Investigation into Simulated Microgravity Techniques Used to Study Biofilm Growth

Angie M. Diaz,[1] Wenyan Li,[2] Tesia Irwin,[3] Aubrie O'Rourke,[4] Christina Khodadad,[5] Mary Hummerick,[6] Jonathan Gleeson,[7] and Luz M. Calle[8]
NASA, Kennedy Space Center, FL, 32899

and

Michael R. Callahan[9]
NASA Johnson Space Center, Houston, TX, 77058

Bacterial growth in liquid media in microgravity conditions is not well understood. Trends such as a shortened lag phase, longer log phase, slower growth rate, and a higher final population concentration have been noted but the underlying cause remains unclear. At the single cell level, it is predicted that bacteria are less gravity-sensitive than larger species. The effects on their immediate environment, including the lack of cell settlement and slower mass transfer of nutrients due to lack of density driven convection, could help explain the trends. Ground-based spaceflight analogs, or simulated microgravity devices, are often employed to achieve different attributes of weightlessness to study effects on bacterial growth. Though these technologies could isolate gravity's role in various biological processes, they cannot replicate all its effects and underlying mechanisms. Hence, interpretation of results could be misleading, even if similar to spaceflight. In this study two common simulated microgravity devices were investigated to determine whether they could simulate relevant microgravity conditions for bacterial growth. A bioreactor, the high aspect ratio vessel (HARV), was used with dyes of different density mounted on a random positioning machine (RP machine) or a rotating wall vessel (RWV). The RP machine displayed higher mixing rates than the RWV. The RWV was further tested at different rotations per minute (RPM). The range to minimize effects of density driven convection (low speeds) or centrifugal forces (high speeds) was between a range of 15-20 RPM. These results will help inform the selection of simulated microgravity device as well as interpretation of subsequent biofilm growth results.

Nomenclature

1G	=	Earth's gravity
g/mL	=	Grams per Milliliter
HARV	=	High Aspect Ratio Vessel
mL	=	Milliliter
RWV	=	Rotating Wall Vessel
RP Machine	=	Random Positioning Machine
RPM	=	Rotations per minute
µL	=	Microliters

[1] Amentum, Research Scientist, Kennedy Space Center, FL, 32899.
[2] Amentum, Research Scientist, Kennedy Space Center, FL, 32899.
[3] The Bionetics Corporation, Research Scientist, Kennedy Space Center, FL, 32899.
[4] NASA, AST, Life Sciences Research, Kennedy Space Center, FL, 32899.
[5] Amentum, Research Biologist, Kennedy Space Center, FL 32899.
[6] Amentum, Research Microbiologist, Kennedy Space Center, FL 32899.
[7] The Bionetics Corporation, Aerospace Engineer, Kennedy Space Center, FL, 32899.
[8] NASA, Senior Research Scientist, Science and Technology Programs Division, Kennedy Space Center, FL, 32899.
[9] NASA, Water Technology Lead, Crew and Thermal Systems Division, Johnson Space Center, TX, 77058.

I. Introduction

MICROBES are predicted to be less gravity-sensitive than larger species, making potential effects from microgravity negligible in comparison to other physical forces both within and on the individual cell.[1] However, the change in gravity could impact the environment of the microbes, affecting microbial growth indirectly. The influence of nutrient concentration on microbial growth is described by Monod's equation, in which there is a relationship between the bulk substrate (nutrient) concentration (S) and the growth rate (μ). In which K_S represents the value of S when μ/μ_{max} is equal to 0.5 (Equation 1).[2] Because of this relationship, when the growth rate is below μ_{max}, it is controlled by the nutrient concentration, which is in turn affected by the rate of mass transfer.

$$\mu = \mu_{max} \frac{[S]}{K_S + [S]} \tag{1}$$

Klaus et al.[3] also suggests that bacterial growth is a function of both the environment of the cell, as well as some intracellular influences. Hence the availability of nutrients and the ability to remove byproducts of the metabolic process could be influential.
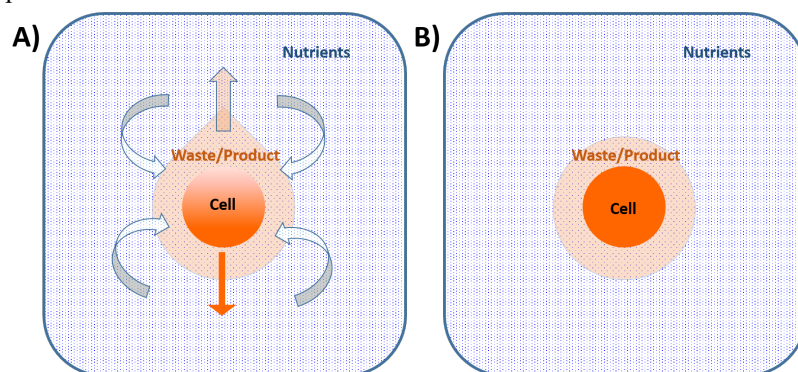

