Worcester Polytechnic Institute

Microbial Community Models for Measuring Survival and Persistence of SynBio Microbes in Soil

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Abstract

Genetically engineered microbes (GEMs) hold the potential for many different soil applications. However, the relationship between GEMs and bacteria of the natural soil microbiome is unknown. This research utilizes growth curves and co-cultures, analyzed by flow cytometry, to develop assays for defining relationships between the engineered bacteria *P. putida* and soil strains *C. freundii* or *B. thailandensis*. Here we show that flow cytometry can be used to correlate monoculture growth with co-culture growth to differentiate neutral or non-neutral relationships under a variety of growth conditions. We observe that the growth temperature and media composition affect the nature of co-culture relationships. However, more experimentation, including addition of a viability dye and cytometric analysis by FlowJo will be critical in producing more concrete definitions of the relationships between the species tested.

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Background

Genetically engineered microbes (GEMs) are used for many different soil applications, such as biosensing, bioremediation, pathogen control, and many more (Sayler & Ripp, 2000). However, the relationships between GEMs and predominant bacterial soil species are unknown. The unknown relationships between bacterial species are an important topic of study because the addition of GEMs to the natural soil microbiome could affect the GEMs ability to perform their designed function(s) and/or lead to unwanted effects, such as horizontal gene transfer and natural microbiome disruption (Stirling & Silver, 2020).

This work specifically focuses on the soil bacterium *Pseudomonas putida* and its relationships with other bacteria of the natural soil microbiome, in particular, *Citrobacter freundii* and *Burkholderia thailandensis*. *P. putida* has been known for its "versatile metabolism and low nutritional requirements" (Weimer et al., 2020). Such features, along with its readily editable genome, make *P. putida* an ideal species for biotechnological applications. This bacterium has been shown to function in biosensing, bioremediation, bio-manufacturing, formation of biopolymers, and plant fertilization (Weimer et al., 2020). The extensive potential of *P. putida* in biotechnology has made it a highly desirable species for genetic engineering.

As with any two species that interact in an ecological system, different bacteria have relationships between them. There are six types of possible ecological relationships that can be shared between two species, which are outlined in Figure 1. Out of those six, there are three relationships that are considered negative interactions: parasitism, amenalism, and competition. Two relationships are considered positive interactions: mutualism and commensalism. A neutral relationship is also possible, in which the presence of one species has no effect on the growth or survival phenotypes of the second species, and vice versa. For a relationship to be considered negative, at least one of the species in it is "losing," that is, there is some negative impact of one species on the growth or survival of the second species. To be considered a positive interaction, at least one is "winning," that is, one species is conferring some benefit from the co-existence from the second species, beyond its performance in monoculture. The exception is parasitism, in which one species is winning and one is losing but is considered a negative relationship.

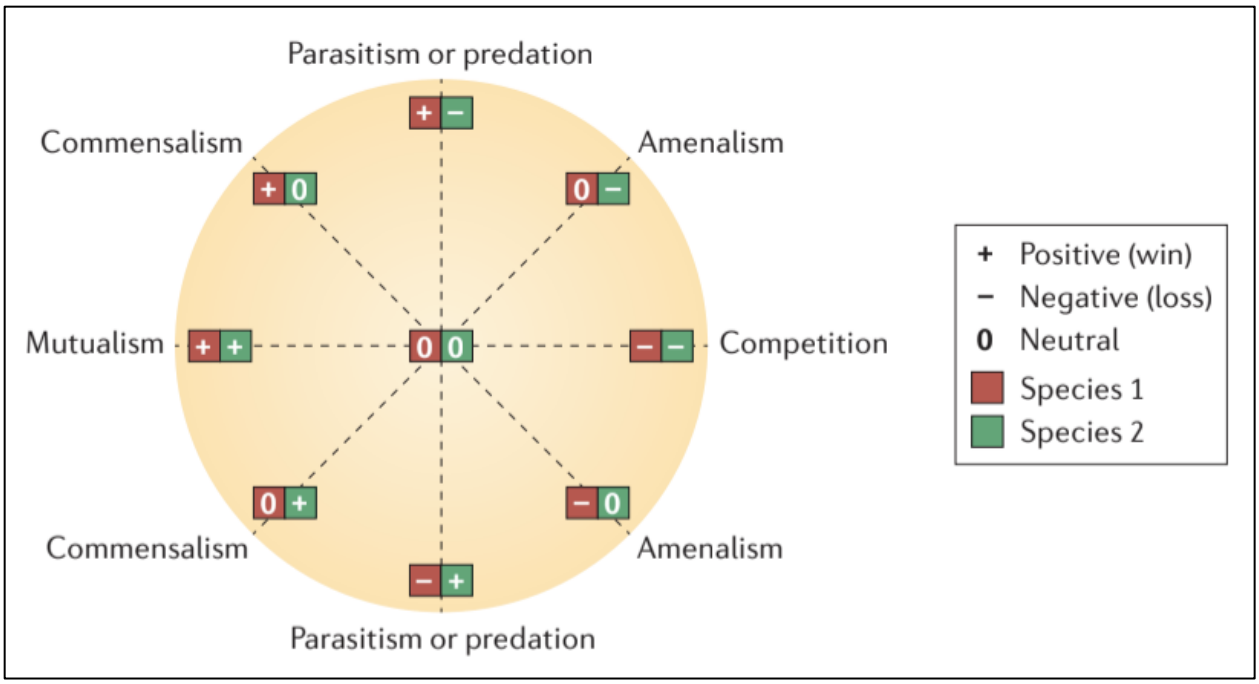

Figure 1: Summarizing chart of ecological relationship types between members of different species. From Faust & Raes (2012).

The measurement of these relationships is not always simple. Figure 2 shows the same six relationships detailed above, displayed as 100% stacked column graphs. As shown in Figure 2, when measuring the percentage of the culture represented by each of two species in a co-culture experiment, a given graphical pattern could represent up to three different types of ecological relationships. Thus, it is not possible to discern relationships simply by measuring the amount of each organism in the sample. Additional research and analysis will be required to determine the exact relationship.

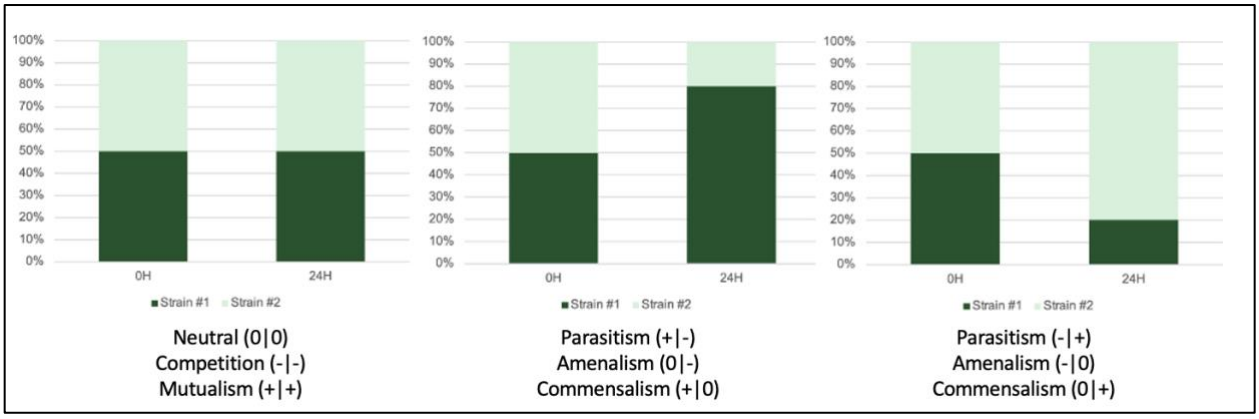

Figure 2: Exemplar 100% stacked column graphs, representing each of the nine possible relationship types highlighted in Figure 1.

Research with *P. putida* has investigated its relationships with species such as *L. monocytogenes* and *E. coli*, however, current studies have not examined its relationships with native soil species

(Giaouris et al., 2013 and Molina-Santiago et al., 2017). Giaouris et al. (2013) found that when *P. putida* was grown in co-culture with *L. monocytogenes* to form biofilms, the resistance of *P. putida* to benzalkonium chloride, a common ingredient in wet wipes, hand, and surface sanitizers, was greatly increased. This example could represent either a mutualistic, commensalistic, or parasitic relationship, because *P. putida* is benefitting from the relationship by developing increased resistance to the biocide. The authors Giaouris et al. do not explicitly discuss the change in *L. monocytogenes* viability between mono- and co-culture conditions (Giaouris et al., 2013). Molina-Santiago et al. (2017) examined the relationship between *P. putida* and *E. coli*. The team found that the growth of those two bacteria in co-culture results in transcriptional changes within the carbon metabolism pathways of both species, and that the two species participated in horizontal gene transfer with each other. Further experimentation would need to be completed looking at the impact of these transcriptional changes and gene exchange on the bacteria's viability and functionality to determine what the exact relationship between *P. putida* and *E. coli* is in the conditions used. More research into the relationships of *P. putida* and bacteria of the natural soil microbiome is needed to unlock the potential for use of engineered *P. putida* for soil applications.

The goal of this research is to define the relationships between engineered *P. putida* and native soil species. In the end, we found that by utilizing data from monoculture and co-culture growth analyses, it is possible to determine the presence of a neutral or non-neutral relationship, under a variety of growth conditions, between engineered *P. putida* and the soil bacterium, *C. freundii* or *B. thailandensis*. These relationships were also shown to be affected by the growth temperature and media composition of the cultures. More experimentation, including the addition of a viability dye, will be essential for defining exactly what type of ecological relationship is present between the two species being tested.

Materials and Methods

Bacterial Strains and Plasmids

The primary strains used in this study are outlined in Table 1 below.

Table 1: Soil-derived bacterial strains used in this study, and their key features.

	Species #1	Species #2	Species #3
Phylum/Class	Gammaproteobacteria	Betaproteobacteria	Gammaproteobacteria
Family	Pseudomonadaceae	Bacillaceae	Enterobacteraceae
Genus	Pseudomonas	Burkholderia	Citrobacter
Species (ATCC#)	Pseudomonas putida (ATCC 700007)	Burkholderia thailandensis (ATCC 700388)	Citrobacter freundii (ATCC 8454)
Optimal Growth Medium	Nutrient agar or nutrient broth	Nutrient agar or nutrient broth	Nutrient agar or nutrient broth
Optimal Growth Temp. (°C)	30	30	37

The *P. putida* strain, KT2440, and plasmids for gene expression and genomic integration, were kind gifts from Dr. Adam Guss of Oak Ridge National Laboratory. Using a genomic integration system created by the Guss lab (Elmore et al., 2017), the fluorescent protein tdTomato was stably integrated into the *P. putida* genome (strain created by Dr. A.F. Carrillo, Farny lab, WPI). The tdTomato signal is used to identify the *P. putida* strain in co-culture by flow cytometry.

To express *mScarlet* in E. coli, the coding region of mScarlet-I (Bindels et al., 2017) fused to the strong promoter J23101 was obtained by gene synthesis and cloned into pSB1C3 using the EcoRI and PstI restriction sites (plasmid created by Dr. Natalie Farny, WPI).

