Development of Fungal Bioreactors for Water Related Treatment and Disinfection Applications

Russell Blake Umstead

Thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science in
Biological Systems Engineering

Zachary M. Easton, Chair
Brian D. Badgley
David J. Sample

June 27, 2016
Blacksburg, Virginia

Keywords: (fungi, fungal bioreactor, *E. coli*, antimicrobial compounds, water treatment, disinfection, filtration, solid-state fermentation)

Development of Fungal Bioreactors for Water Related Treatment and Disinfection Applications

Russell Blake Umstead

Abstract

Wastewater, recycled irrigation water, and agricultural runoff can contain high levels of pathogenic bacteria, which pose a threat to human and ecosystem health. The use of a bioreactor containing mycelial mats of filamentous fungi is a novel treatment technology that incorporates physical, biological, and biochemical processes to remove bacterial pathogens from influent water. Although a relatively new concept, fungal bioreactors have demonstrated the ability to dramatically reduce fecal coliform bacteria in water, but no studies have attempted to explicitly identify the bacterial pathogen removal mechanisms exhibited by the fungi.

This study evaluated several different species of fungi for use in fungal bioreactor systems and aimed to identify the modes of action responsible for the removal of bacterial pathogens. The species evaluated were *Daedaleopsis confragosa*, *Pleurotus eryngii*, and *Piptoporus betulinus*. Experimental results showed that all species of fungi assessed were capable of removing *E. coli* in a synthetic water solution. Significant concentrations of hydrogen peroxide, an antiseptic, were produced by all species of fungi evaluated. The fungal bioreactors containing *P. eryngii* produced the highest concentrations of hydrogen peroxide, generating a maximum concentration of 30.5 mg/l or 0.896 mM. This maximum value exceeds reported minimum concentrations required to demonstrate bacteriostatic and bactericidal effects when continually applied, providing evidence that a major bacterial removal mode of action is the production of antimicrobial compounds. In addition to its promising application to improve water quality,

fungal bioreactors are a low cost and passive treatment technology. The development a hyper-functional system could be a have a substantial impact on the use of recycled irrigation water and on the water/wastewater treatment industry, for both municipal and agricultural wastewater.

I dedicate this work to my family, friends, and colleagues, whose support has helped me persevere

Acknowledgements

I would like to acknowledge the contributions of my colleagues in the effort to complete this work, specifically members of the Easton research group and members of the Badgley lab. I would like to thank my advisor Dr. Zach Easton for his continued support throughout the development and execution of this project. Zach, I appreciate you giving me the unique opportunity to design my own thesis research project and the completion of this study would not have been possible without your encouragement. I also would like to thank Dr. Badgley not only for serving on my committee, but for providing technical support as well. Emily and Andrew, I appreciate your guidance and help with the statistical analysis. Josh, your technical support was instrumental in carrying out this study.

I would like to give a special thanks to Dr. Randy Grayson of the Virginia Tech George Washington Carver program, your support and mentorship has been invaluable. I owe a large part of my success to you and Dr. Saied Mostaghimi, in part for having faith in me. To Dr. Carmine Balascio and Dr. Palaniappa Krishnan, I appreciate your mentorship throughout my years as an undergraduate at the University of Delaware. Dr. Anastasia Chirnside, I valued your mentorship during my time as an undergraduate researcher in your lab, your help and guidance with my thesis project was immense. To Dr. Sample, in addition to thanking you for serving on my committee I would like to thank you for all of your help during my application process to Virginia Tech. To the members of the Biological Systems Engineering Department, in particular Dr. Wolfe, Dumitru, Ling, and Laura, thank you for all of your support. Finally I would like to acknowledge the contributions of my family as well as friends, in particular my mom and brother, who inspired to me to persevere. Thank you.

Table of Contents

List of Figures

List of Tables

1. Introduction

1.1 Bacterial Pollution of Water, Removal Methods, and Technologies

Excessive growth of certain microbes in surface water can deleteriously affect water quality, and some of these microorganisms are pathogenic to aquatic life and humans. Numerous waterborne diseases, such as cholera, typhoid fever, and dysentery have been attributed to the presence of pathogenic microorganisms (Blumenthal and Peasey, 2002). Microbial pollution of drinking water typically receives significant attention; in particular *Giardia lamblia* and fecal coliform indicator bacteria such as *E. coli,* which are emblematic of larger source water pollution problems.

Microbial contamination is not only a health concern, but also an economic one. Pathogenic microorganisms pose a serious threat to the health and viability of commercial crops, potentially causing significant monetary loses if not mitigated. Batz et al. (2012) estimated the cost of foodborne illnesses contracted from the bacterial pathogen *E. coli* 0157:H7 alone to be $255 million annually. For 14 major pathogens the total cost was estimated to be around $14 billion annually (Batz et al., 2012). These figures were just for the United States, likely paling in comparison to global costs and highlight the need to development novel low cost treatment technologies that can eliminate or remove pathogens.

Stormwater runoff is also a critical source of bacterial pathogens. High concentrations of pathogenic microorganisms in stormwater runoff can contribute to the degradation of water quality in receiving water bodies (Hathaway et al., 2011). Haile et al. (1999) investigated the relationship between *E. coli* and *Enterococci* in untreated stormwater discharging in to the ocean and the associated health effects experienced by recreational swimmers. They concluded that

there was increased incidence of illnesses such as upper respiratory and gastrointestinal infections associated with swimming in the ocean water contaminated with untreated stormwater runoff.

There are several different mechanisms of treatment that can be implemented to eliminate or reduce the number of bacterial pathogens in recycled irrigation water, agricultural runoff, wastewater, drinking water, or stormwater. These treatment regimes include but are not limited to physical, chemical, and biological treatment processes (Stewart-Wade, 2011). Physical filtration, as the name suggests, is the physical entrapment of pathogens in a filtration system (Stewart-Wade, 2011). Physical entrapment can work in concert with biological and chemical processes by retaining the pathogen for adequate periods of time to allow for the other processes to remove the pathogens.

Chemical mechanisms primarily consist of using compounds that are toxic to bacterial pathogens. Although chemical disinfection has proven to be exceptionally effective in reducing bacterial pathogens, it can also adversely affect possibly beneficial microorganisms such as *Alphaproteobacteria*, thus allowing pathogens to thrive in a less competitive environment (Pinto et al., 2012).

Biological treatment involves using biological control agents or biofiltration systems such as biologically activated carbon filters that produce biologically stable water. Biologically stable water refers to water that does not support microbial growth due to lack of available nutrients needed by the bacteria (Hammes et al., 2010). Biological control agents are non-pathogenic organisms that are antagonistic to pathogens (Paulitz and Bélanger, 2001). Naturally produced antimicrobial compounds that inhibit the growth of pathogenic organisms can also be used for disinfection. Biological treatment is widely used in Europe for a variety water related

applications including drinking water, and biologically based treatment technologies, such as membrane bioreactors, are increasingly being implemented in the Unites States (Shannon et al., 2008).

1.1.1 Conventional Treatment Technologies: Bacterial Removal and Disinfection

Many treatment processes designed to eliminate pathogens, in particular bacteria, from wastewater and drinking water have been developed over the years. Conventional pathogen treatment methods include chlorine disinfection, ultraviolet light irradiation, and ozone disinfection (Shannon et al., 2008). Chlorine disinfection is the most widely employed treatment process used to eradicate or inactivate bacterial pathogens. It has been credited with greatly reducing previously pervasive diseases, such as typhoid fever, originating from pathogenic bacteria present in un-sanitized water (Ashbolt, 2004). Liquid chlorine is typically applied to drinking or wastewater as free or combined chlorine (i.e. chloramines) (Shannon et al., 2008). Despite the efficacy of using treatment methods such as chlorine or ozone there can be several drawbacks. These drawbacks include cost and the formation of disinfection byproducts, some of which are classified as carcinogens. As a result of these drawbacks the use of ultraviolet irradiation for disinfection has increased. Ultraviolet light damages the DNA of microorganisms eliminating their ability to reproduce and thus persist (Zimmer and Slawson, 2002). Although ultraviolet irradiation is an effective technology there are some drawbacks. The efficacy is primarily dependent on the penetration of ultraviolet light in the water. Solid particles in water can absorb or deflect ultraviolet light and thus reduce the amount of light reaching pathogens, this is an issue for treatment of irrigation or agricultural water, as these sources may have substantial quantities of suspended solids (Christensen and Linden, 2003).

1.1.2 Existing Low-Cost Treatment Technologies Used to Remove Fecal Coliform Bacteria

The conventional treatment technologies described above are typically reserved for municipal or industrial related applications and are rarely implemented for agricultural purposes since the cost is prohibitive (Pescod, 1992). Many of the low-cost treatment technologies currently available have effectively demonstrated the ability to remove pollutants, such as excess concentrations of nitrate or phosphate, from some waters (Hatt et al., 2009). Unfortunately some of these systems have proven to be ineffective in removing bacterial pathogens and in some cases actually exported higher concentrations of bacteria (Hathaway et al., 2009). The treatment technologies that do reduce bacterial pathogens in water, bioretention cells and wetlands, tend to do so through physical filtration or rely on competitive environmental predation (Davies and Bavor, 2000).

Slow sand filters have proven to be an effective treatment option for removing fecal coliform bacteria from runoff (Barrett, 2003). Sand filters are relatively inexpensive, can be easily implemented, and are capable of removing bacteria (Jenkins et al., 2011). However, despite the advantages offered by sand filtration, there are considerations that need to be addressed when implemented, namely filter size and filtration rate (Table 1). Dry and wet detention ponds are common BMPs used to treat stormwater runoff. Wet detention ponds are designed to retain a given volume of water indeterminately, while dry detention ponds are designed to impound water temporarily, slowly releasing treated stormwater. In general pollutants are removed through sedimentation and biological uptake. Wet detention ponds tend to demonstrate higher levels of bacterial removal than dry detention ponds. According to a study conducted by (Hathaway et al., 2009) dry detention ponds actually showed negative fecal coliform removal rates, exporting higher concentrations than wet detention ponds. Bioswales are conventional

BMPs used to attenuate flow and treat stormwater runoff. Sufficient removal rates of nutrient related pollutants have been achieved using bioswales, however fecal coliform bacteria are not adequately eliminated (Clary et al., 2008). Bioretention cells are a treatment technology analogous to bioswales, and customarily are ditches or depressions layered with soil and/or mulch that include a variety of vegetation to increase pollutant removal via biological uptake. In contrast to bioswales, bioretention cells have demonstrated the ability to effectively remove fecal coliform indicator bacteria (Hunt et al., 2008). Irrigation mats are another alternative management practice that is thought to physically filter out pathogens (Van Der Gaag et al., 2001). Irrigation mats are essentially porous material that greenhouse plants or crops are placed on. Water is treated as it flows from the base of the plants through the capillary irrigation mat material and to a drainage collection line.

Wetlands are one of the most efficacious low cost treatment options available for removing bacteria, a high degree of removal for nutrients, pollutants, and fecal coliform bacteria can be attained. Wetlands can be used as a standalone treatment system for some wastewater applications depending on flow and the strength of the wastewater, in addition to being used as polishing step for wastewater that has already gone through primary treatment (i.e. clarification). Although natural and constructed wetlands have proven to be viable options for removing bacteria from wastewater and stormwater (Verhoeven and Meuleman, 1999), the elimination of these contaminants greatly fluctuates as a result of the complex relationships between various ecological parameters. Pollutants are removed through a variety of physical, chemical, and biological processes.

A relatively new treatment technology is the use of biological control agents. Adding selective inhibitory biochemical inhibitors that suppress bacterial pathogens have proven to be an effective

tool for managing plant microbial pathogens. Pagliaccia (2008) demonstrated that by altering recycled hydroponic nutrient solution with a nitrogen stabilizer the resident population of fluorescent pseudomonads increased, while the presence of disease causing microorganisms, *Phytophthora capsici* and *P. aphanidermatum*, decreased on several hydroponically grown fruits. The decrease in the number of microbial pathogens is believed to be attributed to selective inhibition as a result introducing the nitrogen stabilizer.

Table 1: Common low-cost treatment technologies

Treatment Technology	Description	Advantages	Drawbacks
Dry Detention Ponds	Constructed basins used to detain runoff for a specific amount of time to remove pollutants from influent flow. Typically consist of a basin with an inlet and outlet. The outlet structure is sized according to the desired detention time. Pollutant removal is achieved through the sedimentation of soil particles containing chemical pollutants or bacteria that are bound to the surface of soil particles.	* Effective in removing TSS, total phosphorus, total nitrogen, with moderate removal of other pollutants. * Less expensive than wet detention ponds.	* Not effective in removing fecal coliform bacteria or other bacterial pathogens (Hathaway et al., 2009). *Ineffective removing soluble pollutants. * Requires more land area than other treatment options such as bioswales.
Wet Detention Pond	Constructed basin similar to that of a dry detention pond, with the exception that there is a permanent pool of water. Runoff is directed to the detention pond where the influent water is treated until the next storm event occurs. Sedimentation is the primary mechanism responsible to the removal of particulate matter, organic matter, and metals.	* Generally more effective in removing TSS, total phosphorus, total nitrogen, & other pollutants than dry detention ponds. *Effective in removing fecal coliform bacteria.	* More expensive to construct than dry detention ponds. * Requires relatively more land compared to other treatment alternatives.

	Nutrient pollutants are removed through biological uptake.	(Hathaway et al., 2009)	
Treatment Technology	**Description**	**Advantages**	**Drawbacks**
Wetlands	Constructed basin or pond that achieve pollutant removal through sedimentation or biological uptake. The design basis for a wetland is similar to that of a wet detention pond, except with a much larger volume. Plant vegetation is also used to assist in the removal of pollutants.	* Effective in removing bacterial pathogens (Hench et al., 2003). * Effective in removing in removing TSS, total phosphorus, total nitrogen, & other pollutants.	* Requires a large volume relative to other treatment alternatives.
Bioretention Cell	Ditch or depression layered with soil and / or mulch. Typically used for small drainage areas such as parking lots or residential neighborhoods. Often contain a variety of vegetation.	*Effective in removing fecal coliform bacteria (Hunt et al., 2008). *Demonstrates high rates of removal for many pollutants including: metals, phosphorus, ammonium, total nitrogen, and calcium. *Relatively easy to install and typically is inexpensive.	* Not able to treat large drainage areas or basins. * Requires more maintenance than other treatment alternatives.
Bioswale	A longitudinal vegetated depression or ditch similar to a bioretention cell in terms of function. Used to attenuate flow from stormwater runoff. Removal of pollutants is achieved primarily through infiltration.	*Relatively inexpensive to construct and are compact. * Effective pollutant removal, especially metals.	*Not effective in removing fecal coliform bacteria (Clary et al., 2008). *Prone to erosion when faced with high flow.

			* Cannot treat large drainage basins or volumes of water.
Treatment Technology	**Description**	**Advantages**	**Drawbacks**
Media / Sand Filters	Can consist of a basin or ditch filled with sand, or can resemble a filter used for pool water and drinking water. Achieves reduction of pollutants through physical filtration / infiltration.	* Effective removing fecal coliform bacteria (Barrett, 2003). *Effective in removing BOD, TSS, TOC, total nitrogen, total phosphorus, and various metals. * Relatively inexpensive.	*Requires frequent maintenance and performance can decrease significantly due to clogging. *Can be more expensive due to maintenance costs associated with replacing sand every few years. *Relatively slow filtration rates typically are used, which results in requiring large areas for filters.
Lagoons	Holding or maturation ponds commonly used to treat wastewater to remove organic pollutants, metals, and fecal coliform bacteria. Various physical, chemical, and biological processes are responsible for the removal of pollutants.	* Effective removing fecal coliform bacteria (Barrett, 2003). *Effective in removing BOD, TSS, TOC, Total Nitrogen, Total Phosphorus, and various metals.	*Requires a relatively large area.

While some of these methods are initially effective, performance decreases significantly over time as a result of clogging due to suspended solids, thus, frequent maintenance may be required (Barrett, 2003).

1.1.3 Overview of Current Technology Issues and Limitations

The treatment technologies available can essentially be classified into two categories: conventional technologies that can achieve high removal rates of bacteria, but for some applications are cost prohibitive, and passive low-cost treatment technologies that may not achieve the same level of performance but are far less expensive and maintenance intensive. The development of a cost-effect, but robust alternative treatment biotechnology that can bridge the gap between these two types of technologies is a critical component in the effort to provide clean water and promote environmental stewardship. One such technology that could fill such a role is a fungal bioreactor based treatment system.

