*Pseudomonas* spp., *Bacillus* spp., and *Pantoea* sp., on various plant species located at the Lone Mountain portion of the University of San Francisco’s Hilltop Campus

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# Introduction

Classifying plants as standalone entities is inaccurate because they host a diverse array of microorganisms that are essential to their major functions (1). Although microbes are fundamental to plant maintenance, little is known about the exact microbial communities inhabiting different plants species around the world and their impact on plants’ ecological function. In addition, the leaf microbiome, or phyllosphere microbiome, is influenced by abiotic and biotic factors that make this dynamic relationship harder to understand (2). Leaves are a huge contributor to a plant’s system and it is estimated that about 6.4 × microbes can colonize a leaf’s surface area (3). Culture-based and molecular techniques have helped biologists examine isolated microbes in greater detail. These techniques can be applied to examining the phyllosphere and its vast array of microbial communities. Thus far, many different genera of bacteria, fungi, yeasts, and algae have been identified through research (4). Most of the microorganisms which inhabit the phyllosphere are bacteria (5). Of the bacteria identified in various communities, *Pseudomonas*, *Acinetobacter*, and *Bacillus* were found to be among some of the most abundant genera found across various plant species (6). The reasoning as to why these genera predominant the phyllosphere may be linked to the environments in which many plant species are found. The leaf surface is exposed to a rapidly fluctuating environment due to changing temperatures such as rain and humidity and physiochemical interactions (7). These short-scaled fluctuations may select for bacterial species with versatile traits that allows them to better adapt and colonize plant surfaces over other genera.

The focus on my investigation was to examine differences in the colonies and morphotypes present on two plants growing on the Lone Mountain portion of the University of San Francisco’s Hilltop Campus. The two plants I examined were the ice plant (*Carpobrotus edulis*) and the fern-leaf yarrow (*Achillea filipendulina*). I chose these particular plants because of their differing plant structure and environmental status. Fern-leaf yarrows are bright, yellow flowering plants and ice-plant is a succulent with a very low percentage of flowers present. Plant-pollinator relationships are highly important to plant biodiversity and in particular, about 90% of all flowering plants rely on animals for pollination (8). In Northern California, fern-leaf yarrows are naturalized plants and ice plant is considered an invasive species. Plant invasions are known to pose serious threats to native and naturalized plant communities by robbing natural resources and reducing overall biodiversity (9). Due to the structural diversity and environmental impact between these plant species types, I hypothesized that there would be a greater morphotype diversity and higher colony counts present on the leaves of fern-leaf yarrows because of the higher interactions with pollinating insects and its proximity to an invasive plant species requires more microbes to protect and enhance its fitness against the invasive threat.

To test this plant-microbiome relationship, I conducted culture-based techniques from samples obtained on the leaves of each plant species. I grew cultures on tryptic soy agar (TSA) plates at three different dilutions. I found both colony and morphotype counts to be larger in fern-leaf yarrow leaves but there was only a statistical significance between the morphotype counts of each plant. I obtained pure cultures for 7 different observed morphotypes. I created a crystal violet stain, gram stain, and Maneval’s stain slides for each morphotype under a light microscope. After collecting images for stain, I measured the length, width, and area for each microbe using the ImageJ software and Fiji platform. I also analyzed the mean and standard deviation for the length, width, and area of each microbe. My predictions for each morphotypes identities yielded a high concentration *Bacillus* species and *Pseudomonas* species. I performed PCR amplification and Sanger sequencing to obtain successful BLAST results. The advantages to using the Sanger sequencing method is that it’s been the most widely used sequencing method for the past 40 years, it works well for small-scaled projects, and is the easiest method to resolve uncertainties in data (10). Of the 7 morphotypes I investigated, I received successful matches for 4 of them. The identities of the morphotypes belong to either *Bacillus* or *Pseudomonas* genera. A maximum likelihood phylogeny with bootstrap support further supported their identities and relationships to one another.

The second portion of my investigation focused on examining differences in the number of colonies and morphotypes growing on the phyllospheres of five different plant species. In particular, I examined Mexican marigolds (*Tagetes erecta*), wax begonias (*Begonia x semperflorens-cultorum*), lavender (*Lavendula*), California goldfields (*Lasthenia californica*), and California poppies (*Eschscholzia californica*). Due to the significance I observed in morphotype diversity between ice plants and fern leaf yarrows, I hypothesized that species diversity would be more statistically significant as compared to the number of colonies present per plant species. In addition, I expected to encounter *Bacillus* spp. or *Pseudomonas* spp. as I did in the first portion of my experiment. Each plant species is classified as a native or naturalized flowering plant. Flowering plants, or angiosperms, account for approximately 80% of all living plants (11). Microorganisms are integral to maintaining and protecting the health of their host plant. They have the ability to assist in growth, seed germination, nutrient supply via nitrogen fixation, and resistance against abiotic and biotic stress factors (12). Following similar methods to the first portion of my experiment, I cultured a total of 105 samples from all of the plant species and controls I observed onto tryptic soy agar (TSA) plates. For each plant species, I sampled five individual plants located in the same landscaped plot. For each plant individual, I collected samples on 5 different leaves using sterile cotton swabs. I found that both average colony and morphology counts were quantitatively higher in Mexican marigold and wax begonias. I obtain pure cultures for 6 different observed morphotypes. I performed PCR amplification and gel electrophoresis to obtain successful DNA sequence results. Of the 6 morphotypes I investigated, I received a successful identification for the second morphotype. Morphotype #2 is likely a strain belonging to the genus *Pantoea*.

Overall, the microbiota has a profound positive effect on plant and ecosystem health (13). Assessing the microbial communities of the phyllosphere can be accomplished through descriptive approaches. Culturing methods measure species richness and diversity of each community. Unfortunately, this approach is subject to human error. However, with molecular approaches, we can now rapidly survey and describe microbial community structure and composition at an unprecedented level of detail. Microorganisms therefore provide unique opportunities for studying evolutionary relationships through phylogenetic inferences and sequencing technology. Phylogenetic trees are the most common form of displaying inferred relationships that exist between different species (14). Maximum likelihood phylogenies are one of the most effective methods to examine relatedness, but they can be used on relatively small data sets. They also do not provide full functional information about the microbiota. Further developments into sequencing technology has helped resolve some of those limitations. Sequencing technologies, Thanks to the advancement of various sequencing technologies, the complete genomes for 1,554 bacterial species, 112 archaeal species, and 2,675 virus species had been published by 2012 (15). Therefore, uncovering the microbes that inhabit the phyllosphere requires the conjunction of various robust approaches.

# Methods

## Study design

### Field Collection

On January 31st, 2019, I sampled leaves from a fern-leaf yarrow and an ice plant located at the Lone Mountain campus of the University of San Francisco in San Francisco, California (37.776N, 122.4506W). For each plant, I collected samples from 3 different leaves which were located at 3 different elevations from the soil to the tip of the plant. I used a new, sterile cotton swab on each leaf for approximately 10 seconds and then I proceeded to place each swab into a labeled tube filled with 1mL of PBS solution. To obtain the control for my experiment, I used sterile swabs to collect 3 air samples from the area where the plants were located. I exposed each swab to the air for approximately 10 seconds and placed each swab into a labeled tube filled with 1mL of PBS solution. I kept each sample swab in its respective tube for approximately an hour and a half before removing it for culturing.

### Culturing and Dilution Plating

I used 100mm tryptic soy agar (TSA) plates for plating the three sample treatments I collected. I first vortexed each test tube for approximately 10 seconds in order to release remaining microbes from the cotton swab and into the PBS solution. For each tube, I transferred 50 µL into a new sterile tube and added 450 µL of PBS solution to create a 10x dilution sample. I created a 100x dilution sample by transferring 50 µL from the 10x dilution and adding 450 µL of PBS solution. For each sample collected at each plant type and control, I used 27 TSA plates. Each TSA plate was covered with its respective PBS and sample solution mix at a concentration of either 1, 10, or 100. I spread the bacterial liquid onto each TSA plate by swirling approximately 6-8 glass beads until the plate was completely covered. The glass beads were removed and appropriately disposed of. I placed these plates inside an incubator at 37C for a weak to allow colonies to grow. On February 5, 2019, I removed all of the plates from the incubator in order to examine colonial morphology and counts. I distinguished morphotypes based on color, pigmentation, shape, colony margin, and elevation. Any additional features or anomalies such as distinctively emitted smells were noted as well. To count the number of colonies per plate, I used a Sharpie pen to mark each counted colony as to avoid miscounting.