Figure 1. Schematic representation of effects of normal gravity (A) and microgravity (B) on mass transfer of nutrients, waste, and cell products. A) Under 1G, the difference in densities causes density driven convection, mixing a cell's environment and increasing mass transfer. B) Under microgravity conditions, the lack of density-driven convection decreases nutrient, waste, and product mixing and limits it to diffusion around the suspended cells.

In a liquid environment, objects under normal gravity (1G) with higher density settle towards the bottom and lower density bodies float up. This density driven convection causes mixing in a bulk fluid environment, speeding up the process of mass transfer around a settling cell to bring in nutrients and get rid of excreted waste and products (Figure 1A). But under microgravity, objects of different densities travel at the same speed, appearing motionless relative to each other. The lack of density driven convection limits mass transfer, while the lack of gravity maintains cells suspended in place. Diffusion becomes the dominant mixing effect for nutrients and cell products (Figure 1B). Though studies have been done to try and understand the m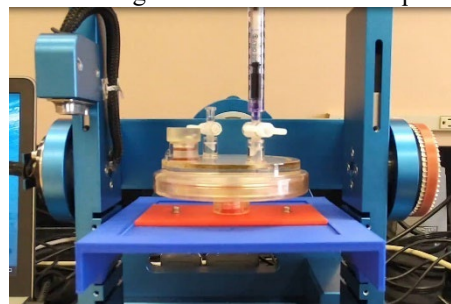echanisms and subsequent effects of microgravity on microbial growth, results have been inconsistent and mechanisms remain unclear.

Studies looking to simulate different attributes of a microgravity environment have utilized instruments and techniques such as the clinostat and its derivative, the rotating wall vessel (RWV) with HARVs (high aspect ratio vessels)(Synthecon). There are also other instruments and methods like the random positioning machine (RP machine), drop-tower/temporary free-fall, neutral buoyancy, and diamagnetic levitation.[4] The two most commonly used are the RWV and clinostats.[5,6] Both are instruments that help control rotational speed by using rotation normal to Earth's gravity to counteract collective sedimentation of the suspended cells in a viscous media. Unfortunately, neither instrument can truly reproduce all aspects of microgravity. For instance, a cell in microgravity could experience changes in the displacement of intercellular components, which under 1G would be subject to density driven convection within the cell. Furthermore, the influence of gravity on the structure of the cell could cause some deformation which would no longer be applicable in microgravity and could be difficult to reproduce in a simulated environment.[7] The mass transfer through the media also cannot be fully reproduced. In a clinostat, relative "motionlessness" of a microbe in comparison to the surrounding media is theoretically achieved through rigid body rotation due to the rotation of the vessel. This keeps cells from sedimenting and creates a low shear environment due to the movement of cells and fluid media in tandem. The RWV has similar physics, maintaining the cells suspended in a low-shear environment and in a continuous fall due to vessel rotation (Figure 2). The HARV allows perfusion of nutrients to and waste removal from cells through the diffused gas exchange membrane on the "back" of it. However, one of the risks is formation of bubbles which could create shear and turbulence, mixing the media when mounted on the RWV.[5] In comparison the RP machine uses independent motors to rotate two frames independently (Figure 3). An experimental bioreactor mounted on a plate fixed to the inner frame would therefore be subjected to randomized movement. The movement, if quick enough, would change the vector for 1G too quickly for the object to react to the new gravity vector and reduce shear forces.[8]
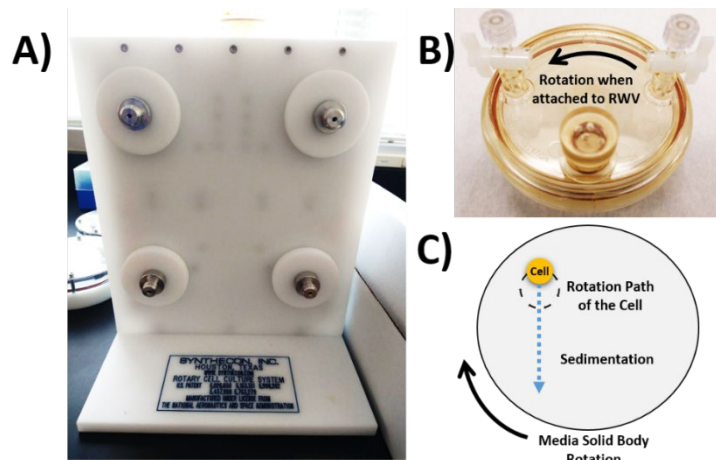
Figure 2. A Synthecon Rotary Cell Culture System or RWV (A) and a HARV bioreactor (B) which could be attached to the RWV. (C) is a schematic of the solid body rotation of the media and a cell in a rotating HARV.

