MYCOREMEDIATION OF STORMWATER USING WHITE-ROT FUNGI:

An in vitro examination of Stropharia rugosoannulata and Pleurotus ostreatus for aqueous Escherichia coli mitigation

by

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ABSTRACT

Mycoremediation of stormwater using white-rot fungi:
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Pathogenic bacteria in waterways can cause health, economic, and environmental problems. *E. coli* is a common pathogenic bacteria pollutant in stormwater and its overabundance can impact downstream natural ecosystems, agricultural food systems, and safe recreation. As cities grow denser and climate change continues to escalate, increased urban flooding will expand these impacts. Research has shown that certain white-rot fungi can degrade *E. coli* and a variety of other xenobiotic pollutants, sparking interest in using fungi to mitigate stormwater pollutants. With this in mind, a laboratory experiment was conducted to test if *Stropharia rugosoannulata and Pleurotus ostreatus* produce a measurable reduction of *E. coli* populations in an aqueous environment. *S. rugosoannulata* and *P. ostreatus* fungi as well as *E. coli* bacteria were cultured in a lab. Petri dishes were used as molds to culture the fungi into pucks weighing 30g each. These pucks were then added to 500mL of sterile DI water that was inoculated with approximately 3,000 CFU of *E. coli*. The water was sampled at 0 hours, 12 hours, 24 hours, and 72 hours. The samples were serial diluted and plated for *E. coli* enumeration. This data was then plotted as CFU over time. The data showed a reduction of *E. coli* concentrations in the *S. rugosoannulata* treatments that was not seen in the control groups. The results of this pilot study show that the addition of floating *S. rugosoannulata* mycelium to water containing *E. coli* can lead to a reduction of *E. coli* at greater rates than that of a control. These findings add support for the use of *S. rugosoannulata* fungi in stormwater mitigation projects and they highlight the importance of further research on white-rot fungi in mycoremediation projects.

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Introduction

Xenobiotic contaminants are chemicals or compounds that are not naturally found in an ecosystem. The term can cover a range of pollutants; these include accumulated herbicides, PAHs (polyaromatic hydrocarbons), PCBs (polychlorinated biphenyls), and pharmaceuticals. These pollutants can cause serious health, environmental, and economic damage. As concerns over xenobiotic and bacterial contaminants grow, people are searching for solutions that are efficient and effective.

As a diverse kingdom, fungi inhabit all ecosystems and utilize a wide spectrum of nutrient sources. The evolutionary processes that have diversified them have resulted in many adaptations that can be useful for restoring degraded natural environments. From breaking down xenobiotic contaminants and harmful bacterial colonies, to aggregating heavy metals and restoring soil health, fungi is being used for a range of restoration projects (Bishnoi, Kumar, & Bishnoi, 2007; Deshmukh, Khardenavis, & Purohit, 2016; Eskander, Abd El-Aziz, El-Sayaad, & Saleh, 2012).

Mycoremediation is a term coined by its proponents to describe the process of restoring a degraded natural area through the use of fungi (Bumpus, Tien, Wright, & Aust, 1985; Eskander et al., 2012; Stamets, 2005). Specifically selected fungal strains are added either directly to the soil of a restoration site, or more commonly, the fungi is densely aggregated in places where stormwater has been diverted to or where it naturally flows through. Depending on the size and scope of the restoration project, these mycoremediation ‘bio filters’ can range in size. They can be as simple as burlap sacks filled with fungal mycelium to constructed boxes the size of a small car that stormwater has been diverted to flow through. In these ‘bio filters,’ fungal mats are installed to filter

the passing water. The constructed box allows for the filters to be monitored and maintained.

Use of fungal mycelium to treat contaminated water systems has been growing in popularity (Stamets, 2005) yet the scientific research to support its growth is lagging behind (Sasek, 2003; Singh, 2006); more rigorous testing is needed to assess its abilities, limitations, and best applications (Sasek, 2003; Singh, 2006). Often cited in bioremediation literature, H. Singh is quick to point out that the majority of mycoremediation studies involving experiments are conducted *in vitro* (this experiment included), meaning that they take place on a small scale, under highly controlled and regulated conditions (Singh, 2006). The success of mycoremediation projects to restore soil health depend on the initial survival of the fungi and many site-specific characteristics such as temperature, moisture levels, native soil fauna populations, and a myriad of other variables (Sasek, 2003). Because of these abiotic and biotic constraints, *in situ* tests can be difficult to run and often produce ambiguous results (Sasek, 2003).

Supporters are often quick to sell mycoremediation's advantages, but in personal communications with several Washington Department of Transportation Stormwater professionals, they expressed a common concern that most people promoting the advantages of mycoremediation are also the same people that are selling it, thus making it hard to find impartial facts.

Some of the traits that make mycoremediation attractive, such as its relatively inexpensive application and its low threshold of knowledge needed for implementation (Singh, 2006; Stamets 2005), are the same traits that can develop a potential for its

overuse or misuse. As more people and organizations turn to mycoremediation as a hopeful solution, further research is needed to better guide these efforts towards solutions that are supported by empirical evidence.

With this tension in mind, a lab experiment was created to test the ability of select fungal mycelium to reduce *Escherichia coli* (*E. coli*) levels in a controlled aqueous environment. *Stropharia rugosoannulata* and *Pleurotus ostreatus* were selected as the fungal species for this experiment. These species were chosen because they are native to most ecosystems and are already often used in mycoremediation projects because of their common availability. While *P. ostreatus* has a rich history of study in the related literature (Bumpus et al., 1985; Fungi Perfecti, 2013), study into *S. rugosoannulata* for mycoremediation is less advanced (Fungi Perfecti, 2013).

Stropharia rugosoannulata and Pleurotus ostreatus fungal mycelium was grown on pucks of Alder wood and then floated on top of *E. coli* inoculated water. *E. coli* concentrations in this water was tested using a spread plate method over a time gradient to look for a potential change in *E. coli* concentrations.

To best understand the growing desire for mycoremediation, it is important to understand the context of stormwater management that it fits into (the scope of current challenges and current best management practices). For this experiment, it is also important to understand why *E. coli* is used as an indicator measurement for contaminated water and the current disputes in the scientific literature regarding mycoremediation. To provide this context, a literature review will be presented before the experiential procedure description. Following this background, the methods and

procedures for the experimental phase of the project will be outlined and discussed. Next, the results will be presented along with their statistical analysis. The final section provides an overview of key finding and an analysis of useful further research.

Literature Review

Introduction

Inhabiting all ecosystems, fungi can be found inside leaf cells at the top of rainforest canopies (Espinosa-Garcia & Langenheim, 1990), hidden in the frozen soils beneath glaciers (Gostinčar, Grube, De Hoog, Zalar, & Gunde-Cimerman, 2009), deep at the bottom of the ocean (Damare & Raghukumar, 2008; Raghukumar, 2008), floating as spores in the air we breathe (Fröhlich-Nowoisky, Pickersgill, Després, Pöschl, & Chameides, 2009), and living in our own gut (Huseyin, O'Toole, Cotter, & Scanlan, 2017). The fungal kingdom represents an ancient and vast group of organisms that play a vital role in regulating the flow of energy and nutrients through the systems they populate (Harris, 2009).

In a separate kingdom from both plants (Viridiplantae) and animals (Animalia), fungi are eukaryotic organisms that can range from simple molds and yeasts to more advanced structures like mushrooms that can be found on the forest floor or in a supermarket isle. These fruiting bodies are formally referred to as basidiocarps. Mycological taxonomy is seemingly in a state of constant flux (most recently due in large part to current DNA sequencing (May & Redhead, 2018)) and as such even the number of phyla in the fungal kingdom is currently debated. While seven phyla is currently the most agreed upon amount (Hibbett et al., 2007), arguments have recently been made for eight phyla (Spatafora et al., 2018), ten phyla (Tedersoo et al., 2018), and elven phyla (Tedersoo et al., 2018; Turland et al., 2017).

Fungi are often broken down into three functional classifications; saprophytic, mycorrhizal, and parasitic. For a simplistic overview: saprophytic fungi consume and

break down dead and decaying material, mycorrhizal fungi grow at the rhizosphere of plant roots and aid in nutrient transfer between plants and their surrounding environments, and parasitic fungi receive their nutrients from other organisms. All three functional types of fungi can and are used in ecological restoration projects: saprophytic fungi to break down and/or aggregate xenobiotic pollutants (Bishnoi et al., 2007); mycorrhizal to favor particular plant assemblages and as a bio-indicator for soil health/successional trajectory (Harris, 2009; Heneghan et al., 2008); and parasitic fungi to combat specific insects, nematodes, bacteria, or plant assemblages (Deshmukh et al., 2016).

Saprophytic Basidiomycetes

The two fungal species that this paper primarily focuses on, and the ones used in the experimental phase, *Stropharia rugosoannulata* (King Stropharia) and *Pleurotus ostreatus* (Oyster), are both saprophytic fungi. This means that both species receive their nutrients from decaying organic matter, through a process of extracellular chemical digestion. Under taxonomic ranking, both *S. rugosoannulata* and *P. ostreatus* fall into the Dikarya subkingdom, the Basidiomycetes phyla, and the Agaricales clade. The Agaricales clade is populated by many gilled mushrooms that occupy a wide variety of ecosystems and niches.

"White-Rot" Fungi

Both *S. rugosoannulata* and *P. ostreatus* also belong to the informal category of "white-rot" fungi. White-rot fungi are known for their decomposition of plant lignin (Hirai, Sugiura, Kawai, & Nishida; 2005). There is a wide diversity of white-rot fungi, some of which are parasitic to their hosts (many *Armillaria* species, like the honey

mushrooms), and some of which are commonly cultivated edible species (such as *Lentinula edodes*, shitake mushrooms) and *Pleurotus ostreatus* (oyster mushrooms). These fungi typically excrete oxidase enzymes that breakdown plant lignin into digestible molecules, leaving behind a characteristic spongy white or yellow rotted wood. This remaining rotted wood is composed primarily of plant cellulose that turns from white to yellow as it oxidizes with age (this same reaction is what turns old newspapers yellowish; as the higher concentration of lignin in cheap newsprint is exposed to air, it yellows more with time).

Fungal Assisted Chemical Decomposition

White-rot fungi secrete a wide array of heme-containing perox-idase enzymes that facilitate the breakdown of plant cell lignin. These diverse enzymes are categorized into three families; lignin peroxidase (LiP), manganese peroxidase (MnP), and a more recently described hybrid peroxide, a mix containing both lignin and manganese peroxidases (MnP-LiP) (Hirai et al., 2005). Lignin is a natural polymer with a complex and heterogeneous structure that is highly resistant to degradation (Orth, Royse, & Tien, 1993). Lignin aids in plant rigidity and structure as wells as instilling some pest and pathogen protection too (Orth, Royse, & Tien, 1993). The wood pulp industry and commercial agriculture are both fields that can benefit from novel ways of breaking down lignin rich waste materials. Incentivized to find cheaper and more efficient lignin processing, they are advancing much of the research around enzymatic waste degradation.

Through both oxidation of non-phenolic aromatic compounds (due to its high redox potential) and oxidation through long range electron transfer pathways, LiP is able

to degrade lignin found in woody plant material (Hirai et al., 2005). MnP oxidizes Mn(II) to Mn(III) through chelation (Hirai et al., 2005). Chelation facilitated mineral absorption is also a critical mechanism that drives *E. coli's* acquisition of iron (Dertz, Xu, Stintzi, & Raymond, 2006). As iron is an important growth limiting nutrient for *E. coli* (Dertz et al., 2006), a potential pathway for *E. coli* reduction could be through resource competition for free iron brought about from the siderophores (high-affinity iron-chelating compounds) that white-rot fungi can excrete.

Bacteriophagous Fungi

Fungi can be found in all ecosystems and as such, many fungal species have evolved an ability to utilize a diverse array of substrates for nutrients. There is a large body of research devoted to the study of predatory fungi. Annette Hervey's 1947 paper, "A Survey of 500 Basidiomycetes for Antibacterial Activity," presented the results of testing 508 unique species of Basidiomycetes fungi against *Staphylococcus aureus* and *E. coli* growth rates. Hervey and her fellow researchers found that "about 55 per cent of the 508 isolations of Basidiomycetes investigated by streak method showed antibacterial activity against *S. aureus* or *S. aureus* and *E. coli* together" (Hervey, 1947, p. 502). Hervey's paper is illuminating both through the amount of fungi tested as well as the date that it was published, its age helps show that such research has been valued and pursued for a substantial amount of time. Laboratory studies that show the efficacy of specific fungal strains to consume bacteria form the theoretical groundwork for supporting the use of fungi in bioremediation projects that are combating bacterial contamination.

Some fungi have gone so far as to evolve snare like traps in their hyphae that 'lasso' passing nematodes. Once a nematode is ensnared, the enzymatic excretions from

the mycelium dissolve the nematode and allow the hyphae to absorb the nutrients (Barron & Thorn, 1986). It is these external 'digestive' enzymes that help fungi to function as useful bioremediation tools. The ability to find and utilize a range of diverse food sources has equipped fungi with enzymes that through extracellular digestion, are capable of degrading a diverse array of bacteria and carbon containing compounds, with many of these bacteria and compounds being labeled as harmful by the EPA (Environmental Protection Agency) (Pointing, 2001).

Mycoremediation

Mycoremediation is the process of remediating environmentally degraded systems through the use of beneficial fungi (Singh, 2006; Stamets, 2005). Typically, in mycoremediation, fungal mycelium is used to filter or amend soils and waters, either to strain out pollutants, actively break them down through metabolism and metabolite by-products, or bio-accumulate them for potential manual removal. Combinations of these techniques are even being utilized and further tested in some remediation efforts for radioactively contaminated soils (Bumpus et al., 1985; Eskander et al., 2012; Stamets, 2005). Mycoremediation applications have proven beneficial at breaking down Polycyclic Aromatic Hydrocarbons (PAHs) (Bishnoi et al., 2007), DDT (Bumpus et al., 1985), some antibiotics commonly used in livestock care such as Oxytetracycline (Migliore, Fiori, Spadoni, & Galli, 2012), beer and wine wastewaters (González et al., 2000; Yagüe et al., 2000), clothing dye contaminates (Cripps, Bumpus, & Aust, 1990), and a diverse range of bacteria (Hervey, 1947; Robbins, Hervey, Davidson, Ma, & Robbins, 1945). Fungal mycelium can also be used to increase water retention in soils and to increase soil stabilization in erosion mitigation (Stamets, 2005). Still a relatively

new subset of ecological restoration, mycoremediation has plenty of room to grow as it becomes an important resource for a diversity of restoration objectives. This growth should be aided and directed by hard science; however, many critics feel that mycoremediation's benefits are prone to exaggeration. To best utilize mycoremediation, more research and understanding around its abilities, limitations, and best applications must be continually sought out.