Soil Extract Production

Liquid soil extract for bacterial culturing was prepared by combining 100g of Miracle-Gro Performance Organics Potting Soil Mix with 500mL of 1X PBS in a 1L Erlenmeyer flask. The flask was then placed into a shaking incubator set at 220rpm and 37°C for at least 2-3 hours. Following the incubation period, the soil/PBS mixture was strained through a French press into Büchner funnel vacuum filtration system. The Büchner funnel was lined with two pieces of Whatman filter paper, one grade 4 piece layered on top of a grade 1 piece. After filtration through the vacuum system, the extract was filtered through a sterilizing 0.2-micron filter unit and the resulting liquid stored at 4°C until used for culturing.

Growth Curves by Spectrometry and Flow Cytometry

For all growth curves, each strain was prepared as an overnight culture at its optimal growth temperature in Luria-Bertani (LB) broth. The OD600 of each overnight culture was measured

using a spectrophotometer, and cultures were diluted, if needed, with LB broth or PBS, depending on the final growth medium, to get a reading within the linear range of the spectrophotometer (OD600 = 0.1-1.0).

Individual cultures were prepared by calculating the volume of overnight culture and LB broth or soil extract needed to get an OD600 of 0.1, at a chosen final volume. Examples of these calculations are shown in Figure 3.

$$\frac{0.1\ (desired\ final\ OD600)}{starting\ OD600\ of\ culture\ (possibly\ diluted)} = X * desired\ final\ volume$$

$$X * desired\ final\ volume = \boldsymbol{amount\ of\ starting\ culture}$$

$$desired\ final\ volume - amount\ of\ starting\ culture = \boldsymbol{amount\ of\ media}$$

Figure 3: Example starting calculations for individual cultures for co-culturing trials.

Cultures were grown at varying growth conditions. These conditions altered media (LB broth or soil extract) or growth temperature (25°C, 30°C, or 37°C). Samples were taken at the 0-, 1-, 2-, 3-, 4-, 5-, and 24-hour time points.

For growth curves that were examined by spectrometry, the machine was blanked with either LB broth or soil extract, depending on the growth medium of that culture, and then an OD600 measurement was taken of a 1mL sample of each culture. If any samples exceeded the linear range of the spectrophotometer, they were diluted and remeasured, and the resulting OD600 value was corrected based on the dilution factor used.

For growth curves that were examined by flow cytometry, each sample taken was prepared in triplicate using the “Flow Cytometry” protocol outlined below, and the stopping rules set to 10uL of sample run. The 0–5-hour time points were prepared on the sample plate, which was stored at 4°C between taken samples each hour to prevent continued growth in the cultures. The 24-hour time point was run on a separate plate the following day.

Co-Culturing

For all co-cultures, each strain was prepared as an overnight culture at its optimal growth temperature and in LB broth. The OD600 of each overnight culture was measured using a spectrophotometer, and cultures were diluted, if needed, with LB broth or PBS, depending on the final growth medium, to get a reading within the linear range of the spectrophotometer (OD600 = 0.1-1.0) and greater than 0.5.

Each individual culture was prepared by calculating the volume of overnight culture and LB broth or soil extract needed to get an OD600 of 0.5, at a chosen final volume. Co-cultures were prepared by calculating the volume of each overnight culture and LB broth or soil extract needed to get each strain at an OD600 of 0.25, a total OD600 of 0.5, at a chosen volume. Examples of these calculations are shown in Figures 4 and 5.

$$\frac{0.5\ (desired\ final\ OD600)}{starting\ OD600\ of\ culture\ (possibly\ diluted)} = X * desired\ final\ volume$$

$$X * desired\ final\ volume = \boldsymbol{amount\ of\ starting\ culture}$$

$$desired\ final\ volume - amount\ of\ starting\ culture = \boldsymbol{amount\ of\ media}$$

Figure 4: Example starting calculations for individual cultures for co-culturing trials.

$$\frac{0.25\ (desired\ final\ OD600)}{starting\ OD600\ of\ P.\ putida(possibly\ diluted)} = X * desired\ final\ volume$$

$$X * desired\ final\ volume = \boldsymbol{amount\ of\ P.putida}$$

$$\frac{0.25\ (desired\ final\ OD600)}{starting\ OD600\ of\ co-culture(possibly\ diluted)} = X * desired\ final\ volume$$

$$X * desired\ final\ volume = \boldsymbol{amount\ of\ co-culture}$$

$$desired\ final\ volume - (amount\ of\ P.putida + amount\ of\ co-culture) = \boldsymbol{amount\ of\ media}$$

Figure 5: Example starting calculations for co-cultures for co-culturing trials.

After individual and co-cultures were prepared at a final, total OD600 of 0.5, a set of 0-hour time point samples were prepared in triplicate for flow cytometry using the “Flow Cytometry” protocol outlined below, and the stopping rules set to 50,000 events counted of 600 seconds of run time. The remaining cultures were diluted 1:10 to an OD600 of 0.05 and left for 24 hours at varying growth conditions. These conditions altered media (LB broth or soil extract) or growth temperature (25°C, 30°C, or 37°C). The 24-hour time point samples were also analyzed by flow cytometry, using the same stopping rules as the 0-hour time point samples.

Live/Dead Cell Preparation

To test the effectiveness of the Live-or-Dye viability cell stain, the following strains were prepared using this protocol, adapted from (Robertson et al., (2019)) and (Molecular Probes Inc., (2004)): *P. putida*, *B. thailandensis*, and *C. freundii*. The strain was cultured overnight in 5mL of LB broth and then diluted to 2.5mL at an OD600 of 0.4-0.6 in 1X PBS. The 2.5mL of culture was transferred to a 15mL conical tube and concentrated by centrifugation at 3,900 rpm for 15 minutes. Without disturbing the pellet, the supernatant was poured off and then resuspended in 0.2mL of fresh 1X phosphate-buffered saline (PBS). The resulting suspension was split equally into two tubes, one containing 2mL 1X PBS (for the live cultures) and one containing 2mL 70% ethanol (for dead cultures). Both samples were then incubated at room temperature (~25°C) for 1 hour, mixing by hand every 15 minutes.

Live/Dead Cell Staining for Discrimination by Flow Cytometry

The following protocol, adapted from (Biotium, 2021), was used after the creation and any experimental incubation of co-cultures to stain for live-dead discrimination by flow cytometry using Biotium's Live-or-Dye™ 488/515 fixable viability staining kit. Each co-culture was diluted to an OD600 of 0.1 in LB Broth at a total volume of 3mL into 50mL conical tubes. The diluted culture was centrifuged at 2,000rpm for 5 minutes and the supernatant poured off. The pellet was washed in 1mL of 1X PBS and once again centrifuged at 2,000rpm for 5 minutes. The cells were then resuspended to an OD600 of 0.01 by adding 30mL of 1X PBS and 30uL of 488/515 Fixable Dead Cell Dye was added to the resulting suspension. The tube was then wrapped in aluminum foil to prevent light exposure and incubated on ice for 30 minutes. Following the incubation, tube tube of cells was washed with 30mL of 1X PBS and centrifuged at 2,000rpm for 5 minutes. The final pellet was resuspended in 30mL of 1X PBS and ready for analysis by flow cytometry.

Flow Cytometry Preparation and Analysis

To prepare samples for flow cytometry, a solution of 25mL of 1X PBS and 100uL of kanamycin (50 mg/mL stock, KAN) was prepared and vortexed. Into a 96-well plate, 150uL of the PBS+KAN solution was added to any well that would contain liquid culture sample. For each sample being run, 50uL of liquid culture was added to a well containing the PBS-KAN solution. After all samples were loaded, the second to last well was loaded with 200uL of flow cytometry calibration beads, and the last well was loaded with 200uL of 10% bleach. The plate was then run on a Beckman Coulter CytoFlex Flow Cytometer.

Raw cytometric data was analyzed primarily by using the TASBE analytics suite (Beal, 2019) in MATLAB; other data sets were examined by using the FlowJo software (BD Bioscience). Data sets analyzed by TASBE gated for only red cells, and the white cell count was calculated by subtracting the number of red cells from the total cell count. Analysis by FlowJo allowed for gating of both red and white cells.

Results

Growth Curves by Spectrometry

Growth curves were needed to measure monoculture growth rates which could be used as a baseline measurement for comparison with the results of co-cultures. The curves were generated for *P. putida*, *B. thailandensis*, and *C. freundii* at varying growth conditions, which included temperature (25°C, 30°C, or 37°C) and medium (LB broth or soil extract). Species growth was measured by optical density at 600nm, via a spectrophotometer, at the 0-,1-, 2-, 3-, 4-, 5-, and 24-hour time points. As shown in Figure 6, all three species of bacteria showed relatively similar trends and levels in growth across the varying conditions tested. However, outliers of these trends were seen in four of the generated graphs. Two of these showed 24-hour growth measurements significantly higher than the other species grown in those conditions. The first, seen in Panel C, is *P. putida* when grown in LB broth at 30°C. Second, shown in Panel F, is *B. thailandensis* when grown in soil extract at 25°C. The other two outliers showed 24-growth measurements significantly lower than the other species grown in those conditions. First, seen in Panel B, is *B. thailandensis* when grown in soil extract at 37°C, and the second, shown in Panel D, is *C. freundii* when grown in soil extract at 30°C. Overall, we observed that all three species grew with similar kinetics at 37°C in LB broth and in both media types at 25°C, while certain species displayed significantly different growth kinetics at the other growth temperatures and media.