### Streak Plating

For further examination, I obtained pure microbial cultures from morphotypes that I observed to be either in high abundance or very rarely present among all of the TSA dilution plates from my samples. To do this, I used an inoculating loop to scoop the desired morphotype colony from the diluted plate. I transferred the single colony on a sterile TSA plate by streaking the loop across one third the agar surface, inoculating the loop with a bacticinerator, dragging the loop through the 1st streak and streaking another third, and repeating once more. This method should isolate pure cultures of microbes. I completed this method 7 times, once for each morphotype observed. I also used the bacticinerator to sterilize the metal loop between each morphotype streaking. After 5 days of incubating the plates at 37C, I restreaked plates that did not produce single colonies or were contaminated. The restreaked plates were incubated for only two days.

### Staining and Microscopy

In order to visualize and identify the different morphotypes, I created 3 stained slides from the pure cultures I obtained. I made a crystal violet stain, gram stain, and Maneval’s stain. These staining methods were used so that I could observe and identity microbes’ different physical features under a light microscope. Crystal violet staining helps to visualize the basic size, shape, and arrangement of the cells. Gram staining helps distinguish between gram-positive and gram-negative bacteria. Maneval’s stain is a negative stain that shows whether the bacterium contains a capsule. After mounting, fixing, and staining each culture, I placed the slide under a Leica DM500 light microscope and observed each slide at 1000x magnification under immersion oil.

### DNA Extraction, PCR Amplification and Gel Electrophoresis

I followed the standard DNA extraction protocol for bacterial cells as provided in the SigmaREDExtract n’ Amp Plant Kit. For the PCR amplification, the PCR recipe per reaction mixture is as follows: 4µL template DNA, 10µL Amp solution, 1µL of 10µM 27f primer, 1µL 10µM 1492r primer, 1µL 1M BSA, and 3µL PCR water. The temperature and run time for each PCR cycle are as follows: 3min at 94C, 30 seconds at 94C (35 times), 30 seconds at 55C (35 times), 2 minutes at 72C (35 times), 10 minutes at 72C, and holding at 4C. I checked for successful PCR amplification using 1% agarose gel. The gel was run for 30 min at 100V. Products were visualized with SYBR Safe and UV light. The ladder in image is a 1kb ladder (Bio-Rad Laboratories, Inc). After checking for successful amplification using gel electrophoresis, PCR products were cleaned with Invitrogen ExoSAP. They were sent for unidirectional Sanger sequencing at MCLAB located in South San Francisco, CA.

### Second Round of PCR Amplification

I performed a second round of PCR amplification with a 1/10 master mix dilution for unsuccessful BLAST sequences with sufficient Qubit DNA concentrations. For the new PCR amplification, the PCR recipe per reaction mixture is as follows: 1µL template DNA, 10µL Amp solution, 0.5µL of 10µM 27f primer, 0.5µL 10µM 1492r primer, 1µL 1M BSA, and 7µL PCR water. For unsuccessful BLAST sequences with very low Qubit DNA concentrations, I performed a second PCR amplification with the 1/10 master mix dilution and reextracted DNA. The annealing temperature was modified to be 1 degree higher (56 C) and I ran the PCR cycles 32 times instead of the original 35 times.

## Computational Analysis

### Culturing Analysis

The process to analyze the data gathered was accomplished using a systematic workflow. First, I created and uploaded data onto a Google Sheet. I added data about the number of different colonies and morphotypes counted per TSA plate at each concentration for each sample treatment. This sheet was then converted and exported as a csv file to the raw data project file folder. Using the R language and RStudio, I uploaded my project and added various software packages such as dplyr and ggplot2 to a Rmd file. I used the csv data file and appropriate packages I added to create the figures for my analysis. I also performed ANOVA analyses on both colony and morphology counts.

### Microscopy Analysis

I connected the light microscope to a computer containing the Leica LAS E2 software in order to directly take photos of each slide from the microscope. Photos were standardized and captured at 2048x1536 resolution. After obtaining my images, I used Fiji and ImageJ to measure and analyze each image. Fiji is an image processing package within the ImageJ software that focuses on biological-image processing (16). I uploaded the captured micrograph and then proceeded to calibrate and scale each micrograph to 10 m. I changed each image to an inverted, binary form and recorded the length, width, and area for 5 cells per morphotype. I created a montage with appropriate labels of each micrograph taken for each staining method per morphotype. Using R and R studio, I uploaded these montages to my report with appropriate captions. I also analyzed the mean and standard deviation for length, width, and cell surface area per morphotype. Finally, I created a markdown table displaying the appropriate characteristics I observed to be present per morphotype.

### Molecular Analysis

I analyzed the sequence data using the program MEGA7. The Molecular Evolutionary Genetic Analysis (MEGA) software is an integrated desktop tool designed to preform sequence alignments, infer phylogenetic trees, mine web-based databases, estimate rates of molecular evolution, and test evolutionary hypotheses (17). Using MEGA7, I manually trimmed raw Sanger sequences as well as noted which sequences failed. I exported the trimmed sequences to Fasta. Using the ClustalW program, I aligned the trimmed sequences along with the sequence for the outgroup, *Thermus aquaticus*. Using the saved alignment, I used the Maximum Likelihood method to infer their phylogeny. Using NCBI Blast and the GenBank nucleotide database, I searched for the identities and accompanying descriptions for each non-failed, trimmed sequence.

### Phylogenic Analysis by Maximum Likelihood Method

The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (18). The tree with the highest log likelihood (-1393.40) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 200.0000)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 6 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 250 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (19).

# Results

## Colony Counts

The first thing I investigated was the number of colonies present for each sample treatment at the original concentration (1x). Looking at each sample, I observed that, qualitatively, the number of colonies found on the leaves of fern-leaf yarrow plants is larger than the number of colonies found on the leaves of ice plants but I found no statistical significance (Figure 1; ANOVA, *P* = 0. 3372).

## Detecting Morphotypes

Because of the differences I observed in colony counts, I decided to investigate whether the sample treatments differed in the number of morphotypes I detected per sample. The average number of morphotypes detected in fern-leaf yarrow plants were larger than the average number found in ice plants (Figure 2; ANOVA, *P* = 0.03303). One of the morphotypes I detected at the highest frequencies were white, circular, glistening, and raised. One of the rarest morphotypes obtained from the 1x sampling plates was present in the fern-leaf yarrow sample which was taken from the leaf closest to the soil of the plant.

# Discussion

## Culturing

The focus of my investigation was to determine whether differences in the colonies and morphotypes present on a fern-leaf yarrow plant and ice plant growing on the Lone Mountain portion of the University of San Francisco’s Hilltop Campus. I hypothesized that the fern-leaf yarrow plant would have higher colony counts and morphotype diversity than the ice plant. The result of this experiment suggests that the plant structure and naturalized status allow fern-leaf yarrows to have greater morphology diversity. Although, qualitatively, fern-leaf yarrows exhibited higher colony counts, there was no statistical difference between the mean counts of the two plants. Some limitations and caveats of the study that are important to mention are the weather on my day of collection and the type of media I used for culturing. There were rain showers the night and morning before I collected my samples which could have potentially added or removed microbes and reduced the amount pollinator visits to each plant type. In addition, I used TSA plates to grow the microbes which may have negatively impacted the morphotypes I observed. My observations may be based on only a subset of microorganisms because tryptic soy agar is an ideal media for bacterial growth, not fungi or plant microbes that are very common in plant species.

