Experimental Evaluation of Mycoremediation of *Escherichia coli* Bacteria in Solution using *Pleurotus ostreatus*

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ABSTRACT

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Waters contaminated with fecal coliform bacteria (FCB) require treatment prior to release into natural systems. The use of fungi to remove bacteria from solution (mycofiltration) been proposed as a possible low-tech method to treat waters contaminated with FCB (Stamets, 2001; Thomas et al., 2009). This thesis reviews background information relevant to FCB contamination, detection, conventional treatment, and mycofiltration applications. In addition, a study was designed and conducted to experimentally evaluate mycofiltration of the bacteria *Escherichia coli* by the fungus *Pleurotus ostreatus.* In this study, an *E. coli* solution was filtered through open ended glass tubes containing sawdust with (treatment) and without (control) mycelium of *P. ostreatus* to determine the effects of mycofiltration on the abundance of *E. coli* within a solution. A significant reduction in *E. coli* abundance was observed in the treatment group and was not observed in the control group. These results were confirmed using both parametric and non-parametric statistics. Refinements to the experimental design and methodology, which reduce data variation and provide further information on the interactions between *E. coli* and *P. ostreatus*, are also presented. The results of this small scale *in vitro* experiment provide evidence that fungi can be used to filter bacteria from a solution and support the application of this approach in larger scale *in situ* studies to test the use of mycofiltration treatment of waters contaminated with animal waste.

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Chapter 1 Introduction

Background

Fecal coliform is the name given to the group of bacteria that originate in the gut of warm-blooded animals. Though the majority of these organisms are harmless to humans and other animals, they exist in great concentrations in feces, meaning that even small amounts of fecal contamination could contain pathogenic organisms. Negative health effects of infection from the fecal coliform group include pneumonia, bronchitis, urinary tract infection, meningitis, compromised immune system, and gastrointestinal diseases ranging from diarrhea to dysentery. Because of the health effects of these bacteria and the viruses and protozoans that may accompany them, fecal coliform bacteria (FCB) are used as indicators of contamination of water and food.

Contamination of waterways with FCB is widely recognized as a major issue of environmental concern for both human and ecosystem health. Numerous methods for the treatment of these waters have been described and they are usually used in combination, however, each treatment has limitations (described below in "Current Methods of FCB Treatment"), leading researchers to examine new treatments, such as bioremediation. Bioremediation is the use of organisms to decontaminate a polluted site. Numerous fungi have been proposed as bioremediation organisms for various types of pollution. For example, Paul Stamets, a mycologist in the Pacific Northwest, has postulated that certain species of fungi may be applicable to the treatment of fecal coliform contamination

(Stamets, 2001). This use of fungi as a bioremediation tool is termed mycoremediation.

The use of bioremediation/mycoremediation rather than conventional treatment offers the benefits of being inexpensive and often as simple as introducing an organism to a pollutant. Bioremediation techniques are also aligned with goals of sustainability, because they do not require the use of toxic or poisonous chemicals. This paper will consider the history of fecal coliform science, current FCB treatment methods, scientific theory supporting mycoremediation of bacteria, mycoremediation applications, and the use of mycoremediation as a tool for treating fecal-contaminated water streams. In addition, an experiment was conducted to test the use of mycoremediation on *E. coli* bacteria within a water stream.

Background information on the history of the study of FCB is important to gain an understanding of the methods and scientific theories that have contributed to FCB science. Scientific perception of FCB and associated pathogenic organisms has changed over time. Knowledge of the progression of FCB science and methodology is necessary to understand treatments and interpret data on detection and enumeration of FCB. It is important to understand both the possibilities and limitations of these techniques in terms of application to human and ecosystem health.

History of Fecal Coliform Detection, Perception, and Monitoring

The birth of sanitary water bacteriology is generally considered to be 1882, when bacteriologist Von Fritsch first described *Klebsiella pnuemoniae* and *K. rhinoscleromatis* as organisms present in human feces. Though these were the first FCB described, it had already been demonstrated that diseases, such as cholera, were spread through contaminated water sources with a link to sewage (Snow, 1855). Then, in 1886, Theodore Escherich presented and published his work entitled *Die Darmbakterien des Säuglings und ihre Beziehungen zur Physiologie der Verdauung* (translated: Enterobacteria of infants and their relation to digestion physiology). In this document, Escherich describes *Bacterium coli commune* (AKA *Bacillus coli comunis*), which would later be renamed *Escherichia coli.* Both Von Fritsch and Escherich considered human feces to be a source of hazardous bacterial contamination while the feces of other warm blooded animals they considered benign.