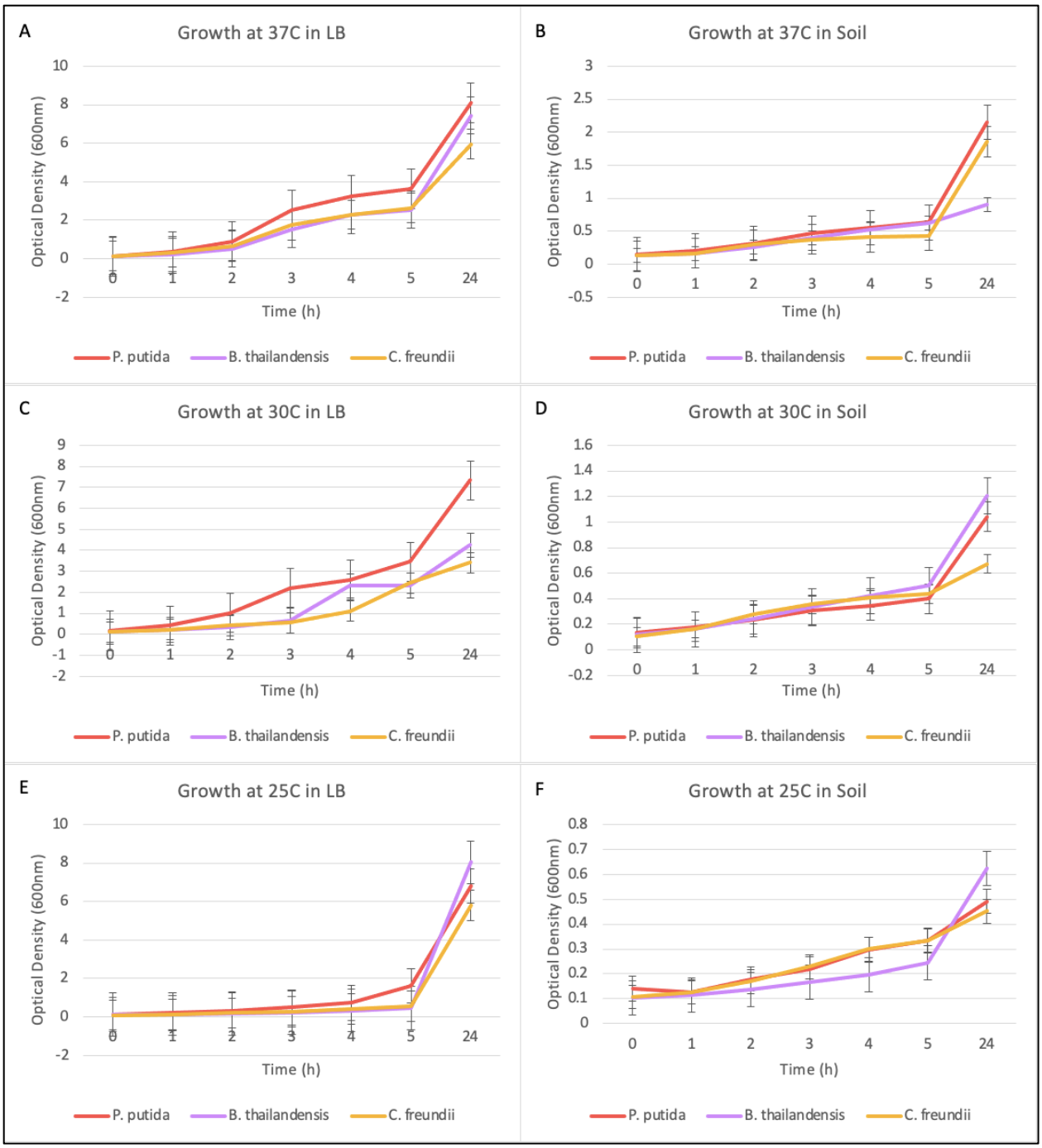

Figure 6: Growth of *P. putida*, *B. thailandensis*, and *C. freundii*, measured by spectrometry at 600nm. Growth conditions varied by temperature (25°C, 30°C, or 37°C) and/or medium (LB broth or soil extract). Measurements were taken at the 0-, 1-, 2-, 3-, 4-, 5-, and 24-hour time points. Each curve represents the averaged values of three biological replicates, each completed in technical triplicate. Error bars represent standard error of the mean.

Growth Curves by Flow Cytometry versus Spectrometry

While growth measurements are traditionally measured via spectrometry here, we developed assays for culture growth measurements via flow cytometry. Spectrometry required lots of time and materials, and other experiments in this paper showed that it is not always a reliable method

for measuring *P. putida* growth (see "Testing RFP's Role in Co-Culturing Results"). Therefore, the goal of utilizing flow cytometry was to circumvent these issues and costliness to produce growth curves similar to those produced by spectrometry. The curves were generated for *P. putida*, *B. thailandensis*, and *C. freundii*, each grown at their optimal growth temperature, reference Table 1, in LB broth. Measurements were taken at the 0-, 1-, 2-, 3-, 4-, 5-, and 24-hour time points. The growth curves generated by spectrometry are shown in Panel A of Figure 7, and the curves generated by flow cytometry are shown in Panel B. The final measurement of *B. thailandensis* taken by spectrometry was greater than the other two species but was lesser than them when measured by flow cytometry. We observed similar growth kinetics across both methods of measurement, other than the apparent inversion of *B. thailandensis*. However, it is important to note that this difference may not be statistically significant and will require further statistical analysis to investigate.

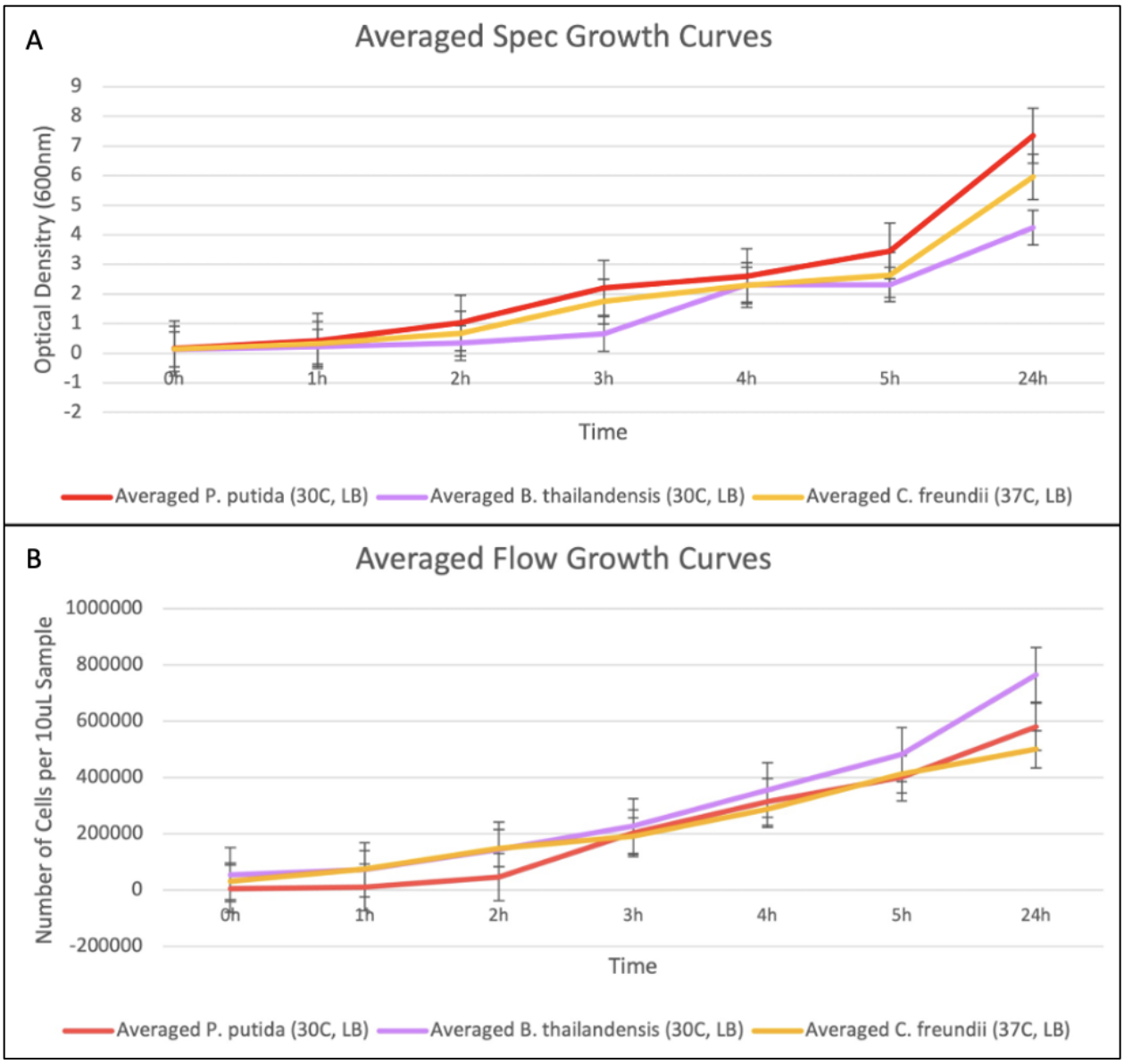

Figure 7: Growth of *P. putida* at 30°C, *B. thailandensis* at 30°C, and *C. freundii* at 37°C in LB broth, measured by spectrometry at 600nm, in Panel A, and by flow cytometry in Panel B. Measurements were taken at the 0-, 1-, 2-, 3-, 4-, 5-, and 24-hour time points. Each curve represents the averaged values of three biological replicates, each completed in technical triplicate. Error bars represent standard error of the mean.

Co-Culturing *P. putida* and *C. freundii*

The goal of co-culturing was to determine if the growth of *P. putida* was altered when placed in co-culture with another strain and begin to define the relationship between the two strains based on the relationships depicted in Figures 1 and 2. This set of co-cultures examined *P. putida* and *C. freundii*. The control 0-hour ratio measurement for *P. putida* and *C. freundii*, grown in both LB broth and soil extract, was not 50/50, and *P. putida* counts were consistently lower than they were calculated to be. When comparing this starting ratio to the 24-hour measurements grown in LB, seen in Panel A of Figure 8, *P. putida* levels increased when grown at both 25°C and 30°C, however, when both species were grown at 37°C, *P. putida* levels dropped. When the co-cultures

were grown in soil extract, seen in Panel B of Figure 8, *P. putida* levels increased at each variable growth temperature. In the end, we observed that *P. putida* increased its growth as a percentage of the co-culture the most in LB broth at 25°C, and decreased as it progressed to 37°C. In soil extract had the least growth at 25°C and increased as it progressed to 37°C.

