Seasonal Microbe Effect on Duckweed

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Introduction:

The complex interaction that occurs between microorganisms and plants plays an important role in supporting the health and functionality of multicellular organisms (O'Brien et al. (2020)). In plants, microbial communities significantly enhance fitness through improving nutrient uptake, enhancing stress tolerance, and protecting against pathogens (O'Brien et al. (2020)). These relationships are central to ecosystem stability and biodiversity preservation. The benefits of these interactions, are influenced by various environmental factors, including temperature, soil composition, and seasonal changes (Ruan et al. (2023); Santoyo et al. (2021)). As climate change continues to reshape Earth's ecosystems, understanding these interactions has become increasingly critical for developing strategies to maintain biodiversity as well as agricultural productivity.

Seasonal changes in particular, can cause shifts in both the composition and functionality of microbial communities. For example, Ma et al. (2021) found that soil microbiomes surrounding hazelnut plants varied across seasons which in turn altered nutrient availability for the host plants (Ma et al. (2021)). While it is understood that environmental conditions can reshape microbial communities, the effects of these shifts on the plant hosts remain unclear. It is important to understand these effects and how the overall dynamic changes to be able to predict plant-microbe interactions in fluctuating environments to be able to improve plant fitness.

To address this knowledge gap, duckweed (*Lemna minor*) was used as a model organism. Duckweed is a small, free-floating aquatic plant with a rapid growth rate and short life cycle. These characteristics make it an ideal model for studying generational changes and effects of environmental variables (Thingujam, Pajerowska-Mukhtar, and Mukhtar (2024)). Because duckweed is sensitive to environmental fluctuations, it provides an excellent system for investigating how seasonal shifts in microbiomes impact plant growth and fitness (Thingujam, Pajerowska-Mukhtar, and Mukhtar (2024)). This study hypothesized that microbiomes collected during different seasons will vary in their growth as well as their impact on duckweed growth, as measured by plant area and optical

density of microbial communities, reflecting the seasonal shifts in microbial community composition.

Methods:

1. Microbe Collection:

Microbes were sampled from two different locations across all four seasons. Seven sample collections were completed at the La Roche sample site, and eight sample collections were completed at the Thompson Farm site. Samples were collected at the edge of a body of water using sterilized metal spatulas . Collection tubes were rinsed in the body of water three times before being used to collect duckweed and the surrounding water.

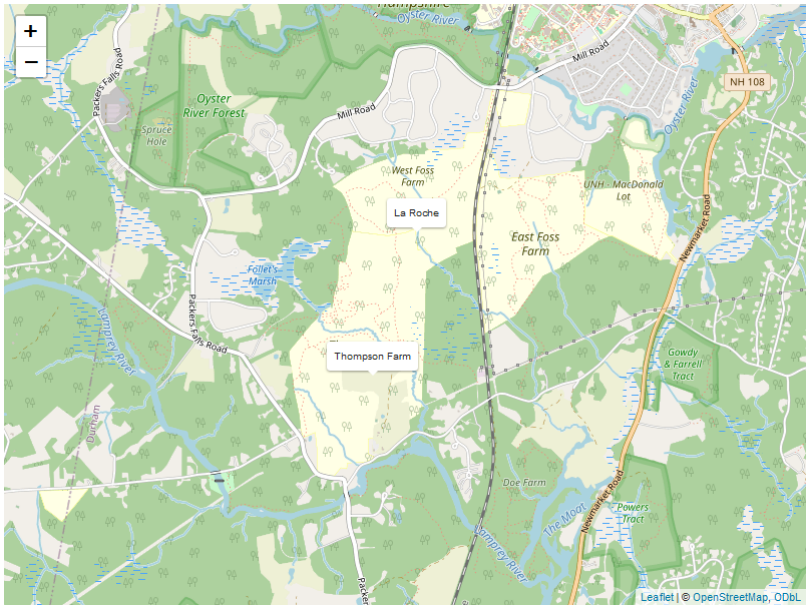

Figure 1: Map showing the locations of Thompson Farm and La Roche sampling areas. Both locations are located Southwest of the University of New Hampshires campus.

