identity in the *Chrondomyces* genus) and OTU.663 (best type strain match outside *Proteobacteria* entirely, *Clostridium* genus, 89% sequence identity). Some *Proteobacteria* responders share high sequence identity with type strains for genera known to posess cellulose degraders including *Rhizobium*, *Devosia*, *Stenotrophomonas* and *Cellvibrio*. One *Proteobacteria* OTU shares high sequence identity with the *Brevundimonas* type strain. *Brevundimonas* has not previously been identified as a cellulose degrader, but has been shown to degrade cellouronic acid, an oxidized form of cellulose (Tavernier *et al.,* 2008).

Verrucomicrobia, a cosmopolitan soil phylum often found in high abundance (Fierer *et al.*, 2013), are implicated in polysaccaride degradation in many environments (Fierer *et al.*, 2013; Herlemann *et al.*, 2013; Chin *et al.*, n.d.). *Verrucomicrobia* comprise 16% of the total cellulose responder OTUs detected. 40% of *Verrucomicrobia* responders belong to the uncultured FukuN18 family originally identified in freshwater lakes (Parveen *et al.*, 2013). The *Verrucomicrobia* OTU with the strongest *Verrucomicrobial* response to ^{13}C-cellulose shared high sequence identity (97%) with an isolate from Norway tundra soil (Jiang *et al.*, 2011) although growth on cellulose was not assessed for this isolate. Only one other ^{13}C-cellulose responding verrucomicrobium shared high DNA sequence identity with a sequenced type strain, OTU.638 with *Roseimicrobium gellanilyticum* (100% sequence identity) and *Roseimicrobium gellanilyticum* grows on soluble celluose (Otsuka *et al.*, 2012). The remaining ^{13}C-cellulose *Verrucomicrobia* responders did not share high sequence identity with any type strains (maximum sequence identity with any type strain 93%).

Chloroflexi are traditionally known for their metabolically dynamic lifestyles ranging from anoxygenic phototropy to organohalide respiration (Hug *et al.*, 2013). Recent studies have focused on *Chloroflexi* roles in C cycling (Goldfarb *et al.*, 2011; Cole *et al.*, 2013; Hug *et al.*, 2013) and several members of this phylum demonstrated cellulose utilization (Goldfarb *et al.*, 2011; Cole *et al.*, 2013; Hug *et al.*, 2013). Four closely related OTUs in an undescribed *Chloroflexi* lineage (closest matching type strain for all four OTUs: *Herpetosiphon geysericola*, 89% sequence identity) responded to ^{13}C-cellulose in this microcosm experiment. One additional OTU also from a poorly characterized lineage (clos-

est type strain match a proteobacterium at 78% sequence identity) responded to ^{13}C-cellulose (Figure 3).

Other notable ^{13}C cellulose responders include a *Bacteroidetes* OTU that shares high sequence identity (99%) to *Sporocytophaga myxococcoides* a known cellulose degrader (Vance *et al.*, 1980), and three *Actinobacteria* OTUs that share high sequence identity (100%) with sequenced type strains. One of the three *Actinobacteria* ^{13}C-cellulose responders is in the *Streptomyces*, a genus known to possess cellulose degraders, while the other two closely match the type strains *Allokutzneriz albata* (Tomita *et al.*, 1993; Labeda and Kroppenstedt, 2008) and *Lentzea waywayandensis* (LABEDA and LYONS, 1989; Labeda *et al.*, 2001) that do not decompose cellulose in culture. Nine *Plantomycetes* OTUs responded to ^{13}C-cellulose but none are within described genera (closest type strain match 91% sequence identity) (Figure 3). Interestingly, one responder is annotated as belonging in the *Cyanobacteria*. The phylum annotation is misleading as the OTU is not closely related to any oxygenic phototrophs (closest type strain match *Vampirovibrio chlorellavorus*, 95% sequence identity). A sister clade to the oxygenic phototrophs that does not itself possess known phototrophs has recently been proposed to constitute its own phylum ("Melainabacteria", Rienzi *et al.* (2013)) although its phylogenetic position is debated (Soo *et al.*, 2014). The catalog of metabolic capabilities associated with *Cyanobacteria* (or candidate phyla previously annotated as *Cyanobacteria*) are quickly expanding (Rienzi *et al.*, 2013; Soo *et al.*, 2014). Our findings provide evidence of cellulose degradation within a lineage closely related to but apart from oxygenic phototrophs. Notably, polysaccharide degradation is suggested by the analysis of a *Melainabacterial* genome (Rienzi *et al.*, 2013). Although we highlight ^{13}C-cellulose responders that share high sequence identity with described genera, by and large ^{13}C-cellulose responders uncovered in this experiment are not closely related to isolates (Table XX).

