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# Cropping Sequence Affects Subsequent Soybean Yield, Soil Microbiome and Soil Health

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## CROPPING SEQUENCE AFFECTS SUBSEQUENT SOYBEAN YIELD, SOIL

### MICROBIOME AND SOIL HEALTH

BY

IZZET BULBUL

A thesis submitted in partial fulfillment of the requirements for the

Master of Science

Major in Plant Science

South Dakota State University

2019

# CROPPING SEQUENCE AFFECTS SUBSEQUENT SOYBEAN YIELD, SOIL MICROBIOME AND SOIL HEALTH

## IZZET BULBUL

This thesis is approved as a creditable and independent investigation by a candidate for the Master of Science in Plant Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

> Shin-Yi Marzano, Ph.D. Thesis Advisor Date



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Dean, Graduate School Date

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# **CONTENTS**



#### ABBREVIATIONS

ACE: Autoclaved Citrate Extractable ANCOM: Analysis of Composition of Microbiomes BCA: Bicinchoninic Acid Assay BSA: Bovine Serum Albumin C: Carbon CaCl <sup>2</sup>: Calcium Chloride CCA: Canonical Correspondance Analysis CC: Continuous corn CCS: Corn/corn/soybean CSC: Corn/soybean/corn CTAB: Cetyltrimethylammonium Bromide EtOH: Ethanol ITS: Internal Transcribed Spacer KMnO <sup>4</sup>: Potassium Permanganate LOC: Labile Organic Carbon Mnm: Monmouth MUB: Modified Universal Buffer N: Nitrogen Pg: Petagram PNG: p-Nitrophenyl β-D-glucoside POXC: Permanganate -oxidizable carbon OTUs: Operational Taxonomic Units rRNA: Ribosomal ribonucleic acid SCS: Soybean/corn/soybean SEWS -M: Salt/Ethanol Wash Solution SOC: Soil Organic Carbon SOM: Soil Organic Matter THAM: Tris Aminomethane T1: Continuous corn (CCCCCCCCCCCCCC -S) T2: 2 -yr of corn (CCSCCSCCSCCSCC -S) T3: 1 -yr of corn (SCSCSCSCSCSCSC -S) T4: 1 -yr of soybean (CSCSCSCSCSCSCS -S) Urb: Urbana

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#### ABSTRACT

# CROPPING SEQUENCE AFFECTS SUBSEQUENT SOYBEAN YIELD, SOIL MICROBIOME AND SOIL HEALTH

## IZZET BULBUL

#### 2019

Rotation is an important cultural practice that farmers use to manage crop growth and diseases. Diversified crop rotations directly affect plant residue inputs that may enrich specific groups of microbes that form beneficial associations with the following crop. At two field sites, yield differences were observed in soybeans planted after the following four long-term (14 year) crop sequences: continuous corn (CC), corn (*Zea mays*)/corn/soybean (*Glycine max*) (CCS), corn/soybean/corn (CSC),

soybean/corn/soybean (SCS). Soybean yields were in decreasing order,

CC>CCS>CSC>SCS, and the yield differences could not be explained by soil chemical and physical properties previously reported by a different study on the same sites. Our goal was to relate soil biological properties, including soil health indicators and soil microbial community composition, with the differences in soybean yields. To assess the soil health, soil protein, permanganate-oxidizable carbon (POXC) and β-glucosidase activity were measured. After 14 years of the above-mentioned rotation regimes, soil protein was significantly higher in continuous corn (CC) plots compared with other rotations ( $p<0.05$ ) in one of the two sites. POXC was also significantly greater in CC plots in one of the two sites (P<0.001). For microbial composition, we found uncultured order JG30-KF-AS9 was associated with higher POXC and protein levels. The taxa of bacteria *Chthoniobacter*, and one taxa of fungi, *Ascomycota*, were associated with higher levels of β-glucosidase and correlated with the lower soybean yield observed in the SCS treatment. We also found a differential abundance of specific bacterial and fungal Operational Taxonomic Units (OTUs) to be informative on predicting yield differences, especially fungal pathogens from the genera *Macrophomina* and *Corynespora* at one location. Informative bacterial OTUs, however, are not pathogens, and belong to an uncultured family.

#### CHAPTER ONE

#### LITERATURE REVIEW

Crop rotation is a series of different crops planted in the same field following a defined order (e.g. Corn-soybean) to manage the soil quality, soil fertility, water, weeds and diseases in an agroecosystem (1). It is a common practice for farmers to "rotate" the type of crops planted in their fields throughout the years (2, 3). Corn (*Zea mays*) and soybeans (*Glycine max*) are the annual main crop species in the Midwest U.S. that cover about 75% of the land surface (4). Several factors are at work to sustain this practice: efficient pesticides and fertilizers, the policies of the government and favorable economics (5, 6). In the 1950s and early 1960s, the use of fertilizers and pesticides were considered as a probable substitution for crop rotation, but that theory was proved wrong (2). Crop rotation has been largely accepted to have many advantages (7). Rotating crops is thought to increase the yield and sustainability (2). One study conducted six types of crop rotations to evaluate corn grain yields and the results showed that the rotations with alfalfa, soybeans, oats were associated with enhanced corn grain yields (6). R. Holliday (8) reported that regular corn and beans crop rotation could help to decrease the effect of *Ustilago maydis* disease, which affects overall corn yields by balancing the nitrogen to phosphorus ratio. Crop rotations play a role in improving the soil nutrient cycle because different crops can be effective in recovering different nutrients lost from soil (9). Rotating crops helps to increase soil microbial functions, soil accumulation and carbon segregation in the soil. Hence, crop rotations have a significant impact on improving soil quality (10). Moreover, crop substitutions have a positive effect on soil organic carbon

(SOC) and microbial biomass, break weed and disease cycles and decrease soil erosion (7, 11, 12).

The long-term crop rotation experiment helps to identify problems that threaten future productivity as an early warning system (13). The properties of soil are affected by crop rotation and tillage management. Therefore, it is important to understand the longterm rotation impacts to sustain optimum soil properties (14). Having sustainable agriculture management by using long-term crop rotation is an imperative helper of our knowledge of practical solutions. Most of the existing agricultural problems may be explained by using long-term experiments (15). Therefore, it is important to evaluate different cultural practices, such as crop rotation, for the response of microorganisms in the soil because soil microorganisms can act as a bioindicator of changes in soil conditions (16).

The structure of the microbial communities associated with soil and plants can be affected by crop rotation (17). A previous study showed that crop rotation had a positive impact on the microbial community structure in the soil by increasing microbial diversity when crops were rotated compared to when the same crop was continuously planted  $(18)$ . It is known that microbial communities respond to factors such as plant host and genetics, soil properties and environmental conditions (1). Additionally, land management influences the biological activity in soil, and soil microbial activity is relevant to soil erosion rates (19). The right management of the soil is one of the cornerstones of agricultural management. Soil provides basic ecosystem services, including nutrient cycling, water regulation and the transformation of organic materials and toxic compounds, alongside pest and disease control (20).

Microorganisms represent the countless and metabolically complex life forms in the soil. On a per gram soil basis, it is estimated that there are at least one billion bacteria, a million fungi, millions of protozoa, thousands to millions of algae and several dozen nematodes present (1). The microbiome is an integral part of almost all soil processes (21). Soil microorganisms producing extracellular enzymes are responsible for the biotransformation that provides the nutrients to plants and for maintaining the soil function (22). Soil microbial communities are particularly important to the support of ecosystems around the world, impacting nutrient cycling (22, 23), carbon cycling (24, 25), suppression of diseases (17) and enrichment and conservation of soil organic matter (SOM) (24, 26, 27). Furthermore, microbial diversity and composition are the main factors that ensure the maintenance of ecological functions (28-30). In this way, elucidating the causes and controls of the soil microbial community's distribution and composition are necessary to reach a better understanding of sustainable agriculture (31, 32). Since soil microbiome plays a critical role in the improvement of soil from degradation (33, 34), the evaluation of microbial properties provides valuable information in soil health and soil quality, which contribute to sustainable agriculture (35).

