Investigation into Space Effects on Biofilm Growth Using Simulated Microgravity

Angie M. Diaz,[1] Wenyan Li,[2] Tesia Irwin,[3] Aubrie O'Rourke,[4] Christina Khodadad,[5] Mary Hummerick,[6] Anirudha Dixit,[7] Jonathan Gleeson,[8] and Luz M. Calle[9]
NASA, Kennedy Space Center, FL, 32899

and

Michael R. Callahan[10]
NASA Johnson Space Center, Houston, TX, 77058

Bacterial growth in liquid media while under 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. 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, results could be misleading even if they are similar to spaceflight. 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 cell settlement and slower mass transfer of nutrients, might help explain the trends seen in liquid media microgravity studies. Therefore, experimental design factors must be carefully considered when selecting a simulated microgravity device for proper underlying mechanisms and interpretation of results. To verify if the simulated microgravity devices simulate the relevant microgravity conditions for bacterial growth, including the changes in cell settlement and mass transfer of nutrients, a high aspect ratio vessel (HARV) was used with dyes of different density in various simulated microgravity setups. The results will help inform the selection of the proper 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
RWV	=	Rotating Wall Vessel
rpm	=	Rotations per minute

[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] Amentum, Research Scientist, Kennedy Space Center, FL 32899.
[8] The Bionetics Corporation, Aerospace Engineer, Kennedy Space Center, FL, 32899.
[9] NASA, Senior Research Scientist, Science and Technology Programs Division, Kennedy Space Center, FL, 32899.
[10] 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]

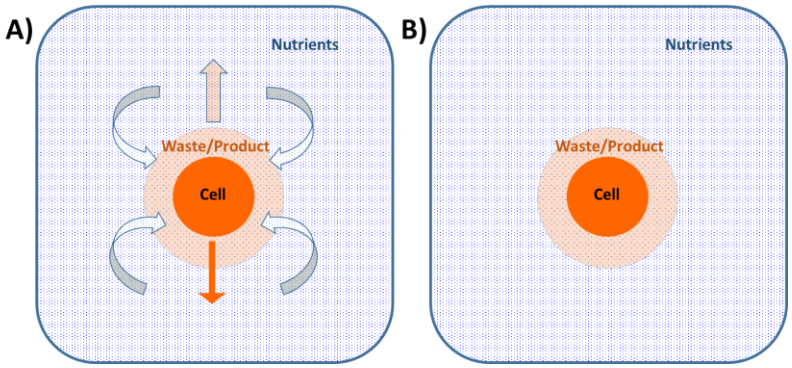


Figure 1. Schematic representation of effects of normal gravity (A) and microgravity (B) on mass transfer of nutrients, waste, and cell products. A) Under 1G, the difference in densities causes density driven convection, mixing a cell's environment and increasing mass transfer. B) Under microgravity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

However, the change in gravity could impact the environment of the microbes, affecting microbial growth indirectly. Klaus et al.[2] suggest that bacterial growth is a function of both the environment of the cell, as well as some intracellular influences. Of these, the availability of nutrients and the ability to remove byproducts of the metabolic process could be the most influencing. Under gravity, a planet pulls smaller bodies towards its center. Hence, 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 mechanisms 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), the random positioning machine (RPM), drop-tower/temporary free-fall, neutral buoyancy, and diamagnetic levitation.[3] The two most commonly used[4,5], the RWV and clinostats, use 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. 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). However, the RWV creates some mixing by allowing perfusion of nutrients to and waste removal from cells through the

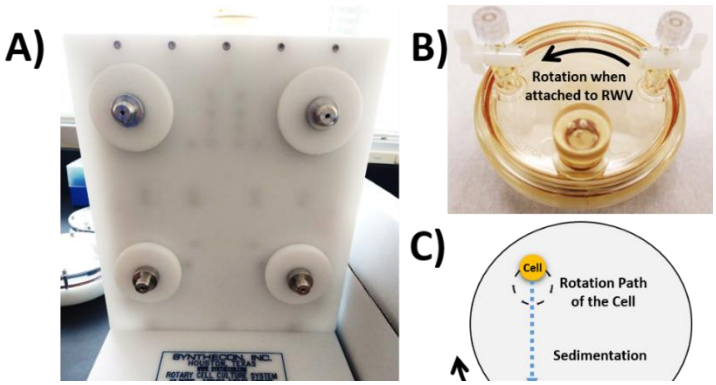


Figure 2. A Synthecon Rotary Cell Culture System or RWV (A), a HARV (B), and a schematic of the solid body rotation of the media and a cell in a rotating HARV (C).

