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

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Abstract

Harmful Algae Blooms (HABs) largely occur because of an increase of Nitrate and Phosphate (N&P) and are an increasing problem globally. The overall goal of this 5-year study is to develop an inexpensive method for preventing HABs. The purpose of the 4th phase of this research was to determine the field implementation potential of Stropharia (*S. rugosoannulata)* Mycelium (SM) when colonized on Organic Substrate, SM when colonized on Alder Sawdust, and Waste Treatment Residual Mixed with Alder Sawdust treatments, at agricultural settings for N&P filtration from agricultural run-off. This was determined by implementing the aforementioned treatments inside of Agricultural Runoff Simulators, where their impact on the Nitrate/Total Dissolved Nitrogen, Phosphate, and pH Levels of Simulated Agricultural Runoff (SAR) along with soil pH and Spinach (S. oleracea) growth was measured over 32 days. Additionally, the effect of SM on Spinach growth and soil pH was studied in Spinach Growing Cups. It was found that that all three treatments can effectively filter N&P from SAR without drastically altering its pH or having any statistically significant impact on Spinach growth or soil pH, suggesting that the treatments have potential for implementation.

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III. Key Words

Harmful Algae Blooms, Mycofiltration, Mushroom Mycelium, Waste Treatment Residual, Nitrate, Phosphate, pH, & *Spinacia oleracea*

IV. Abbreviations and Acronyms

MOS = Stropharia Mycelium colonized on Organic Substrate

MS = Stropharia Mycelium colonized on Alder Sawdust

WTRMS= Waste Treatment Residual Mixed With Alder Sawdust

SAR = Simulated Agricultural Runoff

ARS = Agricultural Runoff Simulators

SGC = Spinach Growing Cups

TDN = Total Dissolved Nitrogen

V. Acknowledgments

I would like to acknowledge Mr. Jeremy Stanton, Dr. Yang Deng, and Mr. Lei Zheng for their time, support, and guidance throughout the course of my research.

VI. Biography

Harshal Agrawal is a junior at Dr. Ronald E. McNair Academic High School in Jersey City, New Jersey. At school, he is the secretary of Epsilon Math Club, Head of Design on the FTC Robotics team, "lead Witness" for the Mock Trial Team, and goalkeeper for the soccer team. In 8th grade, he was inspired by the research of Paul Stamets with Mycofiltration and designed his own experiment to test the groundwater filtration abilities of *Stropharia Mycelium*. After his initial discovery of *Stropharia Mycelium*'s filtration abilities, a cherished local reservoir fell victim to Harmful Algae Bloom and motivated him to embark on his current 5 year research to find a solution to Harmful Algae Blooms.

Introduction

Harmful Algae Blooms, or HABs, occur when there is excessive growth of algae in body of water. The term “Algae” refers to a wide variety of organisms that are all capable of producing oxygen via photosynthesis [1]. Species of algae can range from single-celled microscopic organisms to dense and thick seaweed-like mats [2]. Algae can be found worldwide, in both fresh and salt water bodies and they are very resistant to temperature/pH changes. In limited quantities, Algae are a very beneficial organism to aquatic ecosystems because they are primary producers [3]. Algae are also responsible for producing nearly half of the oxygen in our atmosphere thus they are indispensable [2]. However, in excessive amounts, Algae do more harm than good as they wreak havoc upon delicate aquatic ecosystems, harm human health, and cause massive damages to coastal, water-reliant industries.

Certain species of Algae, such as *Cyanobacteria*, release toxins into the water that that they inhabit which are deadly to both animals and humans. Domoic Acid and Microcystin are two toxins released by *Cyanobacteria* and numerous studies have found that these toxins are responsible for gastrointestinal illness, liver damage, amnesiac shellfish poisoning, seizures, and short-term memory loss [4]. Furthermore, as algae dies and decomposes, it consumes dissolved oxygen that otherwise would have been used by aquatic wildlife, which results in the creation of hypoxic “dead zones,” where life cannot be sustained due to a lack of oxygen, refer to Figure 1 [5]. HABs also have negative impacts on coastal and water-reliant industries as they close beaches, worsen catches from fisheries, and ward away potential tourists. From the years 1987 to 2000, it is estimated that HABs cost 82 million dollars in losses annually to US fishing and tourism industry [6]. A more recent study found that just in Ohio, HABs in lakes caused approximately 42 million dollars in losses

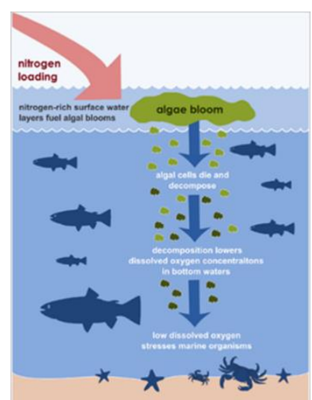


Figure 1: How HABs cause Hypoxia. *Diagram taken Smithsonian Environmental Research Center*

between 2009 and 2010 [7]. Due to increasing frequency and severity of HABs, economic losses should continue to rise.

HABs largely occur because of an unnatural increase of Nitrate and Phosphate in a body of water (also known as Nutrient pollution) due to agricultural runoff, warm water temperatures, and weak water currents [8]. Nitrate and Phosphate are essentially two nutrients that all living organisms need for growth and reproduction. They can be found in human/animal feces and are a large component of most fertilizers [9]. Application of fertilizer on fields and animal waste from CAFOs results in nutrient discharge, which is the downhill flow of excess nutrients due to rainfall. The presence of these excess nutrients in a body of water "overfeeds" the algae in the body of water and causes for large Algae Blooms [2].

