Using Stropharia Mushroom Mycelium (*S. rugosoannulata*) and Waste Treatment Residual for Filtration of Nitrate/Total Dissolved Nitrogen and Phosphate from Agricultural Runoff to Prevent Harmful Algae Blooms - Year 4

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Introduction

- Harmful Algae Blooms (HABs), which are fatal to aquatic ecosystems, health, and water-reliant industries, are an increasing problem worldwide
  - As algae dies and decomposes, it consumes oxygen and creates hypoxic dead zones in water, refer to Figure 1, where no life can survive.
  - Many species of algae release toxins, which are deadly to humans and animals, into the water that they inhabit
  - HABs cause an estimate of $82 million in losses to the tourism, seafood, and restaurant industries annually
- HABs occur because of an unnatural increase of Phosphate and Nitrate, high water temperatures, and weak water currents
  - **The major source of nutrient pollution in water bodies is polluted agricultural runoff from farms and animal feedlots**

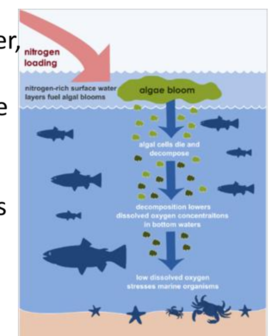


Figure 1: How HABs cause Hypoxia. *Diagram taken Smithsonian Environmental Research Center*

- Current solutions to Harmful Algae Blooms are difficult to implement, not eco-friendly, and costly
  - Sorbtive media, while effective, is expensive and difficult to implement because it requires to be mixed in with soils manually over large surfaces. Once nutrients are absorbed, the media just remains in the ground and has no potential use.
- Results from phases 1, 2, and 3 of this research, refer to Figure 2, suggest that Stropharia Mycelium can effectively filter Nitrate and Phosphate from running, contaminated water in lab settings.
- Recent research with WTR suggests that is has Phosphate filtration abilities

Definitions of Key Terms:

- *Stropharia Mycelium* is the root structure of the *S. rugosoannulata* mushroom and is responsible for providing nutrients to the mushroom for growth/reproduction
- Organic Substrate is an equal parts mixture of dead phragmite leaves (invasive weed) and ground-up corn cobs without the kernels on them (agricultural byproduct) that was created by the author for this study
- Alder Sawdust is a readily available industrial byproduct with no significant use
- WTR is an industrial waste produced from coagulation in water treatment facilities
- Buffer Strips are thin patches of vegetation grown downhill of farmland to filter any runoff

Purpose

Figure 2: Progression of Research

Phase 1 (8th Grade)	Phase 2 (9th Grade)	Phase 3 (10th Grade)	Phase 4 (Current -11th Grade)	Phase 5 (12th Grade)
Initial Discovery of *Stropharia Mycelium's* filtration properties using elementary methodology	Testing of *Stropharia Mycelium's* rapid filtration properties (results inconclusive)	Testing of *Stropharia Mycelium's* filtration abilities using improved methodology	Determination of field implementation potential for agricultural runoff filtration	Outdoor field implementation and testing on a farm

Figure 2: Results from previous phases of this research have led up to the current study

- **Long-Term Purpose:** Develop a cost-efficient, eco-friendly, effective, and easy to implement method of Nitrate/Total Dissolved Nitrogen (TDN) and Phosphate filtration from Agricultural Runoff to prevent HAB
- **Purpose of Current Phase/Year 4:** Determine the field implementation potential of Stropharia Mycelium when colonized on Organic Substrate (MOS), Stropharia Mycelium when colonized on Alder Sawdust (MS), and Waste Treatment Residual Mixed with Alder Sawdust (WTRMS) treatments at agricultural settings for Nitrate/TDN and Phosphate removal from polluted agricultural run-off.
- Field implementation potential was determined by implementing aforementioned treatments as buffer strips inside of self-designed Agricultural Runoff Simulators (ARS), refer to Figure 5, where their immediate and long-term impact on the Nitrate/TDN, Phosphate, and pH Levels of Simulated Agricultural Runoff (SAR) along with soil pH and Spinacia oleracea (Spinach) growth was measured over 32 days
  - Phosphate and Nitrate/TDN levels were studied because they are limiting factors for Algae growth
    - While Nitrate and Phosphate are largely responsible for Harmful Algae Blooms, changes in TDN were also measured to come to a better supported conclusion
  - SAR pH levels were studied because it is vital for any on-site, non-point filtration method to not drastically alter water pH thus not harming any aquatic wildlife
  - Study was conducted over 32 days to determine effectiveness of treatments after periods of prolonged saturation (simulation of real-world scenarios)
    - Treatments will not be effective in the field if they require frequent replacement after extended rainfalls or saturation
  - Spinach growth and soil pH were studied to understand any negative impacts of mycelium spread crops if any mycelium-containing treatments were implemented on farms
- Additionally, the effect of Stropharia Mycelium on S. oleracea growth and soil pH was also studied in self-designed Spinach Growing Cups (SGC), refer to Figure 6.
- **Contribution to Field of Study:** Increased knowledge about mycelium's filtration properties; design of easily-replicable Agricultural Runoff Simulator (ARS) and Spinach Growing Cups (SGC)
- The results from this current phase will lead to field testing of these treatments in the next phase