Figure 3. A HARV bioreactor mounted on an RP machine (Airbus RPM 2.0, www.airbusDS.nl).

Because of the differences in simulation techniques, experimental design must take all features of the method into consideration when attempting to recreate important aspects of microgravity which could affect cells. For a microbial experiment, this would mean an instrument that could cancel out cell settlement in a fluid media, as well as decrease density driven convection to slow down mass transfer. If not considered carefully, results of a study at best could be confusing and lacking the right underlying mechanisms even if results are similar to those of microbial growth in spaceflight studies.[9,10,11,12,13] And at worst, inconsistent.[14]

This study aims to evaluate two of the more utilized ground-based spaceflight analogs: the RWV and the RP machine. Both were tested with HARV bioreactors, using visualization through dyes of varying densities. This will allow observation of mass transfer rates and cell settlement conditions which can then be qualitatively compared to spaceflight conditions. For these studies, water was used as the representative bulk media, and dyes represented potential media components ("nutrients" or "cell byproducts") of either high or low densities with respect to water. A comparison of the two simulated microgravity analogs showed that the RWV provides an environment with slower mass transfer. The RP machine's random motions created a turbulent environment in which the dye was swirled, indicating that spaceflight mass transfer would not be represented well because of the high mixing rate. Further testing was continued with the RWV at differing speeds, as well as different 1G control samples for selection of the most accurate representation of ground control effects. An optimal

speed of rotation between 15-20 RPM was found to minimize the effects of density driven convection (due to gravity effects) and centrifugal motion at higher speeds to create an environment of lower mass transfer.

Finally, three control conditions were tested for both the high- and low-density dyes. A standing static HARV device mounted onto an RWV, a static control HARV placed on the benchtop, and a horizontal rotating control. In the lower density dye, the static control was most representative of a higher mass transfer rate. The higher density dye results were not as clear however, as the gas exchange membrane of the HARV created shear forces and influenced the dye distribution. In terms of biofilm growth,

II. Methods

A. Dye preparation
Low density dye was prepared by making a 1:1 dilution of BD Gram Crystal Violet Stain (BD Diagnostic Systems) to deionized water (DiH2O). This was used as a representative for lower density components in the media relative to DiH2O. Average density of 1ml of dye mix was 0.97 g/mL.

High density dye was prepared by using Rat Dyemore Fabric Dye (Racing Red) in a dilution with DiH2O to a density of 1.03 g/mL. The dyes were utilized as qualitative representatives in terms of the relationship to the media: either denser than, or less dense than the water.

B. HARV Dye Loading
Each 50 mL HARV (Synthecon) was filled with DiH2O, with care taken to avoid leaving bubbles in the bioreactor. A 1 mL Luer lock syringe was loaded with 200 µL of a dilution of either high density or low-density dye, with the HARV ports closed. Once screwed onto the one of the ports, the HARV with the attached syringe was placed on the simulated microgravity instrument.

C. Rotating Wall Vessel
Once the HARV was loaded onto the RWV (customized Synthecon Rotary Cell Culture System), the speed was set on the instrument. It was allowed to rotate for 2 minutes to allow the water to settle from mixing due to beginning of rotation. Once 2 minutes had passed, a timer set for 20 minutes was started and the port on the HARV was opened. The syringe was depressed to introduce dye into the HARV, and cell phone videos were created from which images were pulled for the selected timepoints.

D. Random Positioning Machine
The HARV (with dye-loaded syringe screwed on to port) was attached to the Airbus RPM 2.0 (www.airbusDS.nl) in a position parallel to the lab benchtop. The partial G path file was used to achieve microgravity simulation and allowed to run immediately once the syringe released dye into the HARV. A timer was set for 20 minutes, and videos were taken to capture timepoint images.

III. Results

A. RWV and RP Machine Comparison
The RWV and RP machine were used to test two different methods of simulating microgravity (solid body rotation and random positioning respectively). A low-density crystal violet dye was used to visualize mixing in the HARV loaded on both instruments. In Figure 4, images are compared starting at 0 minutes (T0) and incrementing by two-minute intervals up until ten minutes (T10), followed by a final image after twenty minutes (T20) on each device.