Current large-scale solutions to Harmful Algae Blooms include increased government regulation and clay-cell flocculation. Government organizations like the National Pollutant Discharge Elimination System currently exist to regulate point sources of nutrient pollution, such as wastewater management plants and publicly owned treatment works. Another example would be from Minnesota, where Governor Mark Dayton, in response to worsening water quality throughout the state required for all state waters to have a riparian buffer strip border. However since major sources of nutrient pollution are non-point such as farms and private lawns, which are difficult to regulate, these measures are not entirely effective. Clay-cell flocculation is the application of clay to the surface of water infected with HABs. Through a chemical process, the clay will bind to the algae and cause it to sink to the sea floor. However, a large problem with its use is that the clay used for flocculation contains Phosphate, which further leads to HABs [4]. Recent research with HAB prevention, such as that of Intel Science Talent Search Winner, Paige Brown, uses absorbents to remove nutrients from water. Paige Brown utilized seaweed alginate along with foam bricks to absorb Phosphate from water in a separate "holding pond"

[10]. Her research is still in its early stages and the effect of her filtration method on the physiochemical properties of water along with possible methods of implementation have yet to be studied.

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 on a farm

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

The long-term goal of this 5 year research project is to develop a cost-efficient, eco-friendly, effective, and easy to implement method of Nitrate and Phosphate filtration from Agricultural Runoff to prevent Harmful Algae Blooms. This research started 4 years ago, when the author discovered in 8^{th} grade (Phase 1) that *Stropharia Mycelium* when colonized on woodchips can filter Phosphate and other chemical contaminants from groundwater, using very elementary water testing methods (qualitative color chart test kits). *Stropharia Mycelium* is a network of outward branching hyphae that act like the root structure of the *Stropharia* Mushroom and are responsible for finding and absorbing nutrients for the mushroom[11]. The research in Phase 1 was inspired by the works of Paul Stamets, a mycologist in Washington State. Stamets used *Stropharia Mycelium* to filter *E. Coli* from storm water and coined the term, *Mycofiltration*, which is the use of mushrooms (myco) to conduct filtration of contaminants from water [12]. In the second year of research (Phase 2), the rapid Nitrate and Phosphate filtration abilities of *Stropharia Mycelium* when colonized on corncobs and woodchips, were tested using more reliable water-sampling methods (API reagents and calibration curves). Corncobs were used in Phase 2 as a substrate because they are an agricultural byproduct with no significant use. Due to an unexpected reaction of test samples with the API reagents, the results obtained were largely inconclusive. However, they suggested that *Stropharia Mycelium* has the potential to be a good filter for Nitrate and Phosphate. In the third year of this research (Phase 3), the filtration abilities of *Stropharia Mycelium* when colonized on Alder Sawdust, were further tested using improved equipment and methodology. The statistically significant results that were obtained, suggested that *Stropharia Mycelium* can effectively filter Phosphate and that further research will have to be done regarding Nitrate filtration abilities.

Currently in the 4th year of this research (Phase 4), the purpose was to 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 and Phosphate removal from polluted agricultural run-off. Alder Sawdust and Organic Substrate, which is the name for the author's self-created mix of ground-up corn cobs (without kernels) and dead phragmite leaves (invasive weed), were both used as substrates in this phase because they are industrial/agricultural by-products and contribute to the overall cost-efficiency of the filtration method. Results from phases 1 to 3 have suggested that Stropharia Mycelium can effectively reduce Nitrate and Phosphate from running water. Additionally, recent research conducted Waste Treatment Residual, which is "an industrial waste produced from coagulation in water treatment facilities", suggests that it has Phosphorus filtration abilities [13]. Field implementation potential was determined by implementing the aforementioned treatments as buffer strips (thin patches of filter medium grown downstream of farmland) inside of self-designed Agricultural Runoff Simulators (ARS) where their immediate and long-term impact on the Nitrate/Total Dissolved Nitrogen (TDN), Phosphate, and pH Levels of Simulated Agricultural Runoff (SAR) along with soil pH and *Spinacia oleracea* (Spinach) growth was measured over 32 days. Additionally, the effect of *Stropharia Mycelium* on *S. oleracea* growth was also studied in self-designed Spinach Growing Cups (SGC).

The impact of the treatments on Phosphate and Nitrate/TDN levels were studied because they are limiting factors for algae growth and any filtration method seeking to prevent Harmful Algae Blooms would have to be one that can filter Nitrate and/or Phosphate. The impact of these treatments of SAR pH was also studied because it is vital for any filtration method to not drastically alter water pH. If that filtered water were to re-enter its original environment with a different pH, the wildlife living in that water might not be able to adjust to the pH change and die, thus the filtration system would be doing more harm than good. Additionally, the impact of these treatments on *S. oleracea* growth and soil pH was also studied because both MS and MOS treatments consist of a living organism (*Stropharia Mycelium*). If these treatments were implemented on farms as buffer strips and the mycelium root structure were to spread outwards from the buffer strips, it could interact with the farmer's soil and crops in a negative manner given that it is a fungus. Although most crops share a symbiotic relationship with mycorrhizal fungi, this research is using *S. oleracea* as a model organism to study that mycelium-crop-soil interaction as a pre-cautionary measure prior to field implementation.

Methodology

Phase 4.1

Prior to starting experimentation, MS, MOS, and WTRMS treatments were prepared. For the MS and MOS treatments, equal parts of substrate (Alder Sawdust or Organic Substrate - mix of dead phragmite leaves and corn cobs) and Stropharia spawn were mixed together inside of mesh bags and kept in a dark and moist environment for 4 weeks with frequent watering periods so that the substrates could become fully colonized with *Stropharia Mycelium*. Stropharia spawn is a fertilized mixture of sawdust and woodchips that has a culture of *Stropharia Mycelium* growing upon it. Unlike bacteria or other fungi, mushroom cultures have to be purchased in the form of spawn if one wishes to colonize other substrates with a certain mushroom species. All sawdust/organic substrate used in this research was kept submerged in distilled water for 12 hours prior to use to remove any residues. For the WTRMS treatment, equal parts of powder WTR and Alder Sawdust were mixed thoroughly inside of a plastic container.

