COMPOSITION OF CULTIVATABLE METHYLOTROPHIC COMMUNITIES THROUGH A SOIL DEPTH PROFILE

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ABSTACT:

Methylotrophy is the ability of microorganisms to utilize single carbon compounds such as methanol (CH_3OH), the second most abundant organic compound in the atmosphere. Methylotrophs have been recognized as the main drivers of methane fluxes, but their role with methanol fluxes have been overlooked. Understanding methanol degraders such as methylotrophs can help better our understanding of Earth's systems to help us combat and understand climate change as well as soil ecosystem health. This study creates a basis for future examinations of the physiological attributes of specific methylotrophs; studies that would further our understanding of the methylotroph's role in methanol exchange. We grew methylotrophic bacteria by plating a dilution of the soil onto methylotrophic specific media. The colonies on the plates were counted and sequenced for identification. We found that there was a decrease in the number of methylotrophs, and a difference between the community types of methylotrophs between different depths that were cultivated from a 12 cm deep soil core.

INTRODUCTION:

Methylotrophy is the ability of microorganisms to utilize single carbon compounds such as methanol (CH_3OH). Many of these single carbon compounds are nonmethane biogenic volatile organic compounds (BVOCs). This means they are produced by living organisms, and have high vapor pressure (thus are readily exchanged between the soil and the atmosphere). Methanol is the second most abundant organic compound in the atmosphere (Stacheter et al., 2013), with the primary source of methanol being plant biomass decomposition (Galbally, & Kirstine, 2002). The specific microbes that degrade methanol are called methylotrophs and are found in the phyla of *Actinobacteria*, *Verrucomirobia*, and *Proteobacteria* (Chistoserdova, Kalyuzhnaya, & Lidsrom, 2009). Methylotrophs oxidize methanol to formaldehyde with methanol-specific dehydrogenases (Stacheter et al., 2013). Formaldehyde is then either oxidized to carbon dioxide as a source of energy, or assimilated into biomass via the Ribulose Monophosphate Cycle, or the Serine Cycle (Kelly, Ardley, & Wood, 2014).

Methylotrophy has been recognized in the scientific microbial community since the early 20^{th} century, and many early attempts to cultivate methylotrophs were not successful (Whittenbury & Dalton, 1981). However, in the past thirty years, much has been discovered about methylotrophy. There have been new techniques developed, more genes discovered, and their physiology is better understood (Chistoserdoval, Kalyuzhnaya, & Lidsrom, 2009). Despite this, many methylotrophs have never been cultivated, and their overall impact on the Earth's methanol exchange is unknown. Understanding Earth's methanol flux can help better our understanding of how soil biogeochemistry affects the atmosphere. This study creates a basis for future examination of the physiological attributes of specific methylotrophs to further our understanding of their role in the Earths terrestrial and atmospheric methanol exchange.

Despite the ubiquity of methylotrophs, including in terrestrial habitats (Kelly, Ardley, & Wood, 2014), their roles in terrestrial methanol sinks are poorly understood because many species have not been cultivated (grown in the laboratory), or studied at all; known methanol measurements from methylotrophs are missing from terrestrial ecosystems (Stacheter, et al., 2013). If an organism has not been cultivated in the laboratory, its traits (like metabolism) cannot be studied. Since many methylotrophic metabolisms (specifically their methanol uptake) have been poorly characterized between species, the role that methylotrophs play in terrestrial BVOC

emissions and uptakes is poorly understood (Stacheter et al., 2013) (Gray, Monson, Fierer, 2014). Since the first 10 cm of soil typically have the most bacterial diversity (Eilers, Debenport, Anderson, & Fierer, 2012) we chose to study the first 12 cm of soil for our profile in order to encompass the greatest number of bacteria. The top 15 cm is the most studied profile of bacteria in general, but not necessarily for methylotrophs. Since studies in the area of methylotrophy are lacking, we have decided to look at a depth profile of a soil on the University of Colorado at Boulder's main campus near Varsity Lake with ideal conditions (a relatively undisturbed area with large amounts of degrading plant litter). We studied whether or not there was a difference on the community composition and abundance of methylotrophs throughout a depth of the first 12 cm of soil. Edaphic factors such as pH, nutrients, organic carbon, moisture, and oxygen all change with depth. Since these factors change with depth, it could be expected that a corresponding change would occur with the microbial community (Eilers, Debenport, Anderson, & Fierer, 2012). Because much of the methanol comes from the plant biomass on the surface of the soil, we hypothesize (H1) that the abundance of cultivatable methylotrophs decreases as depth increases. In addition, we also hypothesize (H2) that the community composition will change across the depth profile.