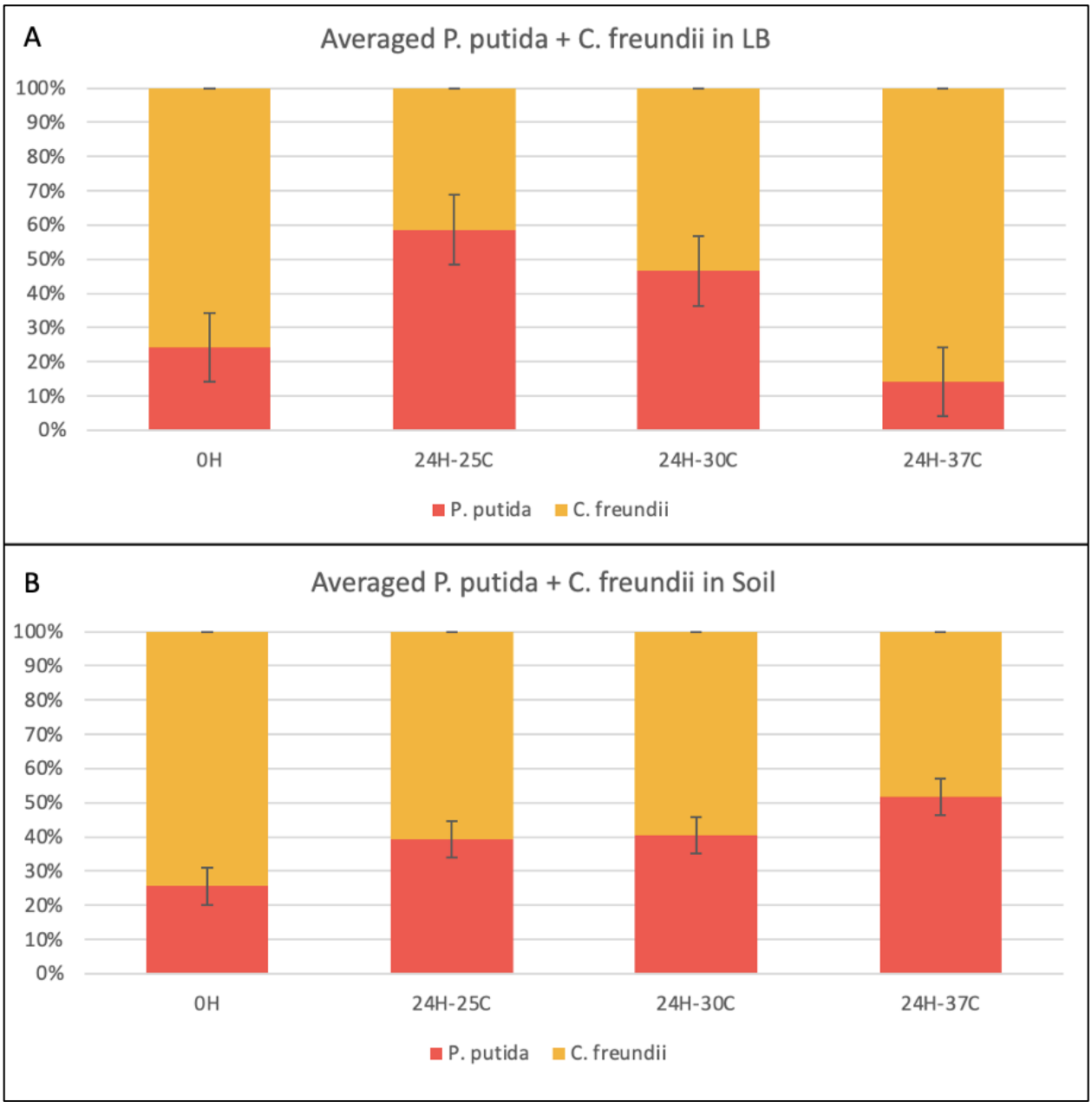

Figure 8: Resulting ratios of *P. putida* and *C. freundii* co-cultures grown in LB broth, in Panel A, and in soil extract, in Panel B. A control ratio was measured at the 0-hour time point and a final measurement taken at the 24-hour time point for each growth temperature variation (25°C, 30°C, and 37°C). Cell counts were calculated via flow cytometry and analyzed with the TASBE analytics suite for MATLAB (Beal et al., 2019). Each bar represents the averaged values of three biological replicates, each completed in technical triplicate. Error bars represent standard error of the mean.

Co-Culturing *P. putida* and *B. thailandensis*

This set of co-cultures sought to understand the dynamics between *P. putida* and *B. thailandensis*, with the same overall goal as with the co-cultures run on *P. putida* and *C. freundii*. Similar to the control 0-hour ratio measurement for *P. putida* and *C. freundii*, starting ratios of *P. putida* and *B. thailandensis* were not 50/50, and *P. putida* was once again consistently lower than expected. When comparing these starting ratios to the 24-hour measurements grown in LB, seen in Panel A of Figure 9, *P. putida* levels decreased when grown at both 25°C and increased when grown at 30°C. When the co-cultures were grown in soil extract, seen in Panel B of Figure 9, *P. putida* levels increased at each variable growth temperature. Overall, we saw that the growth of *P. putida* increased with temperature from 25°C to 30°C in both LB broth and soil extract.

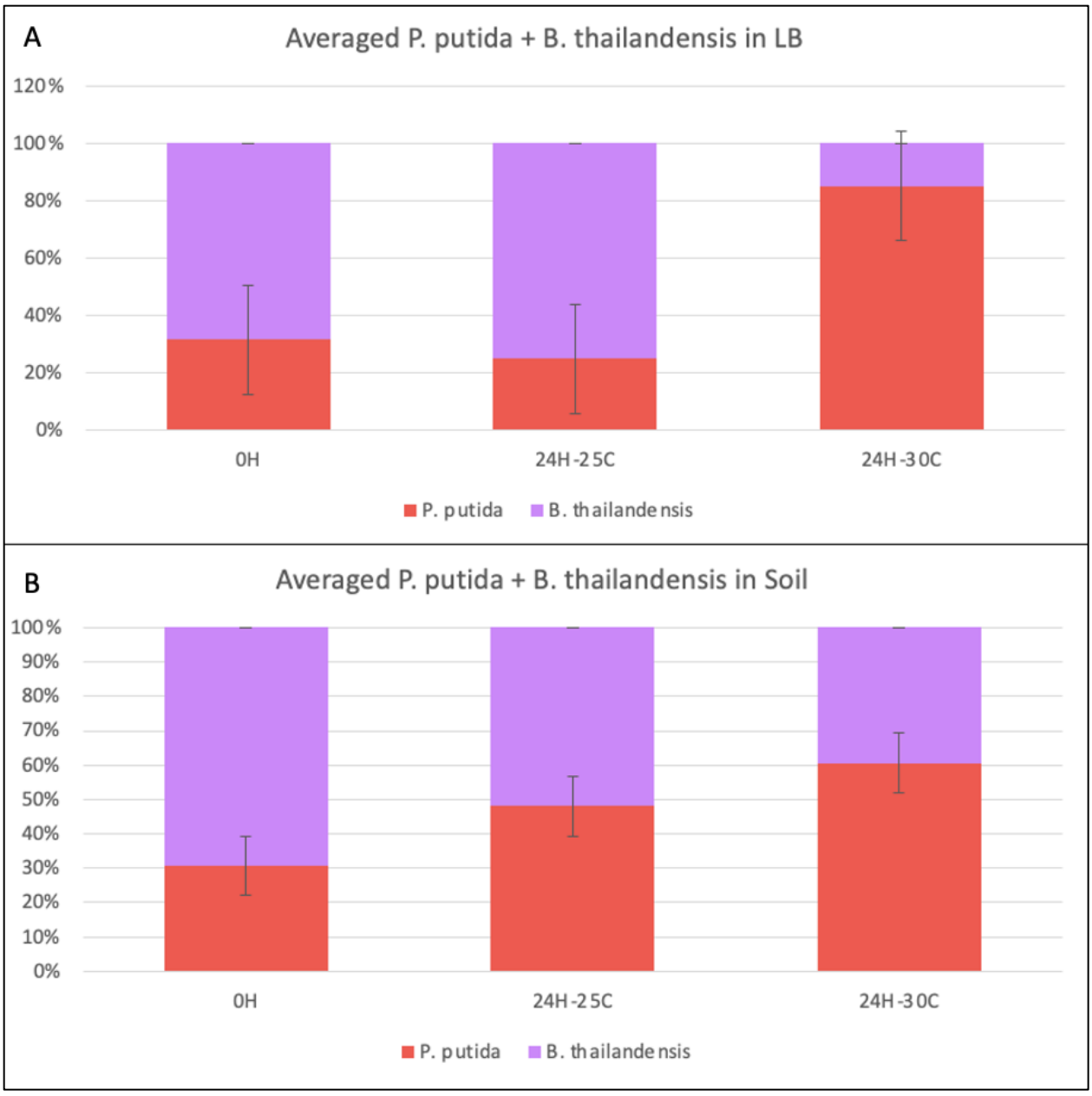

Figure 9: Resulting ratios of *P. putida* and *B thailandensis* co-cultures grown in LB broth, in Panel A, and in soil extract, in Panel B. A control ratio was measured at the 0-hour time point and a final measurement taken at the 24-hour time point for each growth temperature variation (25°C and 30°C). Cell counts were calculated via flow cytometry and analyzed with the TASBE analytics suite for MATLAB (Beal et al., 2019). Each bar represents the averaged values of three biological replicates, each completed in technical triplicate. Error bars represent standard error of the mean.

Testing the Effect of Red Fluorescent Proteins on OD600 Measurements

Throughout the co-culture experiments, it was observed that the number of *P. putida* cells identified by flow cytometry was always significantly less than the 50% target that was attempted to establish using OD600 measurements. As a result of the *P. putida* counts always being less than 50/50 when used in starting co-culture ratios, this set of experiments aimed to

determine if there was a possible interference in the OD600 measurement by the red fluorescent protein (RFP, tdTomato) used to differentiate *P. putida* from the other species, such as described by Hecht et al. (2016), or if the *P. putida* itself was causing issues when performing initial calculations from the OD600 measurements. To test this hypothesis, untagged *P. putida* was co-cultured with a strain of *E. coli* that was tagged with the RFP mScarlet, and the initial ratios were calculated and displayed in Figure 10. The initial ratios for these new co-cultures also showed a less than 50/50 ratio with *P. putida* having counts less than the *E. coli* strain. Therefore, we showed that potential interference with the OD600 measurement by RFP did not explain the consistent underestimation of *P. putida* by spectrometry.

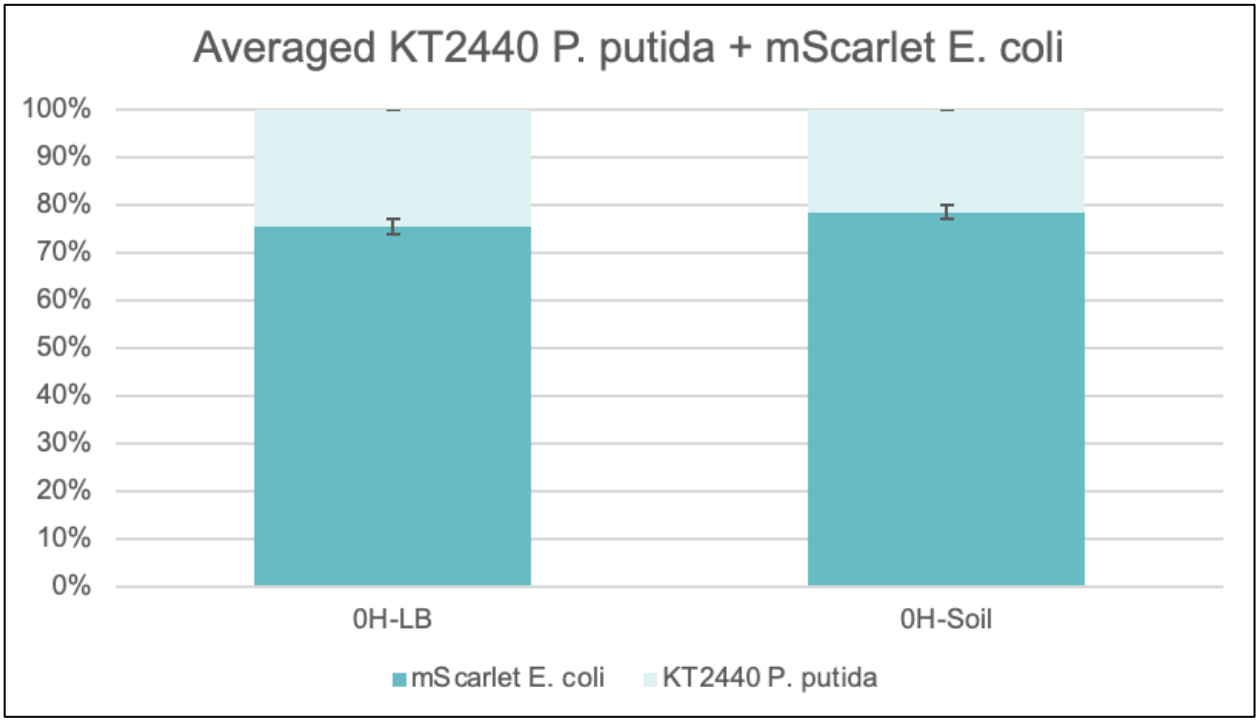

Figure 10: Resulting 0-hour ratios of mScarlet *E. coli* and KT2440 *P. putida* co-cultures grown in LB broth, left bar, and in soil extract, right bar. Cell counts were calculated via flow cytometry and analyzed with the TASBE analytics suite for MATLAB (Beal et al., 2019). Each bar represents the averaged values of three biological replicates, each completed in technical triplicate. Error bars represent standard error of the mean.