After treatments had been prepared, 8 groups of Agricultural Runoff Simulators (ARS) were set up based on Figure 3 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. 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 32 spinach seeds were planted in each of the ARS. Every 4 days for 32, each ARS was watered with 750 ml of diluted 9-4-9 liquid fertilizer to simulate a rainfall and the drainage from each ARS (Simulated Agricultural Runoff) was collected in appropriately labeled tubes. Additionally, the number of spinach sprouts and leaves present in each ARS was also recorded every 4 days for 32 days.

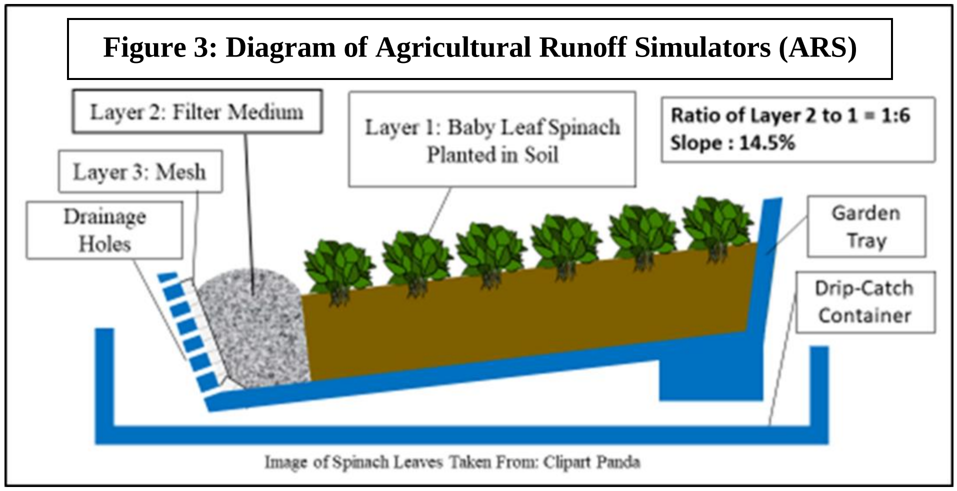


Figure 3: Diagram of Agricultural Runoff Simulators (ARS). *The ARS was designed by the author to simulate an agricultural field inside of a small tray.*

Figure 4: 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 4: Design of Experiment - Content of Agricultural Runoff Simulators (ARS) Layer 2 Filter Mediums. *Groups A, B, and G were the main treatments groups.*

Alongside the three treatment groups (groups A, B, and G in Figure 4), there were other test/control groups that were used as buffer strips or Layer 2 in the ARS. Since Stropharia Mycelium is fungus, it can only exist on a substrate such as sawdust or woodchips. The only way to colonize a substrate with Stropharia Mycelium is to mix it with Stropharia Spawn, which is a commercially available culture of Stropharia Mycelium that has been growing on treated woodchips. Thus, the mycelium can expand from the spawn's treated woodchips onto the desired substrate for colonization. To understand and isolate non-mycelium aspects of the treatment groups, which were comprised of substrate and Stropharia spawn, the study treated Stropharia Spawn as Dead Spawn (autoclaved spawn) + Mushroom culture and had groups with just dead spawn and just substrate. You cannot physically separate the mushroom culture from spawn but you can autoclave spawn to kill the mushroom culture and just get dead spawn. The spawn, which is purchased form commercial growers, itself is fertilized and as observed in previous phases, the fertilizer from the spawn will often leach into the water samples and give inaccurate readings. The same phenomenon could occur with any residues on the substrates themselves. Thus, just dead spawn and just substrate groups were also created to take into account any contamination of water samples from the substrate or dead spawn. Since the soil used to fill the ARS was commercially purchased, it is likely that it had some slow-release fertilizer mixed into it. Thus, to account for any contamination of water samples due to the soil, there was a negative control group comprised of just the soil and spinach seeds.

The ARS was designed by the author to simulate an agricultural field inside of a tray where one could control rain-fall levels, light exposure, slope-gradient, soil-quality, etc. and has been made public online for other researchers to use. For the purposes of this research, the ARS was configured to have an exaggerated buffer strip to farm-field ratio (1:6) and slope gradient (14.5%). By having a large buffer strip to farm-field ratio, the purpose was to ensure that the treatments have enough physical exposure to the runoff. If implemented on farms, the size of the buffer strip would be anywhere from 1-3 meters and have a ratio of approximately 1:200 but that couldn't have been effectively scaled down inside the ARS. To accommodate for the increased ratio, the slope gradient was also exaggerated so that while there would be more exposure between the treatment and the runoff, the runoff would go through the treatment at a faster rate. Traditional farms will have slope gradients between 1-3%.

In comparison to previous phases of this research where the author tested the treatments in lab settings using funnels with limited saturation and soil-particle contamination, the ARS allowed for the author to get more real-world representative data. The ARS allowed for the author to determine the effectiveness of these treatments after they went underwent periods of excessive saturation and faced the high water-flow that follow a rainfall on actual farm-fields. It also let the author determine the filtration effectiveness of these treatments over extended periods of time because these treatments were tested every four days inside of the ARS for a span of 32 days. Long-term effectiveness was an important characteristic to measure because there are numerous Nitrate/Phosphate absorbents available on the market but their potential for implementation on agricultural fields (the largest source of nutrient pollution) is limited because these absorbents will get saturated and thus become ineffective over time. However, with the MS and MOS treatments, since they are comprised of living mycelium which absorbs the nutrients and uses them to grow, they should remain effective over time and thus should have potential for field implementation. In summation, the ARS simulated the conditions the treatments would undergo if actually implemented and allowed for more thorough determination of the treatments' field implementation potential.

