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Protist Facilitated Transport in an Artificial Soil Micromodel

Rebecca L. Rubinstein
University of Connecticut - Storrs, rebecca.l.rubinstein@uconn.edu

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Rebecca L. Rubinstein

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Protist Facilitated Transport in an Artificial Soil Micromodel

Presented By

Rebecca L. Rubinstein, B.S.

Major Advisor _____

Dr. Leslie M. Shor

Associate Advisor_____

Dr. Ranjan Srivastava

Associate Advisor_____

Dr. Emmanouil N. Anagnostou

University of Connecticut

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Objective and Organization of Thesis

The primary goal of this project was to develop techniques and methodologies for testing and quantifying facilitated transport of fluorescent particles, or potentially bacteria, in microfluidic devices. Due to the complexity of the system, extensive troubleshooting was involved to reach the final product. The abstract and the first three sections of this thesis are from a manuscript currently in preparation. The following two sections address additional work to be done for completion of the manuscript and possible future directions for this work. The final section is describes the troubleshooting process, with a description of the key challenges faced and methods used to address them.

Abstract

The soil microhabitat contains a complex network of microbial, biochemical, and physical interactions, all of which have very important implications for remediation technologies as well as agriculture. As a result, observing and quantifying this environment has been of recurring interest to scientists and engineers, but by nature many of these processes are difficult if not impossible to observe *in situ*. To that end, we have developed techniques utilizing soil-structured microfluidic devices for direct observation of soil microbial processes. Specifically, natural soil protists were observed moving through fabricated artificial soil networks, and their impact on the spatial and temporal distribution of fluorescent particles was quantified. Protists were found to significantly enhance the transport of non-motile particles through the microstructured environment, spreading them throughout the area five days before diffusion alone brought them to the beginning of the patterned area.

I. Introduction

Because of its dense microstructure, soil intrinsically limits mobility of particles. Most of the literature focuses on transport of bacteria through soil due to their many applications for remediation and agriculture, but this adds numerous additional complications to the system, to be discussed in detail later. Extensive work was done with bacteria over the course of this project, but ultimately there were too many factors that could not be well controlled at this stage. As a result, fluorescent beads were used as a substitute for bacteria to facilitate study of transport alone, separating out other obfuscating factors. The majority of bacteria in soil exist in biofilms,[1] and have minimal mobility in that form, so beads made a reasonable substitute for bacteria. Although predation and digestion would be significant factors for bacteria, studies have shown that bacteria are able to survive water treatment processes inside of encysted protists, and remain viable after egestion,[2] so it seems feasible that they may also survive and remain viable in soil systems, where there are no disinfection processes in effect.

Soil-dwelling biocontrol bacteria are a key component of the rhizosphere ecosystem. These bacteria prevent root colonization by pathogens by producing antibiotics or iron-sequestering siderophores, competing for substrate nutrients, and reducing the number of available binding sites.[3] Beneficial bacteria also fix nitrogen, mobilize nutrients from soil organic matter[4], and secrete hydrophilic polymers that moderate soil moisture[5], which ultimately results in improved plant health and increased crop yield. These benefits cannot be fully realized, however, without dispersal and colonization along the length of the root. [6]

A study on the motility of two common soil bacteria, *Rhizobium japonicum* and *Pseudomonas putida*, found that the bacteria did not move below 2.7 cm soil depth when the top 2.4 cm were inoculated.[7] Lack of bacterial mobility in soil has also received attention in the

context of bioremediation, and various studies have sought an improved means of delivering the bacteria responsible for remediation directly to the site of contamination, using both biological vectors and abiotic means of directing movement. In a study evaluating electrokinetic transport of polycyclic aromatic hydrocarbon-degrading bacteria in a model aquifer, bacteria only moved in the presence of direct current, though the extent of movement, impact on degradation rates, and viability after prolonged exposure were strain-dependent.[8] Another study investigated earthworms as vectors for polychlorinated biphenyl-degrading bacteria and found that while degradation rates varied by soil treatment, bacteria infiltration rates were clearly improved in the presence of earthworms, and that this effect increased at greater depths.[9] Finally, a recent study considered fungal hyphae as potential vectors for pollutant-degrading bacteria and found that efficacy was highly dependent on the specific organisms used, but for some combinations bacterial dispersal was enhanced.[10]

Meanwhile, it has been shown crop yield is reduced in the absence of protists.[1] The proposed mechanisms of protist-enhanced plant production are the release of nutrients otherwise sequestered in bacterial cells by grazing and grazing-induced shifts in the bacterial composition of the rhizosphere, selectively consuming pathogens and passing over biocontrol stains like *P. fluorescens* or nitrifying bacteria.[11] We propose that an additional mechanism is the facilitated transport of bacteria through the root system by protists acting as vectors. This may occur by surface attachment or very gradual physical pushing of bacteria, and as mentioned above, even successful predation may be a key means of transportation. [2] A more recent study showed that bacteria can avoid digestion and be egested viable from freshwater and soil ciliate *Colpoda*.[12] Ciliate swimming speeds in constricting channels have been measured at over 400 μm s^{-1}, so the distance travelled between ingestion and egestion may be significant.[13]

Many ecosystem-level studies are performed in bench-scale reactors or in field-scale studies, but this often involves disruptive sampling and direct observation of micro-scale processes is limited if possible at all. Microfluidic devices have emerged as a way of studying these processes *in situ*. Microfluidic experiments have been performed to study bacterial chemotaxis in soil,[14] effects of micro-structures on bacterial colony organization,[15] and the impact on physical habitat structure on competitive, multi-species community stability. [16] Studying these processes *ex situ* can lead to an incomplete understanding of the system because the physical micro-structure of soil impacts the microbial communities within. [17] The many complexities of the soil microbial environment make microfluidic devices a very useful tool for studying the micro-scale interactions within. A better understanding of the feedbacks and nonlinearities of the rhizosphere system could lead to improved predictions of biogeochemical processes and enhanced environmental biotechnology for remediating contamination and improving agriculture.

II. Materials and Methods

1 Biological Culture Procedures

1.1 Protist Culture Methods

The protists used for this study were *Colpoda sp.*, naturally-occurring soil ciliates that forms cysts in the absence of a sufficient food source, and excyst when a food source becomes available. Protist stocks were maintained in Page's Saline Solution in Nunc cell culture treated flasks with 25 cm^2 culture area. Page's Saline was prepared and stored as two separate 10 × solutions, as shown in Table 1.

Table 1 - Page's Saline Recipe (10 × solutions in 1 L deionized water)

	Chemical name	**Mass (g)**	**Source**
Solution 1	Sodium chloride	1.2	Fisher Scientific, Pittsburgh, PA
	Magnesium sulfate heptahydrate	0.04	Fisher Scientific, Pittsburgh, PA
	Disodium phosphate	1.42	Fisher Scientific, Pittsburgh, PA
	Monopotassium phosphate	1.36	Fisher Scientific, Pittsburgh, PA
Solution 2	Calcium chloride (anhydrous)	0.04	Fisher Scientific, Pittsburgh, PA

To remove protists from the culture flasks, cysts were scraped loose from the bottom of the flask using a sterile pipette tip and then the required volume was withdrawn from immediately above the bottom. For culture propagation, 1 mL from the old culture was added to 9 mL of sterile Page's Saline, then non-fluorescent bacteria were added for a final concentration of 7×10^7 cells mL^{-1}. The same withdrawal technique was used for protists added to microfluidic devices, which will be discussed in depth later.

1.2 Bacterial Culture Methods

Non-fluorescent *E. coli* were used to propagate cultures and induce protists to excyst for trials. The *E. coli* were grown to stationary phase at 30 °C for 24 hours in LB media (recipe in

Table 2) with no antibiotic. The bacteria were re-suspended in Page's Saline for use. For facilitated transport experiments, 7×10^7 *E. coli* cells mL^{-1} were combined with 1.0 µm Nile Red FluoSpheres carboxylate-modified microspheres (Invitrogen Molecular Probes, Thermo Fisher Scientific, Waltham, MA) at 3.5×10^7 beads mL^{-1} in sterile Page's Saline.

Table 2 – Recipe for LB media (in 1 L deionized water)

	Ingredient	**Mass (g)**	**Source**
Liquid	Tryptone	10	Sigma-Aldrich Corp. St. Louis, MO
	Yeast Extract	5	Fisher Scientific, Pittsburgh, PA
	Sodium chloride	10	Fisher Scientific, Pittsburgh, PA
Plates	Agar	15	Fisher Scientific, Pittsburgh, PA

2 Device Design and Fabrication

The complex, heterogeneous microstructure of soil is an important factor affecting microbe motility so a microfluidic device with an emulated soil pattern as described in Orner et al.,[18] was used to test for facilitated transport. Devices were designed with an area designated for microbial inputs and three distinct channels for travel, each filled with a soil pattern with an approximate porosity of 0.47, which was divided into sections for quantization, as shown in Figure 1B.