METHODS:

Sample Collection

The soil samples were collected at a site on the University of Colorado at Boulder campus. The area is located near Varsity Lake on the northwest area of main campus. Coordinates for this location are 0°00'36.4"N 105°16'25.5"W. The site was chosen for the minimal disturbance it receives: the site is not maintained for landscape, nor is it very accessible to people. The tree type was mixed deciduous and conifer. The sample was collected using a soil corer. The depth taken was 12 cm deep, and was separated into five depth sections: The leaf layer (on top of the soil), 1-3 cm, 4-6 cm, 7-9 cm, and 10-12 cm. Each section was sieved to 2 mm, put into sterile whirl-paks, and labeled accordingly.

Sample Processing and Inoculation

For each depth, sieved soil was added to phosphate buffered saline (PBS; 1% weight/volume), blended (1 min), and iced (5 min) three times. The soil-PBS slurry was serially diluted to a final dilution of 10^{-6}g soil. For each depth, 500 μL of the 10^{-4} g soil, 10^{-5}g soil, and 10^{-6} g soil dilutions were spread on each plate by rolling sterilized beads across the media, which means the plates were inoculated with 5×10^{-5} g soil, 5×10^{-6} g soil, and 5×10^{-7} g soil solution. Five replicates of each dilution of each depth were conducted. The inoculated plates were incubated at 25°C for 8 days, after which, microbial colonies were counted on the 5×10^{-7} plates.

In order to identify taxa grown on each plate, colonies were picked into individual wells of a 96 well plate that contained the sterile liquid growth medium containing identical nutrient amendments as the medium the cultures were originally isolated on. The inoculated 96 well plates were incubated for 1 month.

Media

The media was formulated to accommodate the growth of methylotrophic bacteria. We used aspects of existing methylotrophic growth media preparations (Kelly, Ardley, & Wood, 2014) and added new components such as reducing substrate concentrations, methylotrophic-specific cofactors, and trace elements.

First the salts from **Table 1** were added to a 1L bottle with deionized (DI) water. Gellan gum (9 gL^{-1}) was added at this time. The media was autoclaved on the liquids cycle at 121°C for 90 min. Once out of the autoclave, the media was placed in a hot water bath at 60°C for 60 min. Once cool, the contents of **Table 2** were added to the media. Once all of the compounds were dissolved in the media, it was then poured into petri dishes, which were then stored to cool to room temp.

Once the colonies were counted, the isolated colonies were picked into 96 well plated that contained all components of the media in the plates except for gellan gum and the antibiotics (cycloheximide and nystatin).

Table 1: Pre Autoclave

Compound	Final Concentration (mM)
$MgSO_4$	5.5
(NH4)2SO4	3.8
CaCl2	0.14

Table 2: Post Autoclave

Compound	Final Concentration (mM)
KH2PO4	396
K2HPO4	402
EDTA	53.7
Methanol	246
NiCl2*6H20	0.001
CoSO4*7H2O	0.001
NaMoO4	0.001
ZnSO4*7H2O	0.001
FeSO4	0.005
CuCl2	0.005
LaCl3	0.001
PQQ	0.00006
B1	0.001
B7	0.0001
B12	0.0005
4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP)	0.001
4-amino-5-aminomethyl-2-methylpyrimidine (AmMP)	0.001
Cycloheximide	0.000355