Bacteria are known as the most abundant species among the microorganisms (36, 37) and contribute to many activities, including nitrogen-fixing and carbon cycling in the soil (37, 38). There are several algorithms and databases used to identify microorganisms taxonomically (39). Based on the amplicon sequencing approach, 16S ribosomal RNA (rRNA) gene sequences are commonly used to determine the bacterial classifications (40). Analysis of the 16S rRNA gene sequence is a suitable way to detect the uncultured

bacteria. It can also be used routinely for the identification of mycobacteria and may aid in recognition of new pathogens and uncultured bacteria (41)

#### *Permanganate Oxidizable Carbon (POXC)*

Organic matter content is an indicator of soil health. Permanganate oxidizable carbon (POXC) is a simple procedure for estimating labile organic carbon in soil. POXC is used as an indicator of soil quality parameters to determinate soil labile organic carbon (LOC), which is the most reactive and dynamic driver in soil organic carbon (SOC) (42). The advantages of POXC are that it is a low cost and harmless method for measuring LOC (43). The SOC pool is greater than the atmospheric carbon pool and biomass carbon pool by 2.2 times and 2.8 times, respectively (44, 45). Soils form the greatest terrestrial organic carbon pool with more than 1550 Pg (1 Pg =  $10^{15}$  g) (46). Carbon sequestration in soil is a mechanism to reduce the carbon dioxide concentration in the atmosphere (45). On the other hand, LOC is directly associated with soil carbon (C) and nitrogen (N) mineralization (47), and LOC may promote plant productivity due to influences on enhancing soil health and fertility (48). Positive correlations have further been found between POXC and soil-microbial parameters, comprising microbial biomass and, in particular, organic C (42, 49).

#### *Soil Protein Index*

The Autoclaved Citrate Extractable (ACE) Protein Content refers to the quantity of protein available in the SOM (50). The largest organic N pool in the soil is represented by proteins (51-53). The labile organic N pool is used to evaluate soils capacity to provide N (54). Since labile soil organic matter is an energy source accessible to microorganisms, it provides mineralization by playing a dominant role in N

mineralization in soil (55). Regarding plant growth and development, N mineralization is a critical process in the soil to provide an adequate amount of N for the use of the plant (56). Since protein content is an indicator of biological and chemical soil health, especially for SOM quality, it is directly linked to general soil health status (57). N-linked glycoprotein, which is called glomalin, is produced by arbuscular mycorrhizal fungi hyphae (58, 59), and glomalin is considered an enhancer of soil drainage, microbial activity and carbon sequestration in soil ecosystems (60). Also, there have been many studies that show soil protein is sensitive to crop rotation and tillage (57, 61-63). *β-glucosidase Enzyme Activity*

Soil β-glucosidase, which plays a central role in the carbon cycle as an active enzyme in the soils (64), is one of the important indicators of soil quality (65). In terms of the carbon cycle, the importance of soil microorganisms in many ecosystems hinges on breaking down cellulose in plant cell walls (66). Cellulose is an organic compound widely found in the biosphere (67). β-glucosidase activity, which has a role in the final stage of cellulose degradation in soils, supplies important energy sources, like simple sugar, for microorganisms (68). Microorganisms have an important position for βglucosidase activity in the soils. Several microorganisms are reported for the production of β-glucosidase, especially filamentous fungi, such as *Penicillium brasilianum* (69), *P. decumbens* (70), *Aspergillus niger* (71), *A. oryzae* (72), *Phanerochaete chrysosporium* (73), *Paecilomyces* sp. (74), *Debaryomyces pseudopolymorphus* (75)*, Stachybotrys* sp. (76) and *Fmitopsis palustris* (77). Reports for the production of β-glucosidase from yeast such as *Candida* sp. (78), and from various bacteria such as *Flavobacterium johnsonae* (79) and *Lactobacillus plantarum* (80) are also available.

S. M. Zuber, et al. (14) concluded that long term cropping sequences between continuous corn and corn-soybean rotation do not result in significant differences in soil's physical and chemical properties. Therefore, it was hypothesized that biological properties, including soil health indicators, diversity, and richness of soil microbiome, were correlated with differences in the productivity of the crop. Our objectives were to determine the effect of rotation on soil protein, organic carbon and β-glucosidase, and to identify microbiome composition differences in four different crop rotations that correspond to different soybean yields following the long-term rotation regimes.

#### CHAPTER TWO

# CROPPING SEQUENCE AFFECTS SUBSEQUENT SOYBEAN YIELD, SOIL MICROBIOME AND SOIL HEALTH

#### INTRODUCTION

A crop rotation is a series of different crops planted in the same field following a defined order (e.g. Corn-soybean) to manage the soil quality, soil fertility, water and weeds in an agroecosystem. Crop rotations are useful to the farmers for recycling different types of nutrients from the soil required by different crops. In order to maintain the fertility of the soil, the crops should be changed routinely, since not all the same nutrients are being used at the same rate each season. When planted within corn-soybean systems, diversified cropping rotations can provide important ecosystem services, such as enhancing C concentration and nutrition levels in the soil, which, in turn, provides environmental benefits like reduced soil erosion and nitrate leaching (1).

The observations that yield differences often do not correlate well with soil chemical and physical properties suggest that plant-associated microbiomes, i.e. "phytobiomes," can be the drivers for such differences (14, 81). Soil health is the term used for defining the properties of soil to sustain plant and animal productivity, maintain or enhance water and air quality and support human health and habitation, within the natural or managed ecosystem boundaries. It is vital for agriculture business operations since soil health is degrading at a very fast rate due to modern agricultural practices like aggressive tillage, mono-cropping, excessive usage of inorganic fertilizers, excessive removal of crop residues and usage of broad-spectrum pesticides (1). In order to assess soil health, we used main three biological indicators, POXC, protein content and βglucosidase activity, to investigate the correlation between these parameters and the microbiome. Since soil bacteria and fungi directly mediate the carbon and nitrogen cycle, and regulate the nutrient availability for plants, these three soil biological indicators are expected to be associated with members of the soil microbiome.

#### MATERIALS AND METHODS

#### *Fields Descriptions and Soil Sampling*

Field conditions were described in a published article (82).The Urbana soil site structure is on Flanagan silt loam and the Monmouth site soil structure is on Muscatune silt loam. Soils were sampled at a 0-15 cm depth from 14 year long-term rotation plots (Northwestern Illinois and Agricultural Research Center of the University of Illinois, Urbana-Champaign) with two locations (Monmouth IL (GIS: 40.931 -90.722) and Urbana IL (GIS:40.048 -88.232)). The experiment had 4 treatments: T1: Continuous corn (CCCCCCCCCCCCCC-S), T2: 2-yr of corn (CCSCCSCCSCCSCC-S), T3: 1-yr of corn (SCSCSCSCSCSCSC-S) and T4: 1-yr of soybean (CSCSCSCSCSCSCS-S) ahead of soybean x 4 rep (block) x 3 subsamples/plot in 2016. The field layout followed the random complete block design at both locations. After sampling, soils were kept cool during transportation and stored in a -80°C horizontal freezer until further processing. *Determination of Permanganate Oxidizable Carbon (POXC)* 

The procedure defined by Weil et al. (42) was followed for the measurement of the POXC. The standard stock solutions were prepared as 0.25, 0.50, 0.75 and 1.0 ml of KMnO4. From each sample, 2.5 g of dry soil were weighed in two 50 mL centrifuge tubes. In each tube, 18 ml of deionized water and 2.0 ml of 0.2 M KMnO<sup>4</sup> stock solution were added into the tubes. A control was prepared in the same manner without adding the soil. Tubes were put on a shaker at 240 oscillations per minutes for 2 minutes. After shaking, caps were removed and the soil allowed to settle down for 10 minutes in a dark place. The supernatant from the extracted samples were loaded in 96 well plates at the volume of  $200 \mu l$ , with the same for the standards and control. The colorimetric method was used to measure the absorbance by a microplate reader (BioTek Synergy 2 Multi-Mode Microplate Reader) at the wavelength of 550 nm. If a sample required dilution due to exceeding the range of the standard curve, 0.5 ml of the supernatant from each sample was mixed with 49.5 ml H<sub>2</sub>O into a second set of centrifuge tubes and calculated with the dilution factor accounted for.