Escherich described the bacterium now known as *E. coli* by its characteristics of metabolism in milk, gelatin, grape sugar, and potato (Parr, 1939). This definition would later be updated in order to distinguish *E. coli* from other related bacteria. To aid in the task of coliform bacteria differentiation and classification, new methods were introduced.

Theobald Smith (1895) stated in *Notes on Bacillus col-communis and Related Forms* that the coliform group poses a threat to health without consideration for the environment in which the bacteria is found (including non-

human warm blooded animals). Methods of fecal coliform identification, which, at that time, relied on production of gas within a culture tube exposed to sugar-bullion, lacked the ability to distinguish between related groups. Smith developed a method that combined the production of gas, bacterial response to different carbohydrates, and production of acids to differentiate between related coliform bacteria (Smith, 1895). Smith is given credit for pioneering early methods of bacterial differentiation based on gas production which included analysis of the gases produced and the ratios of those gasses to one another. His methods would later be fine-tuned using more delicate procedures.

As methods of fecal coliform identification progressed, so did the perception of pathogens in this group. C. Eijkman, who, in 1904, developed a restrictive test based on gas formation from lactose or glucose at 46°C, believed that only coliform bacteria known to be of fecal origin (not all coliform bacteria is of fecal origin) represented a threat to human health (ORSANCO, 1971). Eijkman theorized that only the coliform bacteria of fecal origin would grow at an elevated incubation temperature (46°C), thus producing a simple test to determine the source of a coliform bacteria sample. This process is termed the Eijkman reaction.

As the taxonomy of coliform bacteria evolved, so did methods of identification and differentiation. A new method, the IMViC reaction, was described in 1936 to help group these bacteria on a biochemical basis (Parr, 1936). This method involves a diagnostic series of tests, each yielding a positive

or negative result, reported as a series of "+" or "-". IMViC is a pneumonic acronym that dictates the order in which results of the four tests are listed.

The first test, "I", is the indole test. Production of indole yields a "+" result. The "M" represents the methyl red test, a test of pH, in which pH below 4.4 turns the solution red and a pH above 6.2 turns the solution yellow. The next test, "V", is the Voges-Proskauer reaction. In the Voges-Proskauer test, alpha-napthol and potassium hydroxide are added to a bacterial solution grown in Voges-Proskauer broth. A positive result turns the solution red and indicates the presence of acetoin, while a negative result yields a yellow-brown solution. The small "i" is added simply for pronunciation. The last test, "C", is for carbon. More specifically, the test determines if the bacteria could use citrate as its sole carbon source (Parr, 1936). The results of this test are presented as a series of "+" and "-" (e.g. - + - +). This series of tests is still used in diagnostic research but rarely for routine analysis.

Leland Parr also developed a guide for the interpretation of IMViC results. Bacteria that exhibit the profiles (+ + - -), (+ - - -), and (- + - -) would be classified into the *Eschericia* group and considered of fecal origin. The Aerobacter group included types (- - + +), (- - + -), and (- - - +) and designated bacteria of soil origin. The remaining ten combinations constituted the intermediate group considered to be of indeterminate origin. Parr's work with the IMViC reaction also demonstrated that some coliform strains could remain viable for very long periods – 289 days in a saline (salt water) solution and over 550 days stored in a low temperature environment (Parr, 1936). IMViC reactions were considered the

best test for differentiation of the fecal coliform group for some time, but, unexplained discrepancies occurred when determinations were made from small samples (Gelreich, 1964).

As the study of FCB progressed, revised methodology was necessary to expand scientific understanding. Of utmost concern was the classification of coliform bacteria into fecal and non-fecal groups. A review of data and new experimentation in 1964 yielded 5 conclusions:

1) The most acceptable temperature of incubation for the separation of the fecal coliform group is 44.5°C in a water bath.
2) A small percentage of the fecal coliform strains will be excluded and an equal percentage of non-fecal coliform strains will be included.
3) The EC medium, as described by Perry and Hajna (1944), will give the most rapid results, as it requires only 24 hours incubation.
4) The test can only be used as a confirmatory procedure from coliform cultures growing on nonselective medium.
5) In the evaluation of results, all coliforms from the feces of warm blooded animals must be considered as fecal coliform strains, and all cultures isolated from unpolluted sources must be considered as non-fecal coliform strains (Geldreich, 1964, based on data from Clark, et al., 1957, Geldreich, et al., 1958; and Perry and Hajna, 1944).