2. Duckweed Line:

Isogenic duckweed lines were created using duckweed from each respective site. Single fronds from each site were placed into twenty four well plates and allowed to asexually reproduce. From these two lines, axenic cultures were created for each respective line. These cultures were created by sterilizing the duckweed using a 1% bleach solution for one minute and thirty seconds. Following the bleach sterilization, the plants were rinsed three times with sterile water. The first rinse lasted forty five seconds with the following two rinses lasting ten minutes each. The sterile duckweed was then placed into sterile

Krazčič media (K media) for each respective isogenic line. The duckweed within each new axenic culture were then plated on sterile enriched yeast mannitol media to test for microbe growth to ensure sterilization.

3. Culturing Microbes:

Duckweed from the microbe samples were crushed on the inside cover of a plate that contained yeast mannitol agarose using a sterilized inoculation loop. This inoculation loop was then used to streak the plate to culture the microbes from the duckweed. These plates were then incubated until colony growth was sufficient. Once sufficient growth was achieved, they were stored in a 4ºC refrigerator until use.

4. Experiment Design and Set Up:

Both axenic duckweed lines were inoculated with each seasonal collection of microbes from both duckweed lines respective sample site. These inoculations were set up with 4 blocks containing 8 replicates each into six 96 well plates according to a randomized blocking map. This allowed for 32 replicates for each treatment. To create the experimental inocula from the microbe samples, 15 mL culture tubes containing sterile yeast mannitol media were inoculated by swabbing with microbes from each combination of site and date. The experimental combinations of these microbes were cultured for two days at an RPM of 300 and at 30ºC. A BioTek Cytation 5 Plate Reader was used to examine optical density at 600 nm for each microbe inocula to test cell concentration (O'Brien et al. (2020)). Cell concentrations from the OD readings wree used to dilute all inocula to the same concentration (5000 cells/µl). Dilutions were formed by adding a calculated amount of microbe inocula to sterile Hogland's media in the laminar flow hood. Well plates were prepped by placing 600 µl of sterilized Hogland's media into each well, followed by placing duckweed fronds in each well. A seal (BreathEasier Diversified Biotech) was placed onto each plate and then the plates were left in the sterile laminar flow hood overnight. Each microbe inocula (10 µl) were added to the 96 well plates according to the randomized block design. This was completed by puncturing through the Breatheasier seal. Once all wells were inoculated, the BreathEasier seal was removed and a new seal (BreathEasy Diversified Biotech) was placed onto the plate to prevent contamination. Plates were placed into the growth chamber and imaged on day 12. Images were taken using an iPhone 13 cell phone camera. Images were taken through the plexiglass shield that the plate were set upon to ensure uniform images. ImageJ was used for analysis to measure duckweed growth.

5. Post Microbe Analysis:

After the treatment period, the media containing the microbial samples was analyzed for optical density (OD) using a microplate reader (BioTek Cytation 5 Plate Reader)

to assess microbial growth. Samples were transferred from experimental plates into a clear bottom 96-well microplate. The optical density was measured at two different wavelengths: 450 nm (OD450) and 600 nm (OD600), to capture both general microbial growth and specific cell concentration.

6. Statistical Tests:

Statistical tests were conducted after the experiment to analyze the data. Both OD450 and OD600 wavelengths were initially considered for analysis. A Spearman rank correlation test was conducted to assess the relationship between the two wavelengths, and a strong positive correlation was observed (rho = 0.85, $p < 0.001$). Based on this high degree of similarity between the two measurements, and given that OD600 is commonly used to assess microbial growth, it was selected for further analysis as it directly reflects the microbial biomass in the samples (Mira, Yeh, and Hall (2022)). This decision was made to align with standard practice in microbial studies and to ensure the relevance of the measurements to the research goals.

To account for the random effects of replicates, a linear mixed model (LMM) was then applied to the log-transformed optical density data. The log transformation was applied based on model fit, as indicated by the AIC criterion. The LMM was used to compare the effects of seasonal microbial treatments against the control group (which contained no microbes) and to evaluate differences between the two plant locations. While the assumption of normality was slightly violated, this was deemed acceptable due to the large sample size (greater than 500 samples), as supported by the Central Limit Theorem. Additionally, a post hoc Tukey test was performed to assess pairwise comparisons between the groups. Given the large sample size, the slight departure from normality was considered negligible, and the results of the Tukey test were interpreted accordingly.