Using Hach PhosVer Phosphate reagents/spectroscopy, a Vernier Nitrate Ion-Selective Electrode, a Shizmudu Total Organic Carbon Analyzer with a Total Dissolved Nitrogen component, and a Vernier pH probe, the Phosphate, Nitrate/Total Dissolved Nitrogen, and pH of all the waters samples collected was tested and recorded. Note that the experimental design and set-up was conducted independently at the author's high school. Unlike in previous phases, all water-sample testing in this phase was done at Dr. Yang Deng's lab at Montclair State University under adult supervision due to availability of better equipment. While Nitrate and Phosphate are largely responsible for Harmful Algae Blooms, changes in Total Dissolved Nitrogen (TDN) were also measured to further increase confidence in the data collected for Nitrate and come to a better supported conclusion. Although TDN is a measurement of numerous forms of Nitrogen, including Nitrate, the primary form of Nitrogen that the ARS was fertilized with was Nitrate therefore a decrease in TDN would suggest a decrease in Nitrate. At the end of the 32 days, the soil pH of each ARS was measured and recorded using pH probe and a 0.01 M Calcium Chloride Solution (standard procedure for soil pH determination which involves submerging 50g of soil sample in CaCl solution prior to reading using pH probe). Also on Day 32, any spinach biomass present in an ARS was picked out, stored in a foil pan corresponding to that ARS, and placed in a drying oven overnight. The following day, the biomass was measured using an analytical balance and the average biomass per sprout was calculated for each ARS.

In addition to just measuring the impact of the treatments on runoff's physiochemical properties, the ARS allowed for the impact of these treatments on *S. oleracea* growth and soil pH to also be measured. As previously mentioned, the impact of these treatments on *S. oleracea* growth and soil pH were important to measure to ensure that the treatments don't have a negative impact on the farmer's crop yield if implemented on a farm. The ARS placed *S. oleracea* roots in close proximity of the treatments and allowed for interactions to occur between mycelium, roots, and soil. *S. oleracea* were used in this study as opposed to the traditional *Arabidopsis thaliana* (Radishes) that is used in most agricultural studies because radishes are one of the few plants in the world that do not form symbiotic relationship with mycorrhiza fungi. Furthermore,6 *S. oleracea* are known to grow well in narrow trays (ARS) and can withstand excessive watering, which occurred on every 4th day. To compare the effect of treatments on *S. oleracea* growth, average biomass per sprout for each ARS was calculated and compared between test groups and control groups so that the random variability associated with root sprouting could be accounted for. This measure had to be taken since *S. oleracea* seeds cannot survive the stress that comes with transplantation after pre-germination.

Phase 4.2

Figure 5: Design of Experiment - 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 5: Design of Experiment - Content of Spinach Growing Cups (SGC) Layer 2.

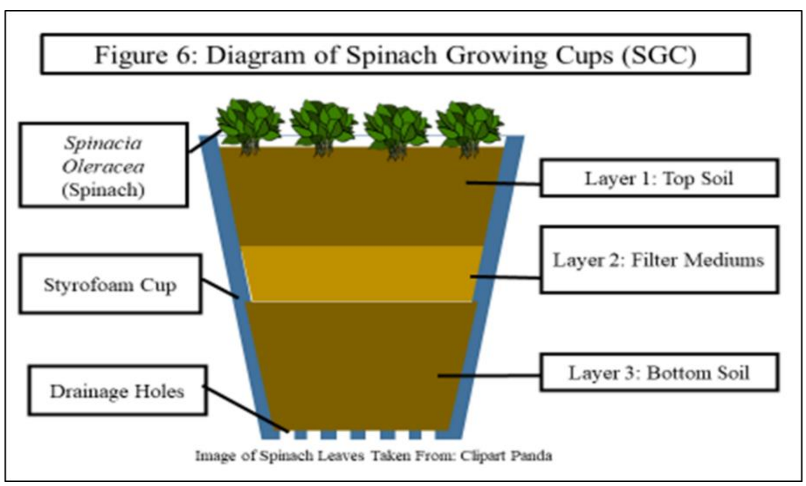
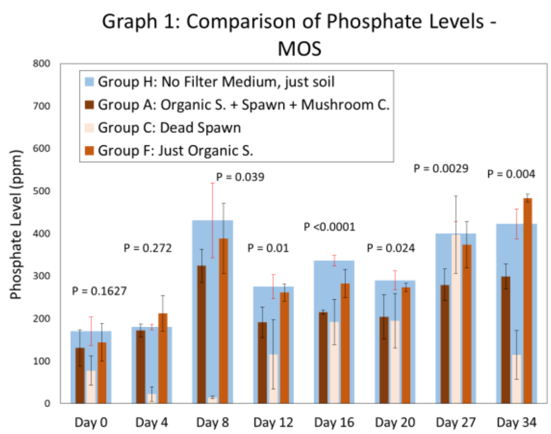


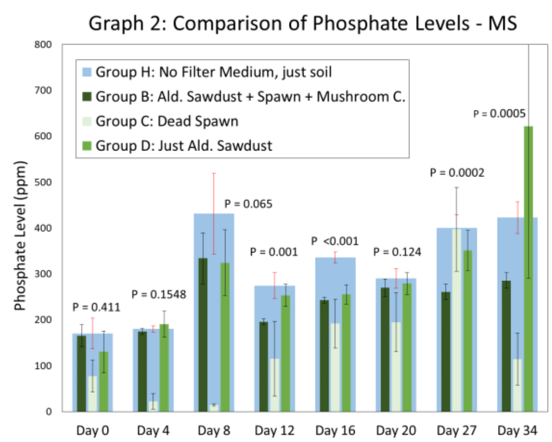
Figure 6: Diagram of Spinach Growing Cups (SGC).). *The SGC was designed by the author to serve as a more controlled environment for determining impact of treatments (Stropharia Mycelium) on Spinach growth.*