Nystatin	0.000108

DNA Extraction, PCR, and Sequencing

The cells from the 96 well plates were processed for PCR using the 515fBC/806r barcodes (515F5'-GTGCCAGCMGCCGCGGTAA-3' & 806R 5'- 309 GGACTACHVGGGTWTCTAAT-3') and the master mix GoTaq Hot Start Colorless Master Mix from Promega. The PCR cycle parameters were 94°C for 3 min, followed by 35 cycles of 94°C 45 s; 50°C 60 s; 72°C 90 s, with a final extension of 72°C for 10 min. The PCR products were then cleaned, pooled, and normalized with ThermoFischer Scientific SequalPrep Normalization Plate kit using the manufacturer's instructions. The 16S rRNA amplicon pools were sequenced on an Illumina MiSeq run, using 151-bp paired end sequencing. The sequencing was done at the University of Colorado, Boulder Next Generation Sequencing Facility. The raw sequence data was then processed to obtain a rarified OTU table by using the mctools R package from J. W. Leff (Getting Started with mctoolsr, found on github.com/leffj/mctoolsr). The relative percents were calculated to 90% or above. This allowed us to see the dominant taxa found in each of the wells. Once this was found, we determined what taxa were cultivated from each depth.

RESULTS:

Abundance:

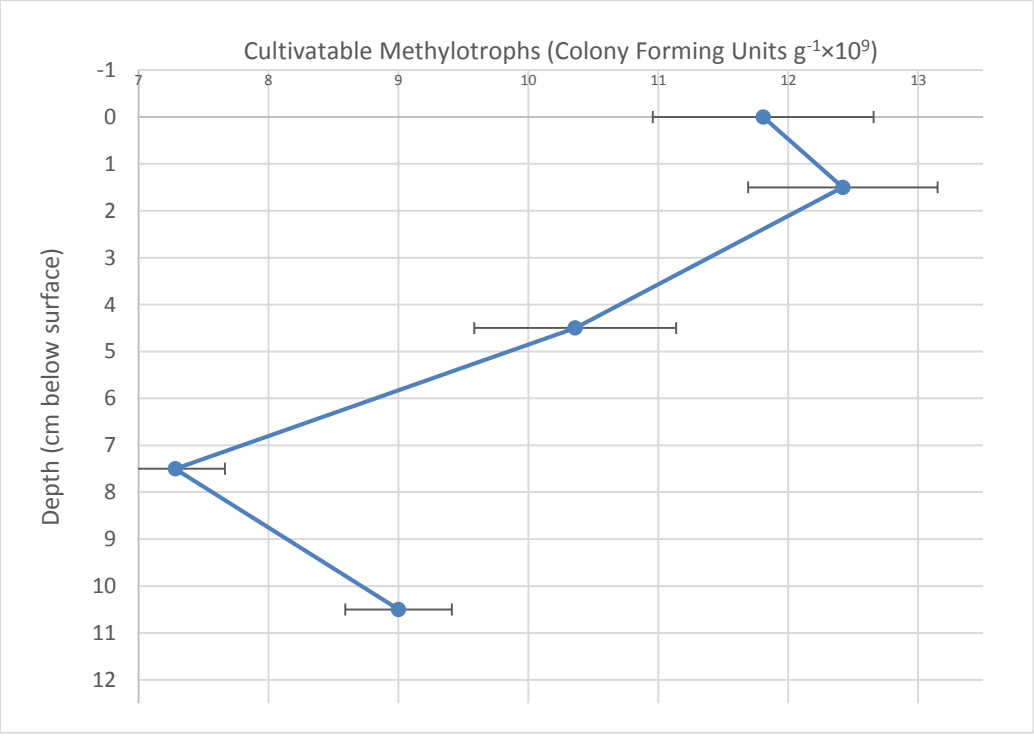

Figure 1*:* The abundance of methylotrophic colony forming units decreases with depth. Points are the mean plus/minus SE CFU g^{-1} soil. The 0 cm mark represents the leaf layer. The points represent an average of the surrounding area, e.g. the 2 cm mark represents the average abundance in the soil from the 1-3 cm section, the 5 cm mark is an average representative of the 4-6 cm section, and so on.