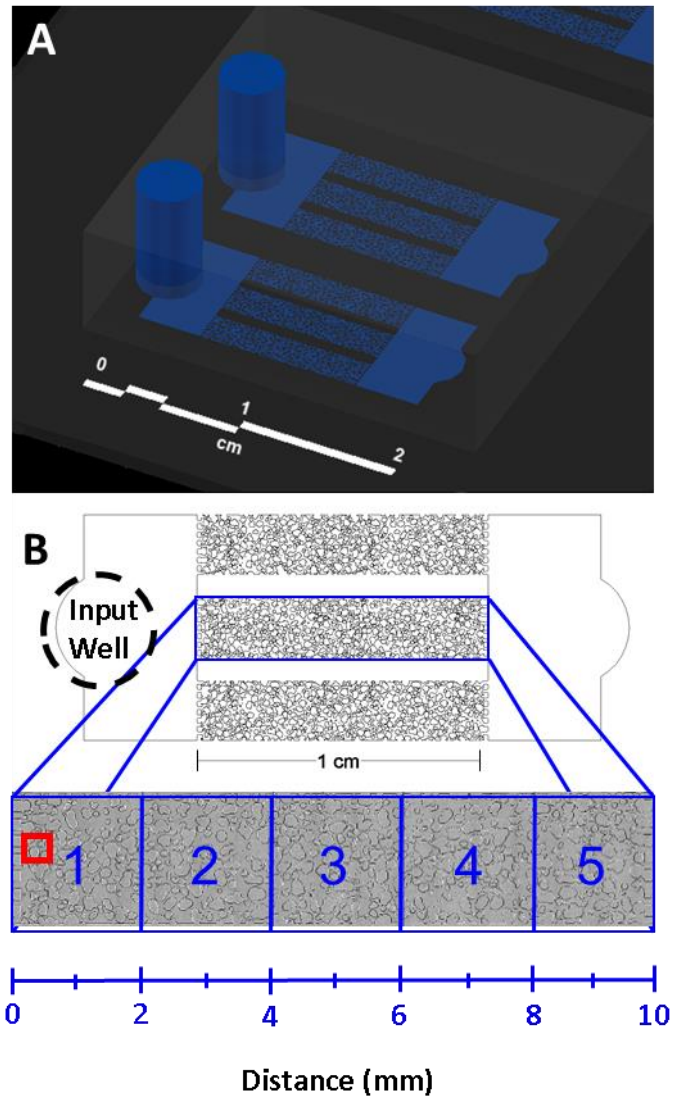

Figure 1 – (A) 3D rendering of microfluidic devices in AutoCAD. (B) Flat view of a single microfluidic device (scale bar below). Detail is microscope photograph of single soil patterned channel, with regions and distances as used in data presentation designated below. The red box indicates the area shown in the zoomed images in Figure 4.

The microfluidic master was fabricated using the procedure detailed in Deng et al., with minor modifications.[19] A 4 inch silicon wafer was spin-coated with SU-8 2025 at a height of 33.5 ± 1.5 µm. It was patterned by exposure to UV light at 26.4 $mWcm^{-2}$ for 6.4 seconds with a

chrome emulsion mask (Advance Reproductions, North Andover, MA). The master was cast with polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) and castings were plasma bonded to clean glass slides using the procedure also described in Deng et al.[19] A 4 mm biopsy punch was used to create the input well. The bonded devices were filled with sterile Page's Saline solution and left undisturbed until no air bubbles remained, as shown in Figure 2B.

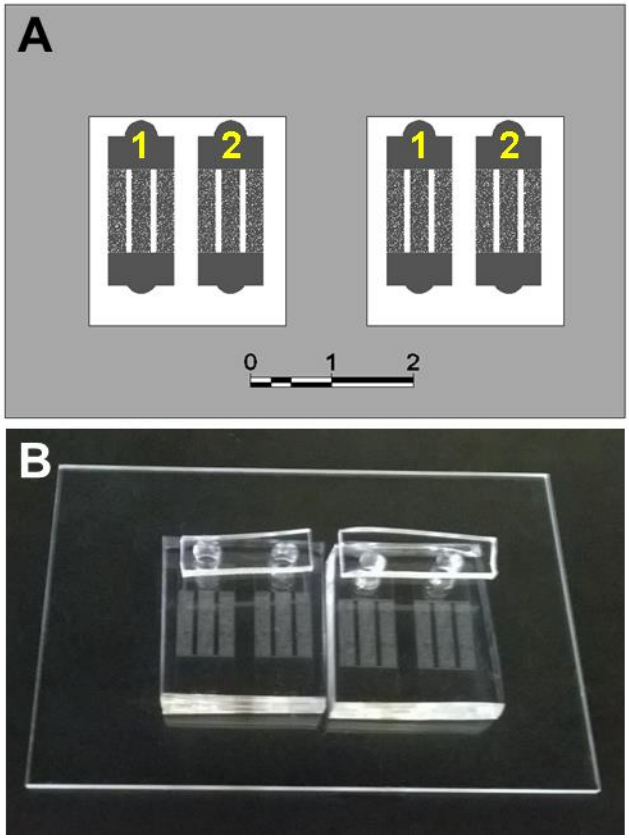

Figure 2 – (A) Schematic of experimental setup. 1 Indicates beads with *Colpoda sp.*, 2 indicates beads alone. (B) Photograph of microfluidic devices bonded to a glass slide with strips of PDMS over wells.

3 Experiment Design and Setup

Experiments were performed evaluating transport of beads both with and without protists present, with 2 replicates of each treatment, arranged as shown in Figure 2A. Once devices were

prepared and filled with Page's Saline, 30 µL was withdrawn from the input wells of devices used for protist treatments and 30 µL of protist cysts, withdrawn from culture flasks as described above, were added. Initial images (process described below) were taken after addition of protists, to allow time for the cysts to settle and to obtain counts of the initial number of protists. After initial images were taken, 10 µL was withdrawn from the top of each input well and 10 µL of the combined beads and *E. coli* were added to each input well. Input wells were then covered with thin strips of PDMS to minimize evaporation. Between time points, devices were stored in the dark at approximately 24 °C and 99 % humidity.

4 Microscopy and Quantification

Images were taken using a Nikon A1R confocal microscope system with an Eclipse Ti microscope and LU4 laser unit. Automated x-y stage movement was used to capture full mosaic images of each soil-patterned channel, and Nikon NIS Elements software was used to stitch the mosaics and apply shading correction. Images were captured using an S Plan Fluor ELWD 20× DIC N1 objective, with resonance scanning capturing at 512 × 512 pixel resolution and the laser emitting at 595 nm. Facilitated transport was quantified by dividing each channel into 5 equal regions, as shown in Figure 1A, and manually counting the number of beads in each section. The raw counts were averaged over the non-soil area in each region to account for the heterogeneity of the soil pattern.

III. Results and Discussion

This experiment shows that protists can drastically increase the transport of discrete particles through heterogeneously micro-structured pathways. Several mechanisms for particle transport have been observed over the course of these experiments. *Colpoda sp.* are voracious grazers and consume both bacteria and beads readily (Figure 3). Since the beads are not digestible, they were carried around the device within the cells and then egested along with other waste products throughout the device. This mode of transport may also apply to bacteria, as the literature shows that bacteria can survive ingestion by *Colpoda sp*. and maintain viability upon egestion.[12]

In addition to direct internal transportation, particles near protists are also pushed along by the micro-currents created by the protists' beating cilia. Finally, though bacterial data is not included in the final version of this work, surface attachment of bacteria to protists was observed, and may be a significant mode of transport for some particles. The contributions of each of these mechanisms were not evaluated separately in this work, though the first is likely the most significant for large-scale transport, but a clear difference was seen in the number of beads present in the soil-patterned channels when protists were present (Figure 4).

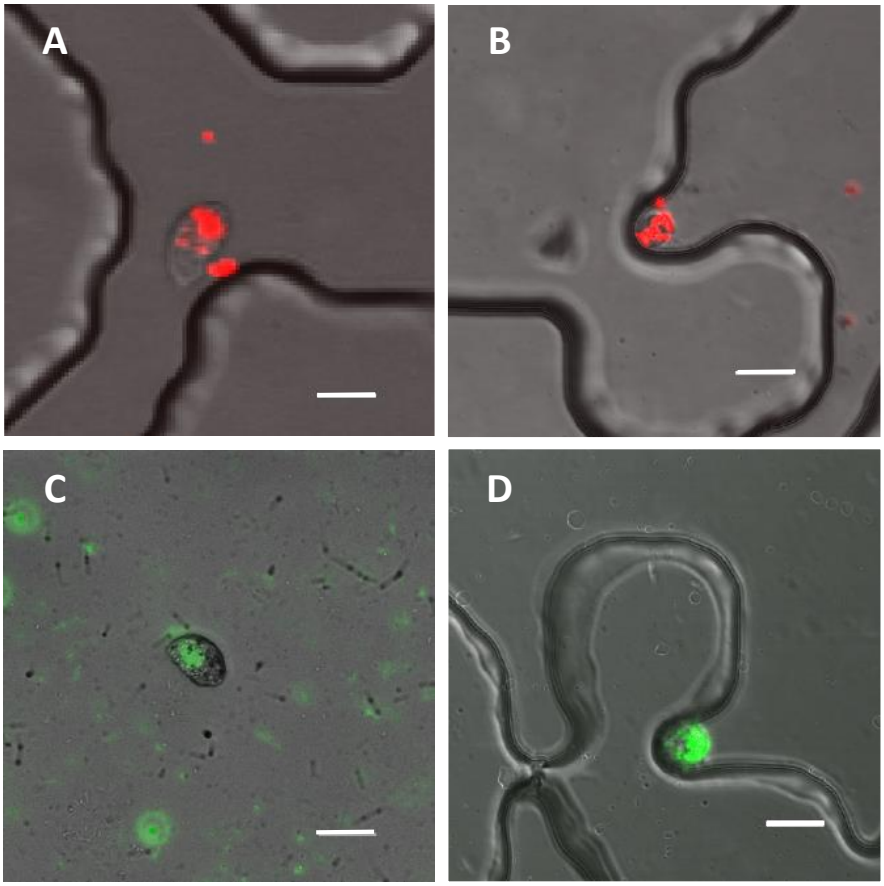

Figure 3 - (A) Active *Colpoda sp.* carrying fluorescent red beads; (B) Encysted *Colpoda sp.* with fluorescent red beads; (C) Active *Colpoda sp.* carrying GFP-labelled bacteria; (D) Encysted *Colpoda sp.* with GFP-labelled bacteria. All scale bars are 20 µm.