By T2, the RP machine demonstrates further dye spreading than in the HARV on the RWV, nearly covering half of the HARV area while the RWV dye seems to only cover about a fourth of it. As time progresses, the HARV on the RP machine continues to have rapid spread of the dye, while the RWV HARV remains more densely packed around the injection site. By T20, the RP machine HARV is almost completely covered while the RWV remains well under half. Considering that the objective was to have slower mass transfer in the media, the dye experiment determined that the RP machine would not be a suitable candidate for simulated microgravity microbiological studies.
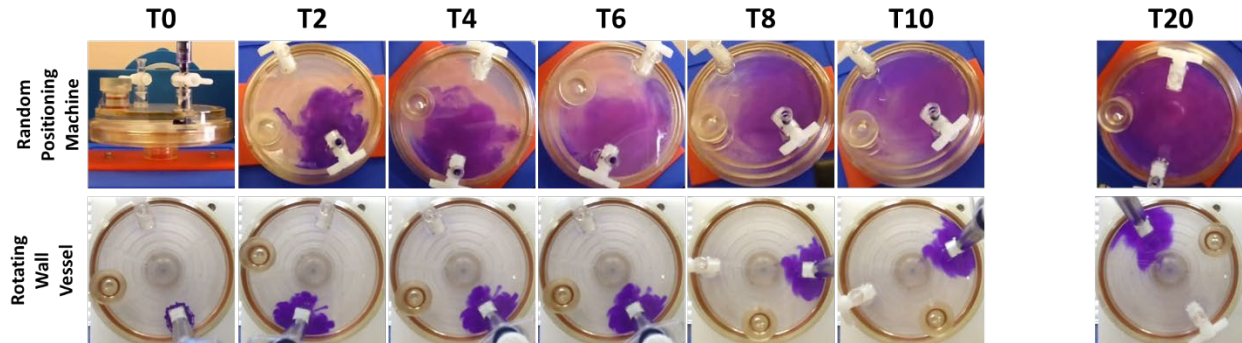
Figure 4. Comparison of a low-density dye in the RP machine and RWV for twenty minutes.

B. RWV With Low Density Dye

Since the RWV was determined to be the more suitable of the two simulated microgravity analogs, the variability of speeds was considered for both low- and high-density dyes. A range of 5 to 40 rotations per minute (RPM) were tested in twenty-minute increments. For the dye with lower density relative to water (Figure 5), if the speed was too slow (5 or 10 RPM), the dye spread upwards due to the influence of density driven convection. When the speed was too high (at 35 or 40 RPM), centrifugal forces would become dominant and cause the dye to move towards the center of the HARV. The intermediate speeds around 15 or 20 RPM had a minimized mixing effect because of the decreased influence of both density driven convection and centrifugal forces. Increasing between 20-30 RPM did show the