Perceived Benefits

There are several aspects of mycoremediation that are helping to drive its popularity. One of these is its relatively inexpensive cost, both of implementation and maintenance (Stamets, 2005). Whereas many options for *E. coli* reduction in polluted stormwater require expensive components such as re-routing water flows or building large catchment pools (National Research Council, 2008), they fail to be practical for smaller stormwater issues. The smaller scale of mycoremediation makes it both less expensive and simpler to implement. Mushroom cultures can be obtained for free from the wild or ordered on-line from inexpensive vendors. Once obtained, cultures only need inexpensive organic supplements such as grain, wood chips, straw, or other economical organic substrates to grow and flourish. Furthermore, these fungal inoculants can be distributed into the targeted watersheds without heavy machinery (Stamets, 2000).

The low threshold of scientific knowledge or experience needed to both grow and implement fungi for use in mycoremediation projects has helped bolster its appeal. Many online communities and local clubs exist to help people learn to grow mushrooms that can be used for mycoremediation. Species such as *Pleurotus ostreatus* (Oyster) and *Stropharia rugosoannulata* (Wine Cap) are popular in mycoremediation treatments and

samples can be readily obtained for less than $20 from many online vendors. Any interested person needs only to type ‘mushroom growing’ into YouTube or Facebook Groups to find active experts conversing with hobbyist and literally thousands of resources ranging from scientific papers to video tutorials.

The growing enthusiasm for mycoremediation, and the ease in spreading its message and footprint, brings with it a growing need for increased scientific study. Further research will not only illuminate new and novel ways to benefit from mycoremediation but it will also protect the field from false claims or over promised benefits. When the loudest voices for mycoremediation are coming mostly from the same people selling the supplies and treatments, it undercuts its credibility. Expanding current scientific research into more aspects of mycoremediation can help mitigate this and other potentially harmful overstatements of the benefits to mycoremediation.

Mycoremediation as a Subset of Restoration Ecology

> Ecological restoration is the process of assisting the recovery of an ecosystem that has been degraded, damaged or destroyed. (McDonald, Gann, Jonson, & Dixon, 2016)

As a process that uses biotic elements to repair degraded natural ecosystems, mycoremediation is a division of bioremediation that is contextually grounded in the larger, overarching field of restoration ecology. While not as young as mycoremediation, restoration ecology is still a relatively new field (Cairns & Heckman, 1996). The Society for Ecological Restoration (SER), the primary group of professionals leading the restoration ecology community, was founded as recently as 1987. Restoration ecology

works to synthesize ecological theories and use them to achieve set objectives (Cairns & Heckman, 1996). Restoration ecology works to position itself in the broader worldly context of economics, sociology, and politics and as such, it works to find solutions to ecological problems that not only work, but are also effective, efficient, and engaging (Keenleyside, Dudley, Cairns, Hall, & Stolton, 2012). Ideal restoration projects have outcomes that involve stakeholders, are equitable, and recognize humans as part of the ecosystem (Palamar, 2010). Another subset of restoration ecology, phytorestoration, uses selected plant communities to achieve ecological restoration goals. This field has a rich history of scientific study and positive results; an attentive review of the lessons that have been learned from phytorestoration could potentially yield valuable and applicable lessons for the younger field of mycoremediation. Because of mycoremediation's democratizing nature (it is frequently inexpensive and often requires only limited science to implement) it fits well as a specialized tool among the broad selection of tools that make up restoration ecology as a field.

Since mycoremediation is still a developing field, there is a pressing need for further lab and field studies exploring the myriad of potential applications for its use. Promisingly, many studies have yielded encouraging results for the ability of fungal mycelium to break down a diverse array of contaminates and some of these were previously covered in the "Bacteriophagous Fungi and Fungal Assisted Chemical Decomposition" discussions earlier in this work (Hervey, 1947; Stamets et al., 2013).

Bacterial contamination from both point and non-point sources often makes its way into stormwater and as such, it can pose a significant health risk (NRC, 2009). The potential for mycoremediation in the context of stormwater management is under-

researched and many studies have been limited in their scope or have tested applications that are different than they would appear in an actual restoration context. Current literature and experiments concerning stormwater filtration using mushroom mycelium have primarily centered around projects that force simulated stormwater through a mycelium filter under pressure (Stamets et al., 2013; Taylor et al., 2014). These fungal bioreactors have been shown to work well in engineered closed systems and studies have proven the ability of some mushroom mycelium to remove *E. coli* from simulated stormwater when used in such a way (Stamets et al., 2013; Taylor et al., 2014). *Stropharia rugosoannulata* is the species that appears to be the most effective from these studies (Fungi Perfecti, 2013; Taylor & Stamets, 2013). Martinez (2016) looked at the effectiveness of using floating rafts of mushroom mycelium to filter stormwater, simulating conditions much closer to what could be expected in real world settings. The experiment in this study looks to address shortcomings in both the common methods used in pressure forced filtration and in the Martinez (2016) project. While the methods in Martinez's study (floating rafts of mycelium) created more natural conditions, they failed to incorporate previous data that suggests the species of mycelium used, *Pleurotus ostreatus*, is less effective than *Stropharia rugosoannulata* at filtering *E. coli*. Thus, as detailed in the methods chapter, this study used floating rafts of mycelium, but with the species *Stropharia rugosoannulata* instead of *Pleurotus ostreatus*.

Many limitations to mycoremediation are ones that it shares in part with the overall field of restoration ecology as a whole. Restoration ecology, and mycoremediation even more so, are both relatively young fields and as such there are only a limited amount of scientifically rigorous lab and field studies. Advancing the field

of restoration ecology as a whole is often hampered by the size and complexity of the problems that it seeks to address (Luoma, 2006). The issues and concepts that restoration ecology sets out to test or solve require results and experiments that can encompass whole ecosystems and large time spans (Luoma, 2006). This same dilemma also plagues the smaller microcosm of mycoremediation. The ability of fungi to degrade environmental contaminants can depend not only on the species of fungi but sometimes even the specific strain. Additionally, many environmental conditions such as nutrient availability, weather conditions, and age of the fungi can all impact the successfulness of mycoremediation projects (Stamets, 2005; Stamets et al., 2013). Most of these projects are tested in situ instead of laboratories and as such these confounding variables can be hard to account for. Like many restoration ecology projects, the results of mycoremediation can often be invisible to the naked eye and thus success can be hard to measure without clearly outlined goals and pre-established methods for monitoring results. Furthermore, because both restoration ecology and mycoremediation are both goal oriented endeavors, testing for results can be difficult. Understandably, many restoration practitioners are predominately focused on fixing the problem at hand, rather than trying to pinpoint the exact solution. Because often many methods are tried at the same time, with the hope that if you throw a lot of solutions at a problem at least one of them will work; making it difficult to determine the exact cause of the results in successful restoration projects.

Mycoremediation also suffers from another side effect of its young age, some people are prone to overstating its abilities. This overselling can make project managers reluctant to use mycoremediation if they have been oversold in the past. In personal

communications with stormwater technicians working with WSDOT, I heard sentiments of weariness among technicians that the people promoting mycoremediation are often the same people that are selling it.

Assessment of Fungi Perfecti's EPA SBIR Research

Paul Stamets' company, Fungi Perfecti, was awarded an EPA Small Business Innovative Research (SBIR) grant to assess the ability of 'mycofiltration biotechnology' to remove stormwater pathogens (Stamets et al., 2013). In conducting their SBIR experiment, Stamets et al. cultured several species of fungal mycelium, stress tested them, selected the most vigorous species, and then used this mycelium and its substrate as a filter to run *E. coli* inoculated water through it. They then tested the effluent for a reduction in *E. coli* concentrations (Stamets et al., 2013). Fungi Perfecti's EPA SBIR project centered around two research objectives; identify an ideal filter media and fungal species that could withstand stress tests for resiliency, and secondly, to "quantify the effects of mycofilters on bacteria" (Stamets et al., 2013, p. 5).

To fulfill the first objective the researchers grew six different fungal species with five different substrate combinations. A total of 510 of these 'mycofilters' were prepared (including 68 controls). These permutations were then exposed to controlled fluctuations in water saturation and temperatures. Measuring growth and contamination rates, the Fungi Perfecti researchers determined that *Stropharia rugosoannulata* displayed the traits they felt were best suited for a fungal species being used for biofiltration (most notably, vigorous growth, resiliency towards environmental stressors, and a high resistance to cross contamination).

To fulfill the second objective, Fungi Perfecti partnered with Washington State University (WSU) researchers. The researchers tested the water permeability of the mycelial filters using a permeameter cell located on their campus. Permeameter cells are a type of testing equipment that is commonly used in soil sciences to determine the coefficient of permeability for the flow of water through a chosen media. The researchers used the permeameter cell to test the water permeability of the mycelial filters. This was done to establish a comparison of water flow rates between the mycelial filters and current standard BMPs in stormwater management. Once this rate was calculated and determined to be within the range of currently used systems (roughly equivalent to loose sand filtration), the researchers cultured *E. coli* and used it to inoculate synthetic stormwater. Then, under pressures simulating an average storm and an above average storm, they passed this *E. coli* dosed synthetic stormwater through the *Stropharia rugosoannulata* mycelium and substrate combination that was previously selected based on data obtained from the first research objective results.

Fungi Perfecti's results showed that *S. rugosoannulata* fungi outperformed the 7 other fungal species in stressed growth testing and that *S. rugosoannulata* is able to have a measured impact on *E. coli* CFU concentrations. These results from the Fungi Perfecti EPA SBIR provide beneficial data on mycoremediation effects, yet also highlight several ambiguities that are latent in many mycoremediation studies.

In fulfilling their first research objective, stress testing several different fungal species, the Fungi Perfecti researchers provided further support to the limited existing literature as to the abilities of *S. rugosoannulata* mycelium to mitigate *E. coli* levels in stormwater (Stamets, 2005; Stamets et al., 2013; Taylor et al., 2014). It is noteworthy that

their results suggest that *S. rugosoannulata* maybe an overall better choice for mycoremediation projects than *P. ostreatus*. While there are numerous studies showing the bioremediation abilities of *P. ostreatus* (Robbins et al., 1945; Singh, 2006; Thorn & Tsuneda, 1995), none have compared *P. ostreatus* vs *S. rugosoannulata* performance in a stressed environment like the Fungi Perfecti EPA SBIR project. Because of this research *P. ostreatus* and *S. rugosoannulata* were the two fungal species selected for the experimentation detailed later in this paper.

Through both faults in their experimental design and a lack of established lab protocols for this type of experimentation, the second stated research objective to "quantify the effects of mycofilters on bacteria" (Stamets et al., 2013, p. 5), had outcomes that were at times unclear and open to greater interpretation. The researchers came to the conclusion that the *Pleurotus ostreatus* mycofilters removed 100% of the loaded *E. coli* while the 'control' removed only 38-45% of the *E. coli*. While this data seems to make microfiltration the clear choice for *E. coli* removal there are a few flaws with its presentation.

In their experimental design, the Fungi Perfecti researchers added an extra confounding variable that diminishes the power of their results. In their experimental design, the researchers used a control that had a different substrate medium than that of the treatment groups (straw was added to the mycofilter using mycelium but not to the control filter which contained only wood chips). This difference (the presence of straw) was later referenced as a reason for potential false positives and as such, if it has potential impact on the results, it should have been included in the control. An even better design would have maintained the wood only control but also simply included an additional

control with the added straw substrate. Doing so would have added factual support for the claim that the false positives were from the addition of the straw.

A further change in the experimental design that would make the results of the research far more illuminating would be the addition of a control that mimicked a current BMP (Best Management Practice) in stormwater filtration, specifically sand. In the first phase of their research, Stamets et al. conducted permeability testing and concluded that the coefficient of permeability was "roughly equivalent to medium grain sand" (Stamets et al., 2013, p. 10). As such, adding an all sand filter with similar permeability to the mycofilters could yield data that would show if the *E. coli* filtration achieved from the mycofiltration was better or worse than a currently used BMP for stormwater filtration. Mycofiltration can vary depending on the rate and amount of fungal colonization of the substrate (Stamets et al., 2013) and, as such, if a filter made of a non-changing substrate such as sand can have the same *E. coli* filtration rates it should be considered as a potentially more viable option. Thus, the inclusion of an all sand filter could have yielded critical data.

Further lessons can be learned from Fungi Perfecti's EPA SBIR by examining their critique of the Coliscan MF method for *E. coli* enumeration. Coliscan MF is an EPA sanctioned method for evaluating *E. coli* levels in water and is a common tool used to determine water quality and safety in the USA.

Fungi Perfecti's examination of the inherent faults of the Coliscan MF *E. coli* enumeration method is arguably one of the most valuable contribution resulting from the whole research experiment.

Stormwater Management

> Urban storm water runoff affects water quality, water quantity, habitat and biological resources, public health, and the aesthetic appearance of urban waterways. (EPA Preliminary Data Summary of Urban Storm Water Best Management Practices, 2015).

Stormwater management is a growing scientific field that can encompass aspects of engineering, hydrology, meteorology, biology, and chemistry. In personal communications with Washington Department of Transportation (WSDOT) stormwater technicians, they stated that stormwater management is a young discipline. That much like wetlands management was 20 years ago, it is a developing field, still carving out its niche. Because of its newness, stormwater management is only just starting to gain the recognition and resources that it warrants. With the increasing impacts of climate change being felt worldwide, scientists are preparing for a changing climate that will likely bring with it increased flooding events (either due to increased severity of some storms or due to storms being redistributed to climates not adapted to process the increased water inundation) (Daniel, Jacobs, Douglas, Mallick, & Hayhoe, 2014; Matthew & Hammill, 2009). Additionally, society's increasing urban development is both taking over space that was previously occupied by natural water systems (wetlands, streams, etc.) and is covering its surfaces in ways that prevent the absorption of water and intensify its rate of flow (Daniel et al., 2014; NRC, 2009). To be well equipped to seek out leverage points for mitigation and to understand possible new control methods, it is important to at least briefly examine the history and current scope of stormwater management.