These conclusions helped develop protocols that allowed for classification of coliform into fecal and non-fecal groups. The result (of #5 specifically) was a circumnavigation of the problems related to differentiation of coliform bacteria source, but, the conclusions did provide solid guidelines for classification. It should be noted that sources considered unpolluted (conclusion #5) are those that are not proven (or known) to be polluted.

Geldreich (1962) rigorously tested Eijkman's hypothesis that only coliform bacteria of fecal origin grow at elevated (46°C) temperatures. The study found that 96% of strains of coliform bacteria isolated from the feces of warm-

blooded animals exhibited a positive reaction (growth) to the elevated incubation test on non-selective media. Likewise, the study found that 91% of coliform bacteria isolated from soils tested negative (no growth) to the elevated incubation technique. These findings underscore conclusion #2, listed above, which states that a small percentage of bacteria would be placed into the wrong group (fecal or non-fecal) based on elevated temperature reactions (Geldreich, 1964).

To further test the origin of coliform bacteria in the environment, Geldreich (1965) conducted another experiment using similar methods. This time, instead of isolating bacteria from soil, Geldreich isolated coliform bacteria from insects, fish, and vegetation using temperature regulated growth to determine the source of coliform bacteria. The results indicated that a small percentage, 14.1% on vegetation and 14.9% on insects, were of fecal origin. This study also considered coliform bacteria on fish, however, found that coliform bacteria associated with fish were closely related to habit and species (and varied greatly based on these characteristics).

Continuing his studies on coliform bacteria, Geldreich (1965) developed another method for coliform testing that would become the standard for water and wastewater examination. Because municipal water and wastewater systems are held to a low bacterial threshold, testing of these waters requires a large volume of water and the detection of very small numbers of bacteria. The most efficient means of testing water at this time involved the use of membrane filtration (MF) to capture bacteria from a large volume. As the membrane filter technique gained acceptance within the discipline of water pollution studies, a need developed for a

MF method that could incorporate the elevated temperature techniques for determination of fecal or non-fecal source of coliform bacteria.

At least two methods had been published. The first method required pretreatment of the water sample with nutrients, a 2-hour incubation period, followed by MF, transfer of the filter to an agar plate, and finally, a second incubation at an elevated temperature for 16 hours (Taylor, Burman, and Oliver, 1955). The second method required that the solution be filtered by MF, added to a tryptose bile agar (TBA) plate, and incubated at an elevated temperature for 20-24 hours. The filter would then be transferred to an absorbent pad soaked with indol reagent (McCarthy, Delaney, and Grasso, 1961). Indol causes type 1 *E. coli* to turn a dark red color and confirms presence of FCB. Both of these methods required multiple steps that were difficult to perform in field situations. With this in mind, Geldreich (1965) developed a new nutrient medium for fecal coliform (MFC) that contained aniline blue. Aniline blue is a reagent that stains coliform bacteria blue and other bacteria gray. Application of the MF pad to this medium followed by incubation for 24 hours at an elevated temperature produced countable *E. coli* colonies without subsequent treatment.

The *Standard Methods for Examination of Water and Wastewater* (1989), the handbook referenced by current U.S. regulations, continues to use Smith's lactose incubation at 35°C on a modified lactose agar as one of the standard detection techniques. The British have adopted a modified version of Eijkman's elevated temperature incubation using growth at 44°C as an indication that the bacteria is more dangerous than bacteria that grows at 35°C (ORSANCO, 1971).

Both American and British methods employ the MF technique, which continues to be the simplest, cheapest, and quickest method for detection and enumeration of FCB in water and wastewater testing. Media containing aniline blue or indol reagents are currently available and may be used with the MF technique to differentiate bacteria.

Regardless of the method of identification used for FCB testing, wastes and wastewaters must be treated before being reintroduced into natural waterways, irrigation systems, or even pumped underground into geothermal electricity generating stations (SRRW GRP, 1998). A wide variety of treatment options exist. The most common methods are discussed below.

Current Methods of FCB Treatment

Treatment of FCB and associated pathogens is diverse and includes chlorination, UV sterilization, filtration, decanting/settling, activated sludge, and lagooning. These methods are often used in combinations and vary greatly from one treatment facility to another. This section will briefly examine the effectiveness and limitations of these treatments.

