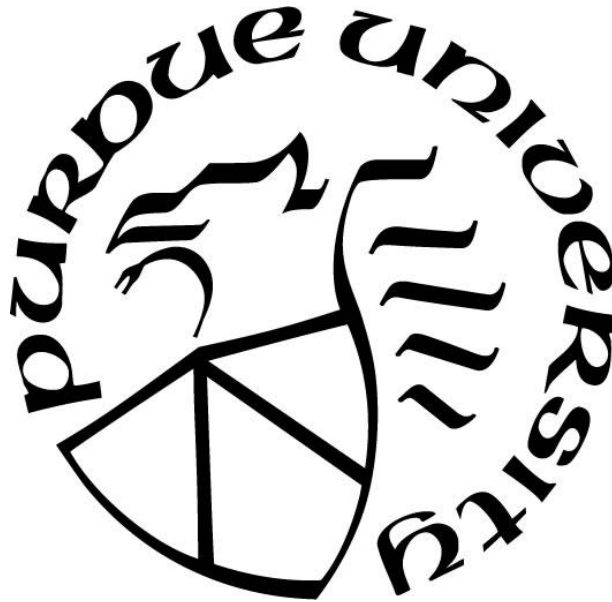# THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE COMMUNITY DYNAMICS OF BIOFERTILIZER MICROORGANISMS

by

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ABSTRACT

Biofertilizers are broths containing beneficial microorganisms that are applied to soils to enhance crop production and soil fertility. The microbes in a biofertilizer enhance and drive natural processes such as nutrient transformation and cycling, organic matter decomposition, and gas emission. Environoc 401 manufactured by Biodyne-USA is described as an agricultural soil enhancer that is comprised of a consortium of beneficial microorganisms. Production of Environoc 401 is achieved by an incubation that begins with a concentrated lyophilized microbial consortium. The focus of this study is to try to understand the community dynamics that occur during the incubation process to help predict the proportions of individual strains and the overall metabolic activity of the microbial community in Environoc 401 under different conditions. In order to quantify individual strains in Environoc 401, species-specific primers were developed for use in quantitative-PCR. These primers were then used to quantify target strains in Environoc 401 broth stored at 22 °C and 27 °C for 1 month and sampled at time 0, 1 week, and 1 month to evaluate the effect of storage conditions on the microbial community. In general, Environoc 401 stored at 22 °C had greater substrate utilization richness compared to Environoc 401 stored at 27 °C, but only after 1 month. The microbial community within Environoc 401 stored at 27 °C after 1 month did not utilize any amines or phenolic compounds, while the communities stored at 22 °C did use these substrates. To evaluate the overall effect of Environoc 401 on plants and on the microbial activity in potting medium, the product was used in the potting soil of soybean plants grown in an environmental growth chamber. This study contained five treatments upon unifoliate emergence: a no treatment control, pesticide and chemical fertilizer, pesticide and biofertilizer (as Environoc 401), biofertilizer only, and chemical fertilizer only. Soil medium samples were collected from each treatment at the time of seed planting, 24 hrs before application, 24 hrs after application, 2

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weeks after application, and 1 month after application. The soybean plants treated with Environoc 401 generally had the highest average total plant height, average number of leaves, average dry weight of leaves, stems, and roots, and the least acidic pH. Samples from both studies were also used to inoculate Biolog EcoPlates to assess changes in carbon-source utilization patterns for each condition and to generate Community-Level Physiological Profiles (CLPPs). Principle Component Analysis was performed on the CLPPs and diversity was also assessed using Shannon's diversity indices for samples from both studies. The CLPPs for the storage samples clustered tightly after 1 week of storage, however, after 1 month of storage the two temperatures diverged greatly. The CLPPS for the soybean plant treatment samples clustered tightly 24 hours prior to treatment but varied greatly after treatment application. These results indicate that treatment application, storage time, and temperature affect carbon utilization within the microbial communities. These results are a reflection on the activity and health of the microbial community and future studies should explore changes taking place on a finer scale by targeting specific carbon sources or conditions.

CHAPTER 1.   INTRODUCTION

Biodiversity, at multiple spatial, taxonomic, and functional scales, is important in maintaining ecological stability. However, knowledge of microbial diversity is not as comprehensive due to limitations in methodology (Xu et al., 2015). Soil microorganisms play vital roles in ecosystem function, and are capable of driving biochemical processes within the soil ecosystem such as nutrient transformation and cycling, organic matter decomposition and production of humus, and gas emission (Dai et al., 2016). The soil microbial community also can modify properties of the soil such as improving drainage and aeration, as well as altering the pH, available nitrogen and phosphorous, and water content of the soil. Although some soil inhabitants are harmful and cause disease, most are beneficial and form essential relationships within the soil complex (Rice & Rice, 2011). Phosphate-solubilizing bacteria, for example, are able to transform phosphorus from an immobilized state to a soluble state, which is essential for cellular metabolism, through release of neutral and alkaline phosphatase enzymes (Yahya & Al-Azawi, 1989).

The microbiota of the soil includes bacteria, archaea, algae, fungi, protozoa, amoebas, and even microscopic animals like nematodes. These organisms are responsible for the addition of a significant amount of organic matter and, though microscopic, in totality their weight can approach half a percent of the soil's weight. In one gram of soil, there is an estimated number of 10_4 to 10_6 species of bacteria (Martin et al., 2012). The rhizobia and mycorrhizal fungi possess symbiotic relationships with the root cells of many plant species. Specifically, the bacterium Rhizobium participates in nitrogen-fixation for legumes through the formation of root nodules (Denison & Kiers, 2011). Mycorrhizal fungi live inside or outside of the roots and can increase the root's capacity to uptake both water and nutrients, such as phosphorous, by ten-fold (Rice & Rice, 2011). The various microbes existing within the rhizosphere of plants stimulate plant growth by countless

mechanisms (Vessey, 2003). For example, rhizosphere microorganisms possess the ability to increase drought tolerance for plants that are growing under water-limited conditions. They achieve this by aiding in the creation of a well-developed root system for the plant by increasing root biomass (Marulanda et al., 2009). With a more developed root system, plants can increase their water content and become more drought tolerant. Rhizosphere microorganisms are also able to enhance root growth and development through indole acetic acid (IAA) and proline production, both of which are directly correlated with root growth (Marulanda et al., 2009).

Nutrients such as nitrogen and phosphorus are necessary for growth, development, and reproduction of plants. For example, although nitrogen naturally occurs within the environment, it exists mostly in its reactively inert form (N2), which is inaccessible to many plants for the biosynthesis of amino acids and nucleic acids (Tang et al., 2018). Therefore, plants are often reliant upon mutualistic microbes, such as rhizobia, to supply these nutrients in forms that are useful, such as ammonium (NH_{4+}) (Denison & Kiers, 2011). Nitrogen-fixing bacteria that convert atmospheric nitrogen into ammonium are important regulators of plant productivity because plants lack the ability to fix atmospheric nitrogen, which together with phosphorus and potassium, are the main limiting factors in plant productivity. Also important are the nitrifying and denitrifying bacteria (Van Der Heijden et al., 2008). Nitrifying microorganisms, such as *Nitrosomonas* and *Nitrobacter*, convert ammonium to nitrite or nitrate while denitrifying microbes, like *Pseudomonas* and *Bacillus*, convert nitrate to nitrogenous gases (Belser, 1979; Gamble et al., 1977; Verbaendert et al., 2011).

While the soil microbial community provides countless benefits, it is easily altered and disturbed. The soil environment directly influences both the diversity and activity of soil microbiota (Wu et al., 2008). Agricultural land management that often entails irrigation, crop rotation, and nutrient application can have a direct effect on the soil microbes. Alteration of pH in

particular, often a result of chemical fertilizer application, selects for only certain species of microbes and changes the availability of nutrients such as lead, copper, zinc, and nickel (Harter, 1983). Therefore, alternative methods, such as administration of a biofertilizer, help to restore soil health.

Biofertilizers are broths containing beneficial microorganisms that are applied to soils to enhance crop production. These microorganisms encourage plants to flourish by increasing the supply or availability of primary nutrients that the plant utilizes, and as a result, many of the microbes create mutualistic relationships with plants in their vicinity (Marulanda et al., 2009). Therefore, biofertilizers have been identified as a viable alternative to chemical fertilizers and they have been shown to increase soil fertility and crop production in sustainable farming (Wu et al., 2005).

While native soil microbes in the environment assist plants in accessing usable forms of both nitrogen and phosphorous, biofertilizer microbes enhance these natural processes through a variety of mechanisms, such as nitrogen-fixation, surfactant production, and cellulose digestion (Parr et al., 1994). Control and manipulation of the soil microflora using inoculants, organic amendments, and soil microflora is not a new concept. Microbiologists have known for decades that introducing inoculants, like biofertilizers, creates a more favorable soil microbiological environment for optimum crop production and protection (Parr et al., 1994). These microbes not only make nutrients more accessible, but also function as biocontrol agents by controlling or suppressing soil-borne plant pathogens through their competitive activities. Furthermore, microbes such as *Streptomyces albidoflavus* produce secondary bioactive metabolites, including antibiotics that can increase their competitive advantage by making the growth of other microbes unfavorable (Narayana & Vijayalakshmi, 2008).

Environoc 401 is a natural consortium of 27 strains of beneficial microorganisms manufactured by Biodyne USA (https://biodyne-usa.com). None of the microbes within this biofertilizer are genetically modified and all of them are non-pathogenic and naturally occurring, making it a valuable resource in organic farming. Organic farming involves the exclusion of pesticides, chemical fertilizers, genetically modified organisms, and growth hormones. Organic farming also does not disturb microbial community diversity and health to the extent observed with chemical fertilizers (Yuan et al., 2015). For example, overuse of chemical fertilizers can result in unintended environmental impacts such as the "dead zone" in the Gulf of Mexico. In this case, nutrients from fertilized farms washing across the Mississippi Basin caused oxygen starvation, resulting in a zone that is essentially uninhabitable in the gulf (Adesemoye et al., 2009). Organic farming not only has a positive public perception but also has demonstrated great benefits for soil microbial health, by increasing both the abundance and activity of soil microbial communities, making it a desirable cultivation technique (Lori et al., 2017).

Environoc 401 biofertilizer is initially produced in small batches using pure lyophilized cultures of each strain combined in specific ratios. These small batches are then used to seed larger batches for production in 100-gallon tanks. The first objective of this study was to identify and quantify several of the constituents of the Environoc 401 biofertilizer in the final batch using quantitative PCR with species-specific primers. These primers were used on DNA taken from Environoc 401 samples exposed to two separate storage temperatures and times to demonstrate their utility in quantifying target species in Environoc 401. The second objective of this study was to assess the effect of treatments with and without Environoc 401 on soybean plants and the microbial activity in the potting medium. To accomplish this, the soybean plants were exposed to treatments with and without Environoc 401, a chemical fertilizer (MiracleGro), and pesticide. For

each treatment, plant health was assessed using measures of plant height, mass, total chlorophyll *a*, and leaf color. The metabolic activity of the microbial consortium in the potting medium was assessed using Biolog Ecoplates (www.biolog.com), which measures the usage patterns of several carbon sources by the microorganisms in the sample. Further, to evaluate changes in the overall activity of the microbial community over time and under a variety of conditions, changes were also examined in the microbial activity of Environoc 401 broth in response to variable temperature and storage time. Overall, biofertilizers are a significant area of research because they play crucial roles in restoring soil fertility and enhancing crop production. With climate change and population growth becoming more problematic, improving agricultural productivity is of utmost importance.

CHAPTER 2.   MATERIALS AND METHODS

2.1   Primer Design and Specificity

To precisely identify the individual constituents within the Environoc 401 biofertilizer, primers were designed which target the 16S rRNA gene specific to each strain of interest. To do this, a sequence alignment of the 16S rRNA gene from all the strains in Environoc 401 was generated and used to identify unique priming regions for each strain as compared to the other strains. Ideally, primers for each individual strain in Environoc 401 should be created. However, since there are some genera with multiple species, and sometimes even more than one strain of the same species present, it was not possible to achieve this level of resolution. NCBI Primer-BLAST tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to initially identify potential forward and reverse priming regions 100 to 300 bases apart in each sequence for consideration. These initial primer sequences were assessed for specificity according to the sequence alignment (Appendix A) and tested against pure culture DNA (positive control), Environoc 401 biofertilizer DNA, and *Escherichia coli* DNA (negative control) using standard PCR. DNA was extracted from each of these sources using the Qiagen DNeasy PowerSoil Kit. Each 20 μl PCR mixture was composed of 10 ng template genomic DNA from each strain, 2 μl 10x DreamTaq buffer, 0.4 μl 10mM dNTP mix, 100 μM of each primer, and 0.1 μl DreamTaq DNA polymerase (5Uμl-1, 200U; Thermo Fisher Scientific). PCR cycling was done in a Bio-Rad T100 thermal cycler starting with 5 min at 94°C then 34 cycles of 94°C for 1 min, annealing for 1 min with the temperature dependent upon the primer set used (Table 1), and 72 °C for 2 min followed by a final extension of 72 °C for 10 min. 16S rRNA gene PCR products were purified using the Thermo Fisher Scientific GeneJET

PCR Purification Kit and sequenced by McLab Laboratories (https://www.mclab.com/) using Sanger sequencing. Sequences were then verified using the NCBI BLAST tool. Primers were successfully generated for *Azospirillum brasilense*, *Bacillus altitudinis*, *Bacillus subtilis subtilis*, and *Rhodopseudomonas palustris*.

2.2 Generation of Standard Curves for Quantitative-PCR

For quantification of the DNA of the four strains listed above, a standard curve containing each species-specific 16S rRNA gene sequence with known values of starting material was constructed for each target sequence for use in quantitative-PCR. To do this, the template genomic DNA was first obtained from pure cultures using the Qiagen DNeasy PowerSoil Kit following overnight growth in TSA at 37 °C. Following PCR amplification and sequence verification as described above, the 16S rRNA gene products were cloned into the pGEM-T vector (Promega) according to the manufacturer's instructions. Blue-white screening was then performed to validate that the genes were successfully incorporated into the vector. Following transformation into *E. coli* and plating onto LB with 1 μg ml-1 ampicillin, 0.5 mM IPTG, and 80 μg ml-1 X-gal, white colonies were selected. The lack of color generation in these colonies indicates successful insertion of the PCR product into the *lacZ* gene, rendering β-galactosidase non-functional and unable to cleave X-gal, which would otherwise produce a blue product (Wehmeier, 1995). Plasmids from the positive clones were purified using Thermo Fisher Scientific GeneJET Plasmid Miniprep Kit. The 16S rRNA gene was sequenced by McLab Laboratories to validate specificity using T7 primers that target priming sites on the plasmid flanking the insertion site. Sequences were verified for specificity to the intended target using the NCBI BLAST tool. The concentration of the plasmids

was determined using the Thermo Fisher Scientific Nanodrop Lite spectrophotometer and the copy number of the 16S rRNA genes was calculated for dilution and use in the standard curves.

2.3   Quantification of Environoc 401 Microorganisms

Four biofertilizer microorganisms (*Azospirillum brasilense*, *Bacillus altitudinis*, *Bacillus subtilis subtilis*, and *Rhodopseudomonas palustris*) were selected for detection via quantitative-PCR (qPCR) using DNA from freshly manufactured Environoc 401 as well as from Environoc 401 stored at various temperatures for varying amounts of time (see Storage Condition Experiment below). qPCR was performed in a Bio-Rad CFX96 real-time thermal cycler. Each 20 µl reaction consisted of 10 µl 2x SYBR Green Master Mix (Bio-Rad), 100 µM of each primer, 10 ng template DNA, and PCR grade water. Reactions were run at 95 °C for 3 min, then 39 cycles of 95 °C for 10 sec and 30 sec at the annealing temperature associated with each primer set (see Table 1). All samples were run in triplicate and standard curves were generated using the plasmids described above in 10-fold dilutions. DNA was extracted from pure cultures of *A. brasilense*, *B. altitudinis*, *B. subtilis subtilis*, and *R. palustris* as described above, and the pure culture DNA served as positive controls. PCR grade water served as the non-template control. Starting quantities, as the log copy number, of each strain in each sample were then calculated by comparison to the standard curve.

2.4   Storage Condition Experiment

Possible changes in the overall activity of the microbial community in Environoc 401 over time and under storage conditions are unknown, some of these changes were examined in the microbial activity of the broth in response to variable temperature and storage time. Six-50 mL Falcon tubes were filled with 30 mL of fresh Environoc 401 and stored at either 22 °C or 27 °C

with constant agitation. Three of the stored Falcon tubes at each temperature were sampled after 1 week of storage while the remaining three were sampled after 1 month of storage. Sampling consisted of DNA extractions for qPCR and the EcoPlate analysis as described below, with 1 ml of sample used for inoculation of the EcoPlates. A control (time 0) sample was also taken using the same batch of Environoc 401 that was used in the rest of the experiment.

Table 1. Primers used in standard and quantitative PCR.

Primer Name	Primer Sequence (5' to 3')	Annealing Temperature (°C)	Organism Name	Product Size (bp)
AbrasF	AGTAACACGTGGGAACCTGC	56	*Azospirillum brasilense*	192
AbrasR	CAGACCAGCTACCGATCGTC			
BaltF	GAGCTTGCTCCCGGATGTTA	60	*Bacillus altitudinis*	122
BaltR	TGAACCATGCGGTTCAAGGA			
BsubF	CCGGATGGTTGTTTGAACCG	60	*Bacillus subtilis subtilis*	166
BsubR	CGTGTCTCAGTCCCAGTGTG			
Rpal2F	GGAAACCCTGATCCAGCCAT	56	*Rhodopseudomonas palustris*	127
Rpal2R	CGGGGCTTATTCTTGCGGTA			

2.5   Soybean Plant Experiment

Soybean plants resistant to the broad-spectrum herbicide Dicamba, were planted in 10.2 cm diameter x 8.9 cm height pots holding a volume of 720.7 cm$_3$ filled with nonsterile sphagnum potting mix (SunGro Horticulture SKU: SUGRMIX4MYCO) up to 2.5 cm from the rim of the pot. For each pot, one soybean seed was planted 1 cm deep into the center of each pot. Plants were housed within an environmental growth chamber with a 16:8 day:night photoperiod throughout the study. Over the course of the study, the mean temperature was 24.1 °C and the mean humidity was 49.7%.