membrane of the high aspect ratio vessel (HARV) used as the bioreactor. Therefore, while an RWV creates a somewhat mixing, fluid environment, a clinostat could reproduce unstirred fluid conditions. In comparison the RPM cancels out the gravity vector through average random movement. This could affect the fluid media by creating a tumbling effect on the cells and lead to increased mass transfer rates.

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.[6,7,8,9,10] And at worst, inconsistent.[11]

This study aims to evaluate two of the more utilized ground-based spaceflight analogs—the RWV and the RPM, using visualization through dyes of varying densities. This will allow observation of mass transfer rates and cell settlement conditions which can then be compared to the expected spaceflight conditions. For these studies, water was used as the representative bulk media, and dyes represented media components ("nutrients" or "cell byproducts") of different densities. A comparison of the two simulated microgravity analogs showed that the RWV is better suited to microbial studies of the two selected. The RPM's random motions created an environment in which the dye was swirled, indicating that spaceflight mass transfer would not be represented well under such conditions. 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.

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 0.97g/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.

B. HARV Dye Loading

Each 50 mL HARV (Synthecon) was filled with DiH2O. 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 HARV port, the HARV with the connected syringe was placed on the simulated microgravity instrument.

C. RWV

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. 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 HARV, and cell phone videos were created from which images were pulled for the selected timepoints.

D. RPM

The HARV (with dye-loaded syringe screwed on to port) was attached to the Airbus RPM 2.0 in a position parallel to the lab benchtop. The software was set to microgravity settings and allowed to run immediately once syringe released dye into the HARV. A timer was set for 20 minutes, and videos were taken to capture timepoint images.

III. Results

E. RWV and RPM Comparison

To verify of the RWV can better simulate microgravity in comparison with the RPM, a low-density dye was used to visualize mixing rates in the HARV loaded on both instruments. In Figure 3, 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 RPM already 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 RPM HARV continues to have rapid spread of the dye, while the RWV HARV remains more densely packed around the injection site. By T20, the RPM 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 RPM would not be a suitable candidate for simulated microgravity microbiological studies.

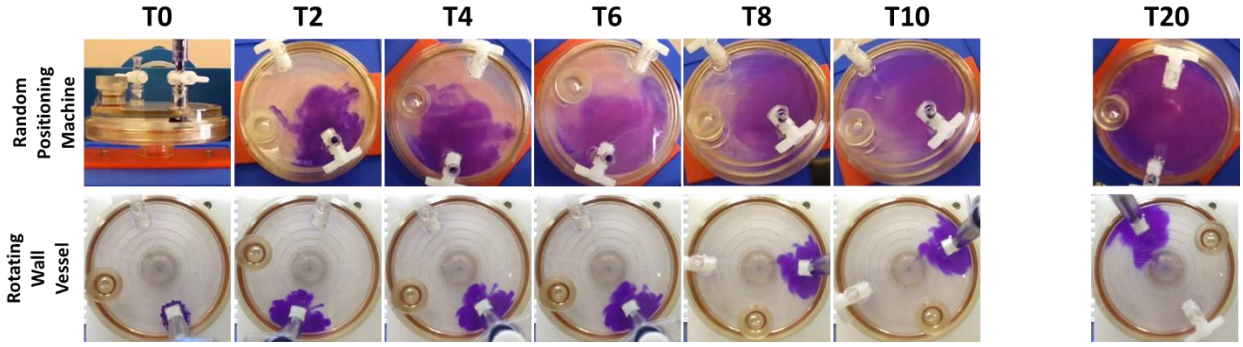


Figure 3. Comparison of a low-density dye in the RPM and RWV for twenty minutes.

F. 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 (Appendix A), 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.

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 4).