Chlorination is the most widely used treatment for wastewater sanitation (EPA, 1999). The effectiveness of chlorine treatments (bleach – NaClO and chlorine dioxide – ClO_2) are well established for the removal and/or inactivation of bacteria (EPA, 1999; Rose et al., 1996). There are, however, two major disadvantages of chlorine disinfection of wastewater. One is that de-chlorination is crucial. Chlorinated water, which was commonly discharged into streams

following the Federal Water Pollution Control Act of 1972 (EPA, 2000), is toxic to aquatic organisms (Brungs, 1973). Therefore, chlorine treated water must be de-chlorinated prior to discharge into natural waterways. Incomplete de-chlorinization results in toxicity and contact with organic matter produces carcinogenic compounds such as trihalomethanes and organochlorides (WEF and ASCE, 1991). Chlorination and de-chlorination are sanctioned by the EPA and are considered to be safe if performed properly, however, treatment methods that do not require the use of toxic compounds may also reduce FCB.

The second limitation of chlorine sanitation is that bacteria are more sensitive to chlorine toxicity than are pathogenic protozoa such as *Giardia lamblia* (Jarroll et al., 1984) and *Cryptosporidium parvum* (Peeters et al., 1989; Korich et al., 1990). This causes a major two-fold issue for water sanitation and examination. First, the pathogenic organisms are not targeted by this treatment and may persist within the water solution. Secondly, the indicator FCB are killed by chlorine treatment, so the most effective method of monitoring for pathogenic organisms is nullified by this treatment. These characteristics of chlorine treatment pose health concerns for both humans and natural ecosystems.

Decanting/settling is another method that is used in nearly all municipal wastewater treatment facilities. This process is designed to remove most of the solid particulates in wastewater, but, also removes FCB. Decanting is usually the first step in the processing of wastewater and it is established that this method removes 10-60% of FCB (Audic, 1990; Dupray et al., 1990; George et al., 2002). It is assumed that the bacteria removed in this manner are attached to solid

particles being targeted by the decanting process. This step is necessary for reduction of suspended solids, but, inadequate for complete treatment of FCB. Filtration is very similar to decanting, but, filtration targets smaller particles. The specifics of filtration vary greatly from facility to facility based on the type of filter, particle size, and effectiveness, but, filtration is generally more effective than decanting (George et al., 2002). Both filtration and decanting/settling reduce FCB, however, an unsatisfactory proportion of bacteria remains.

Activated sludge processing is the most widely used treatment for wastewaters. The sludge method relies on a bacterial solution that removes nitrogen (N) and phosphorus (P) (Gernaey et al., 2004). This process usually occurs over a long period and biologically removes FCB through protozoan grazing, competition with other microorganisms, and sedimentation with flocs (George et al., 2002). Similar to decanting, activated sludge processing is not directly intended to remove FCB, but it does reduce FCB as a side benefit.

Historically, wastewater lagoons were the most popular and inexpensive method of wastewater treatment (Wolverton and McDonald, 1979). A lagoon is basically a large pool that stores wastewaters for extended periods. This process allows microorganisms to break down complex organic compounds. FCB are effectively removed during this process when compared to other treatments (George et al., 2002). Mechanisms of reduction of FCB in lagoons include long retention time (60 days), grazing by protozoa, high pH due to photosynthesis, and UV inactivation (George et al., 2002). This process is a typical component of multi-phased wastewater treatment programs. The limitations of this method are

that it requires substantial space and time. The costs of lagooning are highly variable due to land values.

The most recent development in wastewater treatment is the application of ultra violet (UV) radiation to inactivate bacteria. UV treatment is viewed favorably because it does not require toxins, chemicals, or additives. Studies show, however, that UV sterilization may not be as effective as standard testing methods indicate (George, 2002; Said et al., 2010). In one study, UV treatment resulted in a great reduction of culturable FCB, however, the treatment did not reduce the abundance of bacteria detected using other indicators of life, such as enzymatic activity (George, 2002). These results demonstrate the possibility that UV treatment does not kill FCB, but causes it to enter a viable but not culturable (VBNC) state (VBNC phenomena is further explored in Chapter 2). Said et al. (2010) confirms that UV irradiation results in some proportion of VBNC *E. coli* cells and the proportion varies with dose and recovery time.