Putative spore-formers in the Firmicutes assimilate ^{13}C from xylose within first day after soil amendment. Within the first 7 days of incubation an average 63% of ^{13}C-xylose was respired and only an additional 6% more was respired between days 7 and 30. At the end of the 30 day experiment 30% of the original ^{13}C from xylose remained in the soils. The ^{13}C remaining in the soil from ^{13}C-xylose addition has likely been stabilized by assimilation into microbial biomass and/or microbial conversion into other forms of organic matter, though it is possible that some ^{13}C-xylose remains unavailable to microbes due to abiotic interactions in soil (Kalbitz *et al.*, 2000). All xylose responders were first responsive in first 7 incubation days.

At day 1, 84% of xylose responsive OTUs belong to Firmicutes, 11% to *Proteobacteria* and 5% to *Bacteroidetes*. At day 3 (d3), Firmicutes responders decreased to 5% (from 16 OTUs to 1) while *Bacteroidetes* increased to 63% (from 1 to 12 OTUs) of day 3 responders. The remaining day 3 responders are members of the *Proteobacteria* (26%) and the *Verrucomicrobia* (5%). Day 7 responders were 53% Actinobacteria, 40% Proteobacteria, and 7% Firmicutes. A substantial amount (75%) of xylose responders for day 7 had not previously been identified as responders at earlier time points.

We observe dynamic changes in ^{13}C-xylose assimilation with time at the phylum level. The numerically dominant xylose responder phylum shifts from *Firmicutes* to *Bacteroidetes* and then to *Actinobacteria* across days 1, 3 and 7 (Figure 2). We also see strong and significant correlation between estimated rRNA operon genome copy numbers per ^{13}C-xylose responder OTU genome and time (p-value, Figure XX). ^{13}C-xylose responder rRNA operon geneome copy number is inversely related to time. That is, OTUs that first respond at later time points have fewer estimated rRNA operons per genome than OTUs that first respond earlier. rRNA operon copy number estimation is a recent advance in microbiome science (CITE) ane the relationship of rRNA operon copy number per genome with ecological strategy is well established (CITE). Specifically, microorganisms with a high number of rRNA operons ber genome tend to be fast growers specialized to take advantage of boom-bust environments whereas a low rRNA operon copy number per genome tends to occur in microorganisms that favor slower growth under lower and more consistent nutrient input (CITE). At the beginning of our incubation, OTUs with estimated high rRNA operon copy numbers per genome or "fast-growers" assimilate xylose into biomass and with time slower growers (lower rRNA operon number per genome) begin to respond to the xylose addition. Further, ^{13}C-xylose responders have fewer estimated rRNA operon copy numbers per genome than ^{13}C-cellulose responders suggesting xylose respiring mircrobes are generally faster growers than cellulose degraders.

At any given time soils harbor microorganisms at varying degrees of dormancy depending on nutrient availability (Jones and Lennon, 2010). The sudden addition of our complex C mixture could stimulate dormant microbes.

Cellulose degrader DNA exhibits greater bouyant density shifts upon ^{13}C incorporation than xylose degrader DNA.

DNA BD shifts due to ^{13}C-asimilation differ across phylogenetic types. Temporal dynamics of C-assimilation in soil. The dynamics of ^{13}C-xylose and ^{13}C-cellulose assimilation varied dramatically. Isotope incorporation increases the bouyant density (BD) of DNA and labeled DNA is enriched in 'heavy' fractions of the density gradient. Isotope incorporation by an OTU is revealed by enrichment of the OTU in heavy CsCl gradient fractions containing ^{13}C labeled DNA relative to heavy fractions from control gradients (no ^{13}C labeled DNA). Variation in 16S rRNA gene amplicon pool composition in fractions of ^{13}C-labeled samples and their corresponding controls is readily observed in 'heavy' gradient fractions (partitioning along axis 2, Fig. 1). The amplicon pool composition of 'heavy' fractions of ^{13}C-xylose and ^{13}C-cellulose samples vary from corresponding controls and from each other, indicating that the substrates were assimilated by different members of the microbial community(Fig. 1A).

The ^{13}C-incorporation reveals temporal dynamics of C degradation demonstrated by ^{13}C-xylose incorporation at days 1, 3, and 7 and ^{13}C-cellulose incorporation at days 14 and 30 (Fig. 1B), as expected (Amelung *et al.*, 2008). The microbial community changed significantly (pval) with time in the bulk community supporting the temporal dynamics observed in the gradient fraction amplicons (Fig. S2). Although within a single time point, the bulk community demonstrated no significant difference between treatments.