Differences in performance among plant species can greatly impact ecosystem function so studying the plant microbiome is important. Invasive plant species, like ice plants, are often able to invade and persist in their environment due to their ability in altering soil chemistry and nutrient cycling through the microbes that accompany them (20). This can lead to the decline in species diversity among native and naturalized plants in their respective environment. To try and combat this depletion in resources, native and naturalized plants such as the fern-leaf yarrow may recruit more beneficial microbes that can aid in tolerance or defense from varying environmental stresses. In addition, scientists argue that invasive plants can be released from attack by natural enemies, like fungal and viral pathogens (21). This may have contributed to the differences I observed in the number of morphotypes present on each plant species. Understanding the microbial impact of invasive species on native and naturalized plants and their ecosystem can be an effective tool in restoring habitats that have been negatively impacted by introduction these invasive plants.

## Predicting Species based on Microscopy

The purpose of performing this microscopy analysis was to gain more detailed information about some of the microbial communities present on the leaves of ice plants (*C. edulis*) and fern-leaf yarrows (*A. filipendulina*). The predictions I made for my selected morphotypes based on the staining results are as follows: morphotype #1 is *Azomonas agilis*, morphotype #2 is *Staphylococcus aureus*, morphotype #3 is an *Acidovorax* spp., morphotype #4 is *Pseudomonas aeruginosa*, morphotype #5 is a *Pseudomonas* spp., morphotype #6 is *Bacillus subtilis*, and morphotype #7 is a *Bacilius* spp. (Table 2). The majority of the morphotypes were gram negative, bacillus shaped bacterium. From all of microbes I analyzed, only the and morphotypes were gram positive. In addition, the only cocci shaped bacteria I noted were morphotypes #1 and #2 (Fig.3, Fig.4). Almost of the predictions I made for the identities of the morphotypes fall under two genera, *Bacillus* and *Pseudomonas*. Both of these genera are found globally, in both plants and mammals. Both genera contain species that can benefit and protect or cause infection to the host. For example, *Pseudomonas aeruginosa* is a naturally occurring bacterium that causes infections in immunocompromised humans acts as a pathogen in plants. In plants, *P. aeruginosa* is an opportunistic pathogen in that it exploits a break in the host defenses to initiate an infection (22). Pathogens are harmful in that infections can eventually lead to disease or death in the host.

## Combining Microscopy and Molecular Data

The phyllosphere presents an opportunity to observe the diverse interactions between plants and bacterial communities. Although the phyllosphere supports great microbial diversity, there is still limited information about the exact microbes that inhabit various plant species. Of the sequences which successfully resulted in valid BLAST results, the two most abundant genera present were *Bacillus* and *Pseudomonas*. *Bacillus* is characterized as being rod-shaped, endospore-forming, and gram-positive. Although it is characterized as being gram-positive, some cultures can eventually turn gram-negative with age (23).

The two morphotypes I identified as Bacillus species are morphotype #2 and morphotype #7 (Table 4). The microscopy images obtained contained some anomalies as when compared to its defining characteristics. I categorized morphotype #2 as being gram-positive and cocci-shaped and morphotype #7 as being bacillus and gram-negative. Some *Bacillus* species can be classified as coccobacillus-shaped. In addition, the body sizes of *Bacillus* spp. often range between 0.5-1.0 µm wide by 1.0-4.0 µm long (23). This accounts for why I initially classified the morphotype as cocci-shaped. Although morphotype #7 does clearly exhibit the bacillus shape, it stained gram-negative. This could be explained by either human error or age. In my process of staining, I possibly may have destained the crystal violet completely off of the slide which would have caused the safranin to completely stain the cells, giving the appearance of a gram-negative bacteria. Another possibility is that although *Bacillus* is characterized as being gram-positive, some cultures can eventually turn gram-negative with age (23). A 97% bootstrap support provides further evidence of the morphotypes likely belonging to the same genera (Fig. 11). Bacillus spp. are among some of the most globally dispersed bacteria and produce many biological compounds that affect host plants and their interactions with other organisms. For example, *Bacillus substilis* is isolated to produce the biocontrol product Serenade which can be effective in controlling foliar and soilborne pathways from pathogenic bacteria such as *Pseudomonas* (24).

Morphotypes #4 and #5 were identified as having a *Pseudomonas* origin (Table 4). *Pseudomonas* species, under a light microscope, appear as gram-negative and straight or slightly curved rods with sizes ranging from 0.5-1.0 µm wide to 1.5-5.0 µm long (25). The generalized features of *Pseudomonas* match those that I observed from microscopy as well as predicted in. In addition, a 99% bootstrap support provides further evidence of the morphotypes likely belonging to the same genera (Fig. 11). Further research into the genus yielded interesting information about the interactions between plants and *Pseudomonas* species. Many *Pseudomonas* spp. are considered pathogens. A pathogen, or infectious agent, is any bacterium or virus that can cause disease or illness. Some *Pseudomonas* spp., like *Pseudomonas syringae*, species can attack and infect plants via different mechanisms. *P. syringae* is able to use its flagella and pili to invade a plant via open wounds, release effector proteins to manipulate the host’s immune response and cause disease, and form biofilms which express virulent genes and produce polysaccharides to create an ideal growth environment. In addition, the bacterium is very effective at causing frost damage in plants via ice nucleation (26). Because *P. syringae* has so many different mechanisms of pathogenicity to plants, one may expect that plants would assemble a large immune response against species of *Pseudomonas* but this is not the case. Some species of *Pseudomonas* can actually function to benefit plant growth and protect the host plant against other threats like herbivores. Some studies suggest that *Pseudomonas* can increase plant growth by synthesizing growth hormones, suppressing other microorganisms, and infecting other organisms that attack the host plant. Interestingly, a strain of *Pseudomonas syringae*, *Pseudomonas syringae pv. tomato DC3000* (*Pst DC3000*), has been shown to reduce the body growth of leaf chewing insects while feeding on leaves infected with the strain (27). As a result of this complicated interactions between different *Pseudomonas* spp., plants are tasked with the challenge of trying to recognize with species cause a higher threat to their health versus which ones are beneficial or benign. This most likely explains why *P. syringae*, a destructive pathogen, was found inhabiting the phyllosphere of ice plants (*C. edulis*) and fern-leaf yarrows (*A. filipendulina*).

## Caveats Regarding Microscopy and Molecular Processing

Certain limitations and errors presented along the process of the experiment created some challenges. Regarding microcopy, errors in producing stains and resolution were the most likely to impact the data. The staining process requires some experience and patience in order to obtain well-produced slides. Taking more time and continually practicing the staining methods is beneficial to making better predictions about the different morphotypes. Another issue I encountered in identifying the different features of the microbes involved contamination of one of the staining solutions. The Maneval’s stain was completely contaminated with other microbes so it was impossible to observe whether any of the morphotypes I studied contained capsules. In addition, having more information could possibly helped in narrowing down the species for some of the predictions I made and the BLAST sequence results I obtained. Resolution was also a limitation because many bacterial cells can reach the resolution limit of light microscopes. Some ways to resolve this issue is by enhancing the contrast of the imaging system used to obtain images of the morphotypes or using staining the background of the slides with another dye. Another serious issue I encountered in the experiments was processing the molecular data. The reasoning as to why some the original samples didn’t successfully produce valid BLAST results is because each sample had too high of a concentration of inhibitors in the bacteria and some of the samples did not have a high enough concentration of DNA. This resulted in some issues collecting valid BLAST sequences and results. To correct this error, I performed another round PCR amplification on the initially unsuccessful sequences with a diluted master mix and reextracted DNA depending on the Qubit concentrations. This resolved the issue of having insufficient Blast results after the first round of PCR amplification and sequencing.