Cytometric Analysis of Co-Cultures by TASBE versus FlowJo

Much of this research was analyzed raw flow cytometry data with the TASBE analytics suite for MATLAB (Beal et al., 2019). However, this was a very time-consuming method and there was no easy way to gate for white cells, only red, so it is probably ratios were misrepresentative because debris in samples was likely counted as white cells. Another popular software used for cytometric analysis is FlowJo, which is known for its simple user interface and gating abilities on large, complex samples. To test the analytic abilities of both methods, a data set of both co-cultures, and all their respective variables were analyzed with both tools. The results of the *P. putida* and *C. freundii* co-cultures are shown in Figure 11. From Panel A, LB broth culture analysis by TASBE, to Panel B, LB broth culture analysis by FlowJo, the relative trends of ratio

change from the 0-hour measurement to the 24-hour measurements were the same for each of the different growth temperatures. However, the ratio seen in Panel A for the 37°C culture showed a nearly perfect inversion to the ratio seen in Panel B, where *P. putida* becomes the dominating culture when compared to *C. freundii*. The results from Panel C, soil extract culture analysis by TASBE, to Panel D, soil extract culture analysis by FlowJo, the relative trends are the same across all growth temperatures analyzed.

The results of the *P. putida* and *B. thailandensis* co-cultures are shown in Figure 12. From Panel A, LB broth culture analysis by TASBE, to Panel B, LB broth culture analysis by FlowJo, the relative trends of ratio change from the 0-hour measurement to the 24-hour measurements were the same for the 30°C sample but changed for the 25°C sample. It is important to note, the 30°C sample showed no presence of *B.* thailandensis. The results from Panel C, soil extract culture analysis by TASBE, to Panel D, soil extract culture analysis by FlowJo, the relative trends are the same across all growth temperatures analyzed.

Overall, analysis by FlowJo produced graphs and trends similar to those generated by TASBE analysis; the few outliers that showed drastic changes in the ratio between the species showed an increase in *P. putida* levels. This can likely be attributed to the ability to gate for and get total white cells counts in FlowJo, which is not something that could be done in TASBE. However more analysis by FlowJo will be needed to determine the exact reason for the changes in ratios between analytic techniques.

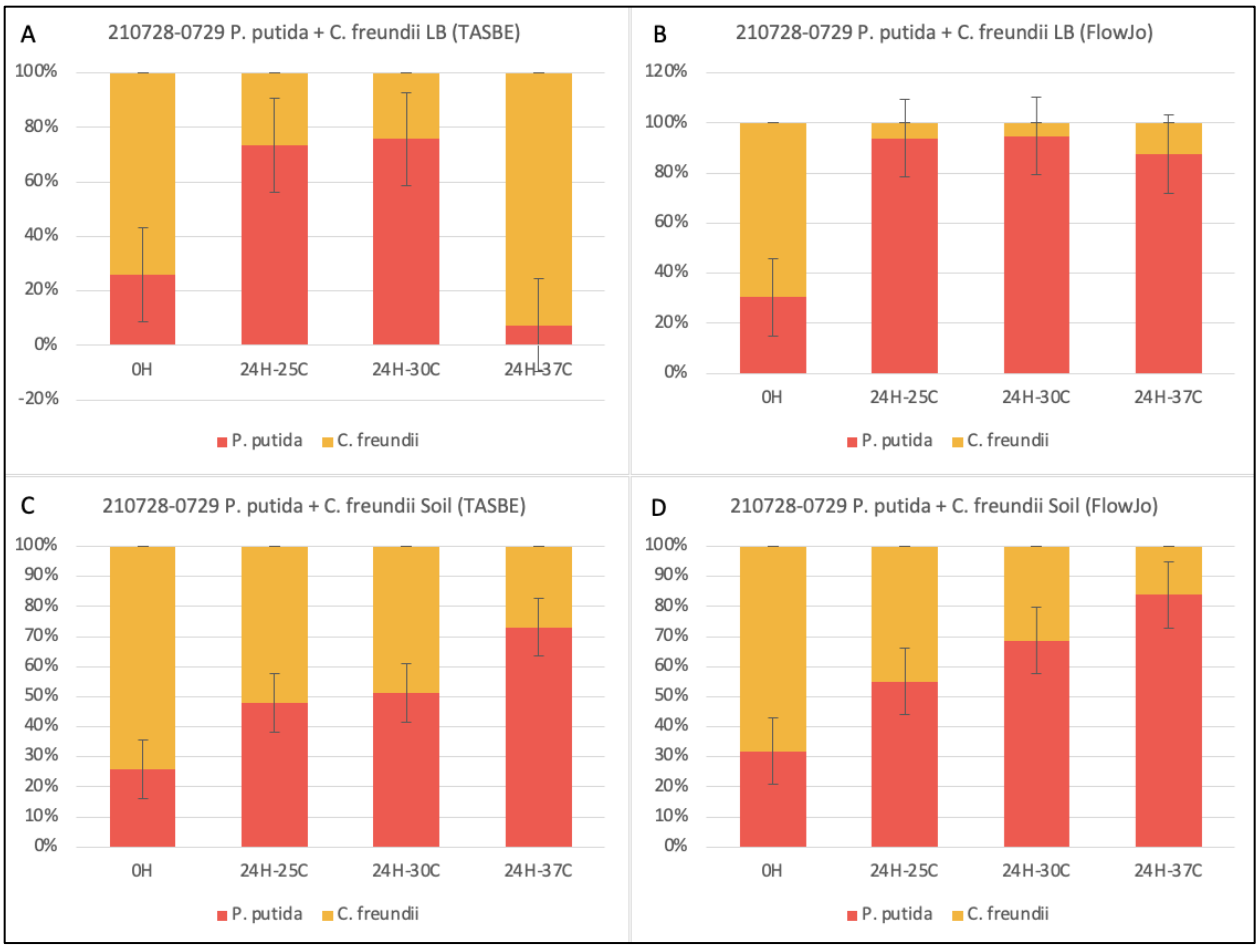

Figure 11: Resulting ratios of *P. putida* and *C. freundii* co-cultures grown in LB broth, Panels A and B, and in soil extract, Panels C and D. Cell counts were calculated via flow cytometry and analyzed with FlowJo, in Panels B and D, or the TASBE analytics suite for MATLAB, in Panels A and C (Beal et al., 2019). Each bar represents the averaged values of a single biological sample measured in triplicate. Error bars represent standard error of the mean.

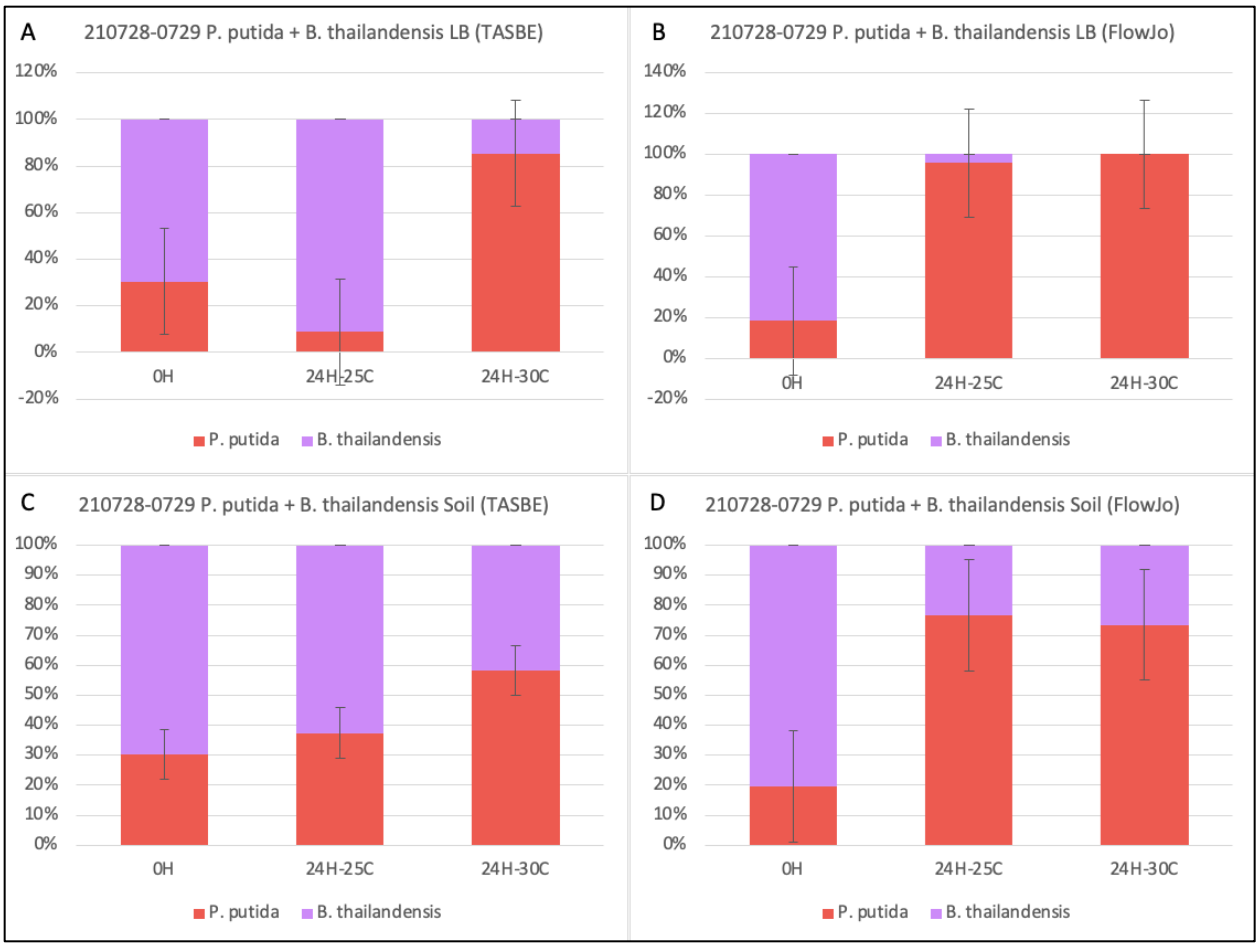

Figure 12: Resulting ratios of *P. putida* and *B. thailandensis* co-cultures grown in LB broth, Panels A and B, and in soil extract, Panels C and D. Cell counts were calculated via flow cytometry and analyzed with FlowJo, in Panels B and D, or the TASBE analytics suite for MATLAB, in Panels A and C (Beal et al., 2019). Each bar represents the averaged values of a single biological sample measured in triplicate. Error bars represent standard error of the mean.

Live/Dead Analysis of Co-Cultures by Flow Cytometry

While the results of the co-cultures and growth curves can provide basic information on the relationships of the species in the co-culture, they cannot give any data on the viability of the cells. Therefore, this set of experiments utilized a Live/Dead cell stain, outlined in Figure 13, to measure viability of the species in the cultures, so in future experiments the relationship between the two species in co-culture can be more specifically defined.

