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Dust is a complex mixture of inorganic and organic materials including diverse microorganisms, which if unattended, accumulates over time. In this study, the microbial content in house dust was tested to determine its forensic detection potential in a model scenario mimicking the conditions of methamphetamine manufacturing. We hypothesized that microorganisms associated with the materials exposed to vapors will respond in a reproducible way. By identifying the microbial communities and any changes that may have occurred we expected to elucidate a correlation between microorganisms and the test chemicals involved, which was supported by the results presented. These findings may provide evidence in otherwise "cold cases" of methamphetamine manufacturer as well as information on the chemistry employed.

CHANGES IN THE MICROBIAL COMMUNITY AS A POTENTIAL INDICATOR OF CLANDESTINE DRUG OPERATIONS

THESIS

Presented to the Graduate Council of the

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By

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CHAPTER I

INTRODUCTION

Dust is a complex mixture of inorganic and organic materials, which is deposited on various surfaces and continues to build up over time. Its sources typically include: outdoor contaminants, food particles, dead insects, animal dander, mold and even human skin particles (1-3). Because bacteria have been found to be present in this mixture, dust has been used in epidemiological studies to determine its microbial contents as an aid in the treatment of health and disease conditions (4). In addition, analyses of dust have been studied to determine the microbial population present in that environment. Information concerning the bacterial community associated with house dust was limited. However recently, the composition of house dust has been investigated in many studies. It has been concluded that the bacterial community of dust is typically dominated by the phyla Proteobacteria, Firmicutes and Actinobacteria. In regards to house dust, carpeted floors are known to be a reservoir for bacteria, molds, endotoxins and allergens (1, 4). It was shown that the genera *Enterococcus, Staphylococcus, Pantoea* and *Pseudomonas* were the most abundant bacteria found in carpet dust (5). As a result of these findings, dust has been considered to be a good environment for microorganisms to grow (3).

Microorganisms are defined as living things, invisible to the human eye, that play important roles in the environment, such as maintaining the atmosphere and keeping people

healthy (6). The typical bacterial genome ranges in size from 0.5 to 10 million base pairs (bp). This genetic material, when obtained from environmental samples (and typically composed of multiple species), is known as the metagenome and the method performed to acquire and analyze this genetic material is known as metagenomics or metagenomic analysis. The process involves the isolation of DNA from environmental samples, sometimes including the cloning of the microbial DNA to obtain many copies in order to study the diversity of the microbial population followed by the sequencing of the clones (6). Alternatively, purified metagenomic DNA samples may be directly sequenced or certain amplicons representative of the community members within the sample may be first amplified by polymerase chain reaction (PCR) and then sequenced. Typically, microorganisms gravitate toward particular areas based on their affinity for available resources in the environment. Groups of bacteria that gather in those areas subsequently form complex microbial communities (7). Metagenomics is used to study these communities, since the samples are taken directly from the environment. This approach is advantageous due to the fact that the majority of bacteria in some environments (up to 99.8%) cannot be cultured under known laboratory conditions (8, 9). Therefore, through metagenomic approaches, information can be provided about the microbial community structure, function and potential interactions (6, 9).

One approach used in many metagenomics studies is the profiling of the 16S ribosomal RNA (rRNA) gene or 16S rDNA (9). The identification of bacteria is frequently determined through the use of this genetic marker in order to study bacterial phylogeny and taxonomy (10). Carl Woese was the first to discover that organisms can be identified by comparing a particular part of this gene (11-13). Comparisons can be made from amongst the 16S rRNA genes in bacteria and archaea and to the 18S rRNA gene of eukaryotes (11, 13). The utility of this

approach arises from certain characteristics of the 16S rRNA gene: 1) it is present in all bacteria and Archaea; 2) it is evolutionary conserved; and 3) it is approximately 1500 base pairs long and therefore large enough to provide sufficient phylogenetic information for discrimination between closely related taxa (10). The 16S rRNA gene is composed of variable regions and conserved regions, the latter including areas of the nucleotide sequence that are common across diverse microorganisms (14). These regions flank the nine variable regions in the gene (15). PCR of the variable regions using primers targeting the flanking conserved regions is a common approach for interrogating a complex community sample (9). The V4 region, used in this study, has an average length of 254 base pairs in most microbial species, though the actual length of this region varies amongst microorganisms (16, 17). Studies have shown the V4 region to be one of the most accurate regions for taxonomic classification (18-21). Next-Generation Sequencing technology has also been applied to this area of research.

The purpose of this study was to develop a microbiological DNA-based method for the detection of previous drug operations through the use of semiconductor sequencing of the V4 region on the 16S rRNA gene. Chemicals commonly used in methamphetamine production, such as benzyl chloride and camp fuel, were used in order to investigate changes in the microbial community as a result of vapor phase exposure to those chemicals. The hypothesis to be tested here is that vapor phase exposure would result in the alteration of the microbial community composition, displaying a reproducible signature. In contrast, our null hypothesis was that vapor phase exposure of the chemicals benzyl chloride and Coleman® camp fuel would not result in the alteration of the microbial community composition relative to unexposed controls. It is anticipated that the chemicals chosen would evaporate, and in their vapor form would come into contact with the microorganisms present in the vacuum dust, leading certain microorganisms,

with the capacity to metabolize or resist the chemical present, to grow. Thus when metagenomic analysis was done, it was expected that the microorganisms that prefer or tolerate a particular chemical would have increased abundance relative to the control.

CHAPTER II

BACKGROUND

In order to grow, a microorganism will exhaust all available resources (7). This includes degrading any substance that may benefit its survival. In regards to volatile liquids, utilization may occur via vapor-phase biodegradation, where absorption of compounds in the gaseous state, are degraded by microorganisms. The evaporation of a liquid depends on its vapor pressure. When the vapor pressure is at or above atmospheric pressure ($\geq$ 1 mmHg), evaporation occurs. A liquid with a low vapor pressure (< 1 mmHg), will take longer to evaporate than one with a high vapor pressure. As a result of exposure, the bacterial community may change depending on the compound. Figure 1 depicts a representation of the process used in this study.

A search of the literature failed to identify microbial communities associated with degradation of the compounds used in this study. Therefore, predictions were made based on published articles that investigated the degradation of the individual components or similar chemicals. Synthesis of benzyl chloride involved the use of toluene and chlorine. Therefore, bacteria associated with toluene were investigated. Previously, studies indicated toluene degradation pathways involving the *Pseudomonas* sp., as a toluene degrading bacterium (22-25). The *Pseudomonas* genus consists of species that can utilize more than 90 different organic compounds as their sole carbon source (26). *Pseudomonas putida* is known to use over 77

carbon-containing compounds as their sole energy source and is said to be highly versatile, suggesting the possible increase in abundance. These findings support a predicted change in abundance of *Pseudomonas* species when benzyl chloride is present.

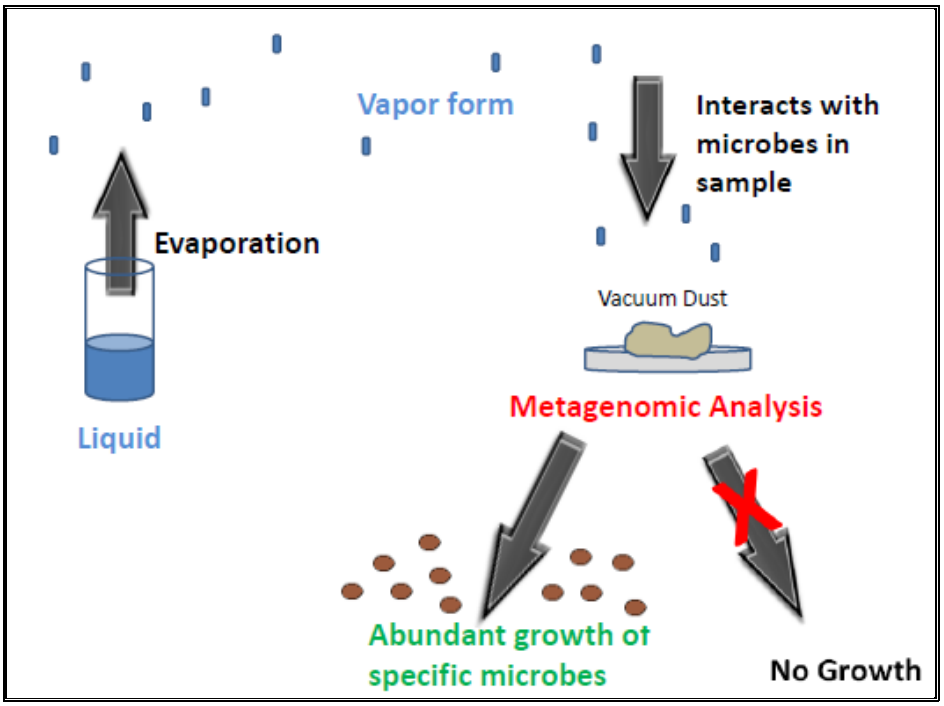


Figure 1: Model depicting vapor-phase biodegradation. This process results in the absorption of compounds in the gaseous state, which are then degraded by microorganisms. Once the chemical liquid evaporates and comes into contact with the microorganisms present in the vacuum dust in its vapor form, certain microorganisms with the capacity to metabolize or resist the chemical present will either have increased abundance or no growth relative to the control. The brown circles indicate the microorganisms from the vacuum dust which grew from their interaction with the liquid chemical's vapor.

Other predictions were made in respect to camp fuel. Coleman® Camp Fuel is used for lighting camp lanterns, stoves and clandestinely as a readily available solvent for the extraction of methamphetamine (27). It is produced from the distillation of petroleum, or crude oil, which is

a mixture of thousands of hydrocarbons (28). Products of crude oil, for example, include naphthalene and naphtha. Naphthalene is the simplest polycyclic aromatic hydrocarbon, consisting of two fused benzene rings and is typically found in fossil fuel. Naphthalene is used in the manufacturing of moth balls, dyes and insecticides (29). Naphtha is a mixed petroleum product in Coleman® Camp Fuel. Many microorganisms can use hydrocarbons as a sole source for carbon and energy (28), which is supported through studies that have shown the growth of *Pseudomonas putida* (NAH7) in the presence of vapor-phase naphthalene (30). Therefore, an abundance of the bacteria *Pseudomonas putida* can be expected for this fuel.

Many microorganisms have versatile metabolisms, which allow the degradation of certain compounds for use as a potential nutrient or energy source. Alkanes are amongst the compounds that particular microorganisms can degrade. Alkanes are saturated hydrocarbons composed of only hydrogen and carbon and can be linear, cyclic or branched. Under standard conditions, alkanes can be found in one of three states: a gaseous form for alkanes containing 1 to 4 carbons; a liquid for alkanes containing 5 to 16 carbons; and a solid form for compounds having more than 17 carbons (31). Alkanes are major components of crude oil, making up more than 50% of the oil depending on its source (31). Crude oil is defined as an unrefined petroleum product consisting of hydrocarbon deposits (32). Microorganisms, known as hydrocarbonoclastic bacteria, specifically degrade hydrocarbons and have played a major role in the removal of hydrocarbons in contaminated environments (33). Similarly, Coleman® Fuel is also a mixture of liquids derived from crude oil: cyclohexane, nonane, octane, heptane and pentane (34, 35). Degradation of vapor-phase compounds from Coleman® Fuel would likely select for alkane-degrading microorganisms. Likely genera include: *Arthrobacter* sp., *Acintobacter* sp., *Candida* sp, *Pseudomonas* sp., *Rhodococcus* sp., *Streptomyces* sp., *Bacillus* sp., *Aspergillus japonicas*,

Arthrobacter sp., *Acinetobacter* sp., *Thalassolitus* sp., *Oleiphilus* sp., *Geobacillus* sp., *Thermus* sp. and *Oleispira* sp. (31).

Clandestine laboratories involve the illegal manufacture of what is commonly known as illegal or illicit drugs. These laboratories exist in a wide variety of places; from homes, or hotels to the trunks of cars. These are often stationed in desolate areas in order to escape law enforcement. Clandestine laboratories can be classified into three types: super laboratories, box laboratories and mobile laboratories. Super laboratories are large facilities that usually produce ten or more pounds of drugs per production cycle (36-38). Box laboratories, also known as "mom-and-pop" laboratories differ in the amount of methamphetamine product that is synthesized. This form of laboratory tends to produce smaller quantities, approximately one to four ounces, and is able to fit inside tiny spaces, such as a bedroom, as shown in Figure 2 (36, 39). Mobile laboratories are typically located in modes of transportation in order to be moved from one place to another, and thereby reduce the chance of discovery. Clandestine laboratories have been identified in all 50 states. Over 80% of those found in the US are involved in the production of methamphetamine (36, 40).

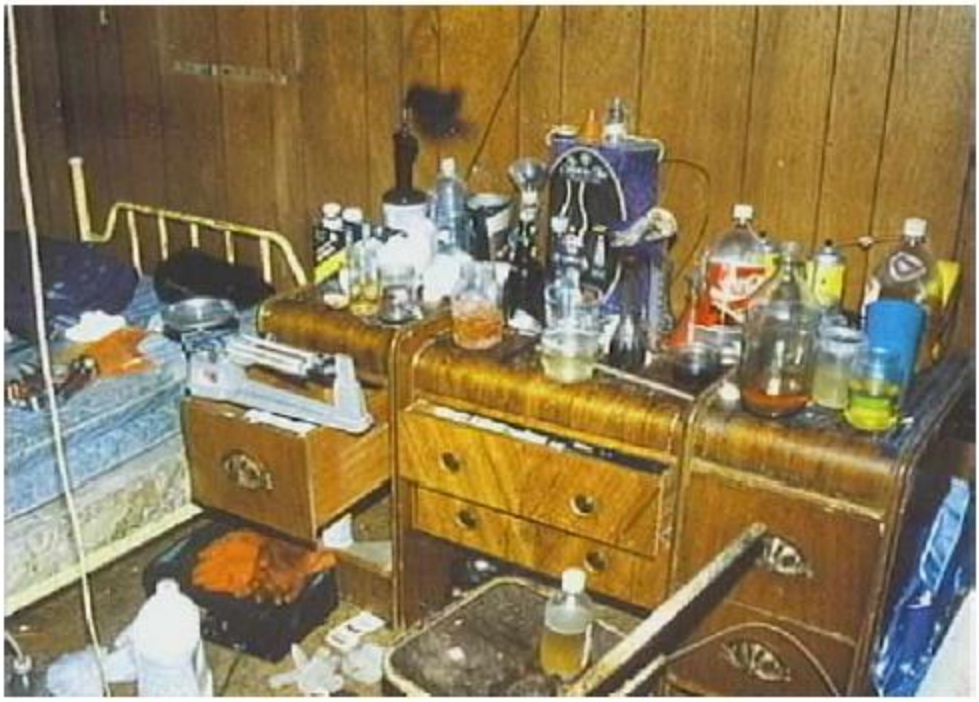


Figure 2: Illegal production of methamphetamine inside a bedroom. This is an example of a box laboratory that was stationed within a bedroom. Box laboratories are typically located in tiny spaces, where approximately one to four ounces of drugs are produced (36).

Methamphetamine is a stimulating agent that differs from amphetamine in that it more rapidly crosses the blood-brain barrier, penetrating the central nervous system. This results in the release of norepinephrine and dopamine from the nerve endings and brain synapses, respectively, leading to a state of euphoria and a desire for more of the drug. Methamphetamine was synthetically developed as an alternative for ephedra, a botanic extract used in Chinese medicines for over 5000 years. The active compound present in ephedra, ephedrine, was studied in 1885. At the beginning of the 20th century, it was discovered that ephedrine was similar to epinephrine. Once it was known how an individual was affected when taken the substance orally, the use of methamphetamine began to expand. In 1919, Japanese chemist Akira Ogata was the first to synthesize methamphetamine using ephedrine as a precursor. Early uses of

methamphetamine included treatments for weight loss, schizophrenia, asthma, morphine addiction, Parkinson's disease, etc. It was also used in World War II as an aid for increasing alertness, reducing fatigue and suppressing the soldiers' appetites (36).

During the 1940s and 1950s, amphetamine-type stimulants such as methamphetamine, were popular medications utilized for diverse purposes (36). It was freely prescribed, and thus large quantities were produced. However, upon recognition of the negative health effects caused by amphetamine, the Comprehensive Drug Abuse Prevention and Control Act of 1970 limited the drug's prescription. This lead to the gradual decrease in the legal production, and as a result initiated the illegal manufacture of the drugs. Since legally manufactured methamphetamine became more difficult to acquire, illegal manufacture of methamphetamine became an increasingly important issue. The first known illicit methamphetamine production was discovered in 1962 in San Francisco, CA. Illicitly produced methamphetamine was often contaminated with numerous impurities. By the mid-1980s, practically all of methamphetamine was produced in clandestine laboratories as opposed to the legal pharmaceutical companies. A relatively recent law, the Combat Methamphetamine Epidemic Act of 2005, regulated the purchase of products that can be used in methamphetamine production. As a result, these precursor products are now stored behind pharmacy counters. The law also limited the amount of pseudoephedrine purchased per day to a maximum of 3.6 g in an attempt to decrease methamphetamine manufacture. However, despite the law, methamphetamine manufacturers have found ways to evade these restrictions and continue their illegal production (36). For instance, since the early part of the 1980s, Mexican drug cartels have been involved in the transportation of methamphetamine and its precursors into the United States. Despite the US and Mexico's efforts to stop importation, methamphetamine production continues to increase. As of

today, the majority of methamphetamine available in the US has been either produced domestically or in Mexico (36).

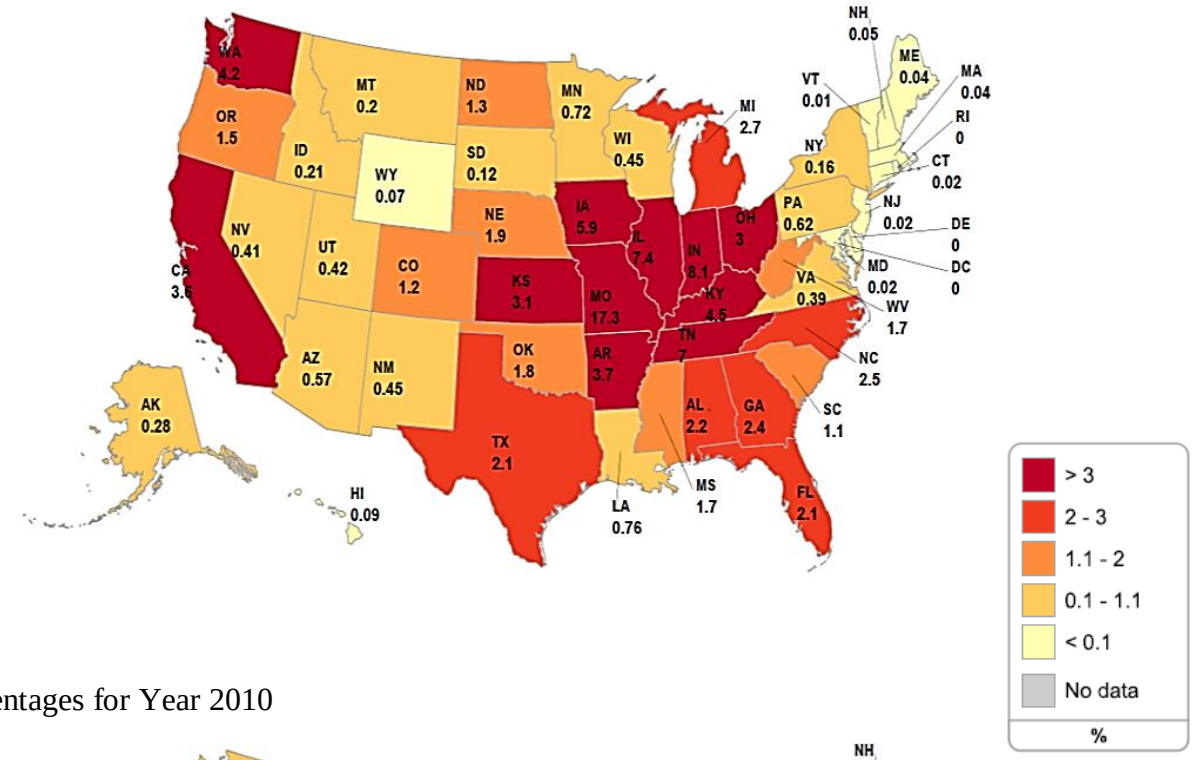
There are several synthesis pathways for the production of methamphetamine. The Red Phosphorus Method is used in 64.6% of known methamphetamine producing laboratories (40). Although there are numerous recipes that have been developed for the production of methamphetamine, the processes are similar (36, 40). Each method begins with the extraction of the precursor drug; followed by the reduction of the ephedrine/pseudoephedrine to methamphetamine; and then finishing with the "salting out stage", which is the extraction of methamphetamine in its solid form from a solution (40). Typically, household chemicals are used as the precursor drug, such as over-the-counter medicines containing ephedrine or pseudoephedrine. Additional chemicals required may include, drain cleaners, hydrochloric acid, Coleman® Camp Fuel, benzene and many others. Despite the variation in chemicals used during production, what remains consistent is that the products used belong to three general classes: precursors, reagents and solvents. Precursors are the basic raw materials used in this process. Typically, these precursors relinquish either a majority or all of their structure to the structure of an intermediate compound. Reagents are chemicals that cause a reaction with the presence of precursors; however, they are not incorporated into the chemical structure of the finished product. Solvents are used to extract the final product (40). For this study, representative chemicals from the following classes were selected: benzyl chloride from the precursor class, and Coleman® Camp Fuel from the solvent class.