Soybean plants were subjected to five treatments (Table 2): 1) a no treatment control, 2) Bayer Insecticide (pesticide) and MiracleGro (chemical fertilizer), 3) Bayer Insecticide and Environoc 401 (biofertilizer), 4) Environoc 401 only, and 5) MiracleGro only. Environoc 401, MiracleGro, and the pesticide were all applied at a rate of 5.6 kg ha$_1$ at unifoliate emergence (approximately 14 days after planting seedlings).

Plants received 0.91 ml of the respective treatment (1.82 ml if pesticide and fertilizer were applied) directly over the unifoliate, and the control treatment received 0.91 ml of sterile DI water. The day of treatment application, Bayer (Complete Insect Killer) and MiracleGro were reconstituted and diluted with sterile DI water following supplier instructions. The soybean plants received 50 ml of sterile DI water every 2 days and were rotated 180° once a week to avoid phototropism. Every week the number of leaves, color of leaves, height (cm) of the soybean plant, and pH of the sphagnum mix (SoilStik pH Meter-Flat Sensor; Spectrum Technologies Inc.) were recorded from all of the individuals within each treatment. The color of the leaves was measured by comparison to a form and foliage color wheel (Fig. 1).

The sphagnum potting mix for each treatment was sampled five times; at the time of seed planting (denoted as time 0), 24 hours before treatment, 24 hours after treatment, 2 weeks after treatment, and 1 month after treatment. For each time point, sphagnum potting mix samples were used to inoculate EcoPlates as described below. All plants were sacrificially harvested one-month post treatment at the conclusion of the experiment. Soybean plants were divided into their principle parts (stem, leaf, and root) and oven-dried at 50 °C to a constant weight (~20 hrs). Dry weight was then recorded for each part. Chlorophyll was also extracted using acetone from leaf-hole-punched disks 6 mm in diameter, in total weighing 1 g, obtained from leaves at both the first and second nodes containing trifoliate leaves (Hiscox & Israelstam, 1979). Chlorophyll *a* content was calculated according to Arnon (1949).

Table 2. Treatments applied to soybean plants.

Treatments	Treatment Number	Treatment Description	Number of Plants
No Treatment Control	1	Control	10
Bayer Insecticide and MiracleGro	2	Pesticide and Chemical Fertilizer	9
Bayer Insecticide and Environoc 401	3	Pesticide and Biofertilizer	8
Environoc 401	4	Biofertilizer	5
MiracleGro	5	Chemical Fertilizer	8

Figure 1. Color wheel utilized to determine the color of leaves (Form & Foliage, 2013).

2.6　Assessment of the Microbial Community Metabolic Activity

Biolog EcoPlates were used to analyze the microbial community function in the Environoc 401 consortium through evaluation of their metabolic activity under different conditions. EcoPlates (www.biolog.com) are specifically designed to investigate environmental samples (Janniche et al., 2012; Xu et al., 2015); they are microtiter plates with 96-wells that are divided into three identical groups, with each group having 31 wells with different single carbon sources (Fig. 2).

Present in all wells is triphenyl tetrazolium chloride, and if any constituents of the Environoc 401 consortium metabolizes the carbon source in any specific well this dye will be reduced by the microbial activity to form a color change from clear to purple. This color change can then be analyzed spectroscopically to yield the Community-Level Physiological Profile (CLPP). The CLPP fingerprint, which is based off the metabolic capabilities of the microorganisms in the sample, can then be compared between samples to assess the activity and health of the microbial community as well as changes over time (Janniche et al., 2012). EcoPlates were inoculated by diluting one gram of the soybean's sphagnum mix (or 1 ml broth for the storage experiment) into 99 mL of sterile DI water. Once homogenized, 1 mL of the homogenate was diluted into 99 mL of sterile 0.8% NaCl resulting in a 10_{-4} dilution. One hundred and fifty µL of the diluted homogenate was inoculated into every well in the first replicon of the plate (this was repeated in triplicates from three separate samples). EcoPlates were incubated at 30 °C for 48 hrs and then read using the accuSkan plate reader (Fisher Scientific) at 590 nm. Absorbance readings were then used to calculated CLPP fingerprints. In order to derive the CLPPs, the absorbance values of each carbon substrate were corrected by subtracting the absorbance value of the control well that contained water (Fig. 2). Any negative values that arose from this correction were

recorded as zero. After the absorbance values were corrected, the Average Well Color Development (AWCD) was calculated by taking the mean of the three replicates, yielding a mean for each of the 31 corrected values (Janniche et al., 2012).

The functional diversity was then calculated based on the substrate utilization richness (S), which is simply the number of metabolized carbon substrates in each EcoPlate. A carbon substrate is considered metabolized if the corrected absorbance value is greater than 0.25 (Janniche et al., 2012). The p_i, which is the approximate use of one specific carbon source, is the ratio between the corrected absorbance value within each well and the summation of the corrected absorbance values of the EcoPlate. Preferential utilization of a carbon source was denoted for those with p_i values above 0.032, thus if each of the 31 carbon sources were utilized equally, then the relative use of each specific carbon would be 0.032 (Janniche et al., 2012). Shannon's diversity index (H'), calculated as $H' = -\sum p_i(\ln p_i)$, was another measure of diversity used. Shannon's diversity index takes in account both the richness and the evenness of the carbon substrates utilized. As both richness and evenness increase, so does the Shannon's diversity index. Therefore, large H' values indicate higher functional diversity in the sample.

2.7   Statistical Analysis

All statistical tests were performed in RStudio software version 3.6.1. Soybean plant physiological data collected, such as the number of leaves, height (cm) of the soybean plant, pH of the sphagnum potting medium, and dry weight were analyzed using one-way ANOVA. Results obtained from qPCR and EcoPlate experiments were analyzed using two-way ANOVA. Response variables were tested for normal distribution using a Shapiro-Wilk test for normality. Tukey-HSD

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was used as a post-hoc analysis for pairwise comparisons of treatments. All statistics were performed at a 95% confidence level.

To determine how the various samples were related to one another based on the CLPP generated, the absorbance values from the storage and treatment assessments were analyzed by principle component analysis (PCA) (Janniche et al., 2012). PCAs were run using Bray-Curtis distance calculations. Therefore, the relative distance between points in the ordination is relative dissimilarity.

A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine	A1 Water	A2 β-Methyl-D-Glucoside	A3 D-Galactonic Acid γ-Lactone	A4 L-Arginine
B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine	B1 Pyruvic Acid Methyl Ester	B2 D-Xylose	B3 D-Galacturonic Acid	B4 L-Asparagine
C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine	C1 Tween 40	C2 i-Erythritol	C3 2-Hydroxy Benzoic Acid	C4 L-Phenylalanine
D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine	D1 Tween 80	D2 D-Mannitol	D3 4-Hydroxy Benzoic Acid	D4 L-Serine
E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine	E1 α-Cyclodextrin	E2 N-Acetyl-D-Glucosamine	E3 γ-Hydroxybutyric Acid	E4 L-Threonine
F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid	F1 Glycogen	F2 D-Glucosaminic Acid	F3 Itaconic Acid	F4 Glycyl-L-Glutamic Acid
G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine	G1 D-Cellobiose	G2 Glucose-1-Phosphate	G3 α-Ketobutyric Acid	G4 Phenylethyl-amine
H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine	H1 α-D-Lactose	H2 D,L-α-Glycerol Phosphate	H3 D-Malic Acid	H4 Putrescine

Figure 2. Carbon sources in a Biolog EcoPlate (www.biolog.com).

CHAPTER 3.   RESULTS

3.1   Primer Specificity

Primer sets Abras, Balt, Bsub, and Rpal2 were all tested for specificity to the target strains (see Table 1) through standard PCR before use in qPCR. Each of the primer sets successfully amplified DNA of the correct size from Environoc 401 (Appendix A) and the correct sequences were confirmed from all the products. Further, each primer set did not amplify a negative control of *E. coli* DNA or water, which serves as a no template control (NTC).

3.2   Quantification of Target Strains in Environoc 401 Storage Samples

The primers designed above were used to quantify each respective strain from Environoc 401 samples obtained in the storage condition experiment described previously. Using DNA extracted from Environoc 401 samples stored at 22 °C or 27 °C after 1 week and 1 month of storage as the template in qPCR, the starting log copy number of the 16S rRNA gene from each strain was successfully calculated using a standard curve (Fig. 3). Storage samples were overall significantly different for *Azospirillum brasilense* ($p < 0.001$), *Bacillus altitudinis* ($p = 0.026$), *Bacillus subtilis subtilis* ($p < 0.001$), and *Rhodopseudomonas palustris* ($p < 0.001$) populations (Fig. 4). For *A. brasilense*, the 22 °C stored samples significantly increased in quantity from 1 week of storage to 1 month of storage while the 27 °C stored samples were not significantly different between 1 week and 1 month of storage. The quantity of *B. altitudinis* significantly increased after 1 month of storage at 27 °C, but was not significantly different from the control or storage at 22 °C. The *B. subtilis subtilis* populations were significantly different amongst all samples except for the sample stored at 22 °C for one month and the time 0 sample, which were not significantly different. The

amount of *R. palustris* was significantly different in all stored samples except for the sample stored at 27 °C for 1 month and the time 0 sample which were not significantly different.

At time 0, all populations had a significantly different starting quantity ($p < 0.001$). After 1 week of storage at 22 °C, none of the populations had a significantly different starting quantity ($p = 0.303$). However, after 1 month of storage at 22 °C, there was a significant difference in starting quantity amongst the different populations ($p < 0.001$). Specifically, *A. brasilense* and *R. palustris* had a significantly higher starting quantity compared to *B. altitudinis* and *B. subtilis subtilis*. After 1 week of storage at 27 °C, all of the populations had a significantly different starting quantity ($p < 0.001$). By 1 month of storage at 27 °C, all populations had significantly different starting quantities except *B. altitudinis* and *A. brasilense*.

A

$y = -3.139x + 40.262$
$R^2 = 0.945$
$E = 108.2\%$

Azospirillum brasilense log starting quantity

B

$y = -3.786x + 46.316$
$R^2 = 0.916$
$E = 83.7\%$

Bacillus altitudinis log starting quantity

C

$y = -3.193 + 39.616$
$R^2 = 0.939$
$E = 105.7\%$

Bacillus subtilis subtilis log staring quantity

D

$y = -2.903x + 38.208$
$R^2 = 0.910$
$E = 121.0\%$

Rhodopseudomonas palustris log starting quantity
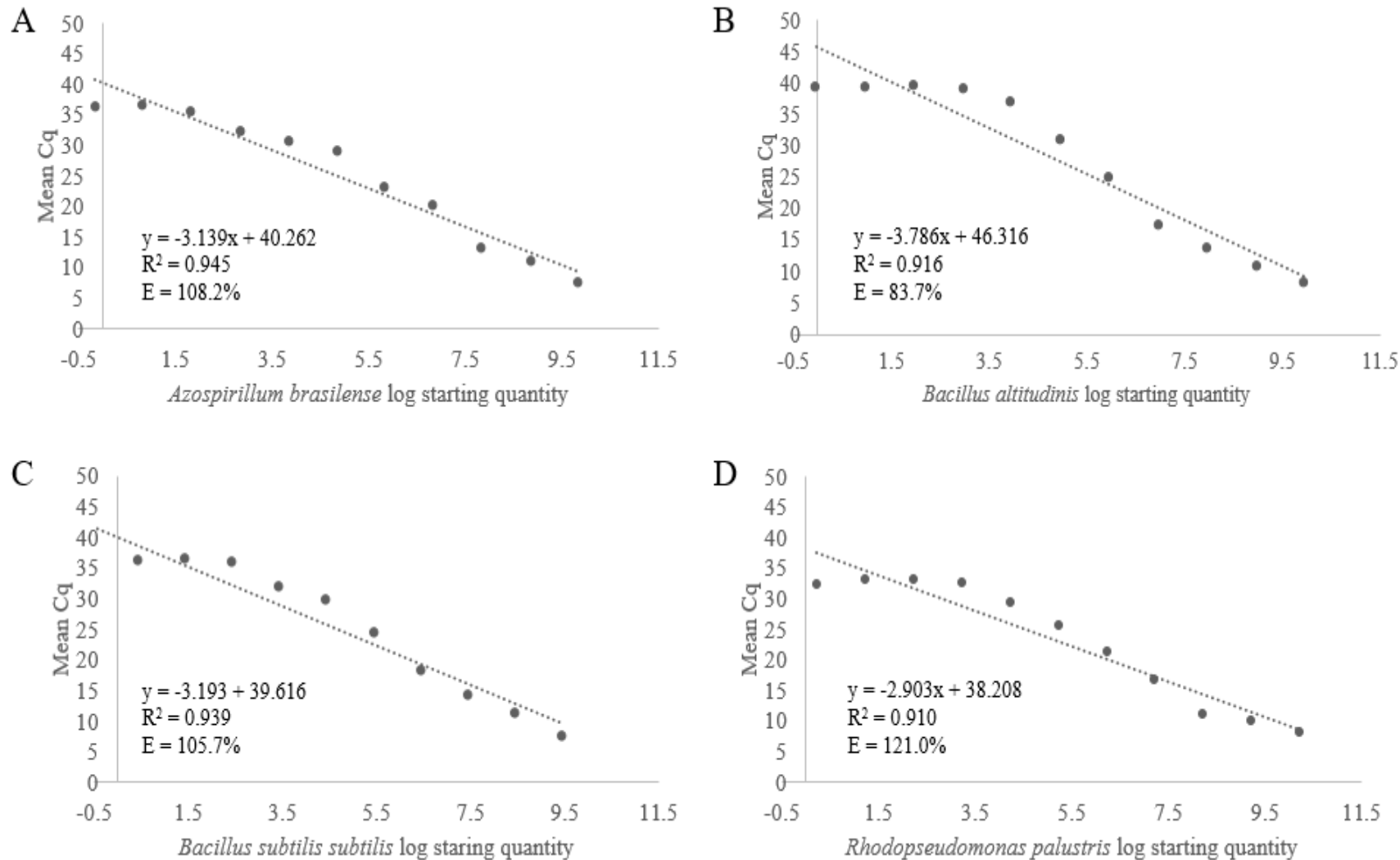
Figure 3. Standard curves used in qPCR on the Environoc 401 storage samples for A: *Azospirillum brasilense*, B: *Bacillus altitudinis*, C: *Bacillus subtilis subtilis*, and D: *Rhodopseudomonas palustris*.
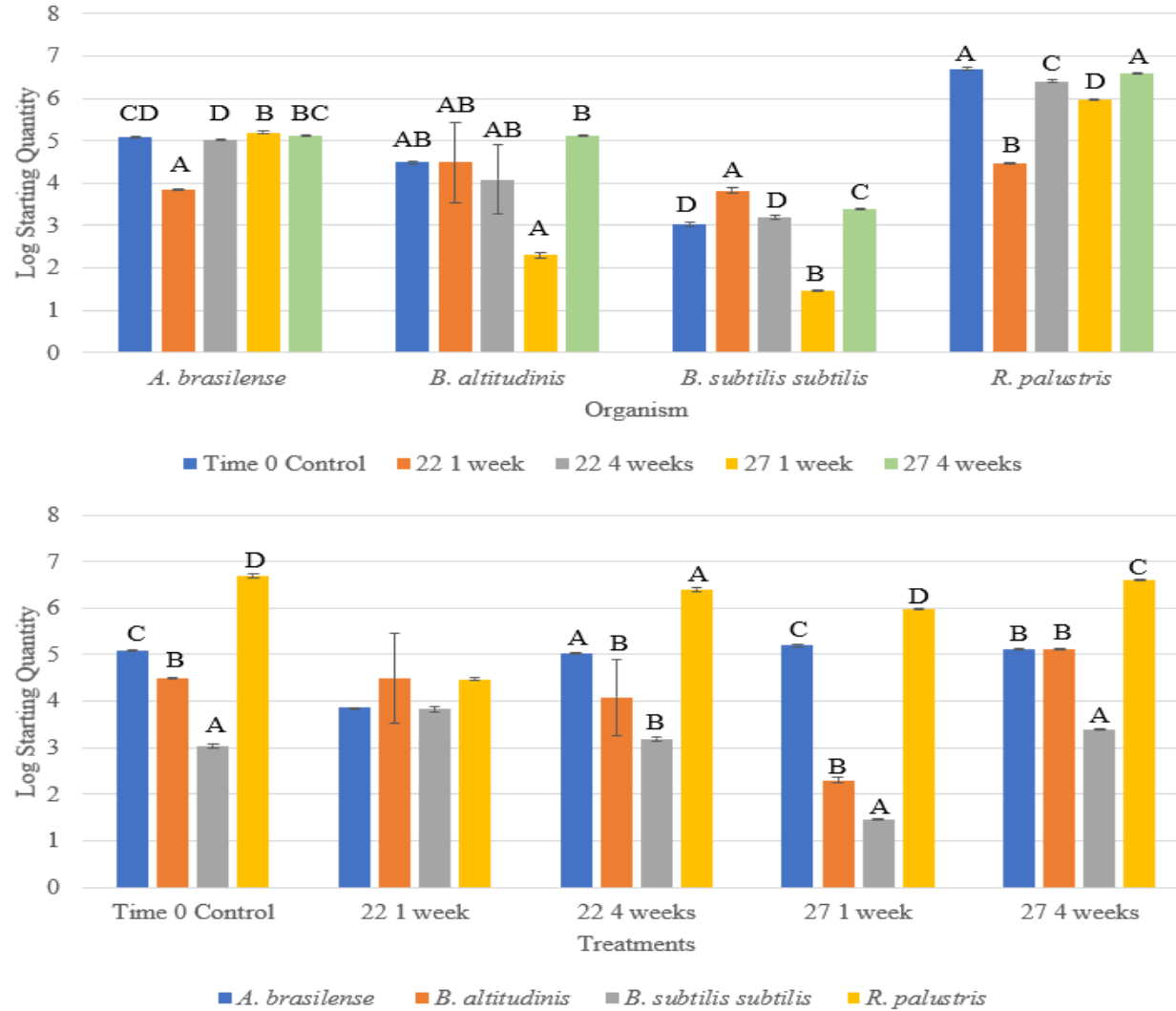
Figure 4. The log starting quantity of *A. brasilense, B. altitudinis, B. subtilis subtilis*, and *R. palustris* in Environoc 401 stored at either 22 °C or 27 °C for up to a month shown as a function of A) each strain and B) each treatment. Error bars represent standard error (n=3). The treatments with different letters are significantly different than each other (α = 0.05).