1.2 Fungal Bioreactors and Removal Mechanisms

1.2.1 Fungal Bioreactor Systems

The use of a bioreactor containing mycelial mats of filamentous fungi to remove pollutants from water and wastewater is a relatively new concept. The mycelium acts as a physical, biological, and biochemical filter, eliminating bacteria, such as *E.coli*, from influent water. Generally, a fungal bioreactor is comprised of a vessel filled with a substrate that supports microbial growth (e.g., woodchips, saw dust or some other alternate carbon based substrate) that is inoculated with a wood-rot fungus as shown in Figure 1, for instance *Piptoporus betulinus* (Taylor et al., 2014). Subsequently, influent water is introduced into the

Figure 1: Example of sawdust and straw media inoculated with a wood-rot fungus

container, where it interacts with the mycelium as it percolates through the bioreactor, resulting in the removal of bacteria present in the influent water.

Traditionally, fungal bioreactors have been used to produce industrial enzymes, proteins, and other value added biochemical compounds by utilizing a biological process known as solid-state fermentation (Pandey, 2003). An elementary solid-state fermentation reactor simply contains solid growth media (i.e. carbon based source such as wheat straw), which is then inoculated with a filamentous fungus. Thenceforth enough water is added to the reactor to create a saturated environment in terms of moisture, but one where there is a limited amount of free water (Pandey, 2003). The moist environment mimics the natural habitat that many species of fungi, used to produce compounds via solid-state fermentation, are found in. After a selected fermentation time has been reached, the reactor is drained, producing a concentrate that contains the desired biochemical compound. In contrast to submerged fermentation reactors, solid state-fermentation reactors typically produce lower volumes of concentrate, but higher yields of the target compound (Pandey, 2003). Fungal bioreactors employed for water treatment are essentially modified derivatives of solid-state fermentation reactors. The main differences are that fungal bioreactors used for water treatment operate in an environment where this is a substantial amount of free water, and the end applications between the two are dissimilar.

Fungal bioreactors geared towards treating water can be used in a variety of applications such as improving the effluent quality of stormwater runoff or agricultural drainage. Although fungal bioreactors have been primarily used in runoff related applications, it is not necessarily limited to treating runoff (Thomas et al., 2009). Other applications for this treatment process include treating reclaimed water to be used for irrigation, agricultural wastewater, and potentially drinking water. While reports in the literature suggest that this method is successful in removing

bacterial pathogens from water, the processes behind the removal are not well understood (Rogers, 2012; Taylor et al., 2014). Currently, it is unknown whether the driving mechanism behind bacterial removal is physical, biochemical, biological, or a combination of all three processes.

The direct interaction of fungi with bacteria in an isolated setting has been studied to some extent (Barron, 1988). Numerous species of fungi, such as *Pleurotus ostreatus* and *Daedaleopsis confragosa*, have been shown to exhibit predatory behavior as well as demonstrate the ability to produce extracellular antimicrobial metabolites (Alves et al., 2012; Frey-Klett et al., 2011; Kalyoncu et al., 2010; Suay et al., 2000). Nevertheless, fungi grown in a liquid substrate with only essential nutrients and known bacterial strains present are not representative of the heterogeneous environment that exists within a fungal bioreactor. Thus, understanding the explicit mechanisms that underlie the removal processes is imperative in order to maximize the potential of this method as an alternative remediation, treatment, and/or purification technology.

1.2.2 Characteristics of Wood-rot Fungi

Wood-rot fungi are a variety of fungi that decay wood, converting dead organic matter into nutrients. The main types of wood-rot fungi are brown-rot and white-rot. Brown-rot fungi primarily break down hemicellulose and cellulose present in wood. White-rot fungi in addition to breaking down hemicellulose and cellulose also break down lignin. As a whole white-rot fungi tend to produce a wider variety extracellular enzymes than brown-rot fungi, which makes them conceivably suitable candidates for use in remediation related applications (Větrovský et al., 2013). The diversity of enzymes as well as secondary metabolites produced by white-rot fungi allows for a wider range of chemical pollutants and possibly bacterial pathogens to be removed.

It should be noted that the production of extracellular enzymes, antimicrobial metabolites, predatory behavior, and the associated control factors varies from species to species. For example, there is literature that suggests the basidiomycete *Fomes fomentarius* will decrease the production of extracellular enzymes in the presence of higher nitrogen concentrations, but other species of fungi will increase the production of enzymes in a nitrogen rich environment (Větrovský et al., 2013). This is of importance because the nutrient content of the media may affect the production of antimicrobial compounds or alter predatory behavior, thus affecting the removal efficiency of bacterial pathogens in a fungal bioreactor. These naturally occurring processes can be harnessed in an engineered system and used to remove or eliminate bacterial pathogens in water.

1.2.3 Antibacterial Functions, Properties, and Behavior of Fungi

The interaction of fungi with bacteria and other microbiota in a given environmental setting is complex and not completely understood. There are several different classifications typically used to characterize the interaction between fungi and bacteria. The primary classifications used to describe these interactions are symbiotic and antagonistic (Frey-Klett et al., 2011). Symbiotic interactions refer to a mutually beneficial relationship between fungi and bacteria. For instance (Artursson et al., 2006) discuss the symbiotic relationships between arbuscular mycorrhizal fungi and *endobacteria*, and hypothesize several advantages of forming these relationships. Antagonistic interactions refer to a hostile or an uncooperative relationship between fungi and bacteria. The antagonistic relationship between fungi and bacteria is one that is well documented (Barron, 2003; Mille-Lindblom and Tranvik, 2003). Competition for vital nutrients within a collective environment and substrate leads to antagonistic associations (Mille-Lindblom et al., 2006). Fungi and bacteria alike have developed unique defensive mechanisms to combat one

another for survival, including secreting secondary extracellular antimicrobial compounds and biological predation (Barron, 1992).

1.2.3.1 Predation of Bacteria by Fungi

Key nutrients, such as nitrogen and phosphorus, necessary for subsisting in a biological environment are not always available in abundance. Millions of microorganisms vying for a limited supply of nutrients within a given environment results in fierce competition for essential compounds. As a result of this competition numerous species of fungi seek alternative sources of nutrients, and one such source is obtained through active predation of other biological organisms. Several species of fungi that actively prey on nematodes and rotifers, have been identified (Barron, 1992; Cooke, 1962a, 1962b). Hyphomycetes that exhibit predatory behavior have been shown to produce a vast network of mycelium containing biological constructs such as adhesive knobs, adhesive branches, rings, and constricting rings (Thorn and Barron, 1984), that can entrap prey. After an organism has been entrapped it is infiltrated by fungal hyphae, where nutrients from the organism are extracted for utilization (Barron, 1992). (Barron and Thorn, 1987) reported that the basidiomycete *P. ostreatus* demonstrated the ability to immobilize and consume nematodes. Essentially the fungus secreted a mycotoxin on the outer tips of exploratory hyphae, stunning nematodes that came into contact with the toxin. Subsequently, hyphae from the fungus infiltrated the immobilized nematode and absorbed its contents for nutrition (Barron and Thorn, 1987).

Initially, it was thought that the predatory behavior of some species of fungi evolved to be the primary mode of obtaining essential nutrients, however this was later disproven to be the case (Barron, 1992). The predatory behavior shown by *P. ostreatus* is thought be controlled by

several environmental factors, specifically the carbon to nitrogen ratio in their given environment and that the nematodes they prey on serve as a supplemental source of nitrogen (Thorn and Barron, 1984). The predatory behavior was theorized to increase or decrease depending on the carbon to nitrogen ratio (Barron, 1988). In several studies, a higher C:N ratio corresponded with the initiation of trapping and predatory behavior, while a lower C:N ratio corresponded to a reduction in predatory behavior (Satchuthananthavale and Cooke, 1967).

Despite evidence that some species of fungi actively prey on other microorganisms given the right habitat, there still was little proof that they prey on bacteria as supplemental source of nutrition. Much of the skepticism in regards to bacterial predation by fungi was in part due to the fact that the biomass of single bacterial cell is miniscule compared to other prey such nematodes. A single nematode or rotifer provides much more nutritional value than a single bacterial cell. Contrary to this skepticism (Fermor and Wood, 1981) showed that under the right environmental conditions some fungi will consume dead bacteria as an auxiliary food source. In their study (Fermor and Wood, 1981) demonstrated the ability of the fungus *Agaricus bisporus* to grow on gram-positive or gram-negative bacteria, which were the sole sources of carbon and nitrogen. They also showed that other species of fungi, in particular basidiomycetes, were able to degrade *Bacillus subtilis* and utilize lysed biological contents for nutritional purposes.

Sparling et al. (1982) showed that fungi could degrade and use microbial biomass as a nutrient source. The biomass was thought to serve as a source of nitrogen, carbon, and other minerals. Though it had been shown that fungi could use bacteria as the sole nutrient source, the ability of fungi to prey on living bacteria had not been confirmed. (Barron, 1988) provided proof that a number of fungal species actively preyed on microcolonies of bacteria under low nutrient conditions. The following species of fungi exhibited the ability to prey on bacterial colonies:

Agaricus brunnescens, *Coprinus quadrifidus*, *Lepista nuda*, and *P. ostreatus*. *P. ostreatus* is the only species to have been evaluated for the removal of bacteria in water. Under low nutrient conditions fungal hyphae were observed to invade microcolonies of bacteria, where bacterial cells were penetrated and lysed. Once the contents from the lysed bacterial cells were released, it was assumed that the hyphae of the fungi absorbed the organic contents of the lysed cells and translocated them to satisfy nutritional demands (Barron, 1992). Thus, the predatory behavior exhibited by some species of fungi could be exploited to facilitate the removal of bacterial pathogens from water via an engineered system.

1.2.3.2 Production of Antimicrobial Compounds by Fungi

The discovery of penicillin in 1928 by Alexander Fleming and subsequent discoveries of other antibiotic metabolites fueled the search to find other novel biopharmaceutical compounds (Scherlach et al., 2013). Despite the "Golden Age" of antibiotics, fungi have long been used for medicinal purposes. Mushrooms have been commonplace in traditional medicine in many Asiatic countries for centuries (Lindequist et al., 2005). Neolithic Europeans were also thought to use mushrooms to remedy a variety of ailments, confirmed with the discovery of the "Iceman" in 1991, who was found to be carrying fruiting bodies of the birch polypore mushroom *P. betulinus* (Lindequist et al., 2005). Antimicrobial metabolites and compounds, such as piptamine as well as numerous phenolic complexes, have been isolated from fungal extracts (Alves et al., 2013; Schlegel et al., 2000).

Customarily fungi of the phylum ascomycota have shown to be the most prolific producers of bioactive substances, and, as a result, are routinely screened for antimicrobial activity. Revolutionary biopharmaceutical compounds such as beta-lactam antibiotics have been isolated

from ascomycota fungal species (Bycroft, 1987). Examples of antibacterial compounds sequestered from ascomycota fungal species includes ampicillin and various cephalosporin derivatives (Bycroft, 1987). The emergence of antibiotic resistant bacteria has spurred researchers to identify other sources of novel antibacterial compounds (Levy and Marshall, 2004). While a significant portion of new antibiotics are still primarily generated via synthetic chemistry, other sources of antibacterial compounds such as allelochemicals derived from mycorrhizal fungi or aquatic plants are proving to be a promising alternative source of antibiotics (Balandrin et al., 1985). Another source of antimicrobial compounds are fungi of the phylum basidiomycota (Lorenzen and Anke, 1998). Wood-rot fungi have been shown to produce hydrogen peroxide, which is an antiseptic (Koenigs, 1974). Screening for antimicrobial activity exhibited by fungi is usually the first step to assessing new species for the production of possible bioactive compounds. This generally involves observing the interaction between mushroom culture extracts and bacteria grown on agar in a petri dish. The inhibition zone diameter is used to quantify the antimicrobial activity of the mushrooms extracts (Kalyoncu et al., 2010; Suay et al., 2000). In order to be classified as demonstrating antimicrobial activity, mushroom extracts must show a defined zone of inhibition against bacteria. The zone of inhibition is simply an area where no bacterial growth is observed due to an inhibitory or biocidal substance and is defined as the length, usually in mm, of the clear zone from edge the antibacterial substance (i.e. antibiotioc tablet) to where bacterial growth is observed (opaque portion of growth plate) (Suay et al., 2000). A larger inhibition zone indicates the presence a more potent antimicrobial substance. Ideally mushroom extracts that demonstrate significant antimicrobial activity are analyzed further, which involves characterizing and isolating the specific antimicrobial molecular complexes (Fakoya et al., 2013).

A substantial number of studies that have screened macrofungi for antibacterial activity, but few of them have attempted to characterize or isolate the compounds responsible for the antimicrobial activity observed. (Gangadevi et al., 2008; Janes et al., 2006; Keller et al., 2002; Suay et al., 2000). Several of the species of fungi screened for antimicrobial activity in the prior studies listed, such as *Daedaleopsis confragosa*, are suitable for use in fungal bioreactor water treatment systems. These species are suitable primarily due to their abundance and the relative ease mass culturing them. Assessing the exact chemical structure of the metabolites being produced by the fungi of interest is critical for fungal bioreactor based water treatment applications to ensure that the natural antimicrobial compounds being produced are not pollutants themselves. Characterizing the metabolites also has implications for filter optimization and performance. One of the approaches that could be used to design a hyper-functional filter is to upregulate the production of the antimicrobial compound or compounds responsible for the inhibition of bacterial pathogens using metabolic engineering. However to do so, the specific structure of the antibacterial metabolite or metabolites must be known in order to determine the metabolic pathway or pathways responsible for the production of the compounds. Another and more straightforward approach is to design a system that provides optimal environmental conditions (i.e. optimal nitrogen levels) for the production of antimicrobial compounds.

Naturally produced antimicrobial compounds could be utilized as a natural disinfection mechanism to eliminate bacterial pathogens in water. The process would be similar to chemical disinfection, but naturally generated biochemical compounds would be employed instead.

1.3 Prior Studies, Current State of the Technology and Applications

The concept of using fungal filtration to eliminate bacterial pathogens in water was first implemented by (Stamets, 2005) on a farm with high concentrations of fecal coliform bacteria in runoff and subsequently has been referred to as "mycofiltration". Taylor et al. (2014) reported that *Stropharia rugoso-annulta* grown on a 100% alder woodchip media reduced *E.coli* up to 22% in synthetic stormwater. Chirnside et al. (2013) assessed the ability of *P. ostreatus* grown in spent mushroom compost to remove *E. coli* from simulated wastewater and observed reductions as high as 75%. Thomas et al. (2009) evaluated a bioretention cell in combination with mycorrhizal fungi, as well as a woodchip bed inoculated with fungi for the removal of fecal coliform bacteria from runoff and a 97% reduction of fecal coliform bacteria was reported after a steady state had been achieved. Rogers (2012) reported up to a 45% reduction of fecal coliform bacteria in simulated runoff filtered through a lab scale fungal bioreactor containing a sawdust growth media inoculated with *P. ostreatus*, and did not find a correlation between retention time and percent reduction of *E. coli*. Figure 2 shows an example of a simple fungal bioreactor containing pasteurized sawdust inoculated with the wood-rot fungus *D. confragosa*.

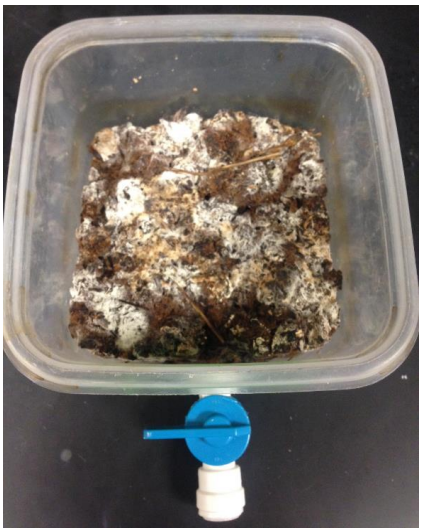

Figure 2: Example of a fungal bioreactor

Although the results from previous studies of fungal bioreactors have exhibited some promising results, there is considerable need to better define the processes responsible for removal. Most of the previous studies focused solely on evaluating suitable species of fungi or fungi growth

substrates, and did not identify the explicit removal mechanisms employed in fungal bioreactors used for removing bacterial pathogens from water.