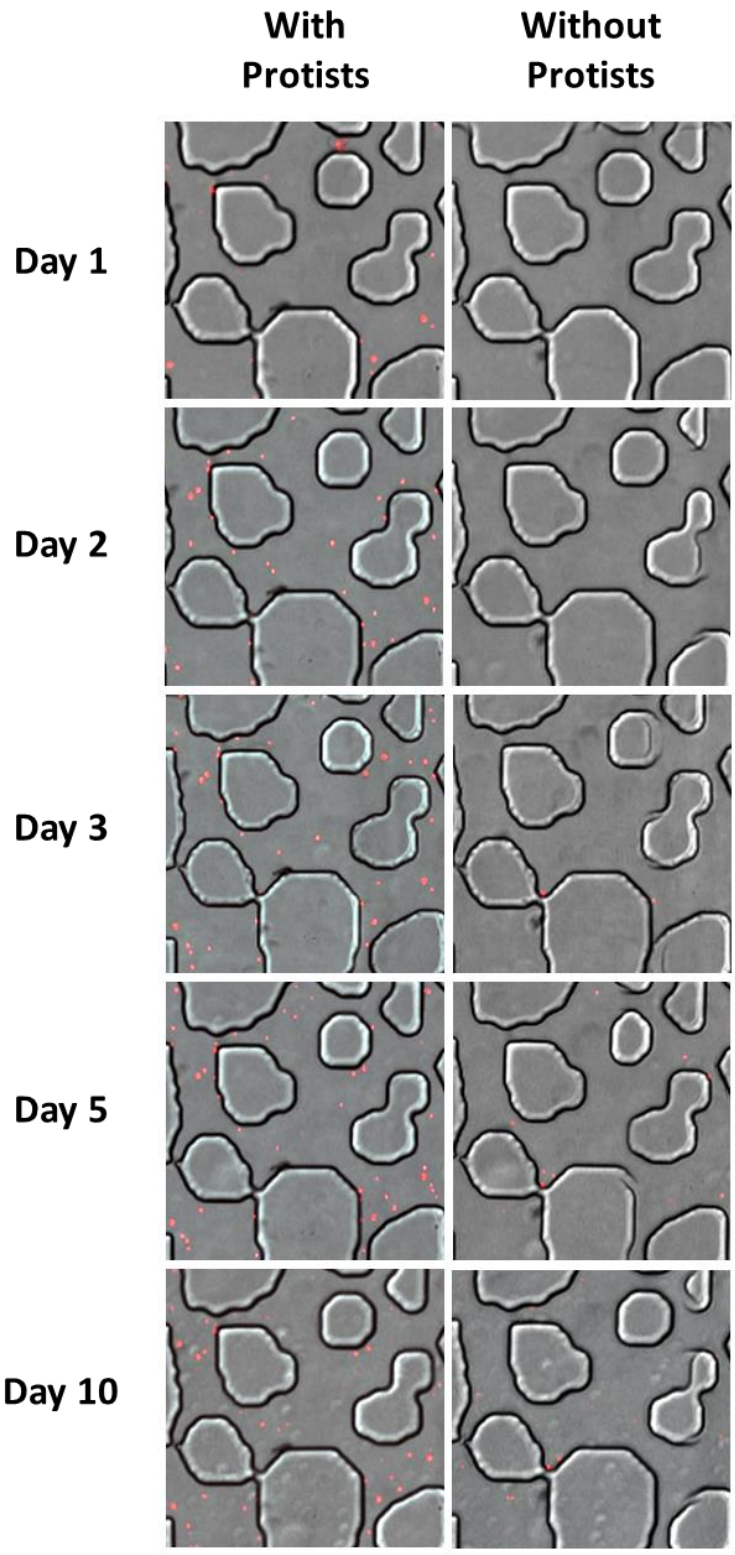

Figure 4 – Zoomed images of beads in the soil-patterned channels, in the region nearest the input wells.

The spatial distribution of beads throughout the soil-patterned channels was greatly enhanced in the presence of protists (Figure 5). Although there was some variation in the concentration of beads in regions of the soil-patterned channel furthest from the input well, only the difference between the first and second regions was statistically significant ($P < 0.05$) based on a two-tailed t-test. By the same method, the difference in spatial distribution between time points was determined not to be statistically significant. It is also worth noting that replication in this experiment was very good, with no statistically significant difference in spatial or temporal distributions between replicates.

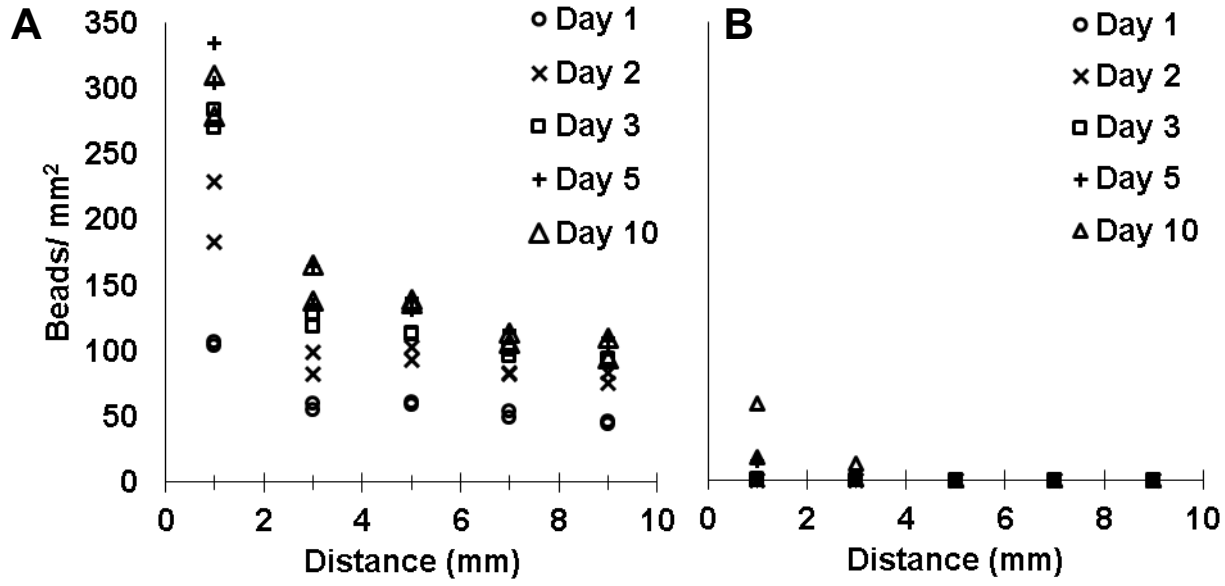

Figure 5 - Spatial distribution of fluorescent beads in soil-patterned region of the microfluidic devices (A) with *Colpoda sp.* present and (B) without protists. Distance was measured from the beginning of the soil-patterned region to the center of each sub-region enumerated.

Framing the data in terms of temporal distribution also yields a useful perspective on the system. Although the change in spatial distribution over time was not found to be statistically significant, a very clear overall increase in the number of beads between the first and last time points was observed at all positions. Initially it was expected that protist swimming speed would be the limiting factor for distribution of beads, but the protists were extremely mobile in these devices, and spread beads throughout within a single day. The actual limiting factors were determined to be ingestion and egestion rates, the latter because protists were often observed to traverse the channel multiple times before egesting anything. Figure 6A shows the temporal distribution of beads in each region of the soil-patterned channel. Average deposition rates were also calculated in terms of beads d^{-1}, and are shown in Figure 6B with linear regression lines superimposed for each data set. The close clustering of the regression lines for all regions except the first, as well as the drastic difference between the first and all subsequent regions, emphasizes well the difference first demonstrated through statistical analysis.

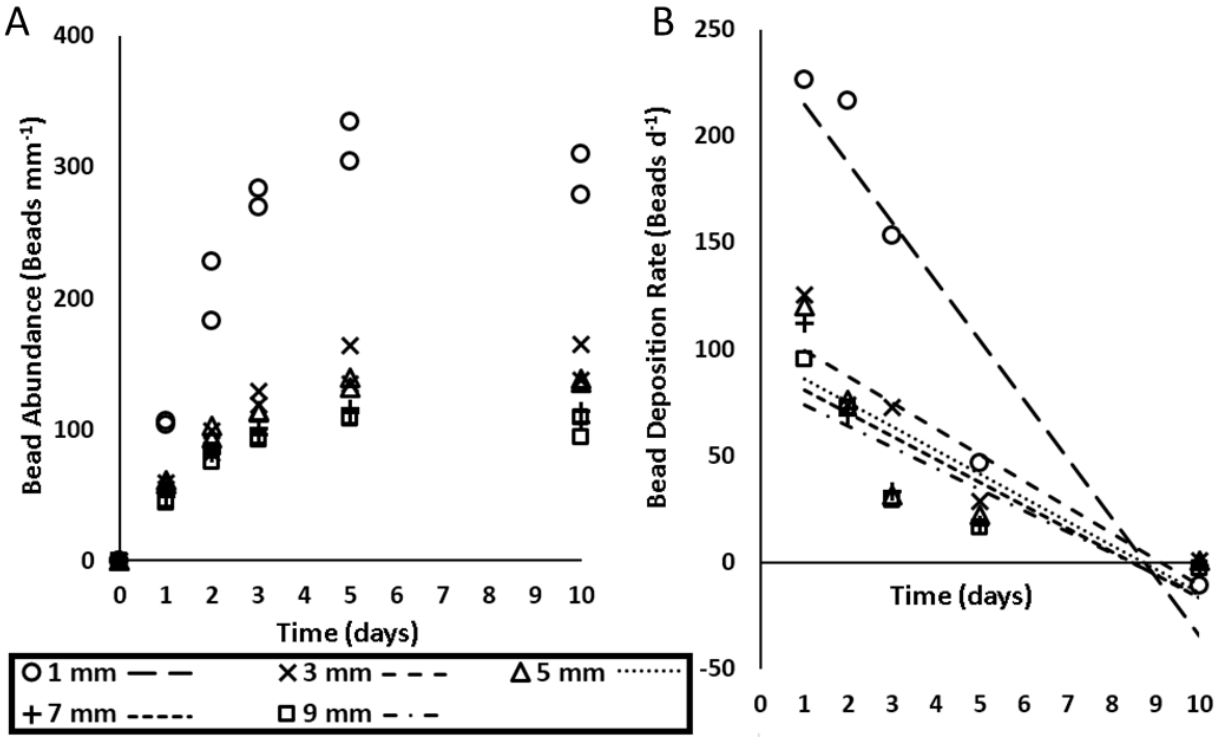

Figure 6 – (A) Bead abundance with time at different positions along the soil-patterned channels. Average abundance for all three channels in a single device shown for each replicate. (B) Overall average deposition rate of beads over time, with linear regression lines superimposed.