3.3 Soybean Plant Results

By the end of the treatment period, there was a significant difference between treatments overall in the sphagnum potting medium pH ($p < 0.001$; Fig. 5). The biofertilizer treatment had the least acidic potting medium pH (6.38 ± 0.05 SE) while the pesticide and chemical fertilizer treatment had the most acidic potting medium pH (5.59 ± 0.11 SE). After analysis with a Tukey HSD post-hoc test, there was no significant difference found between the biofertilizer treatment, the control, and the pesticide and biofertilizer treatment. However, the biofertilizer had a significantly higher pH when compared to the chemical fertilizer treatment and the pesticide and chemical fertilizer treatment. Also, only the biofertilizer treatment increased in pH once the treatment was applied.

In general, at the endpoint of the experiment, there was a significant difference in the average total plant height between the treatments ($p < 0.001$; Fig. 6). By the end of the treatment period, both the biofertilizer treatment (73.4 cm $\pm$ 5.19 SE) and the pesticide and biofertilizer treatment (73.6 cm $\pm$ 1.97 SE) had the largest average total plant heights while the chemical fertilizer treatment had the smallest average total height (54.8 cm $\pm$ 3.8 SE). There was also a significant difference between the chemical fertilizer treatment and the control and both biofertilizer treatments. The average number of leaves between the treatments differed significantly overall by the end of the experiment ($p < 0.001$; Fig. 7). The biofertilizer treatment (6.4 leaves $\pm$ 0.24 SE) had the highest average number of leaves while the chemical fertilizer treatment (4.8 leaves $\pm$ 0.23 SE) had the lowest average number of leaves by day 42. In fact, the biofertilizer treatment had a significantly higher average number of leaves when compared to all treatments except the pesticide and biofertilizer treatment, which was not significantly different.

Even so, the pesticide and biofertilizer treatment had a significantly higher average number of leaves compared to both the control and the chemical fertilizer treatments. All treatments except for the biofertilizer treatment and the pesticide and biofertilizer treatment contained plants that had either yellowing leaves or leaves turning reddish-purple (data not shown) which is likely indicative of either a nitrogen deficiency, a phosphorous deficiency, or perhaps a combination of both (Rice & Rice, 2011).

At the end of the experiment, the soybean plants were sacrificed, and dry weights were recorded for leaves (Fig. 8), stems (Fig. 9), and roots (Fig. 10). Overall, significant differences were found in the dry weight of leaves ($p < 0.001$), stems ($p = 0.030$), and roots ($p < 0.001$) between all treatments. The average dry weight per leaf was lowest for both the control treatment (0.12 g/cm_2 ± 0.01 SE) and the biofertilizer treatment (0.12 g/cm_2 ± 0.01 SE) and highest for the chemical fertilizer treatment (0.16 g/cm_2 ± 0.01 SE) and the pesticide and biofertilizer treatment (0.14 g/cm_2 ± 0.003 SE). The chemical fertilizer treatment had a significantly higher average dry weight per leaf when compared to every treatment except the pesticide and biofertilizer treatment. For the dry weight of stems, only the control and the biofertilizer treatments were significantly different. The biofertilizer treatment (0.51 g ± 0.06 SE) had the largest average dry weight of stems while the control treatment (0.37 g ± 0.02 SE) had the smallest average dry stem weight. The root dry weight of the control treatment was significantly lower compared to all treatments except for the pesticide and chemical fertilizer treatment, for which there was no significant difference. The pesticide and biofertilizer treatment (0.55 g ± 0.02 SE) and the biofertilizer treatment (0.54 g ±0.06 SE) had the greatest average stem weight while the control treatment (0.27 g ± 0.06 SE) had the smallest average stem weights. Chlorophyll *a* was also extracted at the end of the experiment (Table 4). The average chlorophyll *a* content was highest for the pesticide and chemical fertilizer

treatment (7.70 µg/cm$_3$ ± 1.01 SE) and lowest for the control treatment (5.23 µg/cm$_3$ ± 0.72 SE). However, there was not a significant difference in chlorophyll *a* content between any of the treatments ($p = 0.058$; Table 4).
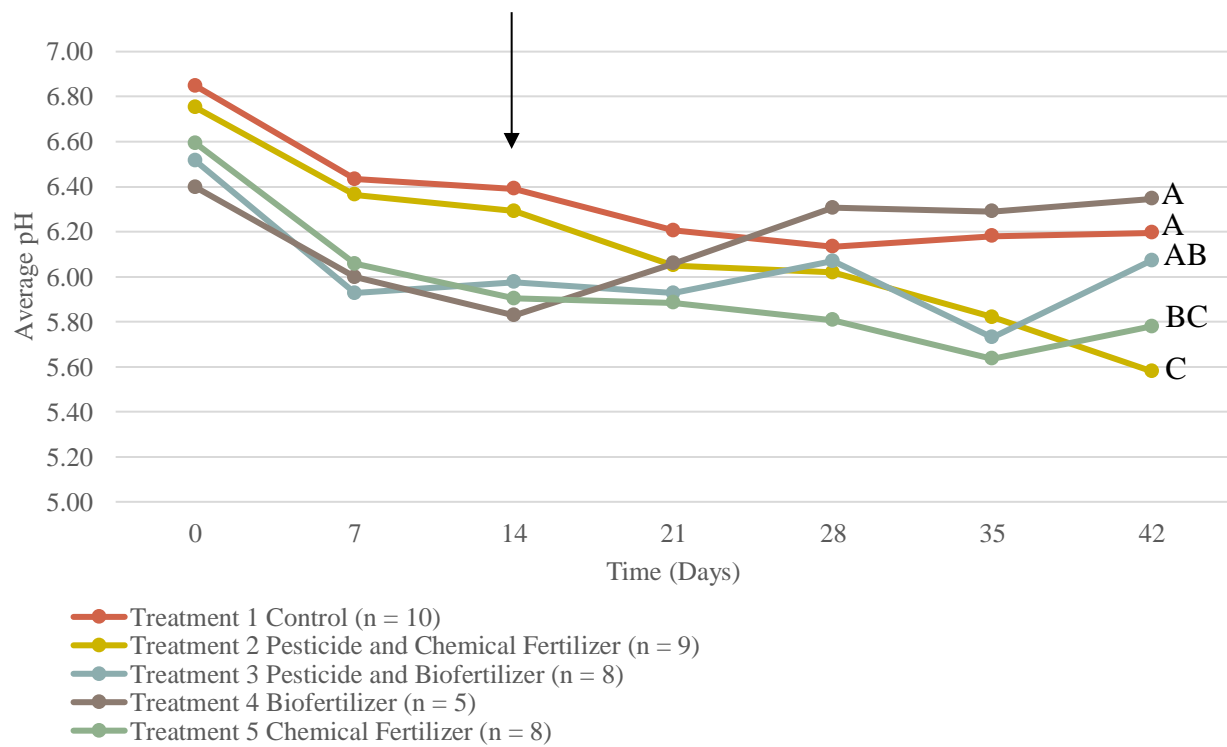
Figure 5. Average pH of the soybean potting medium over time for the various treatments. The treatments with different letters are significantly different than each other at the endpoint (α = 0.05). Treatment application occurred on day 14 as indicated by the black arrow.
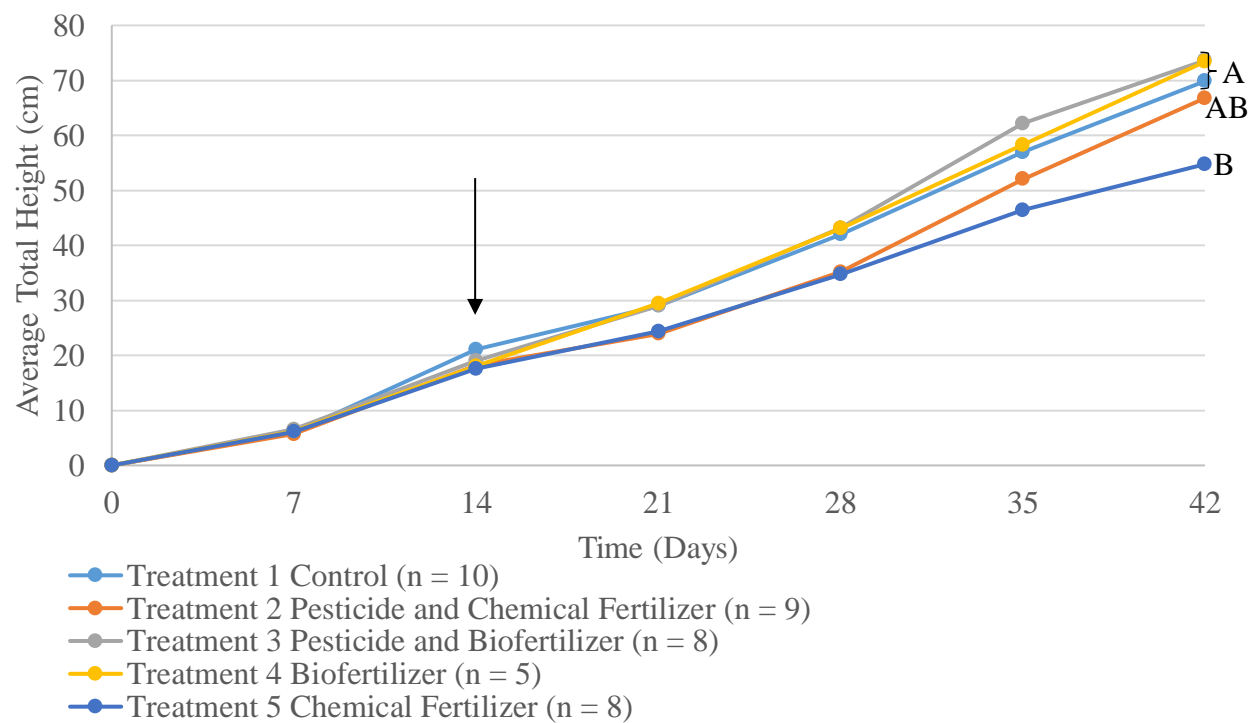
Figure 6. Average total height in cm of soybean plants subjected to various treatments. The treatments with different letters are significantly different than each other at the endpoint (α = 0.05). Treatment application occurred on day 14 as indicated by the black arrow.
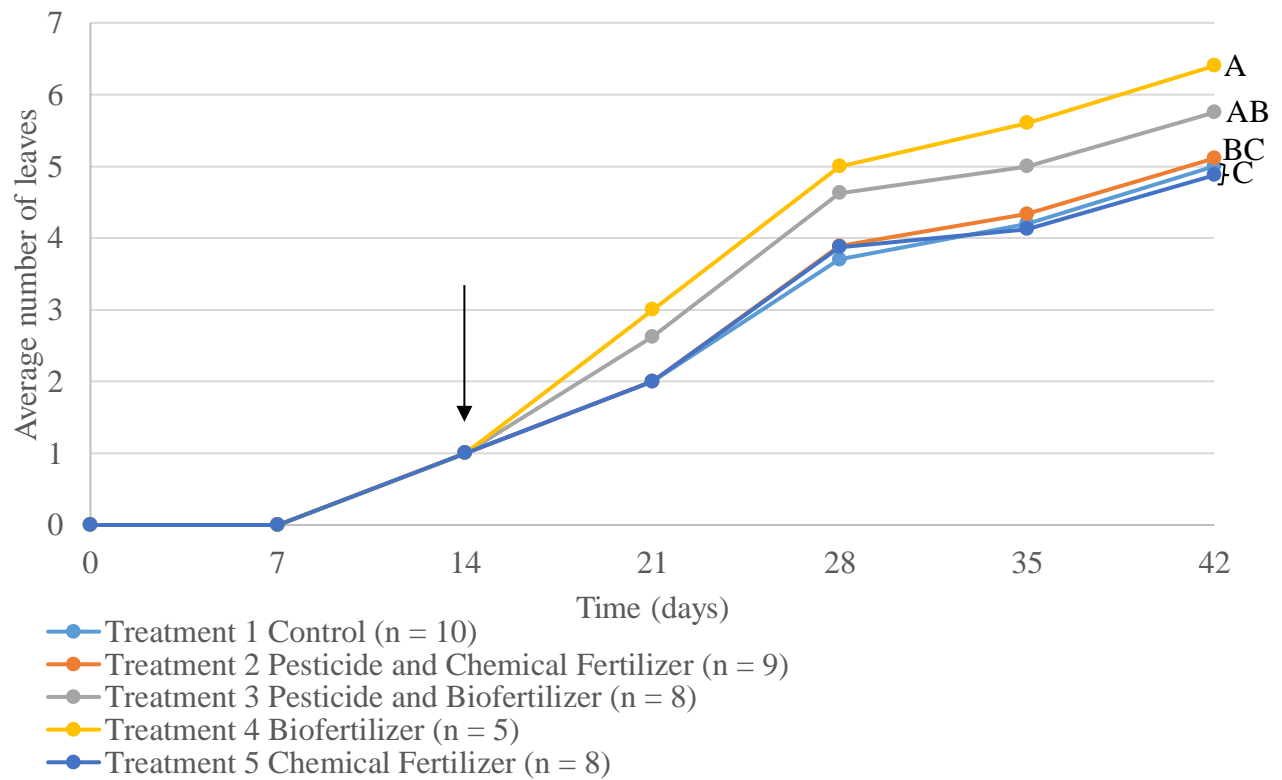
Figure 7. Average number of total leaves for soybean plants subjected to various treatments over time. The treatments with different letters are significantly different than each other at the endpoint (α = 0.05). Treatment application occurred on day 14 as indicated by the black arrow.
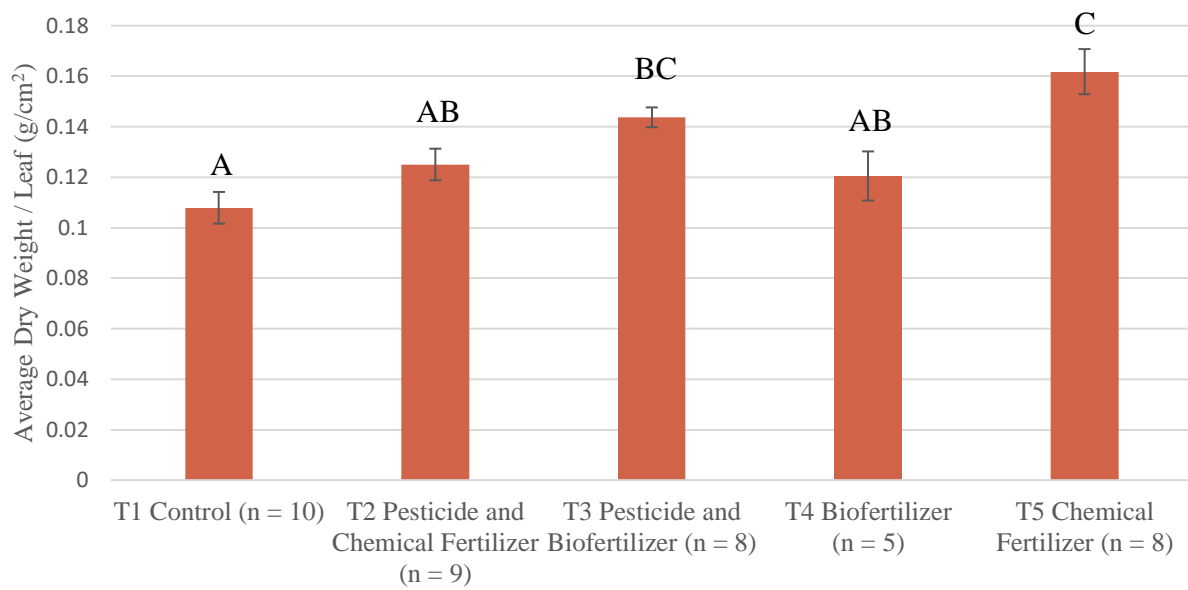
Figure 8. Average dry weight per leaf (g/cm2) from soybean plants subjected to various treatments. The treatments with different letters are significantly different than each other at the endpoint ($\alpha = 0.05$).
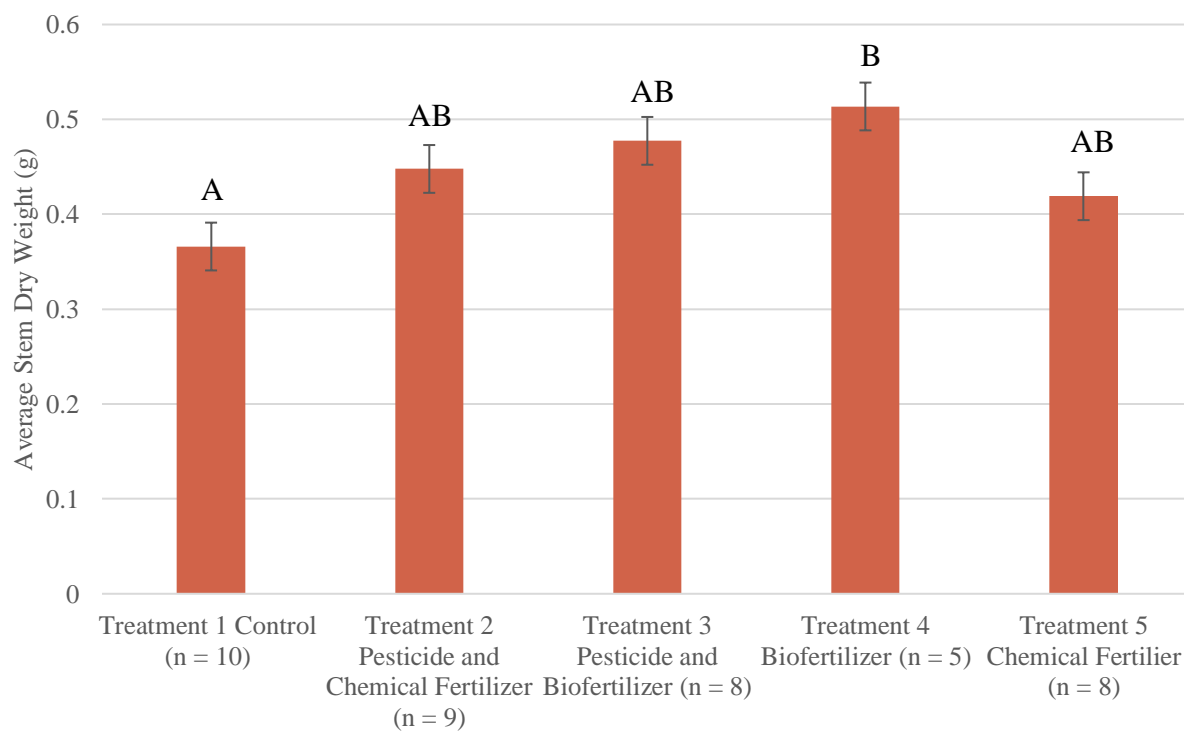
Figure 9. Average dry weight of stems (g) from soybean plants subjected to various treatments. The treatments with different letters are significantly different than each other at the endpoint (α = 0.05).
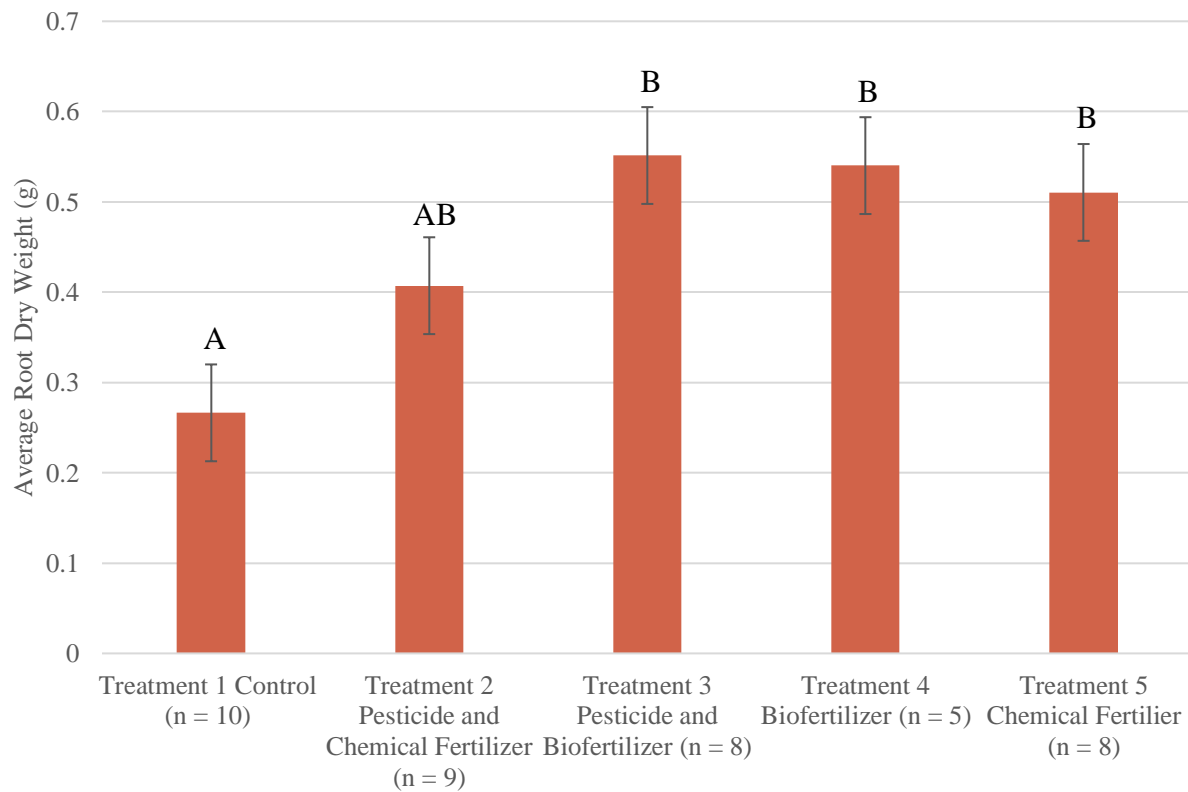
Figure 10. Average dry weight of roots (g) from soybean plants subjected to various treatments. The treatments with different letters are significantly different than each other at the endpoint (α = 0.05).