#### *Soil protein Index*

Soil protein content was measured by following a protocol modified from Wright and Upadhyaya (1996) and Moebius-Clune et al. (2016) (50, 83). Standard solutions were prepared as 0, 25, 125, 250, 125, 250, 500, 750, 1000, 1500, and 2000 µg/ml of Albumin (BSA) (Pierce™ BCA Protein Assay Kit, LOT: TB263610, Thermo Scientific). 3.0 g of soil was weighed for each glass screw-top tube for three replications. 24 ml of sodium citrate buffer (pH 7.0) were added to each tube and mixed well. Samples were placed in a centrifuge at 180 rpm for 5 minutes. The tubes were put in an autoclave at  $121^{\circ}$ C and 15 psi for 30 minutes. After the tubes were cooled, 2 ml of the slurry was transferred to clean microcentrifuge tubes to remove soil particles where samples were centrifuged at 10,000 x gravity. 200 µl of the supernatant for each sample was placed into 96 well plates and incubated at 60°C for 30 minutes. After the incubation, the microplate reader (BioTek Synergy 2 Multi-Mode Microplate Reader) was used to obtain the optical density reading at the wavelength of 562 nm to measure the soil protein index.

#### *Soil β-glucosidase enzyme activity*

Soil β-glucosidase enzyme activity was assayed according to the method described by Deng and Tabatabai (1994) (84). P-nitrophenol standard (4-nitrophenyl-β-Dglucopyranoside, LOT: 001788-20140328, Chem-Impex International, Inc.) solutions were made from a series of dilutions resulting in 0, 10, 20, 30, 40 and 50 mg of pnitrophenol for a standard curve. Each sample as dry soil was weighed into three 50 mL Erlenmeyer flasks as two reps and one control. Subsequently, 2 ml of MUB (pH 6) and 0.5 ml p-Nitrophenyl β-D-glucoside (PNG) solutions (4-nitrophenyl-β-Dglucopyranoside, LOT: 001788-20140328, Chem-Impex International, Inc.) were added to the two reps of the samples, but not the controls. All samples were placed in an incubator at 37°C for 60 minutes. After 60 minutes of incubation, 0.5 ml of 0.5 M calcium chloride (CaCl<sub>2</sub>) and 2 mL 0.1 tris aminomethane (THAM) buffer (pH 12) were added and mixed well. At the same time, 0.5 mL PNG solution was added to the control samples. Then, the soil suspension was filtered through a Whatman filter paper No.2 into pre-labeled 50 ml disposable falcon tubes. THAM buffer (pH 10) was used to dilute samples at the rate of 1:4 (note: the controls were not diluted) and samples were pipetted into 96 well microplates. The absorbance was measured using a microplate reader (BioTek Synergy 2 Multi-Mode Microplate Reader) at the wavelength of 405 nm. *Soil DNA Extraction*

The FastDNA™ Spin Kit (For soil, Cat.No.116560200, MP Biomedicals, Solon, Ohio) was used following the manufacturer's protocol for DNA extraction from soil with some minor modification. 500 mg of soil was placed in each Lysing Matrix E tube. 978  $\mu$ l sodium phosphate buffer and 122  $\mu$ l MT buffer were added to each sample and

allowed to homogenize in a vortex adapter as horizontal at the highest speed for 20 minutes. Samples were centrifuged at the speed of 14,000 rpm for 10 minutes. After centrifugation, the supernatant was transferred to a new 2 ml centrifuge tube and 250 µl protein precipitation solution was added. Then, the tube was inverted to mix and centrifuged at 14,000 rpm for 5 minutes. In order to get a better mixing and DNA binding, the supernatant was transferred to a 15 ml microcentrifuge tube, 1 ml binding matrix solution added to the supernatant, and inverted gently by hand for 2 minutes and the silica beads were left to settle for 3 minutes. After that,  $500 \mu l$  of supernatant was discarded from the samples. The binding matrix in the remaining supernatant was resuspended and 600  $\mu$ l of the mixture was transferred to a spin<sup>TM</sup> filter where it was centrifuged at 14,000 rpm for 1 minute. After centrifuge, catch tubes were emptied and the remaining mixture was added to the same spin<sup>TM</sup> filter and centrifuged again. 500  $\mu$ l of SEWS-M solution was added to the filters with empty catch tubes and were centrifuged at 14000 rpm for 1 minute for washing (note: this step was repeated 3 times). Eventually, filters sitting on top of empty catch tubes were centrifuged at 14,000 rpm for 2 minutes. The catch tubes were discarded and replaced by new catch tubes. Next, spin<sup>TM</sup> filters were left for drying at room temperature for 5 minutes and 100 µl of deionized water was pipetted onto the spin filter. Lastly, tubes were centrifuged at 14,000 rpm for 1 minute to elute the DNA and spin filters were discarded to get the eluted DNA in the new catch tubes. Samples were kept at -20°C until CTAB purification of DNA.

*CTAB (Cetyltrimethylammonium bromide) Purification of DNA (post-extraction)*

The above DNA extract was placed in a pre-labeled 1.5 ml tube and 5 M of 16.25 µl NaCl solution added. 12 µl of CTAB stock solution (0.7 M NaCl, 10% CTAB) was

added to each DNA samples then mixed well and incubated at 65°C for 15 minutes. After 15 minutes of incubation, 128 µl of chloroform: isoamyl alcohol (24:1) was added to each sample and mixed carefully. Samples were placed in a centrifuge at 14,000 rpm for 5 minutes. The top layer of sediment was transferred carefully to a clean 1.5 ml centrifuge tube using filter tips and  $256 \mu l$  of 100 % ice-cold ethanol (EtOH) was added to precipitate DNA. In order to increase DNA precipitation, samples were put in a freezer  $(-20^{\circ}C)$  overnight. After that, samples were placed in a centrifuge at the maximum speed at 4°C for 30 minutes, followed by discarding the supernatant carefully to avoid disturbing the pellet, which was washed by adding 125 µl of 70% EtOH to each sample. Samples were centrifuged at the maximum speed for 10 minutes. After centrifugation, the supernatant was removed, and samples were placed in a speed-vac (Savant ISS100- Thermo Scientific) to dry the pellets for 15 minutes at low setting. 30  $\mu$ l dH<sub>2</sub>O was added to each sample to resuspend the DNA. The DNA concentrations were measured to evaluate ratios of A**260/280** and A**260/230** the purity of DNA using Thermo Scientific NanoDrop<sup>TM</sup>1000 Spectrophotometer. Finally, DNA samples were stored at -20 $\degree$ C in the freezer until further analysis.

#### *Bacterial and Fungal DNA Amplicon Sequencing*

Bacterial 16S rDNA and fungal Nuclear ribosomal internal transcribed spacer (ITS) classifications were amplified to identify the Operational Taxonomic Units (OTUs) and sequenced by University of Minnesota Genomic Center (Minneapolis, Minnesota, US) using MiSeq-V3 chemistry from a published protocol with a dual-index approach (85). The 16S V3-V4 and ITS-1 regions were targeted for the bacterial community and fungal community, respectively.