The temporal and spatial distribution of protists in the soil-patterned areas of the device was also analyzed. The number of protists observed in the images taken of the soil-patterned channels was monitored, separated by both position and time. Further analysis is needed to determine whether the observations made are meaningful, since these images provide only a very brief snapshot of the actual state of the device, approximately one minute out of the 24 hours between images. Because the protists are able to swim very fast, and do so in a random pattern as opposed to any sort of normal distribution, these images may not accurately portray actual protist concentrations in the devices. Additionally, most protists were observed to stay in or around the

input well most of the time, and many may have returned there after depositing beads in the soil-patterned channels, so the number counted in the channels during that one minute may not be representative of the number of protists responsible for transporting the observed beads.

The number of protists observed in each region of the soil-patterned channels is shown in Figure 7. Despite the uncertainty in measurement of the number of protists, the difference in protist concentration between Day 3 and Day 5 was found to be statistically significant, and this also makes sense in terms of observations made during the experiment. Specifically, at extended time points, protists begin to slow down and re-encyst. Although encysted protists were included in the counts, the reduced activity level explains the reduced presence of protists in the soil-patterned area, especially given their observed propensity for returning to the input well and surrounding area. The observed tendency of protists to stay in the earlier sections of the device was not reflected in the counts recorded.

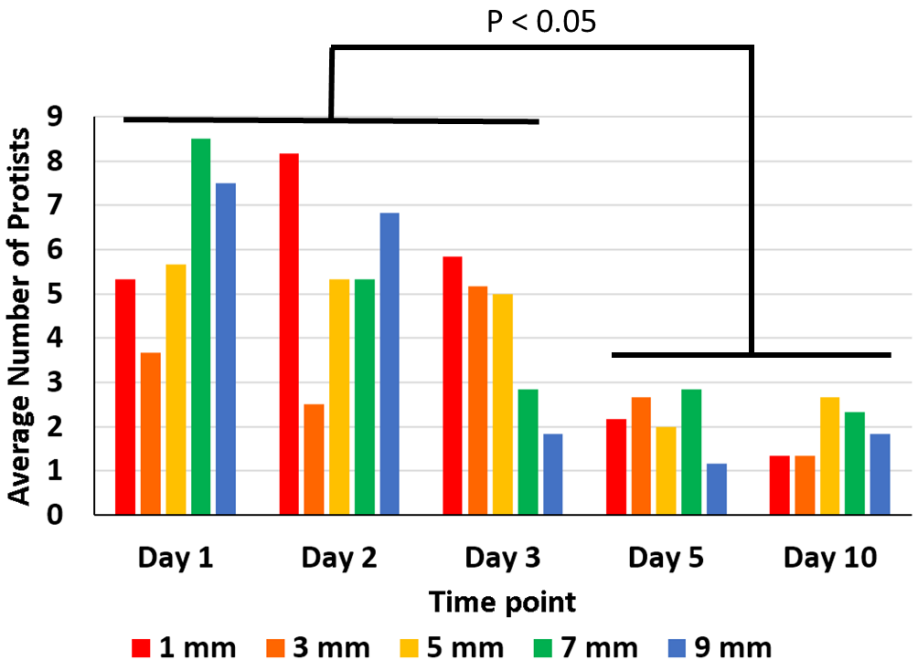

Figure 7 – Average number of protists counted in images in each position, grouped by elapsed time. Only the temporal variation between Day 3 and Day 5 was statistically significant.

The impact of the initial concentration of the particles to be transported, initial number of protist cysts, and variation of the physical characteristics of the soil pattern were not evaluated in this study, but can be evaluated efficiently using the techniques developed. These promising preliminary results also encourage further study on the facilitated transport of bacteria, a much more complicated process dependent not only on retention time but also on grazing rates, digestion resistance of specific bacterial strains, and viability after egestion. That process is also likely to be highly dependent on the species of bacteria used, particularly because gram-positive strains are more resistant to digestion by predators.[12] Nonetheless, these preliminary results together with the literature on the ability of bacteria to survive and thrive after egestion support the conclusion that this could be a very useful delivery method for remediation and agricultural biotechnologies.

IV. Ongoing Work

Ongoing work, which will be included in the manuscript to be submitted on this work, will be primarily comprised of more robust statistical analysis of the data collected. Analysis of variance (ANOVA) will be used to evaluate the relative importance of each factor monitored for this experiment to the overall transport of beads through the soil device. Specifically, the variables in question are shown in Table 3. Treatment type is expected to be the most significant factor related to the number of beads, but the number of protists counted may also be significant.

Table 3 - Variables to be analyzed with ANOVA

Variable	**Values**
Treatment	With Protists, Without Protists
Device	1, 2
Channel	1, 2, 3
Number of Protists	Numerical Input
Number of Beads	Numerical Input

More rigorous analysis of the protist data is also called for, to determine the degree to which the images are representative. As mentioned in the discussion section, these numbers are counts made from images that represent the protists present in the channel during about one minute out of the 24 hours being analyzed. The resulting counts may still provide meaningful insight into the typical distribution of protists in the soil patterned channels, but it bears further analysis.

IV. Future Work

Future work will address computational modelling of this complex system for prediction of spatial and temporal distribution capacity. In order to generate a robust model, additional experiments will need to be carried out to better characterize key parameters. Specific grazing rates will need to be determined for particles of interest, both bacteria and abiotic. Digestion rates of each type of particle will also need to be characterized, as that will be a critical component affecting transport, particularly for bacteria. Finally, retention time of various particles will need to be evaluated, since the preliminary work suggests this may have the greatest effect on facilitated transport.

In addition to quantification of each of the above parameters, protist motility through the microstructured channels will need to be evaluated in greater depth. For example, it was observed that protists had a strong tendency to enter pore spaces only slightly larger than themselves, especially immediately before encysting. This may have a strong impact on spatial distribution of the particles transported, especially on a long time scale since in some cases particles were egested when protists encysted. In order to better understand the system as a whole, and also the potential industrial applications, varying the starting concentration of particles or bacteria to be transported and the initial number of cysts would also be valuable. Finally, testing transport in devices with varying porosities or mean pore throat size may also yield useful insight on the overall transport process, but is likely less critical in the early stages of model development.

V. Troubleshooting

This project brought with it a unique set of complications, and a significant amount of troubleshooting was required to develop a system to effectively test for facilitated transport. The main issue to be addressed was control of factors not related to protist transport capacity, such as bacterial motility, predation effects, and flow-induced movement. These were addressed through a combination of procedural controls, device design, and careful selection of inputs. Determining an appropriate means of quantifying the effects of facilitated transport also required some trial-and-error. Detailed description and a summary of results from previous methodologies follows.

1 Flow

The first serious problem to be addressed was flow through the microfluidic devices. Direct observation of early devices revealed that flow in the microfluidic devices was pushing bacteria through the devices faster than they could swim by themselves, and in larger numbers than the protists could carry. There was no slope or temperature difference between ends to induce flow, nor was there any pumping or suction, so the conclusion was that flow was driven by a difference in hydraulic head between wells. The flow rate was not constant between devices or over time within a given device, so could not be readily subtracted or otherwise accounted for after-the-fact. Fluorescent beads were used as a tracer for fluid flow, as they were clearly visible, approximately the same size as bacteria, and non-motile.

1.1 Gelling Agents

The first attempt at stopping flow in the devices involved gelling the media within the channels with agarose to increase resistance to flow. Although the gel was fully liquid when filling began, it quickly began to cool and solidify when it was loaded into the

devices, more quickly for higher concentrations, which complicated the filling process. Several concentrations of agarose were tried, with results as shown in Table 4.

Table 4 - Agarose concentrations used to fill microfluidic devices, and the results of the attempted filling

% Agarose	Result
0.1	Device filled, but flow was still present at the sides of channels, and still rapid and carrying significant quantities of bacteria.
0.15	Gel was present throughout, but micro-bubbles were present throughout channels, resulting in rapid drying from the inside of channels
0.2	Gel could not be spread through more than 75% of the device
0.25	Gel progressed through less than 10% of the device

In order to completely fill the devices, all wells were loaded as rapidly as possible to allow for multi-point infiltration, and after filling pressure was carefully applied to each well in an attempt to force the gel through. Filling the devices on a hot plate to keep the media liquid for longer was also attempted, but it still was more viscous than media without agarose, and pressure was still required to force the media through. This presented a safety hazard, and also tended to cave some areas of the device that lacked sufficient internal supports. Additionally, the high temperature resulted in extremely rapid drying, such that devices sometimes dried before they could fill completely. Although lower concentrations could be made to fill the devices without the added complications, they were found to pull away from the sides of the channels, resulting rapid flow and bead movement along the edges of channels.

While work was being done to fill the experimental devices with gelled media, protist motility in different gel concentrations was also evaluated. Tests were performed

using experimental protists *Colpoda sp.* Protists were not able to swim through 0.25 % agarose, and could only penetrate a short distance into 0.2 %, They were able to swim in 0.1 % and 0.15 % agarose, though more slowly than normal. In addition to reduced speeds, which would obviously impact facilitated transport results, when protists swam through the gel they carved pathways. Once pathways were carved, those protists generally stayed within those tracks rather than carve new ones, and future protists also tended to follow those paths. Finally, preliminary work with bacteria and protists showed surface attachment as a possible means of transportation, and the gel might also have scraped off attached particles. Ultimately, gel had to be abandoned as a possible solution to flow, due to the many operational concerns and lack of useful results.

1.2 Time

The second means of stopping flow attempted was simply allowing more time for the levels in the various input wells to equilibrate. The volume in a given well was orders of magnitude greater than the volume in the channel network in between, and the very small cross sectional area of channels resulted in significant friction, so this was expected to be a slow process. Devices were bonded and filled up to a week before use, but flow showed no sign of subsiding. A smaller version of the device was designed and fabricated, with nearly identical geometry but a network of channels only about a quarter of the original size (to be discussed in greater detail later), in the hopes of reducing the time necessary for equilibration, but flow still persisted for over a week.