1.4 Broader Impacts, Applications, and Future Research

Few attempts to engineer a more robust system have been made, which is likely due to the lack of information in regards to the specific removal mechanisms responsible for the eradication of bacterial pathogens

Thus I propose to focus on assessing three different species of fungi for use in lab-scale fungal bioreactors, and identifying the processes that are responsible for the removal of pathogens. The removal processes that will be investigated include: physical filtration of bacteria, consumption of bacteria as a substitute nutrient source, and the production of antimicrobial metabolites. While this study will attempt to identify several different removal processes, there will be a focus on evaluating species of fungi that have previously exhibited the ability to produce secondary antimicrobial metabolites. Prior research has shown that many species of fungi are capable of producing secondary extracellular antimicrobial metabolites (Suay et al., 2000). The production of biocidal or inhibitory metabolites can be used as a natural disinfection mechanism. The process is be similar to using chlorine for disinfecting drinking water, but naturally produced biochemical compounds would be utilized instead.

Obtaining a better understanding of the different removal mechanisms at work within a fungal bioreactor will allow for the design of a system or filter that maximizes the aforementioned processes. The ultimate objective, beyond the scope of this project, will be to eventually produce a hyper-functional filter that can be used for a wide range of treatment applications including but

not limited to: agricultural runoff, stormwater runoff, wastewater, recycled irrigation water, and drinking water.

2. Methods

2.1 Experimental Design and Setup

Three different experiments were performed: two experiments assessing the mass removal rates of *E. coli* in fungal bioreactors and one experiment testing for the production of the antimicrobial compound hydrogen peroxide, in lab-scale bioreactors. The bioreactors were constructed out of square plastic containers (liquid volume of 45.6 oz), fitted with ¼" ball valves (Chirnside et al., 2013). To prevent the outlet ball valves from clogging, fiberglass screens were fitted over the valves and secured using zip ties.

The first experiment entailed evaluating the ability of three bioreactor treatments to remove *E. coli* via batch treatment. Bioreactor treatment groups contained three bioreactors each and were permitted to treat influent water for seven discrete treatment times.

The ensuing experiment assessed the ability of five different bioreactor treatments to remove *E. coli* via a flow through treatment mode. The bioreactors were allowed to treat influent water for three distinct treatment times. For both the batch and flow through treatment experiments, influent and effluent concentrations of *E. coli* were measured for each treatment time for every bioreactor. The overall percent reduction ((effluent concentration - influent concentration) / influent concentration) of *E. coli* and treatment time were used to assess the performance of the bioreactors (Rogers, 2012).

The final experiment comprised of assaying for the production of the antimicrobial compound, hydrogen peroxide, by the fungi evaluated in the preceding experiments. For this experiment, there were four bioreactor treatments, each containing three replicate bioreactors. The bioreactors were saturated with enough deionized water to create a moist environment with limited free water in order to replicate a solid-state fermentation environment (Pandey, 2003). Effluent samples from the bioreactors were collected at three different sequential time steps and analyzed for extracellular concentrations of hydrogen peroxide.

2.1.1 Batch Treatment Experiment

For the batch treatment experiment, two species of fungi were assessed, *P. betulinus* and *D. confragosa*. The experimental setup consisted of three treatment groups of bioreactors. The first treatment included triplicate bioreactors containing pasteurized growth media, the second treatment consisted of triplicate bioreactors containing growth media inoculated with *P. betulinus,* and the third treatment consisted of triplicate bioreactors containing growth media inoculated with *D. confragosa*.

At the outset of the experiment bioreactors were loaded with 200 mL of an influent water solution containing an *E. coli* concentration of $1x10^3$ cells/ mL and allowed to treat for 5 min., 30 min., 1 hr., 3 hr., 12 hr., 24 hr., and 48 hr., respectively. Treatment times were increased sequentially, with a 24 hour rest period in between each treatment run. Once the allotted residence time had been reached for an individual treatment run, 100 mL of effluent was collected, and subsequently analyzed for *E. coli* load. The influent and effluent bacterial concentrations were used to calculate the overall percent reduction of bacteria. After each

treatment run the bioreactors were drained completely to eliminate any free water. A summary of the experimental details are provided in Table 2 and Figure 3 depicts the experimental setup.

Table 2: Summary of experimental details - batch treatment experiment

Batch treatment experiment	
# of Treatment Times	**Treatment Times**
7	5 min., 30 min, 60 min, 1 hr., 12, hr., 24 hr., 48 hr.
Bioreactor Treatment Groups	**# of Bioreactors**
Negative Control (Pasteurized)	3
P. betulinus	3
D. confragosa	3
Measured Parameters	
Influent *E. coli* Conc. (cfu/100 ml)	
Effluent *E. coli* Conc. (cfu/100 ml)	

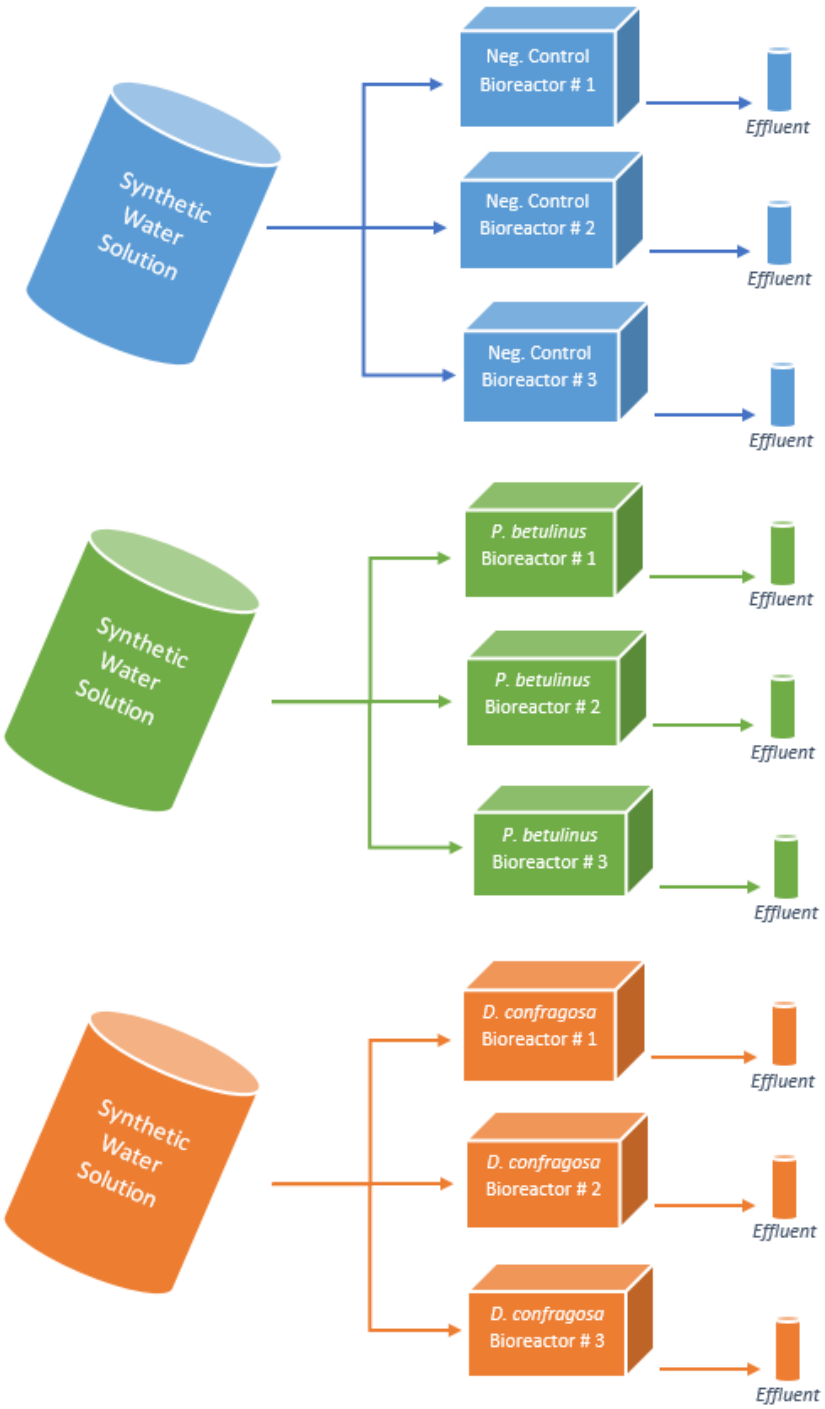

Figure 3: Batch treatment experimental setup

2.1.2 Flow Through Treatment Experiment

Three species of fungi were evaluated, *P. betulinus*, *D. confragosa*, and *P. eryngii* in the flow through treatment experiment. The experimental setup consisted of five bioreactor treatments. The first treatment consisted of a negative control reactor containing only pasteurized growth media. The second treatment included two negative control reactors containing only autoclaved growth media. The third treatment consisted of triplicate bioreactors containing growth media inoculated with *P. betulinus.* The fourth treatment consisted of triplicate bioreactors containing growth media inoculated with *D. confragosa*. The fifth treatment was comprised of triplicate bioreactors containing growth media inoculated with *P. eryngii*. As noted above there were two different negative control treatments, this was done in order to investigate the effect that autoclaved and pasteurized media may have on treatment efficacy.

Pasteurization involves treating media at a temperature of 73°C for 1 hour, which for mycological applications is typically accomplished by immersing growth media in water at 73°C (Stamets, 2005). Although pasteurization eliminates the majority of bacteria, spores and thermotolerant strains of bacteria can remain. Autoclaving is performed at 121°C and results in the destruction of theoretically all microorganisms and spores (Harrigan, 1998). The difference between the two pre-treatment methodologies can be thought of in terms of environmental competition. Autoclaving eliminates all competing microorganisms within a given media, producing a sterile environment. Pasteurization eliminates a significant portion of competing organisms, but not all, resulting in a less competitive non-sterile environment. The reason for intentionally permitting thermotolerant bacteria to remain in pasteurized media is that these species of bacteria are often non-pathogenic, and outcompete virulent strains of pathogens, decreasing the ability of injurious bacteria to establish themselves and survive in the media

(Harrigan, 1998). In contrast autoclaved media lacks this competitive environmental component, therefore allowing virulent strains of bacteria to establish themselves due to a lack of competition.

At the start of the flow through experiment an influent solution of water containing an *E. coli* concentration of $1x10^3$ cells/mL was pumped through the bioreactors at varying rates to achieve residence times of 20 min., 10 min., and 5 min., respectively. In contrast to the batch treatment experiment, treatment times were decreased sequentially (i.e. 20 min. treat time day 1, 10 min. treat time day 2, and 5 min. treat time day 3), though a 24 hour rest period was also permitted between each treatment run. A 100 mL sample of the influent water solution was collected prior to each treatment run and 200 mL of effluent were collected from every bioreactor at the conclusion of each treatment run. Similar to the batch treatment experiment, influent and effluent *E. coli* concentrations were used to calculate the overall percent reduction of *E. coli* which was used to assess the performance of the bioreactors. A summary of the experimental components for the flow through study is provided in the Table 3.

Table 3: Summary of details - flow through treatment experiment

Flow through experiment	
# of Treatment Times	**Treatment Times**
3	20 min., 10 min., 5 min.
Bioreactor Treatment Groups	**# of Bioreactors**
Negative Control (Pasteurized)	1
Negative Control (Autoclaved)	2
P. betulinus	3
D. confragosa	3
P. eryngii	3
Measured Parameters	
Influent *E. coli* Conc. (cfu/100 ml)	
Effluent *E. coli* Conc. (cfu/100 ml)	

A Cole-Parmer FH100M Digital Multichannel Peristaltic Pump (Cole-Parmer, Vernon Hills, IL) was used to pump the synthetic water solutions through the bioreactors. Cole-Parmer Masterflex platinum cured-silicone tubing, suitable for biological applications, was used (Cole-Parmer, Vernon Hills, IL) to convey water to the bioreactors. In order to ensure the even distribution of influent water into the bioreactors and maximize the volume of filter media utilized, the portion of the tubing threaded into the bioreactors was perforated. The flow rates to achieve the desired residence times were determined by calculating the porosity of the different filter medias and establishing appropriate pumping rates through trial and error. Porosity was calculated by loading 50 mL of packed media into a graduated cylinder and adding water until the media was completely saturated. The amount of water added was recorded and used to calculate the pore volume of the filter medias. The pore volumes of the medias were 25 mL for the pasteurized media, 20 mL for the autoclaved media, 19 mL for the *P. betulinus* based media, 18 mL for the *D. confragosa* media, and 17 mL for the *P. eryngii* based media, respectively.

After each of the treatment runs, the pump tubes were completely bled and flushed with deionized water for two minutes to remove any biological contaminants that may have adhered to the walls of the tubing. Periodically samples of DI water flushed through the tubes were collected and tested for *E. coli* cells to verify that any cells that had adhered to the wall of the tubing had been removed. These DI samples tested negative for *E coli* and validated that cells had been removed from the pump tubing. Similar to the batch treatment experiment, all of the bioreactors were drained to eliminate any free flowing water following each trial run. Figure 4

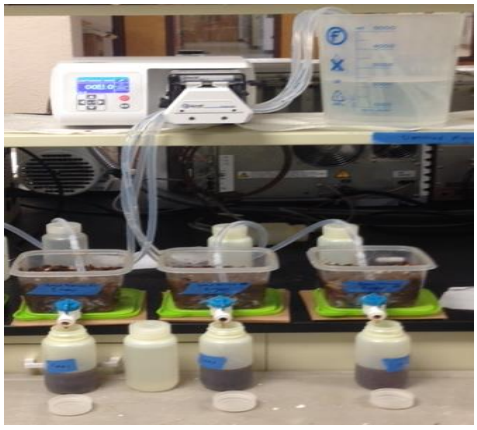

Figure 4: Flow through experimental setup (single group of bioreactors)

depicts the experimental set up for a single group of bioreactors; this setup was replicated for every bioreactor treatment group. Figure 5 illustrates the full flow through treatment experimental setup.