Appropriate stormwater management is one of the cornerstones of a healthy watershed. Stormwater is generated from precipitation that moves overland, draining into adjacent waterways. As this stormwater gathers and travels over the ground, it can build

up pollutants that are then transported into other waterways such as rivers, estuaries, and beaches. These pollutants can include bacterial, environmental, and chemical contaminants and can come from a wide variety of sources such as construction sites, roadways, and other 'urban development' (NRC, 2009). Because both the type and source of stormwater pollutants can vary so much, its effective mitigation requires a diverse suite of solutions carried out by a cooperation of varied stakeholders (Prosser, Morison, & Coleman, 2015).

With the increased urbanization of America, the amount of paved, impervious surfaces has also increased (Daniel et al., 2014; NRC, 2009). This in turn has altered the rate, quantity, and quality of rain water that flows into connected waterways (Daniel et al., 2014; NRC, 2009). Thus, as the US population has grown, so have the impacts of stormwater, both ecologically and politically. Because of stormwater's dispersed nature, its management often involves multiple stakeholders that must work collaboratively to successfully mitigate its negative effects (Prosser et al., 2015; Shandas, 2015).

Contaminates entering waterways can be separated into two different classes: point-source pollution and non-point-source. Stormwater's sweeping impact allows it to amalgamate pollutants from a large array of sources making it the largest vector for non-point-source pollution (NRC, 2009). Pollution or sediment debris that was otherwise dispersed across the landscape is funneled into connected waterways. This stormwater can aggregate roadway pollutants which can include oil and heavy metals that build up from exhaust emissions (NRC, 2009). When stormwater accumulates near construction sites it can easily take on disturbed soil and sediment, adding suspended particulates into waterways, a process that is known to negatively impact aquatic life such as benthic

invertebrates and salmon (NRC, 2009). In addition to this, stormwater can also negatively impact waterways as a force multiplier for point-source pollution when it overwhelms existing infrastructure and causes these systems to overflow and bypass their established treatment regimes. Many cities have wastewater systems that are susceptible to overflowing when inundated with stormwater. Even projects that are deemed as successes by their creators are prone to failures. In 2011, Portland Oregon completed a 1.6-billion-dollar project to prevent stormwater overflows on one of their main sewage pipe ways. While this project reduced stormwater overflows from an average of 50 to an average of 4 each winter, the overflows that still happen create health and economic threats. Even if these massive overhauls were able to fix overflow problems entirely, many cities lack the funds and/or initiatives for such large-scale projects.

The Clean Water Act (CWA) was primarily established to combat point source pollution, primarily through regulation of industrial and municipal sewage discharges (NRC, 2009). Point source pollution can be easier to regulate due to the presence of discrete physical locations and identifiable sources such as pipe outfalls. It was not until 1987 that Congress made stormwater control part of the National Pollutant Discharge Elimination System (NPDES), a program under the scope of the CWA. Initially, these early mitigation efforts were centered around moving stormwater away from urban locations as quickly as possible to prevent flooding and damage to building foundations (NRC, 2009). In 1991 the EPA began to promote stormwater management that took on a "whole watershed approach;" one that sought out stakeholder involvement, was "place-based," and focused more on restoring measureable metrics that mimicked the natural local hydro-cycle (EPA, 2005). Throughout the 1990s, the EPA expanded the scope of

the NPDES and since then stormwater management has become more robust and nuanced, now making up the bulk of the NPDES's work and efforts (NRC, 2009). Currently, there are roughly half a million NPDES stormwater management permits in issue while there are fewer than 100,000 non-stormwater (wastewater) permittees under the NPDES program (NRC, 2009).

Current Best Management Practices

Given that stormwater can collect in a variety of different environments it makes sense that its proper management would be tailored to specific sites (Walsh et al., 2016). BMPs, best management practices, is the main collective term for prescribed treatment options (some literature uses SCMs, stormwater control methods, though this is less common in the US). The physical aspects of mitigation sites can limit the size and options for BMPs while budgets and stakeholder interests can provide further limiting constraints. Unlike municipal drinking water, stormwater is rarely chemically treated and instead BMPs focus on physical treatment, often diverting the flow, either through a type of filtration (such as sand or plant assemblages) or by finding other options to slow or disperse its force and impact (Horner et al., 2001; Mallin, Turner, McIver, Toothman, & Freeman, 2016). Through design elements such as catchment cisterns, bioswales, and retention ponds, slowing or stopping stormwater is a popular management technique (Horner et al., 2001). Causing the flow of storm water to either slow or stop allows for more dissolved solids to drop out of solution and this has been shown to be one of the most significant ways to reduce *E. coli* concentrations (Mallin et al., 2016; Chen & Adams, 2006). Additionally, slowing the speed of moving stormwater can help reduce its potential for eroding landscapes or flooding infrastructure (Horner et al., 2001). These

techniques can be limited by available land size and budgets. These landscape level solutions can be hard to modify if changes in the area such as altered or accelerated urban growth cause flow rates larger than anticipated (Horner et al., 2001). When these systems overflow, water can move through the system without enough time to settle dissolved solids or filter out biological contaminates (Chen & Adams, 2006). Furthermore, there is some concern that the *E. coli* can build up on the floor of the pond or water catchment system (Schillinger & Gannon, 1985). This can then create a risk that concentrations of *E. coli* could be stirred up or otherwise exposed in a future stormwater event (Schillinger & Gannon, 1985).

***Escherichia coli (E. coli*)**

When testing environmental conditions for harmful pathogens it would be nearly impossible to test for every potentially harmful bacterial contaminate. Because of this, it is useful to select indicators to test for, ones whose presence can be statistically linked to a diverse spectrum of concerning contaminates (Edberg, Rice, Karlin, & Allen, 2000). *Escherichia coli* (*E. coli*) is a common pollutant in both urban and rural stormwater discharge and as an organism that is present in all mammal fecal matter its presence in water confirms that mammal waste has entered the water and as such, it serves as a good indicator for the bulk of other potential pathogens (Edberg et al., 2000; van Elsas et al., 2011). *E. coli* can exist in both commensal and pathogenic strains, with some strains passively living in animal guts while other strains impart serious illness (Edberg et al., 2000; van Elsas, Semenov, Costa, & Trevors, 2011). Discovered in 1885 by the then 27-year-old German-Austrian pediatrician Theodor Escherich, *E. coli* was originally named *B. coli commune* until formally changed in 1958 to *Escherichia coli* to honor its

discoverer (National Center for Biotechnology Information, 2015). Since the 1890s, *E. coli* has been a common test indicator for overall water quality (Edberg & Edberg, 1988). Testing for each specific pathogen of concern instead of using *E. coli* as an indicator organism would be labor and cost prohibitive due to the large numbers of all possible waterborne pathogens (additionally, some have yet to be identified) (Edberg et al., 2000). *E. coli* testing has largely replaced testing for fecal coliform in water quality sampling for several reasons. Firstly, it is relatively cheap and easy to test. Secondly, it is a reliable indicator for fecal coliform presence (Environmental Protection Agency, 2015; Mallin et al., 2016).

E. coli in our Waterways

The first major U.S. law to directly tackle water pollution issues was The Federal Water Pollution Control Act of 1948. This helped usher in public awareness for the issue and led to a slew of amendments in 1972 and the resulting law came to be known as the Clean Water Act. The new amendments included giving the EPA the authority to implement control programs for water pollution and to set the basic regulatory structure for permitting and monitoring. Currently, the most recent version of The Federal Water Pollution Control Act was issued in 2002 and is still commonly referred to as the Clean Water Act.

To protect the public health, the Clean Water Act (Environmental Protection Agency, 2005) has set acceptable safe levels of *E. coli* concentration for bodies of water used for recreation or sustenance. According to the EPA National Water Quality Inventory: Report to Congress (2017), 46.1% of U.S. rivers and streams are in poor condition, the main causes for these poor conditions are attributed to pathogens

(bacteria), sediment, and nutrients, in that order; with 23% of the waterways exceeding *E. coli* levels set for safe human health. The American Society of Civil Engineers puts out a report card every four years for all aspects of America's infrastructure and its 2017 findings give wastewater infrastructure a D+ grade, a score that hopefully conveys how large the problem is (American Society of Civil Engineers, 2017). Large flooding events, like the ones predicted to accompany global warming, are linked to increased concentrations of *E. coli* pollution in waterways (ASCE, 2017). Large floods can often cause events such as sewage pipeline overflows and holding tank breaches. As these flooding events increase in frequency and severity, so too will the issues of *E. coli* pollution.

Methods

Conceptual Overview

With mycoremediation practices growing in popularity while the current scientific literature lags behind, more direct experiments must be conducted to ensure that the restoration ecology community is pursuing the most successful and cost-effective solutions.

One way to scientifically support the use of mycoremediation in *E. coli* polluted waterways is to provide documented reductions of *E. coli* concentration levels when contaminated water is exposed to fungal mycelium. Several studies have attempted this with varying degrees of success (Taylor et al., 2014; Taylor & Stamets, 2014) and as seen from close examination of the Fungi Perfecti EPA SBRI project (Fungi Perfecti, 2013), these projects can have enough confounding variables to make it difficult to establish causal mechanisms for reported reductions in *E. coli*. For example, Fungi Perfecti EPA SBRI project's fungal bio-filters reduced *E. coli* populations they did not establish if it was from purely mechanical filtration or if it was the exodigestive enzymes that were reducing the *E. coli* concentrations. Because confounding variables are difficult to control for when performing such tests *in situ*, laboratory experiments are often used to provide a smaller microcosm to test for selected effects. Creating a lab experiment that removes many of the confounding variables allows for greater analysis of potential causations for the observed results. Instead of asking the question "can white-rot fungi remove *E. coli,*" we ask a more specific question, "can white-rot fungi produce a measurable reduction of *E. coli* populations in a static aqueous environment;" the problem is framed in a way that narrows the scope enough to eliminate many variables.

To answer this more specific question, and to closely examine the impacts of white-rot fungal excretions on *E. coli.,* a laboratory experiment was conducted. Measuring for potential changes in *E. coli* concentrations, fungal mycelium was suspended in 500 mL of DI water, the water was then inoculated with a measured *E. coli* dose and then sampled over a time gradient to observe for population growth effects.

Outline of Methods

Two laboratory experiments were conducted to gather data on the efficiency of floating *Stropharia rugosoannulata* and *Pleurotus ostreatus* mycelium to reduce *E. coli* concentrations in *E. coli* inoculated water. A preliminary experiment, Experiment A, was carried out to hone the experimental design of the full experiment, Experiment B.

In experiment A, a total of 40 treatment groups were created; all treatments had 500 mL of deionized and autoclaved water in a plastic beaker, twenty treatment groups contained mushroom mycelium growing on sterilized *Alnus spp.* sawdust wrapped in cotton cloth, ten treatment groups contained sterilized *Alnus spp.* sawdust wrapped in cotton cloth but with no fungi present, and ten treatment groups contained only the 500 mL of deionized water. Of the twenty fungal treatment groups, ten contained a controlled sample of *P. ostreatus* and ten contained an *S. rugosoannulata* fungal sample. *S. rugosoannulata* and *P. ostreatus* mycelium were cultured in 100 mm glass petri dishes, filled with alder sawdust (10 for each species) using standard sterile lab techniques (Stamets, 2000).

A single *E. coli* colony was isolated from a streak plate (Sanders, 2012) and was cultured in 250 mL of Tryptic Soy Broth (TSB) at 37 degrees for 24 hours. This solution

was then used to inoculate all 40 test groups. The *S. rugosoannulata* and *P. ostreatus* mycelium, along with the un-inoculated pucks with only wooden sawdust and cotton wrapping, were all removed from their petri dishes where they were grown and/or shaped and then floated on top of the *E. coli* inoculated water (1 'puck' per beaker; 10 beakers received only the *E. coli* inoculation and no fungal treatment). All 40 groups were subsequently tested on a time metered basis to measure for changes in viable *E. coli* concentrations. Samples were taken at initial inoculation (0 hours), 1 hour, 6 hours, 12 hours, and 24 hours. The samples were serially diluted and the dilutions were tested for *E. coli* concentration levels using the spread plate technique. The design intention was to use simple regression modeling to study the effects of the mycelium exposure on the *E. coli* concentration levels between the two different mushroom species and between the treatments and the control non-treatment groups. However, due to larger amounts of viable *E. coli* colonies and issues with Coliscan MF enumeration, numerical quantification was not possible. While this trial experiment did not yield statically viable data, it did yield useful information that was used to shape the design of Experiment B.

Based on visual performance observations from Experiment A, Experiment B only used one fungal species, *S. rugosoannulata*. While still too dense to enumerate, Experiment A showed visibly fewer colonies of *E. coli* in the *S. rugosoannulata* treatment group results as compared to the *P. ostreatus* treatment results.

The water only control in Experiment A was originally included as a control for the *E. coli* enumeration; in the event that the fungal test samples had removed all of the *E. coli*, water only samples that still tested positive for *E. coli* would have proven that the enumeration technique was working.

Based on this knowledge gained from Experiment A, Experiment B consisted of 16 treatment groups, 8 with *S. rugosoannulata* fungal samples and 8 with sterilized *Alnus spp.* sawdust wrapped in cotton cloth with no fungi present. All treatments had 500 mL of deionized and autoclaved water in a plastic beaker and were sampled at 0 hours, 24 hours, 48 hours, and 72 hours. These samples were plated on agar within 12 hours of sampling, then incubated at 37 degrees Celsius for approximately 24 hours. Once cultured, viable colony counts were performed.

Detailed Methods

Creating the fungal liquid cultures

Two liters of liquid culture media was created and used to grow the mycelium that the experimental pucks were inoculated with. In a 4 L Erlenmeyer beaker, 2 L of distilled water, 40 grams of dextrose, and 40 grams of Light Malt Extract were combined and warmed over a hot plate. After stirring till all ingredients dissolved while avoiding cooking to the point of caramelizing the dextrose, the contents were split evenly into four glass canning jars (1 liter size). These jars were then sealed with a homemade lid that was modified according to specifications from McCoy (2013) to include an air filter and self-healing injection port. All four jars then had their tops covered with foil to help keep the filter from absorbing too much water. Next the canning jars were sterilized at 15 psi for 20 minutes in an All American brand pressure cooker (model type: 921) and allowed to cool for 24 hours (McCoy, 2013).