1.3 Physical Modification of Devices

Since extended waiting time did not remedy the flow problem, physical modifications to the devices were attempted to aid in equilibration. Since flow was

observed not only between the opposite ends of the device but also at times between wells on the same end, connecting wells on the same end was seen as a possible solution. On the reduced-size device, all input wells were connected to one another and all output wells were connected to one another, with separation between the two maintained. First, a scalpel was used to cut trenches across the wells to be connected. The small scale made it difficult to cut consistently sized trenches, but with some practice usable devices were obtained. The large, unconstrained volume of fluid in the trenches resulted in sloshing when the devices were moved, which exacerbated the flow problem. Next, hot glue was used to create tracks on the master connecting the desired wells. These tracks were shallow enough that they were not open to the environment, which seemed to reduce the sloshing, but the hot glue peeled off of the master along with the PDMS after one or two pulls, and the master delaminated after reapplication of hot glue and was no longer usable. Finally, the reduced-size mask was modified by using a scalpel to carefully scrape away the emulsion to connect the wells. The device was fabricated but flow between the input wells and the end wells continued.

1.4 Semi-Closed System

Ultimately, the most effective solution was also the simplest, to simply not punch more than one well in each device. Since there seemed to be no operational controls that would eliminate flow, we chose to forgo the chemo-attractant and signal enhancer at the far end of the device from the inputs. This will be discussed in greater detail in the next section, but it was originally deemed necessary both to encourage microbes to leave the end wells and to prompt a bacterial bloom when they reached the end of the device, to

facilitate detection. With only a single input well and no punches at the far end, there was no opportunity for flow to start once all bubbles had left the device.

2 Microbial Strains & Other Fluorescent Particles

2.1 Protists

Initially, two different species of natural soil protists were used in experiments: *Colpoda sp.* and *Cercomonas sp.* The former are ciliates approximately 30 µm in length, and the latter are flagellates approximately 10-20 µm in length. Later experiments used only *Colpoda sp.* because although the Gage lab had previously recorded a video of *Cercomonas sp.* carrying bacteria around wedged inside their oral cavities, this was not reflected in the microfluidic experiments. In addition, the *Colpoda sp.* consumed all particles presented to them indiscriminately, and in large quantities, whereas the *Cercomonas sp.* grazed much more slowly and were not observed to consume beads.

2.2 Bacteria

1. *Pseudomonas fluorescens*

The first strain of *P. fluorescens* provided was Pf0, which was used for preliminary experiments and the first batch of troubleshooting trials. It was grown in TY media (recipe in Table 5) with 25 µg mL^{-1} Kanamycin monosulfate. For later trials, strain Pf5was used instead because it is a more common biocontrol strain and was simultaneously being used for greenhouse experiments in the Gage laboratory. Pf5 grows in the same media and with the same antibiotic as Pf0, so the switch did not require any operational changes. Both strains are very robust, with both plates and liquid cultures requiring 24 hours at 30 °C to grow. The rapid

growth rate and general robustness of *P. fluorescens* made it more suitable for troubleshooting than the other species evaluated, and thus most bacterial work focused on *P. fluorescens*.

Table 5 – TY Recipe (in 1 L deionized water)

Ingredient	Mass (g)	Source
Tryptone	6	Sigma-Aldrich Corp. St. Louis, MO
Yeast Extract	3	Fisher Scientific, Pittsburgh, PA
Calcium chloride (anhydrous)	0.38	Fisher Scientific, Pittsburgh, PA

2. *Sinorhizobium meliloti*

Several strains of *S. meliloti* were used over the course of these experiments. The strain originally provided was Rm1021/pDG71, which was cultured in TY media with Tetracycline at 5 μg mL^{-1}. The *S. meliloti* grow very slowly, typically requiring several days for a new plate to grow up, and up to five days for a liquid culture to grow to stationary phase, and the cultures were typically not as robust as the *P. fluorescens* cultures. After multiple attempts with no facilitated transport observed, trials with *S. meliloti* were discontinued. Much later in the experimental phase, the non-motile *S. meliloti* strain RU11/011 was used in trials attempting to separate out the effects of planktonic bacterial motility, but no intact cells were egested over the course of a ten day trial.

2.3 Other Fluorescent Particles

1. Liposomes and Nanodiscs

While troubleshooting bacterial protocols, additional transport trials were run using nanodiscs and liposomes filled with Nile red dye. The nanodiscs were 50 – 100 nm in diameter, and the liposomes were spheres approximately 50 nm in

diameter. Due to the very small size of these particles, they were not distinctly visible using any of the available objectives, so the solutions just looked generally red. Various dilutions were used, but nanodiscs and liposomes in the general solution were never distinguishable from those, if any, ingested by protists because of the general washing out of the viewport. At no point was any concentrating effect observed, and over time the discs and liposomes degraded when exposed to light.

2. Fluorescent Beads

Ultimately, the most successful trials performed used FluoSpheres Nile red fluorescent carboxylate-modified microspheres, 1.1 µm actual diameter, from Invitrogen Molecular Probes. While troubleshooting the flow problem, *Colpoda sp.* were observed to consume the beads – in combination with bacteria – and carry them internally. This lead to full transport experiments using beads, which helped separate out many of the obfuscating factors. Most importantly, the beads are completely non-motile, so the only means of transport are diffusion or being carried by protists. The question of digestion rate was also eliminated as the beads are not digested. The process is still dependent on grazing rate, egestion rate, and protist swimming speed, but those factors do not obscure facilitated-transport effects the way intrinsic motility does.

3 Experimental Setup and Device Design

The next major consideration was how experiments should be carried out, and how the device should be designed, to obtain results that were both comparable and replicable.

Separation of predation and bacterial motility effects was also considered in the device design process.

3.1 Preliminary Experiments – Proof of Concept

The first experiment performed used both species of protists, *Colpoda sp.* and *Cercomonas sp.*, and both species of bacteria, *P. fluorescens* Pf0 and *S. meliloti* Rm1021. Each species of bacteria was tested alone and with each protist species. Experiments were carried out using the "Argyle" device, shown in Figure 8, a master that had been fabricated by the lab group in the past but was no longer actively in use. All ports at each end of the device were punched. The wells on one end were loaded with the microbial treatment (bacteria alone or bacteria with protists), and the wells at the far end were filled with TY gelled with 1% agarose. The gelled TY was intended primarily to be a "bell to ring" when bacteria reached the far end of the device, resulting in stronger bacterial growth and thus a stronger signal in that viscinity, but also as a possible chemoattractant to direct movement toward the far end.

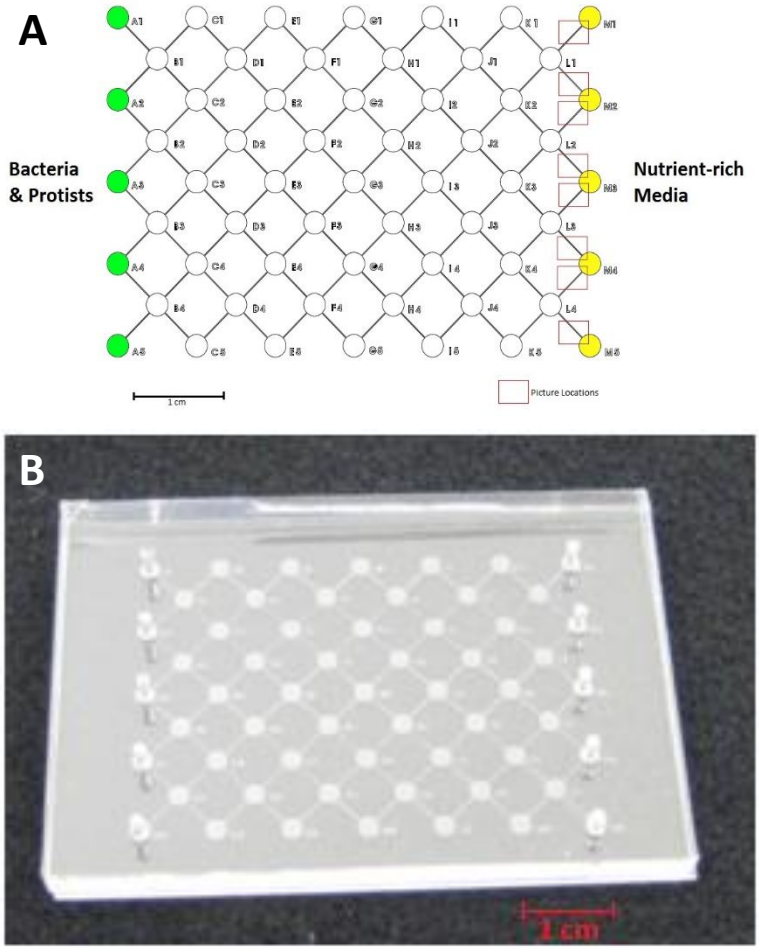

Figure 8 – A) Schematic of preliminary experiment. Green wells indicate microbial inputs, yellow wells indicate wells filled with gelled TY, and the rectangles indicate the locations where images were taken. B) A photograph of the Argyle device bonded to a 2 × 3” glass slide.

Images were taken daily for 5 days at roughly the same time each day. The devices were removed from the stage between time points and stored in square petri dishes filled with deionized water to reduce evaporation. The result was a set of images at each position and time point, as shown in Figure 9.