#### *Data Analyses*

Statistical analyses were done using SAS-JMP version pro 14.0.0 (SAS Institute 2018) to analyze yields, POXC, protein index and  $\beta$ -glucosidase activities using the Analyisis of Varience followed by LSMeans Differences Tukey HSD test. The threshold was designated for probability at  $P < 0.05$ . The classifications of bacteria and fungi were determined using QIIME2 (86) followed by the ANCOM test (87). Also, the multiple analyses module in XLSTAT (v. 2019) was used for the canonical correspondance analysis (CCA).

#### RESULTS

At two field sites, which are Monmouth and Urbana, Figure 1 and Figure 2 showed significant differences in soybean yields planted after the four long-term (14 year) crop sequences - continuous corn (CC), corn/corn/soybean (CCS), corn/soybean/corn (CSC) and soybean/corn/soybean (SCS). Soybean yields were in decreasing order: CC>CCS>CSC>SCS. We observed that there were significant differences between the two locations, so the following statistical analysis was performed separately for each location. Yields from CC (Mnm:5,255 kg ha<sup>-1</sup> and Urb:5,614.25 kg ha<sup>-1</sup>) plots were significantly higher than CCS, CSC and SCS crop rotations with pvalue less than 0.05 at both sites. At the Monmouth site, there were no significant differences among the CCS, CSC and SCS plots, but the average yields were in decreasing order of 4,825.5 kg ha<sup>-1</sup>, 4,676 kg ha<sup>-1</sup> and 4,597.75 kg ha<sup>-1</sup> for each treatment, respectively. At the Urbana site, the yield at CCS  $(5,009.75 \text{ kg ha}^{-1})$  plots were also significantly higher (P<0.05) than CSC  $(4,802.75 \text{ kg ha}^{-1})$  and SCS  $(4,802.75 \text{ kg ha}^{-1})$ 

kg ha<sup>-1</sup>) plots. There was no significant differences between CSC and SCS treatments at the Urbana site.



**Figure 1.** Effect of rotation treatment on soybean yield (kg/ ha−1 ) in Monmouth, IL in 2016.



Figure 2. Effect of rotation treatment on soybean yield (kg/ ha<sup>-1</sup>) in Urbana, IL in 2016.



**Table 1:** Soil Protein, Permanganate Oxidizable Carbon (POXC) and β-glucosidase enzyme analysis for Urbana and Monmouth Location

†CC, continuous corn; CCS, two years corn; CS, corn-soybean; SCS, soybean-corn. <sup>ǂ</sup>Means within the same column followed by different small letters are significantly different at P<0.05 for rotation.

<sup>ǂ</sup>Means within the same row followed by different capital letters are significantly different at P<0.05 for rotation.

Data for soil protein, POXC and β-glucosidase are presented in Table 1. Soil

protein and POXC analyses results showed that there were no significant differences

between Urbana and Monmouth when comparing same treatment for two locations, but

β-glucosidase enzyme analysis displayed that Urbana and Monmouth had significant

differences when comparing the same treatment for each location statistically.

After 14 years of the above-mentioned rotation regimes, shown in Figure 3, we found that POXC was significantly greater in CC (815.4 mg C/kg soil) and CCS (735.7 mg C/kg soil) plots (P<0.001) than CS and SCS plots. Also, there were no difference between CS (605.6 mg C/kg soil) and SCS (585.1 mg C/kg soil) plots when we compared POXC levels at the Monmouth location. At the Urbana site, Figure 4 showed that POXC was significantly higher in CC (646.7 mg C/kg soil) and CCS (776.7 mg C/kg soil) treatments compared to the other plots (P<0.05), and POXC levels were in decreasing order of CCS (776.7 mg C/kg soil) >CC (646.7 mg C/kg soil) >SCS (561.6 mg C/kg soil) >CS (489.7 mg C/kg soil). However, there were no significant differences between CC

and CCS plots, so was there no difference between CC, CS and SCS plots. Both data sets passed the normality test.



**Figure 3.** Effect of rotation treatment on permanganate oxidizable carbon (POXC) of soil samples from Monmouth, IL (mg C/kg of dried soil).





As for the protein index measured from each treatment for two locations, statistical analyses showed that the CC plot had higher protein content when we

compared with other crop treatments at the Urbana plot (Figure 5). CC (7,360 mg/gm−1 per kg soil) treatment was significantly greater than CCS (5,627.3 mg/gm<sup>-1</sup> per kg soil), CS (5,416.8 mg/gm<sup>-1</sup> per kg soil) and SCS (5,612.9 mg/gm<sup>-1</sup> per kg soil) treatments (p<0.05). There was no difference between CCS and SCS plots. As for the Monmouth location, no significant differences in protein content were found among the CC (5,841.7 mg/gm<sup>-1</sup> per kg soil), CCS (5,425.9 mg/gm<sup>-1</sup> per kg soil), CS (5,453.3 mg/gm<sup>-1</sup> per kg soil) or SCS (5,468.7 mg/gm<sup>-1</sup> per kg soil) sequences (Figure 6). However, the CC plot had the highest average of protein. The distribution analysis showed that protein content was normally distributed.



**Figure 5.** Effect of rotation treatment on soil protein of soil samples from Monmouth, IL (mg/gm<sup>-1</sup> per kg soil).



**Figure 6.** Effect of rotation treatment on soil protein of soil samples from Urbana, IL  $(mg/gm^{-1})$  per kg soil).

Soil β-glucosidase enzyme activity was significantly higher in SCS (Urb:1.18 and Mnm:1.72 (mg p-nitrophenol/gm of dried soil)) rotations compared with other rotations (p<0.05) at both sites. When we analyzed each location separately for β -glucosidase enzyme activity, no difference was found among the CC (0.92 mg p-nitrophenol/ gm of dried soil), CCS (0.91 mg p-nitrophenol/ gm of dried soil) and CS (0.86 mg pnitrophenol/ gm of dried soil) rotations at the Urbana site (Figure 7). Also, we observed that SCS (1.72 mg p-nitrophenol/ gm of dried soil) and CS (1.72 mg p-nitrophenol/ gm of dried soil) rotations resulted in more β-glucosidase enzyme produced than CC (1.51 mg p-nitrophenol/ gm of dried soil) and CCS (1.24 mg p-nitrophenol/gm of dried soil) plots at the Monmouth site (Figure 8). The distribution analysis showed that soil β-glucosidase activity values were normally distributed.



**Figure 7.** Effect of rotation on β-glucosidase levels of soil samples from Urbana, IL (mg nitrophenol/gm of dried soil).



**Figure 8.** Effect of rotation on β-glucosidase levels of soil samples from Monmouth, IL (mg nitrophenol/gm of dried soil).

Analysis of the composition of microbiomes (ANCOM) was used as a statistical tool for comparing microbial abundances according to the underlying structure in the data. The ANCOM results showed that three bacterial phyla, *Chloroflexi, Proteobacteria*  and *Verrucomicrobia,* were the differentially abundant taxa at the Monmouth site between different treatments (crop sequences). In the *Chloroflexi* phylum, the uncultured bacterium belonging to the order of JG30-KF-AS9 was observed as having a higher abundance in the CC plot and in a descending relative abundance order of CCS>CS>SCS. Another uncultured bacterium abundance under *Proteobacteria*, belonging to the order of *Ellin329*, had a descending order of abundance in CC>CCS>CS>SCS plots. The third indicative bacteria under *Verrucomicrobia*, belonging to the genus of *Chthoniobacter*, was found in the CS plot as having the highest level of relative abundance with a descending order of other crop sequences of CC>CCS>SCS, respectively (Figure 9).