In addition to determining the impact of these treatments on *S. oleracea* growth and soil pH inside of ARS, a second study was conducted to just study the impact of *Stropharia Mycelium* on *S. oleracea* growth in self-designed Spinach Growing Cups (SGC). First, 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. Layer 2 in each of the SGC were filled with the filter medium corresponding to the SGC's letter group (refer to Figure 5) and spinach seeds were planted in each of SGC. Each SGC was watered with 40ml of distilled water every day and the number of sprouts and leaves present every day was recorded for 21 days. Using previously explained methods, the soil pH and average biomass per sprout was calculated for each SGC. Since the ARS has a lot more variables such as excessive watering, slope-gradient, and fertilization that could impact *S. oleracea* growth, Phase 4.2 was conducted with a simpler experimental set-up so that the impact of *Stropharia Mycelium* could be better studied. Similar to Phase 4.1, this study had test/control groups other than just the treatment group that were used for Layer 2 in the SGC. The purpose of these additional groups was to help isolate the effect of *Stropharia Mycelium* and understand the impact of non-mycelium aspects such as dead spawn and substrate.

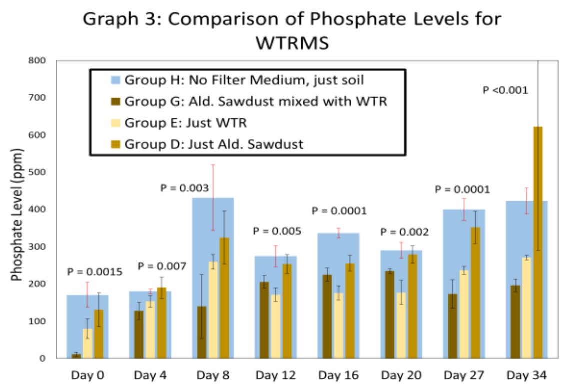
Results and Discussion



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

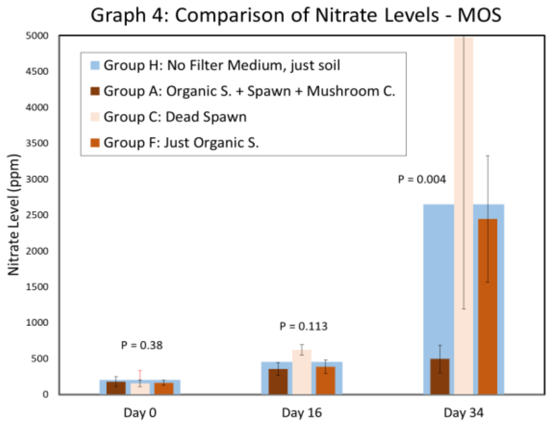


Graph 2: Phosphate Levels for SAR samples from the MS Treatment Groups. P values are from Student's t-Tests between Group H and Group B samples of the same day

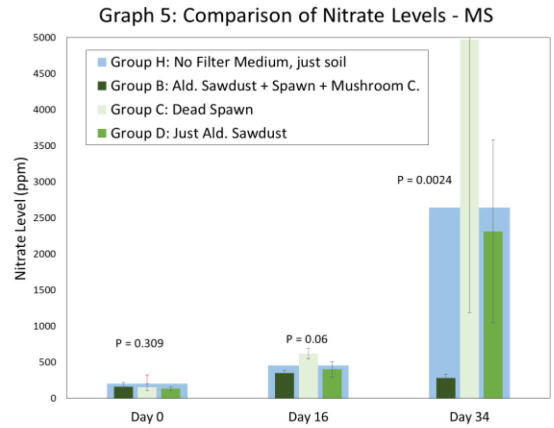


Graph 3: Phosphate Levels for SAR samples from the WTRMS Treatment Groups. P values are from Student's t-Tests between Group H and Group G samples of the same day

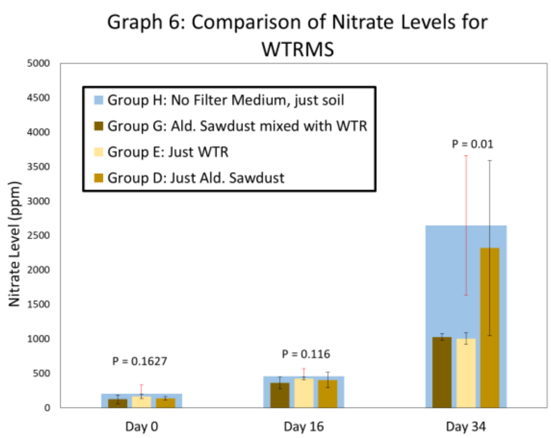
Graphs 1, 2 and 3 show the data for Phosphate levels for Simulated Agricultural Runoff (SAR) samples from ARS for the WTRMS, MS and MOS treatments respectively. In these graphs, the light brown, dark green, and maroon bars symbolizing the treatment groups G, B and A respectively are lower than the overarching blue bars symbolizing the negative control group for most days. Note that the negative control group just had soil in the ARS and lacked a buffer strip whereas the treatment groups respectively had Alder Sawdust mixed with WTR, Alder Sawdust colonized with *Stropharia Mycelium*, and Organic Substrate colonized with *Stropharia Mycelium*. Since the treatment bars are lower than the negative control bars, this suggests that all three treatments can effectively reduce Phosphate from SAR. On average across all days (>0), WTRMS was able to reduce Phosphate levels by 149 ppm daily, MOS by 93 ppm, and MS by 82 ppm. Upon conducting a t-Test analyses between the data for treatments groups and negative control groups individually, the difference was found to be statistically significant. Additionally, across the MS and MOS graphs, the bars symbolizing the non-treatment test groups, which either had just Alder Sawdust or just Organic Substrate, are lower than the negative control bars but higher than the treatment bars. Since they are lower than the negative control bars, all these physical substances, which are agricultural/industrial byproducts, act as Phosphate absorbents and their point of saturation outlasts the duration of this research (> 32 days). Since they are higher than the treatment bars and the difference between these groups and the MS and MOS treatment bars is the presence of *Stropharia Mycelium* (refer to Figure 3), that further decrease in Phosphate levels was due to consumption, not absorption, of Phosphate by *Stropharia Mycelium*. Similar trends in terms of Phosphate consumption by *Stropharia Mycelium* were noticed in previous phases of research. On average, across both MS and MOS treatments, *Stropharia Mycelium* was able to consume approximately 68 ppm of Phosphate daily. For the WTRMS graph, the bars symbolizing just WTR and just Alder Sawdust are lower than the negative control bars but higher than the treatment bars, which symbolize WTR mixed with Alder Sawdust. This suggests that WTR mixed with Alder Sawdust is more effective as a Phosphate Absorbent than just WTR or just Alder Sawdust. While further studies will have to be conducted to determine why this occurs, it is hypothesized that when mixed, there is greater exposure between runoff and WTR than with just WTR due to the slowing down of runoff by sawdust particles.