# Methods: Focused project on the phyllospheres of various flowering plant species

## Study Design

### Field Collection

On April 18th, 2019, I sampled leaves from five different plant species located in various landscaped plots on the Mountain campus of the University of San Francisco in San Francisco, California (37.776 N, 122.4521 W). The plants species I collected from were Mexican marigolds (*Tagetes erecta*), wax begonias (*Begonia x semperflorens-cultorum*), lavender (*Lavendula*), California goldfields (*Lasthenia californica*), and California poppies (*Eschscholzia californica*). For each plant species, I sampled five individual plants located in the same landscaped plot. I selected individuals located in positions that were not in close proximity to other plant species to avoid potential contaminant. For each plant individual, I collected samples on 5 different leaves that were placed at various elevations from the soil to the tip of the plant, being careful not to sample any leaves that were in contact with any petals or soil. To collect my samples, I used a new, sterile cotton swab on leaf for approximately 10 seconds in a continuous vertical motion. I then proceeded to place each swab into an appropriately labeled tube filled with 1mL of PBS solution. To obtain the control for my experiment, I used sterile swabs to collect an air sample in every landscaped plot where each plant species was located. I exposed each swab to the air for approximately 10 seconds and placed each swab into a labeled tube filled with 1mL of PBS solution. Before culturing, the samples for the wax begonias and Mexican marigolds were kept in their respective solutions for approximately 30 minutes and the samples I collected for the lavender, California goldfields, and California poppies were in solution for 15 minutes.

### Culturing

I used 100mm tryptic soy agar (TSA) plates for plating all of the sample treatments I collected. First, I vortexed each test tube for approximately 10 seconds in order to release the remaining microbes from the cotton swab and into the PBS solution. For each sample I collected for each plant type, I used 20 TSA plates, equaling 100 plates total. Additionally, I used 1 TSA plate for each control sample I collected, equaling a total of 5 plates. I covered each TSA plate with 4 µL. I spread the bacterial liquid onto each TSA plate by swirling approximately 6-8 glass beads until the plate was completely covered. The glass beads were removed and appropriately disposed of in a waste container. I placed the TSA plates inside an incubator at 37C for almost a week in order to allow colonies to grow. On April 23rd, 2019, I removed all of the plates from the incubator in order to examine the colonial morphology and counts. I examined the TSA plates for plant species wax begonia on April 23rd. The remaining cultures were placed in a refrigerator to prevent the colonies from growing further. On April 25th I removed the TSA plate cultures for the plant species Mexican marigold, lavender, California goldfield, and California poppy for analyzing. I distinguished morphotypes based on color, pigmentation, shape, colony margin, and elevation. Any additional features or anomalies such as distinctively emitted smells were noted as well. To count the number of colonies per plate, I used a Sharpie pen to mark each counted colony as to avoid miscounting.

### Streaking for Pure Colonies

April 29th, 2019, I obtained pure microbial cultures from morphotypes that I observed to be either in high abundance or very rarely present among all of the TSA dilution plates from my samples for further analysis. To do this, I used an inoculating loop to scoop the desired morphotype colony from the culturing plate. I transferred the single colony on a sterile TSA plate by streaking the loop across one third the agar surface, inoculating the loop with a bacticinerator, dragging the loop through the 1st streak and streaking another third, and repeating once more. This method should isolate pure cultures of microbes. I also used the bacticinerator to sterilize the metal loop between each morphotype streaking. I completed this method 6 times, once for each morphotype observed and labeled each TSA plate with ID labels of Morp. #1-6. I placed all 6 plates in an incubator at 37C. I removed all of the plates from the incubator on April 30th. All plates contained pure colonies so no restreaking was necessary.

### DNA Extraction, PCR Amplification and Gel Electrophoresis

I followed the standard DNA extraction protocol for bacterial cells as provided in the SigmaREDExtract n’ Amp Plant Kit. This PCR kit contains all the necessary reagents necessary to rapidly and simply extract and amplify genomic DNA (28). I created an undiluted and 1/10 diluted PCR reaction master mix. For the undiluted PCR master mix, I added 10 µL of Amp solution, 1 µL of 10µM 27f primer, 1 µL 10µM 1492r primer, 1 of 1M BSA, and 6 µL of PCR water. I added 1 µL of template DNA to a labeled tube for each morphotype and added 1 µL of PCR water for the negative control. The diluted PCR recipe per reaction mixture is as follows: 1 µL of 1/10 diluted template DNA, 10 µL of Amp solution, 0.5 µL of 10M 27f primer, 0.5 µL of 10µM 1492r primer, 1 µL of 1M BSA, and 7 µL of PCR water. Instead of diluted template DNA, I added 1 µL of PCR water for the negative control. The temperature and run time for each PCR cycle are as follows: the initialization cycle for 3min at 94C, denaturing for 30 seconds at 94C (35 times), annealing for 30 seconds at 55C (35 times), extension for 2 minutes at 72C (35 times), a final extension for 10 minutes at 72C, and holding at 4C to cool the products. I checked for successful PCR amplification using 2% agarose gel. I added 4 µL of each PCR product to each well as well as 4 µL of ladder solution to the 8th well. The gel was run for 30 min at 130V. Products were visualized with SYBR Safe and UV light. The ladder in image is a 1kb ladder (Bio-Rad Laboratories, Inc). After checking for successful amplification using gel electrophoresis, PCR products were cleaned with Invitrogen ExoSAP. They were sent for unidirectional Sanger sequencing at MCLAB located in South San Francisco, CA.

## Computational Analysis

### Culturing Analysis

The process to analyze the data gathered was accomplished using a systematic workflow. First, I created and uploaded data onto a data using Microsoft Excel. I added the data regarding the number of different colonies and morphotypes counted per TSA plate at each concentration for each sample treatment. This sheet was then converted and exported as a csv file to the raw data project file folder under the file name “final\_project\_culturing\_counts.csv”. I used the csv data file and appropriate packages I added previously to create the figures and tables for my analysis. I filtered out colony counts over 500 colonies. I performed ANOVA analyses on both colony and morphology counts. I also performed pairwise comparisons for the number of colonies and morphologies based on plant species.

### Molecular Analysis

I analyzed the sequence data using the program MEGA7. Using MEGA7, I manually trimmed raw Sanger sequences as well as noted which sequences failed. I exported the trimmed sequences to Fasta. Using the ClustalW program, I aligned the trimmed sequences along with the sequence for the outgroup, *Thermus aquaticus*. Using the saved alignment, I used the Maximum Likelihood method to infer their phylogeny. Unfortunately, due to insufficient DNA on almost all the sequences and therefore, I could not produce a phylogeny. Using NCBI Blast and the GenBank nucleotide database, I searched for the identities and accompanying descriptions for each non-failed, trimmed sequence. For sequences that could not be successfully matched, I marked as “No Good Match” (Table 11).

# RESULTS: Focused project on the phyllospheres of various flowering plant species

## Culturing for the Focused Portion of the Experiment

The first thing I investigated for this portion of the experiment was the differences in the number colonies I observed for each plant species. I determined that certain plant species had many more colonies grow than other plant species. Qualitatively, the phyllospheres of Mexican marigolds and wax begonias were overall significantly more abundant in the number of colonies present as compared to the other plant species which is quantitatively supported (Figure 13; ANOVA, *P* = 0). Some TSA plates were almost entirely covered in colonies and others that had no colonies that grew (Figure 12). Next, I investigated whether the plant species possessed differences in their morphology counts based on my observations. Again, I observed that the average morphology counts in Mexican marigolds and California goldfields appeared to be the most highly abundant, and I did find statistical support this observation (Figure 14; ANOVA, *P* = 0).

Due to the highly supported data I obtained when observing colony and morphotype counts over all of the plant species, I created pairwise comparisons for each plant species. When comparing colony counts among each plant species, I observed a trend in the average colony counts of the species wax begonia against the other plant species. Mean colony counts in wax begonias are significantly higher than in the California goldfield, the California poppy, and lavender (Table 9; all *P* = 0.00). When comparing pairwise differences among plant species in relation to species diversity, Mexican marigolds and wax begonias dominate. The mean morphotype counts for Mexican marigolds are significantly more abundant than in California goldfields (*P* = 0.02), California poppies (*P* = 0.00), and lavender (*P* = 0.00) (Table 10). Mean morphotype counts are quantitatively greater in wax begonias when compared to California goldfields, California poppies, and lavender (Table 10; all *P* = 0.00). Comparing morphotype abundances between wax begonias and Mexican marigolds suggest that the former contains a higher species diversity.