Methodology

1) Alder Sawdust and Organic Substrate were colonized separately in mesh bags using Stropharia Spawn.

Image 1: Organic Substrate Colonized with Stropharia Mycelium

2) 8 groups of Agricultural Runoff Simulators (ARS) were set up based on Figure 5 with 4 ARS in each group, for a total of 32 ARS. Using letters A-H and numbers 1-4, individual ARS inside the groups were labeled: A1, A2, A3, A4, and so on.

3) Layer 2 in each of the ARS was filled with the filter medium corresponding to the ARS's letter group (refer to Figure 3) and spinach seeds were planted in each of the ARS.

Image 2: 4x Group D Agricultural Runoff Simulators

4) Each ARS was watered with 750 ml of diluted 9-4-9 liquid fertilizer (rainfall simulation) and the drainage from each ARS (Simulated Agricultural Runoff) was collected in appropriately labeled tubes.

5) Using Hach Phosphate reagents/spectroscopy, a Nitrate Ion-Selective Electrode, a Total Organic Carbon Analyzer with a Total Dissolved Nitrogen component, and a pH probe, the Phosphate, Nitrate/Total Dissolved Nitrogen, and pH of all the samples collected was tested and recorded.

6) The number of spinach sprouts and leaves present in each ARS was recorded.

7) Steps 4-6 were repeated every 4 days for 34 days.

8) Using a pH probe and a 0.01 M Calcium Chloride Solution, the soil pH of each ARS was measured and recorded.

9) Any spinach biomass present in an ARS on Day 34 was picked out, stored in a foil pan corresponding to that ARS, and placed in a drying oven overnight.

10) The following day, the biomass was measured using an analytical balance and the average biomass per sprout was calculated for each ARS.

11) Statistical analysis was conducted on the data collected to determine the effect of MOS, MS, and WTRCS treatments on the Nitrate/Total Dissolved Nitrogen, Phosphate, and pH levels of SAR and on the Spinach average biomass/sprout and soil pH inside ARS.

12) 6 groups of Spinach Growing Cups (SGC) were set up based on Figure 6 with 5 SGC in each group, for a total of 30 ARS. Using letters U-Z and numbers 1 to 5, induvial SGC inside the groups were labeled U1, U2,U3, and so on.

Image 3: 16x Spinach Growing Cups

13) Layer 2 in each of the SGC was filled with the filter medium corresponding to the SGC's letter group (refer to Figure 4) and spinach seeds were planted in each of SGC.

14) Each SGC was watered with 40ml of distilled water every day and the number of sprouts present every day was recorded for 21 days.

15) Steps 8-10 were repeated for the SGC.

16) Statistical analysis was conducted on the data collected to determine the effect of Stropharia mycelium on Spinach growth and soil pH inside SGC.