Figure 1 shows that the leaf layer contains similarity with the first 3 cm of soil. This makes sense because of their proximity: the leaves are in direct contact and likely have a similar amount of methanol. As the depth increases, the number of methylotrophic bacteria decreases, except for the last section 10-12 cm. However, the general trend supports the hypothesis (h1) that methylotrophic bacteria will decrease in abundance as depth increases. The increase seen from the 10-12 cm section could stem from a pocket of high nutrient levels, and could be further investigated with more replicate soil core samples in future studies.

Richness

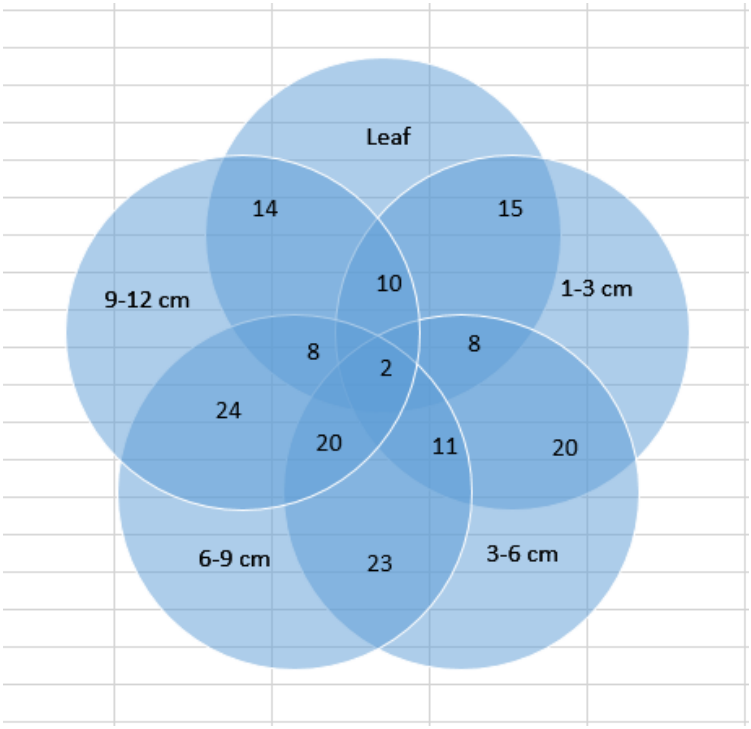

Figure 2: The number of cultivated methylotrophs are not uniform across the depth profile. This Venn Diagram represents the number of shared bacteria present and not present between soil layers.

The cultivated Methylotrophs did not have a uniform community composition across the 12 cm depth. We identified 32 types of methylotrophic bacteria. **Figure 2** shows that the types of bacteria present and not present become more and more similar (or shared between depth sections) as depth increses.

Discussion

We identified 11 unique methylotrophic *Actinobacterial* taxa, 5 *Bacteroidetes* and 16 *Proteobacteria*. Methylotrophic *Actinobacteria* are Gram-positive, and are quite ubiquitous in the human mouth (*Brevibacterium, Gordonia, Leifsonia, Microbacterium, Micrococcus,* and *Rhodococcus*); these can utilize methanol, and methylamine (Hung, Wade, Boden, Kelly, & Wood, 2011). We have cultured *Actinobacteria Rhodococcus* and *Actinobacteria Microbacterium* from our soil. Many different *Actinobacteria Streptomyces* were grown in the soil core. They have similar pathways that methylotrophs have (Korotkova, Chistoserdova, Kuksa, & Lidstrom, 2002), and interestingly, no *Streptomyces* were found in the leaf layer.