## Molecular Analysis for the Focused Portion of the Experiment

Of the six morphotypes I chose to investigate further, I received PCR sequence results for morphotypes #2, #3, #4, and my negative control. I attempted to produce an inferred phylogeny after manually trimming each sequence but could not due to insufficient DNA sequence data from all of the results except morphotype #2. Likewise, I did not receive any matches for those DNA sequences except for morphotype # 2. BLAST results suggested that morphotype #2 is most likely a species strain belonging to the family *Enterobacteriaceae* and genus *Pantoea*.

# DISCUSSION: Focused project on the phyllospheres of various flowering plant species

## Focused Portion of the Experiment

The focus of the second portion of my investigation involved trying to determine whether other plant species, specifically flowering plants, contained differences in the number of colonies and morphotypes growing on their respective phyllospheres. I hypothesized that species diversity, the number of morphotypes present, would be more statistically significant as compared to the number of colonies present per plant species. This difference would translate to dominance of a particular genera present among all of the plant species. In particular, I would expect to encounter *Bacillus* spp. or *Pseudomonas* spp. as I did in the first portion of my experiment. I yielded high statistical support among colony and morphology counts when comparing the plant species overall. Further investigation via pairwise comparisons suggested that the phyllospheres of Mexican marigolds and wax begonias were considerably more abundant than the phyllospheres of California poppies, California goldfields and lavender. These findings partially support my hypothesis. A possible reasoning as to why I observed significant colony abundance among the various plant species could be due to the larger sample set I tested versus the first portion of my experiment. In addition, microbial richness on flowering plants often increases when surrounded by warmer climates and my sampling took place at the end of April, often reaching temperatures around 70F (29). In addition, urban climates, like San Francisco, are often subject to increased temperatures which leads to addition stress on the phyllosphere and overall plant health.

Although I was not able to produce a phylogeny, my molecular analyses using BLAST and the NCBI database yielded the identity of morphotype #2. Morphotype #2 was identified as being a *Pantoea* species strain. *Pantoea* is a highly diverse genus that’s generally associated with plants and they can take the role of either an epiphyte or pathogen. Some species, such as *Pantoea agglomerans* is an epiphyte that helps leaves combat water stress or manages plant health against diseases (30). When in contact with other deadly pathogens, it produces antibiotics that are toxic. However, *P. agglomerans* requires certain environmental factors, like a warm, moist, and well-lit climate in order to effectively survive and work as a biocontrol. Additionally, it requires clean air circulation to survive. Due to the location and elevation of Lone Mountain Hill as well as the placement of the landscaped plot, the plants are provided with a well-lit climate and are not in overwhelming contact with machinery like automobiles.

## Limitations and Caveats

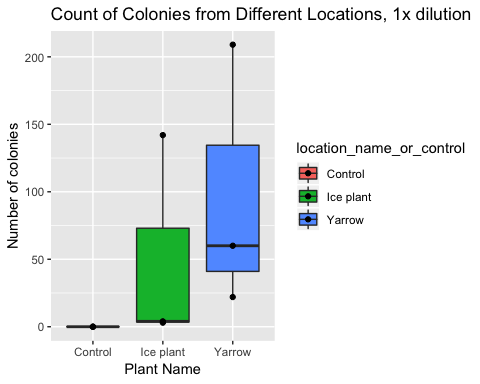
There are some limitations and caveats that may have potentially impacted the observations and results I obtained. Firstly, although I did sample on the same day, I did not have all of my samples in the PBS solution for the same amount of time. The Mexican marigold and wax begonia samples were in solution for upwards of an hour. In contrast, the samples for the other three plant species spent between 15-30minutes in PBS solution. This could have affected the disparities in the number colonies that grew on each plate. If I performed this experiment again, I would more be more consistent with the time I allow my samples to stand in solution. An issue I had regarding the molecular portion of my experiment was that almost all of my sequences were either failed or had insufficient DNA sequence results. This could be a result of a lack of DNA I added in my PCR tubes or not adding enough solution to the gel wells. In a follow-up experiment, I would add more of a concentration of DNA to my PCR reactions and more solution to each gel well.

## Conclusion

Urban areas often create a heat island effect from increased radiation and sparse vegetation cover (31). This thermal accumulation explains why many plants often undergo stress and altered enzymatic processes. As a result, the overall health of the plant can become endangered. Fitness and longevity become reduced, functional traits for survival could be lost or reduced, and in some instances, death may proceed. The combination of invasive species and anthropogenic stressors cause plant health to be under threat at all times. This is why it’s imperative for everyone, scientist and citizen alike, to take measures that preserve plant health. Some ways to accomplish this by planting more native and naturalized plants as they often require less maintenance and care, like succulents. It also preserves overall ecological health because invasive species often deplete the surrounding resources and space (9). Increasing vegetation cover that’s currently present in these urban areas and to combat the rising global temperatures as more cities are being created and populated. The scientific community is only beginning to understand the impacts of how the environment impact plant and their microbes. Altering a plant’s environment can greatly impact the conditions of its respective phyllosphere. These environmental perturbations I previously stated can affect the relative species richness and efficiency of functional microbial groups present. For example, loss or reduction in species abundance and richness can make it easier for invaders like insects and pathogens to attack the microbes inhabiting the phyllosphere. Combining various approaches such as culturing, microscopy, and molecular analyses provides scientists with an understanding of the evolutionary history and relationships of microbes as they adapt to their changing habits today. Culturing provides physical descriptions and overall estimates into species richness. Phylogenies provide inferences on the relatedness among the microbes present in their community. Sequence-based methods like broad-range PCR and metagenomics have been critical in the discovery and of pathogens and microorganisms as well as establish key associations in host-species interactions. When comparing the small scale of microorganism, the large impact that microbes have on their host species and neighboring microorganisms make them one of the most powerful organisms on the planet.

## Figures and Tables

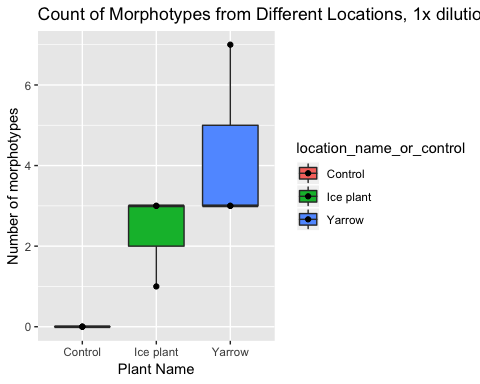
## Culturing Results



**Figure 1:** Number of colonies per sample treatment and control at 1x dilution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| term | df | sumsq | meansq | statistic | p.value |
| location\_name\_or\_control | 2 | 14116.22 | 7058.11 | 1.31 | 0.34 |
| Residuals | 6 | 32326.67 | 5387.78 | NA | NA |

**Table 1:** Statistical analysis of the number of colonies for sample treatments at 1x dilution. P-value of the number of colony counts is 0.3372.



**Figure 2:** Count of different morphotypes present per sample treatment and control at 1x dilution.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| term | df | sumsq | meansq | statistic | p.value |
| location\_name\_or\_control | 2 | 28.22 | 14.11 | 6.35 | 0.03 |
| Residuals | 6 | 13.33 | 2.22 | NA | NA |

**Table 2:** Statistical analysis of the number of morphotypes for sample treatments at 1x dilution. P-value of the number of colony counts is 0.0303.