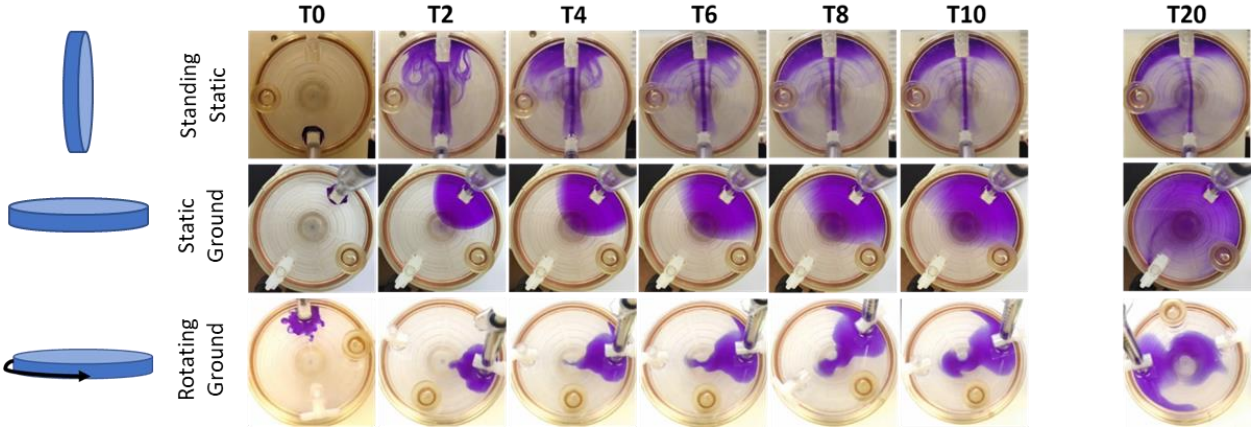


Figure 4. On the left, a representative HARV schematic shows the position of the HARV relative to the ground. The images show a comparison of a standing static HARV, static HARV lying horizontally, and a horizontal rotating ground control HARV at a rotation speed of 25,1 RPM with dye starting at time T0 up until T20 (20 minutes).

The results show that the static standing control experiences density driven convection when the dye is injected at the bottom of the HARV, driving the dye towards the top of the vessel. The static ground control showed the fastest mixing rate after 20 minutes, indicating that this would be the most representative as a ground control. The rotating ground control showed movement of the dye towards the center of the vessel, indicating that solid body rotation and centrifugal forces affected it. Therefore, it would not make an accurate representative of a ground control to compare in a simulated microgravity study.

G. RWV With High Density Dye

For the dye with higher density relative to water (Appendix B), the density driven convection had a more dominant effect at lower speeds (5 and 10 rpm), causing it to spread more downwards and causing increased mass transfer in that way. At the higher end (35 and 40 rpm), the centrifugal force would cause the dye to spread to the outside of the HARV, covering the outer edges. 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.

2. *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 5).

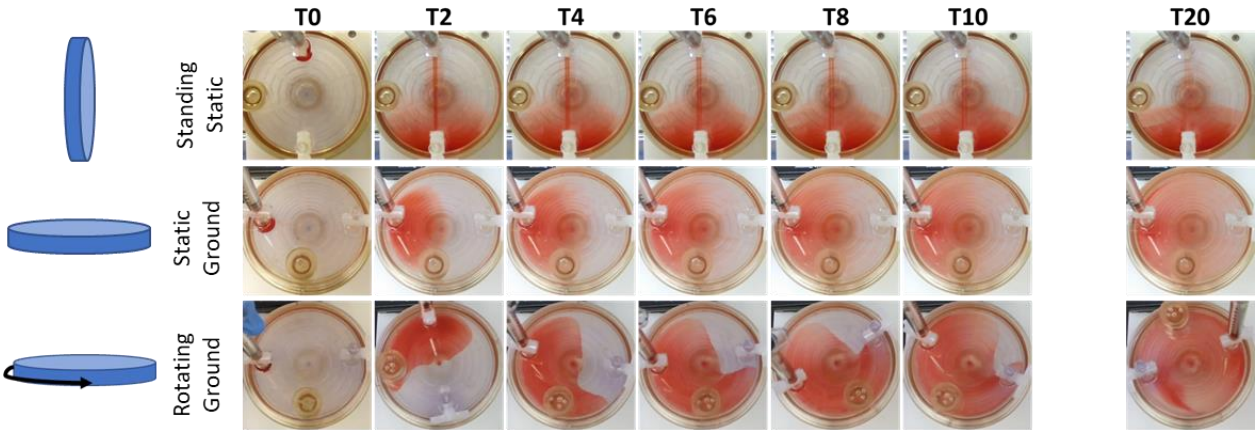


Figure 5. Comparison of different control conditions, including standing static, static ground, and a rotating ground control at 25.1 rpm.