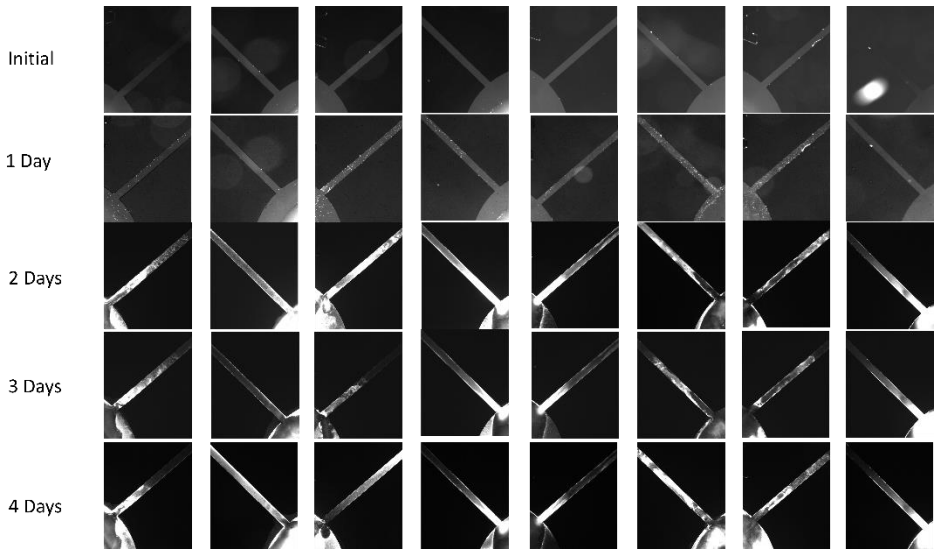

Figure 9 – Sample set of preliminary images, for the treatment of P. fluorescens Pf0 and Colpoda sp.

The preliminary images were not quantifiable, but some issues with the experimental setup were identified, and were addressed in the next round of experiments. Qualitatively, the images did seem to show a beneficial effect from the presence of protists. Those key issues were:

1. Inconsistent image exposure – The goal of this experiment was to determine in a binary manner whether or not protists could facilitate transport. As a result, the images were captured under different lighting conditions, as the ideal exposure varied with the amount of fluorescence in the viewport on a given day. The variance in exposure time between days, however, meant that the results of analysis with image processing software could not be compared.
2. Device design issues – Several issues were encountered with the device itself:

a. No posts in round junction areas – the round junctions within the microfluidic network were large enough that without posts they tended to collapse.
b. Network size – the large size of the microfluidic network required long-term monitoring to see a clear signal for all treatments, which was sometimes infeasible due to equipment availability.
c. Overall device size – the large size of each device meant only one experiment could fit on a single slide, so multiple treatments or replication required a large number of devices. Preparation of that many devices requires significant amounts of both materials and time, and is also not conducive to leaving an experiment on the microscope stage for the duration of an experiment. Leaving experiments on the stage for the full duration is desirable because it allows for more consistent image placement via automated stage movement, which in turn leads to more comparable results from image analysis.

3. Auto-fluorescence of TY – Although not a serious issue, it was observed that the TY media had a low level of fluorescence even before bacteria were introduced, which changed the background fluorescence levels in the device over time as TY diffused out of the gel. Further investigation of the issue was carried out with the next device.

3.2 Second Round

The next round of experiments, and those that yielded the first quantitative results, were performed using the "Mini Argyle" device, shown in Figure 10. The device was a modification of the full Argyle device, made to address the design issues encountered in

the first round of experiments. The same combinations and strains of bacteria and protists were used again, and gelled TY was again used at the far end of the device. Various punching configurations were used with this device while troubleshooting the flow, but the configuration used for the results obtained is shown in Figure 10A. The devices were once again bonded to 2 × 3" slides, but for this version two devices fit on each slide, allowing for simultaneous replication. These experiments were run over much shorter time periods, and were left on the microscope for the duration of the experiment. Devices were placed in a bath of deionized water contained by PDMS bonded to a larger piece of glass, and all punched wells in the device were covered with thin strips of PDMS to further reduce evaporation, as shown in Figure 10B.

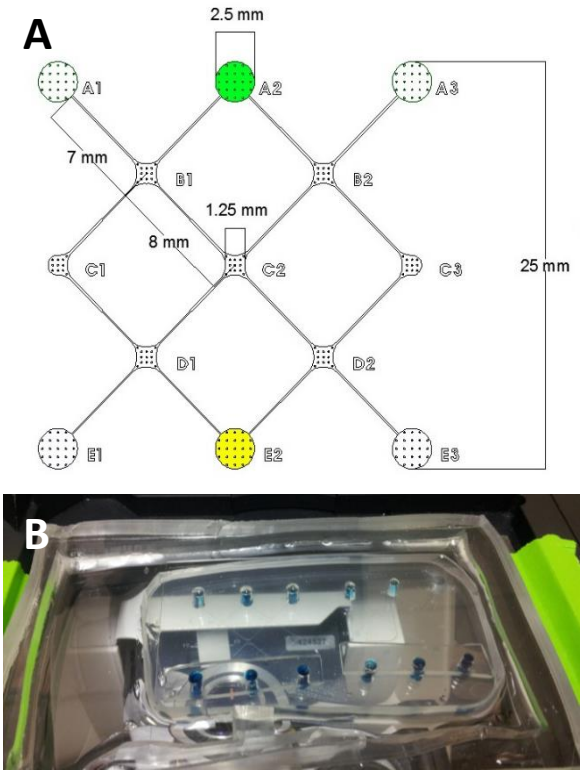

Figure 10 – The device used for the second round of experiments. A) Schematic of experimental setup. As in the previous schematic, the green well received microbial inputs and the yellow well contained gelled TY. The uncolored wells were not punched for the final experiments. B) Photograph of a microfluidic device as it would be placed on the stage for an experiment. Note: the device pictured was used for flow troubleshooting, the punching configuration is not the one used for the experiments.

Images were captured at all channel junctions, and analyzed using the measure function in ImageJ. The exposure was the same for all time points and all treatments, so results were directly comparable. The results of the experiment are shown in Figure 11.

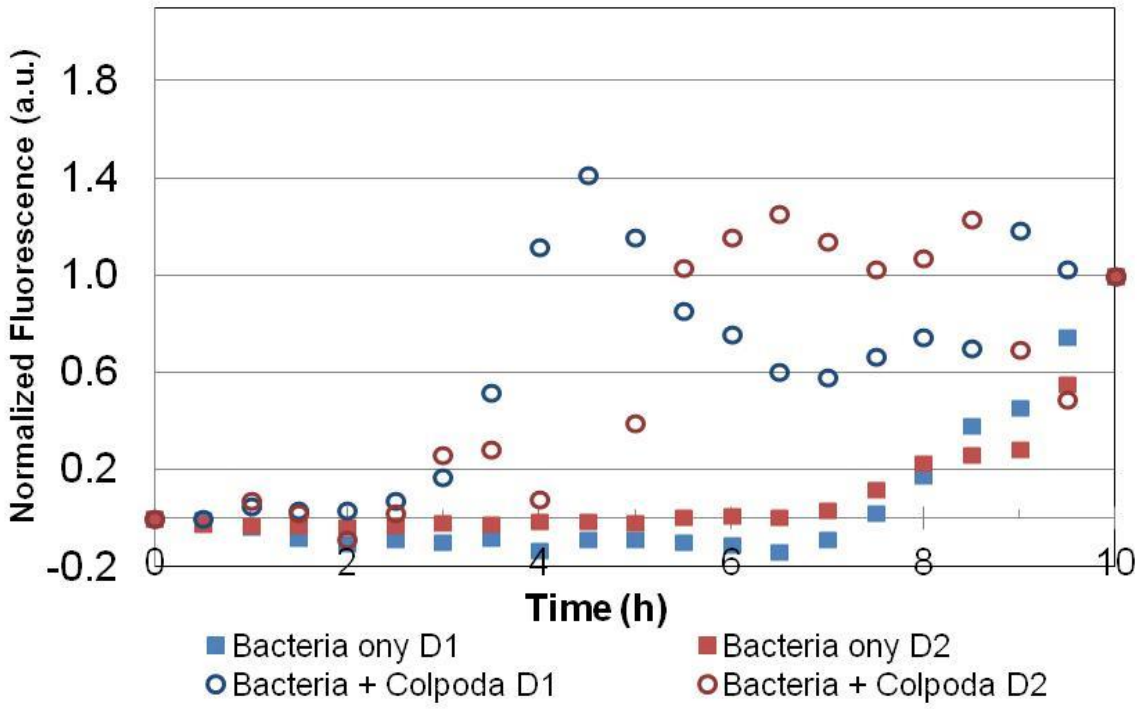

Figure 11 – Results of second-round experiments, as presented by Dr. Leslie Shor at the Bill & Melinda Gates Foundation Grand Explorations Update Meeting.

These results were promising, but replicability was very poor. In some trials, microbes completely failed to leave the input wells. In others flow was not well-controlled and results could not be considered valid. There were some key lessons learned regarding device design by the end of this round:

1. Short networks – The smaller device design allowed for much more efficient testing, and all future devices were designed with that in mind.
2. Predation – One major difficulty with these devices was that there was no shelter for bacteria, so predation effects tended to quickly overwhelm any possible facilitated transport in a way that was not necessarily realistic, since true soil would provide

ample hiding places. We hypothesized that with no way to shelter from protists, bacteria were often not able to gain a foothold in the microfluidic devices.

3. Bacterial Motility – There continued to be difficulties with the bacteria being very motile in the relatively un-constricted channels. In a normal soil setting, bacteria would be effectively non-motile, primarily existing as biofilms, but the planktonic bacteria were able to swim very quickly. This effect seemed to be exacerbated by bacteria entering a reduced-activity state in the presence of *Colpoda sp.* Bacteria were observed to swim rapidly both alone and in the presence of *Cercomonas sp.*, but in the presence of *Colpoda sp.* tended to swim little or not at all, which complicated the results.
4. Fiducial marks – Although image placement was consistent for each location between time points, there was variation between locations in the available area included in the viewport. Future designs incorporated fiducial marks for more consistent image placement.

3.3 Third Round

The third round was focused primarily on design of the "Sawtooth Serpentine" device, shown in Figure 12, which was intended to control for predation and bacterial motility effects. The final design incorporated a sawtooth design along all inner channels, providing many potential hiding places for bacteria in the form of 5 µm wide × 10 µm deep micro-pores. In order to increase throughput, devices were designed to fit up to 6 on a single 2 × 3" slide. To combine high throughput with sufficient distance for protist motility to outstrip bacterial motility, serpentine channels were incorporated, increasing the distance travelled between tiers tenfold. Images were intended to be captured at each

serpentine location, so the serpentines were scaled to fit within a single viewport and fiducial marks were placed to facilitate image placement. Landing pads were placed both in the center of the device and around the edges, to allow for some flexibility in experiment configuration depending on what was most effective.