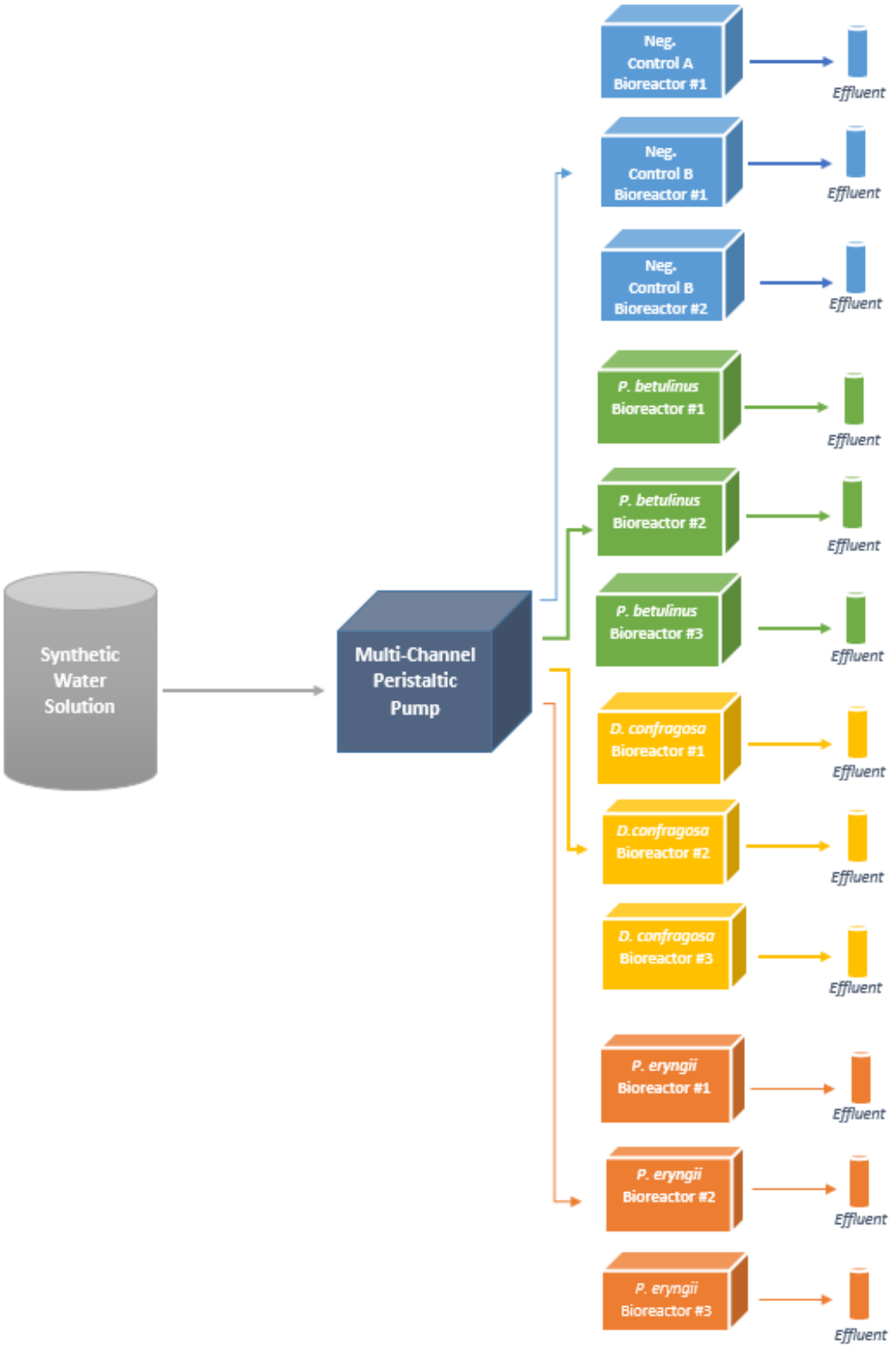

Figure 5: Full flow through treatment experimental setup

2.1.3 Hydrogen Peroxide Production Experiment

The third experiment involved investigating the fungal production of hydrogen peroxide. The motivation behind testing for the production of hydrogen peroxide is that the enzymatic system many species of wood-rot fungi employ to breakdown lignocellulosic biomass involves peroxidase based enzymes, hydrogen peroxide is thought to play a vital role in the redox reactions carried out by these peroxidase based enzymes (Koenigs, 1974). There were four bioreactor treatments for this experiment, each comprised of three replicates: a negative control treatment with bioreactors containing only pasteurized growth media, a treatment group of bioreactors inoculated with *P. betulinus*, a treatment group of bioreactors inoculated with *D. confragosa*, and a treatment group consisting of bioreactors inoculated with *P. eryngii*.

Pasteurized media was used in the negative controls to ensure consistency between the negative control group and the bioreactors inoculated with fungi. The species of fungi used in the experimental bioreactors were cultured on the same bulk growth media present in the negative control reactors. As a result the only difference between the negative control reactors and the inoculated bioreactors was the presence of fungi. Using autoclaved media in the negative control reactors for the hydrogen peroxide production experiment may have invalidated results due to the lack of a resident population of bacteria. Intracellular concentrations of hydrogen peroxide, albeit very low concentrations (μM), have been detected in some species bacteria (Seaver and Imlay, 2001), which is why pasteurized media was used in the negative control reactors to ensure that if hydrogen peroxide was detected it would be due to the presence of fungi and not bacteria.

At the start of the experiment, each of the bioreactors were saturated with enough water to create a moist environment, but not enough where significant amounts of free water were present (Pandey, 2003). The approximate volume of water added to achieve complete saturation was 200

mL, similar to the volume used for the batch treatment experiment. This was done to replicate the conditions used to commercially produce fungal derived extracellular compounds via solid-state fermentation (Pandey, 2003). 40 mL samples of effluent were collected at three different time steps, 5 min., 1 hr., and 4 hr. from the bioreactors in each treatment group, respectively. Consistent with the batch and flow through experiments, a 24 hour rest period was permitted in between each time step. The concentration of hydrogen peroxide produced by each bioreactor was assessed using a hydrogen peroxide test kit and a spectrophotometer. A breakdown of the experimental components for this portion of the study are provided in Table 4.

Table 4: Summary of details - hydrogen peroxide production experiment

Hydrogen peroxide production experiment	
# of Time Steps	**Time Steps**
3	5 min., 1 hr., 4 hr.
Groups of Bioreactors	**# of Bioreactors**
Negative Control (Pasteurized)	3
P. betulinus	3
D. confragosa	3
P. eryngii	3
Measured Parameters	
Hydrogen Peroxide Conc. (mg/l)	

2.2 Culturing Fungi and Filter Media Preparation

Figure 6: Fully colonized grain jar

10 mL syringes containing suspended mycelial cultures of all three species of fungi were purchased from Out-Grow (Out-Grow, McConnell, IL). 4 mL of liquid culture was injected into a one-quart glass jar containing sterilized rye berries that was fitted with a self-healing injector port as well as a 0.22 micron gas exchange filter. The jar was then placed in an incubator set to 25°C and the injected liquid culture was allowed to colonize the sterilized rye berries for 4 weeks. Figure 6 depicts a fully colonized jar that contains sterilized rye berries. Once the jar had been completely colonized the fungus was then transferred to a 4 quart bag fitted with a 5 micron filter patch containing a pasteurized bulk growth substrate purchased from Out-Grow (Out-Grow, McConnell, IL). The bulk substrate consisted of a wood based growth media that included sawdust (50% of volume), straw (10% of volume), and rye berries (40% of volume). The inoculated fungus from the jar was thoroughly mixed with the bulk substrate to ensure even colonization. Subsequently the bag containing the inoculated bulk substrate was resealed, placed in a dark room with a temp of 25°C +/- 0.5°C, humidity level of 55%, and allowed to colonize for four weeks. When the bulk media had been fully colonized, 700 cm^3 was then transferred to a reactor vessel.

Following the transfer of colonized bulk media to the containers, the inoculated fungus was allowed to recolonize for two weeks. This was done to ensure that the fungal filtration media was colonized evenly since portions of mycelium were inadvertently damaged or dislodged during the transferring process. The culturing and fungal filter media preparation steps described above were repeated for all three species of fungi. For the negative controls, there were two different

preparation processes. The bulk growth media purchased from Out-Grow was pre-pasteurized by immersing media in a warm water bath at temperature of 73°C for 1 hour. Upon receiving the pre-pasteurized growth media the appropriate volume was then added to corresponding containers (700 cm^3). The autoclaved media was prepared by autoclaving the bulk growth media for 25 minutes at 121°C. The appropriate volume of autoclaved media was added to the corresponding containers (700 cm^3). Prior to both the batch and flow through mode experiments, all of the bioreactors were saturated with deionized water for 15 minutes and then drained completely (Taylor et al., 2014).

2.3 Culturing Bacteria for Synthetic Water Solutions

E. coli was used as the model pathogen for the treatment experiments described above. Slant tubes of *E. coli* K12 were purchased from Carolina Biological Supply (Burlington, NC). Initially 100 mL of deionized water was dispensed into a 250 mL Erlenmeyer flask. 2.5 g of Fluka Analytical nutrient broth (Sigma-Aldrich, Inc., St. Louis, MO) was added to the Erlenmeyer flask containing the deionized water. A stir bar and plate stirrer were used to mix the deionized water and nutrient broth. The mixture was then sterilized by autoclaving it for 15 minutes. After the nutrient media had been sterilized, 5 mL of the mixture was pipetted into two sterile 50 mL falcon tubes (Fisher Scientific, LLC, Suwanee, GA).

One of the falcon tubes containing the nutrient media was inoculated with *E. coli* K12 using a metal culture loop. The metal culture loop was dipped into a slant tube containing *E. coli* after being flamed, and then immersed into the 50 mL falcon tube. The other falcon tube containing only nutrient media served as a negative control. The tubes were then placed in an incubator-shaker set to 25°C and incubated for 24 hours. When the 24-hour incubation period was reached

the tubes were removed and checked for growth. If cultured properly the falcon tube inoculated with the *E. coli* turned opaque, and the negative control tube would remain clear.

The target concentration of the synthetic bacterial water solution was $1x10^3$ cells/mL, which was selected based off of the simulated wastewater influent concentration of *E. coli* used in the study conducted by Chirnside et al. (2013). To determine the correct dilution, a 1 mL sample of pure *E. coli* culture from the inoculated falcon tube was taken, dyed with trypan blue, and pipetted into a hemocytometer. The number of cells in a given square in the hemocytometer were counted using a microscope and a concentration was calculated. Using the equation $V_1C_1 = V_2C_2$ the appropriate cell dilution volumes needed to achieve the desired concentration in the synthetic water stock solution could be calculated. V_1 is the volume of the pure *E. coli* cell culture required to achieve V_2, C_1 is the concentration of the pure *E. coli* culture, V_2 is the desired volume of diluted *E. coli* solution, and C_2 is the desired *E. coli* concentration of the diluted solution. For example the concentration of C_1 was typically on the order of 1×10^9 cells/ml. The desired synthetic water solution volume (V_2) was 5000 ml with a cell concentration (C_2) of $1x10^3$ cells/mL. By diving $\frac{V_2C_2}{C_1}$ the volume of pure *E. coli* culture required to prepare a 5000 ml synthetic water solution with a cell concentration of $1x10^3$ cells/L could be calculated.

2.4 Bacterial Analysis of Aqueous Samples

Influent and effluent samples were analyzed for bacteria using a method from adopted from Liao et al. (2015) that utilized Idexx Colilert enumeration Kits for bacterial analysis (Idexx Laboratories, Inc., Westbrook, ME). 90 mL of deionized water was pipetted into a plastic bottle fitted with a snap lid. 10 mL of sample was added to the bottle for a total volume of 100 mL to achieve a 1 to 10 dilution. After a reagent pack was added to the bottle containing the diluted

sample the bottle was repeatedly inverted for a few seconds in order to completely mix the contents of the bottle. The reagent pack added to the sample contains compounds that cause *E. coli* to fluoresce when metabolized. The sample containing the enumeration reagent was then added to a custom disposable 97 well tray, which was sealed using an Idexx Quanti-Tray Sealer (Idexx Laboratories, Inc., Westbrook, ME), and then incubated for 24 hours to 28 hours at 35°C. Immediately after the incubation period had been reached the number of wells that fluoresced (under UV light) were counted and recorded. These numbers were then converted to a Most Probably Number (MPN) concentration using software provided by Idexx Laboratories (Hurley and Roscoe, 1983; IDEXX, 2016).

2.5 Testing for Antimicrobial Compounds: Hydrogen Peroxide Assay

Samples collected from the hydrogen peroxide production experiment were analyzed for extracellular concentrations of hydrogen peroxide using an Orion AquaMate 7000 vis spectrophotometer (Thermo Fisher Scientific, Inc., Chelmsford, MA) and a Spectroquant Hydrogen Peroxide Test Kit purchased from EMD Millipore (Billerica, MA). The hydrogen peroxide test utilizes phenathroline, which causes hydrogen peroxide to reduce copper(II) ions to copper(I) ions (Millipore, 2016). During the course of this reduction reaction an orange colored compound is produced and can be measured via a spectrophotometer to determine the concentration of hydrogen peroxide (Millipore, 2016).

The spectrophotometer was prepped by loading a pre-programmed hydrogen peroxide test into the machine. The Orion AquaMate 7000 vis spectrophotometer conveniently comes with a USB drive loaded with numerous pre-programmed analysis methods and tests. The USB drive contains a specific pre-programmed method for the EMD Millipore Hydrogen Peroxide Test Kit,

which includes a pre-programmed and calibrated standard curve. The assay was rated for a measuring range of 0.03 – 6.00 mg/l. Samples with hydrogen peroxide concentrations above the measuring range were serially diluted until a reading within the detection limits could be generated. The true hydrogen peroxide concentration could subsequently be calculated by multiplying the concentration by the dilution factor.

Following the preparation of the spectrophotometer, the effluent samples collected from the bioreactors were prepared for analysis. Samples were filtered using a 0.22 µM syringe filter (Restek Corporation, Bellefonte, PA) to reduce turbidity and were then placed into 50 ml sterile falcon tubes (Millipore, 2016). Following the filtration step, 0.50 mL of H_20_2-1 reagent were pipetted into separate 50 mL falcon tubes. Each of the 50 mL falcon tubes possessing H_20_2-1 reagent corresponded to 50 mL falcon tubes containing filtered effluent samples. 8 mL of the filtered samples were pipetted separately into the falcon tubes containing the H_20_2 reagent-1 and agitated for several seconds. Correspondingly 0.50 mL of H_20_2-2 reagent was then pipetted into the falcon tubes containing the filtered sample/ H_20_2 reagent-1 mixtures and then agitated. In accordance with the manufacturer's guidelines, the reaction between the hydrogen peroxide reagents and filtered samples was allowed to proceed for 10 minutes (Millipore, 2016). Once the allotted reaction time had been reached the samples were then immediately analyzed using the spectrophotometer. Samples were loaded into 10 mm glass cuvettes and then placed in the spectrophotometer. The absorbance was measured, converted to a concentration in mg/l, and then recorded. Prior to analyzing the samples, a cuvette consisting of deionized water and the hydrogen peroxide assay reagents was loaded into the spectrophotometer and analyzed. The cuvette containing deionized water and the hydrogen peroxide assay agents served as a blank.

2.6 Statistical Analysis

Data was analyzed using the R statistical language (R Core Team, 2015). The batch treatment experiment consisted of three different treatments and seven distinct treatment times as previously stated. The treatments are abbreviated as the following: the negative control treatment is abbreviated as **"Neg"**, the *P. betulinus* treatment is abbreviated as **"P. bet"**, and *D. confragosa* treatment is abbreviated as **"D. con"**. The response variable for the batch experiment was the percent *E. coli* reduction. Negative percent *E. coli* reduction values corresponded with the removal of *E. coli* and positive percent *E. coli* reduction values corresponded with the export of *E. coli*. The normality of the data was evaluated by using a Q-Q plot and a Shapiro-Wilk test, with percent *E. coli* reduction as the input variable for both methods. The Q-Q plot provided a visual assessment of normality by showing the distribution of the data and generating a reference line, normality was ascertained by observing how many of the data points deviated from the reference line. If a significant portion of data points deviated from the reference line then the data is non-normally distributed. A quantitative assessment of normality was performed using a Shapiro-Wilk test, with the alpha value set to 0.05. The null hypothesis for a Shapiro-Wilk test is that the data is derived from a normal distribution. A p-value above 0.05 results in the acceptance the null hypothesis, while a p-value below 0.05 results in the rejection of the null hypothesis, indicating that the data is derived from a non-normal distribution. Additional statistical analyses of the data were performed using two different non-parametric approaches. The first approach involved using binomial tests and the Marascuilo procedure. Binomial tests were performed for each group within a factor (i.e. treatment: P. bet group, D. con group, Neg control group) individually using the built in binom.test function in R. The binomial test is a non-parametric technique that predicts the randomness of an event and generates a probability value that predicts

the likelihood of an outcome occurring for one event or trial (similar to a proportion test) (Pereira et al., 2009). Before performing the binomial test, data for each group within a factor was aggregated. For example for the P. bet group within the treatment factor there were triplicate bioreactors each of which were allowed to treat influent water for seven different treatment times, aggregation of the data resulted in a total of 21 values.

There were two input parameters for the binomial test. One input was the total number of observations (i.e. 21) and the other input was the number of successes, which in this case is treatments that successfully removed *E. coli*. The null hypothesis for the binomial test is that the outcomes (removal of *E. coli* and no removal of *E. coli*) are equally likely to occur, indicating a random probability (Pereira et al., 2009). The binomial test generates a p-value and predicts the probability of *E. coli* removal occurring. A low p-value results in the rejection of the null hypothesis that the two events are equally likely to occur.