gradual increase in influence of the centrifugal forces, with the dye making an increasingly larger shift towards the HARV's center.
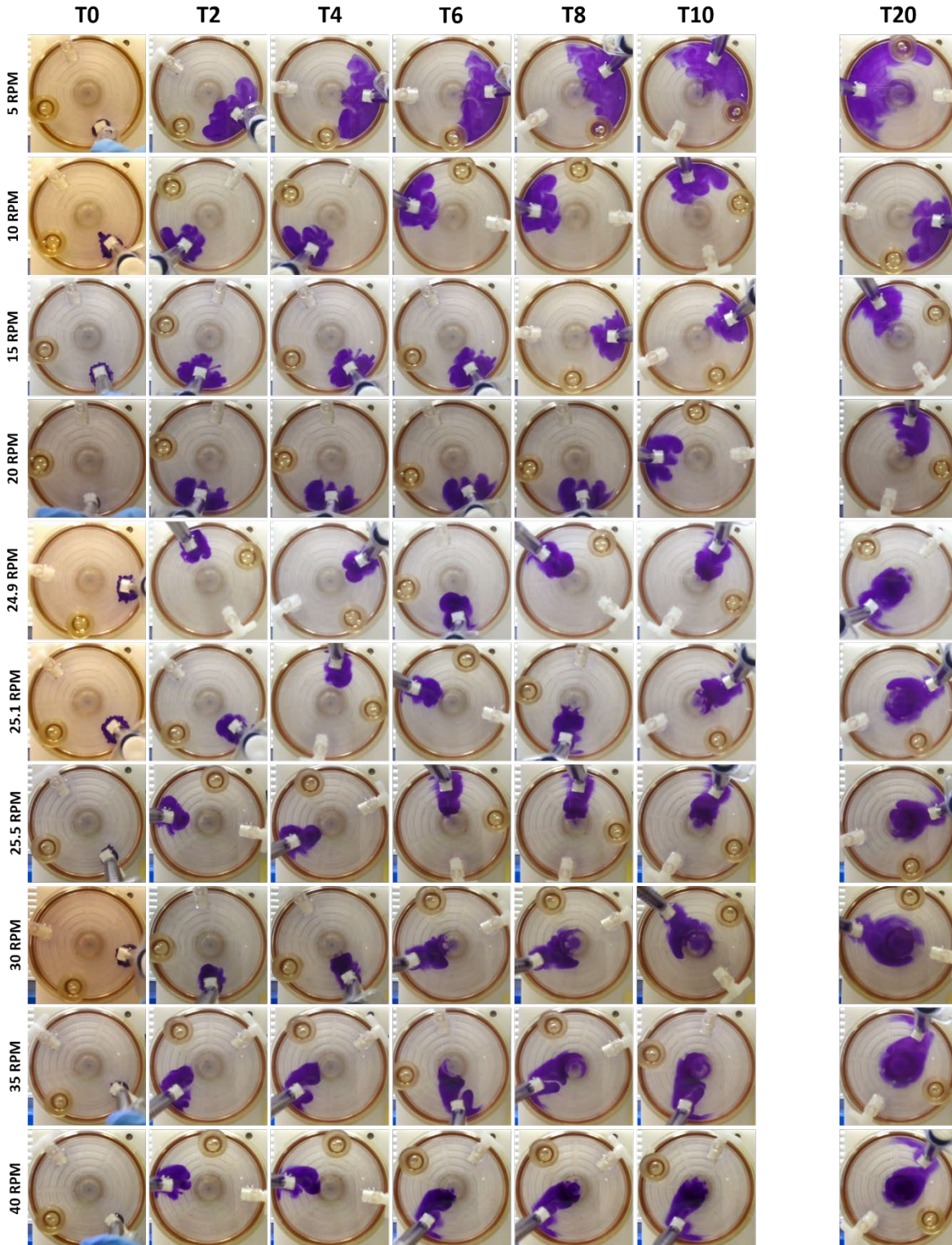

Figure 5. Summary of low-density dye in HARVs at different RPM speeds in two-minute intervals until ten minutes (T10). The final image shows the HARV at twenty minutes (T20).

1. Study on the Lighter Density Control Condition

Previous work done with HARVs references both static or dynamic ground control conditions to compare to spaceflight, though justification as to why either of those conditions is preferred remains unclear. For the low-density dye, three different control conditions were compared (Figure 6).
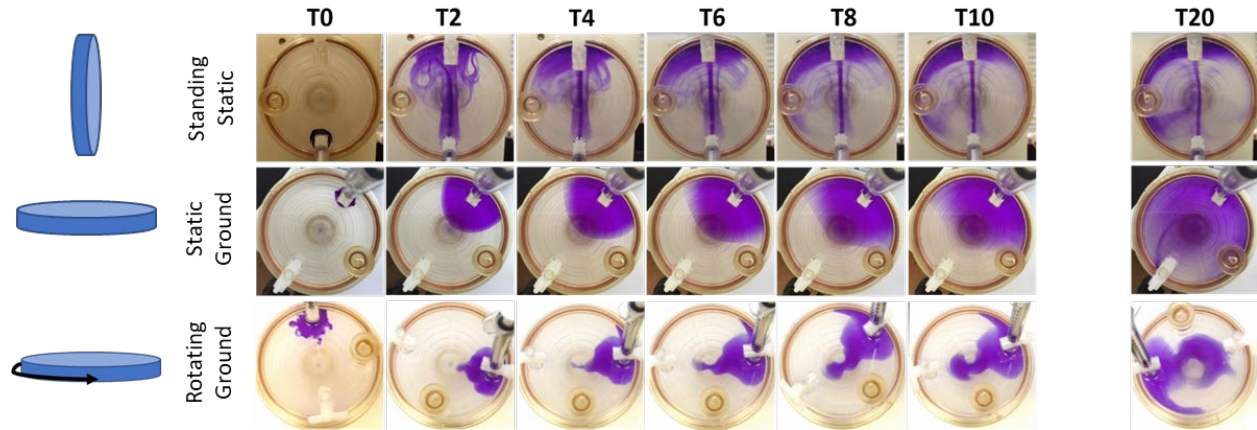

Figure 6. On the left, a representative HARV schematic shows the position of the HARV relative to the ground. The images show a comparison of a standing static HARV, static HARV lying horizontally, and a horizontal rotating ground control HARV at a rotation speed of 25.1 RPM with dye starting at time T0 up until T20 (20 minutes).