'Heavy' fraction amplicon pools from samples that received ^{13}C-xylose diverged from corresponding controls on days 1 through 7 (Fig. 1). Furthermore, amplicon pool composition varied across these days (Fig. 1B) indicating dynamic changes in ^{13}C-xylose assimilation with time. At days 14 and 30 heavy fractions from ^{13}C-xylose labeled samples are no longer differentiated from corresponding controls indicating that ^{13}C is no longer detectable in DNA. The decline in ^{13}C-labelling of DNA is likely due to isotopic dilution resulting from assimilation of unlabeled C and/or due to cell turnover resulting from mortality.

^{13}C-cellulose incorporation isn't detected until day 14 and amplicon composition is consistent for both days 14 and 30 (Fig. 1). The consistency of amplicon composition for cellulose degradation over time compared to xylose suggests a wider array of microorganisms utilize xylose, whereas, cellulose utilization occurs in a select few. This is consistent with long standing notions that more

microorganisms are capable of utilizing simple carbohydrates than complex C substrates.

Differential C utilization by taxa. Individual OTUs that assimilated ^{13}C-substrates were identified using the DESeq framework (Anders and Huber, 2010) to analyze differential representation in 'heavy' fractions (Fig. 2). There were 43 and 35 unique OTUs that significantly (false discovery rate corrected *P*-values <0.10, SI) assimilated ^{13}C-xylose and ^{13}C-cellulose, respectively; herein called 'responders' (Figs. S3, S4,S5).

There were 6 shared responders among all unique responders identified in both the xylose and cellulose treatments (n = 72); Stenotrophomonas, Planctomyces, two Rhizobiaceae, Comamonadaceae, and Cellvibrio. Of these, Stenotrophomonas and Comamonadaceae are the only taxa that are among the top ten l2fc responses measured in both treatments. On the other hand, the only shared responder that is not among the top ten responders for either the cellulose or xylose treatment is Rhizobiaceae. Two of the shared responders corresponded in time between the two treatments (Table S1); Cellvibrio (d3) and Planctomyces (d14).

Responder Characteristics. We found xylose responders were from higher rank abundances than cellulose responders, however, cellulose responders exhibited a greater shift in BD (i.e. assimilated more ^{13}C) than xylose responders in response to isotope incorporation (Fig. 3).

The kernel density estimate (KDE) of BD shifts resulting from ^{13}C-assimilation reveal that cellulose responders exhibit a significantly (p<0.01) greater BD shift than xylose responders (Fig. 3A). A density profile for each responder is generated for the experimental and control treatment at each of the sampling time points using relative abundances from sequence libraries (Figs. S5, S6). The difference in center of mass for each set of density profiles (control and experimental) is measured (supp. MM) and each KDE curve represents the collection of density shifts calculated for all responders in the ^{13}C-cellulose or ^{13}C-xylose treatment (Fig. 3A). We observe xylose utilizers having a smaller density shift (0.008 $\pm$ 0.008 g mL^{-1}) than cellulose utilizers (0.015 $\pm$ 0.009 g mL^{-1}), with few exceptions.

Most xylose responders are found at higher rank abundances than cellulose responders (0.01 <p <0.05), which fall among the rarer taxa in the tail of the RA curve (Fig 3B). This demonstrates that many taxa important to cellulose cycling are present in the rarer fraction of the overall microbial community. Yet, the transitions in abundances of responders is difficult to discern in the bulk community abundances (Figs. S5 , S6) or may not be detected with bulk community sequencing efforts. For example, the increase in Bacteroidetes in the xylose treatment at d3 is not observed in the bulk community abundances. Other instances may result in subtle changes in bulk community abundance that would be difficult to differentiate from natural variation or methodological noise.

Patterns of carbon use vary dramatically within phylum. Dynamic patterns of ^{13}C-assimilation from xylose and cellulose occur at discrete, fine-scale taxonomic units (Fig. 4). Responders for xylose and cellulose are widespread across 6 and 7 phyla, respectively (Fig. 4). There are 5 phyla containing responders for both treatments; of all the responder OTUS detected within those phyla for either xylose or cellulose, there are only six OTUs that respond to both xylose and cellulose (discussed previously). This result suggests that phyla do not represent coherent ecological units with respect to the soil C-cycle, that is, taxa within phyla exhibit differences in substrate use, level of substrate specialization, and dynamics of incorporation.