Table 3. Chlorophyll *a* content.

Treatment	Number of Samples	Mean (μg/cm$_3$) $\pm$ Standard Error	Range (μg/cm$_3$)
Control	10	5.23 ± 0.72	$0.53 - 9.07$
Pesticide and Chemical Fertilizer	9	7.70 ± 1.01	$0.65 - 10.81$
Pesticide and Biofertilizer	8	6.72 ± 0.23	$6.10 - 8.19$
Biofertilizer	5	5.40 ± 0.64	$2.88 - 6.93$
Chemical Fertilizer	8	7.46 ± 0.25	$6.81 - 8.45$

Table 4. Results of ANOVAs performed at a 95% confidence interval for data collected 1-month post treatment in the soybean plant experiment.

Dependent Variable	df	F statistic
pH	4	16.21
Plant Height (cm)	4	5.385
Number of Leaves	4	10.66
Weight of Leaves (g/cm$_2$)	4	9.710
Weight of Stems (g)	4	3.048
Weight of Roots (g)	4	9.899
Chlorophyll a (μg/cm$_3$)	4	2.671

3.4   Carbon Source Utilization by Microorganisms in Soybean Plant Treatments

Two-way ANOVAs were used to analyze substrate utilization richness data obtained from the Ecoplates in response to treatment and time (Table 5). The carbon substrate utilization richness is the number of carbon sources that were considered metabolized ($Abs_{590} > 0.25$). It was found that there was not a significant difference in substrate utilization richness in response to treatment ($p = 0.647$), but there was for time ($p < 0.001$). The 31 potential carbon sources that could be metabolized (Fig. 2) were divided into six carbon types: amines, amino acids, carbohydrates, carboxylic acids, phenolics, and polymers (Janniche et at., 2012). In order to investigate potential differences between treatments with respect to individual carbon types, six linear regressions were generated (one for every carbon type). For each regression, p-values were adjusted using the Holm-Bonferroni method. For all six carbon types there was a significant difference ($p < 0.05$) in substrate utilization between the biofertilizer treatment and all of the other treatments (Table 6).

Table 5. Results of ANOVA performed at a 95% confidence interval of carbon source utilization as determined by the Ecoplate richness data in response to treatment and time.

Dependent Variable	df	F statistic	p-value
Treatment	4	0.62	0.647
Time	4	8.29	< 0.001*

*Indicates significance $p < 0.05$.

Table 6. t statistic and *p*-value for carbon groups utilized as a response to biofertilizer treatment when compared to all other treatments in the soybean plant experiment.

Carbon Groups	Estimate	Standard Error	t value	*p*-value
Amines	1.20	0.35	3.46	0.002
Amino Acids	1.80	0.70	2.56	0.010
Carbohydrates	5.00	1.54	3.25	0.004
Carboxylic Acids	2.80	0.68	4.15	< 0.001
Phenolics	0.60	0.26	2.30	0.030
Polymers	2.00	0.36	5.51	< 0.001

p-values were adjusted using the Holm-Bonferroni method. The estimate represents the distance between two group's means. The t value is the calculated difference in units of standard error.

3.5 Community-Level Physiological Profiles (CLPP) of Soybean Plant Treatments

CLPPs were generated for the soybean plant treatments. A carbon source was considered utilized if it had a corrected $Abs_{590} > 0.25$. In general, carbon substrate utilization richness was highest for all treatments between 24 hours and two weeks post treatment (Figs. 11 and 12). The corrected Abs_{590} values were then used to generate a PCA ordination of the soybean plant treatments (Fig. 13). Overall, the only time the treatments cluster tightly is 24 hours before treatment application (green in Fig. 13). After the treatments are applied, they do not appear to cluster on the basis of time or treatment, except for the control and the chemical fertilizer treatment, which cluster at 24 hours and 1-month post treatment application.
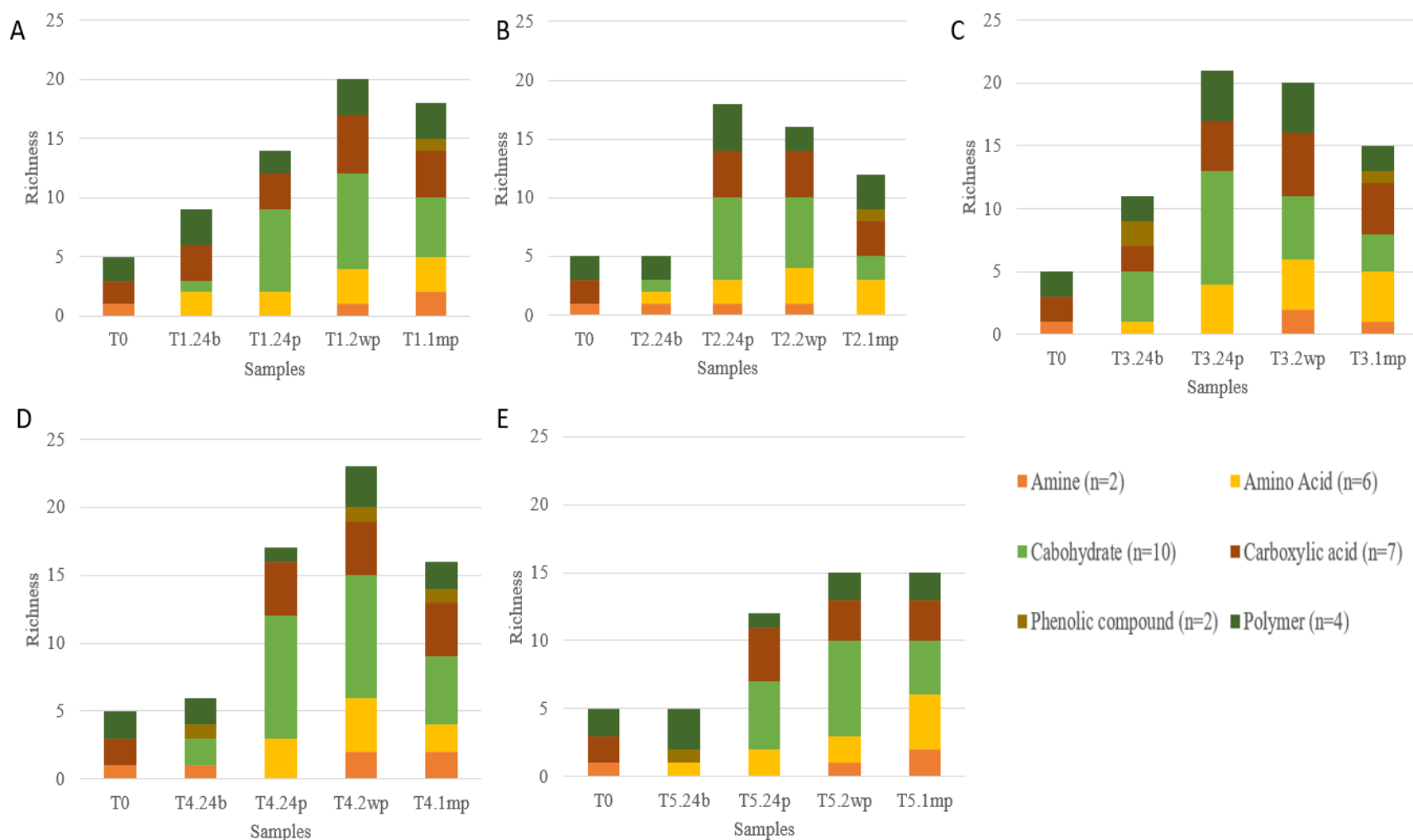
Figure 11. Richness values (Abs$_{590}$ > 0.25) of soil microbial communities from various treatments grouped by treatment. Samples were taken at five different time points: time of soybean planting (T0), 24 hours before treatment (TX.24b), 24 hours post treatment (TX.24p), 2 weeks post treatment (TX.2wp), and 1 month post treatment (TX.1mp). A: no treatment control, B: pesticide and chemical fertilizer treatment, C: pesticide and biofertilizer treatment, D: biofertilizer treatment, and E: chemical fertilizer treatment.
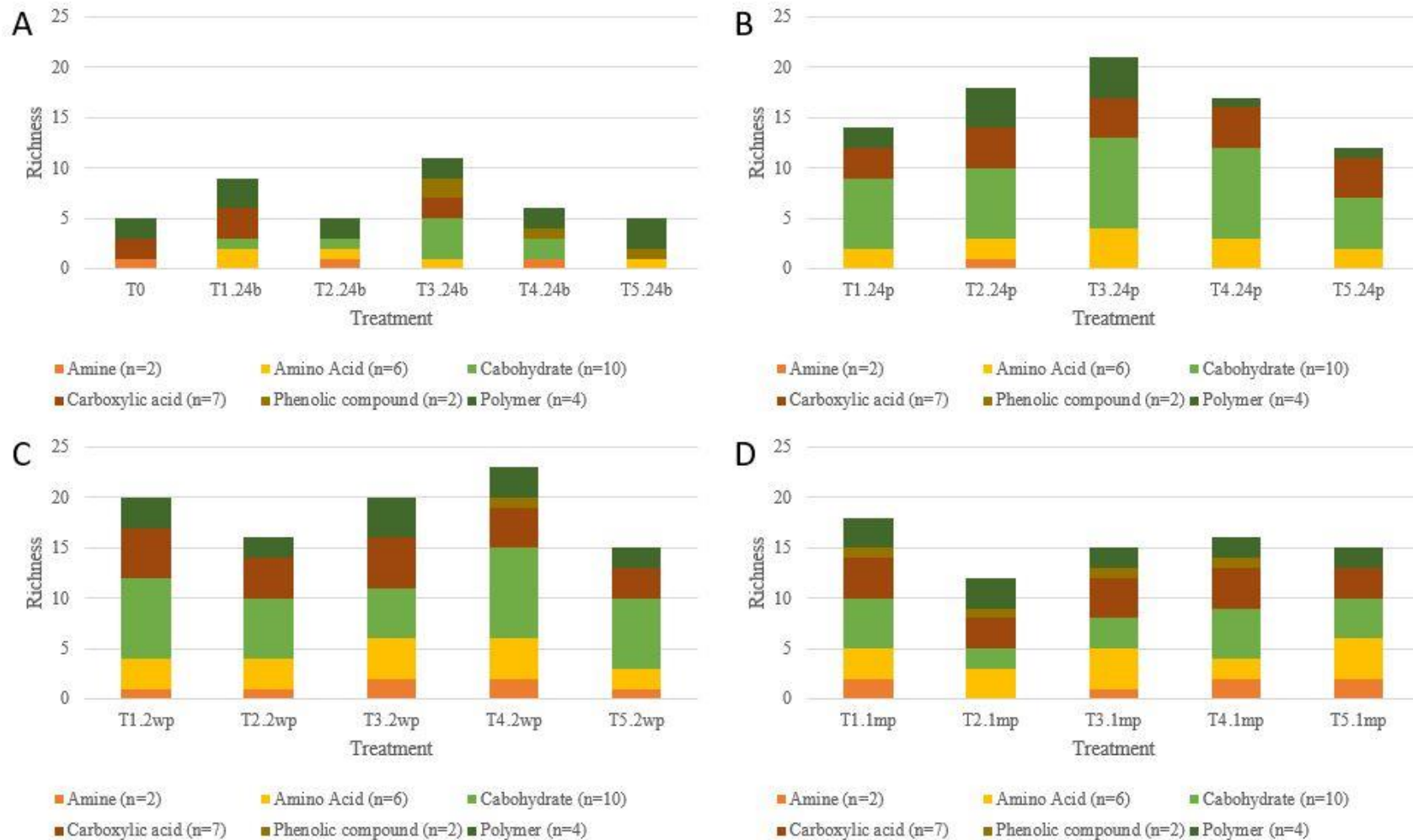
Figure 12. Richness values (Abs$_{590}$ > 0.25) of soil microbial communities subjected to various treatments grouped by time point. The treatments were T1: no treatment control, T2: pesticide and chemical fertilizer treatment, T3: pesticide and biofertilizer treatment, T4: biofertilizer treatment, and T5: chemical fertilizer treatment. A: represents time of soybean planting (T0) and 24 hours before treatment application (TX.24b), B: represents 24 hours post treatment application (TX.24p), C: represents 2 weeks post treatment application (TX.2wp), and D: represents 1 month post treatment application (TX.1mp).
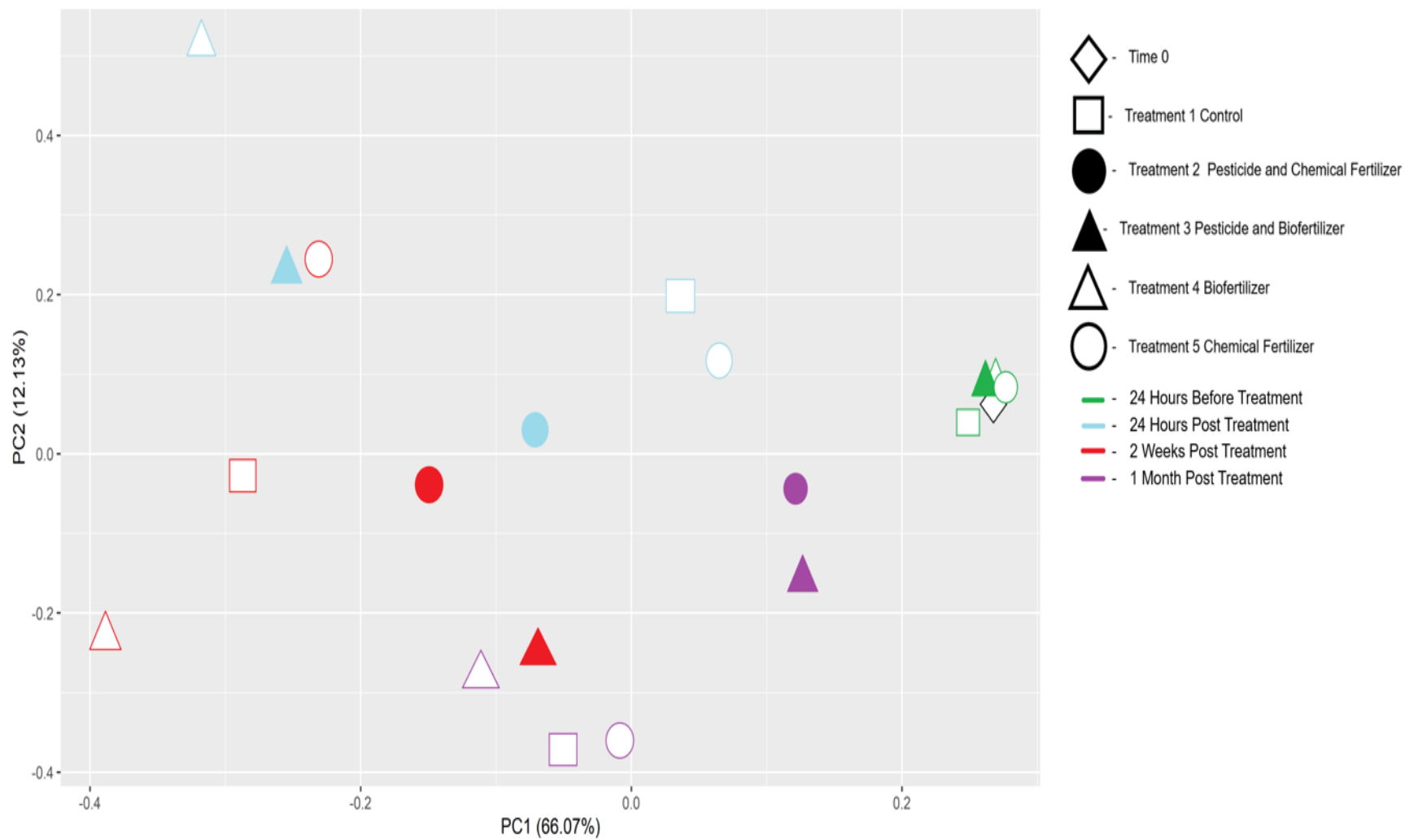
Figure 13. PCA of the CLPPs generated from the soybean plant treatment assessment.