More than 7,500 clandestine methamphetamine laboratories had been seized in 44 states as of 2002 (39). However, in 2005 and 2010, the total number of methamphetamine-related laboratory incidents in the United States was 12,974 and 11,239, respectively. These results,

shown in Figure 3, includes discovery of laboratories, dumpsites or methamphetamine manufacturing equipment used to produce, or "cook", the drug (36). From the 2005 US map (Figure 3a), the general location for the methamphetamine laboratory incidents seemed to be stationed around the borders of Mexico and toward the mid-East part of the United States. However, in the 2010 US map (Figure 3b) there was a decrease in laboratory incidents since by that time, the Combat Methamphetamine Epidemic Act of 2005 was in full force, limiting methamphetamine production. As a result, the methamphetamine incidents migrated more toward the eastern part of the US and its percentage for the states surrounding Mexico decreased.

Illicit drugs manufacturers, or "cooks", have their own "recipes" that they obtain directly from others, the Internet, the chemistry literature or underground resources. Methamphetamine can be synthesized using these different techniques and recipes; however, none are considered safe outside a professional laboratory (36). During "cooking", vapors can spread and be absorbed by nearby materials, such as curtains and carpet. Methamphetamine production can result in areas with potential spills, boil-overs, explosions or chemical fumes. Indoor areas that may be affected include walls, ceilings, carpeting, draperies and other products, while outdoor cooking areas may expose soil, dumpsters, septic systems and/or groundwater to reagents during drug production (41). At these locations, contamination can occur and spread, which requires established lab cleanup teams to secure the site and remove any hazardous materials (41). Cleanup first requires identification of a lab site. The purpose of this study is to develop and test new methods from the field of microbial ecology to address this problem. Results of this study may have important implication in other fields including microbial forensics and criminal justice.

a) Percentages for Year 2005



b) Percentages for Year 2010

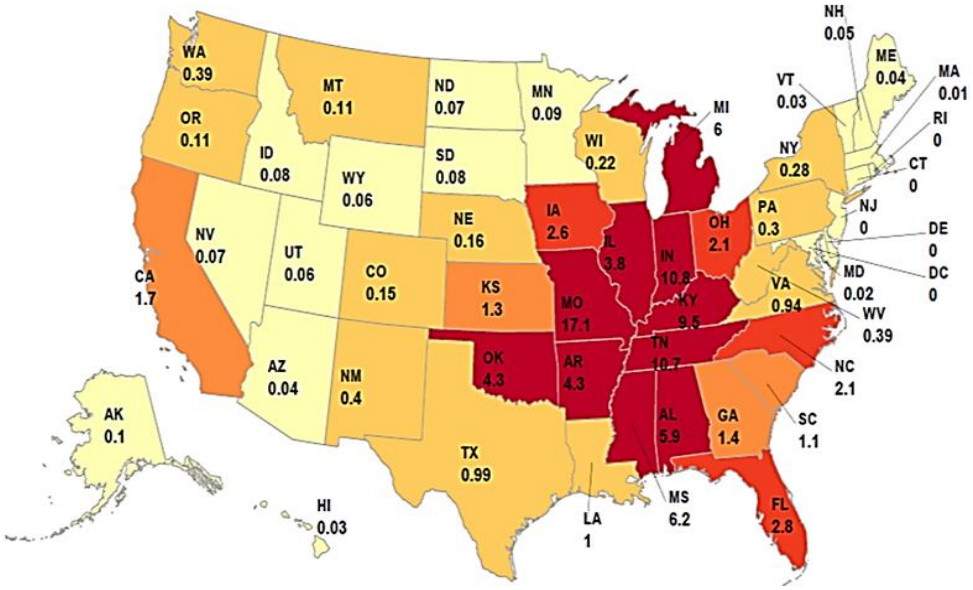


Figure 3: Statistics of clandestine methamphetamine laboratory incidents for 2005 and 2010. Numbers referenced from (36) were used to generate percentages in the map above. From (a) the 2005 US map, the location for methamphetamine laboratory incidents (discovery of labs, dumpsites and equipment) seemed to be stationed around the borders of Mexico and toward the mid-east part of the US. However, in (b) the 2010 US map, there was a decrease in laboratory incidents, which migrated more toward the eastern part of the US, while its percentage for the states surrounding Mexico decreased.

Currently, mass spectrometry is the method used for the detection of volatile chemicals. However, changes in microbial community as a result of exposure to volatile chemicals in methamphetamine production may prove to be more long-lived than the current methods used. The goal is to develop a method that can provide important information in identifying areas used for the clandestine production of methamphetamine. The results from this method may further lend specific information into the particular chemistry used. Since "cooks" often create a signature recipe, identifying the chemistry may link production to a particular perpetrator. The specific aim of this study was to determine if exposure to the chemicals used in methamphetamine manufacture would result in changes in the microbial community in a representative test sample. As a control, the microbial community was also determined for unexposed samples. Test samples were analyzed shortly within 24hrs of collection without any prior treatment (see Materials and Methods). An additional control used in this study included the determination of the microbial community for water-treated samples. Instead of chemical treatment, water was exposed to these samples and served as our negative control. Experimental conditions included the exposure to the chemicals benzyl chloride and a solvent mixture sold under the name Coleman® Fuel. Results were analyzed to determine which bacterial taxa, if any, would increase or decrease in relative abundance following exposure after one, two, or three weeks.

CHAPTER III

MATERIALS AND METHODS

Sample Preparation/DNA Extraction

Carpet dust was collected from an area home using a Dyson DC28 vacuum. Vacuum collection vessel contents were first screened for large debris and trash (e.g. paper, etc), and fine dust was transferred to a sterile 50-mL conical tube and transported the following day to the laboratory for experimentation. For this study, the following three liquids were used: an exposure no treatment control consisting of water, ReagentPlus® 99% Benzyl Chloride (Sigma-Aldrich®, Saint Louis, Mo, USA) and Coleman® Premium Blend Liquid Fuel (Coleman Company Inc., Wichita, Kansas, USA). Twenty-seven autoclaved Ball brand pint and half wide mouth jars with screw cap rings and lids were used in this study: triplicate samples for each chemical treatment, no treatment control, and for each time point consisting of one, two, and three weeks. Approximately 0.3g of the indoor-collected vacuum dust sample was weighed and added to the cap of a 1.5-mL microcentrifuge tube, then placed onto the floor of each jar. Two 16 x 125mm disposable culture test tubes (VWR International, West Chester, PA, USA) were also included, one test tube contained a 5-mL aliquot of one of the test chemicals and the other test tube contained deionized water to maintain some level of humidity. The experimental design is

graphically shown in Figure 4. Both test tubes for the exposure no treatment controls were filled with deionized water. Triplicate jars were set up for each sample treatment and no treatment control, for each time period and remained closed for a period of one, two or three weeks. Another three 0.3 g aliquots of sample was independently sampled to serve as the time period, t=0, giving a total of 30 samples tested. A list of the samples and conditions is shown in Table 1. At the end of the indicated time period, DNA extraction of the vacuum dust proceeded using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA) according to the manufacturer's instructions.

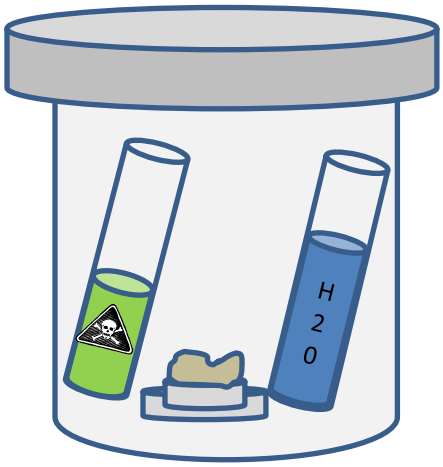


Figure 4: Graphic representation of the experimental set up. The cylinder with green liquid indicates the test tube filled with 5-mL of the tested chemical, which was benzyl chloride, camp fuel, or water. The 5-mL of water was used in the no-treatment control samples. The cylinder with the blue liquid indicates the test tube filled with water to maintain internal humidity.

Samples		
Immediate Samples		
t=0 replicate 1		
t=0 replicate 2		
t=0 replicate 3		
No Treatment Control Samples		
No Treatment Control 1 week Jar 1	No Treatment Control 2 weeks Jar 1	No Treatment Control 3 weeks Jar 1
No Treatment Control 1 week Jar 2	No Treatment Control 2 weeks Jar 2	No Treatment Control 3 weeks Jar 2
No Treatment Control 1 week Jar 3	No Treatment Control 2 weeks Jar 3	No Treatment Control 3 weeks Jar 3
Benzyl Chloride-treated Samples		
Benzyl Chloride 1 week Jar 1	Benzyl Chloride 2 weeks Jar 1	Benzyl Chloride 3 weeks Jar 1
Benzyl Chloride 1 week Jar 2	Benzyl Chloride 2 weeks Jar 2	Benzyl Chloride 3 weeks Jar 2
Benzyl Chloride 1 week Jar 3	Benzyl Chloride 2 weeks Jar 3	Benzyl Chloride 3 weeks Jar 3
Coleman® Camp Fuel-treated Samples		
Fuel 1 week Jar 1	Fuel 2 weeks Jar 1	Fuel 3 weeks Jar 1
Fuel 1 week Jar 2	Fuel 2 weeks Jar 2	Fuel 3 weeks Jar 2
Fuel 1 week Jar 3	Fuel 2 weeks Jar 3	Fuel 3 weeks Jar 3

Table 1: List of samples analyzed in this study. This is a list of samples and conditions that were tested in the indicated time periods. The immediate samples (t=0) had no previous exposure to chemicals and were used as controls. The no treatment control samples were exposed to only sterile water. The remaining samples were exposed to either the chemicals benzyl chloride or Coleman® Camp Fuel.

Quantification

After DNA extraction, 1 µL of DNA solution was pipetted onto the pedestal mode of the NanoDrop 2000c UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The DNA concentrations determined were the amount of DNA present (ng/µL) in the samples.

PCR Amplification

The extracted DNA was amplified using an Accuprime™ Taq DNA Polymerase High Fidelity Kit (Life Technologies, Carlsbad, CA, USA). To target the V4 variable region of the 16S rRNA gene, primers corresponding to base pair 515 (forward) and base pair 806 (reverse) were used, where nucleotides correspond to the numbering of the E. coli gene. Primers were synthesized containing a 10 base pair barcode derived from the Ion Torrent 96 barcode set. Additionally, forward primers contained the A adapter sequence, and reverse primers contained the P1 sequence for use on the Ion Torrent Personal Genome Machine (PGM) (Life Technologies). Primer sequences are listed in the Appendix (21, 42-44). Triplicate DNA extractions were performed using a single set of barcoded primers and amplicons were pooled prior to sequencing. Replicate samples for each time point and treatment contained unique barcoded primers to facilitate downstream data processing. For each PCR reaction, the following was used to create a master mix: 2.5 µL of 10x PCR Buffer II, 0.5 µL of 806R primer, 0.1 µL of Taq enzyme, 2.0 µL of DNA sample with the exception of benzyl chloride-treated samples, which had 10 µL of input DNA, and 19.4 µL of sterile water (11.4 µL of water for the benzyl chloride treated samples). After 24.5 µL of the master mix was aliquoted into each PCR tube, 0.5

µL of one of the unique barcoded 515F forward primers was added. A list relating primer number to sample is shown in Table 2.

The protocol for this amplification begins with an initial incubation of the tubes at 94°C for 2 min. Twenty five cycles of PCR amplification followed with reaction conditions as follows: denaturation at 94°C for 15s, annealing at 52°C for 15s and extension at 68°C for 20s. Then, there was a final extension step at 68°C for 5 min. The reaction was maintained at 4°C after cycling. The amplified products were analyzed via 1.5% (w/v) agarose gel with ethidium bromide, using a 100 bp DNA ladder (Phenix Research Products, Hayward, CA, USA). Electrophoretic separation of the amplified products occurred at 200V for approximately 25 min in 1X TAE buffer. The amplicons were visualized under an EC3 Imaging System (UVP Inc., Upland, CA, USA), using VisionWorks®LS Analysis software (UVP Inc.).

Immediate (t=0) samples	
Forward Primer Assignment	
Replicate 1	1 ionA-515F
Replicate 2	6 ionA-515F
Replicate 3	11 ionA-515F

1 week samples			
Forward Primer Assignment			
	Jar 1	Jar 2	Jar 3
NC	16 ionA-515F	19 ionA-515F	22 ionA-515F
BC	17 ionA-515F	20 ionA-515F	23 ionA-515F
F	18 ionA-515F	21 ionA-515F	24 ionA-515F

2 week samples			
Forward Primer Assignment			
	Jar 1	Jar 2	Jar 3
NC	2 ionA-515F	7 ionA-515F	12 ionA-515F
BC	3 ionA-515F	8 ionA-515F	13 ionA-515F
F	5 ionA-515F	10 ionA-515F	15 ionA-515F

3 week samples			
Forward Primer Assignment			
	Jar 1	Jar 2	Jar 3
NC	25 ionA-515F	28ionA-515F	31 ionA-515F
BC	26 ionA-515F	29 ionA-515F	32 ionA-515F
F	27 ionA-515F	30 ionA-515F	14 ionA-515F

Table 2: List of assigned PCR amplification 515 forward primers. This is a list of the designation of forward primers to samples. The immediate samples (t=0) had no previous exposure to chemicals and were used as controls. The no treatment control and the remaining samples were exposed to sterile water and test chemicals, respectively. NC-No Treatment Control; BC-Benzyl chloride; F- Coleman® Camp Fuel

Ion Torrent™ PGM™ Library Preparation and Sequencing

Three 16S rDNA amplicon replicates from each sample were pooled together and purified using the Agencourt® AMPure® XP PCR magnetic purification beads (Beckman Coulter Inc., Brea, CA, USA) according to the manufacturer's instructions with one minor deviation. A total of 108 µL of AMPure® XP Reagent was used instead of 90 µL, since the pooled samples totaled to 60 µL and not 50 µL. The molar concentration of the pooled amplicon library stock was determined using an Agilent BioAnalyzer® DNA 7500 LabChip® (Agilent Technologies Inc., Santa Clara, CA, USA) on the Agilent 2100 BioAnalyzer® (Agilent Technologies Inc.). Based on the molar concentration from each amplicon library, an equimolar pool of the libraries was generated at the highest possible concentration (26 pmol/L) via dilution with low TE. The diluted library was used in the Ion PGM™ Template OT2 400 Kit (Life Technologies), according to the manufacturer's instructions for an Amplicon library, to set up emulsion PCR (described below).

The DNA quality of the unenriched and enriched Ion Sphere™ Particles (ISPs) were analyzed on the Qubit® 2.0 fluorometer (Life Technologies) through use of the Ion Sphere™ Quality Control Kit (Life Technologies), following the manufacturer's instructions, to determine the percentage of the template-positive ISPs present. Emulsion PCR was then performed on the Ion OneTouch™ 2 System (Life Technologies) for clonal amplification of the template-positive ISPs via emulsion PCR. The ISPs are washed and enriched onto Dynabeads MyOne Streptavidin C1 beads (Life Technologies) and loaded on the Ion OneTouch™ Enrichment System (Life Technologies) to isolate and solely recover the enriched template-positive ISPs. The sequencing libraries were prepared using the Ion PGM™ 400 Sequencing Kit (Life Technologies) and the

entire sample was loaded onto an Ion 316 v2 chip and run on the Ion Torrent™ Personal Genome Machine (PGM), following the manufacturer's instructions.

Data Analysis

Bacterial community analysis was performed using the mothur. Data from the PGM runs were processed using the platform-specific pipeline software Torrent Suite 4.0.2 to check the quality of the resulting raw sequences, discarding poor quality sequences, in order to minimize the effect of sequencing errors (45) . Primers and barcodes were trimmed. The software aligned the sequences using reference sequences from the SILVA reference files database. The sequences were de-noised and then clustered into operational taxonomic units (OTUs) using pre-cluster algorithm (46). After OTUs were generated, the Ribosomal Database Project (RDP) classifier was used for taxon identification. As of 2008, the RDP database contains 33,082 archaeal and 643,916 bacterial rRNA sequences. Of these bacterial sequences, 543,487 were obtained from environmental samples (47). The RDP system is used in order to accurately identify bacterial 16S rRNA sequences by providing taxonomic annotation from domain to genus, and occasionally species (14). Alternatively, 10,000 sequences were subsampled from each sample in order to calculate the unweighted and weighted UniFrac distance, which measures the distance between pairs of samples in order to analyze any similarities/differences between the microbial communities. This displayed using Principal Coordinate Analysis and a clustering tree.

CHAPTER IV

RESULTS

Initial Observation

At both the second and third weeks, the physical appearance of the dust samples in all three no treatment control replicates, had altered compared to time 0 and appeared to have a greenish, mold-like appearance. The no treatment control samples were treated like the other chemical-treated samples, with the exception of using 5-mL of deionized water instead of the chemicals. This possibly produced an artificially high humidity environment, explaining the appearance of the mold. However, the appearance of the benzyl chloride and camp fuel samples seemed unchanged. In addition, jar 1 of the two week benzyl chloride –treated samples contained a dead maggot in the vacuum dust that was not detected prior to its placement of the dust into the jar. This was recorded in case of variation for that sample.