In this study, we have identified Actinobacteria responders for both substrates (Figs. S5 , S6). Although there were no shared Actinobacteria OTUs that responded to both xylose (Microbacteriaceae, Micrococcaceae, Cellulomonadaceae, Nakamurellaceae, Promicromonosporaceae, and Geodermatophilaceae) and cellulose (Streptomycetaceae and Pseudonocardiaceae). This information may suggest that while Actinobacteria exhibit an ability to utilize an array of carbon substrates, substrate use may be more clade specific and not widespread throughout the phylum (Fig. 4). Similarly, Bacteroidetes responders were identified for both substrates, yet, at a finer taxonomic resolution there is a clear differential response for xylose (Flavobacteriaceae and Chitinophagaceae) and cellulose (Cytophagaceae).

Whole phylum responses were not detected for xylose or cellulose yet utilization of these substrates spanned many phylogenetically diverse groups. Within each phylum we observed substrate utilization at the clade or single taxa level with each exhibiting a unique pattern of ^{13}C-assimilation over time (Fig. 4, heatmap). It has previously been suggested that all taxa within a phylum are unlikely to share ecological characteristics (Fierer *et al.*, 2007), and furthermore, within a species population (Hunt *et al.*, 2008; Preheim *et al.*, 2011; Choudoir *et al.*, 2012). Habitat traits of coastal Vibrio isolates were mapped onto microbial phylogeny revealing discrete ecological populations based on seasonal occurrence and particulate size fractionation (Hunt *et al.*, 2008; Preheim *et al.*, 2011). Yet, it has been proposed that the microbial community functionality responsible for soil C cycling appear at the level of phlya rather than species/genera (Schimel and Schaeffer, 2012). The traditional phylum level assignment conventions could in part be due to limitations in finer scale taxonomic identifications or methodological limitations (*i.e.* sequencing depth). Our data in concert with others (Fierer *et al.*, 2007; Hunt *et al.*, 2008; Goldfarb *et al.*, 2011; Preheim *et al.*, 2011; Choudoir *et al.*, 2012) would suggest that assigning substrate utilization of a few OTUs or clades as a phylum level response is not accurate.

Conclusions. We have demonstrated how next generation sequencing-enabled SIP gives an OTU level resolution for substrate utilization. Using this technique, we are able to resolve discrete OTUs that would otherwise be missed using bulk community sequencing efforts. Additionally, this technique provides greater taxonomic resolution than previous techniques (cloning, TRFLP, ARISA) used to determine substrate utilizing community members. While we are currently able to resolve highly responsive OTUs, there is still a need to resolve taxa that are partially responsive which we cannot differentiate from noise with confidence at this time. Although, if we could identify partially responsive taxa, their contributions to the C-cycle would still be difficult to discern. For example, a generalist utilizing many substrates including ^{12}C substrates and the ^{13}C-labeled substrate may exhibit the same partial labeling that a specialist utilizing both the ^{13}C-substrate and the same substrate (unlabeled) that is inherent in the soil. Additionally, partially labeled taxa could be further down the trophic cascade including predators or secondary consumers of waste products from primary consumer microbes that were highly labeled.

OTUs that assimilate xylose and those that assimilate cellulose are largely mutually exclusive. Those OTUs that assimilate xylose are labeled within 1-7 days, while those that assimilate cellulose are labeled primarily after 2-4 weeks. The xylose responders demonstrate a smaller change in BD than the cellulose responders suggesting that xylose responders assimilate multiple C sources (labeled and unlabeled) consistent with a generalist response, while cellulose responders are more heavily labeled suggesting that cellulose is their main source of C, a response more consistent with a specialist lifestyle. Xylose responders include many taxa, such as spore-fomers, known for the ability to respond rapidly to an influx of new nutrients while cellulose responders include many OTUs that are common uncultivated soil organisms. Finally, xylose responders are more abundant in the community while cellulose responders are, on average, more rare as indicated by their rank abundance within the soil community. These results indicate that different bacteria in soil have distinct physio-

logical and ecological responses which govern their interactions with soil C pools.

We did not observe consistent C utilization at the phylum level although both xylose and cellulose utilization were observed across 7 phyla each revealing a high diversity of bacteria able to utilize these substrates. The high taxonomic diversity may enable substrate metabolism under a broad range of environmental conditions (Goldfarb *et al.*, 2011). Other studies of microbial communities have observed a positive correlation with taxonomic or phylogenetic diversity and functional diversity (Tringe, 2005; Gilbert *et al.*, 2010; Philippot *et al.*, 2010; Bryant *et al.*, 2012; Fierer *et al.*, 2012, 2013). The data presented here supports that specific functional attributes can be shared among diverse, yet distinct, taxa while closely related taxa may have very different physiologies (Philippot *et al.*, 2010; Fierer *et al.*, 2012). This information adds to the growing collection of data suggesting that community membership is important to biogeochemical processes. Furthermore, it highlights a need to examine substrate utilization by discrete microbial taxa within a whole community context to better understand how specific community members function within the whole.