Figure 3: Design of Experiment - Content of Agricultural Runoff Simulators (ARS) Layer 2 Filter Mediums

Treatment 1: Stropharia Mycelium Colonized on Organic Substrate (MOS)	Treatment 2: Stropharia Mycelium Colonized on Alder Sawdust (MS)	Treatment 3: Waste Treatment Residual (WTR) Mixed with Alder Sawdust (WTRMS)
Test Groups		
Group A: Organic Substrate + Spawn + Mushroom Culture	Group B: Alder Sawdust + Spawn + Mushroom Culture	Group G: Alder Sawdust + WTR
Group C: Dead Spawn	Group C: Dead Spawn	Group C: Just WTR
Group F: Organic Substrate	Group D: Alder Sawdust	Group D: Alder Sawdust
Negative Control Groups		
Group H: No filter medium, just soil	Group H: No filter medium, just soil	Group H: No filter medium, just soil

Figure 3: Groups A, B, and G (highlighted above) were over-all test groups that consisted of all aspects of the MS/MOS/WTRMS treatment as it would be if implemented in the field. The other test groups within the respective treatment columns were created to test non-mycelium aspects of the treatments in order to come to a better supported conclusion regarding the effectivity of the treatments.

Figure 4: Content of Spinach Growing Cups (SGC) Layer 2

Treatment: Stropharia Mycelium Colonized on Organic Substrate
Test Groups
Group U: Dead Spawn + Mushroom Culture + Organic substrate
Group V: Dead Spawn + Mushroom Culture
Group W: Dead Spawn
Group X: Dead Spawn + Organic Substrate
Group Y: Organic Substrate
Negative Control Groups
Group Z: No filter medium, just soil

Figure 4: Comparison of data was only conducted between Groups U and X, as one had live mycelium and the other didn't. Other groups were used to interpret data.

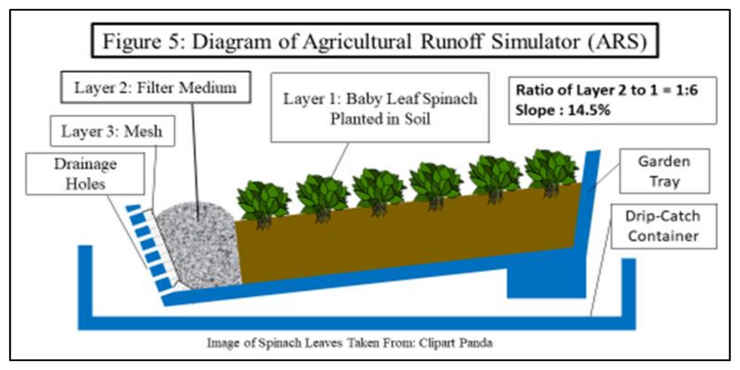


Figure 5: The ARS was designed by the author to simulate an agricultural field inside of a small tray. It was configured with an unrealistically large Layer 2 to 1 ratio to allow enough physical exposure of SAR to treatments. This was compensated for by having an unrealistically large slope gradient to decrease exposure time.

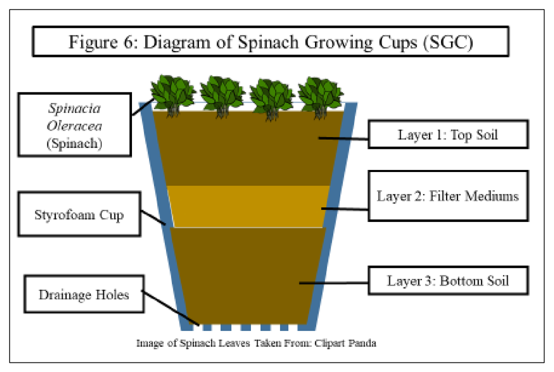
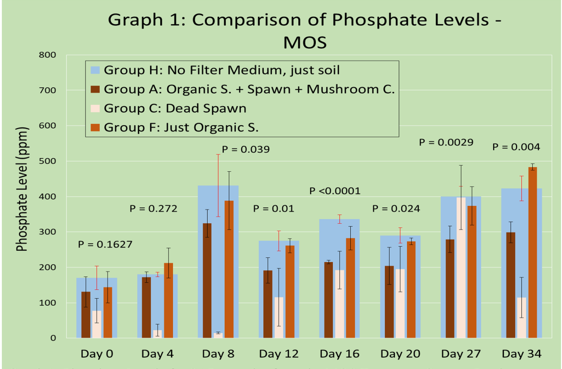
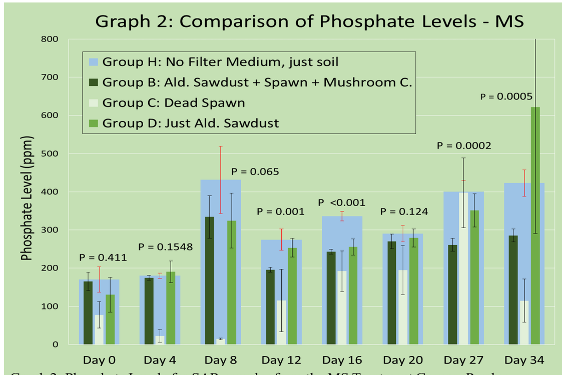