B)

C)Monmouth bacterial community k Bacteria;p Verrucomicrobia;c [Spartobacteria];o ■ [Chthoniobacterales];f [Chthoniobacteraceae];g Chtho niobacter 140 120 abundance Relative 100 80 60 40 20  $\mathbf{I}$  $\mathsf{O}\xspace$  $CC$ CCS CS SCS treatment

**Figure 9 (A, B, C).** Box-and-whisker plots of bacterial relative abundance distributions for the taxa that varied significantly among rotation treatments by the ANCOM tests at the Monmouth site.

In the fungal community, according to ANCOM analysis, *Ascomycota* was found to be the most informative phylum separating the four crop sequences. Specifically, the genius of *Corynespora* was located at higher levels of abundance in the SCS rotation and

21

decreasing relative abundance in order of CCS>CS>CC rotations. The relative abundance of uncultured fungi belonging to the *Mycosphaerellaceae* fungal family was revealed to have the greatest abundance in the SCS crop sequence and showed a decreasing order of CS>CC>CCS crop sequences. Third, the *Macrophomina* genius was detected as most abundant in the SCS crop sequence with decreasing order of abundance as CCS>CS>CC at the Monmouth site (Figure 10).







**Figure 10 (A, B, C).** Box-and-whisker plots of fungal relative abundance distributions for the taxa that varied significantly among rotation treatments by the ANCOM tests at the Monmouth site.

At the Urbana site, ANCOM test showed that only the bacterial genus of *Skermanella*, belonging to family *Rhodospirillaceae* under class *Alphaproteobacteria*, was an informative taxa distinguishing the four treatments, with decreasing relative abundance in order of CS>SCS>CCS>CC plots (Figure 11).



**Figure 11.** Box-and-whisker plots of bacterial relative abundance distributions for the taxa that varied significantly among treatments by the ANCOM tests at the Urbana site.

The uncultured fungus belonging to family *Hyponectriaceae* in order *Xylariales* under class *Sordariomycetes* in the phylum of *Ascomycota* was found to be the only one significantly different in relative abundance separating the four crop sequences, with the order of SCS>CS>CCS>CC at the Urbana site (Figure 12).



**Figure 12.** Box-and-whisker plots of fungal relative abundance distributions for the taxa that varied significantly among treatments by the ANCOM tests at the Urbana site.

At the Monmouth site, the dominant taxa of *Ellin329* in CC and CCS rotations was related to higher levels of soil protein content. The order of JG30-KF-AS9 was found as the most abundant taxa in CC rotation and it was associated with higher levels of POXC. Also the genus of *Chthoniobacter* was related to protein content (Figure13).


**Figure 13.** Canonical correspondence analysis (CCA) for bacteria taxa with differential abundances at the Monmouth location. Only significant taxa are shown. Sites; treatment-Objectives; fungi-Variables; environmental data

Figure 14 showed that, in the soil samples from Monmouth, differentially abundant fungal taxa of *Macrophomina* was not closely related to the soil health indicators measured in this study; however, *Mycosphaerellaceae* and *Corynespora* were associated with β-glucosidase activity, based on the canonical correspondence analysis (CCA).



**Figure 14.** Canonical correspondence analysis (CCA) for fungal taxa with differential abundances at the Monmouth location. Only significant taxa are shown. Sites; treatment-Objectives; fungi-Variables; environmental data

In the soil samples from Urbana, the bacterial genus of *Skermanella* was shown to be highly responsive to the β-glucosidase activity (Figure 15), and the fungal family of *Hyponectriaceae* was observed as the most abundant taxa in SCS rotation for producing higher level of β-glucosidase activity (Figure 16).



Figure 15. Canonical correspondence analysis (CCA) for bacterial taxa with differential abundances at the Urbana location. Only significant taxa are shown. Sites; treatment-Objectives; bacteria-Variables; environmental data



**Figure 16.** Canonical correspondence analysis (CCA) for fungal taxa with differential abundances at the Urbana location. Only significant taxa are shown. Sites; treatment-Objectives; fungi-Variables; environmental data

According to qiime 2 results, the alpha diversity in the bacterial and fungal community, there were no significant differences between the two locations. Therefore, alpha diversity indices from the two locations were pooled and analyzed together for the rotation effect. Figure 17 showed that no difference was found among the four rotations for bacterial community alpha diversity. Likewise, in the fungal community, there were no significant differences among the four treatments in alpha diversity based on the Chao1 index (Figure 18) (P>0.05).



**Figure 17**. Rarefaction curves of the alpha diversity for the bacterial community based on Chao1 index.



**Figure 18**. Rarefaction curves of the alpha diversity for the fungal community based on Chao1 index.

Based on the principal coordinates analysis (PCoA) results of the β-diversity in the bacterial community, the two locations are very dissimilar, represented by two clusters in different colors (Figure 19, left), but there was no separation among the four rotation treatments represented as four colors within each location (Figure 19, right). When analyzed separately by location, no separation was found as well (Figure not shown). Likewise, in the fungal community, the β-diversity between the Monmouth and Urbana locations were dissimilar, but no separation was found among the four treatments (Figure 20) even when analyzed separately by location (Figure not shown).



 **Location & Rotation effects**

**Figure 19.** PCoA plot for β-diversity of bacterial 16S OTU in bulk soil.





**Figure 20.** PCoA plot for β-diversity of fungal OTU in bulk soil.

## DISCUSSION

Crop rotation is a strategy to enhance soybean yield as a cultural management system in agriculture. Many studies have shown that a higher yield was observed in soybeans when rotated with other crops instead of growing soybeans continuously (monoculture) (88-91). Our results showed that after the continuous corn regime, immediately before planting, soybeans had a higher yield when compared with other crop rotations (Figure 1 and Figure 2). A recent study conducted by B. S. Farmaha, et al. (92) stated that soybean yield in corn-corn-soybean rotation was higher compared to soybeancorn-soybean crop rotation. The results from this study explained that two previous years of corn crop sequences resulted in increased soybean yield as compared to one previous year of corn crop sequences. However, the underlying cause for these benefits has been difficult to uncover. One study reported that the rotation of corn and soybeans showed that no effect as positive or negative on above-ground sediment was found (93). K. R. Whiting and R. K. Crookston (94) found that the yield benefit from the rotation of

soybean with corn was not due to the decrease in the incidence of leaf diseases. Rotationrelated increased yield was due to enhanced root function (95-97) or decreased soil pathogenic microorganisms affecting root growth (98, 99). Several studies demonstrated that including corn in rotation helped to decrease soybean cyst nematode (*Heterodera glycines*) composition and raised the yield of soybeans (100, 101). Hence, the increase in soybean yield related to the crop sequences seems to be the result of multiple interactive components in the soil.

Nitrogen and carbon cycling in the soil can be affected by crop rotations (102). Soil microorganisms are central to the carbon cycle (24, 25) and nitrogen fixation (37). POXC is a measurement of LOC and is directly related to soil organic carbon (42). Our POXC results showed that POXC had higher levels in CC and CCS plots (Figure 3 and Figure 4), which corresponded to higher yields of soybean in those plots.

Some free-living *Proteobacteria* may play a role in N fixation (103) and CO<sup>2</sup> fixation process in the soil (104). Indeed, Figure 13 showed that the uncultured order *Ellin329*, belonging to *Proteobacteria*, was associated with higher protein levels. The fungal phylum of *Ascomycota* are known for producing a major amount of β-glucosidase (105). The CCA analysis did reveal that the family of *Mycosphaerellaceae*, belonging to *Ascomycota*, was associated with higher levels of β-glucosidase (Figure 14).

