Seasonal Microbe Effect on Duckweed

Emma Kinerson

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. 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 environements 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 et al., 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 (). 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.

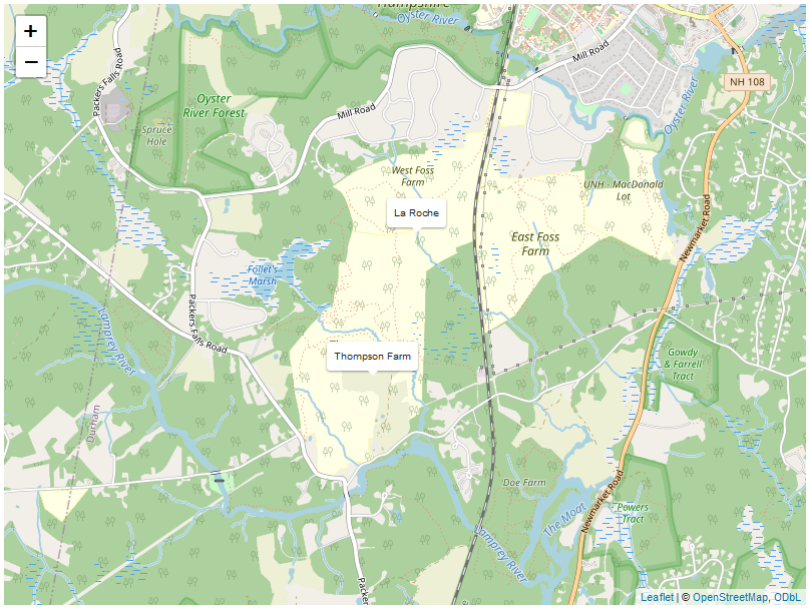

Figure 1: Map showing the locations of Thompson Farm and La Roche.

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. 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. 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. Samples were transferred from experimental plates into a clear bottom 96-well microplate.

6. Statistical Tests:

Statistical tests were run post experiment to analyze the results of the data. Cors Test with a Spearmen method was run on the optical density data to compare the results at two different wavelengths (450 nm and 600 nm). Linear mixed model (LMM) accounting for random effects of replicates was then run on the log values of the optical density data. Optical density data

was log transformed due to the AIC fit of the model. The LMM compared seasonal microbes to the control which contained no microbes, it also compared the two plant locations against one another. The LMM was used and normality was slightly invalidated, but due to the sample size being larger than 500 samples it is okay due to the central limit theorem. A post hoc tukey test was run to analyze the pairwise comparisons of the data. Again due to the sample size the slight invalidation of normality was ignored.

Results:

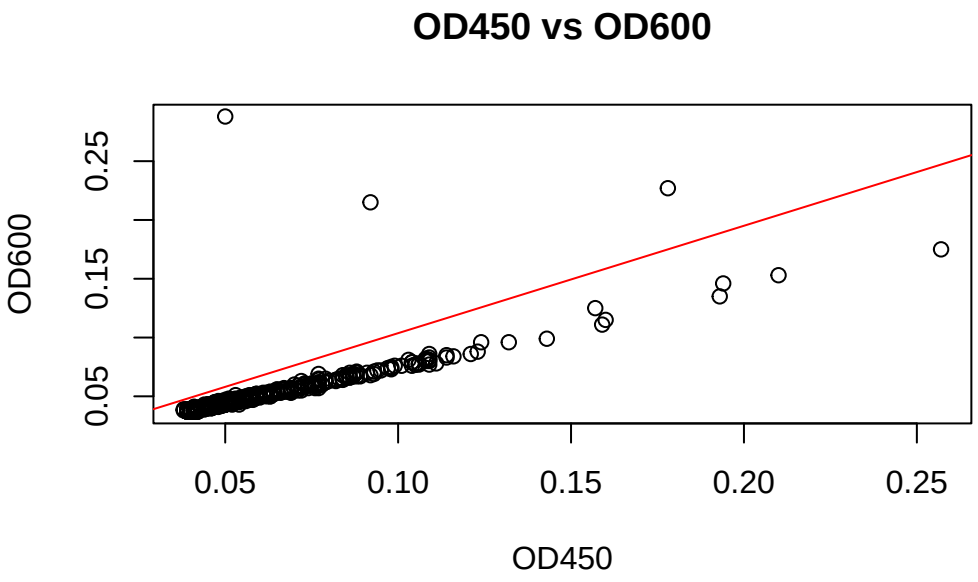

Figure 2: Relationship between OD450 and OD600 measurements

Table 1: Spearman's Rank Correlation Test Results between Variables x and y

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

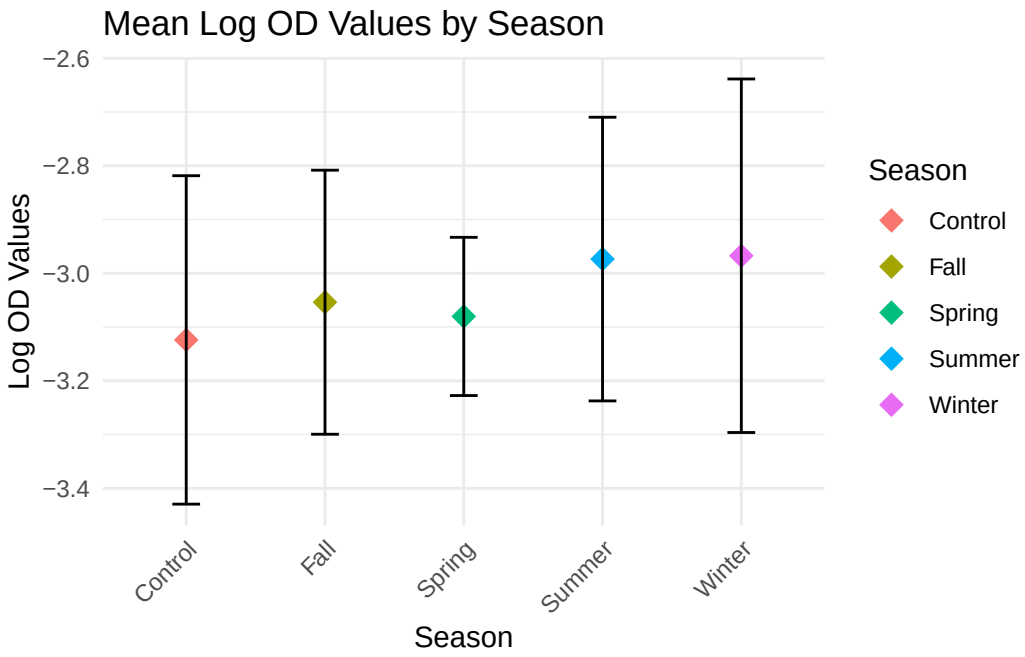

Figure 3: Log mean optical density values by season.

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

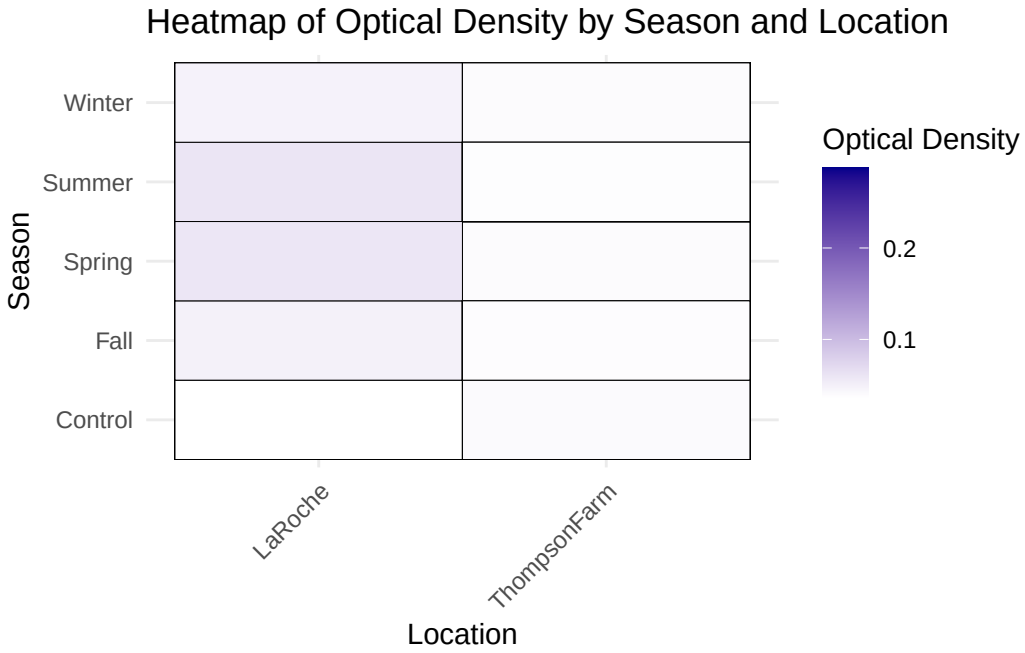

Figure 4: Heatmap of optical density by both season and plant location. Lighter colors indicate a lower optical density, with darker colors representing a higher optical density value. Optical density values are non-log transformed. .

Discussion:

References:

Grady, K.L., Sorensen, J.W., Stopnisek, N. et al. Assembly and seasonality of core phyllosphere microbiota on perennial biofuel crops. Nat Commun 10, 4135 (2019).

Thingujam, D., Pajerowska-Mukhtar, K. M. & Mukhtar, M. S. Duckweed: Beyond an Efficient Plant Model System. Biomolecules 14, 628 (2024).

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

Ma, W., Yang, Z., Liang, L., Ma, Q., Wang, G., & Zhao, T. Seasonal Changes in Soil Microbial Community and Co-Occurrence Network of Species of the Genus Corylus. Microorganisms, 9(11), 2228 (2021).

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Ruan, Y. et al. Elevated temperature and CO2 strongly affect the growth strategies of soil bacteria. Nat Commun 14, 391 (2023).