The standing static control showed effects of the density driven convection but was not the control condition with the fastest mixing rate. The static ground control also showed fast mixing, but the rotating ground control showed the fastest mixing of the three controls. The influence of the speed required consideration here, as the high-density dye results showed that the rpm could have been too high. Also, the "bottom" of the HARV has a membrane allowing gas permeability, which could be influencing the traveling of the denser dye along the bottom in Figure 5.

IV. Discussion

The effect of microgravity on microbial cells could theoretically be minimal compared to its effect on the cell's immediate environment. Such influence on the immediate environment of the cell could affect the mass transfer of nutrients, products, and waste of the cell, limiting such processes 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 RPM, could influence the mass transport of a dye. In the span of a twenty-minute test, the RWV showed solid body rotation, while the RPM revealed fast mixing rate that easily spread the dye to most of the HARV before the time limit had been reached. Therefore, the rest of the studies were conducted with the RWV.

The effects of mass transfer on dyes of both high- and low-density dyes (relative to the water "media") showed that in a range of 5 to 40 rpm, the ideal range for microbial testing would be within 15 to 20 rpm. Speeds tested that were lower than that were dominated by density driven convection, while higher were dominated by centrifugal forces

pushing the lighter density dye towards, the middle and the higher density dye towards the outer edges of the HARV. To minimize the potential mixing caused by either of the dominating forces, the speeds of 15 to 20 rpm were determined to be the optimal range for microbial studies.

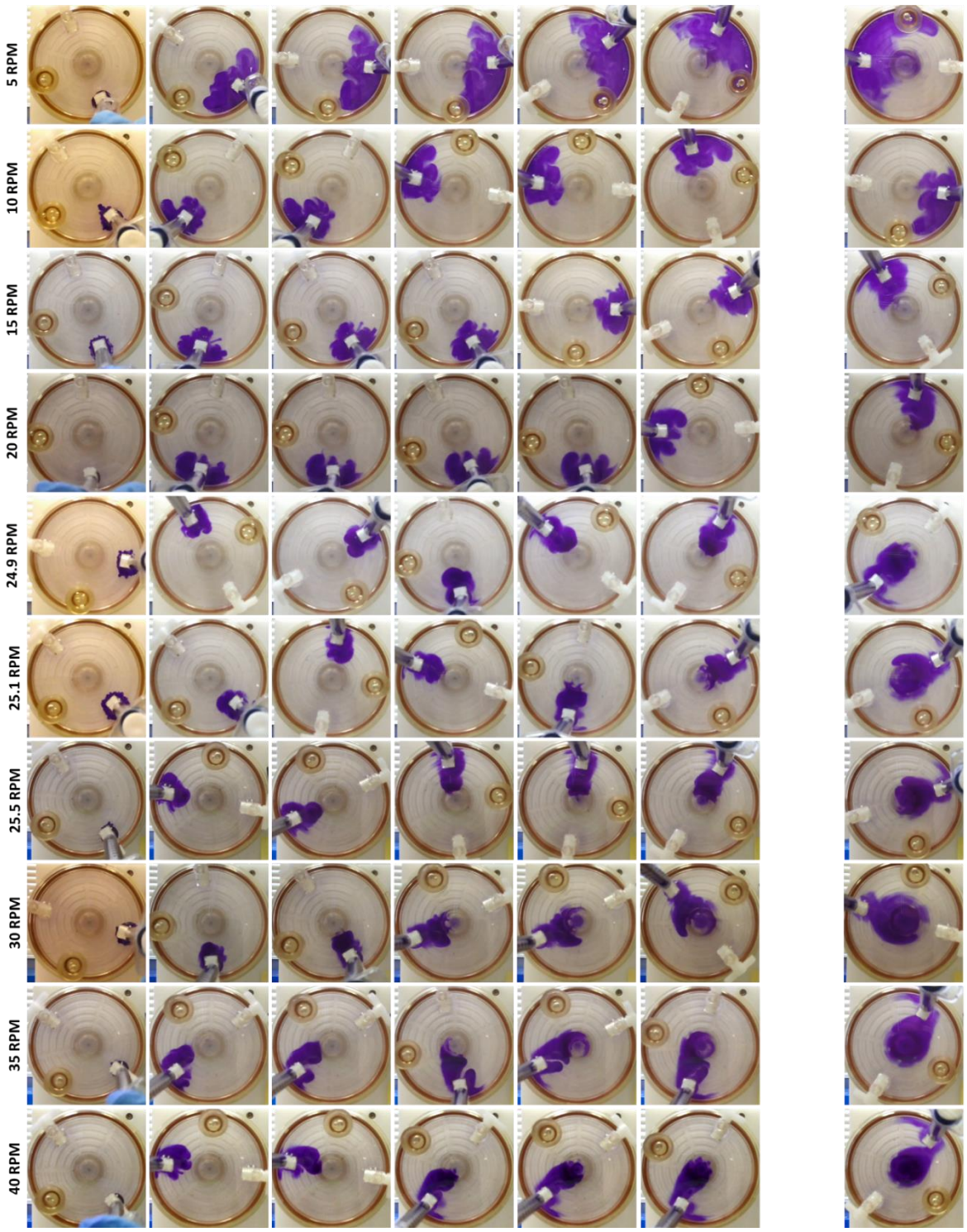
Finally, the control conditions were also studied to determine the most accurate ground control to compare to simulated microgravity for microbial effects. The low-density tests showed that a static, horizontal ground control would be most accurate due to the standing ground control showing density driven convection, but slower mixing, and the rotating ground showing 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, as well as the speed utilized being out of the later determined optimal speed range for the HARV. Further tests utilizing a lower speed would help clarify the high density HARV results, as well as determine whether the lower density results would remain the same in the optimal speed range. In the future, addition of other testing apparatus could further clarify some of the confusion around the best representative 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.