Figure 6: Due to large variability in Spinach growth in the ARS as a result of frequent over-watering (rainfall simulation), the SGC was designed by the author to serve as a more controlled environment for determining impact of treatments (*Stropharia Mycelium*) on Spinach growth.

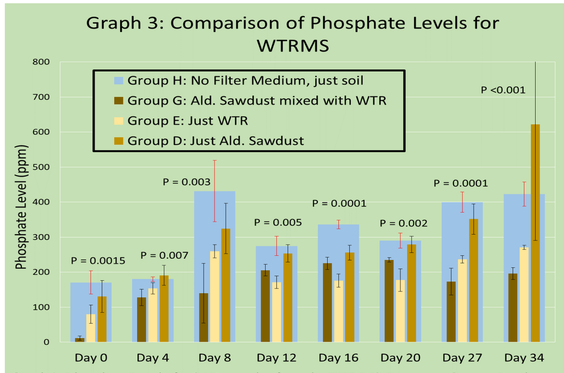
Results



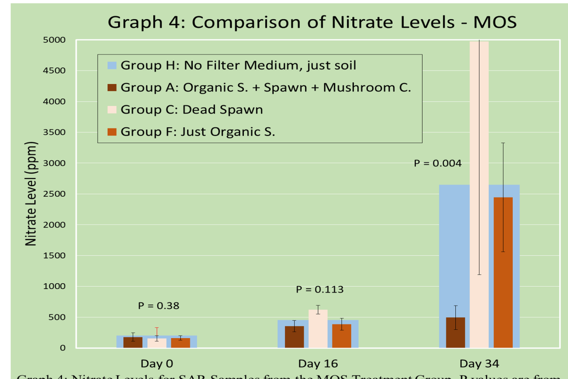
Graph 1: Phosphate Levels for SAR samples from the MOS Treatment Group. P values are from Student's t-Tests between Group H and Group A samples of the same day



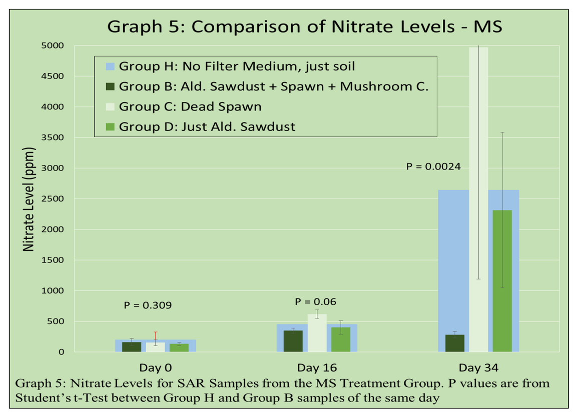
Graph 2: Phosphate Levels for SAR samples from the MS Treatment Group. P values are from Student's t-Tests between Group H and Group B samples of the same day



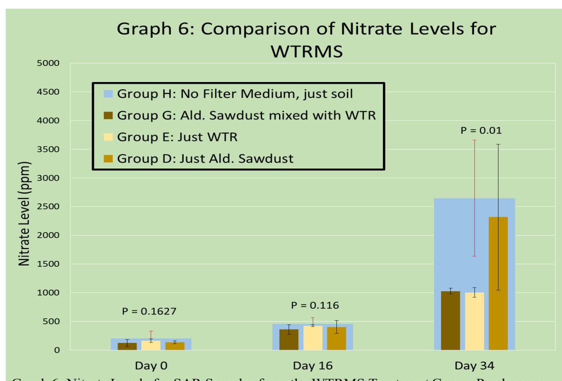
Graph 3: Phosphate Levels for SAR samples from the WTRMS Treatment Group. P values are from Student's t-Tests between Group H and Group G samples of the same day