The class of *Ktedonobacter* is a non-photosynthetic bacteria responsible for carbon monoxide (CO) oxidation and utilizing CO as a carbon source in the soil (106). We observed that *Ktedonobacter* had significantly higher levels of abundance in CC rotation (Figure 9A) and was related to POXC and directly linked to higher soybean yields in CC crop sequences at the Monmouth site.

*Ellin 329* is a member of the *Alphaproteobacteria* class, which is a significant bacterial order for the decomposition of organic matter in the soil (107). In our results, *Ellin 329* was found as significantly dominant taxa in the CC crop sequence (Figure 9B).

*Ktedonobacter* and *Ellin 329* were observed as dominant taxa in CC rotation (Figure 9A,B). When compared to yields difference (Figure 1 and Figure 2), and these two bacterial taxa abundance, both decreased order in the same direction CC>CCS>CS>SCS plots. Therefore, *Ktedonobacter* and *Ellin 329* could be potential soil biological and soil health indicators for future studies.

*Chthoniobacter* is a bacterial genus known to be responsible for the decomposition of organic carbon in the soil (108) but correlated with some of the nematodes as an endosymbiont's life form (109). The highest level of bacterial abundance of *Chthoniobacter* under the CS rotation (Figure 9C) was found to be associated with a lower level of soybean yield. While we did not test the soybean cyst nematode (SCN) population in the study, the endosymbionts form between *Chthoniobacter* and SCN could be a reason for the lower soybean yield in CS rotation.

The fungal pathogen from the genera *Corynespora* is the causal agent of soybean for frogeye leaf spot disease (110). We found that *Corynespora* was significantly more abundant in SCS than the other crop sequences (Figure 10C), which corresponds to a lower soybean yield. Also, another fungal pathogen, genera *Macrophomina*, which infect soybean roots as charcoal rot disease (111) was found to be more abundant in SCS crop rotation (Figure 10A). Lastly, the ANCOM results detected *Mycosphaerellaceae*, which includes many fungal pathogen genera and species (110), as a significantly more abundant taxa in SCS crop rotation (Figure 10C).

## **CONCLUSION**

The results of this 14-year long-term crop study provided insight that crop rotation has a crucial impact on soybean yield. After the continuous corn crop regime, the soybean yield at CC was significantly greater than the other three (CCS, CS, SCS) crop rotation regimes in both locations. The application of crop rotation systems in the field could provide many important benefits enhancing soil C concentration and nutrition, improving soil physical properties, providing diverse bacterial and fungal communities and increasing yield. This study provided evidence that soil biological properties, including POXC, protein content, bacterial 16S rDNA and fungal ITS relative abundances, generally correlated with yield positively but negatively with β-glucosidase. Specific taxa of the microbiome also shed light on the yield differences as a result of the four crop sequences compared when soil chemical and physical properties fall short of providing adequate explanation from other related studies. It is evident that soybean pathogen populations are determinants, as well as some uncultured bacterial taxa, which still require efforts in culturing and further characterization. Culturability of bacteria has been greatly improved in recent years and this study clarifies that bacteria under the orders of Ellin329 and JG30-KF-AS9 should be further focused on for isolation with the goal to improve soybean yields in the future.

## APPENDIX

Microbiome Analysis Report

Taxa Summary Bar charts

Level 2 16S





Level 3 16S





k\_Bacteria;p\_Chloroflexi;c\_Chloroflexi<br>k\_Bacteria;p\_Chloroflexi;c\_Ellin6529 Enteria;p\_Chloroflexi;c\_Gitt-GS-136<br>Enteria;p\_Chloroflexi;c\_Ktedonobacteria ■ Exercision\_Chloroflexic\_resourcestrated<br>
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Exercision\_Chloroflexic\_S085<br>
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k\_Bacteria;p\_Planctomycetes;c\_BD7-11

k Bacteria;p [Thermi];c Deinococci

Level 4 16S





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k\_Bacteria;p\_Gemmatimonadetes;c\_Gemmatimonadetes;o\_N1423WL;f\_

k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Rhodobiaceae **College** k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Xanthobacteraceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhodobacterales;f Hyphomonadaceae Eacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhodobacterales;f\_Rhodobacteraceae<br>
Eacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhodospirillales;f\_ Exacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhodospirillales;f\_Acetobacteraceae k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhodospirillales;f\_Rhodospirillaceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rickettsiales;f Rickettsiales;f Rickettsiaceae<br>Alphaproteobacteria;c Alphaproteobacteria;o Rickettsiales;f Rickettsiaceae Exacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rickettsiales;f\_mitochondria k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Sphingomonadales;Other k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Sphingomonadales;f\_Erythrobacteraceae Example to the protection of the property of the contract of the particle of the particle of the particle of the contract of the particle of t E Racteria;p\_Proteobacteria;c\_Betaproteobacteria;Other;Other k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_;f k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_A21b;f\_EB1003 k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_A21b;f\_UD5 K\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;Other k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;f\_ K\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;f\_Alcaligenaceae k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;f\_Burkholderiaceae<br>k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;f\_Comamonadaceae k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Burkholderiales;f\_Oxalobacteraceae k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Ellin6067;f\_ k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_IS-44;f\_ k Bacteria;p Proteobacteria;c Betaproteobacteria;o MND1;f k Bacteria;p Proteobacteria;c Betaproteobacteria;o MEN)<br>k Bacteria;p Proteobacteria;c Betaproteobacteria;o Methylophilales;f Methylophilaceae k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Nitrosomonadales;f\_ k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Nitrosomonadales;f\_Nitrosomonadaceae k\_\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Procabacteriales;f\_Procabacteriaceae<br>
k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_Rhodocyclales;f\_Rhodocyclaceae k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_SBla14;f\_ k\_Bacteria;p\_Proteobacteria;c\_Betaproteobacteria;o\_SC-I-84;f\_ k Bacteria;p Proteobacteria;c Deltaproteobacteria;o ;f \_<br>k Bacteria;p Proteobacteria;c Deltaproteobacteria;o Bdellovibrionales;f Bacteriovoracaceae E Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Bdellovibrionales;f\_Bdellovibrionaceae k Bacteria;p Proteobacteria;c Deltaproteobacteria;o Desulfobacterales;f Desulfobacteraceae k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Desulfobacterales;f\_Desulfobulbaceae Experience to the contract of the protection of the contract of the protection k Bacteria;p Proteobacteria;c Deltaproteobacteria;o Desulfuromonadales;f Pelobacteraceae k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_FAC87;f\_ k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_MIZ46;f\_ Eacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;Other k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_ k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_0319-6G20 k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_Cystobacterineae k Bacteria;p Proteobacteria;c Deltaproteobacteria;o Myxococcales;f Haliangiaceae<br>
k Bacteria;p Proteobacteria;c Deltaproteobacteria;o Myxococcales;f Myxococcaceae Eacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_Nannocystaceae k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_OM27 k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_Myxococcales;f\_Polyangiaceae K\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_NB1-j;f\_