V. Conclusion

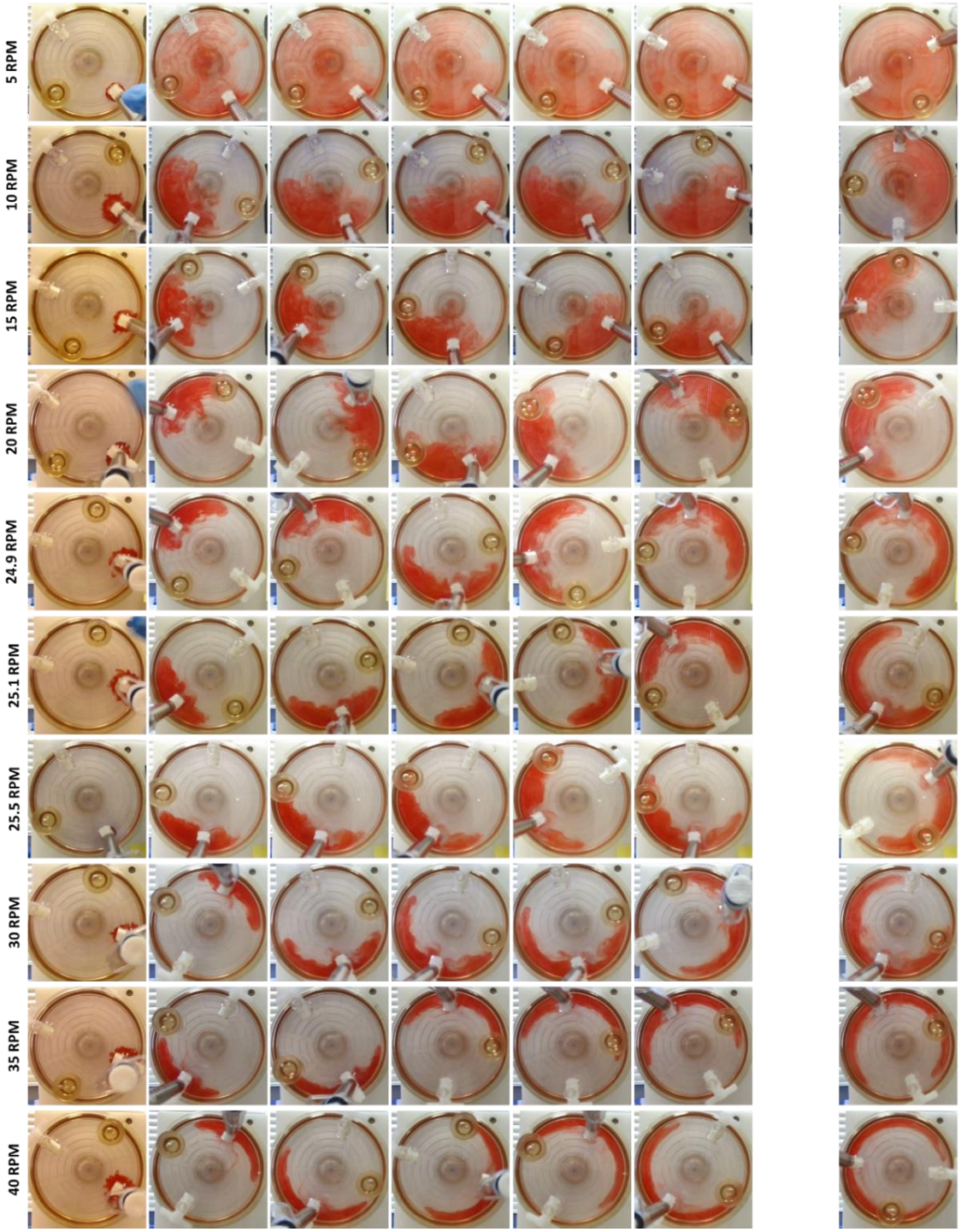
The study of the influence of spaceflight conditions on biofilm growth in ground-based instruments still requires clarification as to what underlying mechanisms are truly influencing the microbes and whether these are representative of their spaceflight counterparts. This study used dyes of different densities in a water media to compare two commonly used instruments—the RPM 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 RPM. 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.