The sensitivity of SIP-NGS provides a means to elucidate substrate utilization by discrete microbial taxa with the hope that we can begin to construct a belowground C food web. We obtained enough information to conclusively determine isotope incorporation for 61% of the more than 6,000 OTUs detected. For those OTUs with enough information (n = 3,825), approximately 2% (n = 72) significantly assimilated ^{13}C from either xylose or cellulose. In the future deeper sequencing will enable us to increase coverage and assess C use by more community members. Using the informations we gain from SIP-NGS, we can expand our knowledge of specific C-cycling OTUs by taking a targeted metagenomic approach in the nucleic acid pools of 'heavy' fractions. Furthermore, we can now expand our knowledge of soil C use dynamics to a wide array of C substrates and increase our grasp on specific community member contributions. Illuminating these microbial contributions associated with decomposition in soil are important because as environments change, there are measurable and functional changes in soil C (Grandy and Neff, 2008) which could cumulatively have large impacts at a global scale.

Methods

Additional information on sample collection and analytical methods is provided in SI Materials and Methods.

Twelve soil cores (5 cm diameter x 10 cm depth) were collected from six random sampling locations within an organically managed agricultural field in Penn Yan, New York. Soils were pretreated by sieving (2 mm), homogenizing sieved soil, and preincubating 10 g of dry soil weight in flasks for 2 weeks. Soils were amended with a 5.3 mg g soil^{-1} carbon mixture; representative of natural concentrations Schneckenberger *et al.*, 2008. Mixture contained 38% cellulose, 23% lignin, 20% xylose, 3% arabinose, 1% galactose, 1% glucose, and 0.5% mannose by mass, with the remaining 13.5% mass composed of an amino acid (in-house made replica of Teknova C0705) and basal salt mixture (Murashige and Skoog, Sigma Aldrich M5524). Three parallel treatments were performed; (1) unlabeled control,(2)^{13}C-cellulose, (3)^{13}C-xylose (98 atom% ^{13}C, Sigma Aldrich). Each treatment had 2 replicates per time point (n = 4) except day 30 which had 4 replicates; total microcosms per treatment n = 12, except ^{13}C-cellulose which was not sampled at day 1, n = 10. Other details relating to substrate addition can be found in SI. Microcosms were sampled destructively (stored at -80°C until nucleic acid processing) at days 1 (control and xylose only), 3, 7, 14, and 30.

Nucleic acids were extracted using a modified Griffiths procotol Griffiths *et al.*, 2000. To prepare nucleic acid extracts for isopycnic centrifugation as previously described Buckley *et al.*, 2007, DNA was size selected (>4kb) using 1% low melt agarose gel and β-agarase I enzyme extraction per manufacturers protocol (New England Biolab, M0392S). For each time point in the series isopycnic gradients were setup using a modified protocol Neufeld *et al.*, 2007 for a total of five ^{12}C-control, five ^{13}C-xylose, and four ^{13}C-cellulose microcosms. A density gradient (average density 1.69 g mL^{-1}) solution of 1.762 g cesium chloride (CsCl) ml^{-1} in gradient buffer solution (pH 8.0 15 mM Tris-HCl, 15 mM EDTA, 15 mM KCl) was used to separate ^{13}C-enriched and ^{12}C-nonenriched DNA. Each gradient was loaded with approximately 5 μg of DNA and ultracentrifuged for 66 h at 55,000 rpm and room temperature (RT). Fractions of $\sim$100 μL were collected from below by displacing the DNA-CsCl-gradient buffer solution in the centrifugation tube with water using a syringe pump at a flow rate of 3.3 μL s^{-1} Manefield *et al.*, 2002 into Acroprep^{TM} 96 filter plate (Pall Life Sciences 5035). The refractive index of each fraction was measured using a Reichart AR200 digital refractometer modified as previously described Buckley *et al.*, 2007 to measure a volume of 5 μL. Then buoyant density was calculated from the refractive index as previously described Buckley *et al.*, 2007 (see also SI). The collected DNA fractions were purified by repetitive washing of Acroprep filter wells with TE. Finally, 50 μL TE was added to each fraction then resuspended DNA was pipetted off the filter into a new microfuge tube.