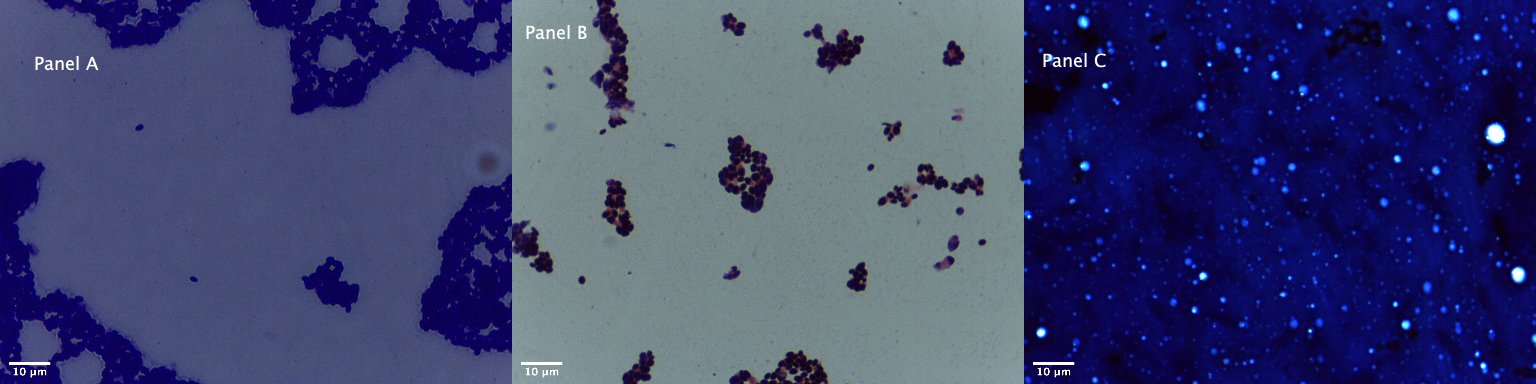
## Microscopy Results

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| MOTU ID | Mean cell length (µm) | SD cell length (µm) | Mean cell width (µm) | SD cell width (µm) | Mean cell area (µm^2) | SD cell area (µm^2) |
| 1 | 0.92 | 0.15 | 0.92 | 0.26 | 0.16 | 0.24 |
| 2 | 1.66 | 0.15 | 1.83 | 0.17 | 0.51 | 0.56 |
| 3 | 0.69 | 0.34 | 2.86 | 0.21 | 0.15 | 0.05 |
| 4 | 0.71 | 0.09 | 1.74 | 0.26 | 0.11 | 0.02 |
| 5 | 1.03 | 0.06 | 2.47 | 0.27 | 0.15 | 0.02 |
| 6 | 0.90 | 0.14 | 4.87 | 0.54 | 0.30 | 0.03 |
| 7 | 0.74 | 0.03 | 2.71 | 0.48 | 0.17 | 0.03 |

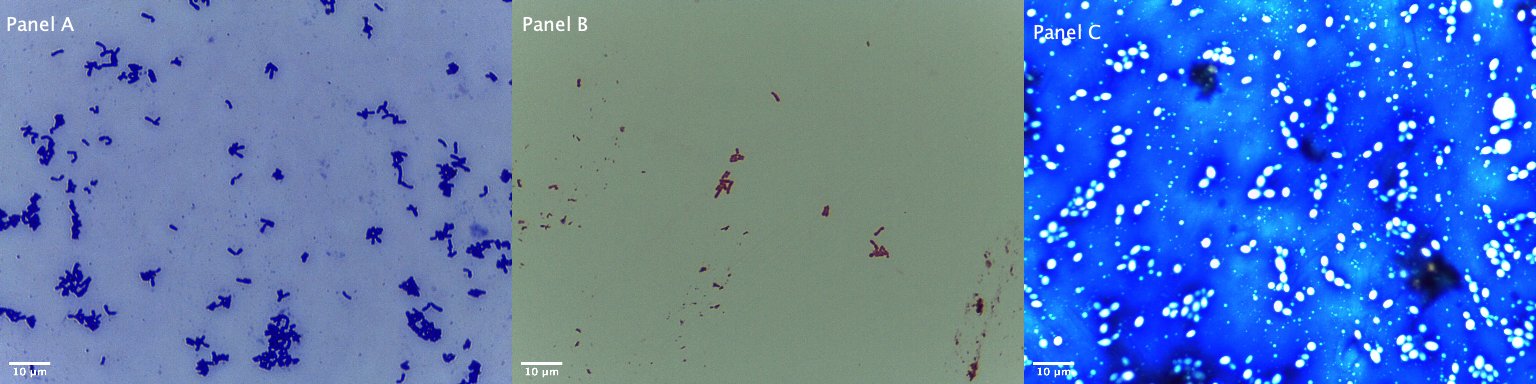
**Table 3:** Mean and standard deviation for the length, width, and cell surface area per morphotype analyzed.



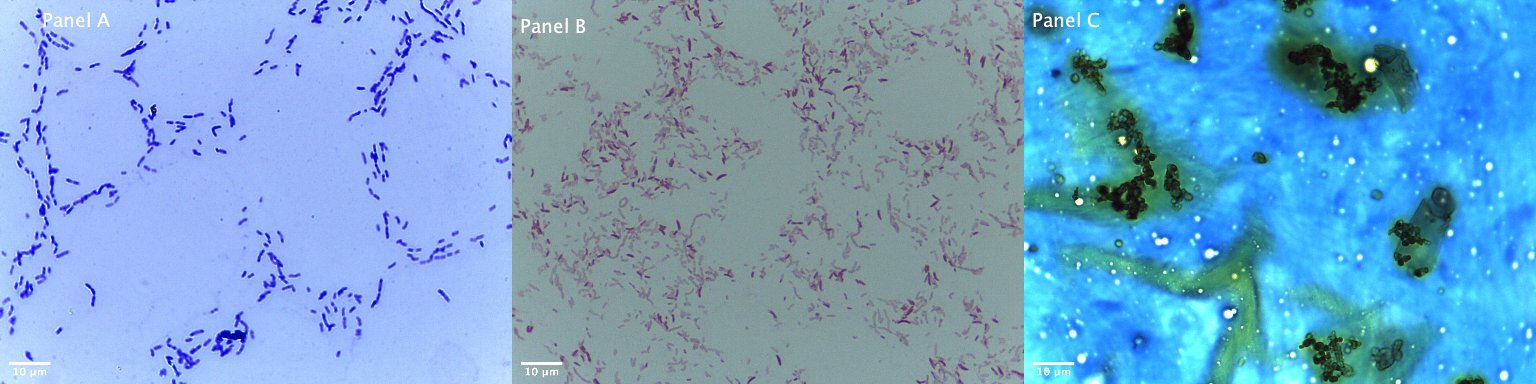
**Figure 3:** Montage imaging of morphotype #1 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



**Figure 4:** Montage imaging of morphotype #2 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



**Figure 5:** Montage imaging of morphotype #3 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



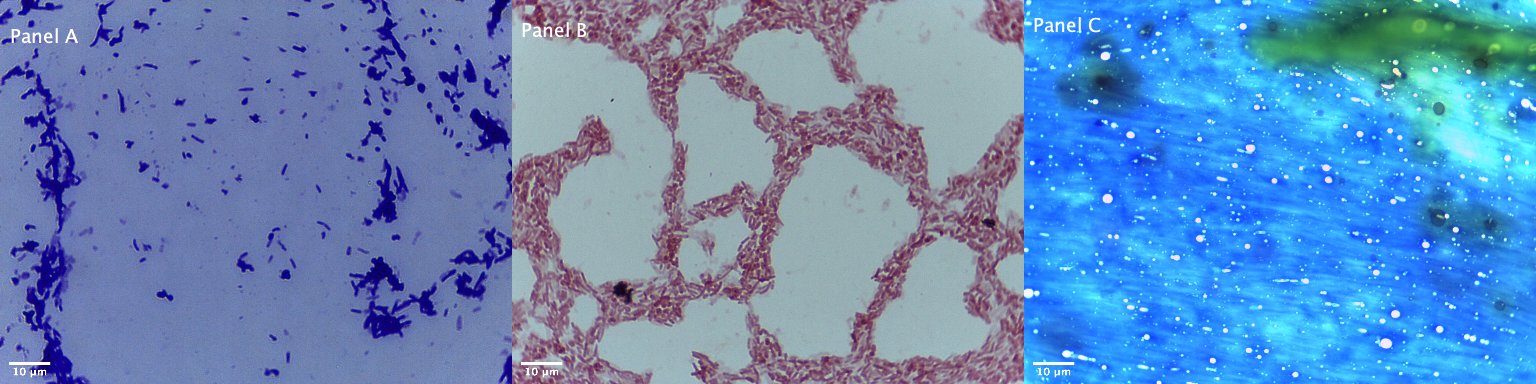
**Figure 6:** Montage imaging of morphotype #4 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



**Figure 7:** Montage imaging of morphotype #5 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



**Figure 8:** Montage imaging of morphotype #6 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).



**Figure 9:** Montage imaging of morphotype #7 stained in crystal violet ( Panel A), gram’s stain (Panel B), and Maneval’s stain (Panel C).