Appendix

A. Low Density Dye in RWV HARVs at Different Speeds



B. High Density Dye in RWV HARVs at Different Speeds



Acknowledgments

The authors would like to acknowledge funding from NASA's Center Innovation Fund (CIF).

References

[1] A. H. Brown, From gravity and the organism to gravity and the cell, ASGSB Bulletin : Publication of the American Society for Gravitational and Space Biology. 1991 Jul;4(2):7-18. PMID: 11537184.

[2] Klaus, D., Simski, S., Todd, P. and Stodieck, L., Investigation of space flight effects on E. coli and a proposed model of underlying physical mechanisms. Microbiology, 143: 449–455 (1997).

[3] Geim, A. 1998. Everyone's magnetism. Physics Today 51:36-39.

[4] Hammond, T. G., and J. M. Hammond. 2001. Optimized suspension culture: the rotating-wall vessel. Am. J. Physiol. Renal Physiol.281:12-25.

[5] Klaus, D. M. 2001. Clinostats and bioreactors. Gravit. Space Biol. Bull.14:55-64.

[6] Baker, P. W., and L. Leff.2005. Intraspecific differences in bacterial responses to modeled reduced gravity. J. Appl. Microbiol.98:1239-1246.

[7] Baker, P. W., M. L. Meyer, and L. G. Leff.2004. Escherichia coli growth under modeled reduced gravity. Microgravity Sci. Technol.15:39-44.

[8] Benoit, M., and D. Klaus.2005. Can genetically modified Escherichia coli with neutral buoyancy induced by gas vesicles be used as an alternative method to clinorotation for microgravity studies? Microbiology151:69-74.

[9] Huitema, C., L. A. Beaudette, and J. T. Trevors.2002. Simulated microgravity (SMG) and bacteria. Riv. Biol.95:497-503.

[10] Kato, Y., Y. Mogami, and S. A. Baba.2003. Responses to hypergravity in proliferation of Paramecium tetaurelia, Zool. Sci. 20: 1373-1380.

[11] Rosa Santomartino, Annemiek C. Waajen, et al., No effect of microgravity and simulated Mars gravity on final bacterial cell concentrations on the international space station: Applications to Space bioproduction, Frontiers in Microbiology, 14 October 2020. https://www.frontiersin.org/articles/10.3389/fmicb.2020.579156/full