One 10 mL liquid culture of *Stropharia rugosoannulata* and one 10 mL liquid culture of *Pleurotus ostreatus* were purchased from www.themyceliumemporium.com for use as starter cultures. (Both of these strains are commonly available from a variety of

different online suppliers.) Working in front of a HEPA filter flow hood and using sterile lab techniques (Stamets, 2000), two jars received 5 mL of *S. rugosoannulata* liquid culture and 2 jars revived 5 mL of *P. ostreatus* liquid culture following the inoculation procedures outlined by McCoy (2013). All four jars were agitated daily and allowed roughly one week at room temperatures to colonize before their use (McCoy, 2013). While one canning jar of colonized liquid culture would be enough for each sample, back up jars were created for each fungal species in case of contamination, though none were actually needed or used.

Culturing the fungal mycelium pucks (Experiment A)

After allowing the liquid fungal cultures to fully colonize, *Alnus spp.* (Alder) sawdust obtained from compressed fuel pellets was soaked in distilled water to allow them to expand from pellets to sawdust and to hydrate. Using sawdust from compressed wood pellets allowed for a uniform particle size and the semi-pasteurization that the sawdust undergoes from the compression helped add an extra step of precaution to combat potential outside contamination. Once decompressed and soaked to "field-capacity" (Stamets, 2000), the *Alnus spp.* sawdust was then wrapped in cotton cheese cloth and packed into glass petri dishes to form 'pucks' of substrate for mushroom colonization (30 grams of sawdust for each 'puck'). The cotton cloth was used to add structure and to help keep the pucks intact.

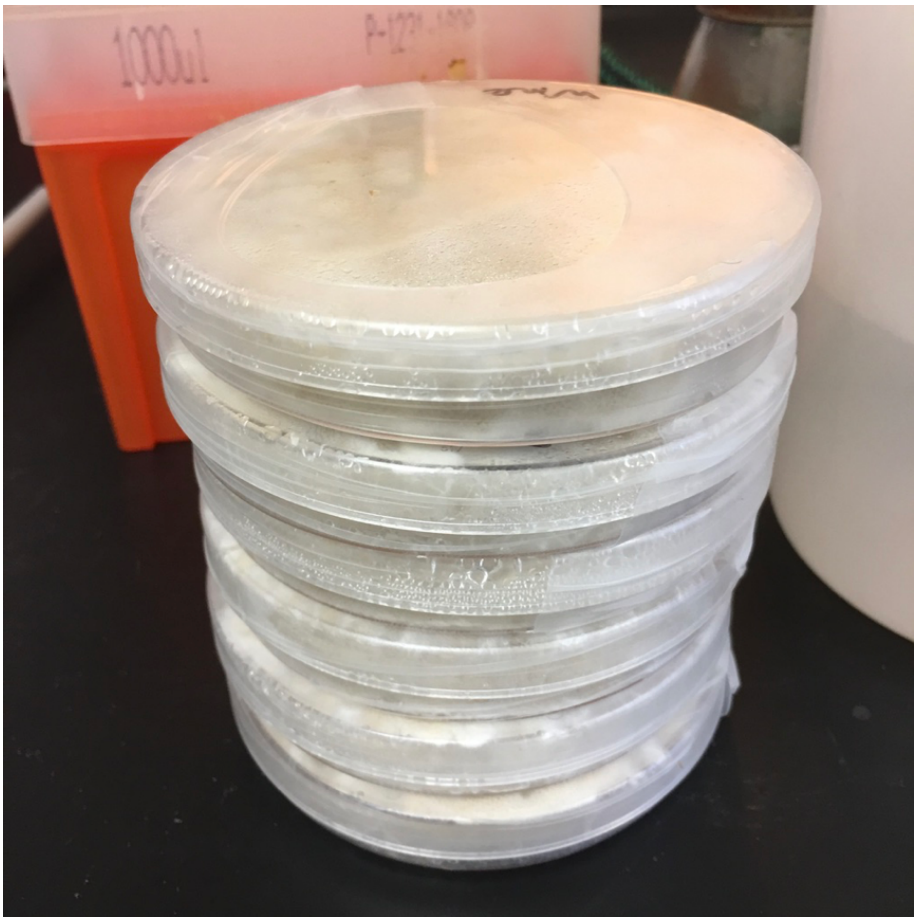

Figure 1: S. Rugosoannulata growing on wooden sawdust packed into petri dishes.

45 of these uniform substrate pucks were created and then wrapped in aluminum foil and sterilized at 15 psi for 1.5 hours in an All American brand pressure cooker (model type: 921). After cooling overnight, the pressure cooker was opened in front of a HEPA filtered flow hood and standard sterile lab protocol for mushroom culturing was used to inoculate 30 substrate pucks with liquid mushroom mycelium cultures (Stamets, 2000). 15 substrate pucks were each inoculated with 2 mL of *S. rugosoannulata* liquid culture mycelium from the culture jars created earlier. 15 substrate pucks were each inoculated with 2 mL of *P. ostreatus* liquid culture mycelium. 15 pucks were left un-inoculated for

use as controls. While only ten pucks from each species of mushroom and ten for controls were needed for the experiment, fifteen of each were cultured or formed to allow for any possible contamination. Though none occurred in the fungal samples, several of the un-inoculated controls did, making it useful to have backups. The pucks were incubated at approximately 26° F for 2 weeks to allow for complete colonization of the substrate by the mycelium (Stamets, 2000).

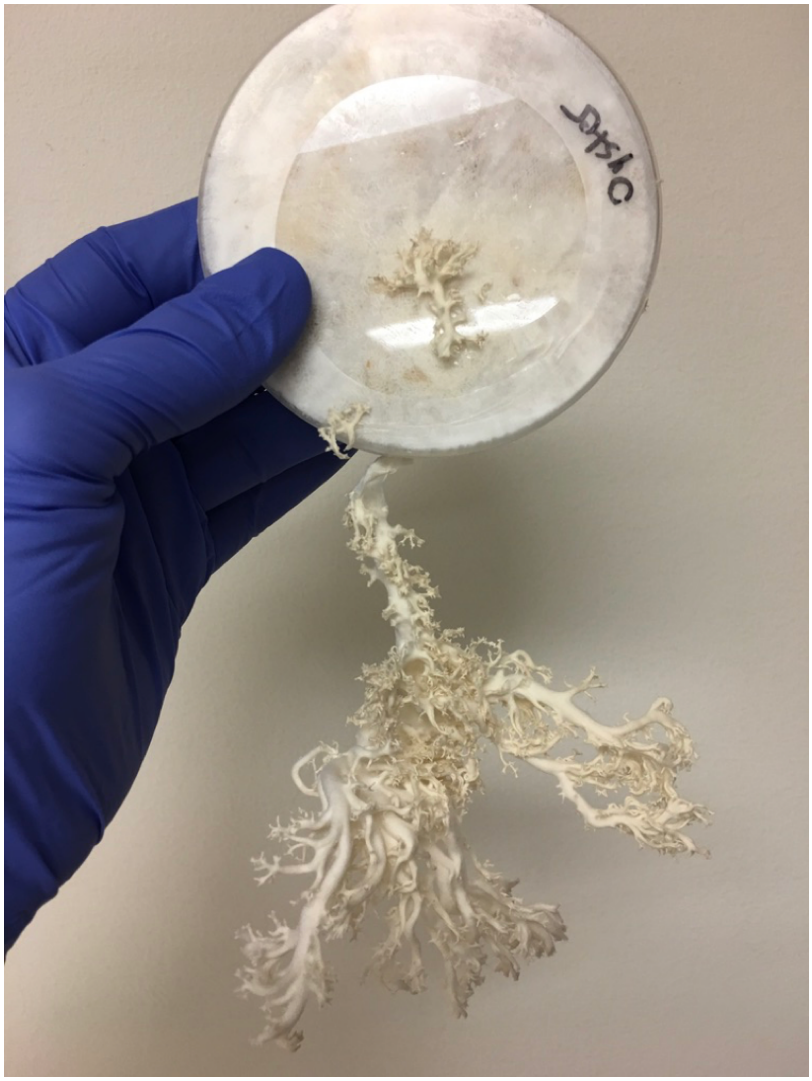

Figure 2: P. ostreatus growing vigorously enough to push through the petri dish lid and a Parafilm™ wrapping to begin fruiting.

Culturing the fungal mycelium pucks (Experiment B)

The methods for culturing the fungal puck in Experiment B varied slightly from those of Experiment A. Growing the fungal mycelium in closed petri dishes was effective but slow due to limited air flow. For Experiment B, a bag of alder wood substrate already colonized by *S. rugosoannulata* was donated by Fungi Perfect. This substrate was then weight into 30 gram units, packed into petri dishes lined with organic cotton cloth and allowed one week to re-colonize and grow denser.

Culturing the *E. coli*

After allowing the fungal samples to fully colonize, 250mL of Tryptic Soy Broth (TSB) was prepared following U.S. Food and Drug Administration guidelines (US FDA, 2015). A pure *E. coli* culture was donated by The Evergreen State College Phage Lab (Strain: W3110) and was used to create a streak plate (Sanders, 2012).

Figure 3: A streak plate of E. coli colonies. A single colony was isolated and used to inoculate the TSB in the pictured beakers.

The streak plate was created to isolate single *E. coli* colonies. After incubating for 24 hours at 37 degrees Celsius, a single *E. coli* colony was used to inoculate the TSB (Tryptic Soy Broth). After incubating the inoculated broth for 24 hours at 37 degrees Celsius, an *E. coli* colony count was performed on the TSB using the spread plate method following US FDA guidelines (US FDA, 2015). This procedure was done directly preceding Experiment A and again two more times directly preceding each trial in

Experiment B. Spread plate results indicated that the TSB contained approximately 3,000 CFU/mL after each culturing.

Figure 4 Culturing the E. coli solution at 37° Celsius in the SteadyState 7 incubating orbital shaker.

Experiment A

Twenty 1,000 mL beakers were filled with 500 mL of DI water and were then covered with foil lids and autoclaved for 25 minutes. Next, the beakers were inoculated

with 1000 uL of the *E. coli* and TSB solution. Five beakers each received one mycelium puck colonized with *S. rugosoannulata,* five beakers each received one *P. ostreatus* mycelium puck, five beakers each received an un-inoculated puck with only the alder sawdust and cotton cloth, and five beakers received no treatment.

Figure 5: Beakers caped with foil and filled with 500 mL of DI water, ready to be sterilized in the autoclave.

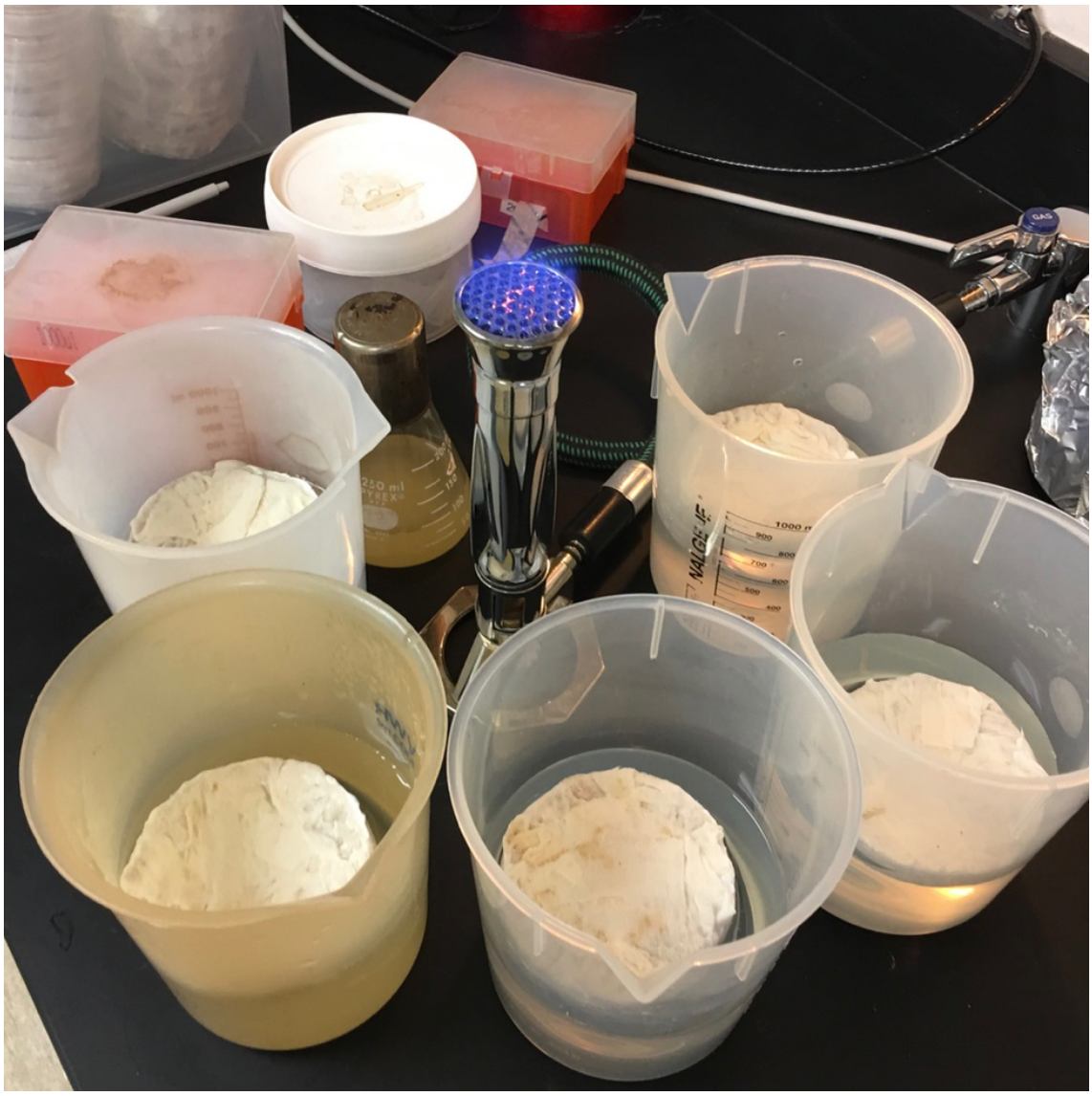

Figure 6: E. coli and S. Rugosoannulata samples being added to the beakers, following aseptic techniques.