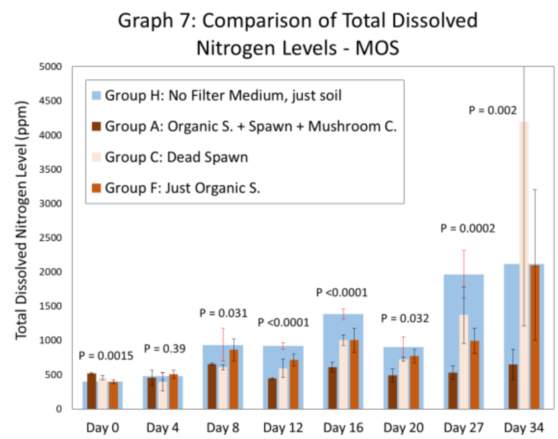
Graph 4: Nitrate Levels for SAR Samples from the MOS Treatment Group. P values are from Student's t-Test between Group H and Group A samples of the same day



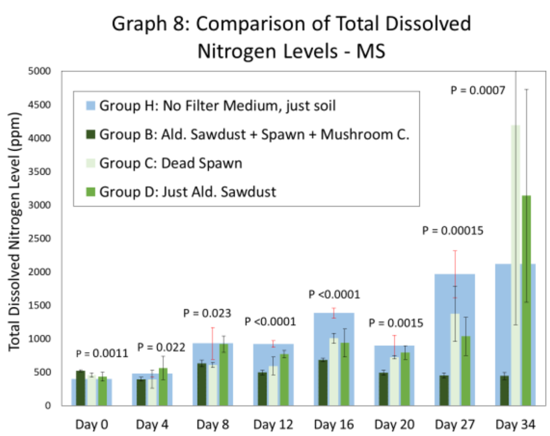
Graph 5: Nitrate Levels for SAR Samples from the MS Treatment Group. P values are from Student's t-Test between Group H and Group B samples of the same day



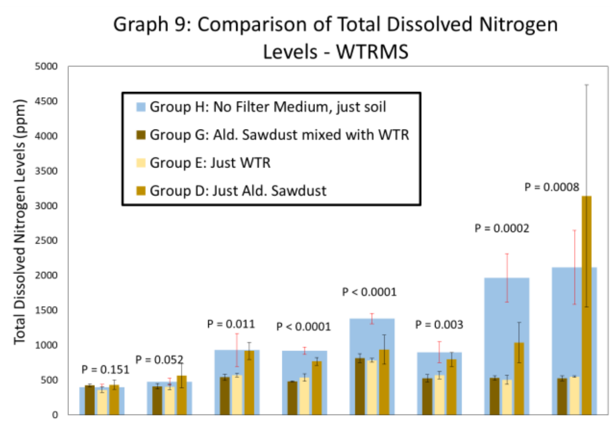
Graph 6: Nitrate Levels for SAR Samples from the WTRMS Treatment Group. P values are from Student's t-Test between Group H and Group G samples of the same day



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



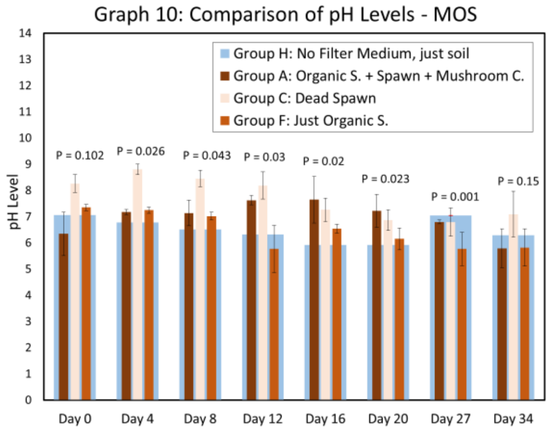
Graph 8: Total Dissolved Nitrogen Levels for SAR samples from the MS Treatment Group. P values are from Student's t-Test between Group H and Group B samples of the same day



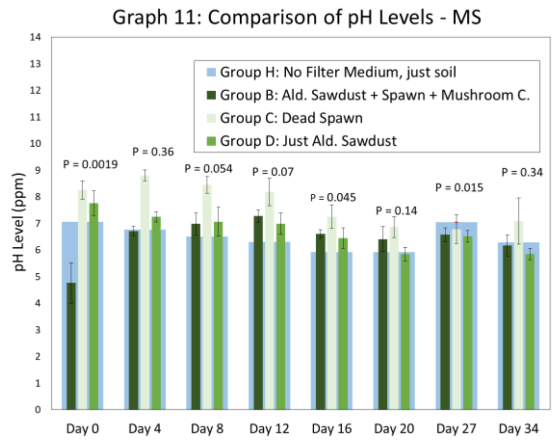
Graph 9: Total Dissolved Nitrogen Levels for SAR samples from the WTRMS Treatment Group. P values are from Student's t-Test between Group H and Group G samples of the same day