3.6   Carbon Source Utilization of Microorganisms in Storage Condition Treatments

Two-way ANOVA was used to analyze substrate utilization richness data obtained from EcoPlates in response to storage temperature and time. There was a significant difference ($p = 0.034$) for substrate utilization richness in response to the temperatures that Environoc 401 was stored in, either 22 °C or 27 °C, but not in response to time ($p =0.899$).

3.7   Community-Level Physiological Profiles (CLPP) of Storage Condition Treatments

CLPPs were generated for the storage condition treatments. Carbon substrate utilization richness was generally consistent except for the Environoc 401 sample stored at 27 °C for one month, which had a lower richness value (Fig. 14). This difference is largely attributed to the fact that the microbial community within Environoc 401 stored at 27 °C did not utilize any amines or phenolic compounds as compared to the other treatments. Corrected Abs_{590} values were used to generate a PCA ordination of the storage condition treatments (Fig. 15). Both temperatures cluster together after 1 week of storage, but then the temperature treatments no longer clustered together after 1 month of storage.
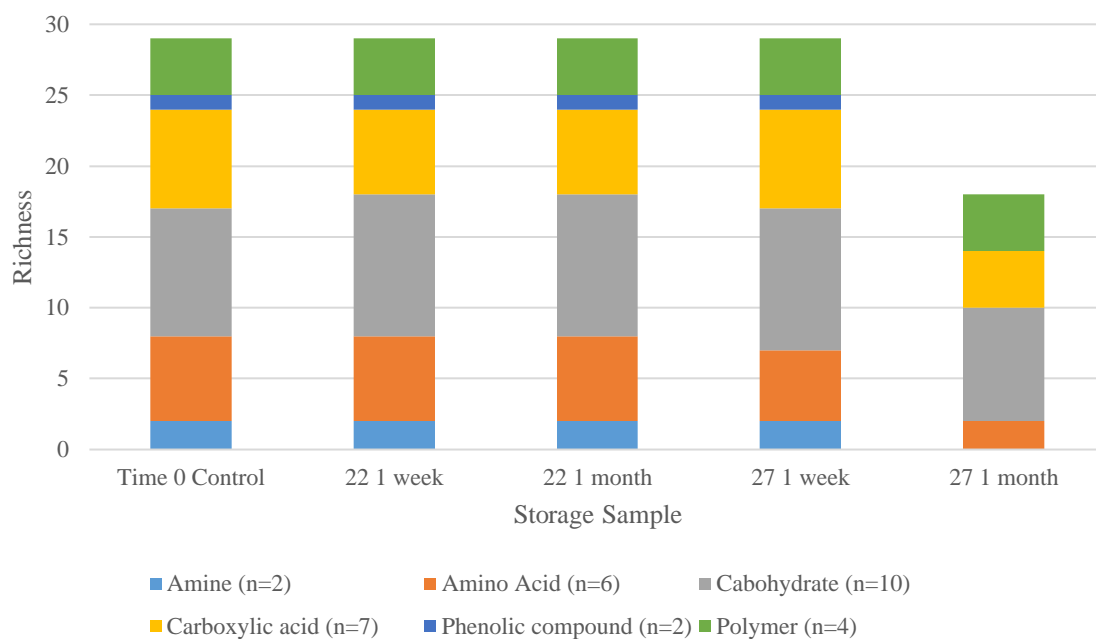
Figure 14. Richness values ($Abs_{590} > 0.25$) of biofertilizer microbial communities in the storage condition experiment. The biofertilizer was stored at either 22 °C or 27 °C and sampled before storing (Time 0 Control), 1 week after storage, and 1 month after storage.
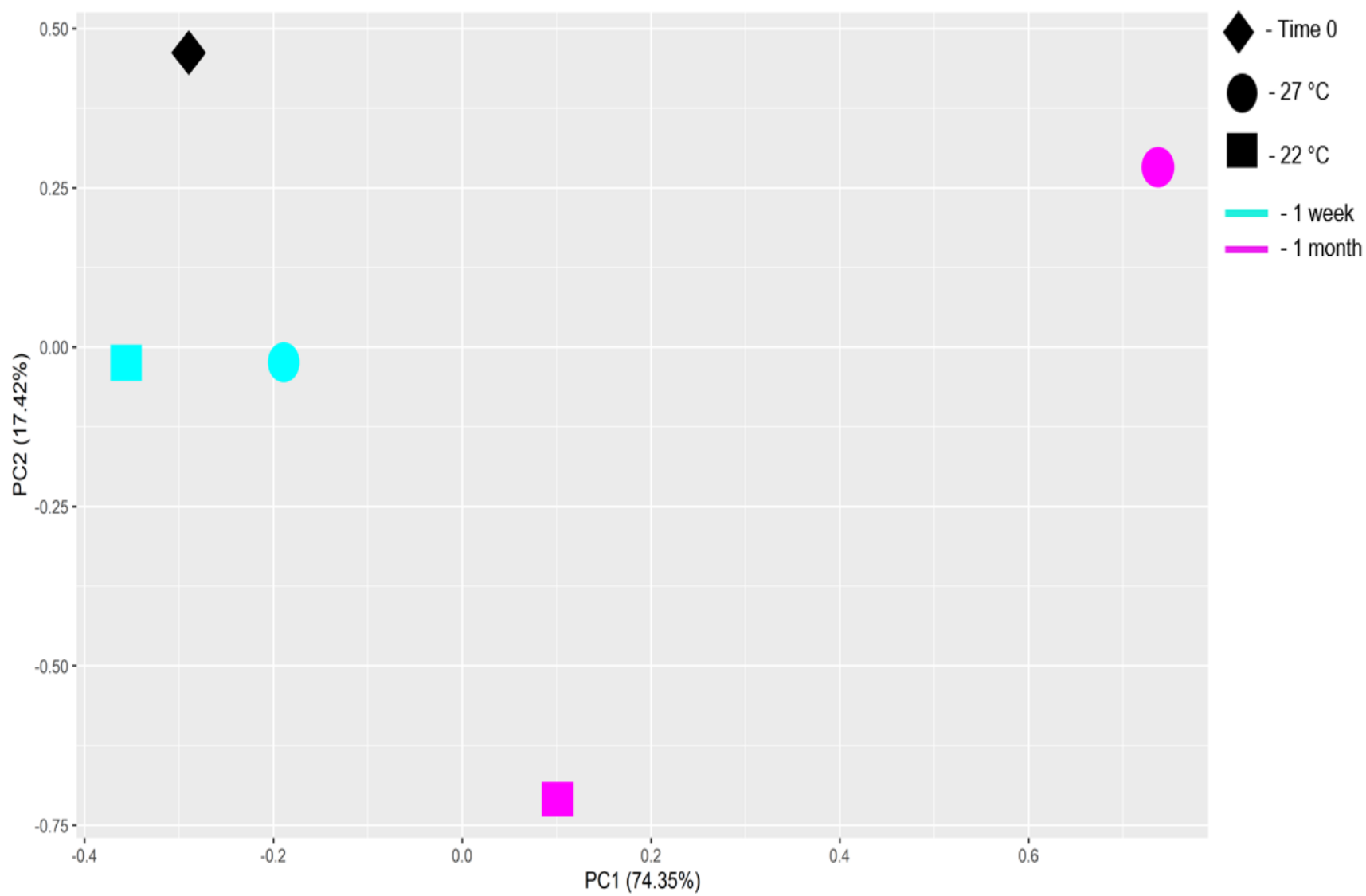
Figure 15. PCA of the CLPPs generated from the storage condition assessment.

CHAPTER 4.   DISCUSSION

In this study, the microbial activity of the biofertilizer Environoc 401 was studied under variable temperature, storage time, and in the potting soil of soybean plants inoculated with Environoc 401. More specifically, population changes of *Azospirillum brasilense*, *Bacillus altitudinis*, *Bacillus subtilis subtilis*, and *Rhodopseudomonas palustris* in Environoc 401 in response to storage temperature and time were analyzed through the use of the strain-specific primers in qPCR. Furthermore, carbon source utilization patterns of the microorganisms in each of these conditions and in the soil medium of soybean plants was compared between various treatments using Biolog Ecoplates. With known carbon source utilization patterns among the various treatments, the activity and health of the microbial community can be inferred, allowing different conditions to be comparatively assessed.

Since Environoc 401 is a community of 27 individual strains, each population must establish itself, which poses difficulty in a changing community. Each of these species much compete for both space and resources, like nutrients. Nutrients such as iron are limited, and different microbial mechanisms are utilized in an attempt to acquire iron (Hibbing et al., 2010). For example, organisms such as *A. brasilense* produce iron-scavenging molecules called siderophores which allow them to sequester iron in iron-poor environments (Tortora et al., 2011). While this is just one example, fluctuations in populations of *A. brasilense*, *B. altitudinis*, *B. subtilis*, and *R. palustris* were expected since each organism has different survival strategies. However, because the populations generally returned to similar starting quantities, it is possible these populations were already well-established in the fresh broth or perhaps these populations were resilient due to their metabolic versatility. *R. palustris*, for example, can utilize numerous substances as sources of carbon and energy. *R. palustris* is also capable of photoautotrophy, photoheterotrophy, and

chemoheterotrophy depending on light availability (Jiangbing et al., 2018). This metabolic adaptability is a reoccurring theme for numerous microorganisms within Environoc 401, such as *Pseudomonas fluorescens*, since *Pseudomonas* spp. populations have revealed enhanced resistance in carbon-starved conditions (Van Overbeek et al., 1995). The metabolic versatility that many microorganisms within Environoc 401 possess is what allows for these populations to persist in the face of competitors and changing conditions.

The EcoPlate analysis of the Environoc 401 storage samples allowed carbon source utilization patterns to be compared between the various conditions imposed on the microbial community. Carbon utilization was determined spectroscopically and Community Level Physiological Profiles (CLPPs) were generated from these absorbance values (Janniche et al., 2012). All the carbon sources were divided into six carbon types: amines, amino acids, carbohydrates, carboxylic acids, phenolic, and polymers. In the storage experiment samples it was found that there was a significant difference in substrate utilization in response to the storage temperature of 22°C or 27°C. Environoc 401 stored at 22°C had greater substrate utilization richness compared to Environoc 401 stored at 27°C, but only after 1 month. The microbial community within Environoc 401 stored at 27°C after 1 month did not utilize any amines or phenolic compounds, while the communities stored at 22°C did use these substrates. This may be indicative of a decline in the microbial community's health, a decrease in microbial diversity, or a number of other things. Previous studies have demonstrated that environmental conditions such as available oxygen, pH, and temperature can affect the degradation of phenolics, for example (Min et al., 2015). Parameters such as oxygen concentration and pH were not monitored during the storage experiment, although these environmental conditions could have impacted a microbial population's ability to degrade phenolics. For example, *Pseudomonas*, one of the constituents of

Environoc 401, is able to degrade phenolic compounds (Min et al., 2015). Although quantification of *Pseudomonas* was not included within this study, it is possible that populations of *Pseudomonas* could have declined resulting in a decrease of phenolic utilization.

Soybean plants were subjected to five treatments. By the end of the experiment, there was a significant difference in potting medium pH between the biofertilizer treatment and both the chemical fertilizer treatment as well as the pesticide and chemical fertilizer treatment at the endpoint of the experiment. The optimum soil medium pH range for most plants is between 6.5 to 7.0 because in this range most nutrients are soluble and available to plants (Rice & Rice, 2011). Because both the pesticide and chemical fertilizer treatment (pH ~5.59 ± 0.11 SE) and the chemical fertilizer treatment (pH ~ 5.88 ± 0.08 SE) are out of the optimum pH range (6.5 to 7.0), nutrients could have been inaccessible to the plant. Since the lack and/or unavailability of both macro- and micronutrients can obstruct typical plant growth and development, this could have contributed to the lower average total plant height and average number of leaves in the chemical fertilizer treatments. This could also explain the lower dry weights (leaf, stem, and root) for the pesticide and chemical fertilizer treatment, which was not statistically different from the control that had the lowest weights.

While nutrients such as nitrogen, phosphorous, and potassium are necessary for growth, development, and reproduction of plants, they often are not in large enough quantities needed for growth. Chemical fertilizers primarily supplement nitrogen, phosphorous, and potassium to the soil and plant, however excessive addition of these nutrients can lead to saturation and uncoupling of these nutrients' cycles (Liang et al., 2012). Chemical fertilizers supplement nitrogen, often in the form of nitrate, which is not bound to soil particles like many other nutrients are such as phosphorus. Since nitrate is a soluble form of nitrogen, and not bound to soil particles, it will

remain dissolved in the water surrounding the soil. Regular watering events can contribute to leaching of nutrients from the soil, thus much of the nitrogen can be lost through this watering (Lehmann et al., 2003). Potassium in chemical fertilizers is also in a soluble, usable form because most potassium is not available to plants otherwise. Leaching of potassium during watering, as a result of its solubility, can also occur, which is problematic because potassium is utilized in starch formation and movement of sugars in the plant. However, unlike nitrogen and potassium, phosphorous is insoluble in water and binds to soil particles, so it was probably not leached from the soil, although insoluble forms are not easily used by plants (Rice & Rice, 2011). Biofertilizers, specifically Environoc 401, alleviate the necessity of providing nutrients in only soluble forms that can be leached from the soil. Biofertilizers contain phosphate-solubilizing, nitrifying, and denitrifying bacteria, making these nutrients available in accessible forms to plants (Parr et al., 1994). As a result, the biofertilizer treatment and the pesticide and biofertilizer treatments were likely to have complete or almost complete nutrient cycles for these critical elements. Perhaps this explains why the average total plant height, average number of leaves, and the average dry weight of the stems and roots was higher amongst both treatments with biofertilizer as compared to those treatments without the biofertilizer. Even though these improvements were not always statistically significant, the biofertilizer treatments consistently provided results that suggested healthier plants than those treatments without biofertilizer. To support this claim, at the conclusion of the experiment, all treatments except for the biofertilizer treatment and the pesticide and biofertilizer treatment contained plants that had either yellowing leaves or leaves turning reddish-purple. This is most likely indicative of either a nitrogen deficiency, a phosphorous deficiency, or perhaps a combination of both (Rice & Rice, 2011).