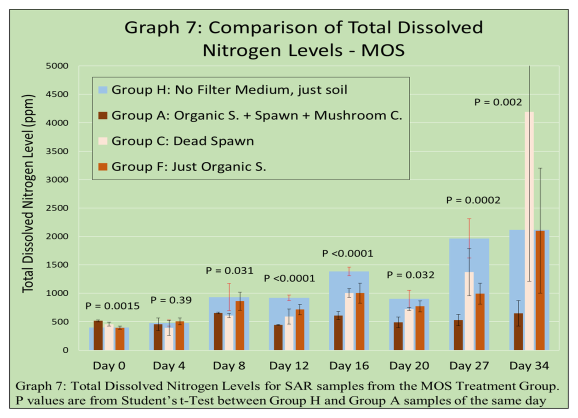
Graph 4: Nitrate Levels for SAR samples from the MOS Treatment Group. P values are from Student's t-Test between Group H and Group A samples of the same day



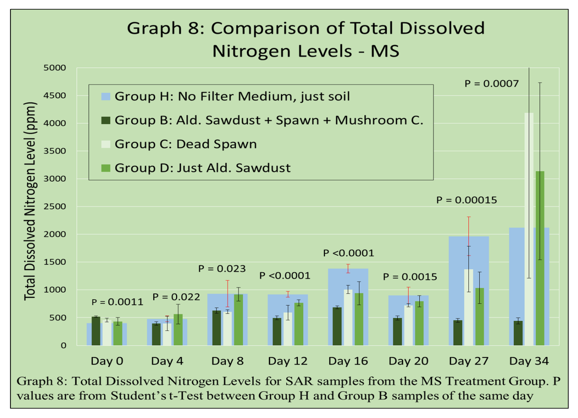
Graph 5: Nitrate Levels for SAR Samples from the MS Treatment Group. P values are from Student's t-Test between Group H and Group B samples of the same day



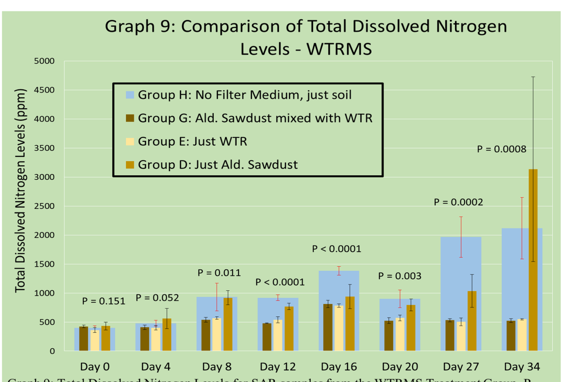
Graph 6: Nitrate Levels for SAR samples from the WTRMS Treatment Group. P values are from Student's t-Test between Group H and Group G samples of the same day



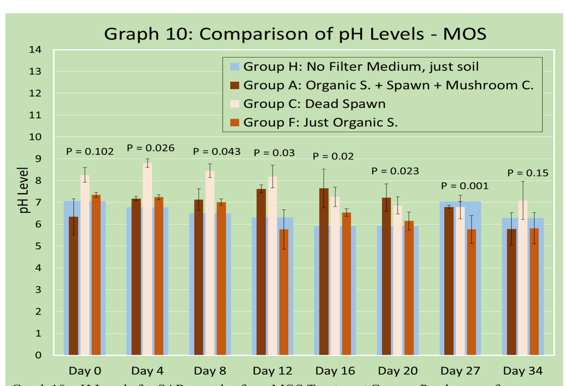
Graph 7: Total Dissolved Nitrogen Levels for SAR samples from the MOS Treatment Group. P values are from Student's t-Test between Group H and Group A samples of the same day



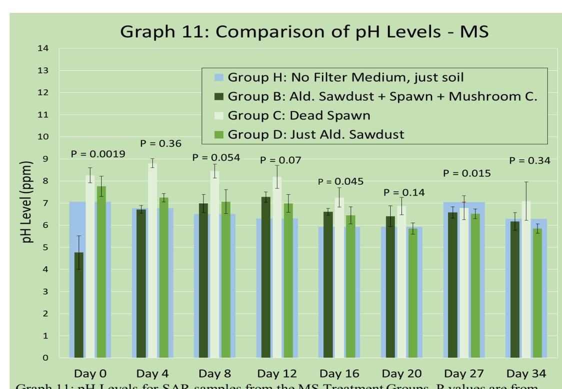
Graph 8: Total Dissolved Nitrogen Levels for SAR samples from the MS Treatment Group. P values are from Student's t-Test between Group H and Group B samples of the same day