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Morphotype ID | Shape | Gram Positive/Negative | Endospore Yes/No | Capsule Yes/No |
| 1 | Cocci | Gram Negative | No | N/A |
| 2 | Cocci | Gram Positive | No | N/A |
| 3 | Bacillus | Gram Positive | No | N/A |
| 4 | Bacillus | Gram Negative | Yes | N/A |
| 5 | Bacillus | Gram Negative | No | N/A |
| 6 | Bacillus | Gram Negative | No | N/A |
| 7 | Bacillus | Gram Negative | No | N/A |

**Table 4:** Detailed descriptions of cell characteristics for all seven morphotypes studied.

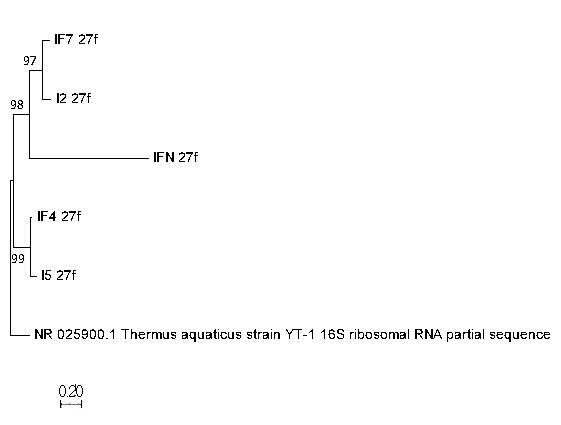
## Molecular Results



**Figure 10:** First round PCR gel image results. The lanes corresponding to each morphotype and negative control are labeled appropriately on the image.

|  |  |
| --- | --- |
| Morphotype ID | Qubit DNA Concentration (ng/μL) |
| 1 | 1.49 ng/μL |
| 2 | 2.7 ng/μL |
| 3 | 0 ng/μL |
| 4 | 1.97 ng/μL |
| 5 | 1.32 ng/μL |
| 6 | 0 ng/μL |
| 7 | 1.69 ng/μL |

**Table 5:** Qubit DNA concentration results for each morphotype tested.

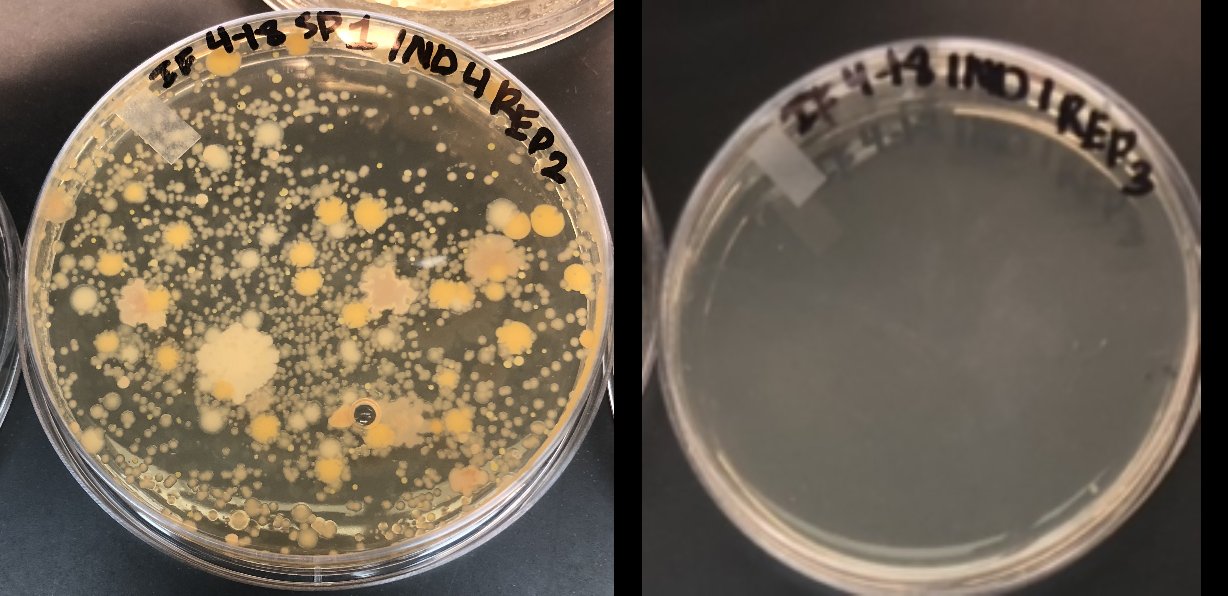


**Figure 11:** Molecular Phylogenetic analysis by Maximum Likelihood method.

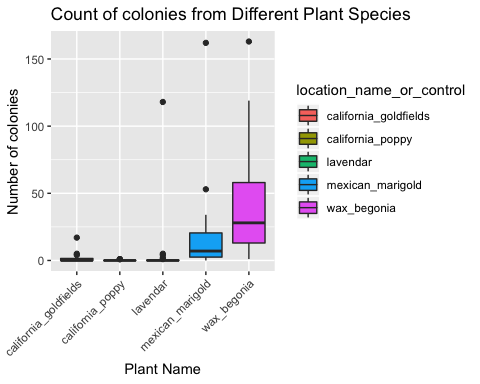
|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Morphotype ID | Morphotype Identity | GenBank ID | Percent Match | Query Cover | E Value |
| 1 | No Good Match | N/A | N/A | N/A | N/A |
| 2 | *Bacillus* sp. | MH260980. 1 | 95% | 80.17% | 8e-94 |
| 3 | Sequence Failed | N/A | N/A | N/A | N/A |
| 4 | *Pseudomonas* sp. | JF313049.1 | 100% | 94.82% | 8e-147 |
| 5 | *Pseudomonas syringae* | MK072676 | 92% | 90.72% | 7e-164 |
| 6 | Sequence Failed | N/A | N/A | N/A | N/A |
| 7 | *Bacillus* sp. | FJ555568.1 | 82% | 89.86% | 9e-138 |
| Negative | Uncultured bacterium | GU510538.1 | 82% | 100.00% | 5e-09 |

**Table 6:** Morphotype identities from BLAST sequence results with accompaning GenBank ID, percent match, query cover, and e value.

## Focused Project: Plant Species Phyllosphere Comparison Results



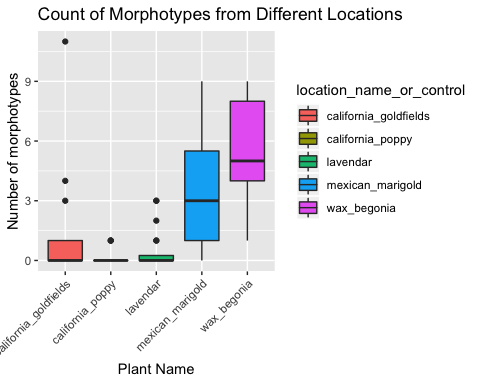
**Figure 12:** Examples of a TSA plates that were considered to be highly abundant (over 500 colonies) and extremely sparse (i.e. no colonies grown).