While Nitrate and Phosphate are largely responsible for Harmful Algae Blooms, changes in Total Dissolved Nitrogen (TDN) were also measured to further increase confidence in the data collected for

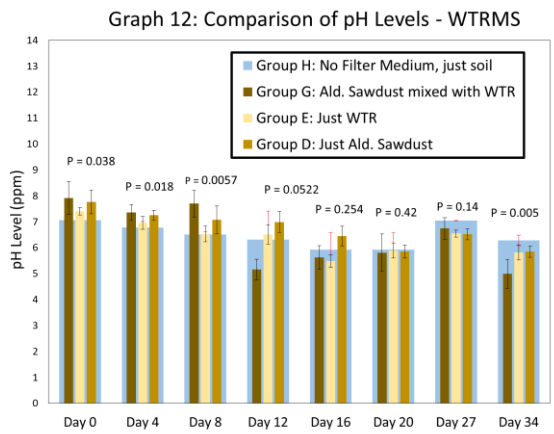
Nitrate and come to a better-supported conclusion. Although TDN is a measurement of numerous forms of Nitrogen, including Nitrate, the primary form of Nitrogen that the ARS was fertilized with was Nitrate therefore a decrease in TDN would suggest a decrease in Nitrate. Graphs 4, 5 and 6 show the data for Nitrate levels and in Graphs 7, 8, and 9 which show the data for TDN levels for Simulated Agricultural Runoff (SAR) samples from ARS for the WTRMS, MS and MOS treatments respectively. In these graphs, the light brown, dark green, and maroon bars symbolizing the treatment groups G, B and A respectively are lower than the overarching blue bars symbolizing the negative control group for most days. Since the treatment bars are lower, this suggests that all three treatments could effectively reduce Nitrate/TDN from SAR. On average across all days (>0), WTRMS was able to reduce TDN levels by 696 ppm daily, MOS by 695 ppm, and MS by 729 ppm. Upon conducting a t-Test analyses between the data for treatments groups and negative control groups individually, the difference was found to be statistically significant. Additionally across the MS and MOS graphs, the bars symbolizing the non-treatment test groups, which either had just Alder Sawdust, or just Organic Substrate, are lower than the negative control bars but higher than the treatment bars for most days. Since they are lower than the negative control bars, all these physical substances, which are agricultural/industrial byproducts, act as Nitrate absorbents and their point of saturation outlasts the duration of this research (> 32 days). Similar trends in terms of Nitrate absorption by Alder Sawdust were noticed in previous phases of research. Since they are higher than the treatment bars and the difference between these groups and the MS and MOS treatment bars is the presence of *Stropharia Mycelium* (refer to Figure 3), that further decrease in Nitrate levels was due to consumption, not absorption, of Nitrate by *Stropharia Mycelium*. On average, across all days and both MS and MOS treatments, *Stropharia Mycelium* was able to consume approximately 670 ppm of Nitrate. Additionally in graphs 8 and 9, the bars symbolizing group C which was just dead spawn, are drastically higher than the negative control bars which suggest that the spawn itself is releasing some form of nitrogen into the SAR. It is hypothesized that spawn, which was purchased from a commercial grower, was likely supplemented with nitrogenous fertilizer as similar trends were noticed in previous years of the study. If implemented in the field, smaller ratios of spawn to substrate would be used for cost purposes, than the 1:1 ratio used in this study. Thus, the effect if any, of nitrogenous leaching from spawn would be minimal. For the WTRMS graphs, there is no significant difference between the bars symbolizing WTR mixed with Alder Sawdust and just WTR which suggests that the addition of Alder Sawdust does not improve or worsen WTR’s absorption abilities. Similar trends were found in the data for TDN which support these conclusions.



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



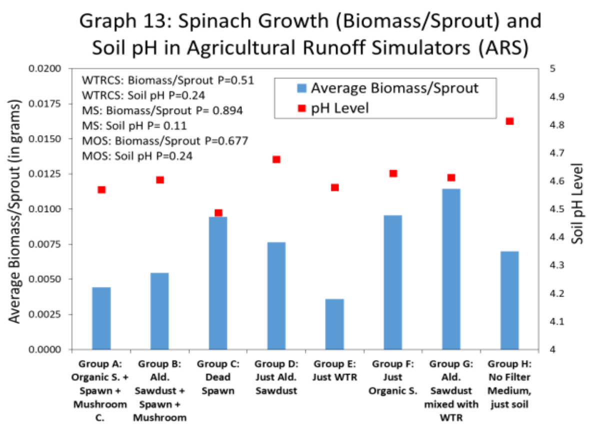
Graph 11: pH Levels for SAR samples from the MS Treatment Groups. P values are from Student's t-Tests between Group H and Group B samples of the same day



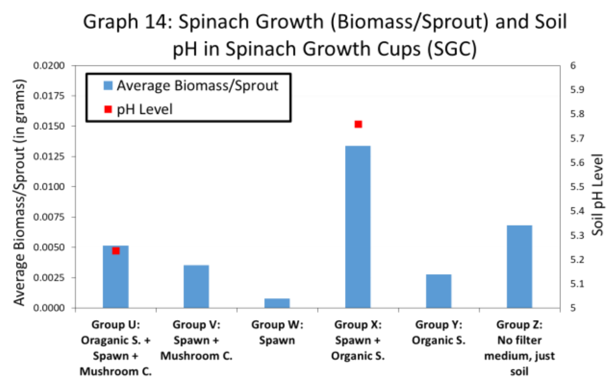
Graph 12: pH Levels for SAR samples from the WTRMS Treatment Groups. P values are from Student's t-Tests between Group H and Group G samples of the same day