k Bacteria;p Kazan-3B-28;c :o ;f k\_Bacteria;p\_NKB19;c\_;o\_;f\_<br>k\_Bacteria;p\_NKB19;c\_TSBW08;o\_;f\_ k\_Bacteria;p\_Nitrospirae;c\_Nitrospira;o\_Nitrospirales;f\_0319-6A21 k\_Bacteria;p\_Nitrospirae;c\_Nitrospira;o\_Nitrospirales;f\_Nitrospiraceae<br>k\_Bacteria;p\_Nitrospirae;c\_Nitrospira;o\_Nitrospirales;f\_[Leptospirillaceae]  $\blacksquare$  k\_Bacteria;p\_OD1;c\_;o\_;f\_ k\_Bacteria;p\_OD1;c\_ABY1;o\_;f\_<br>k\_Bacteria;p\_OD1;c\_Mb-NB09;o\_;f\_ K\_Bacteria;p\_OD1;c\_SM2F11;o\_;f\_ Lackeria:p\_0D1;c\_2B2;o\_;f\_<br>
k\_Bacteria;p\_0D1;c\_2B2;o\_;f\_<br>
k\_Bacteria;p\_0P11;c\_WCHB1-64;o\_;f\_<br>
k\_Bacteria;p\_0P11;c\_WCHB1-64;o\_d153;f\_ k\_Bacteria;p\_OP3;c\_koll11;o\_;f\_ k\_Bacteria;p\_Planctomycetes;c\_BD7-11;o\_;f  $\begin{array}{r} \hline \text{K\_Bacteria:} \text{p\_Planctomycetes:} \text{p\_G5:} \text{mWS-107;f} \\ \text{K\_Bacteria:} \text{p\_Planctomycetes:} \text{p\_G5:} \text{p\_d113;f} \end{array}$ k\_Bacteria;p\_Planctomycetes;c\_OM190;o\_CL500-15;f\_ k\_Bacteria:p\_Planctomycetes;c\_OM190;o\_agg27;f\_<br>k\_Bacteria:p\_Planctomycetes;c\_Phycisphaerae;o\_CCM11a;f\_ Example Plancomycetes;<br/>c\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_Phycisphaerae;o\_P K\_Bacteria;p\_Planctomycetes;c\_Pla3;o\_;f\_ k\_\_Bacteria;p\_\_Planctomycetes;c\_\_Pla4;o\_\_;f\_\_<br>■ k\_\_Bacteria;p\_\_Planctomycetes;c\_\_Planctomycetia;o\_\_B97;f\_\_ Example of Bacteria;p\_Planctomycetes;c\_Planctomycetia;o\_Gemmatales;f\_Gemmataceae E Bacteria;p\_Planctomycetes;c\_Planctomycetia;o\_Gemmatales;f\_Isosphaeraceae<br>
K\_Bacteria;p\_Planctomycetes;c\_Planctomycetia;o\_Pirellulales;f\_Pirellulaceae k\_Bacteria;p\_Planctomycetes;c\_Planctomycetia;o\_Planctomycetales;f\_Planctomycetaceae Entertained the material control of the material control of the Bacterial Control of the Material Cont k\_Bacteria;p\_Proteobacteria;c\_;o\_;f\_ Exacteria; p\_Proteobacteria; c\_Alphaproteobacteria; Other; Other<br>
k\_\_Bacteria; p\_Proteobacteria; c\_Alphaproteobacteria; o\_; f\_ k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_BD7-3;f\_ k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Caulobacterales;f\_Caulobacteraceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Ellin329;f Eacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Kiloniellales;f\_ k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;Other<br>
k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Aurantimonadaceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Bartonellaceae<br>
k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Beljerinckiaceae K\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Bradyrhizobiaceae k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Hyphomicrobiaceae k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Methylobacteriaceae k\_Bacteria;p\_Proteobacteria;c\_Alphaproteobacteria;o\_Rhizobiales;f\_Methylocystaceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Phyllobacteriaceae k Bacteria;p Proteobacteria;c Alphaproteobacteria;o Rhizobiales;f Rhizobiaceae Example: The Lacteria;p\_Verrucomicrobia;c\_Opitutae;o\_[Cerasicoccales];f\_[Cerasicoccaceae] Exacteria;p\_Verrucomicrobia;c\_Verruco-5;o\_WCHB1-41;f\_ Earteria;p\_Verrucomicrobia;c\_Verrucomicrobiae;o\_Verrucomicrobiales;f\_Verrucomicrobiaceae<br>
k\_Bacteria;p\_Verrucomicrobia;c\_[Methylacidjphilae];o\_Methylacidjphilales;f\_ Eacteria;p\_Verrucomicrobia;c\_[Methylacidiphilae];o\_Methylacidiphilales;f\_LD19 k Bacteria;p Verrucomicrobia;c [Methylacidiphilae];o S-BQ2-57;f<br>k Bacteria;p Verrucomicrobia;c [Pedosphaerae];o [Pedosphaerales];Other Exacteria:<br>
Perucomicrobia:
Ceptosphaerae:<br>
Elin515<br>
K\_Bacteria:<br>
Perucomicrobia:
Ceptosphaerae:<br>
Perucomicrobia:
Ceptosphaerae:<br>
Perucomicrobia:
Ceptosphaerae:<br>
Perucomicrobia:
Ceptosphaerae:<br>
Perucomicrobia:
Ceptosphaera k\_Bacteria;p\_Verrucomicrobia;c\_[Pedosphaerae];o\_[Pedosphaerales];f\_OPB35 K\_Bacteria;p\_Verrucomicrobia;c\_[Pedosphaerae];o\_[Pedosphaerales];f\_R4-41B k Bacteria;p Verrucomicrobia;c [Pedosphaerae];o [Pedosphaerales];f [Pedosphaeraceae] k Bacteria;p Verrucomicrobia;c [Pedosphaerae];o [Pedosphaerales];f auto67\_4W<br>
K Bacteria;p Verrucomicrobia;c [Spartobacteria];o [Chthoniobacterales];f 01D2Z36 k\_Bacteria;p\_Verrucomicrobia;c\_[Spartobacteria];o\_[Chthoniobacterales];f\_[Chthoniobacteraceae] k\_Bacteria;p\_WPS-2;c\_;o\_;f\_<br>k\_Bacteria;p\_WS2;c\_SHA-109;o\_;f\_ Example 2011 - Example 2012<br>
2011 - Example 2013 - Example 2013 - Example 2013 - Example 2013 - Example 2014<br>
2014 -

- 
- k\_Bacteria;p\_[Thermi];c\_Deinococci;o\_Deinococcales;f\_Deinococcaceae
- k\_Bacteria;p\_[Thermi]:c\_Deinococci;o\_Deinococcales;f\_Trueperaceae

k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_NB1-j;f\_JTB38<br>== k\_Bacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_NB1-j;f\_MND4 Exacteria;p\_Proteobacteria;c\_Deltaproteobacteria;o\_NB1-j;f\_NB1-i —— sacteria;p\_Proteobacteria;c\_\_Deltaproteobacteria;o\_\_Syntrophobacteriales;f\_Syntrophaceae<br>
- k\_\_Bacteria;p\_\_Proteobacteria;c\_\_Deltaproteobacteria;o\_\_Syntrophobacteriales;f\_\_Syntrophobacteriaceae<br>
- k\_\_Bacteria;p\_\_Proteo k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;Other;Other Example 18 - Exacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_;f\_<br>Example 18 - Exacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Aeromonadales;f\_Aeromonadaceae K\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Alteromonadales;f k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Alteromonadales;f\_125ds10 k Bacteria;p Proteobacteria;c Gammaproteobacteria;o Alteromonadales;f 211ds20 k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Alteromonadales;f\_Alteromonadaceae k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Alteromonadales;f\_OM60 k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Chromatiales;f\_Chromatiaceae Exposition, Proteobacteria; C\_Gammaproteobacteria; C\_Enterobacteriales; f\_Enterobacteriaceae<br>
Exposition = Proteobacteria; C\_Gammaproteobacteria; c\_Enterobacteriales; f\_Enterobacteriaceae<br>
Exposition = Proteobacteria; C\_Ga Reference of the Arcteria Commander Arcteria - HTCC2188;f HTCC2089 k Bacteria;p Proteobacteria;c Gammaproteobacteria;o Legionellales;f Camera in Alexandria;p Legionellales;f Coxiellaceae k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Legionellales;f\_Legionellaceae k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Oceanospirillales;f\_Halomonadaceae<br>k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Pasteurellales;f\_Pasteurellaceae<br>k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria; Experiment and the protection of the action of the set of k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_Xanthomonadales;f\_Xanthomonadaceae<br>k\_Bacteria;p\_Proteobacteria;c\_Gammaproteobacteria;o\_[Marinicellales];f\_[Marinicellaceae]<br>k\_Bacteria;p\_Proteobacteria;c\_TA18;o\_CV90;f\_ k\_Bacteria;p\_Proteobacteria;c\_TA18;o\_PHOS-HD29;f\_ Example SR1;c\_;o\_;f\_<br>
k\_Bacteria;p\_SR1;c\_;o\_;f\_<br>
k\_Bacteria;p\_TM6;c\_SBRH58;o\_;f\_ k\_Bacteria;p\_TM6;c\_SJA-4;o\_;f Example 1981<br>
K\_Bacteria;p\_TM6;c\_SJA-4;o\_S1198;f\_<br>
K\_Bacteria;p\_TM7;Other;Other;Other k\_Bacteria;p\_TM7;c\_;o\_;f k\_Bacteriap\_TM7;c\_MJK10;o\_;f\_<br>k\_Bacteria;p\_TM7;c\_SC3;o\_;f\_<br>k\_Bacteria;p\_TM7;c\_TM7-1;o\_;f\_ k Bacteria;p TM7;c TM7-3;Other;Other<br>k Bacteria;p TM7;c TM7-3;o ..;f<br>k Bacteria;p TM7;c TM7-3;o EW055;f k\_Bacteria;p\_Verrucomicrobia;c\_Opitutae;o\_;f\_