**Figure 13:** Number of colonies observed per plant species.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| term | df | sumsq | meansq | statistic | p.value |
| location\_name\_or\_control | 4 | 21677.97 | 5419.49 | 6.7 | 0 |
| Residuals | 85 | 68781.41 | 809.19 | NA | NA |

**Table 7:** Statistical analysis of the number of colonies. P-value of the number of colony counts for the different plant species is equal to 0.



**Figure 14:** Count of different morphotypes present per plant species.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| term | df | sumsq | meansq | statistic | p.value |
| location\_name\_or\_control | 4 | 319.11 | 79.78 | 19.92 | 0 |
| Residuals | 85 | 340.49 | 4.01 | NA | NA |

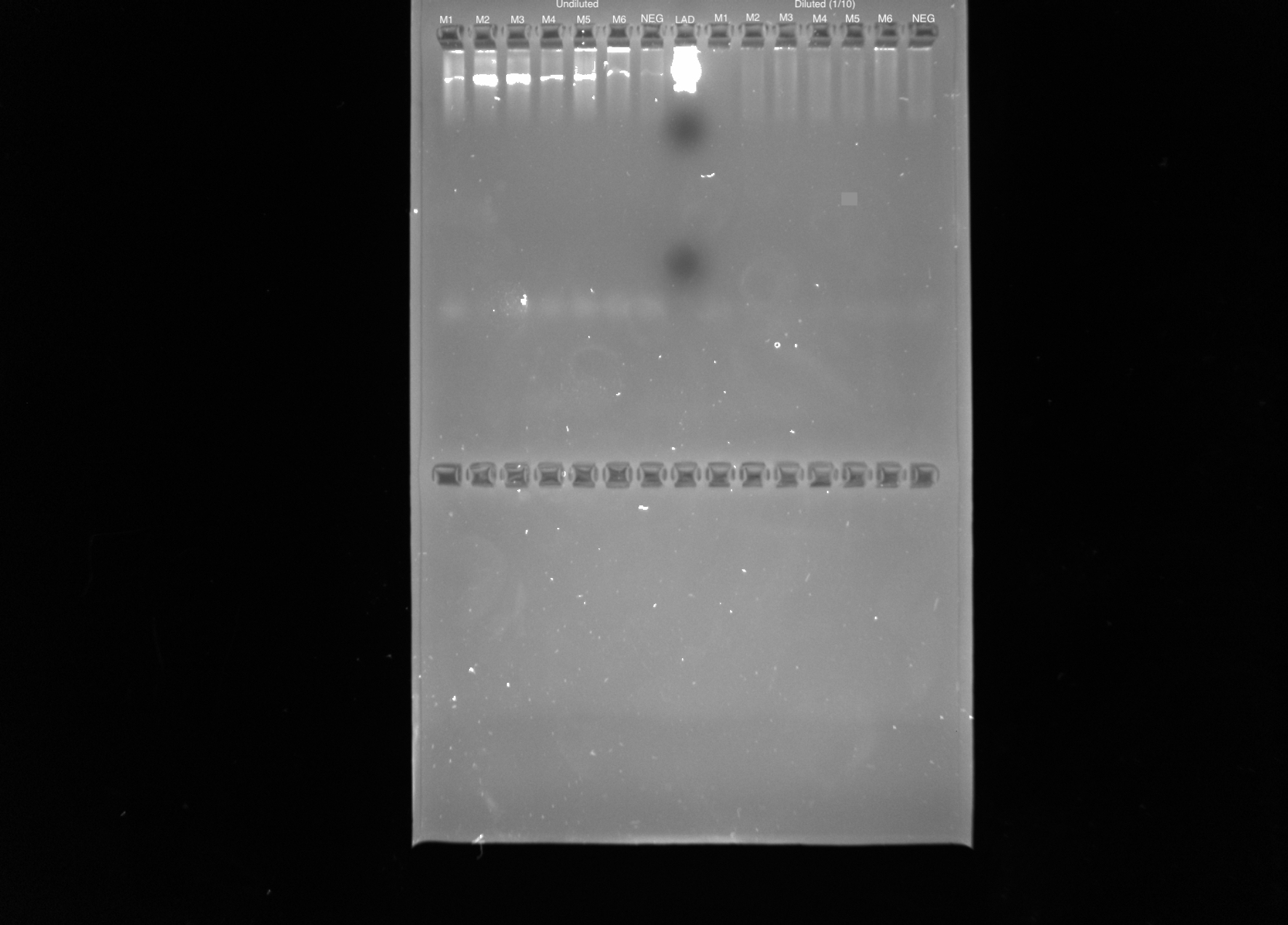
**Table 8:** Statistical analysis of the number of morphotypes. P-value of the morphotype counts for the different plant species is equal to 0.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| comparison | estimate | conf.low | conf.high | adj.p.value |
| california\_poppy-california\_goldfields | -1.58 | -27.30 | 24.14 | 1.00 |
| lavendar-california\_goldfields | 4.76 | -20.64 | 30.16 | 0.98 |
| mexican\_marigold-california\_goldfields | 20.13 | -7.26 | 47.51 | 0.25 |
| wax\_begonia-california\_goldfields | 40.09 | 13.62 | 66.56 | 0.00 |
| lavendar-california\_poppy | 6.34 | -19.06 | 31.74 | 0.96 |
| mexican\_marigold-california\_poppy | 21.71 | -5.68 | 49.09 | 0.19 |
| wax\_begonia-california\_poppy | 41.67 | 15.20 | 68.14 | 0.00 |
| mexican\_marigold-lavendar | 15.37 | -11.71 | 42.45 | 0.51 |
| wax\_begonia-lavendar | 35.32 | 9.17 | 61.48 | 0.00 |
| wax\_begonia-mexican\_marigold | 19.96 | -8.13 | 48.04 | 0.28 |

**Table 9:** Pairwise comparisons of the number of colony counts based on the different plant species sampled.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| comparison | estimate | conf.low | conf.high | adj.p.value |
| california\_poppy-california\_goldfields | -1.05 | -2.86 | 0.76 | 0.49 |
| lavendar-california\_goldfields | -0.71 | -2.50 | 1.08 | 0.80 |
| mexican\_marigold-california\_goldfields | 2.12 | 0.20 | 4.05 | 0.02 |
| wax\_begonia-california\_goldfields | 3.97 | 2.10 | 5.83 | 0.00 |
| lavendar-california\_poppy | 0.34 | -1.44 | 2.13 | 0.98 |
| mexican\_marigold-california\_poppy | 3.18 | 1.25 | 5.10 | 0.00 |
| wax\_begonia-california\_poppy | 5.02 | 3.16 | 6.88 | 0.00 |
| mexican\_marigold-lavendar | 2.83 | 0.93 | 4.74 | 0.00 |
| wax\_begonia-lavendar | 4.68 | 2.84 | 6.52 | 0.00 |
| wax\_begonia-mexican\_marigold | 1.84 | -0.13 | 3.82 | 0.08 |

**Table 10:** Pairwise comparisons of the number of morphology counts based on the different plant species sampled.



**Figure 15:** PCR gel image results for plant species comparison portion of the experiment. The lanes corresponding to each morphotype and negative control are labeled appropriately on the image.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Morphotype ID | Morphotype Identity | GenBank ID | Percent Match | Query Cover | E Value |
| 1 | Sequence Failed | N/A | N/A | N/A | N/A |
| 2 | *Pantoea* sp. strain | MH259986.1 | 86.16% | 91% | 2e-125 |
| 3 | No Good Match | N/A | N/A | N/A | N/A |
| 4 | No Good Match | N/A | N/A | N/A | N/A |
| 5 | Sequence Failed | N/A | N/A | N/A | N/A |
| 6 | Sequence Failed | N/A | N/A | N/A | N/A |
| Negative | No Good Match | N/A | N/A | N/A | N/A |

**Table 11:** Morphotype identities from BLAST sequence results with accompaning GenBank ID, percent match, query cover, and e value.

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