Quantification

The DNA concentrations obtained for the samples in this study are listed in Table 3. The absorbance ratios 260nm/280nm and 260nm/230nm indicate the relative abundance of DNA vs RNA and nucleic acid purity, respectively. The 260/280 ratio should be approximately 1.8 for "pure" DNA. The 260/230 ratio should be around 2 for "pure" nucleic acids (i.e., absence of

contaminating salts, solvents, etc) and are often higher than the 260/280 values (48). The effective range of the Nanodrop in pedestal mode is between 2.0 ng/µL and 15,000 ng/µL (49). Upon analysis of all the sample concentrations, it appeared that the samples were closer to the expected ratio for DNA as opposed to RNA. Compared to the immediate, or t=0, samples, there was a dramatic increase in DNA concentration present in the second and third week for the no treatment control samples. The DNA concentrations for the benzyl chloride-treated samples were lower than the immediate t=0 samples. However, the concentration of the benzyl chloride-treated samples remained consistent over the 3 week time period. The extracted DNA yields for the fuel-treated samples were mostly lower than the immediate t=0 samples, except for one jar in the second and third weeks, which had a similar and higher concentration than the immediate samples, respectively. The values are shown in Table 3 are graphically represented in Figure 5.

a) DNA quantification data for immediate (t=0) and 1 week samples

Sample Name	Nucleic Acid Concentration (ng/µL)	A260	A280	260/280	260/230
t=0 Replicate 1	5.0	0.099	0.048	2.06	1.15
t=0 Replicate 2	7.7	0.154	0.084	1.83	1.24
t=0 Replicate 3	6.2	0.124	0.061	2.03	0.64
NC 1 week Jar 1	4.3	0.086	0.043	2.03	0.72
NC 1 week Jar 2	4.4	0.087	0.042	2.09	0.65
NC 1 week Jar 3	1.9	0.039	0.026	1.45	0.2
BC 1 week Jar 1	2.4	0.047	0.02	2.33	0.32
BC 1 week Jar 2	1.5	0.029	0.007	4.12	0.51
BC 1 week Jar 3	2.7	0.053	0.029	1.83	0.33
Fuel 1 week Jar 1	2.3	0.047	0.019	2.51	0.6
Fuel 1 week Jar 2	3.8	0.076	0.044	1.75	0.53
Fuel 1 week Jar 3	3.3	0.066	0.039	1.68	0.93

b) DNA quantification data for 2 week samples

Sample Name	Nucleic Acid Concentration (ng/µL)	A260	A280	260/280	260/230
NC 2 weeks Jar 1	16.7	0.334	0.186	1.8	0.61
NC 2 weeks Jar 2	16.3	0.326	0.18	1.81	1.02
NC 2 weeks Jar 3	13.5	0.27	0.16	1.69	1.07
BC 2 weeks Jar1	2.6	0.051	0.031	1.65	0.18
BC 2 weeks Jar 2	2.1	0.042	0.029	1.45	0.3
BC 2 weeks Jar 3	1.6	0.033	0.023	1.44	0.2
Fuel 2 weeks Jar 1	2.9	0.058	0.032	1.82	0.26
Fuel 2 weeks Jar 2	9.3	0.185	0.101	1.83	0.72
Fuel 2 weeks Jar 3	2.6	0.051	0.027	1.89	0.37

c) DNA quantification data for 3 week samples

Sample Name	Nucleic Acid Concentration (ng/µL)	A260	A280	260/280	260/230
NC 3 weeks Jar 1	16.7	0.333	0.18	1.85	1.3
NC 3 weeks Jar 2	21.8	0.435	0.231	1.88	0.94
NC 3 weeks Jar 3	10.2	0.204	0.104	1.96	0.81
BC 3 weeks Jar 1	2.4	0.049	0.008	6.09	0.45
BC 3 weeks Jar 2	1.9	0.039	0.024	1.62	0.3
BC 3 weeks Jar 3	1.4	0.029	0.016	1.78	0.21
Fuel 3 weeks Jar 1	2.5	0.05	0.019	2.63	0.41
Fuel 3 weeks Jar 2	5.9	0.118	0.067	1.77	0.37
Fuel 3 weeks Jar 3	3.4	0.067	0.024	2.86	0.48

Table 3: DNA quantification data. Information about the extracted DNA is listed in this table for (a) t=0 and 1 week samples, (b) 2 week samples, and (c) 3 week samples. The absorbance ratios 260nm/280nm and 260nm/230nm indicate the relative abundance of DNA vs RNA and nucleic acid purity, respectively. The 260/280 ratio should be approximately 1.8 for "pure" DNA. The 260/230 ratio should be around 2 for "pure" nucleic acids (i.e., absence of contaminating salts, solvents, etc.). The data in red represents the samples with high DNA concentrations compared to the immediate samples. NC-No Treatment Control; BC-Sigma Benzyl Chloride; Fuel-Coleman® Camp Fuel; t=0- the samples were immediately extracted with no prior treatment. A260, A280- absorbance at 260 and 280 nm, respectively.

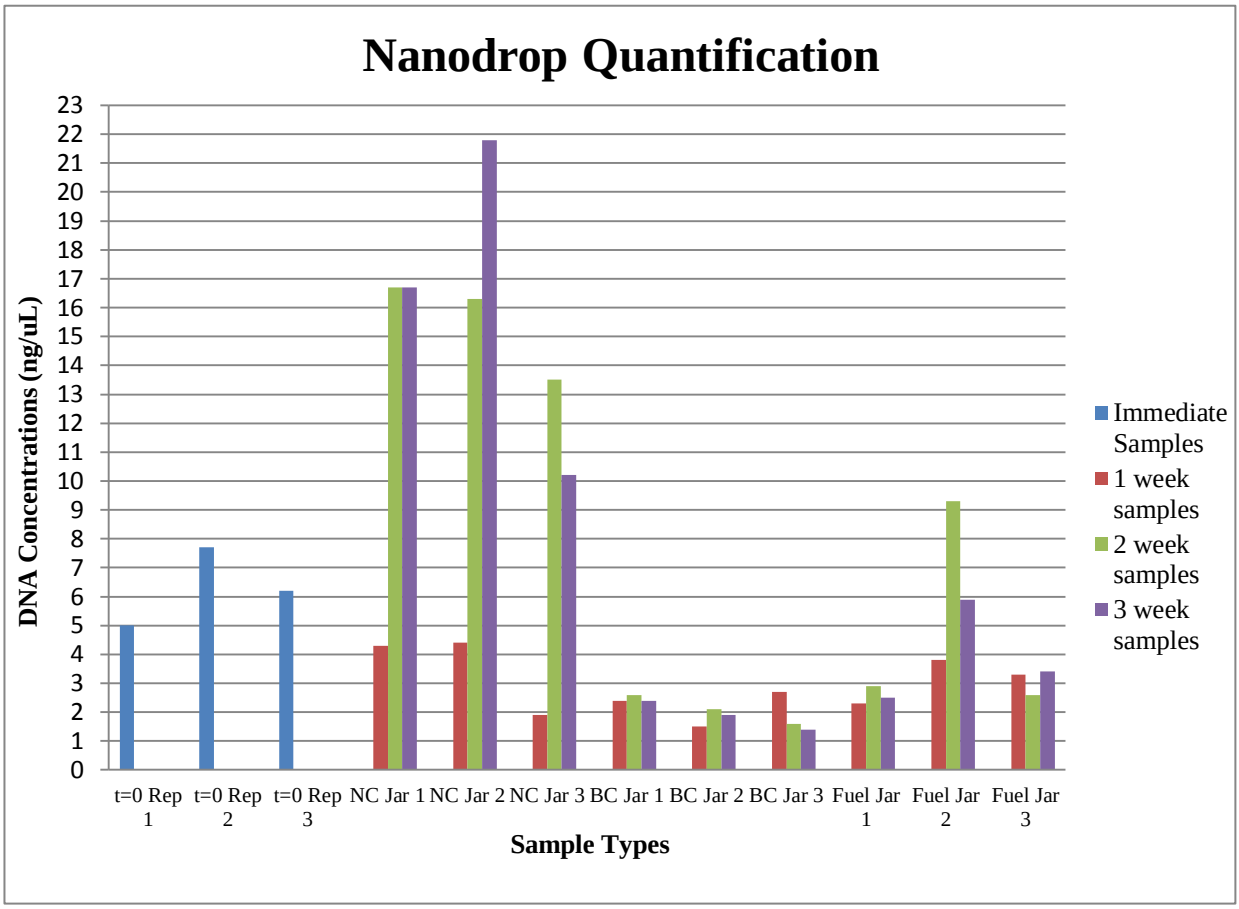


Figure 5: Nanodrop quantification graph. The DNA concentration values that were in Table 3 were used to create this bar graph in order to visually display the differences in the amount of extracted DNA recovered. NC-No Treatment Control; BC-Benzyl Chloride; Fuel-Coleman® Camp Fuel; The t=0 samples were immediately extracted with no prior treatment.

PCR Amplification

The PCR products were analyzed on 1.5% agarose gels. The DNA bands were approximately 300-400 bp in length when compared to the 100 bp ladder and is similar in size to the E. coli positive control used. There was the presence of DNA bands for both the immediate samples and the no treatment control samples. However, the bands for some of the benzyl chloride-treated samples were faintly present and at the third week, there were no visible bands. The bands from the Coleman® Camp fuel samples were mostly visible. The agarose gels are displayed in Appendix B.

Library Preparation/Sequencing

The size and molar concentrations of the pooled amplicon library stock was determined using an Agilent BioAnalyzer® DNA 7500 LabChip® (Agilent Technologies, Inc., Santa Clara, CA, USA) run on an Agilent 2100 BioAnalyzer® (Agilent Technologies, Inc.). A DNA ladder was analyzed first, followed by each sample, according to its migration time, indicating the size of the samples' pooled amplicon technical replicates. A lower mark and upper marker, which serve as internal standards, are used to align the ladder with the samples for analysis. The data between these internal standards correspond to amplicons within the samples; revealing the size, concentration and molarity of each sample. These values were shown in Table 4. In the second and third weeks, the no treatment control samples had two peaks present. This may indicate the presence of two kinds of bacteria with varying sizes of the V4 region, which was mentioned earlier to differ in size among microorganisms. Therefore, at least two kinds of microorganisms were expected to be present for the no treatment control samples. Since this occurs for all the jars in the second and third week of the no treatment controls, it is assumed that the one of the kinds

of microorganisms detected have a correlation to the mold found in these samples. For these samples with the two concentrations, their values were summed and treated as a single concentration when it came to diluting the final library. Benzyl chloride-treated samples in the third week from jar 1 (BC26) and jar 2 (BC29), displayed an absence of DNA and therefore these samples could not proceed for further analysis.

a)

Immediate samples

Sample+Primer	Size (bp)	Agilent concentration (ng/µL)	Molarity (nmol/L)
t1	366	7.25	30.0
t6	368	3.80	15.7
t11	368	5.16	21.2

b)

1 week Samples

Sample+Primer	Size (bp)	Agilent concentration (ng/µL)	Molarity (nmol/L)
NC16	359	8.30	35.0
NC19	368	8.09	33.3
NC22	368	3.39	14.0
BC17	367	2.92	12.1
BC20	365	8.52	35.4
BC23	366	2.92	12.1
F18	368	0.77*	3.2
F21	367	4.74	19.6
F24	366	3.10	12.8

c)

2 week samples			
Sample+Primer	Size (bp)	Agilent concentration (ng/µL)	Molarity (nmol/L)
NC2	335	0.99*	4.5
	370	5.91	24.2
NC7	332	1.68*	7.6
	370	8.09	33.1
NC12	332	3.05	13.9
	367	16.82	69.3
BC3 (5 µL)	365	0.33*	1.4
BC8 (3µL)	363	1.16*	4.8
BC13	362	6.89	28.8
F5	370	7.56	31.0
F10	368	15.95	65.6
F15	360	10.08	42.4

d)

3 week samples			
Sample+Primer	Size (bp)	Agilent concentration (ng/µL)	Molarity (nmol/L)
NC25	330	5.55	25.0
	367	5.71	24.0
NC28	333	10.54	48.0
	370	4.66	19.1
NC31	330	1.64*	7.5
	367	3.52	14.5
BC26	ND	ND	ND
BC29 (5 µL)	ND	ND	ND
BC32 (5 µL)	367	0.28*	1.1
F14	366	13.36	55.4
F27 (3 µL)	364	4.54	19.0
F30	368	8.25	33.9

Table 4: Agilent concentrations. The tables presented show the Agilent data obtained for each sample at (a) the immediate samples, (b) 1 week samples, (c) 2 week samples and (d) 3 weeks samples. The samples highlighted in blue had two peaks present in the sample, concluding that two types of microorganisms were present. The samples in red indicate that no concentration could be calculated. In parentheses is the amount of increased DNA that was used. The samples without the parentheses indicate that the standard 1 µL of DNA was used in the Agilent. NC-No Treatment Control; BC-Benzyl Chloride; F-Fuel. ND- Not Detected. Asterisks denote samples that were used, despite falling below the linear range of the instrument. Sample+Primer is an abbreviation for the chemical treated sample and the forward primers used. The assigned primers were listed in Table 2.

The library was diluted with low TE until an equimolar pool of the libraries with the highest possible concentration (26 pmol/L) was established, so emulsion PCR could take place. Low TE consists of a low volume of EDTA. EDTA chelates, or binds, to metal ions such as Mg^{2+} to inactivate the nucleases from degrading the DNA. However, since PCR typically requires magnesium (Mg^{2+}) in order to proceed, there needs to be a low volume of EDTA present so the Taq polymerase can function.

A naming convention was created to identify the samples (Table 5). An Ion Sphere™ Quality Control Kit (Life Technologies) determined the DNA quality of the unenriched and enriched Ion Sphere Particles™ (ISPs) and the chances for a successful run. The library was analyzed on the Qubit® 2.0 fluorometer (Life Technologies), where every ISP binds to the green-fluorescent dye Alexa Fluor® 488 dye-labeled oligonucleotide (Life Technologies) (50). The beads that are template-positive bind to a complementary red-fluorescent Alexa Fluor® 647 dye-labeled oligonucleotide, as shown in Figure 6. The Qubit® 2.0 fluorometer measures its fluorescent emission and calculates the percentage of template-positive ISPs. The percentage recovered was 47.86% (Table 6) (50). The optimal range for template-positive ISPs is 10-30%. Samples that precede the recommended optimal range are likely to yield a higher amount of polyclonal beads, which will cause an increase in the number of filtered reads (52). Once clonal amplification via emulsion PCR was performed and the enriched template-positive ISPs were recovered, the library was sequenced using the Ion Torrent™ Personal Genome Machine.

Samples	Abbrev.	Samples	Abbrev.	Samples	Abbrev.
t=0 replicate 1	t1	t=0 replicate 2	t2	t=0 replicate 3	t3
No treatment Control, 1 week, Jar 1	NC7j1	No treatment Control, 2 weeks, Jar 1	NC14j1	No treatment Control, 3 weeks, Jar 1	NC21j1
No treatment Control, 1 week, Jar 2	NC7j2	No treatment Control, 2 weeks, Jar 2	NC14j2	No treatment Control, 3 weeks, Jar 2	NC21j2
No treatment Control, 1 week, Jar 3	NC7j3	No treatment Control, 2 weeks, Jar 3	NC14j3	No treatment Control, 3 weeks, Jar 3	NC21j3
Benzyl Chloride, 1 week, Jar 1	BC7j1	Benzyl Chloride, 2 weeks, Jar 1	BC14j1	Benzyl Chloride, 3 weeks, Jar 1*	BC21j1
Benzyl Chloride, 1 week, Jar 2	BC7j2	Benzyl Chloride, 2 weeks, Jar 2	BC14j2	Benzyl Chloride, 3 weeks, Jar 2*	BC21j2
Benzyl Chloride, 1 week, Jar 3	BC7j3	Benzyl Chloride, 2 weeks, Jar 3	BC14j3	Benzyl Chloride, 3 weeks, Jar 3	BC21j3
Fuel, 1 week, Jar 1	F7j1	Fuel, 2 weeks, Jar 1	F14j1	Fuel, 3 weeks, Jar 1	F21j1
Fuel, 1 week, Jar 2	F7j2	Fuel, 2 weeks, Jar 2	F14j2	Fuel, 3 weeks, Jar 2	F21j2
Fuel, 1 week, Jar 3	F7j3	Fuel, 2 weeks, Jar 3	F14j3	Fuel, 3 weeks, Jar 3	F21j3

Table 5: Sample naming abbreviations. The samples were named based on the chemical used, the time period, and the contents of the jar. Asterisks denote samples that were removed. NC- No Treatment Control; BC- Benzyl Chloride; Fuel- Coleman® Camp Fuel.

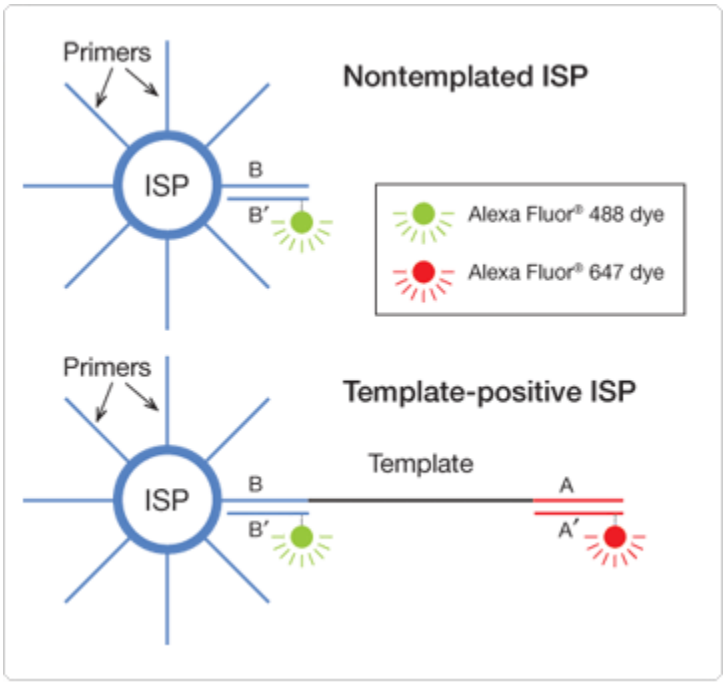


Figure 6: Graphical representation of the ISP assay showing mechanism of determination of template-positive ISPs using the Qubit® fluorometer. "A"- Nucleic acid sequence present only in template-positive ISPs. "B"- Nucleic acid sequence found in both the ISP primer and the template. The "B" nucleic acid sequence is used to initiate amplification onto the bead. A prime (A')-oligonucleotide probe, containing the fluorophore Alexa Fluor® 647, anneals to the primer "A" sites (ISPs with extended templates). B prime (B')-oligonucleotide probe, containing the Alexa Fluor® 488 anneals to primer "B" sites (all ISPs) (51, 52).

Percent Templated ISPs						
	Raw RFU Value		Background RFU (Negative Control Tube)			
Sample ID	AF 488	AF 647	AF 488	AF 647	Conversion Factor	Percent Templated ISPs
400-onetouch2	3005.8	3866.1	64.6	31.5	2.3	47.86%

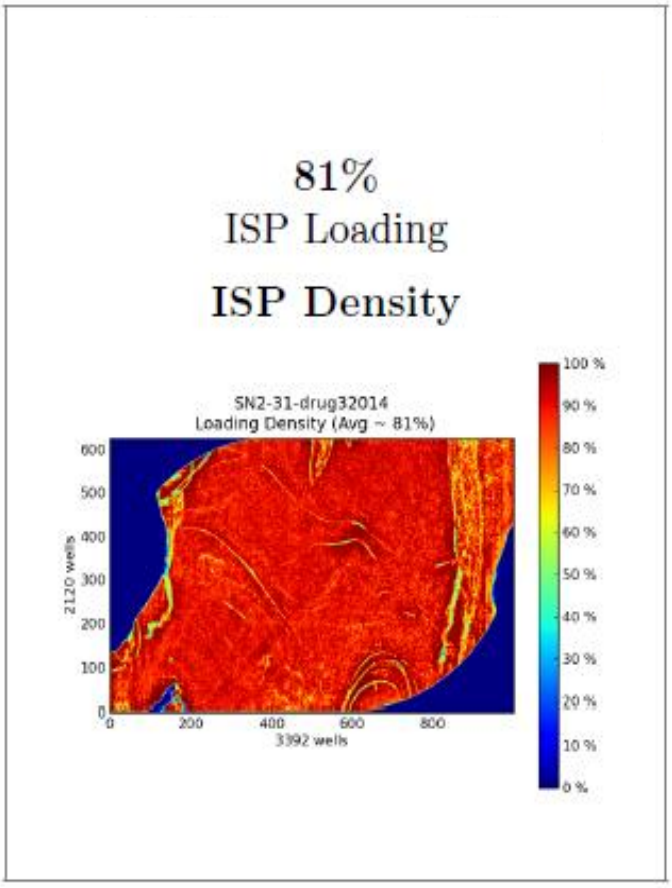
Table 6: Qubit® results. The percentage of template positive ISPs detected after its run on the Qubit®. This value indicates that almost half of the ISPs present have a template strand attached to its particle. The optimal range for template-positive ISPs is 10-30%. ISP-Ion Sphere Particle.