Graphs 10, 11, and 12 show the data for pH levels of Simulated Agricultural Runoff (SAR) samples from ARS for the WTRMS, MS and MOS treatments respectively. In graphs 10, 11, and 12, there is a lack of trends between the dark brown, dark green, and maroon bars symbolizing the treatment groups G, B and A and the overarching blue bars symbolizing the negative control group. On some days, the treatment bars are higher than the negative control bars 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). This suggests that the treatments do not significantly alter the pH of simulated agricultural runoff. In both these graphs, the bars symbolizing group C, which was dead spawn, are higher than both the negative control and treatment bars. Additionally, SAR samples from Group C had high levels of TDN. Since spawn is commonly fertilized by commercial growers, it is hypothesized that the spawn contained ammonium hydroxide (a substance commonly found in fertilizers), which leached from the spawn into the SAR thus explaining the pH increase and elevated TDN levels. Ammonium hydroxide dissociates into OH- and Ammonium. Excess OH- ions cause for a pH increase. If implemented in the field, smaller ratios of spawn to substrate would be used for cost purposes, than the 1:1 ratio used in this study. Thus, the effect if any, of ammonium hydroxide leaching from spawn would be minimal.

As expected, Phosphate, Nitrate, and Total Dissolved Nitrogen levels rose over time across all graphs due the accumulation of contaminants in the ARS after fertilized watering every 4 days. When the ARS would be watered with diluted liquid fertilizer, the soil in Layer 1 would trap some of the nutrients from the fertilized water. Those trapped nutrients wouldn't get released until the soil was re-watered on the next 4-day with more fertilized water, which again would result in the trapping of nutrients. Over the span of 32 days, nutrients were expected to accumulate in the soil and for the general levels of nutrients in the SAR to rise.



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.

Graph 13 shows the data for soil pH and average biomass per *S. oleracea* sprout in ARS. Upon conducting ANOVA analyses between the treatments groups and their respective test groups (refer to Figure 3) for average biomass per sprout, this data was found to be largely insignificant. Due to the over-watering of the *S. oleracea* every four days in the ARS as a simulation of rainfall, there was near random *S. oleracea* growth and thus, any conclusions cannot be made regarding the impact of WTRMS, MS, and MOS treatments on *S. oleracea* growth. Using scientific reasoning, one can assume that WTRMS treatments will not have any impact on crop growth because WTR is not a live organism and will not likely come into contact with crop roots if implemented on a farm. However, for the MS and MOS treatments which do have *Stropharia Mycelium,* any impact that they would have if implemented in the field on crop growth would be due to outward branching of *Stropharia Mycelium* from the point of colonization. This interaction between *Stropharia Mycelium* and *S. oleracea* was studied in Phase 4.2. Graph 14 shows the data for phase 4.2, which tested the impact of *Stropharia Mycelium*, on soil pH and average biomass per *S. oleracea* sprout in Spinach Growing Cups. The presence of *Stropharia Mycelium*, which was the only difference between group X (Dead Spawn + Organic Substrate) and group U (Organic Substrate + Alive Spawn), caused for a decrease in average biomass per *S. oleracea* sprout of 0.075 grams. This decrease was not found to be statistically insignificant after conducting a t-Test analysis. Thus, *Stropharia Mycelium* does not have a significant negative impact on *S. oleracea* growth if any. In terms of soil pH, all treatments had no significant impact on soil pH inside of ARS as shown by near similar pH levels for all groups in Graph 13 and an ANOVA P-Value >0.05. For the SGC, Group U when compared to group X did have a statistically significant however negligible (<0.5 pH) decrease. This suggests that Stropharia Mycelium tends to make soil slightly more acidic. It is hypothesized that Stropharia Mycelium might have an antiport transport protein which “exports” H+ (which would cause the pH to decrease) and in exchange “imports” ammonium/nitrate into the fungi cell. Further studies will have to be conducted to fully determine why this occurs. However, when implemented in the field, this slight acidification of the soil would be negligible as heavy mycelium concentration would only be near a small patch of land downstream of the farm.

Stropharia Mycelium was able to effectively consume Nitrate/TDN and Phosphate from SAR likely due to its biological role in the fungal body, which is to obtain nutrients to help the fungal body grow and reproduce. Fungal mycelium obtains nutrients from its environment by first secreting enzymes into its environment to break down food sources into smaller units and then via facilitated diffusion and active transport, absorbing those broken down food sources back into the fungal body [11]. It is hypothesized that because of this process, *Stropharia Mycelium* was able to consume and reduce Nitrate and Phosphate levels. Given that *Stropharia Mycelium* is a network of outward branching hyphae, as more Phosphate and Nitrate is available for consumption, the more the mycelium will grow and the more Phosphate and Nitrate it will be able to reduce. Thus, unlike currently existing absorbents, this mycelium doesn’t have a point of saturation and if the duration of this experiment were to be extended, its average Nitrate and Phosphate consumption levels would likely increase. Additionally, as a result of mycelium growth, more fruiting bodies (edible, gourmet mushrooms) would also be produced and these fruiting bodies could be harvested by the farmer and sold as additional income.

Conclusion

All three treatments have field implementation potential for further testing

- Stropharia Mycofiltration when colonized on Organic Substrate (MOS), Stropharia Mycofiltration when colonized on Alder Sawdust (MS), and Waste Treatment Residual Mixed with Alder Sawdust (WTRMS) treatments can all effectively reduce Nitrate 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 statistically significant impact on Spinach growth or soil pH

In the next phase of this research (Phase 5), the treatments will be implemented as buffer strips on a test plot inside a farm. Once implemented, this method could last for decades as the mycelium is very resistant to harsh weather conditions. For further information or current updates, visit the author's website, www.HABPrevention.weebly.com In conclusion, this research has potentially discovered a cost-efficient, eco-friendly, and effective method of Nitrate and Phosphate filtration from agricultural runoff to prevent Harmful Algae Blooms.

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