There was no significant difference in the chlorophyll *a* content of the leaves between any of the treatments. Chlorophyll was extracted from leaves at the first and second node containing trifoliate leaves. The second node was only sampled when a plant did not have adequately large enough leaves from the first node. Perhaps sampling from either the first or second node of leaves for the chlorophyll extraction introduced bias because younger leaves most likely do not contain the same amount of chlorophyll as older leaves. Lack of differences between treatments could have also been due to the plants outgrowing their pots by the end of the experiment. When the plants were sacrificed at the endpoint, it was evident that plants were root bound by the pots. However, plants were not transplanted into larger pots because the soil microbial community could have been disturbed in the process. With plants being root bound, this could have greatly restricted plant growth and masked true differences between treatments. In similar studies without the complication of root bound plants, application of a biofertilizer resulted in the highest biomass (Wu et al., 2005) as well as significantly increased chlorophyll *a* and *b* content when compared to a chemical fertilizer (Larimi et al., 2014).

In general, the carbon substrate utilization richness based on the Ecoplate data, was highest for all treatments between 24 hours and two weeks post treatment application. The carbon substrate utilization richness was probably highest during this time because microorganisms within the potting medium were supplemented with more nutrients near the application time. The treatments which included the biofertilizer would have also been supplemented with more microorganisms to utilize the carbon sources. However, after a month post treatment application the carbon substrate utilization richness was not as high as the samples taken closer to treatment application, perhaps this is due to declining populations or nutrient leaching. Although there was not a significant difference in substrate utilization in response to treatment, there was a significant difference in

response to time. Furthermore, when examining the carbon utilization individually for each carbon type, it was found that there was only a significant difference between the biofertilizer treatment and all the other treatments. High substrate utilization richness indicates that the soil microorganisms from the biofertilizer treatment can utilize many carbon sources. In fact, the biofertilizer microorganisms utilized carbon sources from every group (amines, amino acids, carbohydrates, carboxylic acids, phenolic compounds, and polymers) at some point over the course of this study. This is not surprising, since the biofertilizer organism *Rhodopseudomonas palustrus*, for example, is capable of photoautotrophy, photoheterotrophy, and chemoheterotrophy depending on light availability. Thus, this community is metabolically quite versatile. Perhaps the microbial communities from the pesticide treatments were unable to metabolize as many carbon sources compared to the microbial community from the biofertilizer treatment due to pesticide application. The pesticide that was applied contained imidacloprid, and studies have suggested that imidacloprid application can have a negative impact on the biochemical and microbial activity of the soil (Cycoń & Piotrowska-Seget, 2015).

The carbon source Ecoplate absorbance values for each treatment and time point were then analyzed by PCA. The farther points are from one another on a PCA, the more dissimilar those samples are from one another. Overall, the treatments cluster together with one another the tightest 24 hours before treatment application, demonstrating that before treatment application the carbon sources metabolized are more similar between the plants than after treatment. However, once the treatments are applied, the control treatment and the chemical fertilizer treatment cluster 24 hours post treatment and again 1-month post treatment. Both the control treatment and the chemical fertilizer treatment had the smallest average number of leaves and leaves that had similar yellowing or were turning reddish-purple, perhaps indicating they have similar nutrient deficiencies. This

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clustering implies that the microbial communities present in both the control treatment and the chemical fertilizer treatment are metabolizing carbon substrates that do not differ substantially from one another. This could be explained by the fact that the microbial communities found within these two treatments are probably not very different from one another since the only microbes introduced to either of these treatments were the microbes naturally found in the potting medium. Since the plants in both treatments were planted using the same potting medium, they may have similar microbial community structures, which could explain the similar metabolic patterns. The same argument could be made for the pesticide and chemical fertilizer treatment as well. All three of these treatments had the lowest average number of leaves and the lowest average total height.

Overall, biofertilizers are a significant area of research because they play crucial roles in restoring soil fertility and enhancing crop production. The microbes in a biofertilizer enhance and drive natural processes such as nutrient transformation and cycling, organic matter decomposition, and gas emission, making them a valuable area of study in agriculture. In this study it was found that storage temperature affects carbon utilization within microbial communities. In general, Environoc 401 stored at 22°C had greater substrate utilization richness compared to Environoc 401 stored at 27°C, but only after 1 month. The microbial community within Environoc 401 stored at 27°C after 1 month did not utilize any amines or phenolic compounds, while the communities stored at 22°C did use these substrates. The soybean plants treated with Environoc 401 generally had the highest average total plant height, average number of leaves, average dry weight of stems, and roots, and the least acidic pH. These results are a reflection of both the activity and health of the microbial community, which appears to be enhanced in the presence of Environoc 401.

Because of the demonstrated utility of Environoc 401, more research should be conducted regarding the environmental effects on the microbial community dynamics within this biofertilizer.

More storage experiments should be conducted on Environoc 401 for longer periods of time, in dark/ light simulation, and in varying oxygen concentrations with continuous monitoring of pH. Also, temperature could be varied to more extreme temperatures and utilization of the two phenolic compounds (2-hydroxy benzoic acid and 4-hydroxy benzoic acid) could be monitored, specifically, to see how extreme temperatures alter utilization. As for the plant experiment, different plants like alfalfa or corn should be subjected to similar treatments. However, the plants could be planted in sterile potting medium, potting medium containing a time release fertilizer, potting medium containing compost, or potting medium containing manure to see how these conditions alter the microbial activity within the potting medium. Also, after Ecoplates are inoculated and incubated, perhaps DNA could be extracted from each well, via a 96 well plate DNA extraction, and used in qPCR with species-specific primers to see how the presence of individual carbon sources influences the prevalence of certain species over time. Biofertilizers are an important area of research. Biofertilizers, such as Environoc 401, have demonstrated that they are capable of improving agricultural productivity by restoring soil fertility and enhancing crop production. With climate change and population growth becoming more problematic, improving agricultural productivity is of utmost importance.

APPENDIX A. PRIMER VALIDATION

Partial alignment of the 16S rRNA gene of microorganisms within Environoc 401. Highlighted below are the forward and reverse primers from the primer set Abras.

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Abras     - - - - - - - - C C T T - - A G T G G C G C A C G G G T G AGTAACACGTGGG- AACCTGC C T T W Y G G T T C G G G A T A A C G T C T G G A A A C  156
Abras2    - - - - - - - - C C T T - - A G T G G C G C A C G G G T G AGTAACACGTGGG- AACCTGC C T T T C G G T T C G G G A T A A C G T C T G G A A A C  156
Balt      T G C T C C C G G A T G T T A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bamy      T G C T C C C T G A T G T T A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bamy2     T G C T C C C T G A T G T Y A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bamy3     T G C T C C C T G A T G T Y A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Blich     T G C T C C C T T A G G T C A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Blich2    T G C T C C C T T A G G T C A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bsub      T G C T C C C T G A T G T T A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Cellcell  T G C C C T G T C T G A T C A G T G G C G A A C G G G T G A G T A A C A C G T G A G T A A C C T G C C C T T  G A C T C T G G G A T A A C C G C G G G A A A C  156
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Celluda2  T G C T G - G T T G G A T C A G T G G C G A A C G G G T G A G T A A C A C G T G A G C A A C C T G C C C T T  C A C T C T G G G A T A A G C C C T G G A A A C  156
Pflu      T G C T T - C T C T T G A G A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A T A A C G T T C G G A A A C  156
Pflu2     T G C T T - C T C T T G A G A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A T A A C G C T C G G A A A C  156
Ppan      T G C T C - T C T G A T T C A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A C A A C G T T T C G A A A G  156
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Bamy      C G G G G C T A A T A C C G G A T G C T T G T T T G A A C C G C A T G G T T C A R A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
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Bamy3     C G G G G C T A A T A C C G G A T G G T T G T Y T G A A C C G C A T G G T T C A G A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Blich     C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T T A G C T A C C A C T T R C A G A T G G  234
Blich2    C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T Y A G C T A C C A C T T R C A G A T G G  234
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Celluda   G G G G T C T A A T A C C G G A T A T G A C A T C C C T G C G C A T G T G G G G - G T G T G G A A - - - A G A T T - - - - - T A T C G G T G G G G G A T G G  234
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Pstut     G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Pstut2    G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Ptai      G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Rpal      T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G G A - - - A A G - - - - A T T T - - - - - A T C G C C G A A A G A T C G  234
Rpal2     T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G G A - - - A A G - - - - A T T T - - - - - A T C G C C G A A A G A T C G  234
Salb      G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb2     G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb3     T G G G T C T A A T A C C G G A T A G G A C C T T C T G C C G C A T G - G T G G G G G G A A - - - A G G T T - - - - - T T C C G G T G C A G G A T G G  234
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Abras2    G C C C G C G T C C G A T T A G G T A G T T G G T G G G G T A A T G G C C C A C C A A G C C GACGATCG GTAGCTGGTCTG A G A G G A T G A T C A  312
Balt      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy2     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy3     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Blich     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Blich2    A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bsub      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
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Celluda   G C T C G C G G C C T A T C A G C T T G T T G G T G G G G T G A T G G C C T A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Celluda2  G C T C G C G G C C T A T C A G C T T G T T G G T G G G G T G A T G G C C T A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
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Pflu2     G C C T A G G T C G G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Ppan      G C C T A G G T C G G A T T A G C T A G T T G G T G G C G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Pstut     G C C T A G G T C G G A T T A G C T A G T A G G T G G G G T A A T G G C T C A C C T A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Pstut2    G C C T A G G T C G G A T T A G C T A G T T G G T G G C G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Ptai      G C C T A G G T C G G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Rpal      G C C C G C G T C T G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C A G T A G C T G G T C T G A G A G G A T G A T C A  312
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Salb3     G C C C G C G G C C T A T C A G C T T G T T G G T G G G G T A A C G G C C C A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Xflav     A C C C G C G T C G G A T T A G C T A G T T G G T G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A G C T G G T C T G A G A G G A T G A T C A  312
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Highlighted below are the forward and reverse primers from the primer set Balt.

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Pflu2     T G G A G A G T T T G A T C C T G G C T C A G A T T G A A C G C T G G C G G C A G G C C T A A C A C A T G C  A A G T C G A G C G G T A G A G A G A A G - C T  78
Ppan      T G G A G A G T T T G A T C C T G G C T C A G A T T G A A C G C T G G C G G C A G G C C T A A C A C A T G C  A A G T C G A G C G G A T G A A A G G A G - C T  78
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Ptai      T G G A G A G T T T G A T C C T G G C T C A G A T T G A A C G C T G G C G G C A G G C C T A A C A C A T G C  A A G T C G A G C G G A T G A C G G G A G - C T  78
Rpal      T G G A G A G T T T G A T C C T G G C T C A G A G C G A A C G C T G G C G G C A G G C T T A A C A C A T G C  A A G T C G A A C G G G C G T A G C A A - - - -  78
Rpal2     T G G A G A G T T T G A T C C T G G C T C A G A G C G A A C G C T G G C G G C A G G C T T A A C A C A T G C  A A G T C G A A C G G G C G T A G C A A - - - -  78
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Salb2     T G G A G A G T T T G A T C C T G G C T C A G G A C G A A C G C T G G C G G C G T G C C T A A C A C A T G C  A A G T C G A A C G A T G A - - A C C G - - C T  78
Salb3     T G G A G A G T T T G A T C C T G G C T C A G G A C G A A C G C T G G C G G C G T G C C T A A C A C A T G C  A A G T C G A A C G A T G A A G C C C A G - C T  78
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Bamy      T G C T C C C T G A T G T T A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bamy2     T G C T C C C T G A T G T Y A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bamy3     T G C T C C C T G A T G T Y A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Blich     T G C T C C C T T A G G T C A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Blich2    T G C T C C C T T A G G T C A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Bsub      T G C T C C C T G A T G T T A G C G G C G G A C G G G T G A G T A A C A C G T G G G T A A C C T G C C T G T  A A G A C T G G G A T A A C T C C G G G A A A C  156
Cellcell  T G C C C T G T C T G A T C A G T G G C G A A C G G G T G A G T A A C A C G T G A G T A A C C T G C C C T T  G A C T C T G G G A T A A C C G C G G G A A A C  156
Celluda   T G C T G - G T T G G A T C A G T G G C G A A C G G G T G A G T A A C A C G T G A G C A A C C T G C C C T T  C A C T C T G G G A T A A G C C C T G G A A A C  156
Celluda2  T G C T G - G T T G G A T C A G T G G C G A A C G G G T G A G T A A C A C G T G A G C A A C C T G C C C T T  C A C T C T G G G A T A A G C C C T G G A A A C  156
Pflu      T G C T T - C T C T T G A G A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G G A T A A C G T T C G G A A A C  156
Pflu2     T G C T T - C T C T T G A G A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A T A A C G C T C G G A A A C  156
Ppan      T G C T C - T C T G A T T C A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A C A A C G T T T C G A A A G  156
Pstut     T G C T C - Y C K G A T T C A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A C A A C G T T C G A A A A G  156
Pstut2    T G C T C - C A T G A T T C A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A C A A C G T T T C G A A A G  156
Ptai      T G C T C - C T T G A T T C A G C G G C G G A C G G G T G A G T A A T G C C T A G G - A A T C T G C C T G G  T A G T G G G G G A C A A C G T T T C G A A A G  156
Rpal      - - - - - - - - T A C G T C A G T G G C A G A C G G G T G A G T A A C G C G T G G G - A A C G T A C C T T T  T G G T T C G G A A C A A C A C A G G G A A A C  156
Rpal2     - - - - - - - - T A C G T C A G T G G C A G A C G G G T G A G T A A C G C G T G G G - A A C G T A C C T T T  T G G T T C G G A A C A A C A C A G G G A A A C  156
Salb      T T C G G G C G G G G A T T A G T G G C G A A C G G G T G A G T A A C A C G T G G G C A A T C T G C C C T G  C A C T C T G G G A C A A G C C C T G G A A A C  156
Salb2     T T C G G G C G G G G A T T A G T G G C G A A C G G G T G A G T A A C A C G T G G G C A A T C T G C C C T G  C A C T C T G G G A C A A G C C C T G G A A A C  156
Salb3     T G C T G - G G T G G A T T A G T G G C G A A C G G G T G A G T A A C A C G T G G G T G A T C T G C C C T G  C A C T C T G G G A T A A G C C T G G A A A C  156
Xflav     - - - - - - - - T G G G - - A G C G G C A G A C G G G T G A G T A A C A C G T G G G - G A T C T A C C C A A  T G G T A C G G A A T A A C C C A G G G A A A C  156

Abras     G G A C G C T A A C A C C G G A T R C G T C C C C C R G A G R G A T T T G T G C G G A - - - G A A - - - - A G T T - - - - - - T A C G C C R WG A G A G G G  234
Abras2    G G A C G C T A A C A C C G G A T A C G T C C C C C R G A G G G A T T T K K G C G G A - - - G A A - - - - A G T T - - - - - - T A C G C C G A G A G A G G G  234
Balt      C G G A G C T A A T A C C G G A T A G T TCCTTGAACCGCATGGTTCA A G G A T G A A A G A C G G T T T - C G G C T G T C A C T T A C A G A T G G  234
Bamy      C G G G G C T A A T A C C G G A T G C T T G T T T G A A C C G C A T G G T T C A R A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Bamy2     C G G G G C T A A T A C C G G A T G C T T G T T T G A A C C G C A T G G T T C A G A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Bamy3     C G G G G C T A A T A C C G G A T G G T T G T Y T G A A C C G C A T G G T T C A G A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Blich     C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T T A G C T A C C A C T T R C A G A T G G  234
Blich2    C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T Y A G C T A C C A C T T R C A G A T G G  234
Bsub      C G G G G C T A A T A C C G G A T G G T T G T T T G A A C C G C A T G G T T C A A A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Cellcell  G G C G G C T A A T A C C G G A T A T G A G A C G T A C A G G C A T C T G T T G C G T C T G G A A - - - A G A T T - - - - - T A T C G G T C A A G G A T G G  234
Celluda   G G G G T C T A A T A C C G G A T A T G A C A T C C C T G C G C A T G T G G G G - G T G T G G A A - - - A G A T T - - - - - T A T C G G T G G G G G A T G G  234
Celluda2  G G G G T C T A A T A C C G G A T A T G A C A T C C C T G C G C A T G T G G G G - G T G T G G A A - - - A G A T T - - - - - T A T C G G T G G G G G A T G G  234
Pflu      G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Pflu2     G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Ppan      G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Pstut     G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Pstut2    G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Ptai      G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Rpal      T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G G A - - - A A G - - - - A T T T - - - - - - A T C G C C G A A A G A T C G  234
Rpal2     T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G G A - - - A A G - - - - A T T T - - - - - - A T C G C C G A A A G A T C G  234
Salb      G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb2     G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb3     T G G G T C T A A T A C C G G A T A G G A C C T T C T G C C G C A T G - G T G G G G G G T G G A A - - - A G G T T - - - - - T T C C G G T G C A G G A T G G  234
Xflav     T T G G A T T A A T A C C G T A T G T G C C C - - - - - - - - - - - T T C G G G G G A - - - A A G - - - - A T T T - - - - - - A T C G C C A T T G G A T G A  234
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Highlighted below are the forward and reverse primers from the primer set Bsub.