k\_Bacteria;p\_Verrucomicrobia;c\_Opitutae;o\_Opitutales;f\_Opitutaceae

Level 2 ITS



k Fungi;p unidentified

Level 3 ITS



Level 4 ITS





Level 5 ITS



Unassigned:Other:Other:Other:Other k\_Fungi;Other;Other;Other;Other \_Fungi;p\_\_Ascomycota;Other;Other;Other k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;Other;Other k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Botryosphaeriales;f\_Botryosphaeriaceae k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Capnodiales;f\_Mycosphaerellaceae k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Incertae\_sedis;f\_Myxotrichaceae k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Pleosporales;f\_Incertae\_sedis k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Pleosporales;f\_Sporormiaceae k\_Fungi;p\_Ascomycota;c\_Dothideomycetes;o\_Pleosporales;f\_Tubeufiaceae K\_Fungi;p\_Ascomycota;c\_Eurotiomycetes;o\_Chaetothyriales;f\_Chaetothyriaceae k\_Fungi;p\_Ascomycota;c\_Eurotiomycetes;o\_Chaetothyriales;f\_Herpotrichiellaceae k\_Fungi;p\_Ascomycota;c\_Eurotiomycetes;o\_Eurotiales;f\_Trichocomaceae k\_Fungi;p\_Ascomycota;c\_Incertae\_sedis;o\_Incertae\_sedis;f\_Incertae\_sedis k\_Fungi;p\_Ascomycota;c\_Leotiomycetes;o\_Helotiales;Other k\_Fungi;p\_Ascomycota;c\_Leotiomycetes;o\_Helotiales;f\_Helotiaceae k\_Fungi;p\_Ascomycota;c\_Leotiomycetes;o\_Helotiales;f\_Sclerotiniaceae k\_Fungi;p\_Ascomycota;c\_Leotiomycetes;o\_Helotiales;f\_unidentified K\_Fungi;p\_Ascomycota;c\_Leotiomycetes;o\_Leotiales;f\_Leotiaceae k\_\_Fungi;p\_\_Ascomycota;c\_\_Leotiomycetes;o\_\_unidentified;f\_\_unidentified k\_Fungi;p\_Ascomycota;c\_Pezizomycetes;o\_Pezizales;f\_Helvellaceae k\_Fungi;p\_Ascomycota;c\_Pezizomycetes;o\_Pezizales;f\_Pezizaceae k\_\_Fungi;p\_Ascomycota;c\_\_Saccharomycetes;o\_\_Saccharomycetales;f\_\_Saccharomycetaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;Other;Other k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Chaetosphaeriales;f\_Chaetosphaeriaceae K\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Coniochaetales;Other k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Coniochaetales;f\_Coniochaetaceae ■ k\_\_Fungi;p\_\_Ascomycota;c\_\_Sordariomycetes;o\_\_Coniochaetales;f\_\_unidentified k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Diaporthales;f\_Valsaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;Other k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;f\_Bionectriaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;f\_Clavicipitaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;f\_Hypocreaceae k\_\_Fungi;p\_\_Ascomycota;c\_\_Sordariomycetes;o\_\_Hypocreales;f\_\_Incertae\_sedis k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;f\_Nectriaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Hypocreales;f\_unidentified k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Incertae\_sedis;f\_Glomerellaceae k Fungi;p Ascomycota;c Sordariomycetes;o Incertae\_sedis;f Incertae\_sedis K\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Incertae\_sedis;f\_Magnaporthaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Incertae\_sedis;f\_Plectosphaerellaceae k\_\_Fungi;p\_Ascomycota;c\_\_Sordariomycetes;o\_\_Phyllachorales;f\_\_Phyllachoraceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Sordariales;Other k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Sordariales;f\_Chaetomiaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Sordariales;f\_Lasiosphaeriaceae k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Sordariales;f\_unidentified k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_Xylariales;Other k\_Fungi;p\_Ascomycota;c\_Sordariomycetes;o\_unidentified;f\_unidentified k\_Fungi;p\_Ascomycota;c\_unidentified;o\_unidentified;f\_unidentified k\_Fungi;p\_Basidiomycota;Other;Other;Other k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Boletales;f\_Coniophoraceae k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Cantharellales;f\_Ceratobasidiaceae

k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Corticiales;f\_Corticiaceae

- k Fungi;p Basidiomycota;c Agaricomycetes;o Geastrales;f Geastraceae
- Employeer Agaricomycetes;o\_Polyporales;f\_unidentified
- 
- Engip\_Basidiomycota;c\_Agaricomycetes;o\_Sebacinales;Other<br>
k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Sebacinales;Other<br>
k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Sebacinales;f\_Sebacinaceae
- k\_Fungi;p\_Basidiomycota;c\_Agaricomycetes;o\_Thelephorales;f\_Thelephoraceae
- k\_Fungi:p\_Basidiomycota;c\_Agaricomycetes;o\_unidentified;f\_unidentified
- Fungi;p\_Basidiomycota;c\_Cystobasidiomycetes;o\_Cystobasidiales;f\_Cystobasidiaceae
- 
- Entry and the product of the control of the product of the Fungity Design and the control of the contr
- k\_Fungi;p\_Basidiomycota;c\_Tremellomycetes;o\_Filobasidiales;f\_Filobasidiaceae
- k\_Fungi;p\_Basidiomycota;c\_Tremellomycetes;o\_Tremellales;f\_Cuniculitremaceae<br>k\_Fungi;p\_Basidiomycota;c\_unidentified;o\_unidentified;f\_unidentified
- 
- Europhycliaceae
- k\_Fungi;p\_Chytridiomycota;c\_Chytridiomycetes;o\_Rhizophydiales;f\_unidentified
- k\_Fungi;p\_Chytridiomycota;c\_unidentified;o\_unidentified;f\_unidentified
- Entrying Comeromycota;c\_Glomeromycetes;o\_Paraglomerales;Other<br>
k\_Fungi;p\_Glomeromycota;c\_Glomeromycetes;o\_Paraglomerales;Other<br>
k\_Fungi;p\_Zygomycota;c\_unidentified;o\_unidentified;f\_unidentified
- k\_Fungi;p\_unidentified;c\_unidentified;o\_unidentified;f\_unidentified
- 

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