The micro-pores were at the lower limit of our micro-fabrication capabilities, and fabricating the master required some trial and error, but a usable master was produced and devices were bonded and filled. Several experiments were performed using these devices, with all 6 combinations tested on a single glass slide. Some experiments were performed with TY added to the outer ring and microbes added in the center, and some with the reverse, but both caused operational difficulties. The TY gelled with 1% agarose did not spread well around the outer ring, as it solidified before it could move far. On its own this was not necessarily a problem, but the resulting air bubbles induced significant flow. Filling the center well with TY was more straightforward, but there was no way to add microbes to the entire outer ring in a way that would produce a homogenous spread. Spaces were made for four punches on the outer ring and filling all four helped with homogenization, though the time elapsed between loading each well sometimes induced flow. The first row of serpentines were placed with a 90° offset from those wells to inhibit flow during the filling process, but they did not prevent it entirely.

The difficulties in loading TY into this device resulted in the closest-to-successful configuration yet. By omitting the TY entirely, and punching only the center well for microbe addition, flow was entirely eliminated, and microbes were still observed to move through the device. No results were obtained with this device, but gelled TY was omitted for the remainder of the experiments.

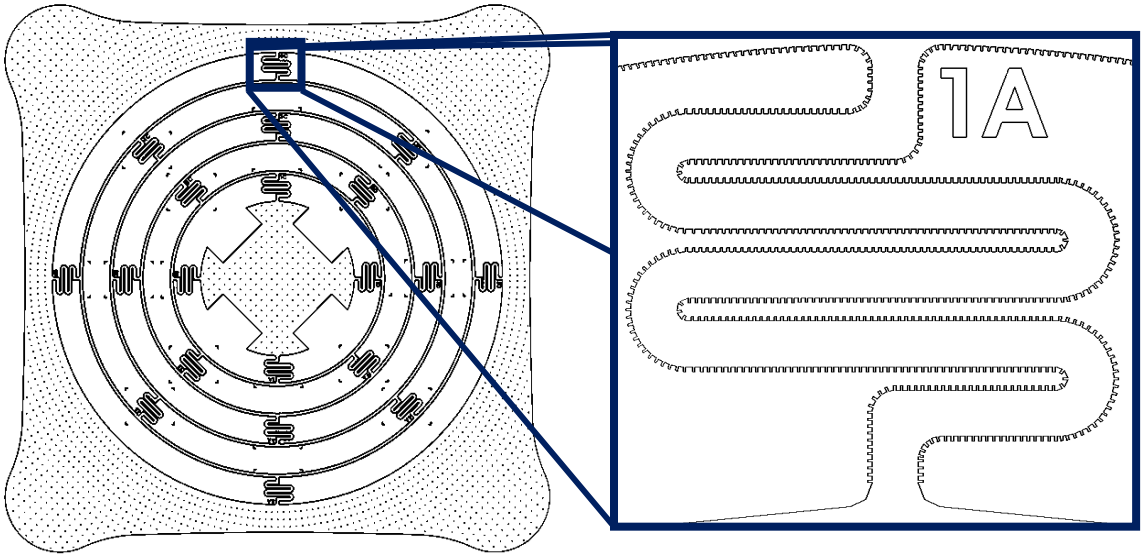

Figure 12 – Sawtooth Serpentine device design, with expanded view of a single serpentine pathway.

3.4 Fourth Round

The second-to last round of experiments performed utilized several similar but not identical devices. With all the complications resulting from the very complex Sawtooth Serpentine design, the next round of experiments utilized a much simpler design, with a previously-fabricated device as the starting point. The original device, the "Block Matrix" was as large as the original Argyle, but the cast PDMS devices were sliced into smaller sections using a scalpel, to test the pattern before redesigning a smaller version, as shown in Figure 13. PDMS, both bonded alongside the slices and applied to openings before curing, was used in an attempt to seal the sliced channel openings, but neither method completely prevented leaking. The main goal of the trials using these devices was to determine if protists could move through the microhabitats before designing a smaller version.

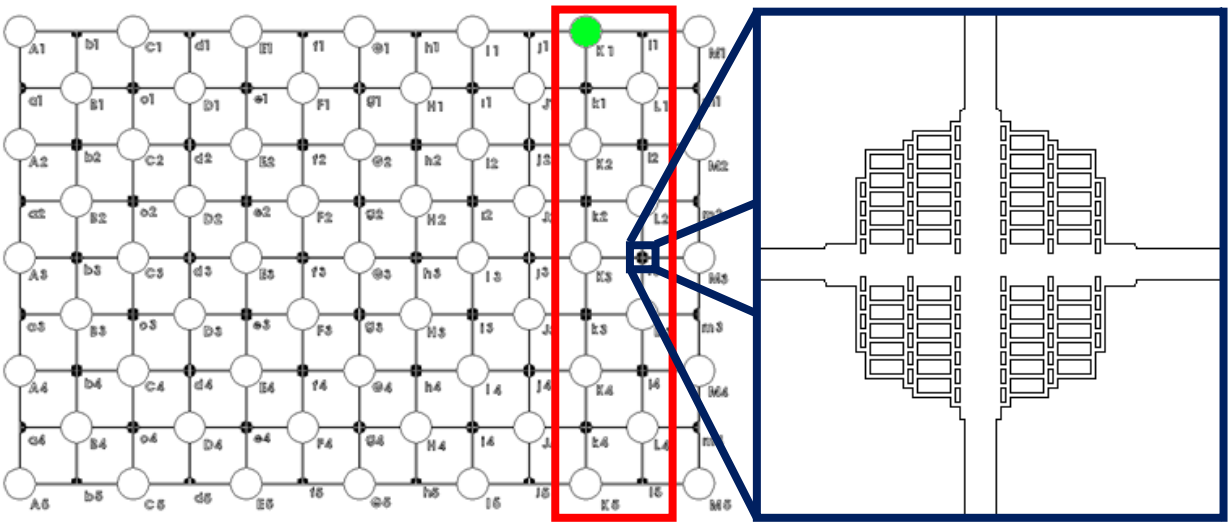

Figure 13 – Original Block Matrix device. The red rectangle indicates the border of a single section that was cut out for the first set of trials, such that five devices could be obtained from each Block Matrix. The green well is where microbial inputs were made, and the detail image is the microhabitat that was intended to provide some shelter for bacteria.

There were several key considerations when designing the second generation block matrix:

1. Designated microbial input – The new generation needed to have a designated area for microbial inputs, evenly spaced between travel channels and with some buffer area so that a slightly shifted punch (since punching was done manually) would not alter the network to be travelled.
2. Posts in round habitats – as with the Argyle device, the round areas in the original design were too large to maintain their form without posts.
3. Equal available area – in the original design, the area available to microbes was very different between the two types of habitats. The new version was designed with exactly the same available area within the fiducial marks for all habitats.

4. Ease of fabrication – the smallest squares in the microhabitats of the original design approached the limits of fabrication and inhibited protist mobility to an extent, so they were omitted in some variants of the new design, including the one ultimately used to obtain the results shown in Figure15.

The resulting device was sized to fit up to six devices on a single 2 × 3" slide, though typically experiments were run with only three at a time to simplify the loading process. Devices were kept on the microscope stage for the duration of experiments in a glass-and-PDMS bath like the one shown in Figure 10. The same exposure was used for all imaging, and the automated stage was set to capture images lined up with the fiducial marks. Experiment set up was as shown in Figure 14.

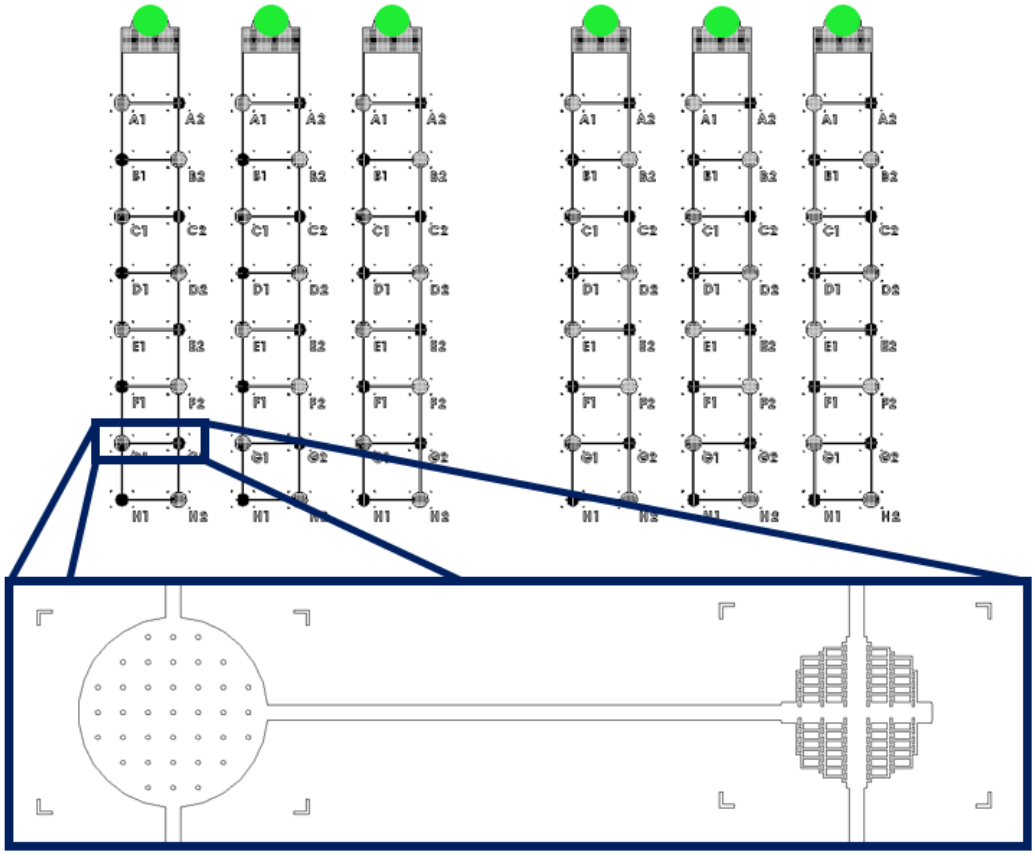

Figure 14 – Modified Block Matrix device. As before, green wells indicate microbial inputs.