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Abras     G G A C G C T A A C A C C G G A T R C G T C C C C C R G A G R G A T T T G T G C G G A - - - G A A - - - - A G T T - - - - - T A C G C C R W G A G A G G G  234
Abras2    G G A C G C T A A C A C C G G A T A C G T C C C C C R G A G G G A T T T K K G C G G A - - - G A A - - - - A G T T - - - - - T A C G C C G A G A G A G G G  234
Balt      C G G A G C T A A T A C C G G A T A G T T C C T T G A A C C G C A T G G T T C A A G G A T G A A A G A C G G T T T - C G G C T G T C A C T T A C A G A T G G  234
Bamy      C G G G G C T A A T A C C G G A T G C T T G T T T G A A C C G C A T G G T T C A R A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Bamy2     C G G G G C T A A T A C C G G A T G C T T G T T T G A A C C G C A T G G T T C A G A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Bamy3     C G G G G C T A A T A C C G G A T G G T T G T Y T G A A C C G C A T G G T T C A G A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Blich     C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T T A G C T A C C A C T T R C A G A T G G  234
Blich2    C G G G G C T A A T A C C G G A T G C T T G A T T G A A C C G C A T G G T T C A A T C A T A A A A G G T G G C T T T Y A G C T A C C A C T T R C A G A T G G  234
Bsub      C G G G G C T A A T A CCGGATGGTTGTTTGAACCG C A T G G T T C A A A C A T A A A A G G T G G C T T - C G G C T A C C A C T T A C A G A T G G  234
Cellcell  G G C G G C T A A T A C C G G A T A T G A G A C G T A C A G G C A T C T G T T G C G T C T G G A A - - - A G A T T - - - - - T A T C G G T C A A G G A T G G  234
Celluda   G G G G T C T A A T A C C G G A T A T G A C A T C C C T G C G C A T G T G G G G - G T G T G G A A - - - A G A T T - - - - - T A T C G G T G G G G G A T G G  234
Celluda2  G G G G T C T A A T A C C G G A T A T G A C A T C C C T G C G C A T G T G G G G - G T G T G G A A - - - A G A T T - - - - - T A T C G G T G G G G G A T G G  234
Pflu      G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Pflu2     G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Ppan      G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Pstut     G G A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Pstut2    G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G T G G G G G A T C T T C G G - - - - A C C T - - - - - - C A C G C T A T C A G A T G A  234
Ptai      G A A C G C T A A T A C C G C A T A C G T C C T A C G G G A G A A A G C A G G G G A C C T T C G G - - - - G C C T - - - - - - T G C G C T A T C A G A T G A  234
Rpal      T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G A - - - A A G - - - - A T T T - - - - - A T C G C C G A A A G A T C G  234
Rpal2     T T G T G C T A A T A C C G G A T A A G C C C - - - - - - - - - - - T T A C G G G A - - - A A G - - - - A T T T - - - - - A T C G C C G A A A G A T C G  234
Salb      G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb2     G G G G T C T A A T A C C G G A T A T G A C T G T C C A T C G C A T G - G T G G A T G G T G T A A - - - A G C T C - - - - - C G G C G G T G C A G G A T G A  234
Salb3     T G G G T C T A A T A C C G G A T A G G A C C T T C T G C C G C A T G - G T G G G G G G T G G A A - - - A G G T T - - - - - T T C C G G T G C A G G A T G G  234
Xflav     T T G G A T T A A T A C C G T A T G T G C C C - - - - - - - - - - - T T C G G G G A - - - A A G - - - - A T T T - - - - - A T C G C C A T T G G A T G A  234

Abras     G C C C G C G T C C G A T T A G G T A G T T G G T G G G G T A A T G G C C C A C C A A G C C G A C G A T C G G T A G C T G G T C T G A G A G G A T G A T C A  312
Abras2    G C C C G C G T C C G A T T A G G T A G T T G G T G G G G T A A T G G C C C A C C A A G C C G A C G A T C G G T A G C T G G T C T G A G A G G A T G A T C A  312
Balt      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy2     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C G A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bamy3     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Blich     A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Blich2    A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Bsub      A C C C G C G G C G C A T T A G C T A G T T G G T G A G G T A A C G G C T C A C C A A G G C R A C G A T G C G T A G C C G A C C T G A G A G G G T G A T C G  312
Cellcell  G C T C G C G G C C T A T C A G C T T G T T G G T G G G G T G A T G G C C T A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Celluda   G C T C G C G G C C T A T C A G C T T G T T G G T G G G G T G A T G G C C T A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Celluda2  G C T C G C G G C C T A T C A G C T T G T T G G T G G G G T G A T G G C C T A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Pflu      G C C T A G G T C G G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Pflu2     G C C T A G G T C G G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Ppan      G C C T A G G T C G G A T T A G C T A G T T G G C G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Pstut     G C C T A G G T C G G A T T A G C T A G T A G G T G G G G T A A T G G C T C A C C T A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Pstut2    G C C T A G G T C G G A T T A G C T A G T T G G C G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Ptai      G C C T A G G T C G G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C C G T A A C T G G T C T G A G A G G A T G A T C A  312
Rpal      G C C C G C G T C T G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C A G T A G C T G G T C T G A G A G G A T G A T C A  312
Rpal2     G C C C G C G T C T G A T T A G C T A G T T G G T G A G G T A A T G G C T C A C C A A G G C G A C G A T C A G T A G C T G G T C T G A G A G G A T G A T C A  312
Salb      G C C C G C G G C C T A T C A G C T T G T T G G T G A G G T A G T G G C T C A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Salb2     G C C C G C G G C C T A T C A G C T T G T T G G T G A G G T A G T G G C T C A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Salb3     G C C C G C G G C C T A T C A G C T T G T T G G T G G G G T A A C G G C C C A C C A A G G C G A C G A C G G G T A G C C G G C C T G A G A G G G C G A C C G  312
Xflav     A C C C G C G T C G G A T T A G C T A G T T G G T G A G G T A A A G G C T C A C C A A G G C G A C G A T C C G T A G C T G G T C T G A G A G G A T G A T C A  312

Abras     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G G G C A A C C C T  390
Abras2    G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G G G C A A C C C T  390
Balt      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy2     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy3     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Blich     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Blich2    G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Bsub      G C CACACTGGGACTGAGACACG G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A A T C T T C C G C A A T G G A C G A A A G T C T  390
Cellcell  G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G C A A G C C T  390
Celluda   G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G C A A G C C T  390
Celluda2  G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G C A A G C C T  390
Pflu      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Pflu2     G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Ppan      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Pstut     G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Pstut2    G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Ptai      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G A A A G C C T  390
Rpal      G C C A C A T T G G G A C T G A G A C A C G G C C C A A A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G G G A A A C C C T  390
Rpal2     G C C A C A T T G G G A C T G A G A C A C G G C C C A A A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G G G A A A C C C T  390
Salb      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G C A A G C C T  390
Salb2     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G A A A G C C T  390
Salb3     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G C A C A A T G G G C G C A A G C C T  390
Xflav     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A A T A T T G G A C A A T G G G C G C A A G C C T  390
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Highlighted below are the forward and reverse primers from the primer set Rpal2.

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Abras     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G G G C A A C C C T  390
Abras2    G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G G G C A A C C C T  390
Balt      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy2     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Bamy3     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Blich     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Blich2    G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Bsub      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T A G G G A  A T C T T C C G C A A T G G A C G A A A G T C T  390
Cellcell  G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G C A A G C C T  390
Celluda   G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G C A A G C C T  390
Celluda2  G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G C A A G C C T  390
Pflu      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Pflu2     G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Ppan      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Pstut     G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Pstut2    G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Ptai      G T C A C A C T G G A A C T G A G A C A C G G T C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G A A A G C C T  390
Rpal      G C C A C A T T G G G A C T G A G A C A C G G C C C A A A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G [GGAAACCCT]  390
Rpal2     G C C A C A T T G G G A C T G A G A C A C G G C C C A A A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G [GGAAACCCT]  390
Salb      G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G A A A G C C T  390
Salb2     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G A A A G C C T  390
Salb3     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G C A C A A T G G G C G C A A G C C T  390
Xflav     G C C A C A C T G G G A C T G A G A C A C G G C C C A G A C T C C T A C G G G A G G C A G C A G T G G G G A  A T A T T G G A C A A T G G G C G C A A G C C T  390

Abras     G A T C C A G C A A T G C C G C G T G A G T G A T G A A G G C C T T A G G G T T G T A A A G C T C T T T C G  C A C G C G A C G A T - G A - - - - - - - - - -  468
Abras2    G A T C C A G C A A T G C C G C G T G A G T G A T G A A G G C C T T A G G G T T G T A A A G C T C T T T C G  C A C G C G A C G A T - G A - - - - - - - - - -  468
Balt      G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A G C T C T G T T G  T T A G G G A A G A A C A A G T G C A A G A G T  468
Bamy      G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A G C T C T G T T G  T T A G G G A A G A A C A A G T G C C G T T C A  468
Bamy2     G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A G C T C T G T T G  T T A G G G A A G A A C A A G T G C C G T T C A  468
Bamy3     G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A G C T C T G T T G  T T A G G G A A G A A C A A G T G C C G T T C A  468
Blich     G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A A C T C T G T T G  T T A G G G A A G A A C A A G T A C C G T T C G  468
Blich2    G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A A C T C T G T T G  T T A G G G A A G A A C A A G T A C C G T T C G  468
Bsub      G A C G G A G C A A C G C C G C G T G A G T G A T G A A G G T T T T C G G A T C G T A A A G C T C T G T T G  T T A G G G A A G A A C A A G T A C C G T T C G  468
Cellcell  G A T G C A G C G A C G C C G C G T G A G G G A T G A A G G C C T T C G G G T T G T A A A C C T C T T T C A  G C A G G G A A G A A - - - G C G A A A G - - -  468
Celluda   G A T G C A G C G A C G C C G C G T G A G G G A T G A C G G C C T T C G G G T T G T A A A C C T C T T T C A  G C A G G G A A G A A - - - G C G A A A G - - -  468
Celluda2  G A T G C A G C G A C G C C G C G T G A G G G A T G A C G G C C T T C G G G T T G T A A A C C T C T T T C A  G C A G G G A A G A A - - - G C G A A A G - - -  468
Pflu      G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G R G C A G T T A C C T  468
Pflu2     G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G G G C A T T A A C C T  468
Ppan      G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G G G C A G T A A G T T  468
Pstut     G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G G G C T G C A A G T T  468
Pstut2    G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G G G C A G T A A G T T  468
Ptai      G A T C C A G C C A T G C C G C G T G T G T G A A G A A G G T C T T C G G A T T G T A A A G C A C T T T A A  G T T G G G G A G G A A - G G G C A G T A A G T T  468
Rpal      [GATCCAGCCAT] G C C G C G T G A G T G A T G A A G G C C C T A G G G T T G T A A A G C T C T T T T G  T G C G G G A A G A T - A A - - - - - - - - - -  468
Rpal2     [GATCCAGCCAT] G C C G C G T G A G T G A T G A A G G C C C T A G G G T T G T A A A G C T C T T T T G  T G C G G G A A G A T - A A - - - - - - - - - -  468
Salb      G A T G C A G C G A C G C C G C G T G A G G G A T G A C G G C C T T C G G G T T G T A A A C C T C T T T C A  G C A G G G A A G A A - - - G C G A A A G - - -  468
Salb2     G A T G C A G C G A C G C C G C G T G A G G G A T G A C G G C C T T C G G G T T G T A A A C C T C T T T C A  G C A G G G A A G A A - - - G C G A A A G - - -  468
Salb3     G A T G C A G C G A C G C C G C G T G A G G G A T G A C G G C C T T C G G G T T G T A A A C C T C T T T C A  G T A C C G A C G A A - - - G C G C A A G - - -  468
Xflav     G A T C C A G C C A T G C C G C G T G A G T G A T G A A G G C C T T A G G G T T G T A A A G C T C T T T C G  C C G G T G A A G A T - A A - - - - - - - - - -  468

Abras     - - - - - - - - - - - - - - - - - T G A C G G T A G C G T G A G A A G A A G C C C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Abras2    - - - - - - - - - - - - - - - - - T G A C G G T A G C G T G A G A A G A A G C C C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Balt      A A C T G C T T G - C A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Bamy      A A T A G G G C G G C A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Bamy2     A A T A G G G C G G C A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Bamy3     A A T A G G G C G G C A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Blich     A A T A G G G C G G Y A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Blich2    A A T A G G G C G G Y A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Bsub      A A T A G G G C G G T A C C T T G A C G G T A C C T A A C C A G A A A G C C A C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Cellcell  - - - - - - - - - - - - - - - - - T G A C G G T A C C T G C A G A A G A A G C G C C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Celluda   - - - - - - - - - - - - - - - - - T G A C G G T A C C T G C A G A A G A A G C G C C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Celluda2  - - - - - - - - - - - - - - - - - T G A C G G T A C C T G C A G A A G A A G C G C C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Pflu      A A T A C G T G A T T G T T T T G A C G T T A C C G A C A G A A T A A G C A C C G G C T A A C T C T G T G C  C A G C A G C C G C G G T A  536
Pflu2     A A T A C G T T G G T G T C T T G A C G T T A C C G A C A G A A T A A G C A C C G G C T A A C T C T G T G C  C A G C A G C C G C G G T A  536
Ppan      A A T A C C T T G C T G T T T T G A C G T T A C C G A C A G A A T A A G C A C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Pstut     A A T A C C T T G T A G T T T T G A C G T T A C C A A C A G A A T A A G C A C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Pstut2    A A T A C C T T G C T G T T T T G A C G T T A C C G A C A G A A T A A G C A C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Ptai      A A T A C C T T G C T G T T T T G A C G T T A C C G A C A G A A T A A G C A C C G G C T A A C T C T G T G C  C A G C A G C C G C G G T A  536
Rpal      - - - - - - - - - - - - - - - - - T G A C G G [TACCGCAAGAATAAGCCCCG] G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Rpal2     - - - - - - - - - - - - - - - - - T G A C G G [TACCGCAAGAATAAGCCCCG] G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
Salb      - - - - - - - - - - - - - - - - - T G A C G G T A C C T G C A G A A G A A G C G C C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Salb2     - - - - - - - - - - - - - - - - - T G A C G G T A C C T G C A G A A G A A G C G C C G G C T A A C T A C G T G C  C A G C A G C C G C G G T A  536
Salb3     - - - - - - - - - - - - - - - - - T G A C G G T A G G T A C A G A A G A A G C A C C G G C C A A C T A C G T G C  C A G C A G C C G C G G T A  536
Xflav     - - - - - - - - - - - - - - - - - T G A C G G T A A C C G G A G A A G A A G C C C C G G C T A A C T T C G T G C  C A G C A G C C G C G G T A  536
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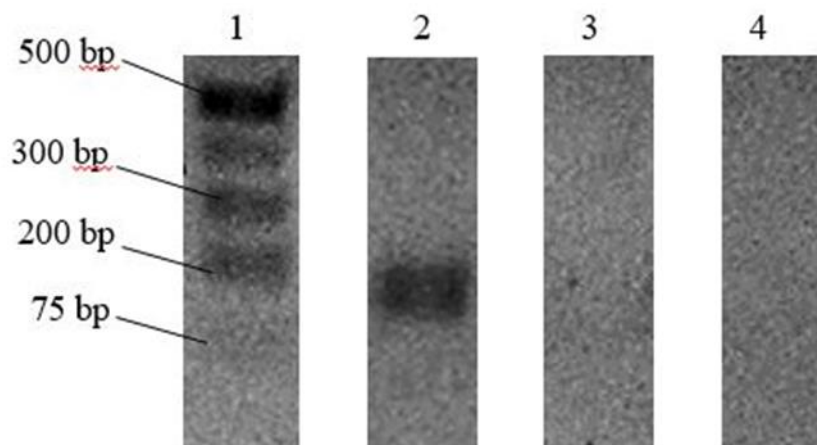
Figure A1. Validation of the primer set Abras for *A. brasilense*. 1% agarose gel ran at 100V for 30 min. Lane 1: O'GeneRuler 1kb Plus DNA Ladder. Lane 2: Abras primer tested on Environoc 401. Lane 3: Abras primer tested on the negative control *E. coli*. Lane 4: Abras primer tested on NTC.
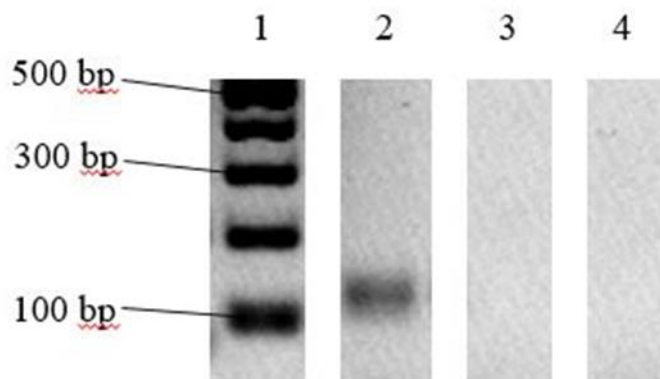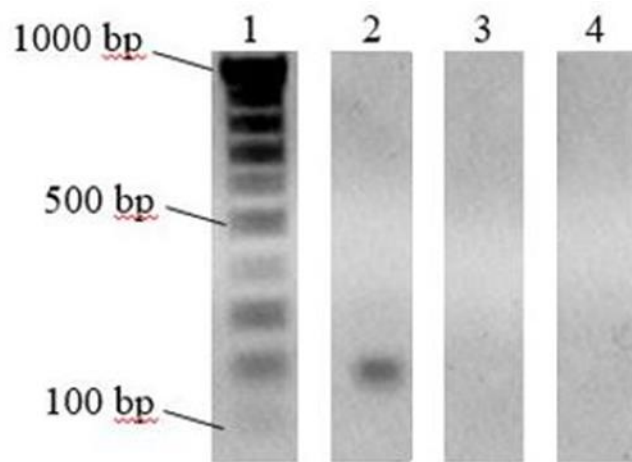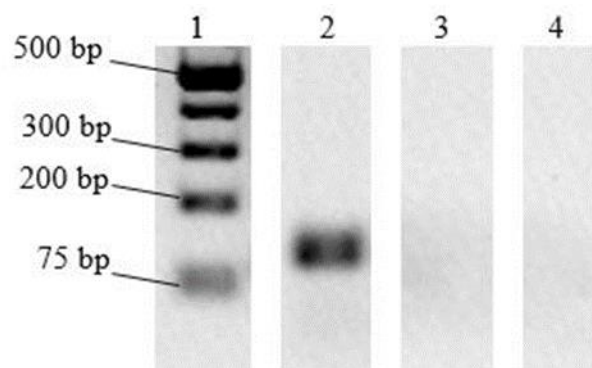

Figure A2. Validation of the primer set Balt for *B. altitudinis*. 1.5% agarose gel ran at 100V for 40 min. Lane 1: O'GeneRuler 100 bp Plus DNA Ladder. Lane 2: Balt primer tested on Environoc 401. Lane 3: Balt primer tested on the negative control *E. coli*. Lane 4: Balt primer tested on NTC.