For every gradient, 20 fractions were chosen for sequencing between the density range 1.67-1.75 g mL^{-1}. Barcoded 454 primers were designed using 454-specific adapter B, 10 bp barcodes Hamady *et al.*, 2008, a 2 bp linker (5'-CA-3'), and 806R primer for reverse primer (BA806R); and 454-specific adapter A, a 2 bp linker (5'-TC-3'), and 515F primer for forward primer (BA515F). Each fraction was PCR amplified using 0.25 μL 5 U μl^{-1} AmpliTaq Gold (Life Technologies, Grand Island, NY; N8080243), 2.5 μL 10X Buffer II (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.5 μL 25 mM $MgCl_2$, 4 μL 5 mM dNTP, 1.25 μL 10 mg mL^{-1} BSA, 0.5 μL 10 μM BA515F, 1 μL 5 μM BA806R, 3 μL H_2O, 10 μL 1:30 DNA template) in triplicate. Samples were normalized either using Pico green quantification and manual calculation or by SequalPrep^{TM} normalization plates (Invitrogen, Carlsbad, CA; A10510), then pooled in equimolar concentrations. Pooled DNA was gel extracted from a 1% agarose gel using Wizard SV gel and PCR clean-up system (Promega, Madison, WI; A9281) per manufacturer's protocol. Amplicons were sequenced on Roche 454 FLX system using titanium chemistry at Selah Genomics (formerly EnGenCore, Columbia, SC)

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Figures

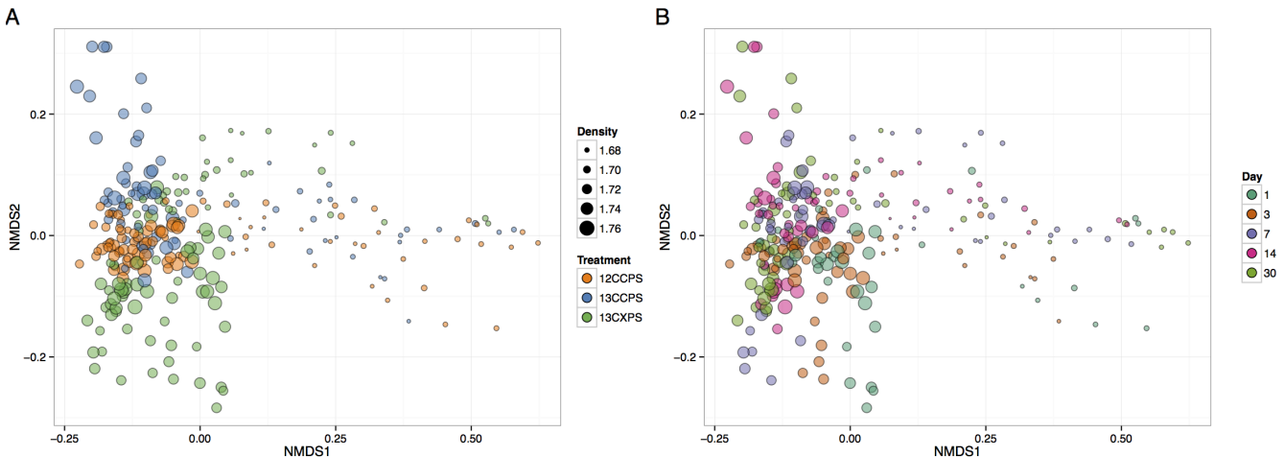

Fig. 1. NMDS analysis from weighted unifrac distances of 454 sequence data from SIP fractions of each treatment over time. Twenty fractions from a CsCl gradient fractionation for each treatment at each time point were sequenced (Fig. S1). Each point on the NMDS represents the bacterial composition based on 16S sequencing for a single fraction where the size of the point is representative of the density of that fraction and the colors represent the treatments (A) or days (B).