Data Analysis

Loss of information from the chip can be due to three occurrences. The first involves the proper loading of the ISPs into the wells of the chip (53). The area inside the chip is shaped like a football. Therefore, for complete loading, the ISPs must be inserted into all the wells with in the football area. According to the results displayed in Figure 7, 81% of the wells were successfully loaded, visible as a reddish orange color in the heat map. Based on the shape of the inner chip, the left side of the chip, (19% of the wells) did not receive amplicon-positive ISPs and wasn't sequenced. Another occurrence for potential loss of information is from the presence of polyclonal reads (53). Polyclonal reads tend to increase in number when there is too much library present during clonal amplification. This results in the attachment of two or more different DNA strands onto a single ISP (53). Upon amplification, should more than one DNA

strand be present on ISP, then there was the possibility that the additional DNA strands would not be amplified effectively or at all. Alternatively, simultaneous sequencing of both results in numerous ambiguous calls. In our results, of the total 4,471,180 reads available, 3% of the reads were determined to be polyclonal in the sequencing run (Figure 8a). The final occurrence for potential loss of information involves filtering low quality sequences (53). Once a sequence has been given a low-quality score, these sequences are filtered out in the final file. Therefore, the data may include representative amplicons of bacterial community members that were lost due to poor quality and are no longer present in the final data. These wells contain template-positive ISPs (Figure 8b).

An important indicator for the quality of the clonal amplification and sequencing run involves the distribution of the read lengths, which are displayed in a histogram shown in Figure 9 (53). The read length distribution histogram was generated after Ion Torrent™ sequencing run. The number of reads was depicted on the y-axis (count) and read length is plotted on the x-axis. The average read length was at 241 bp, while the median was 302 bp and mode was 303 bp. These data indicated that the majority of data resulted from near full length amplicons. Taxonomic classification was performed on 28 of the 30 samples tested in this study, using the Ribosomal Database Project (RDP) classifier to display its classification from domain to genera (Figures 10-19) (18, 54). The two samples removed, due to a lack of DNA, were the third week benzyl chloride-treated samples from Jars 1 and 2.



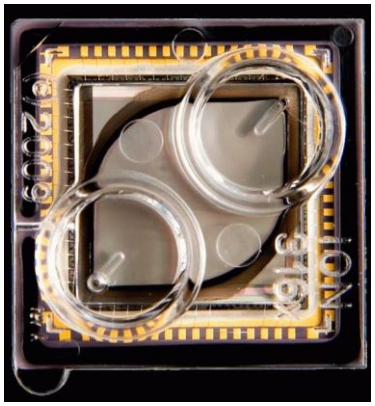
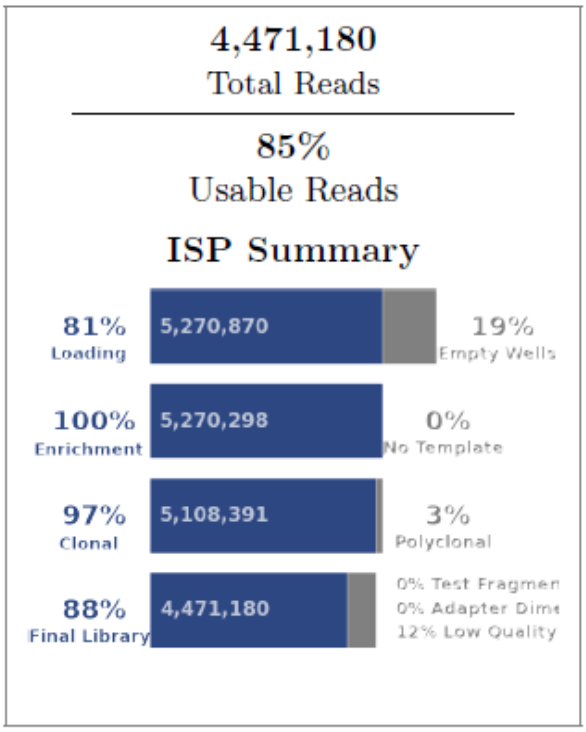


Figure 7: Heat map indicating ISP loading density on the sequencing chip. Red coloring denotes higher ISP loading rate in the wells of the chip. Wells are distributed in a football-shaped array. A full sequencing chip is shown at right for clarity.

a)



b)

Addressable Wells	6,482,371	
With ISPs	5,270,870	81.3%
Live	5,270,298	100.0%
Test Fragment	7,714	00.1%
Library	5,262,584	99.9%

Library ISPs	5,262,584	
Filtered: Polyclonal	161,907	03.1%
Filtered: Low Quality	629,466	12.0%
Filtered: Primer Dimer	31	00.0%
Final Library ISPs	**4,471,180**	**85.0%**

Figure 8: Summary of sequencing run performance. (a) The ISP summary for the chip. The data show that 3% of the reads were polyclonal in the sequencing run. The presence of the polyclonal reads were anticipated when the Qubit® data were higher than the optimal range, indicating the possibility of multi-template, or polyclonal beads. (b) The statistics show the filtering of the data to recover the high quality sequences for analysis. The following were filtered and removed from the remaining data: polyclonal ISPs, low quality ISPs and ISPs with primer dimers. The remaining 85% after filtering was analyzed.

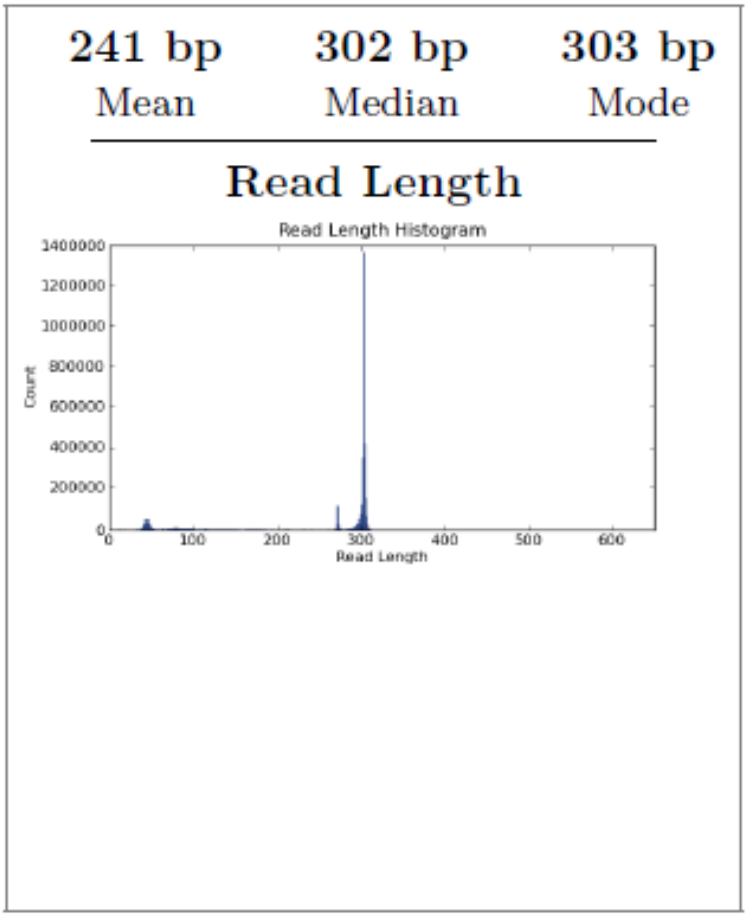


Figure 9: Sample read length histogram. The histogram shows the range of the sequence lengths for the samples in the chip. The majority of the read lengths obtained are 302 bp in length (approximately the full amplicon length).

The complete data set obtained from the chip consisted of 24 phyla, 64 classes, 123 orders, 276 families and 911 genera. Relative abundances were shown for the groups of bacteria consisting of more than 2%. Five phyla of bacteria were discovered at this level for the samples. For the immediate samples (t=0), the dominant bacteria included the phyla Firmicutes (29.4%), Proteobacteria (37.0%) and Actinobacteria (15.7%), which are consistent with previous studies reported for indoor samples (Figure 10) (55). The bar graph shows the number of sequence counts per treatment normalized to 100%. The community detected in the immediate samples

served as a positive control to see if the microbial community had altered in the chemical-treated samples. At the phylum level for the no treatment control samples, the microbial community was dominated by the phylum Firmicutes, with an average of 93.1%, 95.5% and 88.5% for the first, second and third week samples, respectively (Figure 10). The presence of this bacterium was shown to have increased in abundance in relation to the immediate samples. The values presented are the averages of the three replicates for each time point. The benzyl chloride-treated samples were shown to be dominated by the same phyla as the immediate samples: Firmicutes, Proteobacteria and Actinobacteria (Figure 11). The abundance of Firmicutes increased for the benzyl chloride samples to an average of 33.8% for the first week and 38.2% for the second week. However, the abundance of Proteobacteria decreased to an average of 28.2% for the first week and 23.0% for the second week. The third week benzyl chloride sample (BC21) consisted of only one jar sample and therefore an average was not calculated nor compared to the other week samples. The camp fuel-treated samples were shown, in Figure 12, to be dominated by the phylum Firmicutes in the full three week period. Its abundance increased to 60.4% in the first week, 96.3% in the second week and 95.9% in the third week, resulting in the decrease of the phyla Proteobacteria and Actinobacteria.

Since the no treatment control samples and the camp fuel-treated samples were both dominated by the phylum Firmicutes, taxonomic classification was analyzed at the genus level to determine any variation in the microbial community. At the genus level, the immediate samples appeared evenly distributed for most of the present taxa. However, upon analysis of the no treatment control samples, the genus *Staphylococcus* was dominate in the first, second and third week samples with an average of 85.8%, 81.4% and 74.6%, respectively (Figure 13). Since *Staphylococcus* was highly abundant in the no treatment control samples, its presence was

removed from the data set and the remaining genera were normalized to 100% to analyze the communities in the absence of *Staphylococcus* (Figure 14). The results showed that the communities appeared to be the same, though in varying volumes. The genera *Oceanobacillus* and *Salinicoccus* dominated the first, second and third week no treatment control samples. *Oceanobacillus* began to decrease after the second week, while *Salinicoccus* continued to increase in abundance. An alternative representation for Figure 15 was shown, highlighting the increase of these genera (Figure 15). The benzyl chloride-treated samples showed increased levels of *Staphylococcus* throughout, although at much lower levels than the no treatment controls (Figure 16). In addition, there was increased abundance in *Streptococcus* for the first and second weeks. The third week contained data from a single jar and was not compared. *Staphylococcus* was then removed from the data set and the remaining taxa were normalized to 100%, which are presented in Figure 17. The results showed increased levels of *Streptococcus* for the first and second week. However, the remaining bacterial community for the first and second week samples appeared highly similar to the immediate samples. The fuel treated samples were similar to the no treatment control samples, in which S*taphylococcus* was dominant in the first, second and third week samples (Figure 18). However, in the absence of S*taphylococcus*, shown in Figure 19, the remaining taxa suggest increase abundance for *Salinicoccus* and *Lactobacillus*, with concomitant decreases in *Lactococcus* and unclassified *Lachnospiraceae*, and *Enterobacteriaceae*.

The distance between the communities within the samples was then calculated using the UniFrac distance to determine its similarities and/or differences. The unweighted UniFrac distance, which looks at the presence and absence of taxa was determined and represented using Principal Coordinate Analysis, or PCoA (Figure 20), and in a clustering tree (Figure 21). In PCoA, if

there is a difference in communities between the tested samples and the starting samples, in this case the immediate samples (t1,t2, and t3) , then a shift will occur, which is displayed in the PCoA figures (Figures 20 and 22). The PCoA Analysis of microbial communities using unweighted UniFrac revealed a difference of 20.6% between the communities, enabling clear differentiation between treatments. Clustering of the samples revealed that by looking solely at the microorganisms present, there were differences in the community between chemical treatments and similarities of the community within the same chemical treated samples. Alternatively, the weighted UniFrac distance, which includes the presence and absence of taxa in relation to its relative abundance, was represented in a weighted PCoA (Figure 22) and displayed in a weighted UniFrac clustering tree (Figure 23). An increase to 32.5% of the differences between communities was shown using weighted UniFrac. When the samples were clustered, the results showed that the majority of the no treatment control and the fuel-treated samples show similarities in the microbial community, which supports the reason for the inability to differentiate between the two treatments at the phylum and genus levels (Figure 23). However, using these calculations, the unweighted and weighted UniFrac similarly show clear differentiation between treatments. Based on the information provided, it would seem that the microbial community was indeed altered due to the presence of the particular chemicals and that the majority of the community increases are Gram-positive bacteria, which studies have already shown as being dominant in house dust (56). At this moment, for understanding the reason for which these organisms grew as opposed to the others, the only explanation at this time is that the organisms degraded the test chemicals either out of necessity or preference.

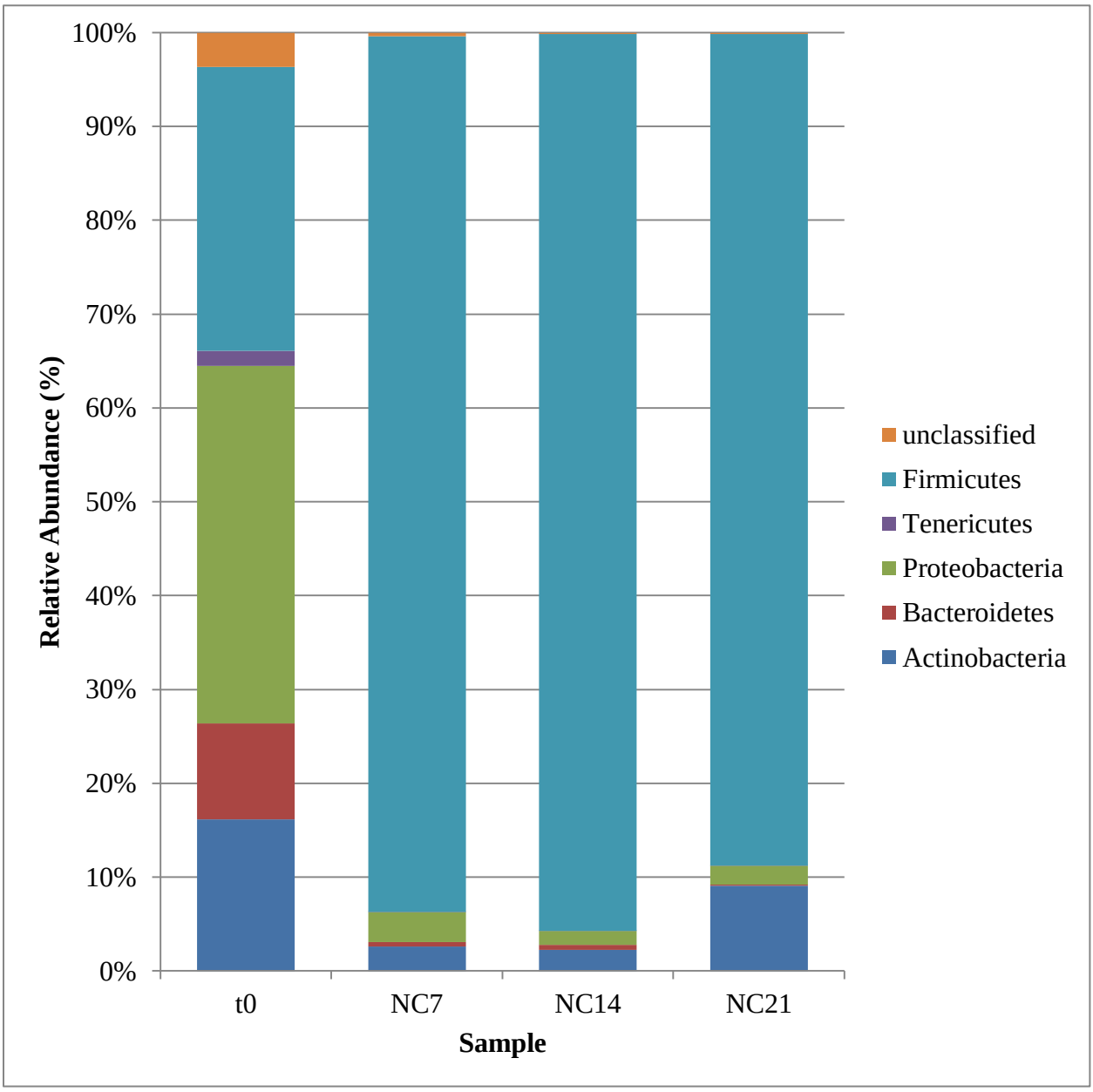


Figure 10: Relative abundance for the no treatment samples at the phylum level. Taxa with a presence greater than 2% are shown. The values presented averages of three independent replicates. NC7- average of no treatment control samples for the first week; NC14-average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples.

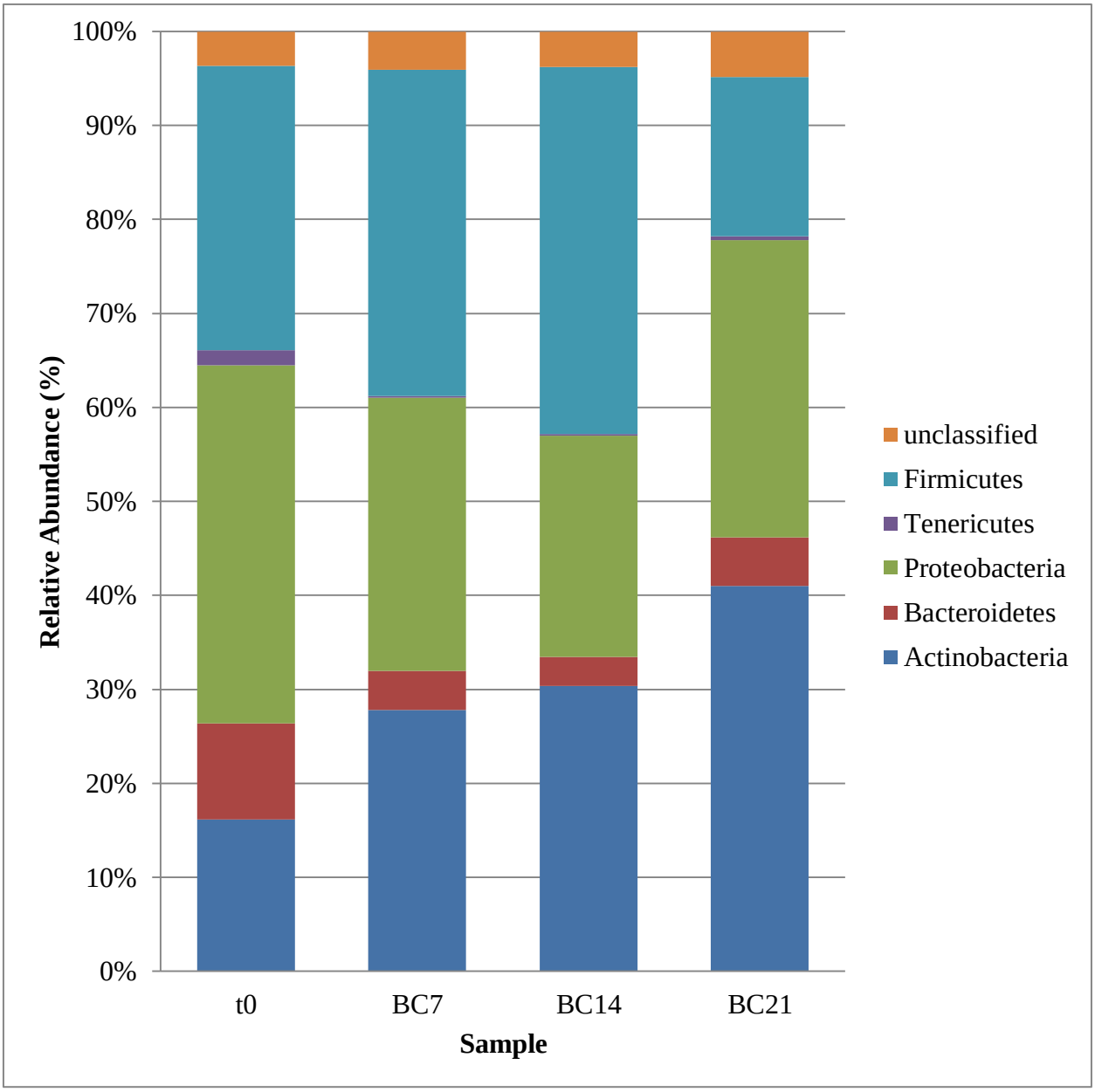


Figure 11: Relative abundance for benzyl chloride samples at the phylum level. Taxa with a presence greater than 2% are shown. The values presented averages of three independent replicates.BC7- average of no treatment control samples for the first week; BC14-average of no treatment control samples for the second week; BC21-benzyl chloride-treated sample for the third week (Note: BC21 represents a single sample); t0-average of immediate (t=0) samples.