The static standing control experienced density driven convection when the dye was injected at the bottom of the HARV, driving it towards the top of the vessel before beginning to diffuse through the rest of the area. The rotating ground control showed movement of the dye towards the center of the vessel, indicating that solid body rotation and centrifugal forces were affecting it. However due to the studies done on the speed variations, 25.1 RPM could be seen to have affected the control condition. Therefore, it would not make an accurate representative of a ground control to compare in a simulated microgravity study. For the static ground control, it had the fastest mixing rate after 20 minutes. Since the objective was to have high mass transfer, of the three conditions tested, the static HARV would be the most representative as a ground control.

C. RWV With High Density Dye

For the dye with higher density relative to water (Figure 7), the density driven convection had a more dominant effect at lower speeds (5 and 10 RPM), causing it to sink before spreading. At the higher end (35 and 40 RPM), the centrifugal force would cause the dye to spread to the outside rim of the HARV. From 15 to 20 RPM was where the mixing once again was most minimized, while speeds between 20 and 30 RPM showed increasing spread of the dye along the edges because of the increasingly dominant centrifugal forces.
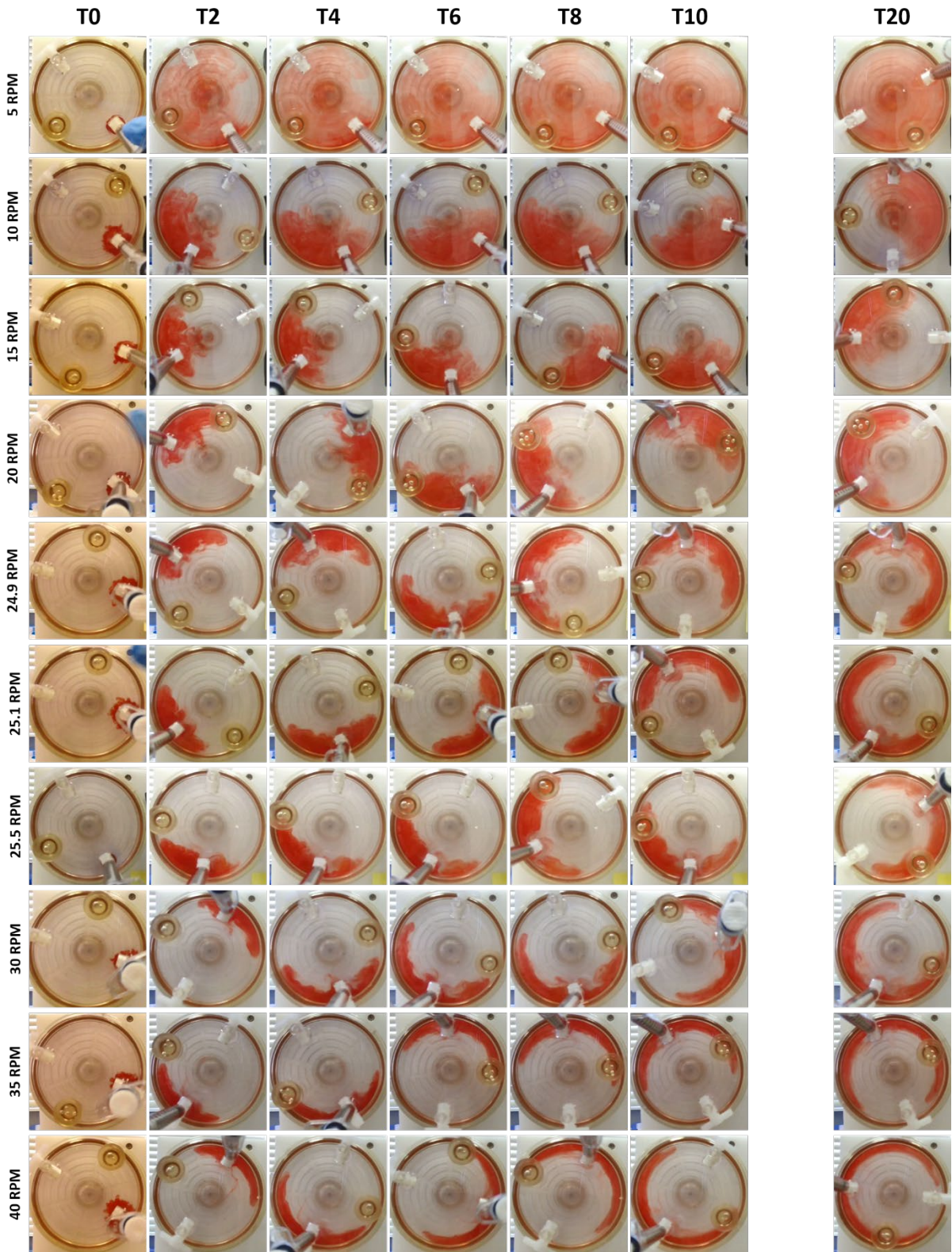
Figure 7. Summary of high-density dye in HARVs at different RPM speeds in two-minute intervals until ten minutes (T10). The final image shows the HARV at twenty minutes (T20).