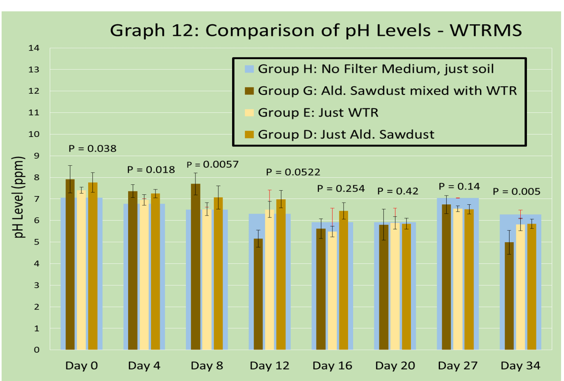
Graph 9: Total Dissolved Nitrogen Levels for SAR samples from the WTRMS Treatment Group. P values are from Student's t-Test between Group H and Group G samples of the same day



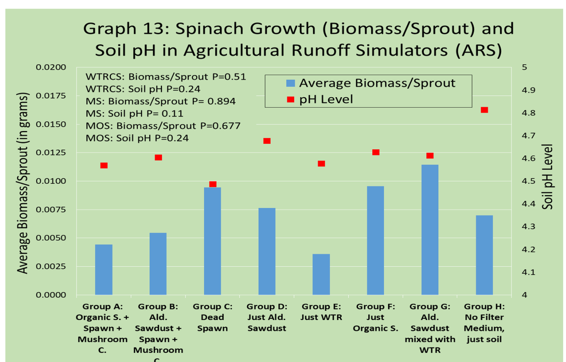
Graph 10: pH Levels for SAR samples from MOS Treatment Group. P values are from Student's t-Tests between Group H and Group A samples of the same day



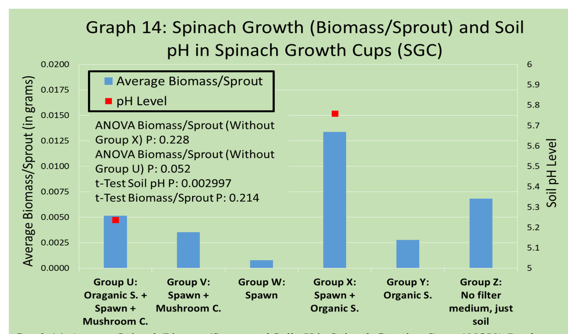
Graph 11: pH Levels for SAR samples from the MS Treatment Group. P values are from Student's t-Tests between Group H and Group B samples of the same day



Graph 12: pH Levels for SAR samples from the WTRMS Treatment Group. P values are from Student's t-Tests between Group H and Group G samples of the same day



Graph 13: Average Spinach Biomass/Sprout and soil pH in Agriculutral Runoff Simulators. P values for each treatment are from one-way ANOVAs between the average biomass/sprout and soil pH of samples from the treatments' groups (WTRCS = H, G, E, & D || MS = H, B, C, & D || MOS = H, A, C, F).



Graph 14: Average Spinach Biomass/Sprout and Soil pH in Spinach Growing Cups. ANOVA P values are from one way ANOVAs between the average biomass/sprout of all samples first, without group X and second, without group Y. t-Tests P-Values are from Student's t-Tests between the average biomass/sprout and soil pH of samples from Group U and Group X. Group U contained live, Stropharia mycelium whereas Group X did not.