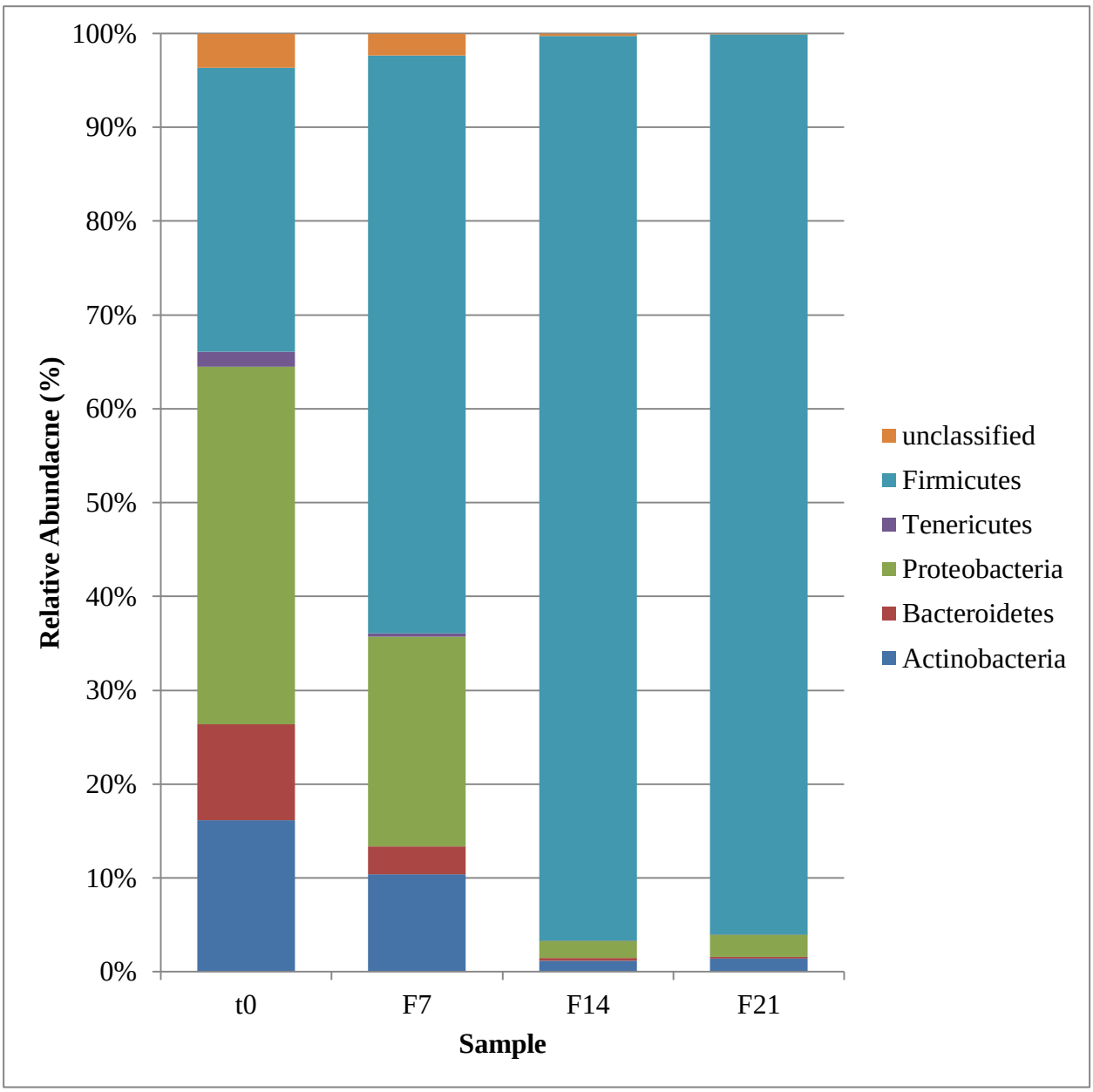


Figure 12: Relative abundance for camp fuel samples at the phylum level. Taxa with a presence greater than 2% are shown. The values presented averages of three independent replicates. The values presented are averages of the three independent replicates. F7- average of fuel-treated samples for the first week; F14-average of fuel-treated samples for the second week; F21-average of fuel-treated samples for third week; t0-average of immediate (t=0) samples.

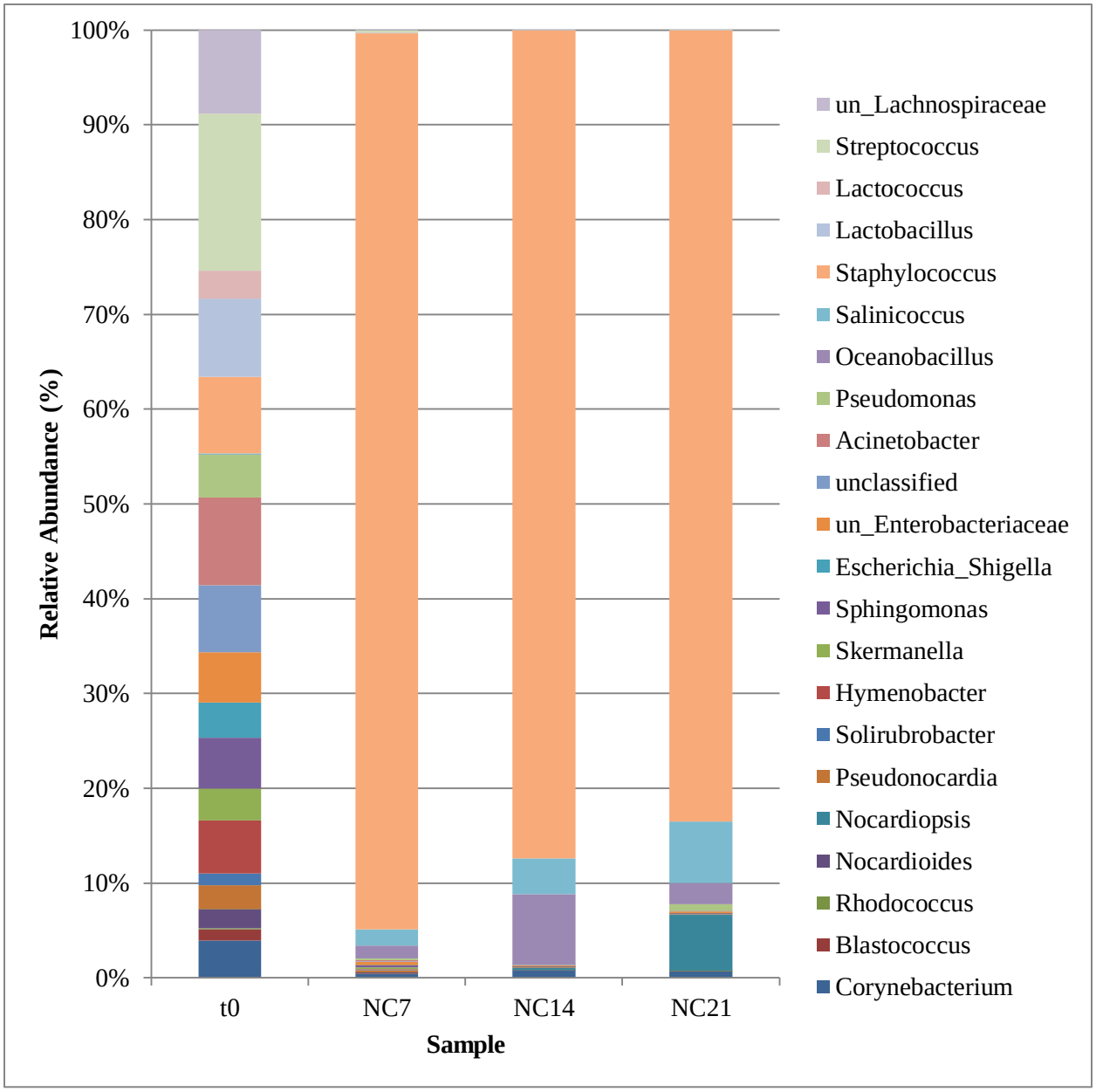


Figure 13: Relative abundance for no treatment control samples at the genus level. The bar graph shows the number of sequence counts per treatment normalized to 100%. Taxa with a presence greater than 2% are shown. The values presented averages of three independent replicates. NC7- average of no treatment control samples for the first week; NC14-average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples.

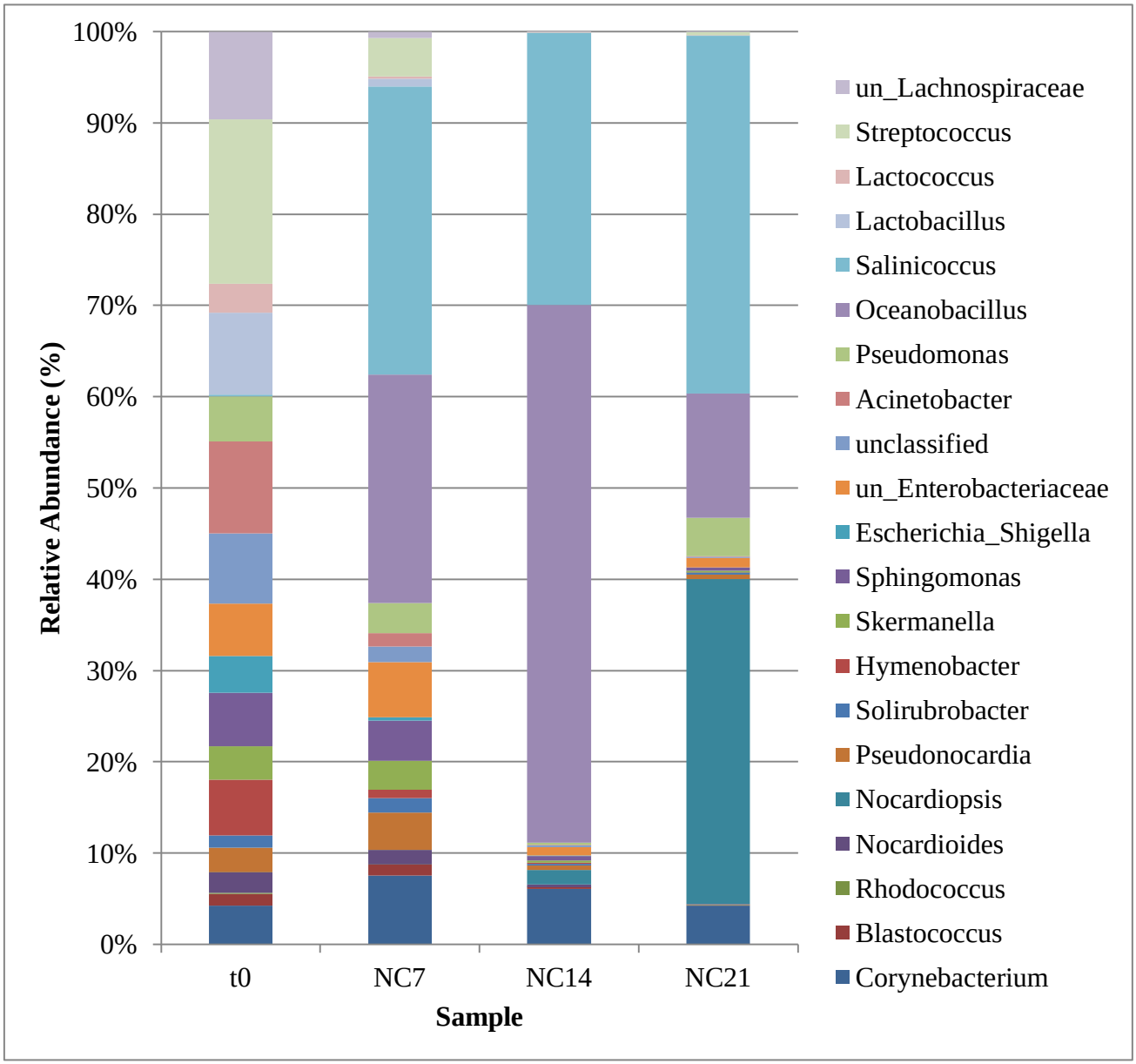


Figure 14: Relative abundance for no treatment control samples at the genus level without *Staphylococcus*. *Staphylococcus* was deleted from the dataset and the remaining genera normalized to 100%. Remaining taxa with a presence greater than 2% in abundance are shown. The values presented averages of the three independent replicates. NC7- average of no treatment control samples for the first week; NC14-average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples.

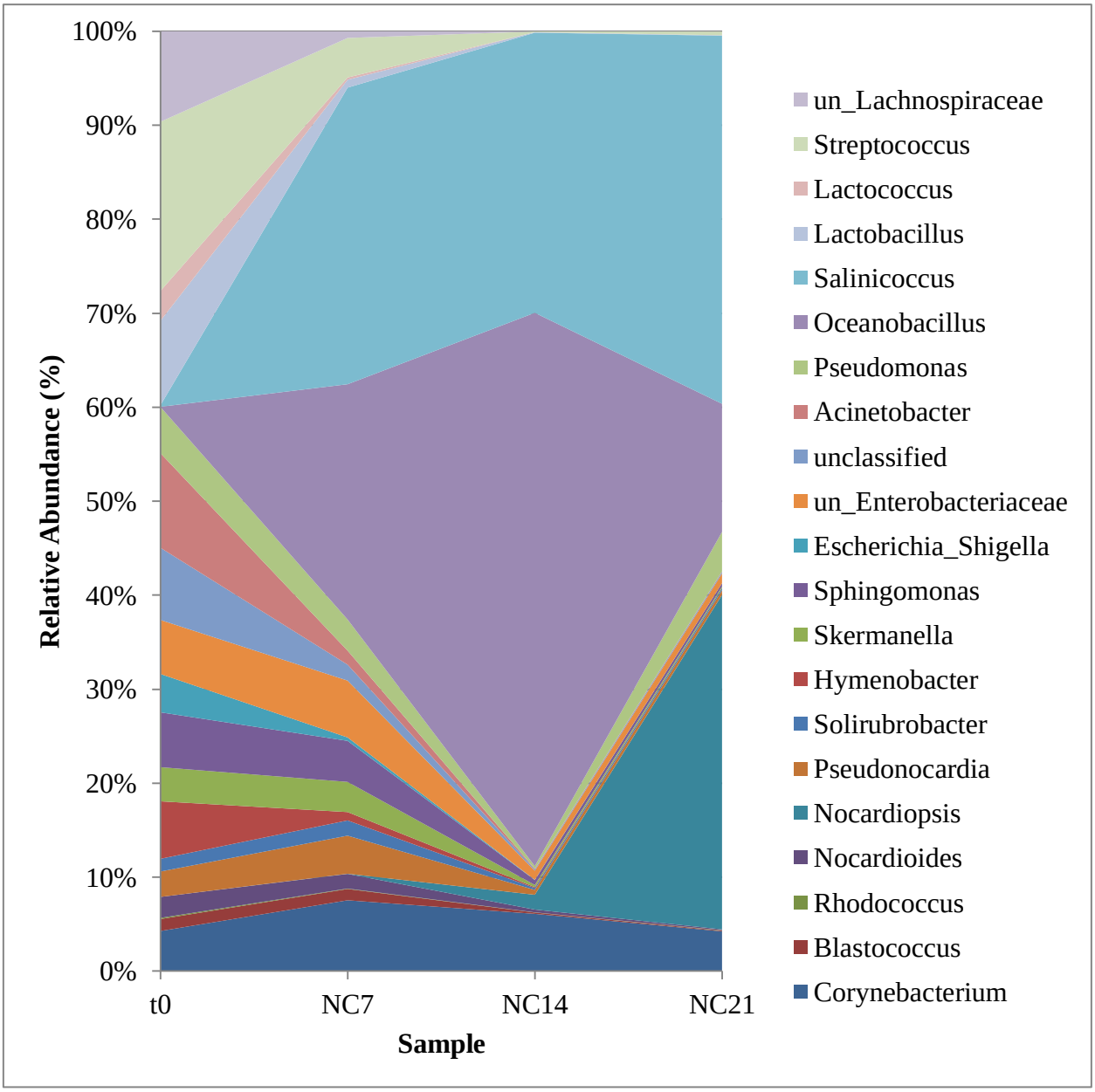


Figure 15: Alternative representation of immediate (t=0) and no treatment control samples. Taxa with a presence greater than 2% are shown, with *Staphylococcus* deleted from the dataset and the remaining genera normalized to 100%. The values presented averages of three independent replicates. NC7- average of no treatment control samples for the first week; NC14- average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples.