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1. *Study on the Higher Density Control Condition*
To determine which of the three control conditions would be best suited as the ground control, the HARVs were tested with higher density dye as a standing static, horizontal static, and rotating horizontal conditions (Figure 8).
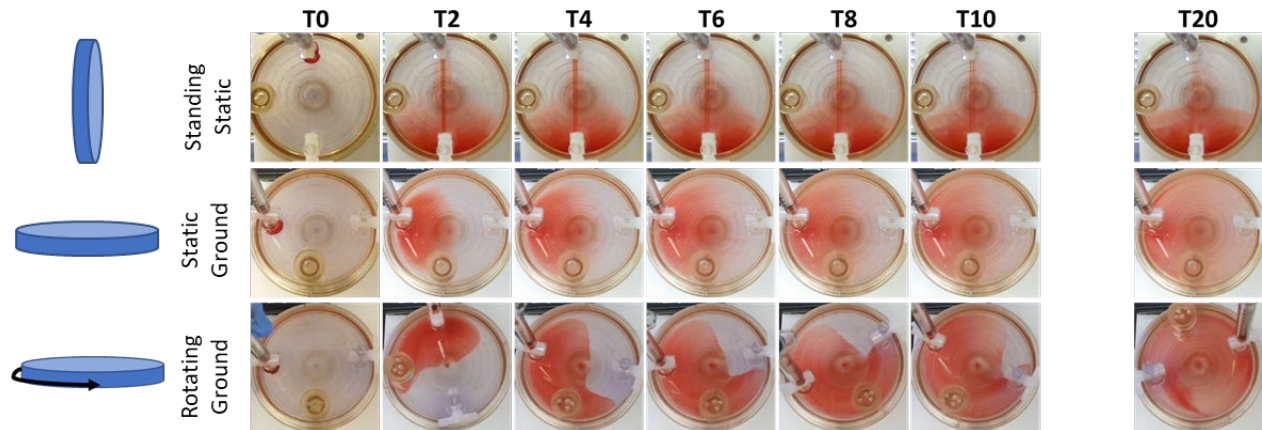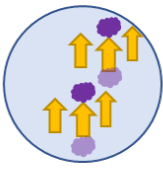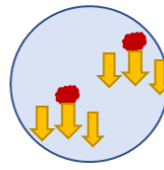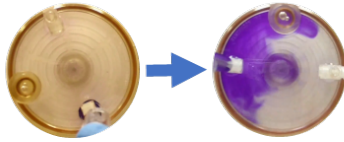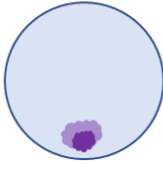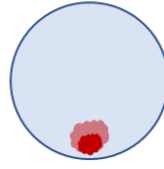

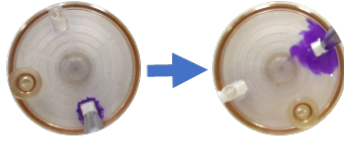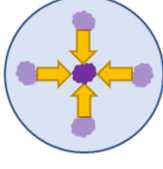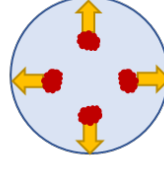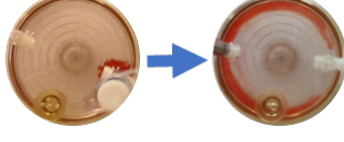**Figure 8. Comparison of different control conditions, including standing static, static ground, and a rotating ground control at 25.1 RPM.**

The standing static control showed effects of the density driven convection through the immediate sinking of the dye but was not the control condition with the fastest mixing rate. Instead, the rotating ground control was determined to be the fastest mixing of the three test conditions. The influence of the speed required consideration however, as the high-density dye results showed that the RPM could have been too high. Also, the back of the HARV, which for the static and ground conditions would be considered the bottom of the bioreactor, contained the membrane allowing gas permeability. This membrane is textured and could have been influencing the spread of the denser dye along the bottom over time due to increased shear force resistance. In the rotating control, though the higher RPM was apparent in the advancement of the dye leading along the outer rim of the HARV, the shear force could also be apparent in the shape of the leading edge forming a border where the water met the dye.