Results:

Relationship Between Microbial OD450 and OD600 Measurements:

To visualize the correlation between microbial optical density (OD) readings at 450 nm and 600 nm, a scatter plot was generated (Figure 2), accompanied by a Spearman's rank correlation test (Table 1). The scatter plot showed a positive correlation between the reading at 450 nm and the reading at 600 nm. This positive correlation suggests that as OD values increase at the 450 nm wavelength that they also increase in the 600 nm wavelength read. The Spearman's correlation coefficient (= 0.98) indicated a strong correlation between the two read wavelengths. However, a p-value could not be generated in the test due to ties in the data set, which can be caused by identical values in the data. Despite this the OD450 and OD600 values were generally consistent

and the strong correlation supports the decision to move forward with OD600 values for further analysis.

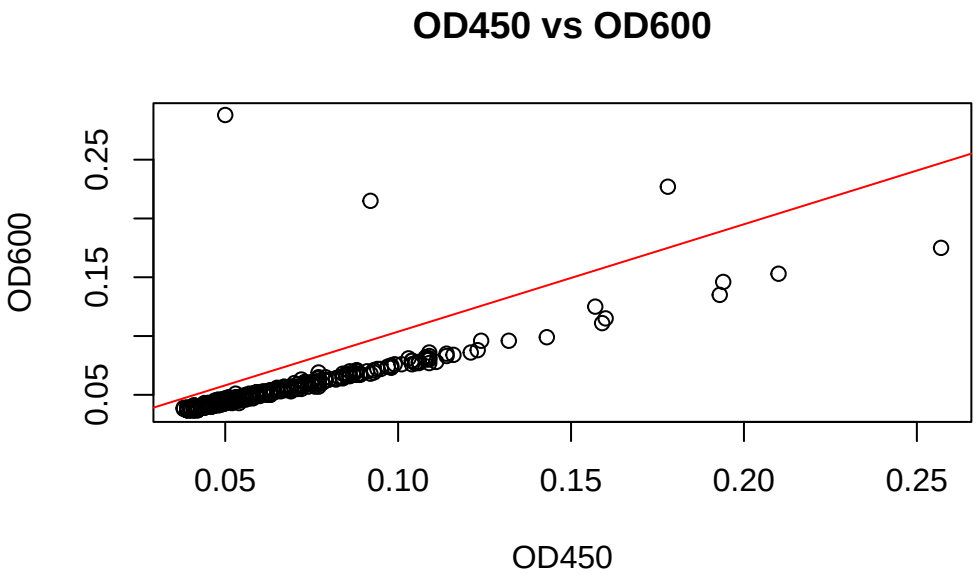

Figure 2: Correlation between OD450 and OD600 readings across all samples. The strong positive correlation (visualized by the red regression line) indicates consistent measurement trends between the two wavelengths

Table 1: Spearman's Rank Correlation Test Results between Variables OD450 and OD600

Statistic	Value
Correlation Coefficient	0.982597526961954
p-value	0
Test Statistic	459250.915424584
Method	Spearman's rank correlation rho

Seasonal and Plant Location Variation in Microbe OD Value:

The mean log-transformed OD600 values were plotted for each season (Figure 3). The values were log-transformed due to AIC value (AIC = 104) determining that it was the best fit for the data. The control with no microbes exhibited the lowest mean OD600

value, with seasonal microbe treatments displaying more variation. Summer and winter treatments appear to have slightly higher mean OD values, with winter displaying the greatest variability. This suggests differences across seasons in microbial growth or activity.