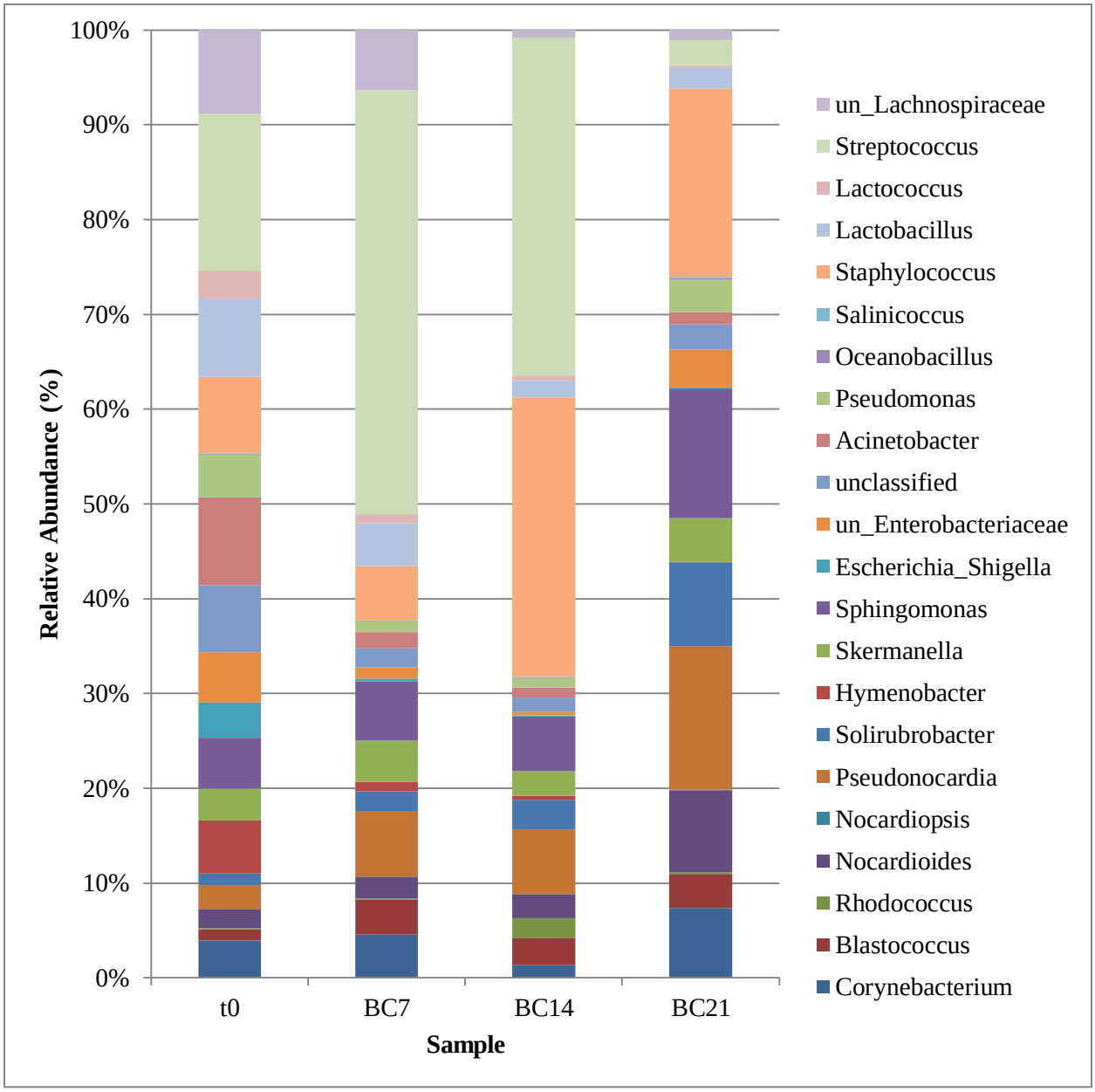


Figure 16: Relative abundance for benzyl chloride samples at the genus level. Taxa with a presence greater than 2% are shown. The values presented averages of the three replicates. BC7-average of no treatment control samples for the first week; BC14-average of no treatment control samples for the second week; BC21-benzyl chloride-treated sample for the third week (Note: BC21 has no average as only one sample was analyzed); t0-average of immediate (t=0) samples.

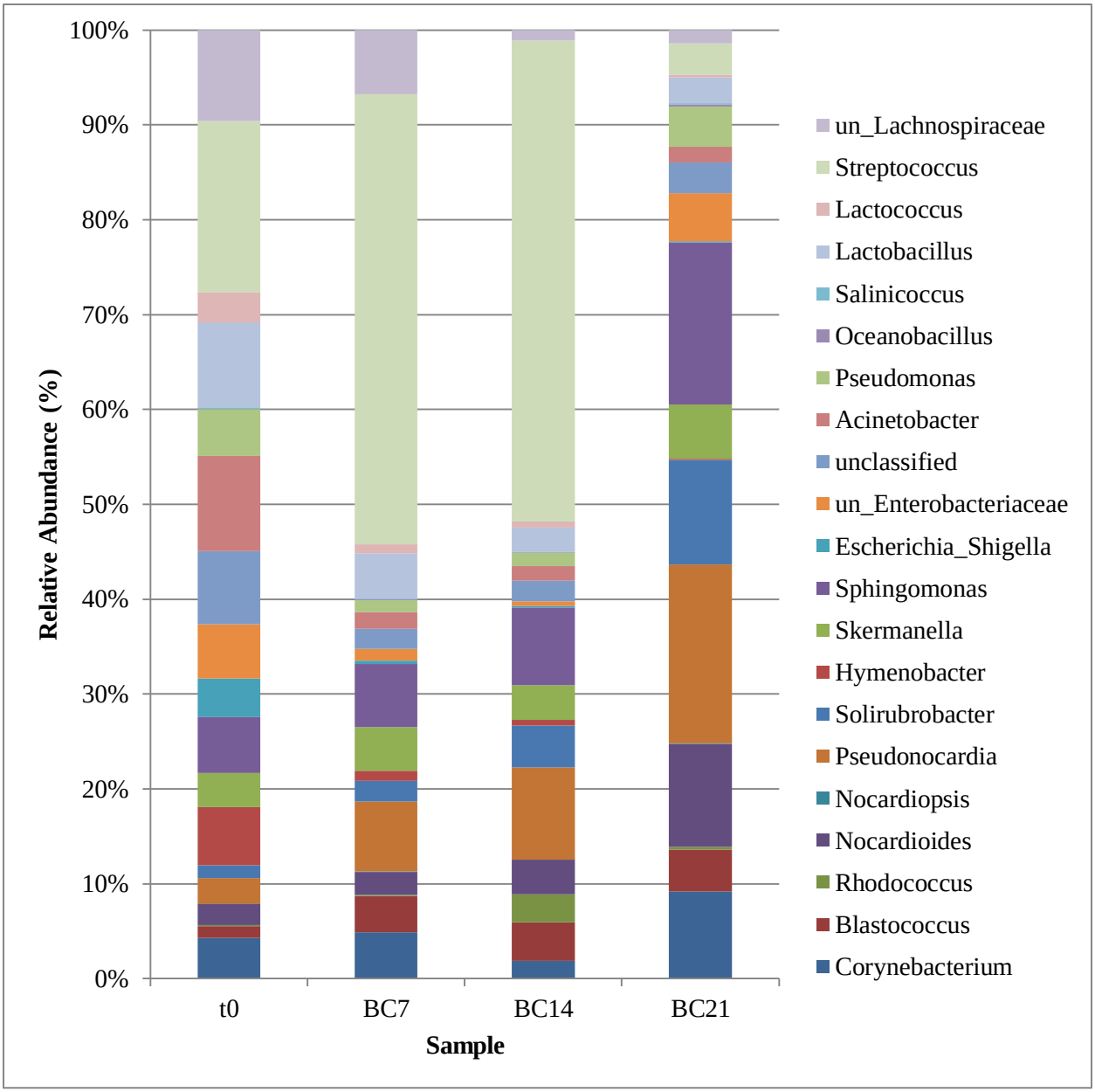


Figure 17: Relative abundance for benzyl chloride samples at the genus level, without *Staphylococcus*. Taxa with a presence greater than 2% are shown, with *Staphylococcus* deleted from the dataset and the remaining genera normalized to 100%. The values presented averages of three independent replicates. BC7- average of no treatment control samples for the first week; BC14-average of no treatment control samples for the second week; BC21-benzyl chloride-treated sample for the third week (Note: BC21 has no average as there was only one sample); t0-average of immediate (t=0) samples.

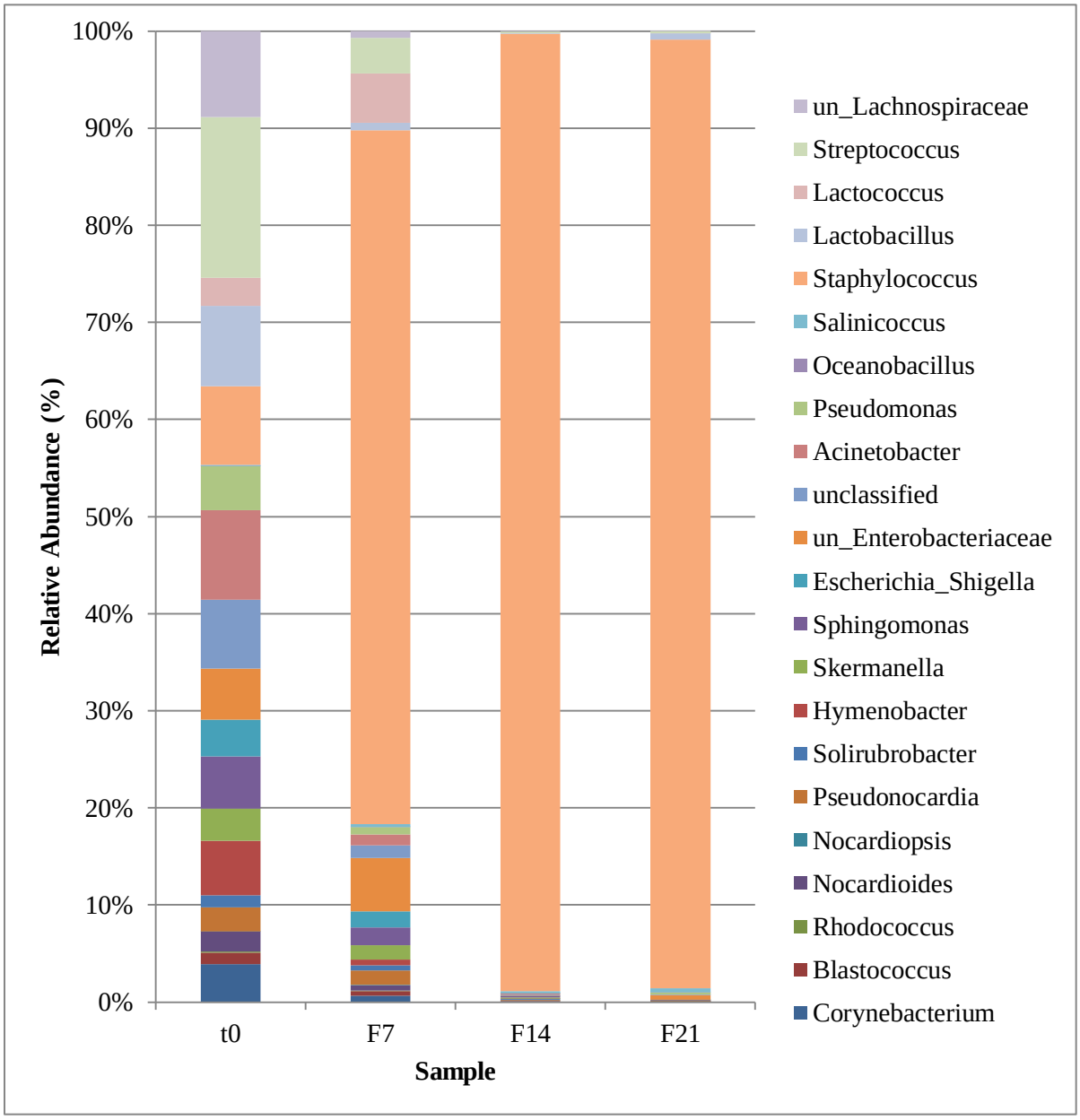


Figure 18: Relative abundance for bacteria classified at the genus level for the camp fuel-treated samples. Taxa with a presence greater than 2% are shown. The values presented averages of three independent replicates. F7- average of fuel-treated samples for the first week; F14-average of fuel-treated samples for the second week; F21-average of fuel-treated samples for third week; t0-average of immediate (t=0) samples.

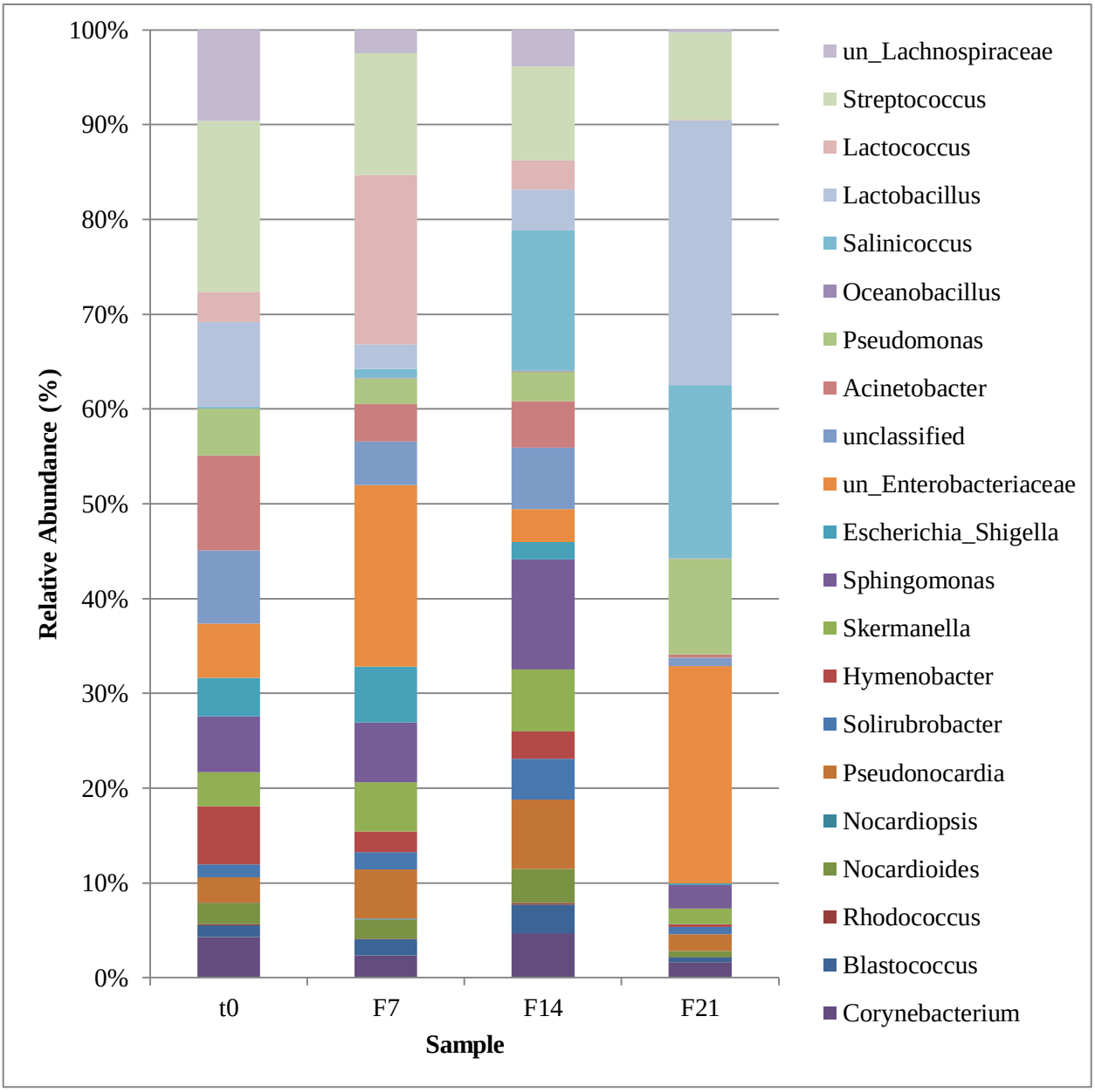


Figure 19: Relative abundance for bacteria classified at the genus level for the camp fuel-treated samples, without *Staphylococcus*. Taxa with a presence greater than 2% are shown, with *Staphylococcus* deleted from the dataset and the remaining genera normalized to 100%. The values presented averages of three independent replicates. F7- average of fuel-treated samples for the first week; F14-average of fuel-treated samples for the second week; F21-average of fuel-treated samples for third week; t0-average of immediate (t=0) samples.

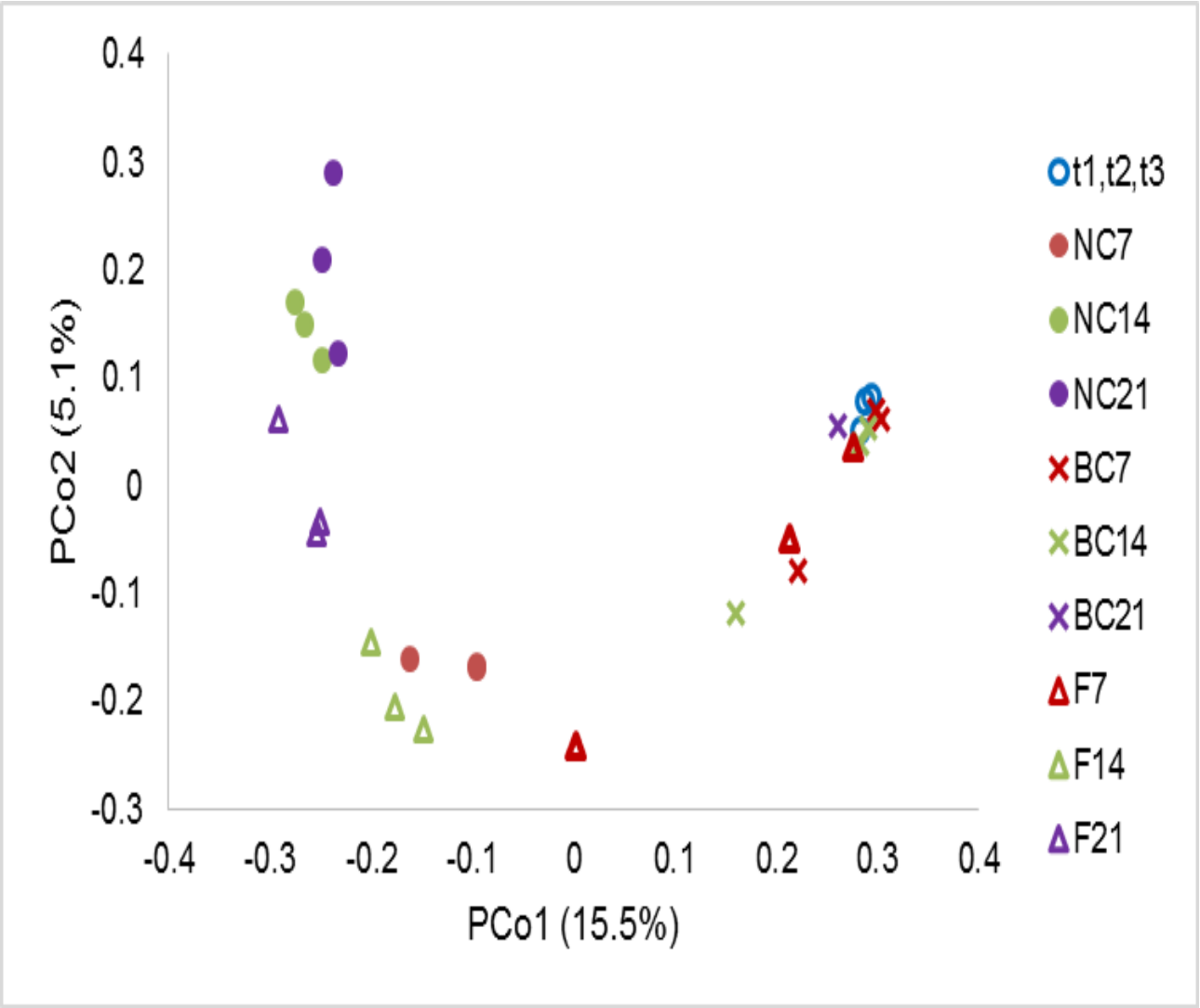


Figure 20: PCoA analysis of microbial communities using unweighted UniFrac, incorporating presence/absence of taxa only. Over time, the samples shift from the immediate samples (t1, t2, and t3) if the communities are different from the starting samples. 20.6% of the difference between treatments is shown in the two components. NC7- average of no treatment control samples for the first week; NC14-average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples; BC7- average of no treatment control samples for the first week; BC14-average of no treatment control samples for the second week; BC21-benzyl chloride-treated sample for the third week (Note: BC21 has no average as there's only one sample that was analyzed); t0-average of immediate (t=0) samples; F7- average of fuel-treated samples for the first week; F14-average of fuel-treated samples for the second week; F21-average of fuel-treated samples for third week; t0-average of immediate (t=0) samples.