100 uL samples were taken from each of the 20 beakers over a period of 24 hours. Samples were taken at the time of inoculation and after 1, 6, 12, and 24 hours. Logarithmic serial dilutions were then performed on each of the samples and these were aseptically plated onto petri dishes with Tryptic Soy Agar, using the spread plate method (Sanders, 2012; US FDA, 2015).

All *E. coli* inoculations and subsequent samplings were done using sterile lab techniques to avoid potential contamination (Stamets, 2013; US FDA, 2015). Petri dishes were incubated at 37 degrees Celsius for 24 hours.

Figure 7: Beakers with E. coli and fungal samples along with control beakers – the experiment is underway.

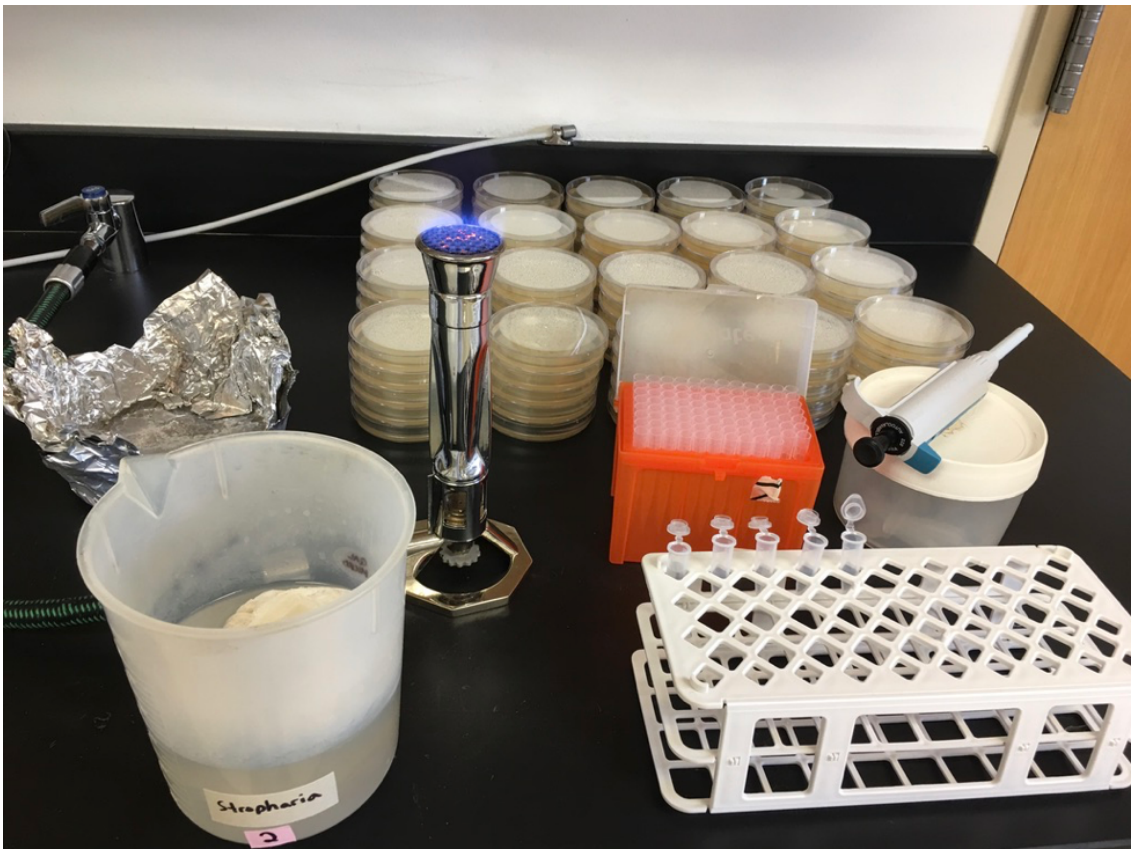

Figure 8: Aseptically conducting sampling on one of the S. Rugosoannulata test beakers. Petri dishes in the background are ready to be spread with the serially diluted samples.

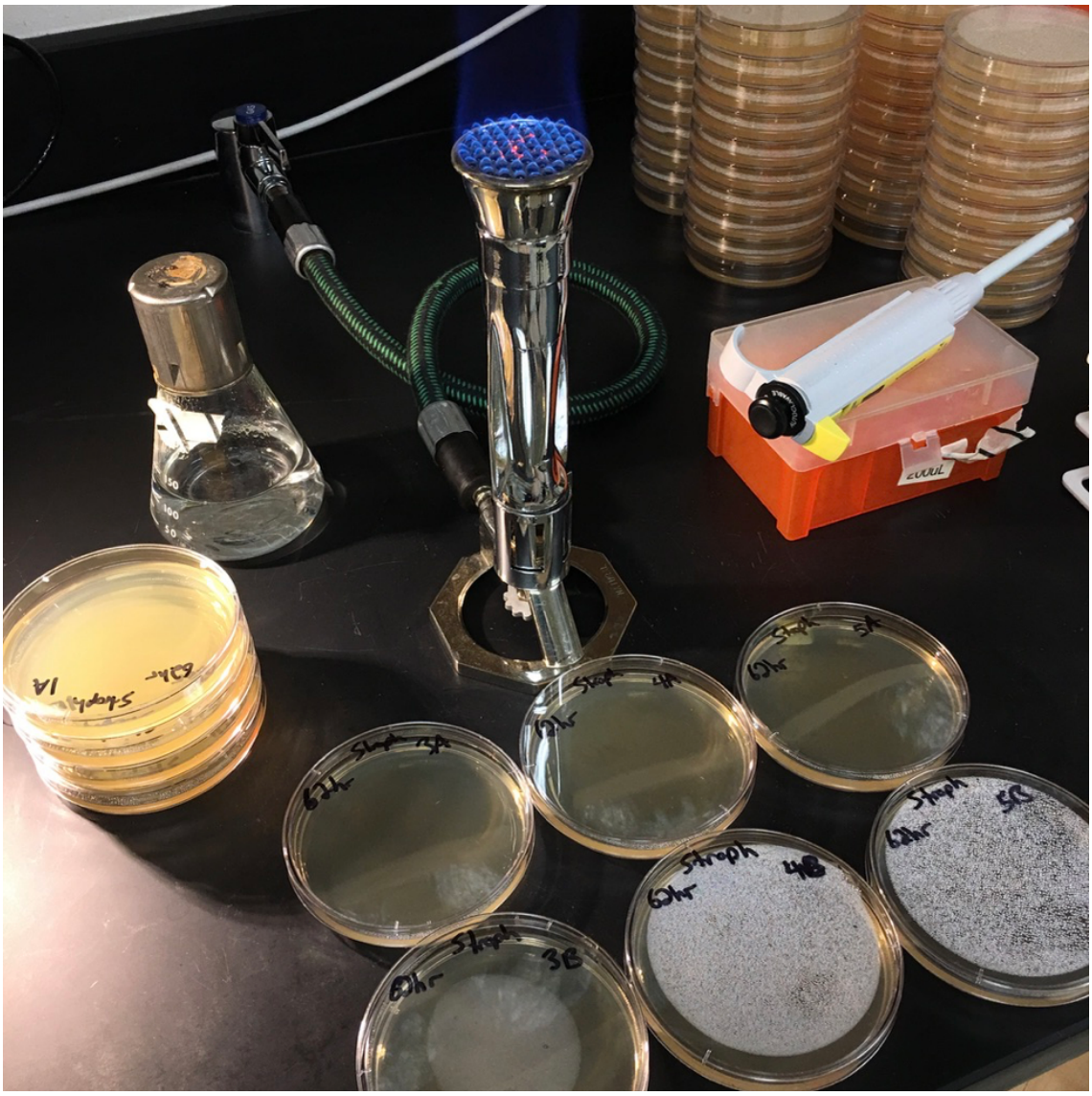

Figure 9: Water samples from one of the S. Rugosoannulata treatments being plated, using aseptic techniques.

The intended objective was to use simple regression modeling to study the effects of the mycelium exposure on *E. coli* concentration levels between the two different mushroom species and between the treatments and the control non-treatment groups, however, all colonies remained too dense to count even after 6 logarithmic serial dilutions so visual analysis was the only method available to evaluate the results.

Experiment B

Sixteen 1,000 mL beakers were filled with 500 mL of DI water and where then covered with foil lids and autoclaved for 25 minutes. Next, the beakers were inoculated with 100 uL of the *E. coli* and TSB solution. (This was a reduction in concentration from Experiment B to make enumeration clearer.) Eight beakers each received one mycelium puck colonized with *S. rugosoannulata* and eight beakers each received an un-inoculated puck with only the alder sawdust and cotton cloth. 100 uL samples were taken from each of the sixteen beakers at the time of inoculation and after 24, 48, and 72 hours. Logarithmic serial dilutions were then performed on each of the samples and these were aseptically plated onto petri dishes with Tryptic Soy Agar, this time using the drop plate method instead of the spread plate method (Herigstad, Hamilton, & Heersink, 2001; Hoben & Somasegaran, 1982). As outlined in papers by both Herigstad et al. and Hoben & Somasegaran, the spread plate method is as statistically valid as the spread plate method, yet it saves a great amount of time and resources (Herigstad et al., 2001; Hoben & Somasegaran, 1982). Using the drop plate method, it is possible to culture 6 samples on one agar petri dish while the spread plate method would take 6 separate petri dishes (See Figures 10 and 11 for visuals). All *E. coli* inoculations and subsequent samplings were done using sterile lab techniques to avoid potential contamination (Stamets, 2013; US FDA, 2015). Petri dishes were incubated at 37 degrees Celsius for 24 hours.

After incubation, viable Colony Forming Units (CFUs) were counted using an illuminated plate counter. Simple regression modeling was used to study the effects of the mycelium exposure on *E. coli* concentration levels between the treatment and control groups.

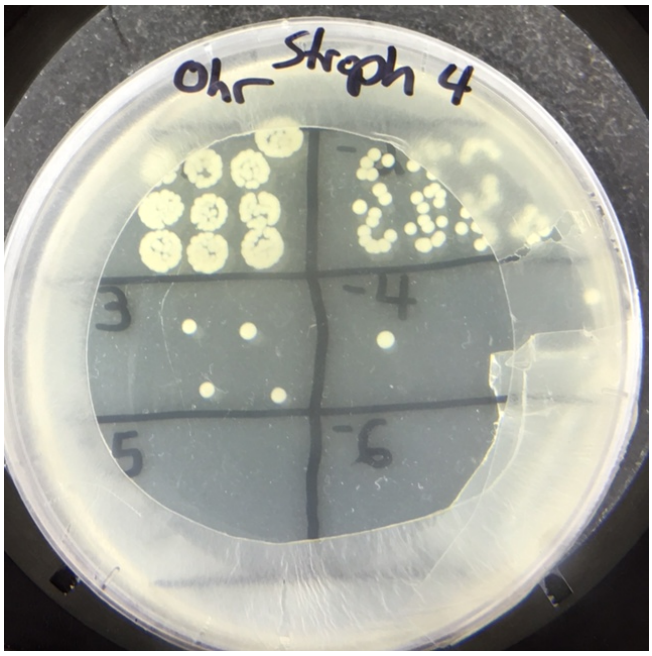

Figure 10: Example of a drop plate sample. Note that you can fit 6 serial dilutions on one petri dish instead of the 6 dishes it would take using the spread plate method, thus saving time and resources.

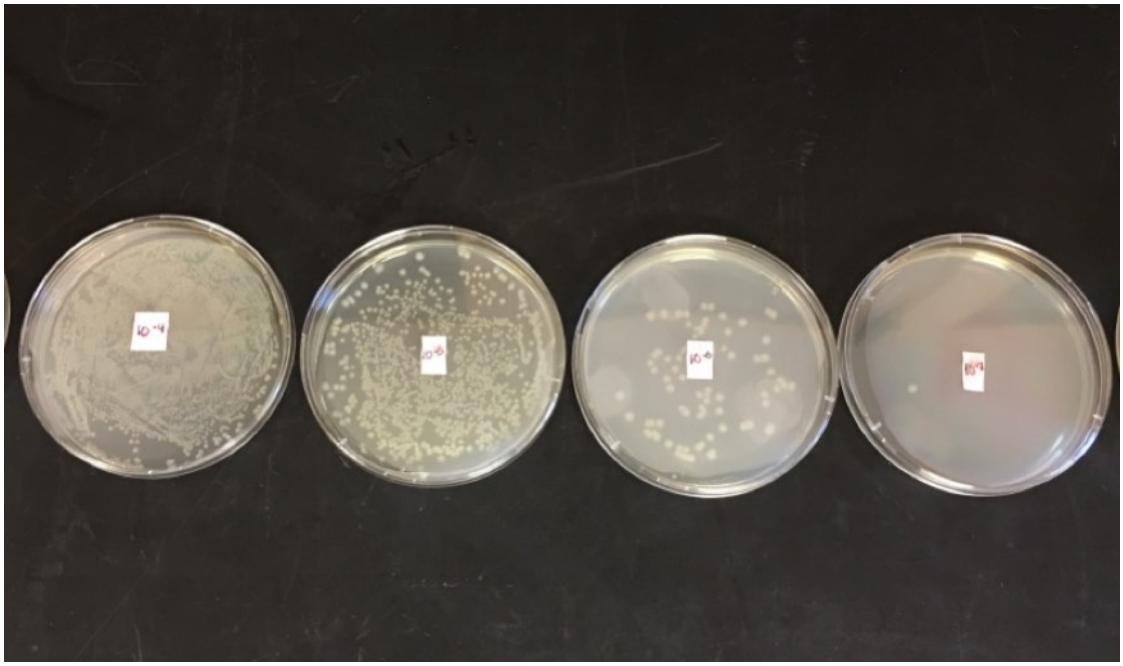

Figure 11: An example of 4 serial dilutions plated using the spread plate method.