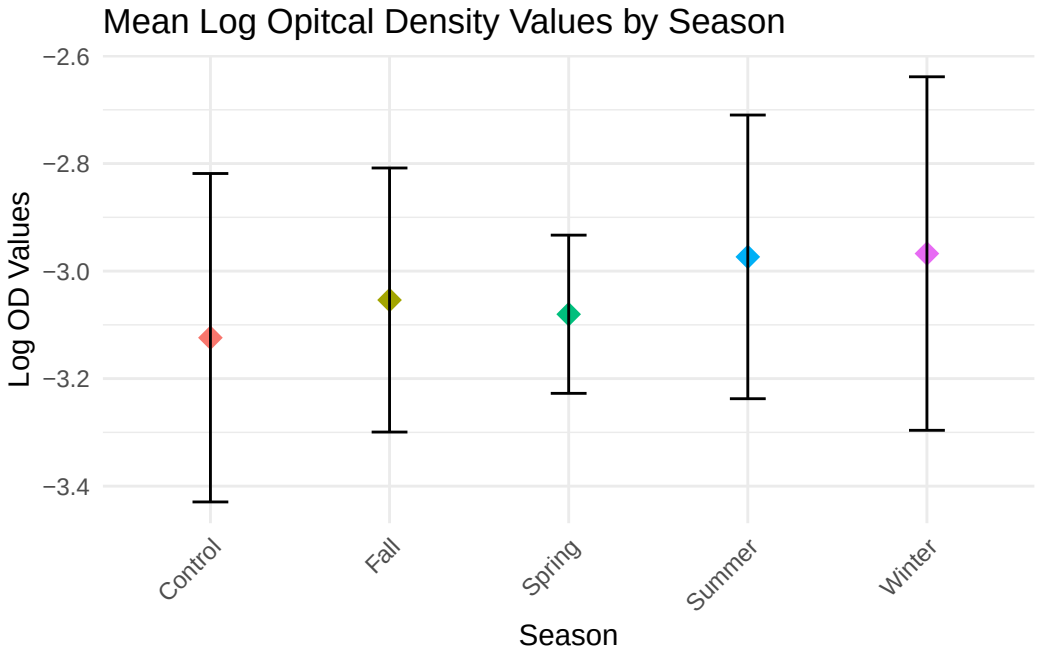

Figure 3: Mean log-transformed optical density (OD) values of microbial growth across different seasons, including a control with no microbes. Error bars represent the standard deviation. Seasonal microbial communities were analyzed for their effect on optical density, highlighting variations in microbial growth between seasons.

A heat map displayed in Figure 4, compares OD600 values of microbe samples by both season and plant location providing a more nuanced perspective into differences in values (Figure 4). Patterns between plant location and season emerged. There was a clear visual difference between OD600 values for certain plant-season combinations, with higher OD600 values being present in microbes that were associated with plants from the LaRoche sample site. Comparisons of microbes across seasons from the sampling site at LaRoche visually depict slightly higher OD600 values in spring and summer. This continues to suggest seasonal differences in microbial growth or activity. This also suggests that plant location could play a role in microbial composition and its susceptibility to environmental changes such as seasons.

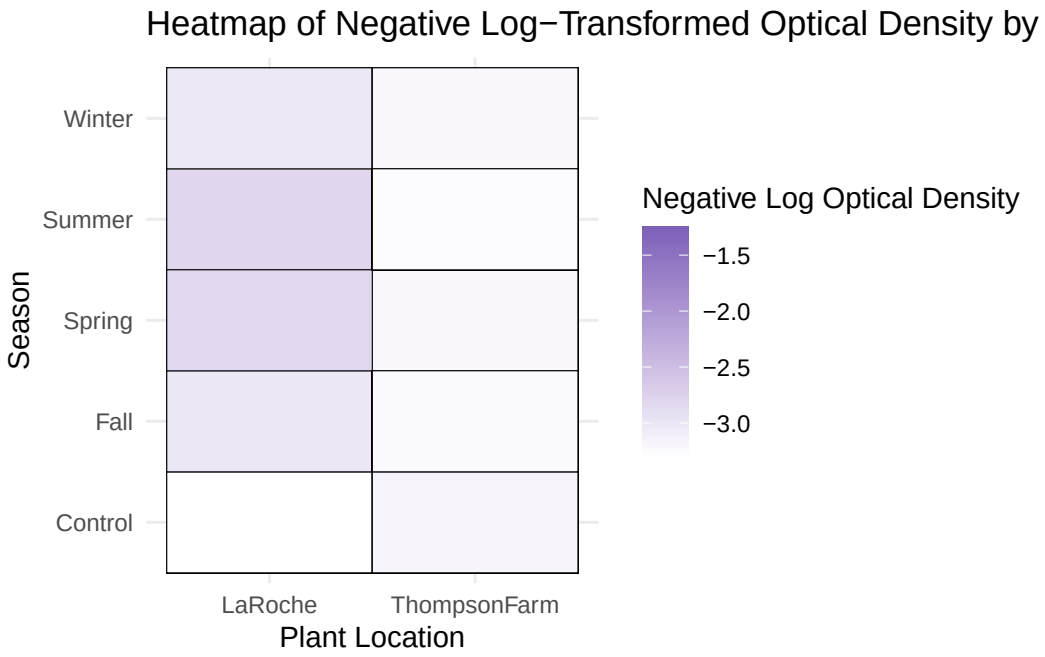

Figure 4: Heatmap of microbe log-transformed optical density by both season and plant location. Lighter colors indicate a lower optical density, with darker colors representing a higher optical density value.