Discussion

All three treatments can effectively filter Nitrate/TDN and Phosphate from Simulated Agricultural Runoff (SAR) despite periods of prolonged saturation (>32 days)

- Graphs 1, 2, and 3 show Phosphate levels, graphs 4, 5, and 6 show Nitrate levels, and graphs 7, 8, and 9 show TDN levels for the MOS, MS, and WTRMS treatments respectively
- In those graphs, the maroon, dark green, and dark brown bars symbolizing the treatment groups A , B and G respectively for the three treatments, are lower than the overarching blue bars symbolizing the negative control (just soil, no filter medium) group for most days thus suggesting filtration of Nitrate/TDN and Phosphate by the treatment
  - Differences between the neg. control and treatment groups were found to be statistically significant for most days (Students t-Test)
- The differences between treatment groups and negative control groups did not shorten significantly throughout the duration of the study which suggests that they have long term effectivity (>32 days)
- MOS and MS treatments likely have no point of saturation because they utilize Stropharia Mycelium for filtering Phosphate and Nitrate/TDN
  - As Stropharia Mycelium absorbs nutrients, it grows/expands. As it grows/expands, its ability to further absorb nutrients increases (POSITIVE FEEDBACK)
    - Gaps between mycelium containing treatment groups and negative control groups tend to increase over time
  - If implemented in the field, these treatments would not need habitual replacement due to saturation
- As expected, Phosphate and Nitrate/TDN levels rose over time across all graphs due to the accumulation of nutrients in the ARS after watering with fertilized water every 4 days.

All three treatments do not significantly alter SAR pH

- Graphs 10, 11, and 12 show pH levels for the MOS, MS, and WTRMS treatments respectively
- In these graphs, there is no clear trend between the respective treatment groups - the maroon dark green, and dark brown bars -and the negative control group (overarching blue bar - just soil, no filter medium) across the duration of this research for all three treatments. On some days, treatments had higher pH than negative control, and on other days, vice versa
  - Additionally, differences between the treatment's and negative control's pH were found to be statistically insignificant for most days (Student's t-Test)

All three treatments do not have a negative impact on Spinach growth or Soil pH

- Conclusions cannot be made from graph 13, which shows the data for soil pH and average biomass per Spinach sprout in ARS, as one-way ANOVAs conducted for each treatment and its respective experimental groups (refer to Figure 3) yielded P-values >0.05
  - Due to the over-watering of the Spinach every four days in the ARS as a simulation of rainfall, there was near random Spinach growth. Thus, Spinach Growing Cups (SGC) were designed to better study mycelium-spinach interactions
- In graph 14, which shows the data for soil pH and average biomass per Spinach sprout in SGC, Group U has live Stropharia mycelium whereas Group X does not
  - Differences in average biomass per sprout between Group U and X was found to be statistically insignificant (Students t-Test)
  - Group U had a statistically significant however negligible (<0.5 pH) decrease

Conclusion

All three treatments have field implementation potential for further testing:

- Stropharia Mycelium when colonized on Organic Substrate (MOS), Stropharia Mycelium when colonized on Alder Sawdust (MS), and Waste Treatment Residual Mixed with Alder Sawdust (WTRMS) treatments can all effectively filter Nitrate/TDN and Phosphate from Simulated Agricultural Runoff without drastically altering its pH
- These treatments remain effective despite prolonged saturation (>32 days)
- None of the treatments nor Stropharia Mycelium has any significant impact on Spinach growth or soil pH

In the next phase of this research (Phase 5), the treatments will be implemented and tested as buffer strips on a farm as depicted in Figure 7. In summary, this research has potentially discovered a cost-efficient, eco-friendly, and effective method of Nitrate/TDN and Phosphate filtration from agricultural runoff to prevent Harmful Algae Blooms.

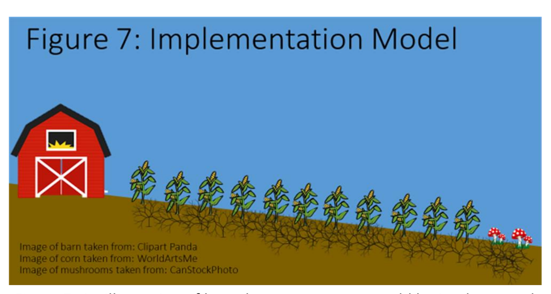


Figure 7: An illustration of how these treatments would be implemented on farms as buffer strips

Figure 8: Cost Analysis

1) Dead Organic Matter: $0 (Agricultural Byproducts)
2) Mushroom Spawn: $150 (Once sufficient growth is obtained, it lasts a life time and can easily be transferred)
3) Seasonal Mushroom Harvest: $300 profit

Total: No net cost, long-term profit

Figure 7: Due to the low cost of materials, these treatments are cost effective

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