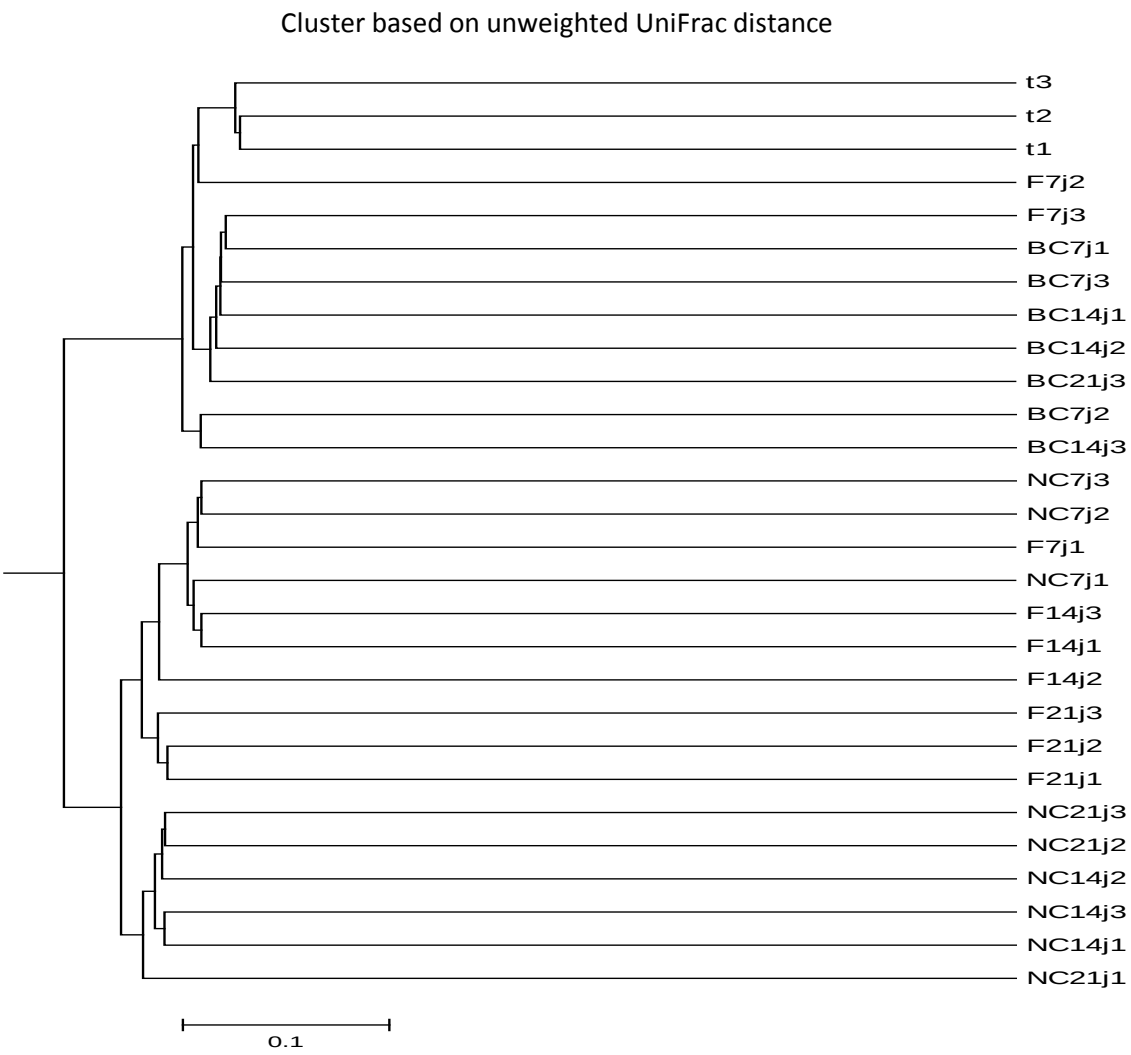


Figure 21: Cluster analysis based on unweighted UniFrac distances. Based solely on the presence and absence of taxa, the samples clustered by similarities in the treatment. The closer the branches connect between samples, the more closely related the microbial communities. The figure shows that the second and third week samples are clustered into groups based on the chemical treatment exposure. However, the first week samples for the no treatment control samples and fuel-treated samples show a variation at that time period. For example, the F7j2 sample is the closest in similarity to the immediate samples (t1, t2, and t3). However, the F7j1 sample and F7j3 sample are better related to other samples instead of F7j2. This differed from the trend the other samples displayed. NC-No treatment control; BC-benzyl chloride; F-camp fuel; j represents the jar from which the sample originated; 7, 14, and 21- represents the time period of 1 week, 2 weeks and 3 weeks, respectively. An example is that F7j2 depicts the fuel sample from Jar 2 of the first week.

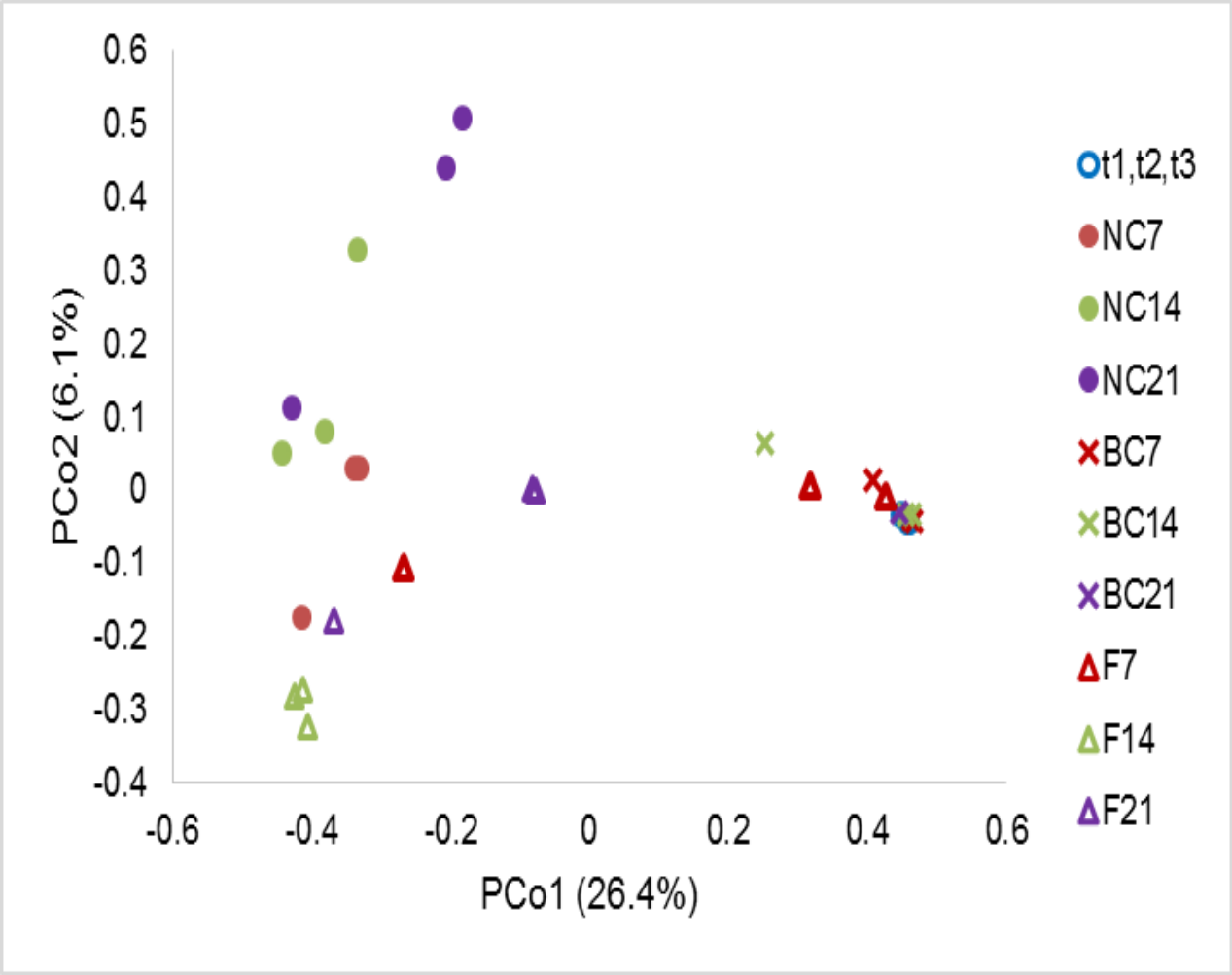


Figure 22: PCoA analysis of microbial communities using weighted UniFrac. Over time, the samples shift from the immediate samples (t1, t2, and t3) if the communities are different from the starting samples. An increase to 32.5% of the differences between communities is explained using weighted UniFrac, which incorporates relative abundance of community members into the analysis. The results similarly show clear differentiation between treatments. NC7- average of no treatment control samples for the first week; NC14-average of no treatment control samples for the second week; NC21-average of no treatment control samples for the third week; t0-average of immediate (t=0) samples; BC7- average of no treatment control samples for the first week; BC14-average of no treatment control samples for the second week; BC21-benzyl chloride-treated sample for the third week (Note: BC21 has no average as there's only one sample that was analyzed); t0-average of immediate (t=0) samples; F7- average of fuel-treated samples for the first week; F14-average of fuel-treated samples for the second week; F21-average of fuel-treated samples for third week; t0-average of immediate (t=0) samples.

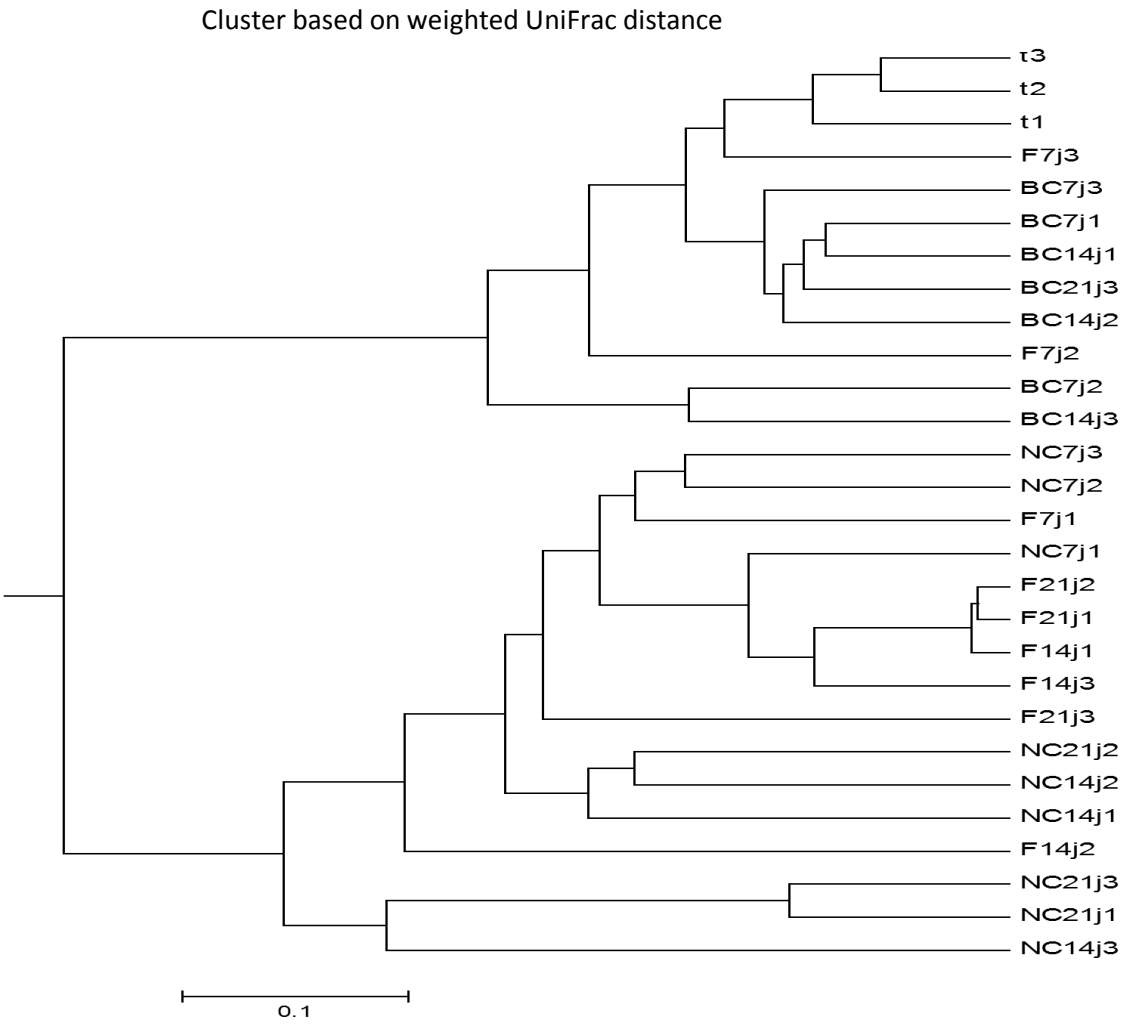


Figure 23: Cluster analysis based on weighted UniFrac distances. The samples are clustered by presence and absence of taxa by their relative abundances. Similar abundance further strengthens the grouping of samples with similar community composition. The community between the no treatment control samples and fuel samples are similar, while the benzyl chloride samples cluster together. For example, although NC21j1 has a similar community to F14j2, it is more closely related to NC21j3. NC-No treatment control; BC-benzyl chloride; F-camp fuel; j represents the jar from which the sample originated; 7, 14, and 21- represents the time period of 1 week, 2 weeks and 3 weeks, respectively. An example is that NC21j1depicts the no treatment control sample from Jar 1of the third week.

CHAPTER V

CONCLUSION

House dust has been known to be a good source for microbial growth due to its composition of organic and inorganic materials (3). These substances bring a carbon source to the microorganisms and with its absence, the community depletes. Therefore, in this study, a similar concept was tested, involving three different volatile liquids to determine if microorganisms had a preference to the carbon presented to it. It was hypothesized that the vapor phase exposure of these chemicals would result in changes in the microbial communities, where the microorganisms that prefer or tolerate the chemical would increase in abundance, displaying a reproducible signature. The results revealed that the microbial communities reproduced the signatures over the determined time periods. However, upon analysis of the samples at a lower level, there was increased abundance of the genus *Staphylococcus* for all three treatments over the one-week, two-week, and three-week time periods. Published articles were researched in order to understand the reason the increased abundance of *Staphylococcus*. Studies have shown that certain *Staphylococcus* strains are separately involved in the degradation of naphthalene, benzene, toluene, and diesel oil (57). Since nearly all samples possessed an increase in Staphylococcus spp., this is most likely due to the availability of water vapor and endogenous degradation of materials within the dust. Further research will be required to determine their use

of the hydrocarbon compounds used in this study. When *Staphylococcus* was removed and the remaining taxa were examined, it seemed that typically the majority of taxa were similar to the immediate or unexposed samples, though the relative abundances were altered. In addition, with regards to the no treatment control samples, the presence of *Oceanobacillus* and *Salinicoccus* increased upon exposure to the sterile water. Removal of *Staphylococcus* in the samples treated with the benzyl chloride revealed an increase in *Streptococcus* for the samples in the first two weeks. For the samples with the chemical Coleman® Camp Fuel, increased levels of *Salinicoccus* and *Lactobacillus* were present. At this moment, there is no reason to explain the increased abundance for these specific microorganisms, other than the assumption that the organisms preferred the materials that the dust was exposed to prior to collection or that its increase was due to the degradation of the test chemicals out of necessity. Future research is needed for further explanations.

The data provided supports these specific aims that were challenged in the course of this study. It has been shown that the chemicals used in vapor form could influence the microbial community compositions. In addition, there was the discovery of particular microbial communities for each chemical, which support the aims. Some of these communities of bacteria that were detected were even included in the earlier predictions (stated in Background). However, in regards to no treatment control samples and the Coleman® Camp Fuel-treated samples, no single taxon was found sufficient to differentiate them, even when looking down to the genus level. Nevertheless, PCoA analyses did successfully differentiate the total community structures among treatments. Since the populations of microorganisms are changing depending on factors in its environment, it seems likely that this concept can be used in diverse ways. For instance, as this study provided, it may be possible to determine the chemistry of drug synthesis.

In addition, this method can be broadly used as a way to determine environmental conditions, such as the presence of kids, pets, diseases or health problems within the environmental setting. If applied to a criminal justice aspect, this could be useful in determining the perpetrator's condition, providing aid to track the perpetrator. Future research will be needed to capitalize on these findings for microbial forensic applications.

Limitations

A limitation to this method used involves determining the microbial diversity based on sampling. As previously stated, when microorganisms, in house dust come into contact with vapor-phase chemicals, if they prefer or tolerate the chemical, then there will be an increase in its relative abundance. However, the microbial community for one sample may vary from another if the locations of the sampling were different. Thus, microorganisms detected from one sample may vary compared to another. This correlates to how microorganisms may be specific for certain environments depending on the conditions. Therefore, the microbial community present may vary depending on the environment involved. A second limitation is that all treated samples exhibited an increase in growth for the genus *Staphylococcus*. Inclusion of water for humidity in the experimental design may have unduly influenced the metabolism and community structures within the experiments, leading to an increase in this group. It may be necessary to look at the species level, or even further down to the strain level, to see if there is a difference in bacteria among the chemicals. However, the data received using RDP Classifier rarely goes down to the species level and is not consistently accurate. By restricting the resolution to the genus, significant information is lost about the microbial community. Further analysis is suggested in order to determine if this method can aid the detection of drug laboratories. In order to determine

changes in the microbial community, a background control is needed to relate our findings back to in order to use this technique effectively. A possible solution to this problem could be a set of interpretation guidelines for use of 16S rRNA sequencing, so that results can be compared to other studies (10). In spite of these difficulties, PCoA analyses did identify changes in community composition for each chemical tested. Further analyses will be required to characterize and develop these changes into a diagnostic tool.