Results

Experiment A

Experiment A yielded only descriptive results. On average, the plated samples had too many CFU (colony forming units) to count (Sanders, 2012). From visual analysis, it was clear that both the *S. rugosoannulata* and *P. ostreatus* fungal treatments had reduced the *E. coli* concentrations to a larger degree than the two controls. It was also evident that the *S. rugosoannulata* treatments outperformed the *P. ostreatus* treatments. Based on this knowledge, Experiment B was conducted using only the *S. rugosoannulata* and a lower concentration of *E. coli* CFU for the initial inoculation. Figure 12 shows spread plates from 4 sampling events for an *S. rugosoannulata* treatment and a water control. Looking at the top row, an *S. rugosoannulata* treatment, a reduction of *E. coli* CFU (the yellowish specks) can been seen as one looks from left to right (moving from the first sample at 0 hours, to the 12-hour plating, the 24-hour plating, and the 48-hour plating). The bottom row of plates, a water-only control, fails to demonstrate a reduction in *E. coli* concentration, with all plates containing too many *E. coli* CFU to enumerate.

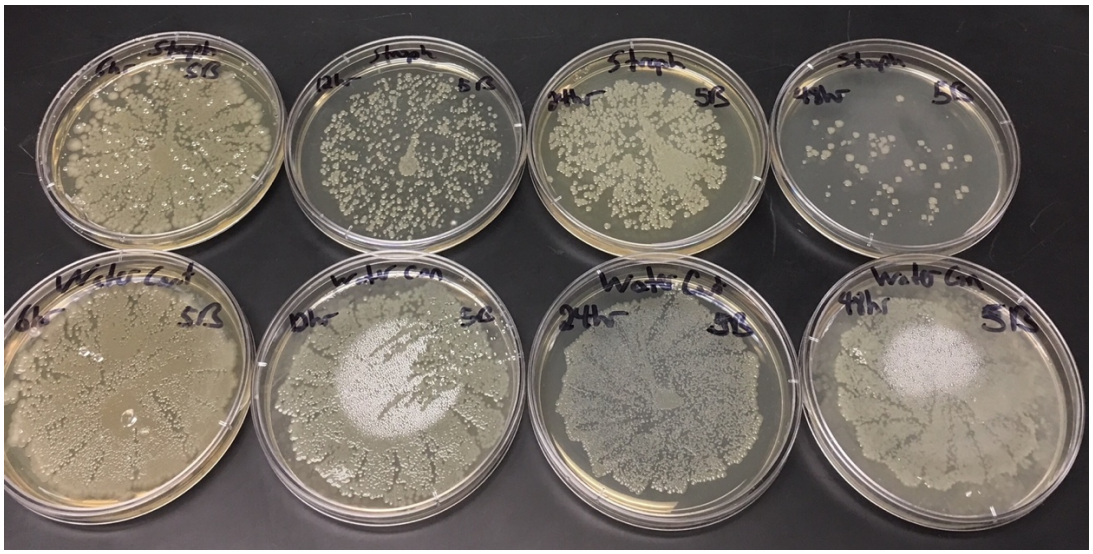

Figure 12: Plated petri dish samples from Experiment A. The top row is from one S. rugosoannulata treatment, the bottom row is from one water control. Each column represents a different hour of sampling; from left to right - 6 hours after E. coli inoculation, 12 hours, 24 hours, & 48 hours.

A further noteworthy observation from Experiment A was a noticeable reduction in tannins leached from the alder wood substrate. Tannins leaching from industrial plants such as paper mills and dyeing factories are large sources of water pollutions (Cripps et al., 1990; Yagüe, et al., 2000). All of the wood-only controls yielded water that was tawny to amber colored while the fungal treatments containing the same wood substrate did not discolor their waters. These visual observations mirror similar visual results seen in Fungi Perfecti's research conducted on *S. rugosoannulata* (Taylor & Stamets, 2014) and corroborate that white-rot fungi can be beneficial for xenobiotic tannin remediation.

Experiment B

Experiment B was performed two times: Trial 1 and Trial 2. Each trial contained 4 replicates of the *S. rugosoannulata* treatment and 4 replicates of the control treatment. Enumerating the exact CFU in the inoculation solution was difficult because samples require 24 hours to incubate before counting, thus only resulting in a CFU number for the

E. coli that were in the solution 24 hours in the past and not their immediate numbers. Therefore, data from the two trials was not simply combined; each trial started with a different *E. coli* inoculating solution and the differences in concentration between these two was enough that it could disrupt pattern analysis.

Trial 1

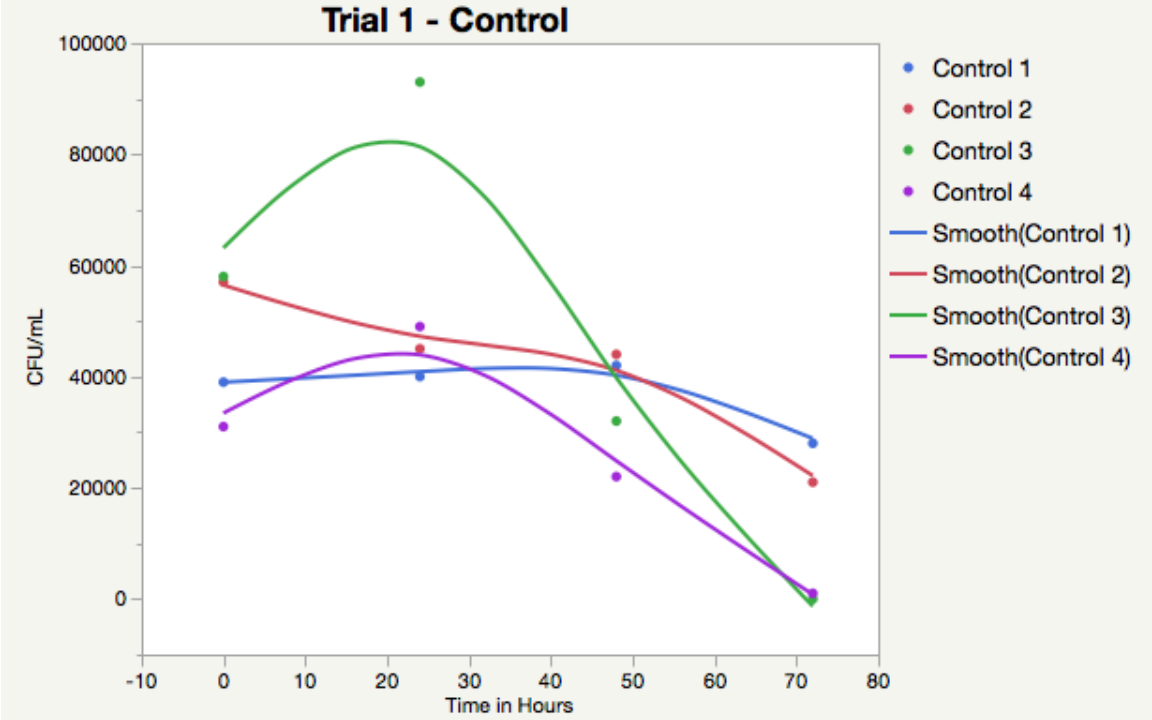

Figure 13: Bivariate display of E. coli Colony Forming Units per mL of water in the Trial 1 Control treatment replicates, plotted over time in hours. A smoothing spline has been calculated for each control replicate and matches the color of its corresponding plot points.

Trial 1 contained 4 replicates of the control treatment and 4 replicates of the *S. rugosoannulata* treatment; Figure 13 displays the CFU/mL sampling results for the 4 control replicates, plotted against time in hours. In these control replicates, half showed an increase in *E. coli* CFUs at the 24 hour sampling and all replicates showed a moderate reduction of *E. coli* over time (Figure 13). At the final 72-hour sampling event, 2 of the 4 controls reached 0 CFU/mL of *E. coli*.

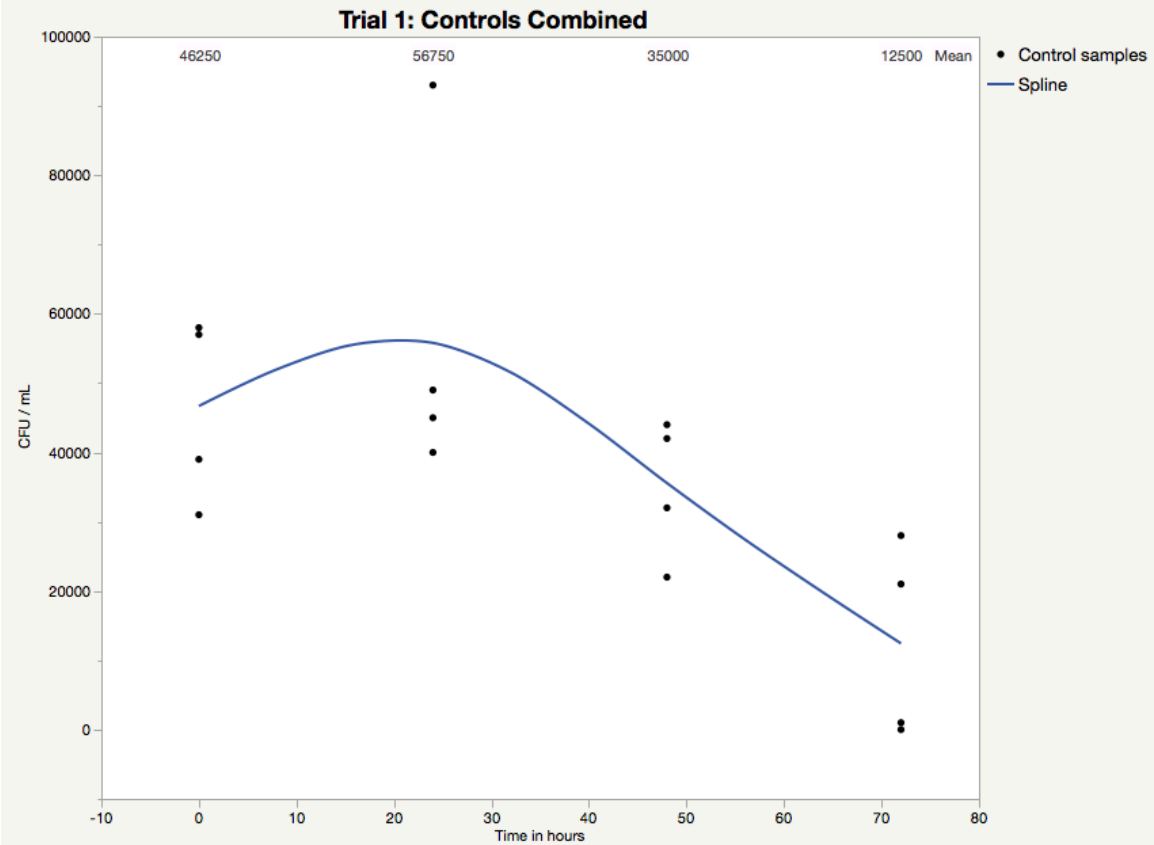

Figure 14: E. coli Colony Forming Units per mL of water, plotted over time in hours for all Trial 1 Control sampling measurements.

The combined controls showed an overall increase in *E. coli* CFU/mL of water at the 24-hour sampling event followed by a gradual decline over time (Figure 14).

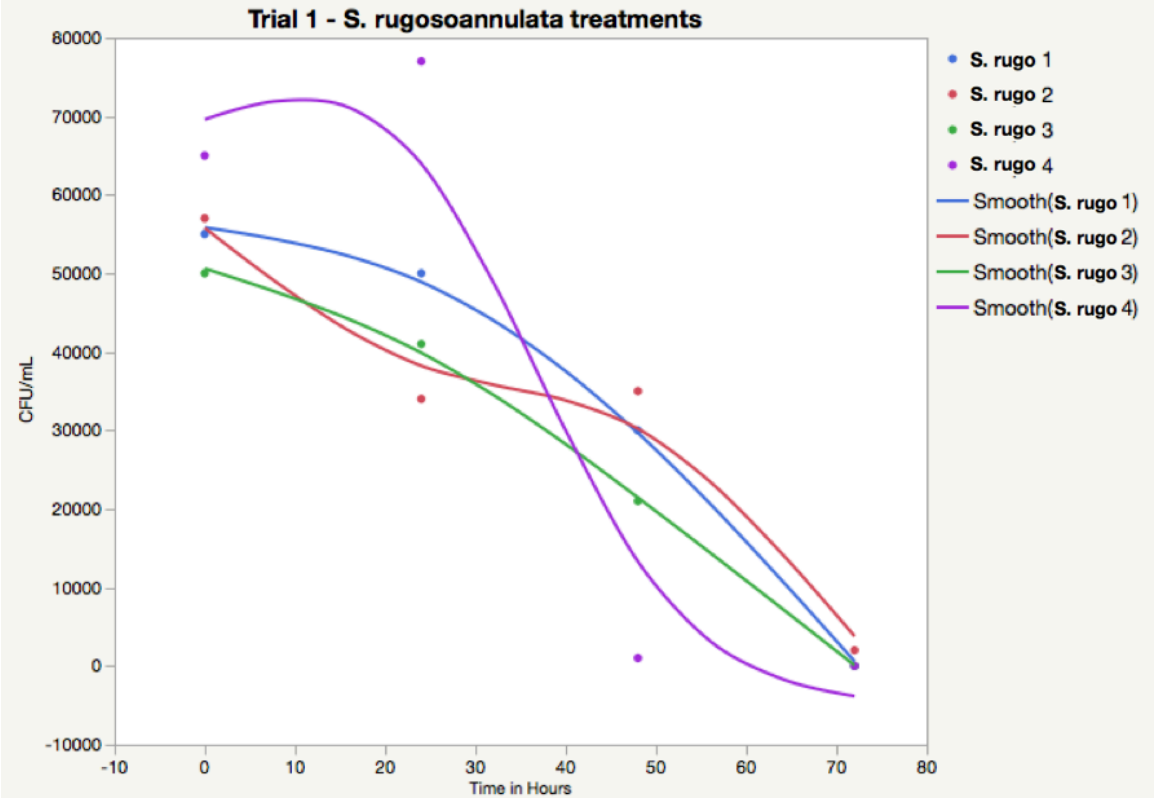

Figure 15: Bivariate display of the number of E. coli Colony Forming Units per mL of water in the Trial 1 S. rugosoannulata treatment replicates, plotted over time in hours.