Proteobacteria can also be found in the mouth (*Achromobacter*, *Klebsiella, Methylobacterium, Pseudomonas,* and *Ralstonia*). These can utilize methanol and methylamine (Hung, Wade, Boden, Kelly, & Wood, 2011) as well. We only cultured *Proteobacteria Methylobacterium,* and *Proteobacteria Pseudomonas umsongensis* from our soil. *Proteobacteria Methylophilaceae* are Gram-negative, and have been found to be methylotrophic (Lapidu et al., 2011). *Proteobacteria Labrys, Proteobacteria Rizhobiales, Proteobacteria Aminobacter, Proteobacteria Methylobacterium, Proteobacteria Methylopila* (Gram-negative, facultative (can also utilize multi-carbon compounds) methylotroph, and colorless (Doronina, Trotsenko, Kraudova, Boulygina, & Tourova, 1998)) have all been identified as containing methylotrophic bacteria (Beck et al., 2015). *Proteobacteria Mesorhizobium* also contains known methylotrophs (Kumaresan, Wisher, Hillebrand-Voiculescu, & Murrell, 2015). *Proteobacteria Methylobacterium adhaesivum* is Gram-negative, pink pigmented, and is also a known methylotroph (Gallego, García, & Ventosa, 2006). *Bacteroidetes Sphingomonas* is Gram-negative, has yellow pigmented colonies, and one strain (sp. 2MPII) is known to degrade 2-methylphenanthrene (Ni'matuzahroh, Gilewicz, Guiliano, & Bertrand, 1999). The Phyla *Bacteroidetes* contains a genus known to have methylotrophs, *Flavobacterium,* which we have cultivated (*Bacteroidetes Flavobacterium succinicans*) from our soil core in the leaf layer and the 1-3 cm section. The literature reads that few *Bacteroidetes Flavobacterium* species have been isolated from soil environments: *Bacteroidetes Flavobacterium glycines* (from the rhizosphere of soybeans), *Bacteroidetes Flavobacterium pharmagmitis* (from roots of reeds) (Irzykowska, 2014), and now *Bacteroidetes Flavobacterium succinicans* which we cultivated from our soil core. *Bacteroidetes Pedobacter* is also know to grow on methanol in soil (Aydin, 2012). *Dyadobacter* contains the metabolic pathways to metabolize methanol (KEGG, Dyadobacter fermentans). In general, methylotrophy is widespread in the three Phyla we have cultivated.

Only two methylotrophs were present in all five depths, and they are in the Phylum *Actinobacteria* (genus *Rhodococcus*), and *Proteobacteria* (genus *Mesorhizobium*). The *Actinobacteria Rhodococcus* is gram-positive, non-sporulating, and thrive in a broad range of environments, like soil – it can catabolize a wide range of compounds. *Proteobacteria Mesorhizobium* are gram-negative soil bacteria. Eight bacteria were found specifically only at the leaf layer: *Proteobacteria Sphingomonas, Bacteroidetes Dyadobacter, Proteobacteria Mycoplana* (contains strains that are pink pigmented facultative methylotrophs (PPFMs) (Green, 2006)), *Proteobacteria Methylobacterium adhaesivum, Actinobacteria Nocardioidaceae, Proteobacteria Aurantimonadaceae, Proteobacteria Methylophilaceae,* and *Proteobacteria Pseudoxanthomonas*. This is representative of all three Phyla found in our study. There were three bacteria that were found throughout the soil core, but not in the leaf layer, each of which represented the phyla found in our study: *Bacteroidetes Pedobacter* (although, this taxa has been identified as a contaminant of DNA extraction kit reagents and ultra-pure water systems, which could be the reason it appears in metagenomic datasets (Salter et al., 2014)), *Proteobacteria Phyllobacterium,* and *Actinobacteria Streptomycetaceae*.

There were five different types of bacteria found solely in the 1-3 cm section of soil; these bacteria represent all of the phyla that has been identified in this study: *Actinobacteria Microbacterium, Actinobacteria Streptomyces mirabilis, Actinobacteria Solirubrobacteraceae, Sphingobacterium faecium* (some other species in *Sphingobacterium* were found to utilize methanol (KEGG, Sphingobacterium sp. 21)), *Proteobacteria Methylopila*. This section of soil

had the most diversity with a total of 14 different organisms that were identified. This makes sense because the uppermost layers of soil typically contain the most diversity.

Only two types of bacteria were purely found in the 4-6 cm section: *Proteobacteria Methylibium*: The known sp. *Petroleiphilum* is Gram-negative, methyl tert-butyl ether – degrading methylotroph and is non-pigmented (18), so we know *Proteobacteria Methylibium* contains methylotrophs, but *Petroleiphilum*, specifically, was not cultivated from our soil core, and *Proteobacteria Yersinia*: a Gram-negative bacteria capable of methane utilization: it degrades methane to methanol, and then utilizes the methanol (KEGG,Yerisina ruckeri)). A total of ten different bacteria were found in the 4-6 cm section.