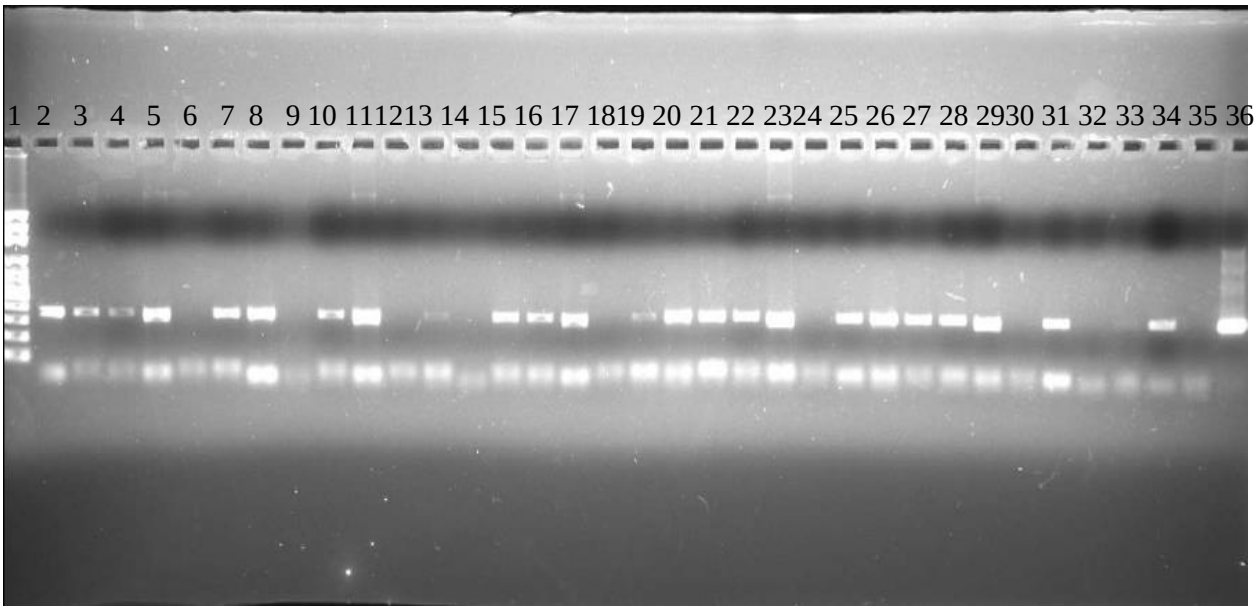
APPENDIX A

Sequence Name		DNA Sequence (5'-3')	Barcode
Forward	Primer A-key	5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG{barcode}{template-specific-primer}-3'	
Reverse	Primer P-key	5'-CCTCTCTATGGGCAGTCGGTGATTCAG{ template-specific-primer}-3'	
Forward Primers			
1ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTAAGGTAACGTGCCAGCMGCCGCGGTAA-3'	CTAAGGTAAC
2ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAAGGAGAACGTGCCAGCMGCCGCGGTAA-3'	TAAGGAGAAC
3ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGAAGAGGATTCGTGCCAGCMGCCGCGGTAA-3'	AAGAGGATTC
4ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTACCAAGATCGTGCCAGCMGCCGCGGTAA-3'	TACCAAGATC
5ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGAAGGAACGTGCCAGCMGCCGCGGTAA-3'	CAGAAGGAAC
6ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGCAAGTTCGTGCCAGCMGCCGCGGTAA-3'	CTGCAAGTTC
7ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGTGATTCGTGCCAGCMGCCGCGGTAA-3'	TTCGTGATTC
8ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCCGATAACGTGCCAGCMGCCGCGGTAA-3'	TTCCGATAAC
9ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTGAGCGGAACGTGCCAGCMGCCGCGGTAA-3'	TGAGCGGAAC
10ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGACCGAACGTGCCAGCMGCCGCGGTAA-3'	CTGACCGAAC
11ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCTCGAATCGTGCCAGCMGCCGCGGTAA-3'	TCCTCGAATC
12ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTAGGTGGTTCGTGCCAGCMGCCGCGGTAA-3'	TAGGTGGTTC
13ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAACGGACGTGCCAGCMGCCGCGGTAA-3'	TCTAACGGAC
14ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTGGAGTGTCGTGCCAGCMGCCGCGGTAA-3'	TTGGAGTGTC
15ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTAGAGGTCGTGCCAGCMGCCGCGGTAA-3'	TCTAGAGGTC
16ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTGGATGACGTGCCAGCMGCCGCGGTAA-3'	TCTGGATGAC
17ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTATTCGTCGTGCCAGCMGCCGCGGTAA-3'	TCTATTCGTC
18ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGAGGCAATTGCGTGCCAGCMGCCGCGGTAA-3'	AGGCAATTGC
19ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTAGTCGGACGTGCCAGCMGCCGCGGTAA-3'	TTAGTCGGAC
20ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGCAGATCCATCGTGCCAGCMGCCGCGGTAA-3'	CAGATCCATC
21ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGCAATTACGTGCCAGCMGCCGCGGTAA-3'	TCGCAATTAC
22ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCGAGACGCGTGCCAGCMGCCGCGGTAA-3'	TTCGAGACGC
23ionA-515F		5'-CCATCTCATCCCTGCGTGTCTCCGACTCAGTGCCACGAACGTGCCAGCMGCCGCGGTAA-3'	TGCCACGAAC

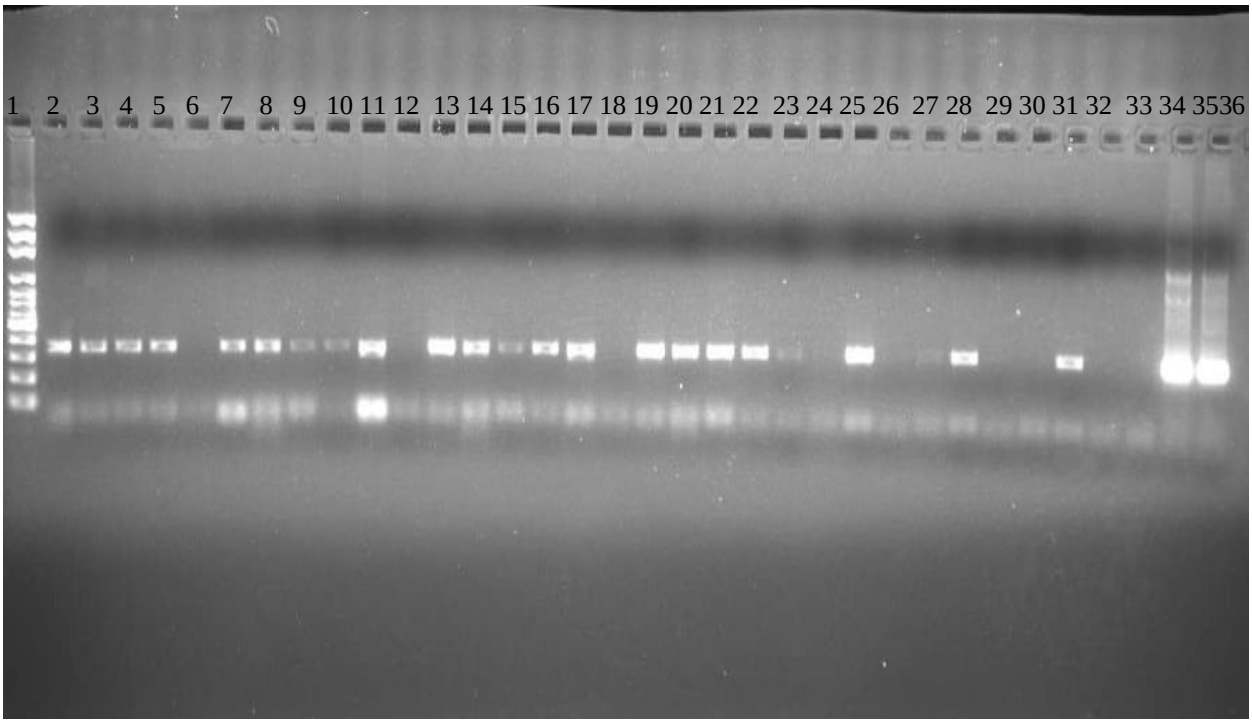
24ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCTCATTCGTGCCAGCMGCCGCGGTAA-3’	AACCTCATTC
25ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCCTGAGATACGTGCCAGCMGCCGCGGTAA-3’	CCTGAGATAC
26ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTTACAACCTCGTGCCAGCMGCCGCGGTAA-3’	TTACAACCTC
27ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGAACCATCCGCGTGCCAGCMGCCGCGGTAA-3’	AACCATCCGC
28ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGATCCGGAATCGTGCCAGCMGCCGCGGTAA-3’	ATCCGGAATC
29ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCGACCACTCGTGCCAGCMGCCGCGGTAA-3’	TCGACCACTC
30ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGCGAGGTTATCGTGCCAGCMGCCGCGGTAA-3’	CGAGGTTATC
31ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCCAAGCTGCGTGCCAGCMGCCGCGGTAA-3’	TCCAAGCTGC
32ionA-515F	5’-CCATCTCATCCCTGCGTGTCTCCGACTCAGTCTTACACACGTGCCAGCMGCCGCGGTAA-3’	TCTTACACAC
Reverse Primer		
IonP1-806R	5’-CCTCTCTATGGGCAGTCGGTGATGGACTACHVGGGTWTCTAAT-3’	No barcode

List of the Ion Torrent™ 515F and 806R barcode sequences used in this study. The primers were assigned to the samples and displayed in Table 2 (see Materials and Methods). Primers 4 and 9 were assigned to the negative and positive control, respectively, used for amplification and gel imaging.

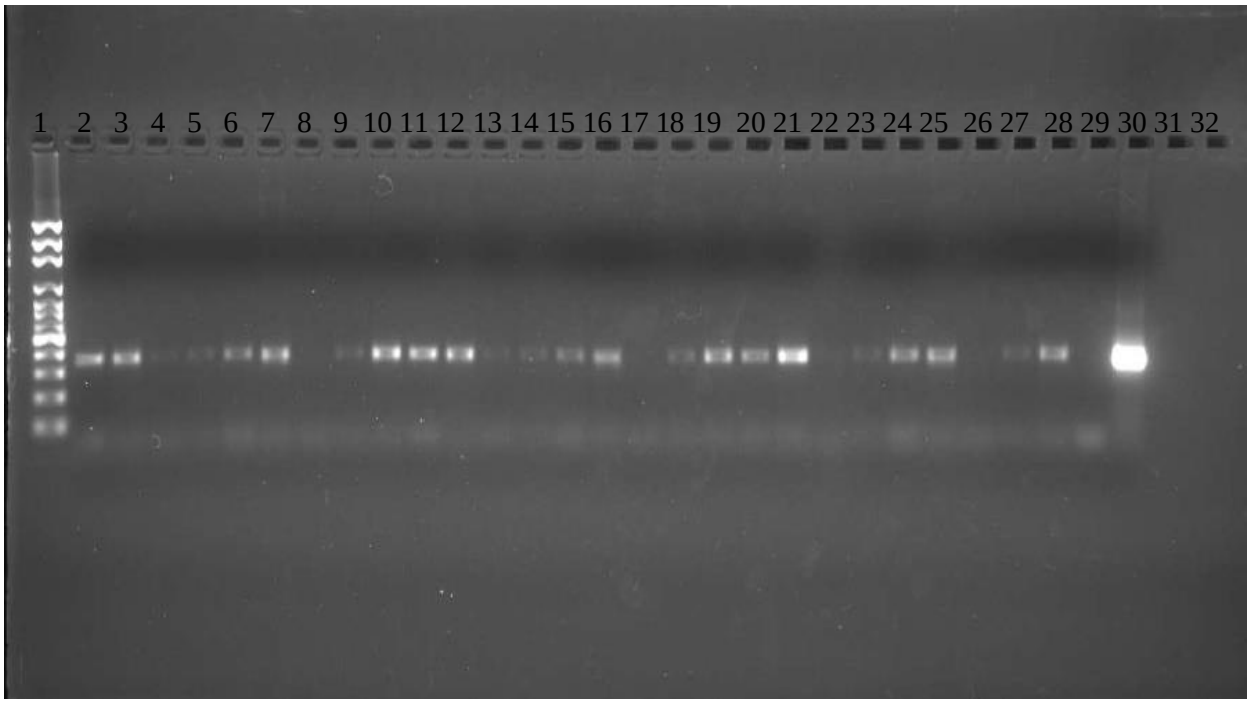
APPENDIX B



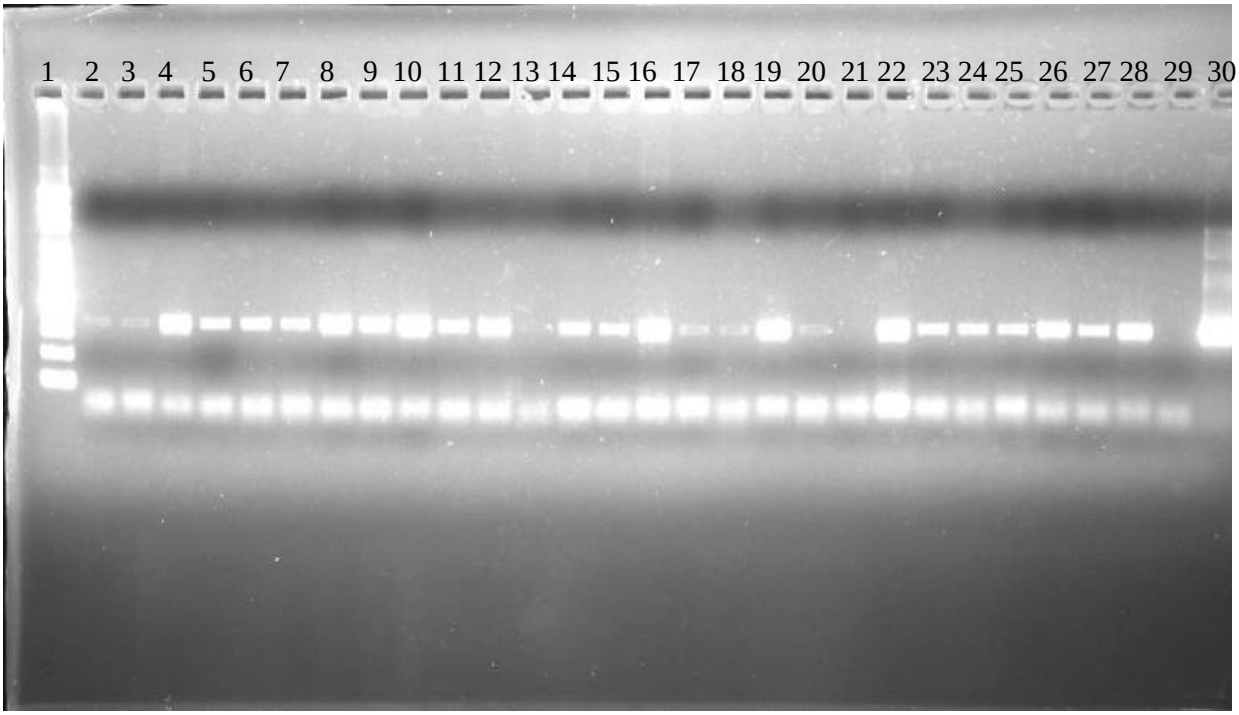
1.5% agarose gel representation for first and third week samples. 100bp ladder- Well 1; NC 1 week Jar 1 replicates- Wells 2,8, and 14; BC 1 week Jar 1 replicates- Wells 3,9, and 15; Fuel 1 week Jar 1 replicates- Wells 4,10, and 16. NC 3 week Jar 1 replicates- Wells 5, 11, and 17; BC 3 week Jar 1 replicates- Wells 6,12, and 18. Fuel 3 week Jar 1 replicates- Wells 7, 13, and 19. NC 1 week Jar 2 replicates- Wells 20 and 26; BC 1 week Jar 2 replicates- Wells 21 and 27; Fuel 1 week Jar 2 replicates- Wells 22 and 28; NC 3 week Jar 2 replicates- Wells 23 and 29; BC 3 week Jar 2 replicates-Wells 24 and 30; Fuel 3 week Jar 2 replicates- Wells 25 and 31 (Note: Only two replicates are shown for the samples in Jar 2 in this gel. The remaining replicates are shown on the following gel); Negative Control (water)- Well 35; Positive Control (*E.coli*)- Well 36. NC- No treatment control; BC-Benzyl chloride; Fuel- Coleman® Camp Fuel. The wells not mentioned were either samples not used in this study or empty wells.



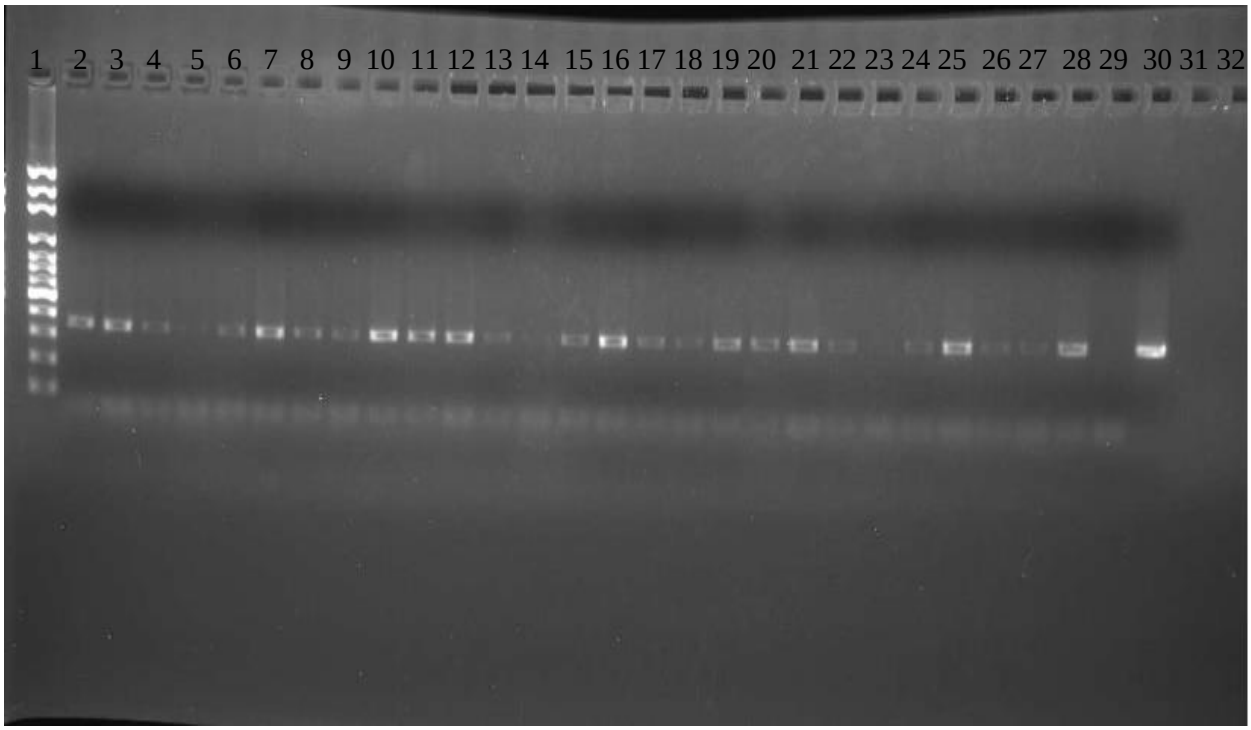
1.5% gel representation for the remaining first and third week Jar 3 samples and remaining replicate for Jar 2. 100bp ladder- Well 1; NC Jar 2 1 week replicate- Well 2; BC Jar 2, 1 week replicate- Well 3; Fuel Jar 2, 1 week replicate- Well 4; NC Jar 2, 3 week replicate- Well 5; BC Jar 2, 3 week replicate- Well 6; Fuel Jar 2, 3 week replicate- Well 7; NC Jar 3, 1 week replicates- Wells 8,14, and 20; BC Jar 3, 1 week replicates- Wells 9, 15, and 20; Fuel Jar 3, 1 week replicates- Wells 10, 16, and 22; NC Jar 3, 3 week replicates- Wells 11, 17, and 23; BC Jar 3, 3 week replicates- Wells 12, 18, and 24; Fuel Jar 3, 3 week replicates- Wells 13, 19, and 25; Negative control (water)- Well 33; Positive control (*E. coli*)- Well 34. The wells not mentioned were either samples not used in this study or empty wells. NC-No treatment control; BC-Benzyl chloride; Fuel- Coleman® Camp Fuel



1.5% agarose gel representation for second week Jar 1 samples and immediate samples. 100bp ladder- Well 1; Immediate samples, or unexposed samples- Well 2, 11, and 20; NC 2 week replicates- Wells 7, 16, and 25; BC 2 week replicates- Wells 8, 17, and 26; Fuel 2 week replicates- Wells 10, 19, and 28. Negative control (water)- Well 29; Positive control (*E.coli*)- Well 30. NC-No treatment control; BC-Benzyl chloride; Fuel- Coleman® Camp Fuel. The wells not mentioned were either samples not used in this study or empty wells.



1.5% agarose gel representation of second week Jar 2 samples. 100bp ladder- Well 1; NC 2 week replicates- Wells 4, 16, and 22; BC 2 week replicates- Wells 3, 17, and 21; Fuel 2 week replicates- Wells 10, 19, and 28. Negative control (water)- Well 29; Positive control (*E.coli*)- Well 30. NC-No treatment control; BC-Benzyl chloride; Fuel- Coleman® Camp Fuel. The wells not mentioned were samples not used in this study.



1.5% agarose gel representation of second week Jar 3 samples. 100bp ladder- Well 1; NC 2 week replicates- Wells 7, 16, and 25; BC 2 week replicates- Wells 8, 17, and 26; Fuel 2 week replicates- Wells 10, 19, and 28. Negative control (water)- Well 29; Positive control (*E.coli*)- Well 30. NC-No treatment control; BC-Benzyl chloride; Fuel- Coleman® Camp Fuel. The wells not mentioned were either samples not used in this study or empty wells.

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