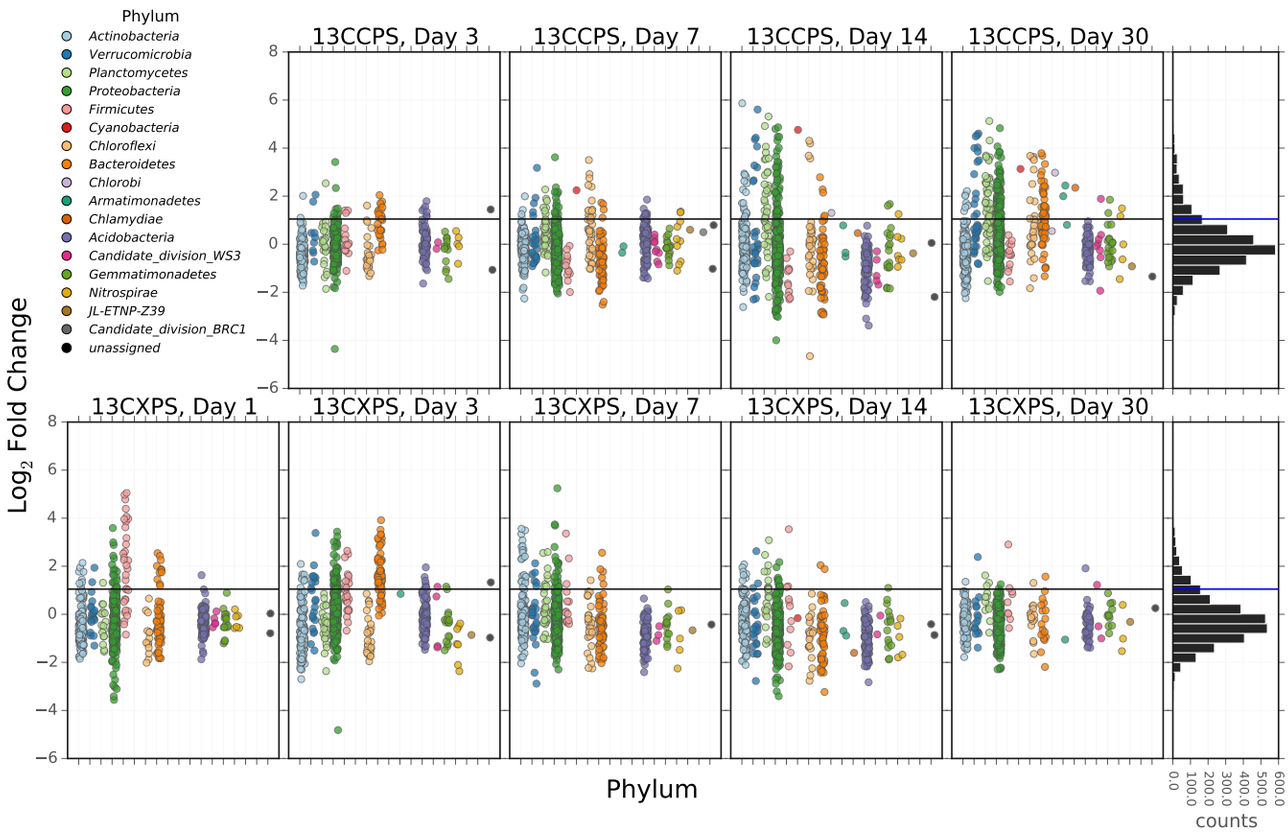

Fig. 2. Log_2 fold change of ^{13}C-responders in cellulose treatment (top) and xylose treatment (bottom). Log_2 fold change is based on the relative abundance in the experimental treatment compared to the control within the density range 1.7125-1.755 g ml^{-1}. Taxa are colored by phylum. 'Counts' is a histogram of number of sequences for each log_2 fold change value.

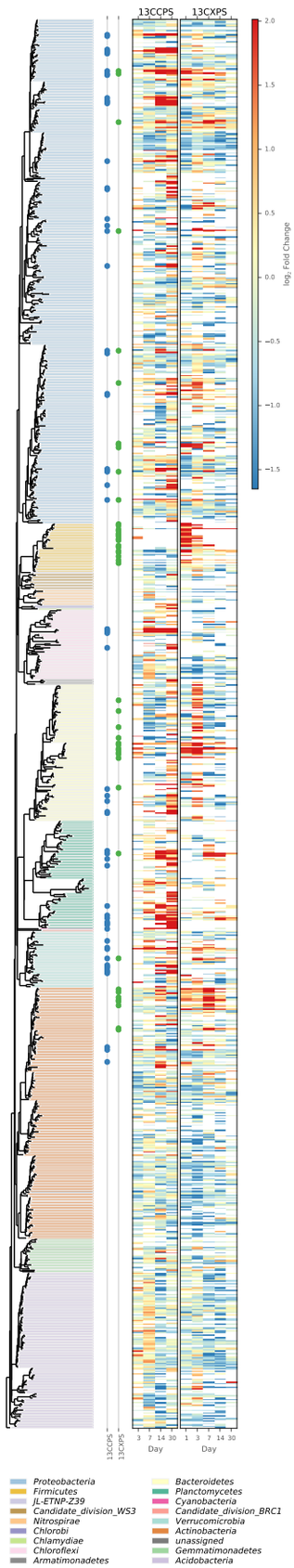

Fig. 3. Phylogenetic tree of sequences passing a user defined sparcity threshold (0.6) for at least one day of the time series. Branches are colored by phylum. ^{13}C-responders for cellulose (blue) and xylose (green) are indicated by a point beside the respective branch. Heatmap demonstrates $\log_2$ fold change of each taxa through the full time series for both treatments (cellulose, left; xylose, right).