While the binomial test can determine differences within a single treatment it cannot determine differences between treatments. The Marascuilo procedure is a binary statistical technique that compares the probability of success between treatments (i.e. P. bet treatment vs. D. con treatment or 5 min. time treatment vs. 12 hr. time treatment) (Zwick and Marascuilo, 1984). Statistical parameters incorporated in the Marascuilo procedure include the critical value and critical range, both of which are calculated for each pair of probability. The critical value is calculated by subtracting the two probability of a pair from one another and taking the absolute value, whereas the critical range is calculated by multiplying the square root of the chi-square statistic by the square root of the observed difference between the two probabilities. If the calculated critical value is greater than the critical range then the difference between the probability is deemed to be significant (Zwick and Marascuilo, 1984). The Marascuilo procedure was performed in R for

both treatment and time, individually. Other non-parametric approaches used to analyze the data from this portion of the study included the Kruskal-Wallis rank sum test and the Kruskal-Wallis multiple comparison test. The Kruskal-Wallis rank sum test was performed in R using the built in kruskal.test function, with the significance level set to 0.05, and the Kruskal-Wallis multiple comparison test was performed in R using the kruskalmc function from the package 'pgirmess" (Giraudoux, 2014). For the two factors, treatment and time, the Kruskal-Wallis rank sum test and the Kruskal-Wallis multiple comparison test were performed individually. The primary difference between the Kruskal-Wallis multiple comparison test and the Marascuilo procedure is that numeric data is used for the former and binary data is used for the latter (Giraudoux, 2014; Zwick and Marascuilo, 1984). Initially data values were binned into a binary format for the Marascuilo procedure, and were categorized as either "No *E. coli* Removal" or "*E. coli* Removal". This was needed as a result of the heavily binary distribution visually observed in the Q-Q plot generated from the original numeric data. However the original numeric data was used in the Kruskal-Wallis multiple comparison test.

Analogous to the batch treatment experiment, percent *E. coli* reduction served as the response variable for the flow through treatment experiment, however in contrast there were five different treatments and three unique treatment times. Percent *E. coli* reduction was used to account for any variations in the influent E. coli concentration, which could affect the absolute *E. coli* reduction value calculated (i.e. influent concentration – effluent concentration). Influent concentrations of *E. coli* did vary between treatment runs primarily due to varying concentrations of the *E. coli* stock culture solutions used to make the synthetic water solutions. The treatments are abbreviated as the following: the negative control treatment containing only pasteurized media is abbreviated as **"Neg A"**, the autoclaved negative control treatment is abbreviated as

"Neg B", the *P. eryngii* treatment is abbreviated as **"P. ery"**, the *P. betulinus* and *D. confragosa* treatments are abbreviated as cited earlier. Normality was assessed using, a Q-Q plot and a Shapiro-Wilk test (alpha value 0.05) with percent *E. coli* reduction as the input variable.

The analysis of the flow through treatment experimental data was conducted using quantitative non-parametric methods, the Kruskal-Wallis rank sum test and Kruskal-Wallis multiple comparison test, which are suitable for non-normally distributed data sets with unequal sample sizes (Giraudoux, 2014). An alpha value of 0.05 as used to determine significance. Identical to the batch treatment analyses, Kruskal-Wallis rank sum tests were performed using the built in kruskal.test function and Kruskal-Wallis multiple comparison tests was performed in R using the kruskalmc function from the package 'pgirmess' (Giraudoux, 2014). Comparable to the batch treatment analysis, the Kruskal-Wallis rank sum test and Kruskal-Wallis multiple comparison test were performed individually for the two factors, treatment and time.

The hydrogen peroxide production experiment consisted of three treatments, with the *P. betulinus*, *D. confragosa*, and *P. eryngii* treatments abbreviated as previously defined. Normality was assessed using a Q-Q plot and a Shapiro-Wilk test with hydrogen peroxide concentration as the input variable, the alpha value was 0.05. A parametric statistical approach was used to analyze the data, specifically a repeated measures two-way ANOVA and was performed utilizing the ezANOVA function from the R package 'ez' (Lawrence, 2013). The significance level for the two-way ANOVA test was 0.05. Successively Tukey's HSD test was employed post-hoc to perform pairwise comparisons of the different treatments for each time step, an alpha value of 0.05 was used to ascertain significance.

3. Results

3.1 Batch Experiment *E. coli* Reduction

The normality of the data from the batch treatment experiment was evaluated using a Q-Q plot and a Shapiro-Wilk test, with percent *E. coli* reduction as the input variable. As shown in Figure 7, the Q-Q plot illustrates that the data is not normally distributed, instead exhibiting a binary distribution.

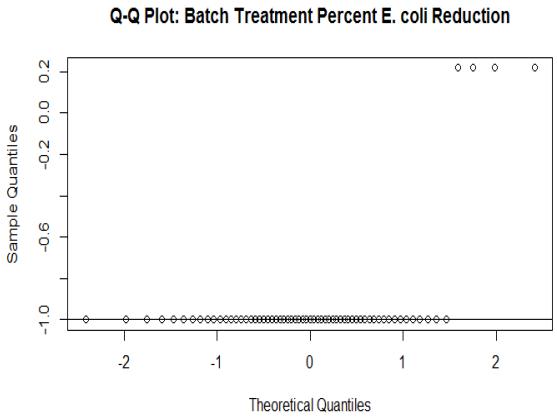

Figure 7: Q-Q Plot for batch treatment percent *E. coli* reduction

The Shapiro-Wilk test further confirmed the non-normality of the data, generating an extremely low p value of 3.768^{-16} (alpha value of 0.05), resulting in the rejection of the null hypothesis that the data is derived from a normal distribution. Initial statistical analyses involved performing binomial tests for the individual treatment groups and time groups, the results are provided below in Tables 5 and 6.

Table 5: Binomial test for individual bioreactor **treatment** groups

Binomial test for individual treatment groups		
Group	**P-value**	**Probability of Removal**
Neg	9.54×10^{-7}	1.00
P. bet	7.20×10^{-3}	0.81
D. con	9.54×10^{-7}	1.00

Key: Neg = Negative control with pasteurized growth media, P. bet = *P. betulinus*, D. con = *D. confragosa*, P. ery = *P. eryngii*

Table 6: Binomial test for individual bioreactor **time** groups

Binomial test for individual bioreactor Groups		
Group	**P-value**	**Probability of Removal**
5 min.	4.00×10^{-2}	1.00
30 min.	0.18	0.78
1 hr.	0.18	0.78
3 hr.	4.00×10^{-2}	1.00
12 hr.	4.00×10^{-2}	1.00
24 hr.	4.00×10^{-2}	1.00
48 hr.	4.00×10^{-2}	1.00

Table 7: Summary of Marascuilo procedure results for **treatment**

Marascuilo procedure results for treatment			
Pair	**Critical Value**	**Critical Range**	**Significant**
Neg – P. bet	0.19	0.21	No
Neg – D. con	0.01	0.05	No
P. bet – D. con	0.19	0.21	No

Table 8: Summary of Marascuilo procedure results for **time**

Marascuilo procedure results for time			
Pair	**Critical Value**	**Critical Range**	**Significant**
5 min. – 30 min.	0.21	0.23	No
5 min. – 1 hr.	0.01	0.05	No
5 min. – 3 hr.	0.01	0.05	No
5 min. – 12 hr.	0.01	0.05	No
5 min. – 24 hr.	0.01	0.05	No
5 min. – 48hr.	0.01	0.05	No
30 min. – 60 min.	0.00	0.01	No
30 min. – 3 hr.	0.21	0.23	No
30 min. – 12 hr.	0.21	0.23	No
30 min. – 24 hr.	0.21	0.23	No
30 min. – 48 hr.	0.21	0.23	No
1 hr. – 3 hr.	0.00	0.01	No
1 hr. – 12 hr.	0.00	0.01	No
1 hr. – 24 hr.	0.00	0.01	No
1 hr. – 48 hr.	0.00	0.01	No
3 hr. – 12 hr.	0.00	0.01	No
3 hr. – 24 hr.	0.00	0.01	No
3 hr. – 48 hr.	0.00	0.01	No
12 hr. – 24 hr.	0.00	0.01	No
12 hr. – 48 hr.	0.00	0.01	No

24 hr. – 48 hr.	0.00	0.01	No

The p-values in Table 5 illustrate that the null hypothesis can be rejected for all of the treatment groups, implying that they are all likely to remove *E. coli*. Outlined in Table 6 are the results from the binomial tests performed for each individual time group, with p-values indicating the rejection of the null hypothesis for all of the time groups except for the 30 min. and 60 min. groups. The p-values for the 30 min. and 60 min. time groups (0.18 for both) implies that there is some reduced performance during these two time periods, though the calculated probability (0.78 for both) points to the removal of *E. coli* as the likely outcome to occur for a single event. Table 7 summarizes the results from the Marascuilo procedure performed for the groups within the treatment factor, which compares the probability or proportion of removal for each treatment group with one another. These results insinuate that the probability of removal does not differ between the treatment groups, signifying that the treatment groups are all likely to remove *E. coli*. Additionally the results from the Marascuilo procedure (Table 8) for the time effect shows that all of the time groups are equally likely to remove *E. coli*, denoting that the probability of success between the time groups are not statistically different. These results are substantiated by the fact that all of the bioreactors removed *E. coli* at each time step, reducing an average influent *E. coli* concentration of 19,863 cfu/ 100 mL to a concentration just above or below the lower detection limit, with the exception of two fungal bioreactors containing *P.* betulinus, which only failed to remove *E. coli* at the 30 and 60-minute time steps. At the 30 minute and 60 minute time steps the effluent from two fungal bioreactors inoculated with *P. betulinus* contained concentrations of *E. coli* above the maximum detection limit of 24,196 cfu/ 100 mL. Nonetheless at subsequent time steps the *E. coli* concentrations in the effluent from these two bioreactors reverted back to values below the lower detection limit. These breakthroughs may be related to

temporary changes in the metabolism of the fungi. To further validate these statistical findings Kruskal-Wallis rank sum tests and Kruskal-Wallis multiple comparison tests incorporating a significance level of 0.05 were performed. On a fundamental level the Kruskal-Wallis rank sum test evaluates the overall effect of a factor, while the Kruskal-Wallis multiple comparison test assesses the difference between groups within a factor (i.e. different species of fungi within the treatment factor). For the Kruskal-Wallis multiple comparison test, pairwise relationships that are found to be significant are labeled as "True" and pairs that are not found to be significant are labeled as "False". Results for the Kruskal-Wallis rank sum and multiple comparison tests are provided in Tables 9-11.

Table 9: Summary of batch treatment data results from Kruskal-Wallis rank sum test for **treatment** and **time**

Batch treatment Kruskal-Wallis rank sum test for treatment and time			
Factor	**Chi-Squared**	**Df**	**P-value**
Treatment	0.11	2	0.95
Time	4.77	6	0.57

Table 10: Summary of batch treatment data results from Kruskal-Wallis multiple comparison test for **treatment**

Batch treatment Kruskal-Wallis Multiple Comparison test for treatment			
Pair	**Observed Difference**	**Critical Difference**	**Difference**
D. con – Neg	1.10	13.54	FALSE
D. con – P. bet	1.33	13.54	FALSE
Neg – P. bet	0.24	13.54	FALSE

Key: Neg = Negative control with pasteurized growth media, P. bet = *P. betulinus*, D. con = *D. confragosa*, P. ery = *P. eryngii*

Table 11: Summary of batch treatment data results from Kruskal-Wallis multiple comparison test for **time**

Batch treatment Kruskal-Wallis multiple comparison test for time			
Pair	**Observed Difference**	**Critical Difference**	**Difference**
5 min. – 30 min.	5.22	26.25	FALSE
5 min. – 1 hr.	5.22	26.25	FALSE
5 min. – 3 hr.	10.33	26.25	FALSE
5 min. – 12 hr.	10.33	26.25	FALSE
5 min. – 24 hr.	7.11	26.25	FALSE
5 min. – 48hr.	0.66	26.25	FALSE
30 min. – 60 min.	0.66	26.25	FALSE
30 min. – 3 hr.	5.11	26.25	FALSE
30 min. – 12 hr.	5.11	26.25	FALSE
30 min. – 24 hr.	1.89	26.25	FALSE
30 min. – 48 hr.	4.56	26.25	FALSE
1 hr. – 3 hr.	5.11	26.25	FALSE
1 hr. – 12 hr.	5.11	26.25	FALSE
1 hr. – 24 hr.	1.89	26.25	FALSE
1 hr. – 48 hr.	4.56	26.25	FALSE
3 hr. – 12 hr.	0.00	26.25	FALSE
3 hr. – 24 hr.	3.22	26.25	FALSE
3 hr. – 48 hr.	9.67	26.25	FALSE
12 hr. – 24 hr.	3.22	26.25	FALSE
12 hr. – 48 hr.	9.67	26.25	FALSE
24 hr. – 48 hr.	6.44	26.25	FALSE

The results from Kruskal-Wallis multiple comparison test indicate that the treatment groups are not statistically different. Furthermore the Kruskal-Wallis rank sum test provides statistical evidence that treatment as an overall factor did not influence the removal of *E. coli* (Table 10). Likewise, time as an overall factor and the difference between time groups were found to not be statistically significant (Tables 9 and 11). The complete data set for the batch treatment experiment can be found in Appendix A. Sub-Section I.

3.2 Flow Through Experiment *E. coli* Reduction

Similar to the batch treatment data analysis, the normality of the flow through experimental data was assessed using a Q-Q plot and a Shapiro-Wilk test. Percent *E. coli* reduction was used as the input variable for the normality tests stated above. As shown in Figure 8, the Q-Q plot indicates that the data is not normally distributed. The Shapiro-Wilk test further confirmed that the data is not normally distributed, producing a p-value of 1.02×10^{-11}, which is well below the significance threshold value of 0.05, resulting in the rejection of the null hypothesis that the data is derived from a normal distribution. Analogous to the batch treatment experiment, the data for the flow through experiment is stratified and exhibits a binary distribution. Consequently, non-parametric Kruskal-Wallis rank sum tests and Kruskal-Wallis multiple comparison tests were used to analyze the data. The Kruskal-Wallis rank sum test is a non-parametric alternative to a one-way ANOVA test, and is applicable to unbalanced data with a non-normal distribution, which was the case for this experimental data set (Siegel and Castellan, 1988). Tables 12, 13 and 14 below summarize the results from the Kruskal-Wallis rank sum test and the Kruskal-Wallis multiple comparison tests, significant p-values and pairs are in bold.

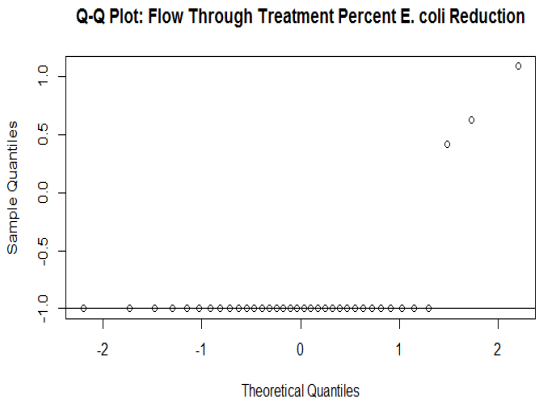

Figure 8: Q-Q Plot for flow through treatment percent *E. coli* reduction

Table 12: Summary of flow through data results from Kruskal-Wallis rank sum test for **treatment** and **time**

Batch treatment Kruskal-Wallis rank sum test for treatment and time			
Factor	**Chi-Squared**	**Df**	**P-value**
Treatment	27.62	4	**1.49×10^{-5}**
Time	0.38	2	0.83

Table 13: Summary of flow through data results from Kruskal-Wallis multiple comparison test for **treatment**

Kruskal-Wallis multiple comparison test – treatment			
Pair	**Observed Difference**	**Critical Difference**	**Difference**
D. con – Neg A	18.50	17.54	True
D. con – Neg B	0.00	13.87	False
D. con – P. bet	1.83	12.40	False
D. con – P. ery	0.00	12.40	False
Neg A – Neg B	18.50	18.61	False
Neg A – P. bet	18.50	17.54	True
Neg A – P. ery	18.50	17.54	True
Neg B – P. bet	1.83	13.87	False
Neg B – P. ery	0.00	12.40	False
P. bet – P. ery	1.83	12.40	False

Key: P. bet = *P. betulinus*, D. con = *D. confragosa*, P. ery = *P. eryngii*, Neg A = Pasteurized growth media negative control, Neg B = Autoclaved growth media negative control

Table 14: Summary of flow through data results from Kruskal-Wallis multiple comparison test for **time**

Kruskal-Wallis multiple comparison test – time			
Pair	**Observed Difference**	**Critical Difference**	**Difference**
5 min. – 10 min.	9.83	9.15	**True**
5 min. – 20 min.	3.67	9.15	False
10 min. – 20 min.	6.17	9.15	False

Treatment was found to be statistically significant, while time was not as shown in Table 12. For the Kruskal-Wallis multiple comparison test three treatment pairs, all including Neg A, were found to be statistically significant as shown in Table 13. Interestingly the Neg A - Neg B pair was found to be marginally not significant (observed difference 18.50 and critical difference 18.61), despite the fact that the Neg B reactors removed *E. coli* completely, while the Neg A reactor failed to remove any. Further investigation of the data set revealed that the lower number of Neg B reactors compared to the fungal reactors (e.g. D. con group of bioreactors) resulted in the Neg A – Neg B pair being classified as not significant. Replicates for each group of

bioreactors were not used due to technical issues with splitting the flow to achieve uniform flow rates for 15 reactors. Uniform flow rates could be achieved using 12 reactors, which is why an unbalanced design was incorporated. Overall the D. con, P. bet, P. ery, and Neg B treatments removed *E. coli* completely, while the Neg A treatment failed to remove any *E. coli* and actually exported higher concentrations. On average the D. con bioreactors achieved an *E. coli* reduction of 100%, the P. bet group attained an average *E. coli* reduction of 99.9%, the P. ery group of bioreactors achieved an average *E. coli* reduction of 100%, and the Neg B group of bioreactors attained an average *E. coli* reduction of 100%, while the Neg A bioreactor on average exported a 70% increase in *E. coli* concentration. The essential finding in terms of treatment is that all of the reactors removed *E. coli*, producing effluent concentrations at or below the lower detection limit, with the exception of the negative control reactor containing only pasteurized growth media, which exported *E. coli*.