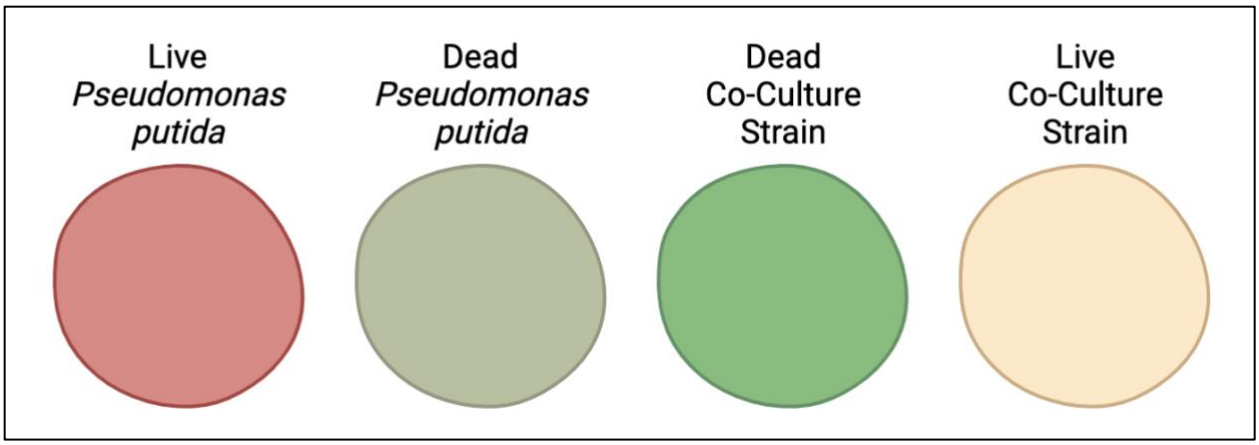

Figure 13: Four different cells differentiated by color using integrated fluorescent tagging (*P. putida*), and the use of a green-channel cell permeable viability dye.

In order to visualize both live and dead cells within a culture, cultures of *P. putida* and *C. freundii* were ethanol fixed to kill the cells, then mixed back into live cultures, stained with the live/dead stain, and analyzed by flow cytometry. Seen in Figure 14, the use of this dye was able to produce four, distinct populations in a co-culture sample, and each of these populations can define a species type and its viability state as predicted in Figure 13. While this stain was only used on one sample in this research, there are future implications for its use in defining bacterial relationships. The population representing living *P. putida* is noticeably smaller than the others, however visualization of the pellet of these cells was lost during the centrifugation process, so this was not unexpected. In the end, the ability to produce four, distinct populations indicates that we will be able to implement this viability dye into all future co-culture experiments to further inform the defined relationship between species in said co-culture.

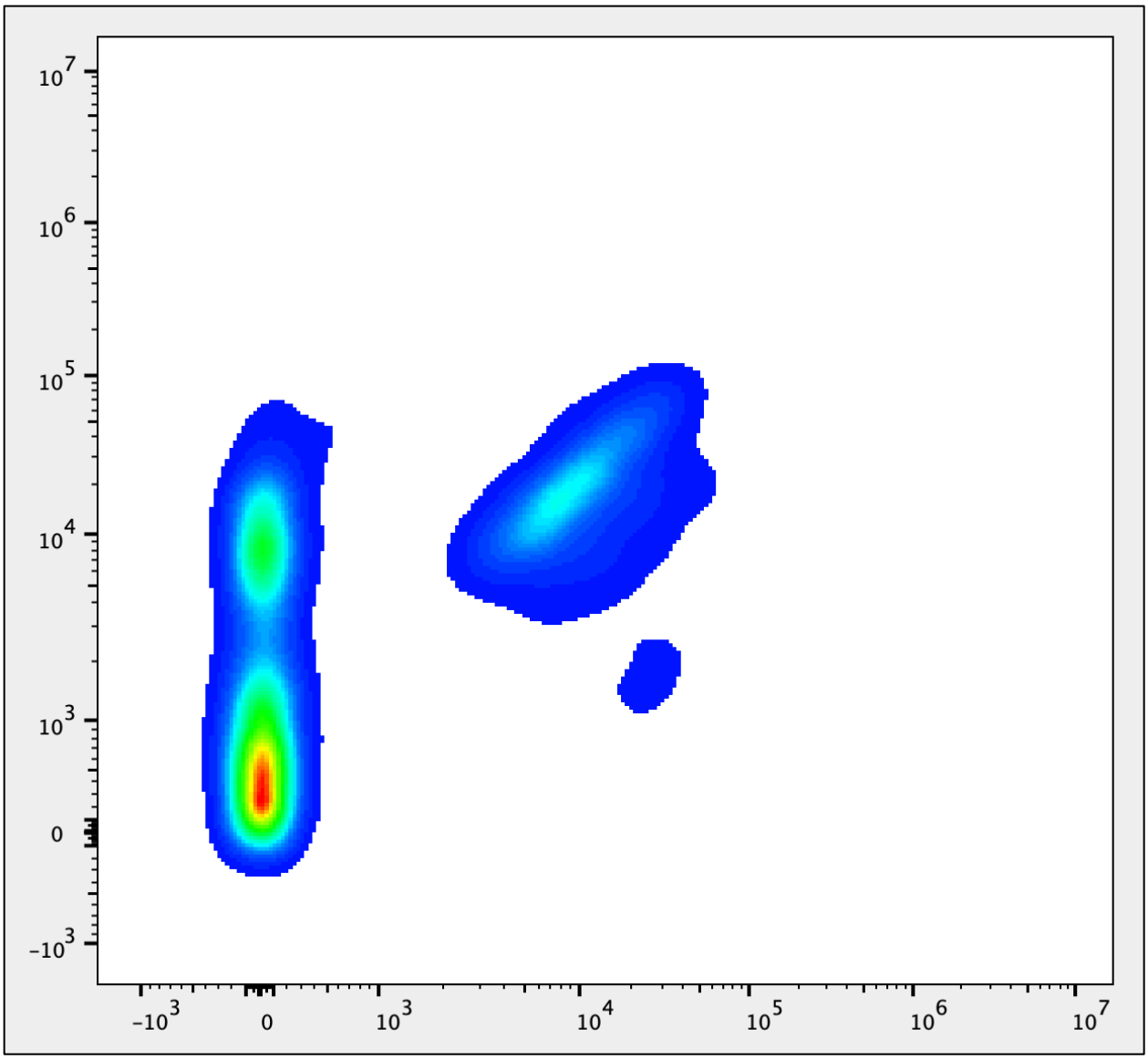

Figure 14: Results of Live/Dead trial collected from flow cytometry and analyzed in FlowJo. Four distinct populations are present in the field. The bottom left indicates living white cells, the top left is dead white cells, the top right is dead *P. putida*, and the bottom right is living *P. putida*.

Discussion

Relationship Analysis of *P. putida* and *C. freundii* in Co-Culture

Based on the results of the growth curves displayed in panels A, C, and E of Figure 6, it was expected, in a neutral relationship, that the amount of *P. putida* would increase compared to *C. freundii* when grown for 24h at 30°C and 37°C in LB broth, and that the ratio between the two species would remain relatively the same when grown in the same medium for 24h at 25°C. These expected results were not shown in the ratios produced in panel A of Figure 8. Instead, *P. putida* levels, when grown in LB broth for 24h, increased significantly at 25°C and decreased slightly at 37°C. However, *P. putida* levels did increase, as expected, when grown in LB broth for 24h at 30°C, but not as drastically as what was predicted by Figure 6.

When grown in soil extract, the growth curves in panels B, D, and F of Figure 6 predicted that there would be no significant difference in the ratio of *P. putida* to *C. freundii* when grown in soil extract for 24h at 25°C and 37°C, and that there would be in increase in *P. putida* levels when grown in the same medium for 24h at 30°C. Panel B of Figure 8 shows that there was an increase in *P. putida* at all temperatures tested.

Relationship Analysis of *P. putida* and *B. thailandensis* in Co-Culture

The growth curves in panels A, C, and E of Figure 6 show an expected increase, in a neutral relationship, in the levels of *P. putida* compared to *B. thailandensis* when grown for 24h in LB broth at 30°C, and that the ratio between the two species would have no change when grown in the same medium for 24h at 25°C. The expected increase of *P. putida* levels at 30°C was seen in the ratios in panel A of Figure 9, however, an unexpected slight decrease in *P. putida* levels was exhibited at 25°C.

Growth curves seen in panels B, D, and F of Figure 6 predicted an increase in *B. thailandensis* compared to *P. putida* when grown in soil extract for 24h at 25°C and no change in the ratio between the two species when grown the same medium for 24h at 30°C. The resulting ratios in panel B of Figure 9 show an increase in *P. putida* at both 25°C and 30°C, which was unexpected for both sets of growth conditions tested.

Defining Microbial Relationships

Expected results of co-culturing based on growth curves suggest a neutral, competitive, or mutualistic relationship between the two species being cultured in those growth conditions. Any unexpected results, suggest that there is a parasitic, amenalistic, or commensalistic, relationship between the two species in those growth conditions. However, at this stage of experimentation it cannot be determined what the exact relationship is between the two species, even if the results were expected. This can be determined by further research implementing the Live/Dead dye to determine the viability of each species throughout growth in co-culture.

Kehe et al. (2021) showed that microbes of the *Pseudomonadaceae* and *Enterobacteraceae* families show a greater than expected prevalence of positive interactions (i.e., mutualism and commensalism) between species when grown in varying carbon conditions. This contradicted previous work by Foster & Bell (2012), which suggested that microbes in co-culture exhibited more negative interactions (i.e., parasitism, amenalism, and competition). Continuing experimentation with the strains used in this study could give more information on the exact type of relationships between these species when grown in different environmental conditions.

Similar to the other studies in the research of soil derived microbes, this work has yet to generate a growth environment similar to that of the real-world which contains many diverse bacteria. There is a limited supply of readily available species that could be easily cultured in the media and conditions used here. As this research continues, the movement to more than two species per co-culture will be critical in determining how the interplay of multiple species affects the growth and viability of target microbes and GEMs. Further, the implementation of Live/Dead viability dye in all future co-cultures will allow for more specific definition of the relationships between species in co-culture. Other future additions to this research include continued analysis with FlowJo cytometric analytic software, with emphasis on gating white and colored cells to remove debris counts from the final ratios, which, in theory, will help with correcting the difference in ratios between FlowJo and TASBE. Additional experiments may involve the addition of a defined carbon source to the soil extract cultures to determine if the carbon in LB broth plays a role in the ratios produced and finding a work-around the challenges encountered when taking OD600 measurements of *P. putida*.

The results of this work and future research from it, will allow for large scale models of soil environments to be developed, in which potential SynBio microbes' viability and environmental impact can be tested prior to their use in the real world. Knowing this, researchers can make hypotheses and test the engineered microbes for potential negative effects they may have when used in the environment.

References

Beal, J., Overney, C., Adler, A., Yaman, F., Tiberio, L., & Samineni, M. (2019). TASBE Flow Analytics: A Package for Calibrated Flow Cytometry Analysis. *ACS Synthetic Biology*. https://tasbe.github.io/

Bindels, D. S., Haarbosch, L., van Weeren, L., Postma, M., Wiese, K. E., Mastop, M., Aumonier, S., Gotthard, G., Royant, A., Hink, M. A., & Gadella, T. W., Jr (2017). mScarlet: a bright monomeric red fluorescent protein for cellular imaging. *Nature methods, 14*(1), 53–56. https://doi.org/10.1038/nmeth.4074

Biotium. (2021). *Product Information: Live-or-Dye™ Fixable Viability Staining Kits*. Fremont, California.