S. rugosoannulata fungal treatment replicates showed rapid declines in *E. coli* concentrations over time. In the Trial 1 *S. rugosoannulata* treatments the *E. coli* did not have the same increase in CFU around the 24-hour sampling that was observed in the control group trials (Figure 15). At the final 72-hour sampling event, 3 of the 4 controls reached 0 CFU/mL of *E. coli*.

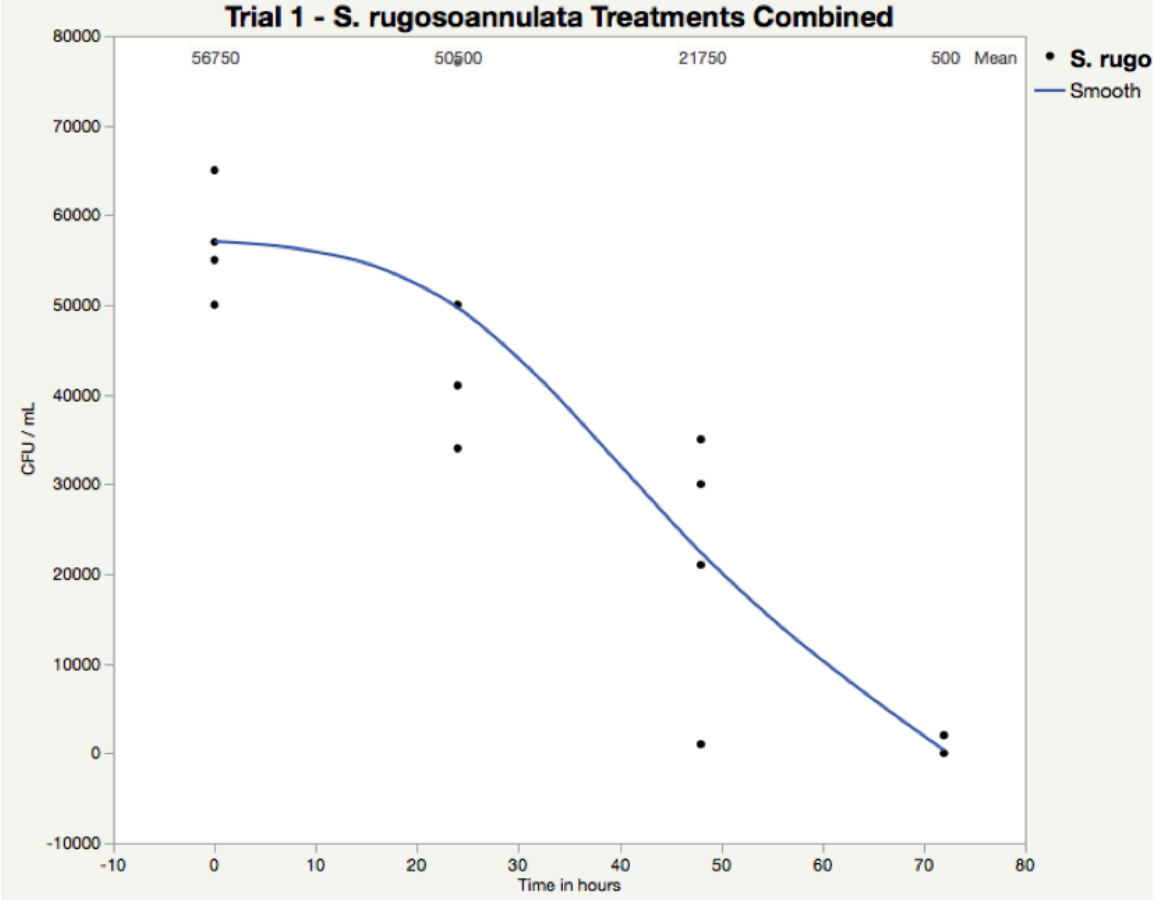

Figure 16: The number of E. coli Colony Forming Units per mL of water, plotted over time in hours for all Trial 1 S. rugosoannulata sampling measurements.

Figure 16 displays the sampling results from the Trial 1 fungal treatment replicates with the smoothing spline averaging all of the data points. The averaged spline shows the lack of an increase in *E. coli* concentrations at the 24-hour sampling event even more clearly. The averaged spline also shows a faster rate of decrease for the *S. rugosoannulata* Trial 1 treatments than seen in the control trials.

Trial 2

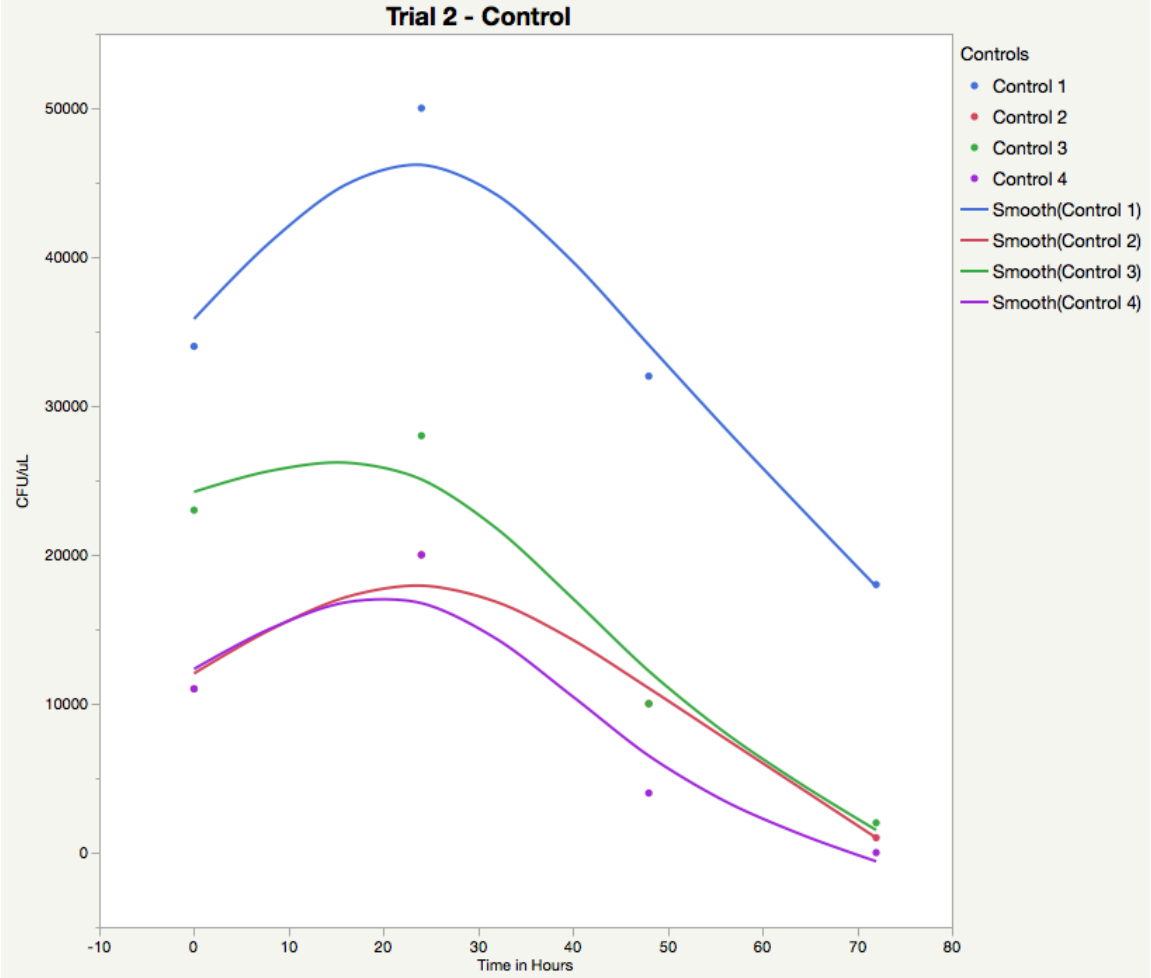

Figure 17: Bivariate display of the number of E. coli Colony Forming Units per mL of water in the Trial 2 Control treatment replicates, plotted over time in hours.

Control Trial 2 shows an initial increase in *E. coli* CFUs at the 24-hour sampling event followed by a gradual reduction in CFUs. These results follow the same pattern of *E. coli* concentrations as observed in Control Trial 1 (Figure 13), an initial *E. coli* colony increase, followed by a gradual decline. At the final Control Trial 2 sampling event, only 1 of the 4 controls reached 0 CFU/mL of *E. coli*.

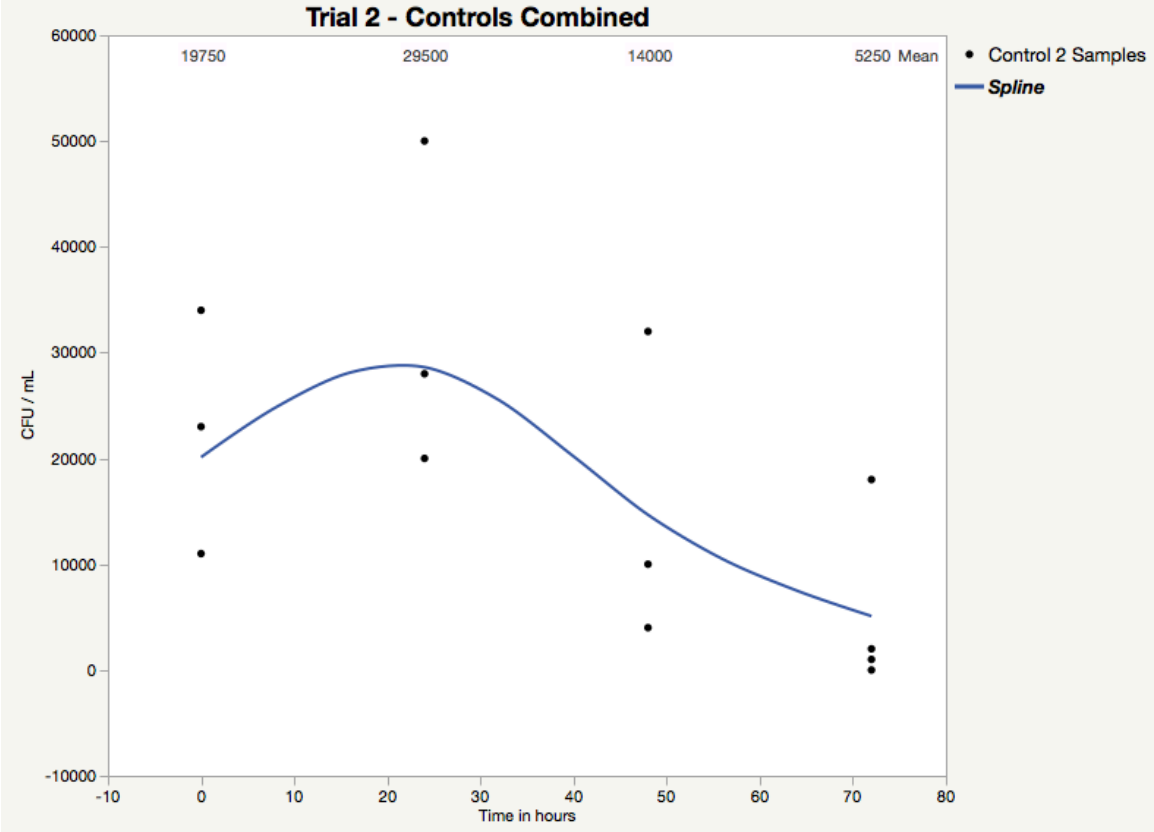

Figure 18: The number of E. coli Colony Forming Units per mL of water, plotted over time in hours for all Trial 2 Control sampling measurements.

Figure 18 displays the sampling results from the Trial 2 controls. A smoothing spline averaging all of the data points once again shows the overall trend of an increase in *E. coli* CFU/mL of water at the 24-hour sampling event followed by a gradual decline, just as was seen in the Trial 1 results for the control treatments. With both control trials showing a similar increase in *E. coli* concentrations at the 24-hour sampling, it can be seen as noteworthy that such a spike does not occur in the *S. rugosoannulata* treatment results.

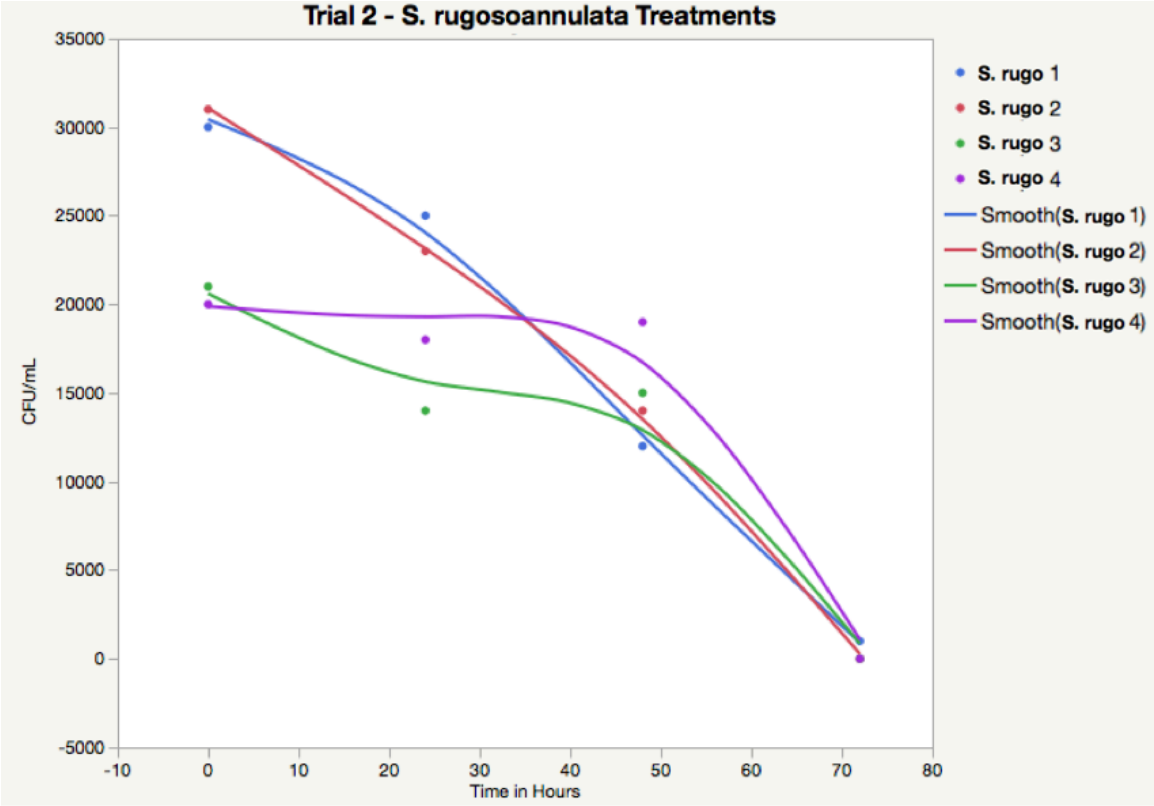

Figure 19: Bivariate display of the number of E. coli Colony Forming Units per mL of water in the Trial 1 S. rugosoannulata treatment replicates, plotted over time in hours.