Experiments were run using both *P. fluorescens* Pf5 and *S. meliloti* Rm1021 (separately) and both *Colpoda sp.* and *Cercomonas sp.* (separately). In all cases, devices loaded with *Cercomonas sp.* were indistinguishable from those loaded with bacteria alone, with no apparent enhancement of transport but also lacking the reduced-activity effect observed with the *Colpoda sp*. present. Once this trend was shown to be replicable, trials with *Cercomonas sp.* were discontinued. Similarly, no facilitated transport effects were observed using *S. meliloti*. Fewer trials were performed than with the *P. fluorescens*, but this was partly due to great difficulty getting the bacteria to grow well enough for use, an issue that was simultaneously being evaluated by the Gage

Laboratory. As a result, all additional trials focused on only *Colpoda sp.* and *P. fluorescens*.

Replicability of results from these experiments was poor, with microbes often failing to leave input wells. This may have been due to variation in temperature or humidity in the microscope room, or simply because microbes do not always behave in a predictable manner. Even so, the results collected when the microbes did leave the input wells were once again promising. Predation and bacterial motility still were significant factors, but the results of image analysis were normalized to aid in direct comparison of the difference in spatial distribution between treatments.

Figure 15 shows fluorescence intensity over time at two successive locations along the microfluidic devices, normalized to the maximum value reached for a given treatment. Treatments with protists present produced a "rolling curve" showing that protists helped bacteria reach their peak concentration before the end of the time trial, though predation then reduced the population. If the experiments had been run over a very long time period, it seems probable that the concentration would have stabilized, especially as protists began to re-encyst. It is worth noting that the bacteria observed may have been dropped off well after the first time a protist entered a given area, so transport was not as strongly dictated by protist swimming speed as originally expected. For bacteria alone, however, transport was entirely dictated by bacterial swimming speed and growth kinetics, as illustrated by the continuous increase until the end of the experiment.

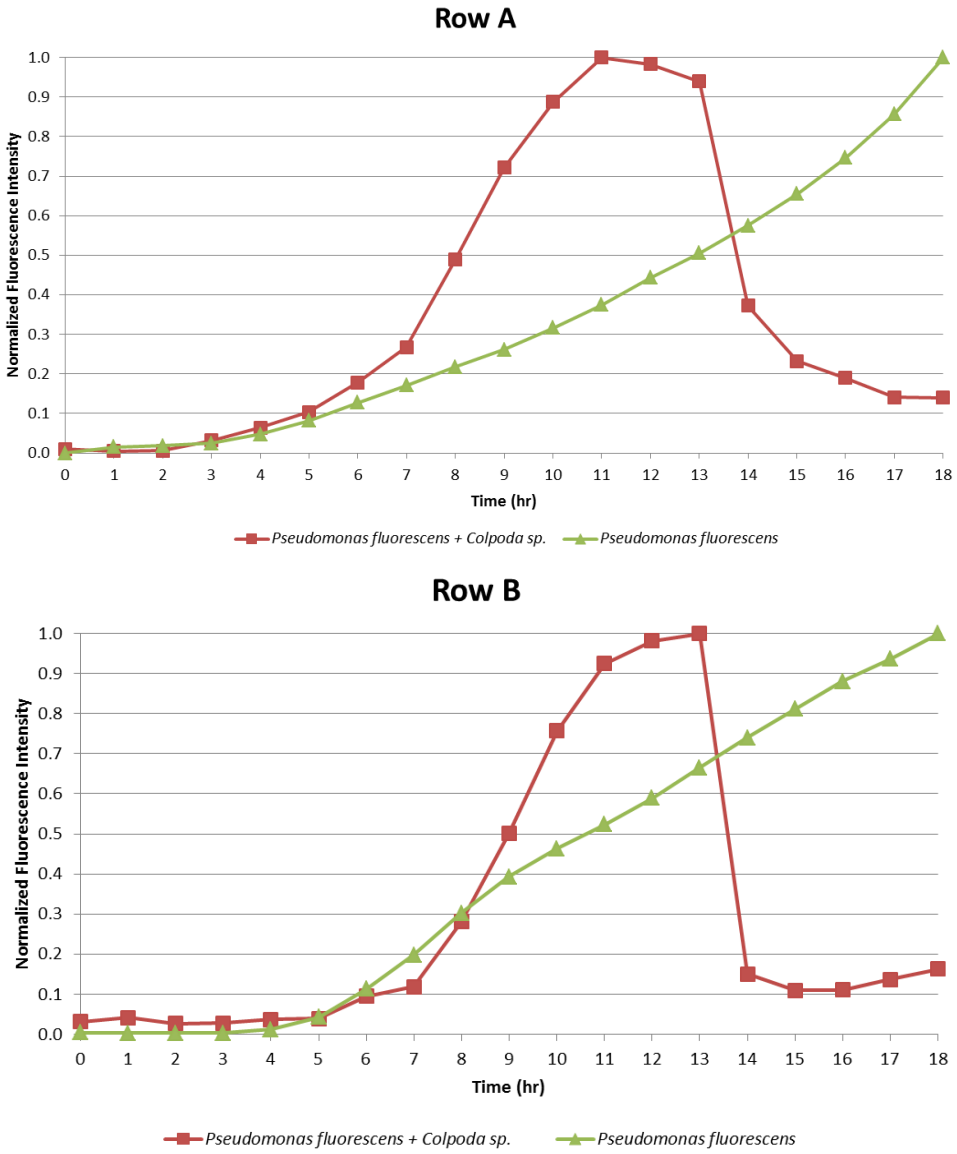

Figure 15 – Results using Modified Block Matrix with *P. fluorescens* and *Colpoda sp*. Row A is the first row after the input wells, and Row B is the second.

Although the results did show a clear difference between bacteria alone and bacteria with protists, the poor replicability prompted further modification of the experimental procedure and setup, leading to the final device and experimental design as described in the first section. Manual counting was done with many images from experiments that had appeared successful but after computer analysis yielded nothing, but this did not change the results.

4 Image Capture and Analysis

4.1 In-Lab Microscopy

For all experiments prior to the final round, the Carl Zeiss AXIO-observer Z1 automated inverted microscope equipped with an AxioCam MRmRev.3 camera (Carl Zeiss Inc., Germany) in the Shor lab was used. Brightfield and fluorescence (470 nm, 62 HE B/G/HR reflector, Carl Zeiss Inc., Germany) images were taken using a 5x objective (Zeiss ECPlan-NEOFLUAR; 5×/0,16 ∞/0,17, Carl Zeiss Inc., Germany). Time lapse, multi-position experiments were set up using the Axiovision 4.8 software. The ideal exposure was found to be 1.5 seconds for *P. fluorescens* Pf5, yielding clear images of bacteria at measurement points but producing minimal background fluorescence. Protist cysts were loaded before putting the devices on the stage, but bacteria were not added until after the experiment parameters and image locations were set, as this was quite time consuming.

4.2 Biotech/ Bioservices Microscopy

Some troubleshooting was done to determine the final image capture parameters used for the the confocal microscope discussed in Section II. The microscope system was capable of imaging with 10 ×, 20×, and 40 × objectives, and using either resonance or galvano scanning. Resonance scanning was much faster, able to capture mosaic images of an entire soil-patterned channel in about 1 minute, but produced lower quality images with resolution 512 × 512 pixels. Galvano scanning produced much higher quality images, up to 4096 × 4096 pixels, but took far longer (the time depends on the resolution selected, but up to 30 minutes to capture a single channel).

The beads were brighter and more distinct than bacteria, so resonance scanning produced sufficiently clear images for quantification. For bacteria, however, concerns were raised as to whether cells could be counted at lower resolutions. A single area of a test microfluidic device containing bacteria in a soil patterned channel was imaged using resonance scanning with both the 20 × and 40 × objectives, and using galvano scanning with the 20 × objective at several different resolutions. Each field was counted manually and the results were compared. The resonance images were too pixelated, and the highest resolution galvano images too so long to capture a single frame that capturing full mosaic images of all tracks was not feasible. The final microscope configurations selected for each type of fluorescent particle are shown in Table 6.

Table 6 – Image capture details for confocal microscopy

Particle Type	Resolution (px)	Scanning Type	Laser emission wavelength (nm)
Beads	512 × 512	Resonance	595
Bacteria	2048 × 2048	Galvano	488

4.3 Image Analysis and Quantification

The last aspect of the project that required troubleshooting was image analysis. The first batch of experiments were analyzed using the measure function in ImageJ. Most experiments yielded several hundred images, so various macros were written to allow automated processing of the full set more rapidly than could be done manually. There was significant discussion regarding thresholding of images. Although it is a widely accepted practice, in this experiment it tended to give results that were not representative of what was actually observed. Because the cells were so small, thresholding typically either completely eliminated the signal or increased the bright area, either of which resulted in inaccurate portrayal of the actual bacterial distribution. Many different

thresholding levels were tried, but there was also random variation in fluorescence between cells, resulting in inconsistent removal or enhancement of cells regardless of the threshold set point.

Setting the appropriate exposure time for fluorescence imaging was key to reducing the need for thresholding. Too long of an exposure resulted in images with significant background signal, sometimes difficult to differentiate from the fluorescent cells. The total fluorescence measured in the initial images, before bacteria arrived, could be subtracted from those that followed, but background lighting was not consistent over the course of an experiment so this sometimes yielded negative values. Too short of an exposure resulted in the cells appearing very dark, sometimes barely visible. The 1.5 second exposure was long enough for cells to be clearly visible, but short enough that the background fluorescence was typically minimal. Ultimately it was determined that simple manual counting of fluorescent particles made the most sense, as it eliminated all uncertainty regarding quantification. All counting was performed by the same person, which ensured consistency between replicates and trials.

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