Elmore, J. R., Furches, A., Wolff, G. N., Gorday, K., & Guss, A. M. (2017). Development of a high efficiency integration system and promoter library for rapid modification of *Pseudomonas putida* KT2440. *Metabolic Engineering Communications, 5*, 1-8. https://doi.org/10.1016/j.meteno.2017.04.001

Faust, K., & Raes, J. (2012). Microbial interactions: from networks to models. *Nature reviews. Microbiology, 10*(8), 538–550. https://doi.org/10.1038/nrmicro2832

Foster, K. R., & Bell, T. (2012). Competition, not cooperation, dominates interactions among culturable microbial species. *Current biology: CB, 22*(19), 1845–1850. https://doi.org/10.1016/j.cub.2012.08.005

Giaouris, E., Chorianopoulos, N., Doulgeraki, A., & Nychas, G. J. (2013). Co-culture with Listeria monocytogenes within a dual-species biofilm community strongly increases resistance of Pseudomonas putida to benzalkonium chloride. *PloS one, 8*(10), e77276. https://doi.org/10.1371/journal.pone.0077276

Hecht, A., Endy, D., Salit, M., & Munson, M. S., When Wavelengths Collide: Bias in Cell Abundance Measurements Due to Expressed Fluorescent Proteins. *ACS Synth. Biol., 5*(9), 1024-1027. https://doi.org/10.1021/acssynbio.6b00072

Kehe, J., Ortiz, A., Kulesa, A., Gore, J., Blainey, P. C., & Friedman, J. (2021). Positive interactions are common among culturable bacteria. *Science advances, 7*(45), eabi7159. https://doi.org/10.1126/sciadv.abi7159

Molecular Probes Inc. (2004). *LIVE/DEAD® BacLight™ Bacterial Viability Kits*. Eugene, Oregon.

Molina-Santiago, C., Udaondo, Z., Cordero, B. F., & Ramos, J. L. (2017). Interspecies cross-talk between co-cultured Pseudomonas putida and Escherichia coli. *Environmental microbiology reports, 9*(4), 441–448. https://doi.org/10.1111/1758-2229.12553

Robertson, J., McGoverin, C., Vanholsbeeck, F., & Swift, S. (2019). Optimisation of the Protocol for the LIVE/DEAD® BacLight™ Bacterial Viability Kit for Rapid Determination of Bacterial Load. *Front Microbiology. 10,* 1-13. https://doi.org/10.3389/fmicb.2019.00801

Sayler, G. S., & Ripp, S. (2000). Field applications of genetically engineered microorganisms for bioremediation processes. *Current opinion in biotechnology, 11*(3), 286–289. https://doi.org/10.1016/s0958-1669(00)00097-5

Stirling, F., & Silver, P. A. (2020). Controlling the Implementation of Transgenic Microbes: Are We Ready for What Synthetic Biology Has to Offer?. *Molecular cell, 78*(4), 614–623. https://doi.org/10.1016/j.molcel.2020.03.034

Weimer, A., Kohlstedt, M., Volke, D. C., Nikel, P. I., & Wittmann, C. (2020). Industrial biotechnology of Pseudomonas putida: advances and prospects. *Applied microbiology and biotechnology, 104*(18), 7745–7766. https://doi.org/10.1007/s00253-020-10811-9

Appendix

Appendix A: Raw data from growth curves by spectrometry

	210624_LB_25C			210629_LB_25C			210630_LB_25C			Averaged_LB_25C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.139	0.121	0.095	0.16	0.119	0.12	0.092	0.112	0.091	0.13033	0.117333333	0.102
1	0.249	0.145	0.119	0.278	0.13	0.156	0.094	0.147	0.111	0.207	0.140666667	0.128667
2	0.432	0.2	0.198	0.433	0.184	0.262	0.143	0.134	0.146	0.336	0.172666667	0.202
3	0.686	0.282	0.291	0.527	0.233	0.387	0.278	0.156	0.195	0.497	0.223666667	0.291
4	0.981	0.461	0.419	0.737	0.325	0.521	0.493	0.211	0.299	0.737	0.332333333	0.413
5	3.23	0.691	0.546	0.92	0.448	0.712	0.693	0.251	0.462	1.61433	0.463333333	0.573333
24	9.22	7.91	2.51	5.23	9.96	7.39	5.96	6.25	7.45	6.80333	8.04	5.783333

	210624_Soil_25C			210629_Soil_25C			210630_Soil_25C			Averaged_Soil_25C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.103	0.11	0.101	0.127	0.111	0.119	0.193	0.093	0.103	0.141	0.104666667	0.107667
1	0.114	0.108	0.125	0.174	0.107	0.123	0.092	0.126	0.129	0.12667	0.113666667	0.125667
2	0.152	0.152	0.185	0.292	0.14	0.183	0.092	0.118	0.138	0.17867	0.136666667	0.168667
3	0.197	0.186	0.245	0.353	0.183	0.249	0.106	0.132	0.191	0.21867	0.167	0.228333
4	0.297	0.216	0.328	0.473	0.223	0.319	0.121	0.155	0.25	0.297	0.198	0.299
5	0.299	0.275	0.365	0.549	0.27	0.353	0.153	0.193	0.285	0.33367	0.246	0.334333
24	0.518	0.639	0.432	0.646	0.692	0.472	0.307	0.539	0.449	0.49033	0.623333333	0.451

	210617_LB_30C			210622_LB_30C			210624_LB_30C			Averaged_LB_30C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.152	0.158	0.133	0.208	0.128	0.122	0.139	0.121	0.095	0.16633	0.135666667	0.116667
1	0.368	0.309	0.279	0.549	0.194	0.244	0.356	0.173	0.162	0.42433	0.225333333	0.228333
2	0.55	0.342	0.504	1.62	0.33	0.467	0.898	0.349	0.312	1.02267	0.340333333	0.427667
3	*0.75466	*0.453666666666	*0.676333	2.05	0.668	0.501	3.82	0.834	0.498	2.20822	0.651888889	0.558444
4	1.56	1.88	1.67	2.59	2.27	0.796	3.64	2.78	0.864	2.59667	2.31	1.11
5	1.72	1.71	1.37	3.63	2.31	0.9075	5.02	2.94	5.04	3.45667	2.32	2.439167
24	4.87	3.87	3.55	6.01	2.730933333	2.8	11.14	6.13	3.91	7.34	4.243644444	3.42

	210617_Soil_30C			210622_Soil_30C			210624_Soil_30C			Averaged_Soil_30C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.108	0.114	0.108	0.187	0.129	0.106	0.103	0.11	0.101	0.13267	0.117666667	0.105
1	0.143	0.172	0.167	0.274	0.17	0.153	0.125	0.139	0.171	0.18067	0.160333333	0.163667
2	0.2	0.267	0.288	0.355	0.265	0.28	0.162	0.196	0.264	0.239	0.242666667	0.277333
3	*0.24233	*0.33733333333	*0.367666	0.44	0.409	0.366	0.234	0.261	0.338	0.30544	0.335777778	0.357222
4	0.219	0.393	0.409	0.591	0.542	0.439	0.228	0.343	0.374	0.346	0.426	0.407333
5	0.226	0.433	0.44	0.665	0.665	0.468	0.312	0.409	0.397	0.401	0.502333333	0.435
24	0.807	0.925	0.655	1.77	2.05	0.595	0.548	0.642	0.77	1.04167	1.205666667	0.673333

	210722_LB_37C			210727_LB_37C			210728_LB_37C			Averaged_LB_37C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.117	0.126	0.114	0.102	0.112	0.154	0.162	0.164	0.172	0.127	0.134	0.146667
1	0.36	0.216	0.277	0.39	0.184	0.35	0.348	0.213	0.335	0.366	0.204333333	0.320667
2	0.914	0.485	0.555	0.89	0.545	0.806	0.913	0.56	0.638	0.90567	0.53	0.666333
3	2.91	0.949	0.947	1.44	0.97	1.97	3.2	2.7	2.33	2.51667	1.539666667	1.749
4	2.96	3.54	3.3	3.82	1.07	1.45	3.02	2.22	2.14	3.26667	2.276666667	2.296667
5	3.56	3.19	3.16	3.88	1.44	1.99	3.43	3.01	2.77	3.62333	2.546666667	2.64
24	8.26	9.24	6.12	8.7	7.55	4.55	7.31	5.5	7.2	8.09	7.43	5.956667

	210722_Soil_37C			210727_Soil_37C			210728_Soil_37C			Averaged_Soil_37C		
	Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm			Optical Density at 600nm		
Hour	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii	P. putida	B. thailandensis	C. freundii
0	0.089	0.119	0.106	0.108	0.094	0.098	0.258	0.2	0.186	0.16633	0.135666667	0.116667
1	0.18	0.154	0.169	0.171	0.135	0.155	0.249	0.18	0.175	0.42433	0.225333333	0.228333
2	0.309	0.225	0.331	0.33	0.231	0.275	0.312	0.328	0.291	1.02267	0.340333333	0.427667
3	0.482	0.342	0.429	0.482	*0.29033333333	*0.353	0.447	0.55	0.345	2.20822	0.651888889	0.558444
4	0.542	0.455	0.454	0.632	0.473	0.388	0.501	0.665	0.4	2.59667	2.31	1.11
5	0.647	0.554	0.48	0.699	0.574	0.393	0.553	0.74	0.422	3.45667	2.32	2.439167
24	3.01	0.804	3.74	0.878	0.945	0.846	2.56	0.968	0.993	7.34	4.243644444	3.42

*Estimated value

Appendix B: Raw data from growth curves by spectrometry versus flow cytometry

P. putida_LB_30C_Spec				
	Optical Density at 600nm			
Hour	210617	210622	210624	Averaged
0	0.152	0.208	0.139	0.166333
1	0.368	0.549	0.356	0.424333
2	0.55	1.62	0.898	1.022667
3	*0.75466(	2.05	3.82	2.208222
4	1.56	2.59	3.64	2.596667
5	1.72	3.63	5.02	3.456667
24	4.87	6.01	11.14	7.34

P. putida_LB_30C_Flow				
	Number of Cells per 10uL Sample			
Hour	210913	211013	211013	Averaged
0	3007	5638.33	7880.33	5508.556
1	3662	14730.7	13102.7	10498.44
2	16530	40431.3	83704.7	46888.67
3	197147	203761	207611	202839.7
4	390189	266277	286857	314441
5	587661	315499	299560	400907
24	959719	365708	417913	581113.1

B. thailandensis_LB_30C_Spec				
	Optical Density at 600nm			
Hour	210617	210622	210624	Averaged
0	0.158	0.128	0.121	0.135667
1	0.309	0.194	0.173	0.225333
2	0.342	0.33	0.349	0.340333
3	*0.45366(	0.668	0.834	0.651889
4	1.88	2.27	2.78	2.31
5	1.71	2.31	2.94	2.32
24	3.87	2.73093	6.13	4.243644

B. thailandensis_LB_30C_Flow				
	Number of Cells per 10uL Sample			
Hour	210913	211013	211013	Averaged
0	18511.3	69785	77607	55301.11
1	22259.3	91222.3	103750	72410.67
2	84432.7	166897	183116	144815.2
3	169135	254574	258849	227519.3
4	391561	333345	343440	356115.2
5	741962	354619	348764	481782
24	1595967	348850	352553	765790.2