Trial 2 *S. rugosoannulata* treatment replicates showed a steep decrease in *E. coli* CFUs over time. None of the treatment replicates showed the initial increase in colony numbers that was observed in both Control Trial 1 and Control Trial 2. Furthermore, 3 of the 4 *S. rugosoannulata* treatments in Trial 2 resulted in 0 CFUs at the final sampling (the 4th treatment was reduced to 1,000 CFU, the smallest countable amount). The Trial 2 treatment results show that the *S. rugosoannulata* fungal treatment replicates reduced *E. coli* CFUs at a greater rate of speed and to a greater degree of total elimination than in either of the two control trials.

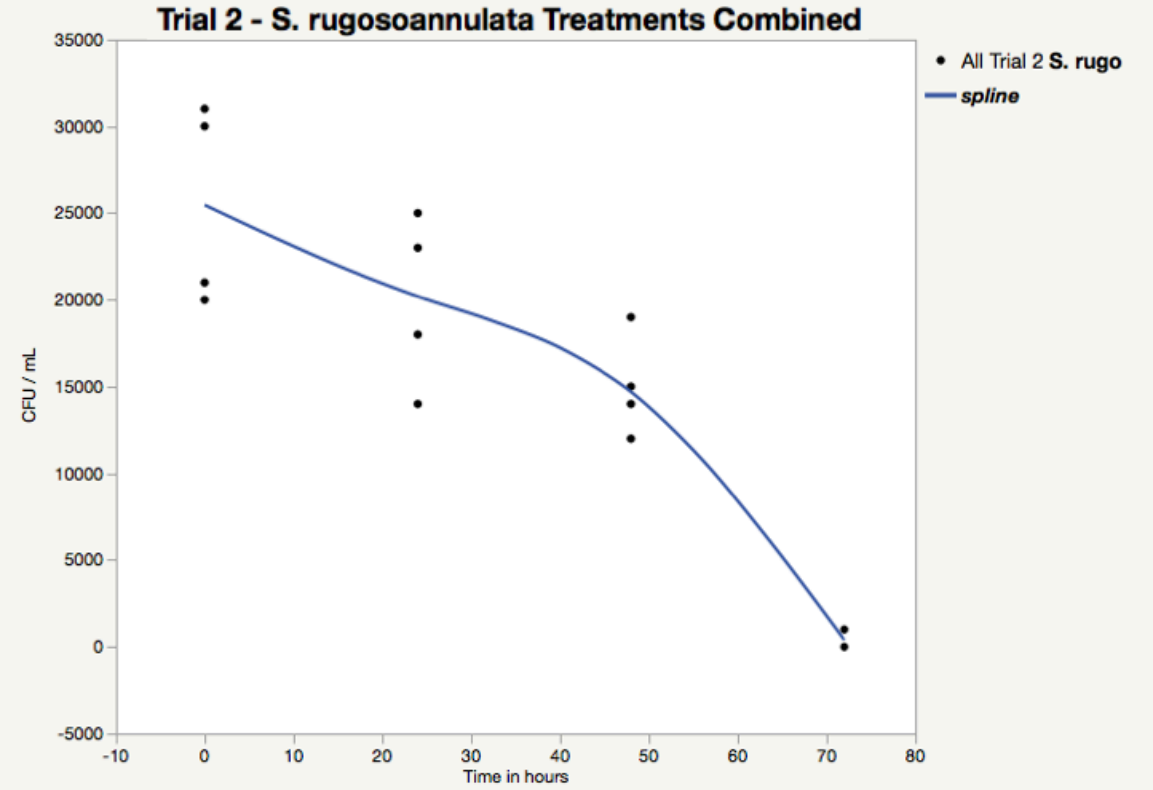

Figure 20: The number of E. coli Colony Forming Units per mL of water, plotted over time in hours for all Trial 2 S. rugosoannulata sampling measurements.

Figure 20 displays the sampling results from the Trial 1 fungal treatments with the smoothing spline averaging all of the data points. Like in Trial 1, the spline highlights the lack of an increase in *E. coli* concentrations at the 24-hour sampling event and shows a faster rate of decrease than found in the control data.

Regression Line modeling

Both the Control and the *S. rugosoannulata* treatments in Trial 1 and Trial 2 showed an overall decrease of *E. coli* concentrations over time. However, the *S. rugosoannulata* treatment replicates showed a substantially greater decrease in *E. coli* concentrations over the observed time. This difference is illustrated by examining the influence of time on *E. coli* concentrations for each of the treatment replicates using

linear regression modeling. For the control treatment, Trial 1 yeilded an R^2 value of .40 and Trial 2 yeilded an R^2 value of .24 (Figure 21). Simply put, elapsed time from inoculation explained 40% and 24%, respectively, of the variation in *E. coli* concentrations, suggesting minimal effect of the control environment on the reduction in *E. coli* concentrations.

In contrast, for the *S. rugosoannulata* treatment, Trial 1 yielded an R^2 value of .78 for Trial 1 and an R^2 value of .81 for Trial 2 (Figure 22). That is, elapsed time following inoculation explained 78% and 81% of the variation in *E. coli* concentration, respectively. This data suggests substantial effect of the *S. rugosoannulata* treatment on the decrease in *E. coli* concentrations.

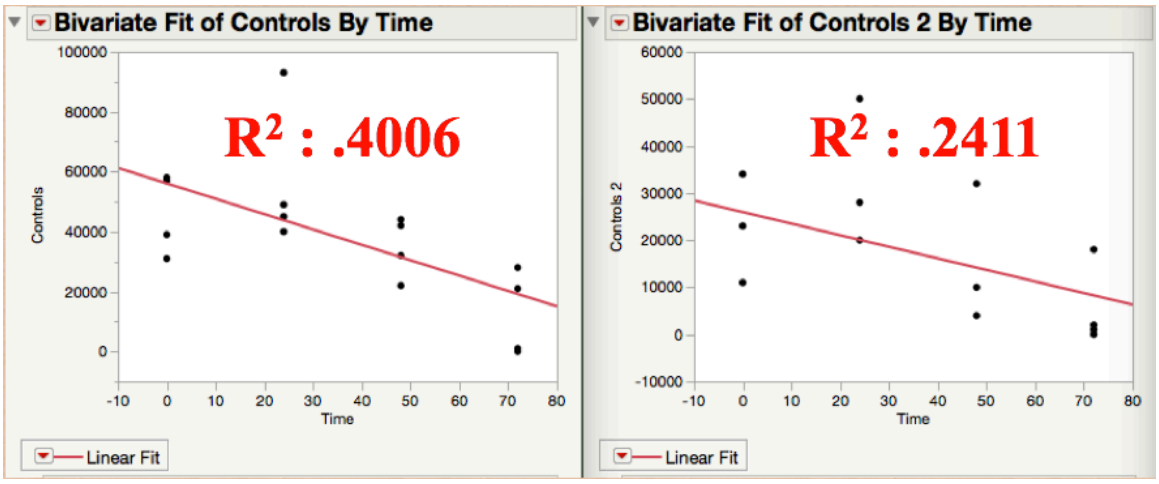

Figure 21: Graphs of linear regression lines for Control Trial 1 and Control Trial 2, with their R^2 results, ($\alpha = .05$).

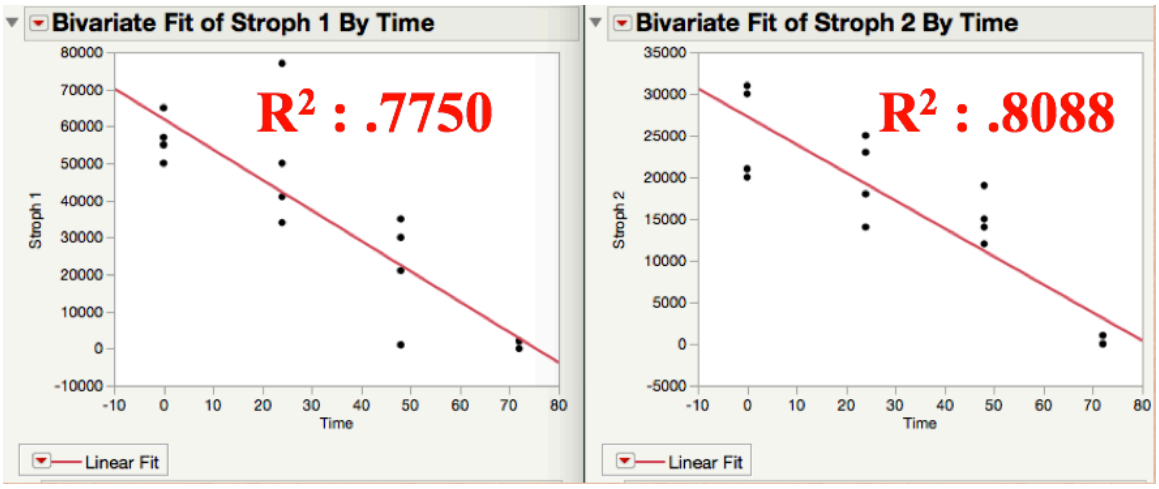

Figure 22: Graphs of linear regression lines for S. rugosoannulata Trial 1 and Trial 2 treatments, with their R^2 results, ($\alpha = .05$).

Discussion

Data gathered from this experiment shows promising results for the use of *S. rugosoannulata* for *E. coli* CFU reduction in an aqueous environment. The inclusion of *S. rugosoannulata* mycelium in the treatment groups resulted in reduced *E. coli* growth and accelerated the *E. coli's* decay when compared to the control groups. Such results add support for the continued use of *S. rugosoannulata* in mycoremediation projects. Expanding this test into a larger, in situ, experiment would give the experiment more power. With the results of this study suggesting a measurable impact of *S. rugosoannulata* on *E. coli* CFU concentrations, an in-situ experiment could see if these same results are measurable in an actual mycoremediation site. Continuing this experiment with a larger number of treatments and controls would give the statistics more weight and descriptive power.

Conclusions

Overall

Evolutionary adaptations have allowed the fungal kingdom to flourish in a diverse array of habitats while utilizing a broad spectrum of nutrient sources. These adaptations have primed the fungal kingdom to be well suited for bio-restoration work. The extracellular chemical digestion of woody material that *S. rugosoannulata* and *P. ostreatus* naturally facilitate can be exploited for targeted reduction of many xenobiotic pollutants, *E. coli* being a prime example.

Experiment Derived Conclusions

Data from Experiment B shows *E. coli* inoculated water exposed to a control of wood sawdust tended to have an increase in CFUs, 24 hours after inoculation. This uptick in growth was not present at the same sampling time in the *E. coli* inoculated water exposed to the *S. rugosoannulata* fungal sample. Through preventing this initial increase of *E. coli* growth in the first 24 hours, the treatment groups exposed to *S. rugosoannulata* mycelium reduced the *E. coli* concentrations at a slightly faster rate.

Future Research

There are a variety of modifications and additions that could be built onto the framework of the experiment outlined here. With more time and resources, additional trials could be run allowing for even more robust statistical analysis.

Furthermore, a second dosing event could be performed after the first dose of *E. coli* has died off. There is potential that priming the system, conditioning the mycelium to

the target pollutant, could increase the ability of the fungal mycelium to degrade the targeted pollutant (McCoy, 2013). A second inoculation of *E. coli* and subsequent sampling could measure for any potential changes in rates of *E. coli* death that might be brought on from priming the system with the first round of testing.

Continuing to add onto this experiment, samples of the treatment and control waters could be taken to measure concentration levels of peroxidases such as lignin peroxidase (LiP) and manganese peroxidase (MnP). Finding a measured increase in peroxidase levels in the treatment and not the controls would help quantify the amount of potential extracellular bacterial digestion the fungi would be performing.

Performing this experiment on a larger scale, such as a 3m x 3m garden plot could yield data that closely represents outcomes in real-world systems. Such an experiment would also open up other variables for testing. Testing for bacterial splash-back resilience in soils treated with mycoremediation techniques highlight an additional novel way that fungi might be used to mitigate bacterial pollution.

From the observational data outlined in Experiment A, a difference in performance rates existed between the *S. rugosoannulata* and *P. ostreatus* mycelium treatments. As such, this experiment could be built upon to test different unproven fungal species for their abilities to mitigate *E. coli*. Many species of fungi, especial the saprophytic 'white rot' fungi, warrant further testing. Using this experiment as an inexpensive test that also uses few resources could allow for such future research. If a uniform test for such characteristics was developed, it could allow for *in situ* testing of native fungi collected directly from the targeted remediation site. Some research has put

forth that fungi cultivated from samples native to the remediation site outperform those that aren't cultured from native sources (Kardol, Bezemer, & van der Putten, 2006; Wubs, van der Putten, Bosch, & Bezemer, 2016).

Broader Impacts

While the benefits of fungal mycelium in reducing *E. coli* concentrations continue to be difficult to statistically support, there are other benefits conferred from implementing mycoremediation projects into contaminated watersheds that can extend beyond measured biological contaminate levels. Studies have shown that engaging local shareholders can have benefits that extend beyond the direct project they were involved in (Suding, 2011). By simply engaging in programs to benefit their local environments, active participants can become better aware of how their own actions can impact waterways. As such, mycoremediation projects that engage their participants and foster a sense of community and engagement could confer benefits outside the scope of those traditionally measured. Moderate statistical advantages, combined with minimal costs and extra community engagement could be enough to tip the scales in favor of particularly well planned and implemented mycoremediation projects.

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