Four different types of bacteria were found purely in 7-9 cm: *Actinobacteria Xylanimicrobium*, *Actinobacteria Streptomyces*, *Proteobacteria Pseudomonas umsongensis* (a Gram-negative, yellow-white, isolated from soil. (Kwon et al, 2003)), and *Proteobacteria Lysobacter*. A total of thirteen types of bacteria were found in the7-9 cm section. However, no organisms were found to only exist purely in the 10-12 cm section, but there was a total of nine different bacteria found.

Methylotrophs have been studied since the beginning of the 20th century, and we are continually learning more. Many methylotrophs are not obligate, (i.e. do not only thrive on C1 compounds), and are facultative (Chistoserdova, 2011). New knowledge of all the possible types of methylotrophs has led to a broader inclusion of what could be a methylotroph (Chistoserdova, 2011), and many bacteria have yet to be identified as such. Many of the unique taxa identified in this study have not been identified to a genus or species level. The lack of identifying the numerous methylotrophic pathways in bacteria could be the reason for the lack of specified identification in this study. The bacteria taxa that were found were researched, and methylotrophy was not conclusively found in seven taxa: *(Phylum) Actinobacteria (Family) Nordioidacaeae, (Phylum) Actinobacteria (Genus) Agromyces, (Phylum) Actinobacteria (Genus) Xylanimicrobium, (Phylum) Bacteroidetes (Genus) Sphingobacterium (species) faecium, (Phylum) Proteobacteria (Genus) Lysobacter and Pseudoxanthomonas, (Phylum) Proteobacteria (Genus) Phyllobacterium*, and *(Phylum) Proteobacteria (Family) Rhizobiaceae.*

Nordioidacaeae, which was found in the leaf section, is known to be found in soils (Dastager et al, 2008). *Argromyces*, is in the family of *Microbacteriaceae*, which has known methylotrophs (found in the 10-12 cm and 3-6 cm sections) has been known to be found in soil (Lee, Ten, Woo, & Park, 2011). *Xylanimicrobium* was found in the 7-9 cm section. *Sphingobacterium* (species) *faecium* (found in the 1-3 cm section) has many similarities to bacteria in the family *Flavobacteriaceae* which has lots of methylotrophs, and is also a genus known to be found in soil (Tronel et al, 2006). *Rhizobiaceae and Phyllobacterium* (found in all sections but the leaf section) share the Order *Rhizobiales* (found in sections 3-6 cm, and 6-9 cm) has many methylotrophs (Pini et al, 2012). *Lysobacter* (6-9) and *Pseudoxanthomonas* (leaf) are of the order Xanthomonadaceae which is known to use formate, a c1 compound (even though not methanol), and is thus a methylotroph (Nercessian et al, 2005).

The data we obtained from our CFU calculations supported our hypothesis that the abundance of cultivatable methylotrophs decreases as depth increases. The standard error bars do not overlap for the mean calculations for all the depths, except for the leaf layer which overlaps with the 1-3 cm section of soil. This makes sense because they are juxtaposed to the topmost

portion of soil which many studies have found to have to most amount of bacteria (Eilers, Debenport, Anderson, & Fierer, 2012).

Of the 32 bacteria found, we calculated the number of similar bacteria present and not present between the soil sections (seen in **Figure 2**). The leaf layer and the first soil depth ,1-3 cm, have a 15 overlap in found taxonomies, though other depths have 20 (between 1-3 cm and 4-6 cm) 23 (between 4-6 cm and 7-9 cm), and 24 (between 7-9 cm and 10-12 cm). The similarity between the depths seem to increase the deeper the soil. This would seem to support our second hypothesis that the community composition changes across a depth profile, though it also looks like they become more similar the deeper the along a depth profile. In order to obtain a more comprehensive result, more cores should be sampled in future studies.

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