3.3 Hydrogen Peroxide Production Experiment

Following previous approaches, a Q-Q plot and a Shapiro-Wilk test were used to assess the normality of the data from the hydrogen peroxide production study. Initially the data appeared to be skewed, however a log transformation resulted in a normal distribution (Figure 9). The Shapiro-Wilk test generated a p-value of 0.467, confirming a normal distribution of the transformed data indicating that the data is derived from a normal distribution since the calculated p-value was above the predetermined significance level of 0.05.

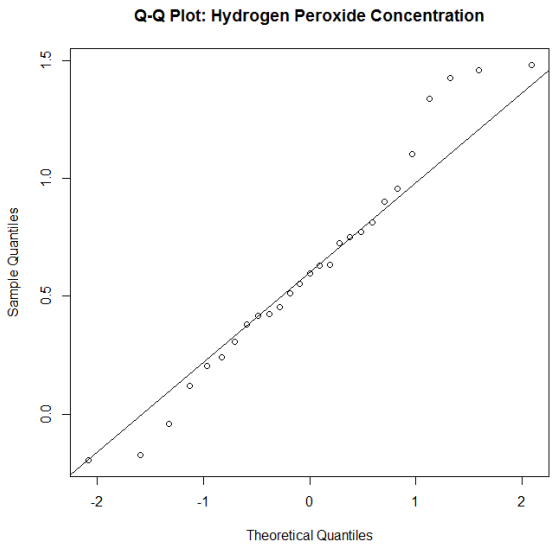

Figure 9: Q-Q Plot for hydrogen peroxide concentration

A two-way ANOVA was performed in R and an alpha value of 0.05 was used as the threshold value for determining significance.

Table 15: Two-Way ANOVA table for hydrogen peroxide concentration with **treatment and time as factors**

Effect	DFn	DFd	F-statistic	P-value
Treatment	2	6	79.980	**4.725×10^{-5}**
Time	1	6	17.367	**5.896×10^{-3}**
Treatment:Time	2	6	2.115	0.202

As shown in Table 15 both treatment and time were significant, but the interaction between the two was not. A Tukey's HSD test was employed post-hoc to perform pairwise comparisons of the different treatment groups to determine their effect on hydrogen peroxide production at each time step. Tables 16, 17 and 18 summarize the results from the Tukey's HSD tests, p-values that are significant are in bold.

Table 16: Tukey's HSD test comparison values for hydrogen peroxide production – **5 Min**. time step

Pair	95 % Lower Conf. Interval	95 % Upper Conf. Interval	P-value
P. bet – D. con	-0.774	-0.1262	**0.01**
P. ery – D. con	-0.0996	0.7469	**0.02**
P. ery – P. bet	-0.5495	1.1968	**4.0×10^{-4}**

Key: P. bet = *P. betulinus*, D. con = *D. confragosa*, P. ery = *P. eryngii*

Table 17: Tukey's HSD test comparison values for hydrogen peroxide production - **1 hr**. time step

Pair	95 % Lower Conf. Interval	95 % Upper Conf. Interval	P-value
P. bet – D. con	-0.6629	0.0212	0.06
P. ery – D. con	0.1130	0.7971	**0.02**
P. ery – P. bet	0.4338	1.1180	**1.0×10^{-2}**

Table 18: Tukey's HSD test comparison values for hydrogen peroxide production - **4 hr**. time step

Pair	95 % Lower Conf. Interval	95 % Upper Conf. Interval	P-value
P. bet – D. con	-0.6277	0.0847	0.16
P. ery – D. con	0.2381	0.9504	**9.74×10^{-4}**
P. ery – P. bet	0.5095	1.2219	**8.35×10^{-6}**

The majority of the comparisons are significant at every time step with the exception of one pair, the P. bet – D. con pair at the 1 hr. and 4 hr. time steps. Moderate to high concentrations of hydrogen peroxide were detected in all of the bioreactors inoculated with fungi. It should be noted that the out of the three negative control reactors two of them did not produce any hydrogen peroxide, while one reactor did (max conc. of 6 mg/L at the 4 hr. time step). Further investigation and inspection of the negative control reactor that did produce hydrogen peroxide revealed growth of an unidentified species of fungi, and as a result was considered to be an outlier. The highest concentrations of hydrogen peroxide were detected in the effluent from the fungal bioreactors containing P. ery as shown in Table 19.

Table 19. Average hydrogen peroxide concentration per treatment group for each time step

Average Hydrogen Peroxide Concentration		
Bioreactor Group	**Time**	**Avg. Concentration (mg/l)**
P. bet	5 min.	0.74
	1 hr.	2.33
	4 hr.	3.27
D. con	5 min.	2.10
	1 hr.	4.84
	4 hr.	4.38
P. ery	5 min.	5.73
	1 hr.	14.57
	4 hr.	28.69

Key: P. bet = *P. betulinus,* D. con = *D. confragosa,* P. ery= *P. eryngii*

4. Discussion

The concept of using fungal bioreactors or "mycofilters" to remove bacterial pathogens from water has been explored in previous studies, however none of these studies attempted to identify the explicit modes of action responsible for the removal of bacteria (Chirnside et al., 2013; Rogers, 2012; Stamets et al., 2013; Taylor et al., 2014; Thomas et al., 2009). The results from this study reveal some possible modes of action responsible for the removal of *E. coli*. Significant levels of hydrogen peroxide were detected in the effluent of all of the fungal bioreactors, with the fungal bioreactors containing P. ery producing the highest concentrations, up to 30.5 mg/l or 0.896 mM. Although brown-rot fungi tend to be more prolific producers of hydrogen peroxide, P. ery a white-rot fungus, was theorized to have produced the highest concentrations due to the fact that it was the most aggressive colonizer of the growth media (Koenigs, 1974). The other species, P. bet, a brown-rot fungus, and D. con, a white-rot fungus, produced moderate levels of hydrogen peroxide (6.52 mg/l or 0.19 mM and 3.94 mg/l or 0.116 mM, respectively). Hyslop et al. (1995) investigated the effects of continuously exposing *E. coli* cells to a constant stream of hydrogen peroxide and reported that during a 1 hr. exposure, a time-dependent killing effect was observed at concentrations greater than 0.5 mM. The hydrogen peroxide concentrations detected in the effluent from the fungal bioreactors in this study were close to or exceeded this value (maximum concentration of 0.896 mM detected). An interesting component of Hyslop's experiment that diverges from other studies was that hydrogen peroxide was constantly applied. This is relevant these results because it is likely that the fungi evaluated here were continuously producing hydrogen peroxide. Hydrogen peroxide is the compound that the fungi use to de-polymerize the wood-based growth substrates during colonization (Koenigs, 1974). The hydrogen peroxide portion of this study demonstrates a proof of concept that these

species of wood-rot fungi can produce antimicrobial agents in high enough concentrations to inhibit bacteria, thus providing some evidence of a natural disinfection mechanism in the fungal bioreactors. However it should be noted that the required bacteriostatic or bactericidal concentrations of hydrogen peroxide will vary by species and by strain. Although inhibitory concentrations of hydrogen peroxide were detected, it is highly probable that other compounds with antibacterial properties were being produced by these species of fungi as well. *P. betulinus* has been shown to produce the antibiotic piptamine, although little information about the antibiotic compound is known (Schlegel et al., 2000). During the bulk culturing phase several different colored dyes and pigments were observed in the growth media being colonized by the fungi. When culturing *P. eryngii* in the bulk growth media, a violet/red pigment was visually observed in significant quantities.

A common class of compounds associated with colored pigments in numerous species of fungi are quinones (Velíšek and Cejpek, 2011). While at this point the colored pigment observed cannot be determined, the presence of this violet/red pigment does likely indicate the production of phenolic compounds. The oxidation of phenolic compounds to quinones is a common chemical reaction. Numerous species of wood-rot fungi, including *P. eryngii*, have been shown to produce antimicrobial phenolic compounds such as 2,4-Dihydroxybenzoic acid (Alves et al., 2013). It is possible that multiple antimicrobial compounds are being produced, and that the production of these complexes is heavily influenced by factors such as the growth stage of the fungus. For example hydrogen peroxide, a growth associated compound, may be present in higher concentrations during the colonization stage, and once the fungus has reached a stationary growth phase or reproductive stage (i.e. fruiting bodies begin to form), antimicrobial secondary

metabolites may be the active antibacterial agents. Nevertheless, the detection of inhibitory concentrations of hydrogen peroxide shows a plausible mode of action.

In terms of operational components, treatment time did not correlate with the removal of *E. coli* for the batch or flow through treatment experiments, supporting the results reported by (Rogers, 2012). For the batch treatment experiment there was not a distinct difference in terms of bacterial removal between the negative control reactors and the reactors containing fungi. Contrastingly, there was a significant difference between the different treatment groups for the flow through experiment; the autoclaved negative control and the bioreactors inoculated with fungi removed *E. coli*, the pasteurized negative control reactor failed to remove any *E. coli* and even exported higher concentrations. The absence of bacteria in the bioreactors containing autoclaved growth media may have played a part in the enhanced performance of these reactors relative to the negative control with pasteurized media due to a greater adsorption capacity as a result of the media matrix being free of bacteria. However it is theorized that both groups of the negative control reactors would eventually start exporting bacteria due to the absence of an antagonistic biological component preventing bacteria from subsisting, while the fungal bioreactors would not, as a result of producing antibacterial or antimicrobial compounds. Evidence of this was supported by a follow up experiment that involved inundating the reactors with only deionized water, flushing them after a 15 minute saturation period, and enumerating the *E. coli* concentration of the flushed effluent samples. *E. coli* was not detected in any of the effluent samples from the bioreactors inoculated with fungi. However out of the three negative control reactors (one containing pasteurized growth media and two containing autoclaved growth media), *E. coli* was detected in the effluent of the pasteurized growth media negative control and in one of the autoclaved growth media negative controls. The *E. coli* concentration in the effluent

from these two reactors were 7,701 cfu/ mL and 14,136 cfu/ 100 mL, respectively. Results from the follow up experiment suggest that *E. coli* was unable to subsist in the bioreactors containing fungi due to an active antibacterial component (i.e. production of hydrogen peroxide), while two of the negative control reactors exported *E. coli* due to a lack of an antimicrobial agents.

One of the most significant findings from this study was the difference in removal between the pasteurized negative control reactor and the bioreactors inoculated with fungi for the flow through treatment experiment. To reiterate, the only difference between the fungal bioreactors and the negative control containing only pasteurized growth media was the presence of fungi, since the species of fungi evaluated were cultured in bulk on the pasteurized growth media. The fungal bioreactors performed extremely well, while the negative control reactor did not. This relationship suggests that the presence of fungi plays an important role in the removal of *E. coli*.

5. Conclusion

This study provides insight into the modes of action responsible for the removal and elimination of bacteria in fungal bioreactors used for water treatment applications. In addition to uncovering plausible bacterial removal mechanisms, namely the production of the hydrogen peroxide, an antimicrobial agent, several new species suitable for use in water treatment fungal bioreactors have been identified. One of the most important results from this study is the proof of concept that species of fungi capable of producing extracellular antimicrobial compounds can be exploited in fungal bioreactor systems for removing bacteria from water. This is significant because harnessing this natural disinfection mechanism may be more effective in removing bacteria than employing a predatory removal mechanism due to the fact that soluble extracellular antimicrobial compounds can diffuse through the water to eradicate bacterial pathogens, while a

predatory mechanism relies on bacteria coming into direct contact with fungal mycelium. Previous studies have evaluated species of fungi that have been thought to operate under the latter mechanism (Rogers, 2012; Taylor et al., 2014), while this study assessed species that operate under the former mechanism.

Despite the fact that sizable concentrations of hydrogen peroxide were detected further studies need to be done confirm that the antimicrobial compound being produced are indeed responsible for the *E. coli* reduction. This can be done by simply concentrating effluent from a fungal bioreactor via lyophilization (freeze-drying and evaporating) and assaying serial dilutions of the concentrated fungal effluent against bacteria. Dose-response curves can be generated to determine the efficacy of the fungal effluent concentrate against bacteria. Ideally if there are any extracellular antibacterial or antimicrobial compounds produced by the fungus, they should be present in the effluent. If present, the fungal bioreactor effluent concentrate should exhibit either bacteriostatic or bactericidal behavior when in contact with the bacteria of interest, which would provide firm evidence that a natural disinfection mechanism is being employed.

Significantly more research needs to be conducted in regards to investigating other operational characteristics of fungal bioreactors in order to improve performance. Examples include investigating whether or not there is a priming effect, the occurrence of bacterial breakthrough over the lifetime of fungal bioreactors, and the ability of fungal bioreactors to remove species of bacteria other than *E. coli*.

Obtaining a better understanding of the underlying functional principles of fungal bioreactors used for water treatment will allow for the development of an optimized system. This study provides further support for the concept of using fungal based systems for water treatment and will hopefully contribute to the advancement of this technology. The ultimate objective is to

eventually produce a hyper-functional system that can treat recycled irrigation water, agricultural drainage, domestic wastewater, and possibly, drinking water.

6. Acknowledgements

This work was supported by a grant from the United State Department Agriculture - Natural Resource Conservation Service

7. References

Alves, M. J., Ferreira, I. C., Dias, J., Teixeira, V., Martins, A., & Pintado, M. (2012). A review on antimicrobial activity of mushroom (Basidiomycetes) extracts and isolated compounds.

Alves, M. J., Ferreira, I. C., Froufe, H. J., Abreu, R., Martins, A., & Pintado, M. (2013). Antimicrobial activity of phenolic compounds identified in wild mushrooms, SAR analysis and docking studies. *Journal of Applied Microbiology, 115*(2), 346-357.

Artursson, V., Finlay, R. D., & Jansson, J. K. (2006). Interactions between arbuscular mycorrhizal fungi and bacteria and their potential for stimulating plant growth. *Environmental microbiology, 8*(1), 1-10.

Ashbolt, N. J. (2004). Microbial contamination of drinking water and disease outcomes in developing regions. *Toxicology, 198*(1), 229-238.

Balandrin, M. F., Klocke, J. A., Wurtele, E. S., & Bollinger, W. H. (1985). Natural plant chemicals: sources of industrial and medicinal materials. *Science, 228*(4704), 1154-1160.

Barrett, M. E. (2003). Performance, cost, and maintenance requirements of Austin sand filters. *Journal of Water Resources Planning and Management, 129*(3), 234-242.

Barron, G. (1988). Microcolonies of bacteria as a nutrient source for lignicolous and other fungi. *Canadian Journal of Botany, 66*(12), 2505-2510.

Barron, G. (2003). Predatory fungi, wood decay, and the carbon cycle. *Biodiversity, 4*(1), 3-9.

Barron, G., & Thorn, R. (1987). Destruction of nematodes by species of Pleurotus. *Canadian Journal of Botany, 65*(4), 774-778.