C. freundii_LB_37C_Spec				
	Optical Density at 600nm			
Hour	210722	210727	210728	Averaged
0	0.114	0.154	0.172	0.146667
1	0.277	0.35	0.335	0.320667
2	0.555	0.806	0.638	0.666333
3	0.947	1.97	2.33	1.749
4	3.3	1.45	2.14	2.296667
5	3.16	1.99	2.77	2.64
24	6.12	4.55	7.2	5.956667

C. freundii_LB_37C_Flow				
	Number of Cells per 10uL Sample			
Hour	210913	211013	211013	Averaged
0	20232.3	37794	38670.3	32232.22
1	59988	83078.3	82525.3	75197.22
2	166081	142361	140029	149490.4
3	262234	166251	148879	192454.4
4	381030	239082	244482	288197.9
5	626891	313921	295412	412074.7
24	811856	342755	348640	501083.6

*Estimated value

Appendix C: Raw data from co-cultures of *P. putida* and *C. freundii*

0h_P. putida + C. freundii_LB									0h_P. putida + C. freundii_Soil							
	Number of Cells per 50,000 Events									Number of Cells per 50,000 Events						
	210722-0723		210726-0727		210728-0729					210722-0723		210726-0727		210728-0729		
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells			Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	
1	15850	34150	11187	38813	12994	37006			1	11425	38575	13236	36764	13049	36951	
2	8953	41047	10594	39406	13091	36909			2	13033	36967	13123	36877	12812	37188	
3	13024	36976	10622	39378	12707	37293			3	11987	38013	13369	36631	12922	37078	
Averaged	12609	37391	10801	39199	12930.6667	37069.3333			Averaged	12148.3333	37851.6667	13242.6667	36757.3333	12927.6667	37072.3333	
24h_25C_P. putida + C. freundii_LB									24h_25C_P. putida + C. freundii_Soil							
	Number of Cells per 50,000 Events									Number of Cells per 50,000 Events						
	210722-0723		210726-0727		210728-0729					210722-0723		210726-0727		210728-0729		
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells			Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	
1	12467	37533	39562	10438	36630	13370			1	7054	42946	27928	22072	22423	27577	
2	12050	37950	39593	10407	36972	13028			2	8077	41923	26359	23641	24662	25338	
3	12769	37231	37154	12846	36638	13362			3	8901	41099	26847	23153	24894	25106	
Averaged	12428.6667	37571.3333	38769.6667	11230.3333	36746.6667	13253.3333			Averaged	8010.66667	41989.3333	27044.6667	22955.3333	23993	26007	
24h_30C_P. putida + C. freundii_LB									24h_30C_P. putida + C. freundii_Soil							
	Number of Cells per 50,000 Events									Number of Cells per 50,000 Events						
	210722-0723		210726-0727		210728-0729					210722-0723		210726-0727		210728-0729		
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells			Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	
1	13608	36392	20049	29951	38776	11224			1	17728	32272	18397	31603	25573	24427	
2	12883	37117	18528	31472	37011	12989			2	16680	33320	19812	30188	25689	24311	
3	12957	37043	17907	32093	37706	12294			3	15834	34166	17051	32949	25703	24297	
Averaged	13149.3333	36850.6667	18828	31172	37831	12169			Averaged	16747.3333	33252.6667	18420	31580	25655	24345	
24h_37C_P. putida + C. freundii_LB									24h_37C_P. putida + C. freundii_Soil							
	Number of Cells per 50,000 Events									Number of Cells per 50,000 Events						
	210722-0723		210726-0727		210728-0729					210722-0723		210726-0727		210728-0729		
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells			Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	
1	15160	34840	3098	46902	3108	46892			1	18250	31750	26738	23262	35814	14186	
2	12891	37109	2844	47156	4112	45888			2	11232	38768	29172	20828	36605	13395	
3	15563	34437	3085	46915	3894	46106			3	9911	40089	27395	22605	37252	12748	
Averaged	14538	35462	3009	46991	3704.66667	46295.3333			Averaged	13131	36869	27768.3333	22231.6667	36557	13443	

Appendix D: Raw data from co-cultures of *P. putida* and *B. thailandensis*

0h_P. putida + B. thailandensis_LB						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	18514	31486	13860	36140	14747	35253
2	18575	31425	13776	36224	15313	34687
3	18530	31470	13316	36684	15452	34548
Averaged	18539.6667	31460.3333	13650.6667	36349.3333	15170.6667	34829.3333

24h_25C_P. putida + B. thailandensis_LB						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	18691	31309	15719	34281	4456	45544
2	18889	31111	11825	38175	4753	45247
3	18197	31803	15058	34942	4144	45856
Averaged	18592.3333	31407.6667	14200.6667	35799.3333	4451	45549

24h_30C_P. putida + B. thailandensis_LB						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	40744	9256	44281	5719	42852	7148
2	40522	9478	43130	6870	42666	7334
3	43149	6851	43009	6991	42431	7569
Averaged	41471.6667	8528.33333	43473.3333	6526.66667	42649.6667	7350.33333

0h_P. putida + B. thailandensis_Soil						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	17226	32774	13858	36142	15104	34896
2	16948	33052	14008	35992	15102	34898
3	16662	33338	14136	35864	15198	34802
Averaged	16945.3333	33054.6667	14000.6667	35999.3333	15134.6667	34865.3333

24h_25C_P. putida + B. thailandensis_Soil						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	20772	29228	23625	26375	18186	31814
2	32711	17289	24215	25785	18734	31266
3	35022	14978	23553	26447	19127	30873
Averaged	29501.6667	20498.3333	23797.6667	26202.3333	18682.3333	31317.6667

24h_30C_P. putida + B. thailandensis_Soil						
	Number of Cells per 50,000 Events					
	210713-0714		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	27812	22188	27924	22076	28894	21106
2	33842	16158	28075	21925	29121	20879
3	38611	11389	29281	20719	29351	20649
Averaged	33421.6667	16578.3333	28426.6667	21573.3333	29122	20878

Appendix E: Raw data from testing RFP's role in co-culturing results

0h_KT2440 P. putida + mScarlett E. coli_LB

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	37417	12583	37415	12585	37836	12164
2	37732	12268	37192	12808	38274	11726
3	37952	12048	37951	12049	38309	11691
Averaged	37700.333	12299.667	37519.333	12480.667	38139.667	11860.333

0h_KT2440 P. putida + mScarlett E. coli_Soil

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	42267	7733	37828	12172	37901	12099
2	42186	7814	37918	12082	37635	12365
3	42044	7956	37863	12137	37992	12008
Averaged	42165.667	7834.3333	37869.667	12130.333	37842.667	12157.333

24h_25C_KT2440 P. putida + mScarlett E. coli_LB

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	13707	36293	15732	34268	16900	33100
2	14243	35757	15956	34044	17366	32634
3	13382	36618	15958	34042	17172	32828
Averaged	13777.333	36222.667	15882	34118	17146	32854

24h_25C_KT2440 P. putida + mScarlett E. coli_Soil

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	38331	11669	20878	29122	34428	15572
2	41515	8485	21862	28138	25977	24023
3	41833	8167	21816	28184	26576	23424
Averaged	40559.667	9440.3333	21518.667	28481.333	28993.667	21006.333

24h_30C_KT2440 P. putida + mScarlett E. coli_LB

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	17576	32424	1694	48306	19282	30718
2	17491	32509	1730	48270	18742	31258
3	18657	31343	1683	48317	18741	31259
Averaged	17908	32092	1702.3333	48297.667	18921.667	31078.333

24h_30C_KT2440 P. putida + mScarlett E. coli_Soil

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	45160	4840	29369	20631	28137	21863
2	44917	5083	30475	19525	28592	21408
3	45343	4657	30087	19913	27950	22050
Averaged	45140	4860	29977	20023	28226.333	21773.667

24h_37C_KT2440 P. putida + mScarlett E. coli_LB

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	22239	27761	19600	30400	16520	33480
2	22163	27837	19839	30161	16391	33609
3	23809	26191	20287	29713	16035	33965
Averaged	22737	27263	19908.667	30091.333	16315.333	33684.667

24h_37C_KT2440 P. putida + mScarlett E. coli_Soil

	Number of Cells per 50,000 Events					
	210720-0721		210726-0727		210728-0729	
Replicate #	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells	Estimated Red Cells	Estimated White Cells
1	28949	21051	17563	32437	15690	34310
2	28915	21085	17907	32093	16470	33530
3	28815	21185	18097	31903	16667	33333
Averaged	28893	21107	17855.667	32144.333	16275.667	33724.333

Appendix F: Raw data from cytometric analysis of co-cultures by TASBE versus FlowJo

0h_P. putida + C. freundii_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	12994	37006	15033	34951
2	13091	36909	15014	34931
3	12707	37293	15423	34426
Averaged	12930.667	37069.333	15156.667	34769.333

0h_P. putida + C. freundii_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	13049	36951	15020	31338
2	12812	37188	15722	34248
3	12922	37078	15918	34029
Averaged	12927.667	37072.333	15553.333	33205

0h_P. putida + B. thailandensis_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	14747	35253		
2	15313	34687		
3	15452	34548		
Averaged	15170.667	34829.333		

0h_P. putida + B. thailandensis_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	15104	34896		
2	15102	34898		
3	15198	34802		
Averaged	15134.667	34865.333		

24h_25C_P. putida + C. freundii_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	36630	13370	46244	3393
2	36972	13028	46524	2935
3	36638	13362	46839	2881
Averaged	36746.667	13253.333	46535.667	3069.6667

24h_25C_P. putida + C. freundii_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	22423	27577	20443	16762
2	24662	25338	23350	18681
3	24894	25106	23690	19581
Averaged	23993	26007	22494.333	18341.333

24h_25C_P. putida + B. thailandensis_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	4456	45544		
2	4753	45247		
3	4144	45856		
Averaged	4451	45549		

24h_25C_P. putida + B. thailandensis_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	18186	31814		
2	18734	31266		
3	19127	30873		
Averaged	18682.333	31317.667		

24h_30C_P. putida + C. freundii_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	38776	11224	45671	4308
2	37011	12989	48265	0
3	37706	12294	46468	3461
Averaged	37831	12169	46801.333	2589.6667

24h_30C_P. putida + C. freundii_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	25573	24427	31010	14354
2	25689	24311	31549	14513
3	25703	24297	32051	14468
Averaged	25655	24345	31536.667	14445

24h_30C_P. putida + B. thailandensis_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	42852	7148		
2	42666	7334		
3	42431	7569		
Averaged	42649.667	7350.3333		

24h_30C_P. putida + B. thailandensis_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	28894	21106		
2	29121	20879		
3	29351	20649		
Averaged	29122	20878		

24h_37C_P. putida + C. freundii_LB

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	3108	46892	41837	6408
2	4112	45888	42964	5980
3	3894	46106	43310	5814
Averaged	3704.6667	46295.333	42703.667	6067.3333

24h_37C_P. putida + C. freundii_Soil

Replicate #	Estimated Red Cells	Estimated White Cells	Number of Cells per 50,000 Events (TASBE) 210728-0729	Number of Cells per 10uL Sample (FlowJo) 210728-0729
1	35814	14186	38611	7961
2	36605	13395	40013	7468
3	37252	12748	41159	7477
Averaged	36557	13443	39927.667	7635.3333