IV. Discussion

The effect of microgravity on microbial cells could theoretically be minimal compared to its effect on the cell's immediate environment. The mass transfer of nutrients, products, and waste of the cell would hence be slowed down. Limitation of such processes could occur through the lack of density driven convection and cell settling. Several simulated microgravity instruments and techniques have been developed, but determination of which instrument/method could be most accurate to the true effects of microgravity on the bulk fluid and component transportation has been unclear. This study aimed to compare how two of the more common simulated microgravity instruments, an RWV and an RP machine, could influence the mass transport of a dye representative of components either less or more dense than the bulk media. In the span of a twenty-minute test, the RWV showed solid body rotation while the RP machine revealed a fast mixing rate that easily spread the dye to most of the HARV before the time limit had been reached. Therefore, further studies were conducted with the RWV. A summary of the speed test results for both the high- and low-density dyes is shown in Figure 9.

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		Lighter Density	Heavier Density	HARV Image (0 to 20min)	Summary
Speed	Slow (5 or 10 rpm)				Density driven convection moves dye either up or down
	Middle (15 or 20 rpm)				Mixing effect minimized; density driven convection no longer dominating effect
	Fast (35 or 40 rpm)				Centrifugal force drives dye either towards center or outer circumference of HARV

Figure 9. A summary of the effects of changes in speed of the RWV on both light and heavier density dye with respect to a bulk water media.

Finally, the control conditions were also studied to determine the most accurate ground control to compare to simulated microgravity for microbial effects. The low-density tests showed that a static, horizontal ground control would be most accurate due to the standing ground control displaying density driven convection, but slower mixing, and the rotating ground having indication of solid body rotation. The higher density dye controls were not as clear, seeing as the rotating ground control showed the fastest mixing of the three controls. However, it should be noted that since the dye is heavier than the water media, it could have been influenced by the rough texture of the HARV membrane on the bottom surface. The speed utilized for both rotating ground controls could have also affected the results since testing of speed variation was done after the control test. Further tests utilizing a lower speed within the optimal range will help clarify the rotating control HARV results. In the future, addition of other testing apparatus could also reveal more candidates for the best representative of a slow mass transfer rate for a simulated microgravity environment. Such an instrument would require allowing the least relative motion between the bulk media, the cell, and all other components of different densities such as nutrients, products, and waste.

Qualitatively, the design of such test methods allowed visual representation of the relationship between different density components in the simulated microgravity environment. However, the fact that these results were done in a shorter amount of time than a full growth curve should also be taken into consideration. How this could affect movement of the dyes would require a much more extended timeframe and would be difficult to determine once the dye fully spreads. Since the aim of this study was to note which instrument had faster mass transfer rates and how speed affected the instrument chosen, the work done could at least offer an initial look into which instruments could be moved on as potential candidates, such as the RWV.

V. Conclusion

The study of the influence of spaceflight conditions on biofilm growth in ground-based instruments still requires clarification as to what underlying mechanisms are truly influencing the microbes and whether these are representative of their spaceflight counterparts. Of these, mass transfer rates of components in the cell environment seem to be influencing. This study used dyes of different densities in a water media to compare two commonly used instruments, the RP machine and RWV for mass transfer conditions. Of the two, the RWV's solid body rotation allowed for slower mass transfer in comparison to the faster mixing rates of the RP machine. Furthermore, study of the effects of different speeds indicated that at lower rotation speeds on the RWV, density driven convection dominated, while centrifugal forces had a larger influence at high speeds. However, between 15 and 20 RPM, the mixing effect caused by either force was minimized. Finally, the control conditions were also considered. The lower density dye studies indicate that a static horizontal ground control would be most accurate for comparison to a simulated microgravity test. The higher

density dye had higher mixing in the rotating horizontal control, which could have been influenced by the texture of the bottom membrane of the HARV, as well as the speed outside of the later determined optimal range. To determine which control would work best for both higher and lower density components, further testing of the rotating ground controls within the optimal RPM range are necessary. Overall, these studies aimed to address the gap in knowledge for the ground-based analogs to create conditions more accurate to the spaceflight conditions, with careful consideration of the effects which the techniques used could cause on microbial studies.

Acknowledgments

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