Barron, G. L. (1992). Lignolytic and cellulolytic fungi as predators and parasites. *The fungal community: its organization and role in the ecosystem, 9*, 311-326.

Batz, M. B., Hoffmann, S., & Morris Jr, J. G. (2012). Ranking the disease burden of 14 pathogens in food sources in the United States using attribution data from outbreak investigations and expert elicitation. *Journal of Food Protection, 75*(7), 1278-1291.

Blumenthal, U. J., & Peasey, A. (2002). Critical review of epidemiological evidence of the health effects of wastewater and excreta use in agriculture. *unpublished document prepared for World Health Organization, Geneva, www. who. int/water_sanitation_ health/wastewater/whocriticalrev. pdf*.

Bycroft, B. W. (1987). *Dictionary of Antibiotics & Related Substances*: CRC Press.

Chirnside, A. E., Harris, J. P., & Gregory, N. (2013, June). Degradation of harmful bacteria in simulated wastewater by the white rot fungus Pleurotus ostreatus. In *International Symposium on Bioremediation and Sustainable Environmental Technologies ,Jacksonville, FL*.

Christensen, J., & Linden, K. G. (2003). How particles affect UV light in the UV disinfection of unfiltered drinking water. *American Water Works Association. Journal, 95*(4), 179.

Clary, J., Jones, J., Urbonas, B., Quigley, M., Strecker, E., & Wagner, T. (2008). Can stormwater BMPs remove bacteria? New findings from the international stormwater BMP database. *Stormwater, 9*(May (3)).

Cooke, R. C. (1962a). Behaviour of nematode-trapping fungi during decomposition of organic matter in the soil. *Transactions of the British Mycological Society, 45*(3), 314-320.

Cooke, R. C. (1962b). The ecology of nematode‐trapping fungi in the soil. *Annals of Applied Biology, 50*(3), 507-513.

Davies, C., & Bavor, H. (2000). The fate of stormwater‐associated bacteria in constructed wetland and water pollution control pond systems. *Journal of Applied Microbiology, 89*(2), 349-360.

Fakoya, S., Adegbehingbe, K. T., & Ogundiimu, A. A. (2013). Biopharmaceutical Assessment of Active Components of Deadaleopsis confragosa and Ganoderma lucidum. *Open Journal of Medical Microbiology, 03*(02), 135-138.

Fermor, T., & Wood, D. (1981). Degradation of bacteria by Agaricus bisporus and other fungi. *Journal of General Microbiology, 126*(2), 377-387.

Frey-Klett, P., Burlinson, P., Deveau, A., Barret, M., Tarkka, M., & Sarniguet, A. (2011). Bacterial-fungal interactions: hyphens between agricultural, clinical, environmental, and food microbiologists. *Microbiology and Molecular Biology Reviews, 75*(4), 583-609.

Gangadevi, V., Sethumeenal, S., Yogeswari, S., & Rani, G. (2008). Screening endophytic fungi isolated from a medicinal plant, Acalypha indica L. for antibacterial activity. *Indian journal of Science and Technology, 1*(5), 1-6.

Giraudoux, P. (2014). Data analysis in ecology. *R Package version, 1*(9).

Haile, R. W., Witte, J. S., Gold, M., Cressey, R., McGee, C., Millikan, R. C., . . . Harmon, P. (1999). The health effects of swimming in ocean water contaminated by storm drain runoff. *Epidemiology, 10*(4), 355-363.

Hammes, F., Berger, C., Köster, O., & Egli, T. (2010). Assessing biological stability of drinking water without disinfectant residuals in a full-scale water supply system. *Journal of Water Supply: Research and Technology-AQUA, 59*(1), 31-40.

Harrigan, W. F. (1998). *Laboratory methods in food microbiology*: Gulf Professional Publishing.

Hathaway, J., Hunt, W., Graves, A., & Wright, J. (2011). Field evaluation of bioretention indicator bacteria sequestration in Wilmington, North Carolina. *Journal of Environmental Engineering*.

Hathaway, J., Hunt, W., & Jadlocki, S. (2009). Indicator bacteria removal in storm-water best management practices in Charlotte, North Carolina. *Journal of Environmental Engineering, 135*(12), 1275-1285.

Hatt, B. E., Fletcher, T. D., & Deletic, A. (2009). Hydrologic and pollutant removal performance of stormwater biofiltration systems at the field scale. *Journal of Hydrology, 365*(3), 310-321.

Hench, K. R., Bissonnette, G. K., Sexstone, A. J., Coleman, J. G., Garbutt, K., & Skousen, J. G. (2003). Fate of physical, chemical, and microbial contaminants in domestic wastewater following treatment by small constructed wetlands. *Water research, 37*(4), 921-927.

Hunt, W., Smith, J., Jadlocki, S., Hathaway, J., & Eubanks, P. (2008). Pollutant removal and peak flow mitigation by a bioretention cell in urban Charlotte, NC. *Journal of Environmental Engineering*.

Hurley, M. A., & Roscoe, M. (1983). Automated statistical analysis of microbial enumeration by dilution series. *Journal of Applied Bacteriology, 55*(1), 159-164.

Hyslop, P. A., Hinshaw, D. B., Scraufstatter, I. U., Cochrane, C. G., Kunz, S., & Vosbeck, K. (1995). Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. *Free Radical Biology and Medicine, 19*(1), 31-37.

IDEXX. (2016). MPN Generator Software Program. Retrieved 3/2/2016, from https://www.idexx.com/water/mpn-generator.html

Janes, D., Umek, A., & Kreft, S. (2006). Evaluation of antibacterial activity of extracts of five species of wood- colonizing fungi. *Journal of Basic Microbiology, 46*(3), 203-207. doi: http://dx.doi.org/10.1002/jobm.200510035

Jenkins, M. W., Tiwari, S. K., & Darby, J. (2011). Bacterial, viral and turbidity removal by intermittent slow sand filtration for household use in developing countries: Experimental investigation and modeling. *Water research, 45*(18), 6227-6239.

Kalyoncu, F., Oskay, M., Sağlam, H., Erdoğan, T. F., & Tamer, A. Ü. (2010). Antimicrobial and antioxidant activities of mycelia of 10 wild mushroom species. *Journal of medicinal food, 13*(2), 415-419.

Keller, C., Maillard, M., Keller, J., & Hostettmann, K. (2002). Screening of European fungi for antibacterial, antifungal, larvicidal, molluscicidal, antioxidant and free-radical scavenging activities and subsequent isolation of bioactive compounds. *Pharmaceutical biology, 40*(7), 518-525.

Koenigs, J. W. (1974). Production of hydrogen peroxide by wood-rotting fungi in wood and its correlation with weight loss, depolymerization, and pH changes. *Archives of Microbiology, 99*(1), 129-145.

Krishnadevi, K., Selvaraj, V., & Prasanna, D. (2015). Thermal, mechanical and antibacterial properties of cyclophosphazene incorporated benzoxazine blended bismaleimide composites. *RSC Advances, 5*(2), 913-921.

Lawrence, M. A. (2013). Package 'ez': Sep.

Levy, S. B., & Marshall, B. (2004). Antibacterial resistance worldwide: causes, challenges and responses. *Nature medicine, 10*, S122-S129.

Liao, H., Krometis, L.-A. H., Hession, W. C., Benitez, R., Sawyer, R., Schaberg, E., . . . Badgley, B. D. (2015). Storm loads of culturable and molecular fecal indicators in an inland urban stream. *Science of The Total Environment, 530*, 347-356.

Lindequist, U., Niedermeyer, T. H., & Jülich, W.-D. (2005). The pharmacological potential of mushrooms. *Evidence-Based Complementary and Alternative Medicine, 2*(3), 285-299.

Lorenzen, K., & Anke, T. (1998). Basidiomycetes as a source for new bioactive natural products. *Curr Org Chem, 2*(4), 329-364.

Mille-Lindblom, C., & Tranvik, L. J. (2003). Antagonism between bacteria and fungi on decomposing aquatic plant litter. *Microbial ecology, 45*(2), 173-182.

Mille‐Lindblom, C., Fischer, H., & J Tranvik, L. (2006). Antagonism between bacteria and fungi: substrate competition and a possible tradeoff between fungal growth and tolerance towards bacteria. *Oikos, 113*(2), 233-242.

Millipore, E. (2016). Spectroquant Hydrogen Peroxide Test. Retrieved 5/3/2016, 2016, from http://www.emdmillipore.com/US/en/product/Hydrogen-Peroxide-Test,MDA_CHEM-118789#documentation

Pagliaccia, D., Merhaut, D., Colao, M., Ruzzi, M., Saccardo, F., & Stanghellini, M. (2008). Selective enhancement of the fluorescent pseudomonad population after amending the recirculating nutrient solution of hydroponically grown plants with a nitrogen stabilizer. *Microbial ecology, 56*(3), 538-554.

Pandey, A. (2003). Solid-state fermentation. *Biochemical Engineering Journal, 13*(2), 81-84.

Paulitz, T. C., & Bélanger, R. R. (2001). Biological control in greenhouse systems. *Annual review of phytopathology, 39*(1), 103-133.

Pereira, F., Mitchell, T., & Botvinick, M. (2009). Machine learning classifiers and fMRI: a tutorial overview. *Neuroimage, 45*(1), S199-S209.

Pescod, M. (1992). *Wastewater treatment and use in agriculture*. Rome, Italy: Food and Agriculture Organization

Pinto, A. J., Xi, C., & Raskin, L. (2012). Bacterial community structure in the drinking water microbiome is governed by filtration processes. *Environmental science & technology, 46*(16), 8851-8859.

Rogers, T. (2012). *Experimental evaluation of mycoremediation of Escherichia coli bacteria in solution using Pleurotus ostreatus.* The Evergreen State College.

Satchuthananthavale, V., & Cooke, R. (1967). Nitrogen nutrition of some nematode-trapping fungi. *Transactions of the British Mycological Society, 50*(3), 423-428.

Scherlach, K., Graupner, K., & Hertweck, C. (2013). Molecular bacteria-fungi interactions: effects on environment, food, and medicine. *Annual review of microbiology, 67*, 375-397.

Schlegel, B., Luhmann, U., Haertl, A., & Graefe, U. (2000). Piptamine, a new antibiotic produced by Piptoporus betulinus Lu 9-1. *Journal of antibiotics, 53*(9), 973-974.

Seaver, L. C., & Imlay, J. A. (2001). Hydrogen peroxide fluxes and compartmentalization inside growing Escherichia coli. *Journal of bacteriology, 183*(24), 7182-7189.

Shannon, M. A., Bohn, P. W., Elimelech, M., Georgiadis, J. G., Mariñas, B. J., & Mayes, A. M. (2008). Science and technology for water purification in the coming decades. *Nature, 452*(7185), 301-310.

Siegel, S., & Castellan, N. (1988). *Nonparametric Statistics For The Behavioral Sciences* New York: McGraw-Hill.

Sparling, G., Fermor, T., & Wood, D. (1982). Measurement of the microbial biomass in composted wheat straw, and the possible contribution of the biomass to the nutrition of Agaricus bisporus. *Soil biology and biochemistry, 14*(6), 609-611.

Stamets, P. (2005). *Mycelium running: how mushrooms can help save the world*: Random House LLC.

Stamets, P., Beutel, M., Taylor, A., Flatt, A., Wolff, M., & Brownson, K. (2013). *Comprehensive Assessment of Mycofiltration Biotechnology to Remove Pathogens from Urban Stormwater*.

Stewart-Wade, S. M. (2011). Plant pathogens in recycled irrigation water in commercial plant nurseries and greenhouses: their detection and management. *Irrigation Science, 29*(4), 267-297.

Suay, I., Arenal, F., Asensio, F. J., Basilio, A., Cabello, M. A., Díez, M. T., . . . Hernández, P. (2000). Screening of basidiomycetes for antimicrobial activities. *Antonie van Leeuwenhoek, 78*(2), 129-140.

Taylor, A., Flatt, A., Beutel, M., Wolff, M., Brownson, K., & Stamets, P. (2014). Removal of Escherichia coli from synthetic stormwater using mycofiltration. *Ecological Engineering*. doi: 10.1016/j.ecoleng.2014.05.016

Team, R. C. (2015). A language and environment for statistical computing. . from R Foundation for Statistical Computing

Thomas, S. A., Aston, L. M., Woodruff, D. L., & Cullinan, V. I. (2009). Field demonstration of mycoremediation for removal of fecal coliform bacteria and nutrients in the Dungeness watershed, Washington. Final Report. *Pacific Northwest National Laboratory. PNWD-4054-1*.

Thorn, R., & Barron, G. (1984). Carnivorous mushrooms. *Science, 224*(4644), 76-78.

Van Der Gaag, D. J., Kerssies, A., & Lanser, C. (2001). Spread of Phytophthora root and crown rot in Saintpaulia, Gerbera and Spathiphyllum pot plants in ebb-and-flow-systems. *European journal of plant pathology, 107*(5), 535-542.

Velíšek, J., & Cejpek, K. (2011). Pigments of higher fungi: a review. *Czech J Food Sci, 29*(2), 87-102.

Verhoeven, J. T., & Meuleman, A. F. (1999). Wetlands for wastewater treatment: opportunities and limitations. *Ecological Engineering, 12*(1), 5-12.

Větrovský, T., Baldrian, P., & Gabriel, J. (2013). Extracellular enzymes of the white-rot fungus Fomes fomentarius and purification of 1, 4-β-glucosidase. *Applied biochemistry and biotechnology, 169*(1), 100.

Zimmer, J., & Slawson, R. (2002). Potential repair of Escherichia coli DNA following exposure to UV radiation from both medium-and low-pressure UV sources used in drinking water treatment. *Applied and Environmental Microbiology, 68*(7), 3293-3299.

Zwick, R., & Marascuilo, L. A. (1984). Selection of pairwise multiple comparison procedures for parametric and nonparametric analysis of variance models. *Psychological Bulletin, 95*(1), 148.

Appendix A.