Figure A3. Validation of the primer set Bsub for B. subtilis subtilis. 1.5% agarose gel ran at 100V for 40 min. Lane 1: O'GeneRuler 100 bp Plus DNA Ladder. Lane 2: Bsub primer test on Environoc 401. Lane 3: Bsub primer tested on the negative control E. coli. Lane 4: Bsub primer tested on NTC.

Figure A4. Validation of the primer set Rpal2 for R. palustris. 1.5% agarose gel ran at 100V for 40 min. Lane 1: O'GeneRuler 1kb Plus DNA Ladder. Lane 2: Rpal2 primer tested on Environoc 401. Lane 3: Rpal2 primer tested on the negative control E. coli. Lane 4: Rpal2 primer tested on NTC.

APPENDIX B. CARBON SUBSTRATE UTILIZATION

Table C1. Carbon substrate utilization of microorganisms from soybean potting medium 24 hours before treatment application as corrected Abs590 values by treatment. Highlighted absorbance values represent carbon sources that were utilized preferentially (Abs > 0.25). Absorbances represent the average of triplicate samples. T1: no treatment control, T2: pesticide and chemical fertilizer treatment, T3: pesticide and biofertilizer treatment, T4: biofertilizer treatment, and T5: chemical fertilizer treatment.

	Time 0	T1.24b	T2.24b	T3.24b	T4.24b	T5.24b
β-Methyl-D-Glucoside	0.0043	0.0010	0.0011	0.0000	0.0009	0.0013
D-Galactonic Acid γ-Lactone	0.0004	0.0029	0.0014	0.0066	0.0034	0.0045
L-Arginine	0.0000	0.0102	0.0000	0.0061	0.0013	0.0016
Pyruvic Acid Methyl Ester	0.0025	0.0000	0.0010	0.0097	0.0036	0.0033
D-Xylose	0.0082	0.0052	0.0114	0.0235	0.0157	0.0131
D-Galacturonic Acid	0.0024	0.0118	0.0000	0.0497	0.0036	0.0013
L-Asparagine	0.0023	0.0158	0.0011	0.0070	0.0031	0.0031
Tween 40	0.0240	0.2869	0.0071	0.0115	0.0075	0.0168
i-Erythritol	0.0007	0.0115	0.0000	0.0111	0.0069	0.0042
2-Hydroxy Benzoic Acid	0.0020	0.0022	0.0005	0.0084	0.0022	0.0043
L-Phenylalanine	0.0000	0.0326	0.0128	0.0065	0.0013	0.0037
Tween 80	0.0007	0.0841	0.0034	0.0116	0.0034	0.0044
D-Mannitol	0.0007	0.0004	0.0001	0.0086	0.0024	0.0020
4-Hydroxy Benzoic Acid	0.0000	0.0308	0.0012	0.0108	0.0128	0.0081
L-Serine	0.0001	0.0476	0.0000	0.0071	0.0033	0.0013
α-Cyclodextrin	0.0059	0.0223	0.0024	0.0014	0.1207	0.1041
N-Acetyl-D-Glucosamine	0.0007	0.1172	0.0007	0.0083	0.0028	0.0066
γ-Hydroxybutyric Acid	0.1233	0.0715	0.0000	0.0067	0.0020	0.0009
L-Threonine	0.0000	0.0055	0.0003	0.0120	0.0031	0.0008
Glycogen	0.0150	0.1151	0.0103	0.0030	0.0096	0.0179
D-Glucosaminic Acid	0.0039	0.0779	0.0000	0.0055	0.0011	0.0014
Itaconic Acid	0.0880	0.0048	0.0011	0.0073	0.0025	0.0006
Glycyl-L-Glutamic Acid	0.0000	0.0418	0.0000	0.0003	0.0016	0.0138
D-Cellobiose	0.0019	0.0223	0.0023	0.0059	0.0163	0.0058
Glucose-1-Phosphate	0.0000	0.0017	0.0020	0.0064	0.0029	0.0001
α-Ketobutyric Acid	0.0000	0.0564	0.0000	0.0022	0.0031	0.0026
Phenylethyl-Amine	0.0581	0.0111	0.0532	0.0011	0.0054	0.0034
α-D-Lactose	0.0010	0.0109	0.0000	0.0012	0.0039	0.0034
D,L-α-Glycerol Phosphate	0.0028	0.0111	0.0000	0.0002	0.0082	0.0010
D-Malic Acid	0.0038	0.0100	0.0008	0.0000	0.0030	0.0013
Putrescine	0.0000	0.0034	0.0075	0.0007	0.0160	0.0014

Table C2. Carbon substrate utilization of microorganisms from soybean potting medium 24 hours after treatment application as corrected Abs590 values by treatment. Highlighted absorbance values represent carbon sources that were utilized preferentially (Abs > 0.25). Absorbances represent the average of triplicate samples. T1: no treatment control, T2: pesticide and chemical fertilizer treatment, T3: pesticide and biofertilizer treatment, T4: biofertilizer treatment, and T5: chemical fertilizer treatment.

	T1.24p	T2.24p	T3.24p	T4.24p	T5.24p
β-Methyl-D-Glucoside	0.6411	1.3532	0.7403	1.2678	0.6441
D-Galactonic Acid γ-Lactone	0.5206	0.9706	1.6801	1.5792	0.5751
L-Arginine	0.1233	0.1175	0.0809	0.1300	0.0619
Pyruvic Acid Methyl Ester	0.3843	0.5140	0.8821	0.9069	0.7825
D-Xylose	0.3026	0.2846	0.3278	0.5243	0.0251
D-Galacturonic Acid	0.5886	0.8413	1.4024	1.0560	0.2495
L-Asparagine	0.2784	0.6465	0.6595	1.0974	0.0861
Tween 40	0.1203	0.7440	0.8244	0.1512	0.1598
i-Erythritol	0.0605	0.1871	0.0993	0.0070	0.0229
2-Hydroxy Benzoic Acid	0.0000	0.0000	0.0000	0.0000	0.0000
L-Phenylalanine	0.0898	0.2733	0.2762	0.0817	0.2405
Tween 80	0.2909	0.5685	0.6253	0.3690	0.2785
D-Mannitol	0.5417	0.9931	1.3920	1.5193	0.7037
4-Hydroxy Benzoic Acid	0.0680	0.2131	0.1986	0.0732	0.1928
L-Serine	0.3264	0.0661	0.3960	0.5317	0.3054
α-Cyclodextrin	0.0049	0.4153	0.5291	0.2251	0.0415
N-Acetyl-D-Glucosamine	0.7293	0.8568	1.6064	1.4488	0.9858
γ-Hydroxybutyric Acid	0.1788	0.5243	0.3016	0.3030	0.2799
L-Threonine	0.0000	0.0479	0.3719	0.0000	0.0664
Glycogen	0.2387	0.2864	0.3452	0.2272	0.0383
D-Glucosaminic Acid	0.0105	0.2110	0.1492	0.1454	0.2300
Itaconic Acid	0.0146	0.0836	0.0653	0.0938	0.0188
Glycyl-L-Glutamic Acid	0.0323	0.2292	0.0963	0.3533	0.1718
D-Cellobiose	0.7890	0.7442	1.4494	2.1907	0.0095
Glucose-1-Phosphate	0.5966	0.4032	0.5331	1.1489	0.6434
α-Ketobutyric Acid	0.0260	0.1060	0.1495	0.1845	0.0027
Phenylethyl-Amine	0.0286	0.1327	0.1743	0.1947	0.0956
α-D-Lactose	0.1055	0.1193	0.4676	1.5265	0.0041
D,L-α-Glycerol Phosphate	0.1597	0.1707	0.4367	0.3874	0.0089
D-Malic Acid	0.0033	0.2964	0.2699	0.4750	0.4885
Putrescine	0.0628	0.3335	0.2415	0.0000	0.0900

Table C3. Carbon substrate utilization of microorganisms from soybean potting medium 2 weeks after treatment application as corrected Abs590 values by treatment. Highlighted absorbance values represent carbon sources that were utilized preferentially (Abs > 0.25). Absorbances represent the average of triplicate samples. T1: no treatment control, T2: pesticide and chemical fertilizer treatment, T3: pesticide and biofertilizer treatment, T4: biofertilizer treatment, and T5: chemical fertilizer treatment.

	T1.2wp	T2.2wp	T3.2wp	T4.2wp	T5.2wp
β-Methyl-D-Glucoside	0.5765	0.7092	0.0168	1.3217	0.5910
D-Galactonic Acid γ-Lactone	0.9998	0.8919	0.6658	0.6171	0.9762
L-Arginine	0.2384	0.3581	0.3646	0.6397	0.0018
Pyruvic Acid Methyl Ester	0.9256	0.6119	0.6843	0.7286	0.6678
D-Xylose	0.5553	0.0003	0.5863	0.6078	0.7355
D-Galacturonic Acid	1.7586	1.5426	1.3006	1.3440	1.5169
L-Asparagine	1.5602	1.1154	0.8815	1.7661	0.6766
Tween 40	0.8168	0.6743	0.7943	0.8766	0.4078
i-Erythritol	0.2699	0.1522	0.0998	0.1445	0.1074
2-Hydroxy Benzoic Acid	0.0066	0.0042	0.0019	0.0138	0.0008
L-Phenylalanine	0.3364	0.3958	0.3114	0.3739	0.1157
Tween 80	0.3179	0.7241	0.7063	0.7229	0.4316
D-Mannitol	1.9881	1.4615	0.5815	1.2023	0.8313
4-Hydroxy Benzoic Acid	0.2194	0.1046	0.2251	0.8682	0.1422
L-Serine	0.5248	0.2330	0.8885	1.0376	0.9936
α-Cyclodextrin	0.1094	0.0822	0.3450	0.0324	0.0673
N-Acetyl-D-Glucosamine	1.5407	0.8280	0.8216	1.5774	1.4893
γ-Hydroxybutyric Acid	0.3947	0.5239	0.4499	1.4791	0.0827
L-Threonine	0.0306	0.0082	0.1391	0.0216	0.0059
Glycogen	0.4502	0.0623	0.6965	0.2572	0.1192
D-Glucosaminic Acid	0.3849	0.3473	0.4552	0.1782	0.0005
Itaconic Acid	0.0309	0.1427	0.3127	0.2147	0.0649
Glycyl-L-Glutamic Acid	0.2479	0.0957	0.1719	0.0591	0.1642
D-Cellobiose	1.0160	0.8491	0.0516	2.1784	1.7075
Glucose-1-Phosphate	0.6229	0.5301	0.2762	0.7012	1.2859
α-Ketobutyric Acid	0.0000	0.1261	0.0771	0.1908	0.0053
Phenylethyl-Amine	0.0491	0.0306	0.3690	0.7556	0.0064
α-D-Lactose	0.0022	0.0864	0.1271	0.9546	0.0380
D,L-α-Glycerol Phosphate	0.1184	0.0269	0.1510	0.4773	0.2200
D-Malic Acid	0.4132	0.0907	0.1969	1.1481	1.0374
Putrescine	0.3371	0.3281	0.4902	0.5707	0.5340

Table C4. Carbon substrate utilization of microorganisms from soybean potting medium 1 month after treatment application as corrected Abs590 values by treatment. Highlighted absorbance values represent carbon sources that were utilized preferentially (Abs > 0.25). Absorbances represent the average of triplicate samples. T1: no treatment control, T2: pesticide and chemical fertilizer treatment, T3: pesticide and biofertilizer treatment, T4: biofertilizer treatment, and T5: chemical fertilizer treatment.

	T1.1mp	T2.1mp	T3.1mp	T4.1mp	T5.1mp
β-Methyl-D-Glucoside	0.2871	0.0739	0.0267	0.6934	0.0166
D-Galactonic Acid γ-Lactone	0.1138	0.4908	0.3526	0.5900	0.0918
L-Arginine	0.4915	0.3668	0.1897	0.1881	0.7051
Pyruvic Acid Methyl Ester	0.4326	0.1973	0.4754	0.6793	0.6976
D-Xylose	0.3010	0.0319	0.0500	0.0243	0.2501
D-Galacturonic Acid	1.6465	0.5977	0.3128	0.7080	0.1718
L-Asparagine	1.0016	0.6224	0.2188	1.2816	1.0898
Tween 40	0.9209	0.3931	0.8228	0.7745	1.0713
i-Erythritol	0.3070	0.0112	0.0354	0.0787	0.1784
2-Hydroxy Benzoic Acid	0.0416	0.0000	0.0000	0.2041	0.0579
L-Phenylalanine	0.1197	0.0175	0.1885	0.1243	0.3031
Tween 80	0.6031	0.3019	0.3428	0.5853	0.7905
D-Mannitol	0.7836	0.1432	0.3634	0.4708	0.3492
4-Hydroxy Benzoic Acid	0.3727	0.2419	0.2712	0.3541	0.1721
L-Serine	0.5575	0.2949	0.2630	0.7108	0.5917
α-Cyclodextrin	0.0115	0.2563	0.0087	0.0114	0.0221
N-Acetyl-D-Glucosamine	0.7734	0.0509	0.1983	1.3581	0.6008
γ-Hydroxybutyric Acid	0.5192	0.2986	0.6543	1.2104	0.7217
L-Threonine	0.0547	0.0282	0.0057	0.0076	0.0303
Glycogen	0.2676	0.1461	0.0498	0.0679	0.0197
D-Glucosaminic Acid	0.1142	0.1108	0.0086	0.0287	0.0035
Itaconic Acid	0.0932	0.0282	0.1042	0.1129	0.0595
Glycyl-L-Glutamic Acid	0.0616	0.0888	0.1933	0.0380	0.1350
D-Cellobiose	0.0574	0.0778	0.0565	0.9131	0.7773
Glucose-1-Phosphate	0.0080	0.4949	0.0196	0.0143	0.0129
α-Ketobutyric Acid	0.0197	0.0096	0.0067	0.0020	0.0000
Phenylethyl-Amine	0.2927	0.0224	0.0102	0.3942	0.5853
α-D-Lactose	0.0042	0.0000	0.0087	0.0259	0.0077
D,L-α-Glycerol Phosphate	0.1242	0.0372	0.0182	0.0079	0.0121
D-Malic Acid	0.5738	0.0961	0.3535	0.3948	0.3294
Putrescine	0.4355	0.1140	0.2580	0.4579	0.4832

Table C5. Carbon substrate utilization of microorganisms from the storage condition experiment as corrected Abs590 values by treatment. Highlighted absorbance values represent carbon sources that were utilized preferentially (Abs > 0.25). Absorbances represent the average of triplicate samples. Samples were stored at either 22°C or 27°C for 1 week or 1 month.

	Time 0	22 1 week	22 1 month	27 1 week	27 1 month
β-Methyl-D-Glucoside	1.1645	1.4011	1.6643	1.3963	0.8453
D-Galactonic Acid γ-Lactone	0.9740	1.2719	1.6660	1.2074	0.9248
L-Arginine	0.7158	0.5188	0.5037	0.5618	0.1159
Pyruvic Acid Methyl Ester	0.9277	1.0778	0.8153	0.8361	0.3032
D-Xylose	1.0590	1.4253	0.5165	0.9796	0.1311
D-Galacturonic Acid	0.9826	1.4249	1.6325	1.1799	0.9549
L-Asparagine	1.2598	1.4831	1.2068	1.6773	0.4962
Tween 40	1.0398	1.0200	0.8036	0.8779	0.6441
i-Erythritol	0.1386	0.3944	0.2534	0.5103	0.0895
2-Hydroxy Benzoic Acid	0.0000	0.0000	0.0314	0.0201	0.0459
L-Phenylalanine	0.2858	0.5133	0.2612	0.1605	0.1040
Tween 80	0.6934	0.6366	0.5333	0.5294	0.3252
D-Mannitol	1.2524	1.3194	1.1827	1.2631	0.3130
4-Hydroxy Benzoic Acid	1.0527	0.9419	0.4388	0.6811	0.1031
L-Serine	1.1009	1.1862	0.7402	1.1396	0.4091
α-Cyclodextrin	1.0267	1.1160	0.5275	0.9636	0.3066
N-Acetyl-D-Glucosamine	1.3829	1.5874	1.9183	1.3744	1.0701
γ-Hydroxybutyric Acid	1.2034	1.2247	0.7561	1.1698	0.3064
L-Threonine	0.7228	0.4594	0.3798	0.3844	0.1800
Glycogen	1.3394	1.1713	0.9226	1.1603	0.5155
D-Glucosaminic Acid	1.2699	0.7274	0.3203	0.4123	0.3886
Itaconic Acid	0.3520	0.2058	0.1978	0.3958	0.0545
Glycyl-L-Glutamic Acid	0.7877	0.6804	0.4613	0.5024	0.2486
D-Cellobiose	1.4925	1.6169	1.7293	1.4749	0.6929
Glucose-1-Phosphate	1.0770	1.4022	1.0538	1.3854	0.5764
α-Ketobutyric Acid	0.6205	0.4306	0.3330	0.4559	0.1081
Phenylethyl-Amine	0.6894	0.5999	0.4094	0.5881	0.1175
α-D-Lactose	1.1352	1.1999	1.4773	1.1007	0.5605
D,L-α-Glycerol Phosphate	0.6943	0.7554	0.6409	0.6579	0.4464
D-Malic Acid	1.1761	1.1917	0.2841	1.0395	0.1610
Putrescine	0.8995	0.7876	0.5244	0.7260	0.1523

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