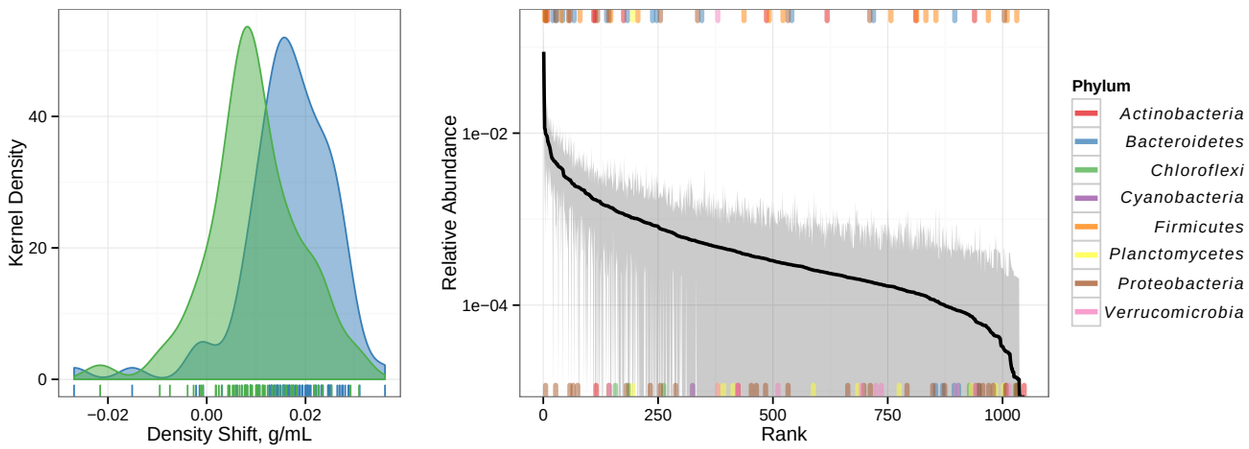

Fig. 4. ^{13}C-responder characteristics based on density shift (A) and rank (B). Kernel density estimation of ^{13}C-responder's density shift in cellulose treatment (blue) and xylose treatment (green) demonstrates degree of labeling for responders for each respective substrate. ^{13}C-responders in rank abundance are labeled by substrate (cellulose, blue; xylose, green) and the phylum which it belongs to.

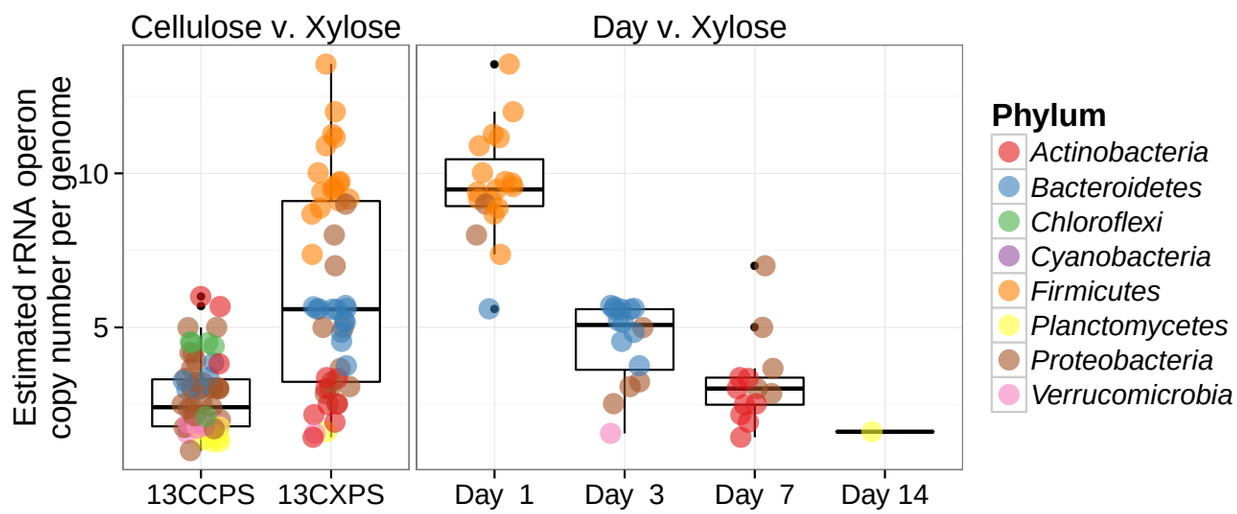

Fig. 5. Estimated rRNA operon copy number per genome for ^{13}C responding OTUS. Panel titles indicate which labeled substrate(s) are depicted.