Linear Mixed Model (LMM) Results:

To quantify the effects of season as well as plant location on OD600 values from microbial samples while accounting for random variation among replicates, a linear mixed model (LMM) was applied (Table 2). The OD values were log transformed before the LMM was applied, as an AIC value of 104 confirmed that it was a good fit for the model. The model revealed mutliple significant fixed effects for season. Fall ($p = 0.041$), winter ($p = 0.00049$), and summer ($p = 0.00005$) were all significantly different from the intercept which was the control condition. The model also revealed that the plant locations were significantly different ($p = 0.0024$). This model suggests that both season as well as plant location impact microbial growth.

Pairwise Comparisons Using Tukey Adjustments:

Post hoc comparisions were completed to determine differences in OD600 values across seasons and plant location (Table 3, Table 4). There were two significant differences for seasons. The log-transformed OD was significantly lower in the control compared to summer, with an estimated mean difference of -0.151 ($SE = 0.037$, $df = 532$, $t = -4.09$, $p = 0.0005$). The control was also significantly lower than winter with an estimated mean

Table 2: Fixed Effects of the Linear Mixed Model

	Estimate	Standard Error	Degrees of Freedom	t Value	p Value
(Intercept)	-3.0900	0.0447	12.7	-69.200	0.00e+00
SeasonFall	0.0778	0.0379	532.0	2.050	4.09e-02
SeasonSpring	0.0446	0.0452	532.0	0.987	3.24e-01
SeasonSummer	0.1510	0.0370	532.0	4.090	5.05e-05
SeasonWinter	0.1590	0.0454	532.0	3.510	4.89e-04
PlantThompsonFarm	-0.0673	0.0220	532.0	-3.060	2.35e-03

difference of -0.159 (SE = 0.045, df = 532, t = -3.51, p = 0.0044). When compared to fall and spring there were no significant differences. Between seasons, the only significant comparison was between spring and summer with spring having significantly lower OD values based on an estimated mean difference of -0.107 (SE = 0.037, df = 532, t = -2.9, p = 0.032). The post hoc Tukey test revealed a significant difference in the log-transformed OD values between the two plant locations, LaRoche and Thompson Farm (Table 4). The estimated mean difference was 0.0673 (SE = 0.022, df = 532, t = 3.06, p = 0.002). This indicates that microbial communities collected from plants at the LaRoche site had higher OD values compared to those from Thompson Farm.

Table 3: Tukey Post Hoc Pairwise Comparisons for Season (Estimated Marginal Means)

Comparison	Estimate	Standard Error	df	t Ratio	p Value
Control - Fall	-0.07780	0.0379	532	-2.050	0.244000
Control - Spring	-0.04460	0.0452	532	-0.987	0.861000
Control - Summer	-0.15100	0.0370	532	-4.090	0.000483
Control - Winter	-0.15900	0.0454	532	-3.510	0.004440
Fall - Spring	0.03320	0.0377	532	0.879	0.904000
Fall - Summer	-0.07340	0.0274	532	-2.680	0.057900
Fall - Winter	-0.08140	0.0379	532	-2.150	0.202000
Spring - Summer	-0.10700	0.0368	532	-2.900	0.031800
Spring - Winter	-0.11500	0.0452	532	-2.540	0.084400
Summer - Winter	-0.00799	0.0370	532	-0.216	1.000000

Table 4: Tukey Post Hoc Pairwise Comparisons for Plant (Estimated Marginal Means)

Comparison	Estimate	Standard Error	df	t Ratio	p Value
LaRoche - ThompsonFarm	0.0673	0.022	532	3.06	0.00235

Discussion:

The study evaluated microbial impacts on duckweed growth across seasons, with data analysis conducted so far focusing on efect of season and plant location on microbial growth post experimental exposure with duckweed. OD600 measurements showed strong correlations with OD450 measurements. Due to the positive correlations between the two measures as well as previous studies using OD600 measurements confirmed its reliability for further analysis (O'Brien et al. (2020)). The further analysis observed statistically significant growth in the microbial sample for the summer months. This may be due to increased microbial activity during favorable environmental conditions. There is also a potential of higher diversity within the community from the summer months that could cause enhanced microbial growth. The lack of significance for the spring months could potentially be explained by unfavorable environmental conditions transitioning from winter to spring. Overall, the initial findings align with prior research that shows seasonal variations influence functions of microbial communities (Santoyo et al. (2021)).

The largest limitation of the analysis within this study was the slight non-normality present within the optical density data which could potentially affect the results of the statistical models. However, the large sample size ($n > 500$) helpts to mitigate the error this limitation could cause under the central limit theorem. Additionally, there were tied/identical OD values within the data set which may have impacted the results of the Spearman's correlation test. Another notable limitation was the high variability observed in OD values for the controls, which could be attributed to incomplete plant sterilization, leaving residual microbes that elevated OD values.

Further analysis will be completed to analyze the impacts of these microbe samples from the various combinations of seasons and plant location on duckweed growth. To analyze the impact on growth the mean frond area will be compared between the control and experimental conditions. The results from this complementary analysis will provide a holistic understanding of how seasonal microbes and plant location influence plant growth. To further contextualize these results, microbial sequencing data will also be added into the analysis to understand the differences in community composition across seasons and how that correlates with plant growth outcomes. Future studies to further the understanding of these dynamics as well as their application should examine the long term stability and consistency of beneficial plant growth promoting bacterial communities across seasons.

This study contributes to understanding the role of environmental factors on microbial communities and, in turn, their effects on plant growth. Seasonal variability in microbial efficacy can help to enhance bio-remediation strategies and agricultural applications. Insights gained from this research can help to inform microbial community selection in aquatic systems, with potential extension to soil applications. By enhancing knowledge

of how environmental factors influence microbial functionality, this study helps to form the foundation for future innovations in sustainable agricultural practices.

References:

Ma, Wenxu, Zhen Yang, Lisong Liang, Qinghua Ma, Guixi Wang, and Tiantian Zhao. 2021. "Seasonal Changes in Soil Microbial Community and Co-Occurrence Network of Species of the Genus Corylus." *Microorganisms* 9 (11): 2228. https://doi.org/10.3390/microorganisms9112228.

Mira, Portia, Pamela Yeh, and Barry G. Hall. 2022. "Estimating Microbial Population Data from Optical Density." *PLOS ONE* 17 (10): e0276040. https://doi.org/10.1371/journal.pone.0276040.

O'Brien, Anna M., Jason Laurich, Emma Lash, and Megan E. Frederickson. 2020. "Mutualistic Outcomes Across Plant Populations, Microbes, and Environments in the Duckweed Lemna Minor." *Microbial Ecology* 80 (2): 384–97. https://doi.org/10.1007/s00248-019-01452-1.

Ruan, Yang, Yakov Kuzyakov, Xiaoyu Liu, Xuhui Zhang, Qicheng Xu, Junjie Guo, Shiwei Guo, Qirong Shen, Yunfeng Yang, and Ning Ling. 2023. "Elevated Temperature and CO2 Strongly Affect the Growth Strategies of Soil Bacteria." *Nature Communications* 14 (1): 391. https://doi.org/10.1038/s41467-023-36086-y.

Santoyo, Gustavo, Carlos Alberto Urtis-Flores, Pedro Damián Loeza-Lara, Ma Del Carmen Orozco-Mosqueda, and Bernard R. Glick. 2021. "Rhizosphere Colonization Determinants by Plant Growth-Promoting Rhizobacteria (PGPR)." *Biology* 10 (6): 475. https://doi.org/10.3390/biology10060475.

Thingujam, Doni, Karolina M. Pajerowska-Mukhtar, and M. Shahid Mukhtar. 2024. "Duckweed: Beyond an Efficient Plant Model System." *Biomolecules* 14 (6): 628. https://doi.org/10.3390/biom14060628.