A.I. Complete Data Set for Batch Treatment Experiment

Treatment	Time (min)	E. coli In (cfu/100 ml)	E. coli Out (cfu/100 ml)	Net E. coli Reduction	Categorical E. coli Removal	Percent Reduction
Neg	5	19863	10	19853	Yes	-0.999
Neg	5	19863	10	19853	Yes	-0.999
Neg	5	19863	0	19863	Yes	-1.000
Neg	30	17329	0	17329	Yes	-1.000
Neg	30	17329	0	17329	Yes	-1.000
Neg	30	17329	0	17329	Yes	-1.000
Neg	60	19863	0	19863	Yes	-1.000
Neg	60	19863	0	19863	Yes	-1.000
Neg	60	19863	0	19863	Yes	-1.000
Neg	180	19863	10	19853	Yes	-0.999
Neg	180	19863	0	19863	Yes	-1.000
Neg	180	19863	0	19863	Yes	-1.000
Neg	720	19863	0	19863	Yes	-1.000
Neg	720	19863	10	19853	Yes	-0.999
Neg	720	19863	0	19863	Yes	-1.000
Neg	1440	19863	0	19863	Yes	-1.000
Neg	1440	19863	0	19863	Yes	-1.000
Neg	1440	19863	0	19863	Yes	-1.000
Neg	2880	19863	10	19853	Yes	-0.999
Neg	2880	19863	0	19863	Yes	-1.000
Neg	2880	19863	10	19853	Yes	-0.999
P. bet	5	19863	10	17339	Yes	-0.999
P. bet	5	19863	0	17329	Yes	-1.000
P. bet	5	19863	0	17329	Yes	-1.000
P. bet	30	17329	24196	-6867	No	0.218
P. bet	30	17329	24196	--6867	No	0.218
P. bet	30	17329	0	17329	Yes	-1.000
P. bet	60	19863	24196	-4333	No	0.218
P. bet	60	19863	24196	-4333	No	0.218
P. bet	60	19863	0	19863	Yes	-1.000
P. bet	180	19863	0	19863	Yes	-1.000
P. bet	180	19863	0	19863	Yes	-1.000

P. bet	180	19863	0	19863	Yes	-1.000
P. bet	720	19863	0	19863	Yes	-1.000
P. bet	720	19863	0	19863	Yes	-1.000
P. bet	720	19863	0	19863	Yes	-1.000
P. bet	1440	19863	0	19863	Yes	-1.000
P. bet	1440	19863	0	19863	Yes	-1.000
P. bet	1440	19863	0	19863	Yes	-1.000
P. bet	2880	19863	0	19863	Yes	-1.000
P. bet	2880	19863	0	19863	Yes	-1.000
P. bet	2880	19863	0	19863	Yes	-1.000
D. con	5	19863	0	19863	Yes	-1.000
D. con	5	19863	0	19863	Yes	-1.000
D. con	5	19863	20	19843	Yes	-0.999
D. con	30	17329	0	17329	Yes	-1.000
D. con	30	17329	0	17329	Yes	-1.000
D. con	30	17329	0	17329	Yes	-1.000
D. con	60	19863	0	19863	Yes	-1.000
D. con	60	19863	0	19863	Yes	-1.000
D. con	60	19863	0	19863	Yes	-1.000
D. con	180	19863	0	19863	Yes	-1.000
D. con	180	19863	0	19863	Yes	-1.000
D. con	180	19863	0	19863	Yes	-1.000
D. con	720	19863	0	19863	Yes	-1.000
D. con	720	19863	0	19863	Yes	-1.000
D. con	720	19863	0	19863	Yes	-1.000
D. con	1440	19863	0	19863	Yes	-1.000
D. con	1440	19863	10	19853	Yes	-0.999
D. con	1440	19863	10	19853	Yes	-0.999
D. con	2880	19863	0	19863	Yes	-1.000
D. con	2880	19863	10	19853	Yes	-0.999
D. con	2880	19863	10	19853	Yes	-0.999

A. II. Complete Data Set for Flow Through Treatment Experiment

Treatment	Time (Min)	Influent E. coli (cfu/100 ml)	Effluent E. coli (cfu/100 ml)	E. coli Reduction (cfu/100 ml)	Percent Reduction
Neg A	5	6867	11199	-4332	0.631
Neg B	5	6867	0	6867	-1.000
Neg B	5	6867	0	6867	-1.000
Neg A	10	1392	2909	-1517	1.090
Neg B	10	1392	0	1392	-1.000
Neg B	10	1392	0	1392	-1.000
Neg A	20	1585	2247	-662	0.418
Neg B	20	1585	0	1585	-1.000
Neg B	20	1585	0	1585	-1.000
P. bet	5	6867	0	6867	-1.000
P. bet	5	6867	0	6867	-1.000
P. bet	5	6867	0	6867	-1.000
P. bet	10	1392	0	1392	-1.000
P. bet	10	1392	0	1392	-1.000
P. bet	10	1392	0	1392	-1.000
P. bet	20	1585	0	1585	-1.000
P. bet	20	1585	10	1575	-0.994
P. bet	20	1585	0	1585	-1.000
D. con	5	6867	0	6867	-1.000
D. con	5	6867	0	6867	-1.000
D. con	5	6867	0	6867	-1.000
D. con	10	1392	0	1392	-1.000
D. con	10	1392	0	1392	-1.000
D. con	10	1392	0	1392	-1.000
D. con	20	1585	0	1585	-1.000
D. con	20	1585	0	1585	-1.000
D. con	20	1585	0	1585	-1.000
P. ery	5	1222	0	1222	-1.000
P. ery	5	1222	0	1222	-1.000
P. ery	5	1222	0	1222	-1.000
P. ery	10	2247	0	2247	-1.000
P. ery	10	2247	0	2247	-1.000
P. ery	10	2247	0	2247	-1.000
P. ery	20	5172	0	5172	-1.000
P. ery	20	5172	0	5172	-1.000
P. ery	20	5172	0	5172	-1.000

A. III. Complete Data Set for Hydrogen Peroxide Assay Experiment

Treatment	Time (Min)	Peroxide Con. (mg/l)
P. bet	5	0.64
P. bet	5	0.67
P. bet	5	0.91
P. bet	60	2.4
P. bet	60	1.74
P. bet	60	2.85
P. bet	240	3.27
P. bet	240	2.61
P. bet	240	3.94
D. con	5	2.65
D. con	5	1.6
D. con	5	2.03
D. con	60	5.94
D. con	60	4.29
D. con	60	4.28
D. con	240	1.32
D. con	240	6.52
D. con	240	5.3
P. ery	5	8.01
P. ery	5	5.62
P. ery	5	3.56
P. ery	60	21.92
P. ery	60	9.07
P. ery	60	12.72
P. ery	240	26.67
P. ery	240	30.5
P. ery	240	28.89

Appendix B.

B.I. Batch Data R Code

```
library(VIF)



file_loc ='mod_batch_data_cata.csv'


## glm (assumptions might be bad?) I think they are bad. But, worth asking.
batchmod = read.csv(file_loc)
batchmods = batchmod[,c(1,4,8,9,10)]
head(batchmod)



fit = glm(ecoli_removal_bi ~  treat + time + 0, data = batchmods, family=binomial(link='logit'))


summary(fit)


plot(fit)


#Binomial test --- works, simple way decide if single treat has an equal change to filter or not filter.
## one for each treatment
# Ho is the treatment is equally likely to successfully filter or not to successfully filter out bacteria.


neg = binom.test(21, 21)# reject null, more likely to filter successfully 9.537e-07
neg


pbet = binom.test(17, 21) # reject null, more likely to filter successfully  p-value = 0.007197
```

```
pbet

dcon = binom.test(21, 21) # reject null, more likely to filter successfully 9.537e-07
dcon


### Binomial Test for Time Groups
fivemin = binom.test(9,9)# reject null, more likely to filter successfully 9.537e-07
fivemin

thirtymin = binom.test(7, 9) # reject null, more likely to filter successfully  p-value = 0.007197
thirtymin

onehr = binom.test(7, 9) # reject null, more likely to filter successfully 9.537e-07
onehr

threehour = binom.test(9, 9) # reject null, more likely to filter successfully 9.537e-07
threehour

twelvehour = binom.test(9, 9) # reject null, more likely to filter successfully 9.537e-07
twelvehour

twofourhour = binom.test(9, 9) # reject null, more likely to filter successfully 9.537e-07
twofourhour

foureighthour = binom.test(9, 9) # reject null, more likely to filter successfully 9.537e-07
foureighthour
```

```
# all treatments are more likely to filter out bacteria successfully compared to equally likely to filter or not filter

# Statistically all treatments are likely to successfully filter within a 95% confidence interval.


## Proportion test (Use Individual Prop Values), Critical chi-square vale for df 1 is 3.84

## Negative Control Group

prop_neg = prop.test(x = 21, n = 21, alternative = c("two.sided", "less", "greater"), conf.level=0.95, correct=TRUE)

prop_neg

## P. betulinus Group

prop_bet = prop.test(x = 17, n = 21, alternative = c("two.sided", "less", "greater"), conf.level=0.95, correct=TRUE)

prop_bet

## D. confragosa Group

prop_dcon = prop.test(x = 21, n = 21, alternative = c("two.sided", "less", "greater"), conf.level=0.95, correct=TRUE)

prop_dcon


### Proportion Test Matrix


batchprop <-matrix(c(21,17,21,21,4,21), ncol=2)

colnames(batchprop) <- ('Removal','No Removal')

rownames(batchprop) <- ("Neg","P.bet","D.con")


prop.test(batchprop, alternative=c("two.sided", "less", "greater"), conf.level=0.95, correct=FALSE)


### Kruskal Wallace Test (For Treatment)

library(pgirmess)

kt_treat = kruskalmc(batchmod$percent_red ~ batchmod$treat, probs = 0.05)
```

```
kt_treat

### Kruskal Wallace Test (For Time)
library(pgirmess)
kt_red = kruskalmc(batchmod$percent_red ~ batchmod$time, probs = 0.05)
kt_red

### Marascuillo Procedure for Treatment
p = c(1.0, 0.8095, .99)
N = length(p)
value = critical.range = c()
for (i in 1:(N-1))
{ for (j in (i+1):N)
{
  value = c(value,(abs(p[i]-p[j])))
  critical.range = c(critical.range,
                     sqrt(qchisq(.95,2))*sqrt(p[i]*(1-p[i])/21 + p[j]*(1-p[j])/21))
}
}
round(cbind(value, critical.range),2)

### Marascuillo Procedure for Time
p = c(0.99, 0.78,0.9999, 0.9999, 0.9999, 0.9999, 0.9999)
N = length(p)
value = critical.range = c()
for (i in 1:(N-1))
{ for (j in (i+1):N)
{
```

```
  value = c(value,(abs(p[i]-p[j])))
  critical.range = c(critical.range,
                     sqrt(qchisq(.95,2))*sqrt(p[i]*(1-p[i])/21 + p[j]*(1-p[j])/21))
}
}
round(cbind(value, critical.range),2)

### Traditional Kruska-Wallis Test
overall_kt_time = kruskal.test(percent_red ~ time,data = batchmod)
overall_kt_time

overall_kt_treat = kruskal.test(percent_red ~ treat, data = batchmod)
overall_kt_treat



### Q-Q Plot
qqnorm(batchmod$percent_red, main="Q-Q Plot: Batch Treatment Percent E. coli Reduction")
qqline(batchmod$percent_red)



### Shapiro-Wilk TEst
shapiro.test(batchmod$percent_red)
```

B.II. Flow Through Data R Code

```
library(ggplot2)
library(GGally)
library(MASS)
library(glmnet)
library(pgirmess)
library(ggplot2)
library(afex)

# Read in Data
flo = read.csv('flowthrough_data.csv')

treatment = as.factor(flo$treatment)
id = as.factor(flo$id)

ggpairs(flo)

# Flow Through Boxplot for Treatment (Original Data) - Shows the Means!
plot(flo[,3], flo[,7])

title(main="Net E. coli Reduction vs Treatment", xlab="Treatment", ylab="Net E. coli Reduction (cfu/100 ml)")


# Linear Regression Model
fit = lm(ecoli_out ~  ecoli_in + treatment + time + treatment*time, data = flo)


## Using 'afex' Package!!!
```

```
## Used a repeated measures ANOVA using Type 3 and contrasts
aov_ez("id", "ecoli_red", flo, between = c("treatment"), within = c("time"))
print(aov_ez)


### Tukey's HSD
T1<-subset(flow_through, time==5)
T2<-subset(flow_through, time==10)
T3<-subset(flow_through, time==20)

#
T1N1<-aov(T1$ecoli_red~T1$treatment)
T1N1.HSD<-TukeyHSD(T1N1)
T1N1.HSD
T2N2<-aov(T2$ecoli_red~T2$treatment)
T2N2.HSD<-TukeyHSD(T2N2)
T2N2.HSD
T3N3<-aov(T3$ecoli_red~T3$treatment)
T3N3.HSD<-TukeyHSD(T3N3)
T3N3.HSD

### Q-Q Plot
qqnorm(flo$percent_red, main="Q-Q Plot: Flow Through Treatment Percent E. coli Reduction")
qqline(flo$percent_red)

### Shapiro-Wilk Test
shapiro.test(flo$percent_red)

##Kruska-Wallis Test (Non-parametric Version of Tukey's Pairwise Test)
```

```
### use for pigmess package

dt = flo[,c(1,7,3)]
dt[,1] = as.character(dt[,1])
dt_no_p = dt[1:27,]
dt_no_p[,1] = as.factor(dt_no_p[,1])
d_no_5 = dt_no_p[-which(dt_no_p[,3] == 5),]

#test = dt$ecoli_red[1:27]
#test[1:3] = -100000

### Overall Kruskal-Wallis Test
kt_treat = kruskal.test(flo$percent_red ~ flo$treatment)
kt_treat
kt_time = kruskal.test(flo$percent_red ~ flo$time)
kt_time

### Multiple Comparison Kruskal-Wallis Test Treatment
kt_treat = kruskalmc(flo$percent_red ~ flo$treatment, cont = “two-tailed”, probs = 0.05)
kt_treat

### Multiple Comparison Kruskal-Wallis Test Time
kt_time = kruskalmc(flo$percent_red ~ flo$time, cont = “two-tailed”,  probs = 0.05)
kt_time

### Kruskal - Wallis Test with Modified Data (90% Confidence Interval) Treatment!
dt = flo[,c(1,7,3)]
dt_no_p = dt[1:27,]
dt_no_p[,1] = as.factor(dt_no_p[,1])
```

```
kt_no_p = kruskalmc(dt_no_p$ecoli_red ~ dt_no_p$treatment, cont = NULL, probs = 0.1 )

### Kruskal - Wallis Test with Modified Data (90% Confidence Interval) Treatment!
kt

## Krusak - Wallis Test with No P. Eryngii or 5 minute Treatment
kt_no_5 = kruskalmc(d_no_5$ecoli_red ~ d_no_5$treatment, cont = NULL, probs = 0.5 )
#kt_test = kruskalmc(test ~ dt_no_p$treatment,cont = NULL, probs = 0.5 )

kt
kt_no_p
kt_no_5
#kt_test


###Basic Boxplot with Modified Flow Through Data (No. P. ery)!!
plot(dt_no_p$treatment, dt_no_p$ecoli_red)
title(main="Net E. coli Reduction vs Treatment", xlab="Treatment", ylab="Net E. coli Reduction (cfu/100 ml)")


###Basic Boxplot with Modified Flow Through Data (No P. ery or 5 min. Treat)
rev_flo = read.csv('rev_flowthrough_data.csv')

treatment = as.factor(rev_flo$treatment)
id = as.factor(rev_flo$id)

ggpairs(rev_flo)
```

```
plot(rev_flo[,1], rev_flo[,7])

title(main="Revised E. coli Removal vs Treatment Boxplot", xlab="Treatment", ylab="E. coli Reduction (cfu/100 ml)")


## Normal Boxplot

p <- ggplot(dt, aes(x=dt_no_p, y=d_no_5)) +

  geom_boxplot()


# Fancy Boxplot

xlabs <- paste(levels(d_no_5$treatment),"\n(N=",table(d_no_5$treatment),")",sep="")

ggplot(d_no_5,aes(x=treatment,y=ecoli_red,color=treatment))+geom_boxplot()+scale_x_discrete(labels=xlabs)

title(main="Revised E. coli Removal vs Treatment Boxplot", xlab="Treatment", ylab="E. coli Reduction (cfu/100 ml)")


###Basic Boxplot with Modified Flow Through Data (No. P. ery)!!

plot(dt_no_p$time, dt_no_p$ecoli_red)

title(main="Net E. coli Reduction vs Time", xlab="Time (Min.)", ylab="Net E. coli Reduction (cfu/100 ml)")
```

B.III. Hydrogen Peroxide Data R Code

```
install.packages(ez)
library(ez)
library(muStat)
file<-read.csv("peroxide_data_modified.csv")

File<-within(file, {
        treat<-factor(treat)
        id<-factor(id)
})

##### One-Way ANOVA Repeated Measures
print(ezANOVA(data=file, dv=Log_peroxide, wid=id, within=time, between=treat))
######

##### Tukey's HSD
T1<-subset(file, time==5)
T2<-subset(file, time==60)
T3<-subset(file, time=240)
#
T1N1<-aov(T1$Log_peroxide~T1$treat)
T1N1.HSD<-TukeyHSD(T1N1)
T1N1.HSD
T2N2<-aov(T2$Log_peroxide~T2$treat)
T2N2.HSD<-TukeyHSD(T2N2)
T2N2.HSD
T3N3<-aov(T3$Log_peroxide~T3$treat)
T3N3.HSD<-TukeyHSD(T3N3)
```

T3N3.HSD

```
##### Histogram
hist(file$Log_peroxide, main="Histogram for Hydrogen Peroxide Production", xlab="Log Hydrogen Peroxide Concentration (mg/l)")

##### QQ plot
qqnorm(file$Log_peroxide, main="Q-Q Plot: Hydrogen Peroxide Concentration")
qqline(file$Log_peroxide)

### Shapiro-Wilk Normality Test
shapiro.test(file$Log_peroxide)

##### Kruskal-Wallis Rank Sum Test
kruskal.test(Log_peroxide~treat, data=file) # Peroxide by Treatment

##### Friedman Test (Unreplicated Block Design)
friedman.test(Peroxide_con ~ time|treat, data=file)
attach(file)
friedman.test(Log_peroxide, time, treat)

#### muStat - Prentice Rank Sum Test (Non-parametric Replicated Block Design)
library(muStat)
attach(file)
prentice.test(Log_peroxide, time, blocks = treat)
```