All of these treatment options have specific benefits and drawbacks. The cost and effectiveness of treatments varies by location, scale, and design. Many treatments such as decanting and lagooning provide multiple benefits in a single stage of treatment. Though current wastewater treatment methods have been proven effective, areas for possible improvement are vast. Further examination into bioremediation/mycoremediation of FCB contaminated waterways is warranted.

Theory Supporting Mycoremediation of FCB

Though the application of fungi to kill, filter, or otherwise reduce FCB has not been described in the published literature (unpublished field reports are described in "Applications of Mycoremediation to Reduce Fecal Coliform Pollution" below), the literature does support the theory that fungi attack bacteria, sometimes in a predatory manner. It is well known that bacteria and fungi are often in direct competition for resources. Fungi often kill bacteria to gain a competitive advantage (Gramss, 1987), however, fungi also often rely on nitrogen fixing bacteria to meet nitrogen demands (Spano et al., 1982). Fungi have developed numerous strategies for obtaining limited nutrients from microorganisms.

One method fungi use to acquire nutrients is predation of small, multi-cellular organisms, such as nematodes. There is a wealth of literature on nematophagous (nematode eating) fungi including physiological, ecological, and morphological information on the fungi, and the process of capture and digestion (Cooke, 1962a and 1962b; Barron and Thorn, 1987; Barron, 1992). For example, predatory fungi produce an extensive mycelial network of anastomosing hyphae that may form traps. These traps take a number of forms including adhesive knobs, adhesive branches, adhesive networks, rings, and constricting rings, all of which capture, penetrate, colonize, and digest the organism (Barron, 1992).

Cooke (1962) postulated that the great majority of these nematophagous fungi were not obligate nematode-trappers because they could be grown in axenic

(only one organism present) culture. Cooke argued that if nematodes and nematode-destroying fungi were in dynamic equilibrium, then one would expect predatory activity to continue as long as nematode populations were high. Cook designed an experiment in which nutritionally equivalent amounts of decomposing cabbage and sucrose were added to separate soils. Decomposing cabbage produced a nematode population explosion while sucrose produced substantially fewer nematodes. Sucrose amended soils, however, produced a longer and more intense period of nematode predation than did decomposing cabbage. Also, Cooke noted that fungi were only able to trap nematodes for a short period following soil amendments. Furthermore, he found that increasing soil amendments beyond certain levels resulted in a decrease in predatory activity despite an increase in nematode population. Based on these results, Cooke (1962) concluded that nematodes were an alternative nutrient source rather than the sole nutrient source for nematophagous fungi.

Cooke suggested that nematode trapping fungi were only predatory during competition, possibly to escape the competition for available substrates during decomposition. Cooke indicated that carbon may be the limiting nutrient determining fungal predation, but others have theorized that nitrogen is the limiting nutrient. Blackburn and Hayes (1966) tested this hypothesis using a variety of carbon and nitrogen sources to grow fungi in culture. They found the nutritional requirements of nematophagous fungi to be similar to other fungi and concluded that there was no specific benefit of nematode predation aside from an alternate nutrient source.

Continuing investigations into C/N ratios and the response of nematophagous wood-rotting fungi, Thorn and Barron (1987) examined *Pleurotus* species. *Hohenbuehelia*, a group that was previously classified as a *Pleurotus* species (and is still considered closely related) was also included in this study. Thorn and Barron examined the activity of *Pleurotus* species in the presence of nematodes and noted that while *P. ostreatus* demonstrated predatory activity, the method of predation was unique.

> On a water agar base, the hyphae of *Pleurotus* produced tiny droplets of toxin from minute spathulate secretory cells. Nematodes touching such droplets showed a sudden dramatic response. The head region appeared to shrink considerably; this was accompanied by displacement of the esophagus and disruption of the adjacent tissue. Within a few minutes the nematode became more or less paralyzed but not killed and showed feeble movements if disturbed or transferred to water. Stimulated by leakage products from the immobilized nematode, hyphae in the vicinity produced directional hyphae which converged on the body orifices of the victims. On reaching the host, directional hyphae penetrated through the orifices and colonized and digested the victim (Barron and Thorn, 1987).

Does this predation extend beyond nematodes, possibly to organisms of human health concern? It is well established that fungi attack nematodes. Fungi also attack yeasts (Hutchinson and Barron, 1996), rotifers (Barron, 1980), other macro-fungi (Griffith and Barnett, 1967; Owens et al., 1994), and other microorganisms (Tsuneda and Thorn, 1995). Bacteria constitute a large proportion of biomass and contribute to the ecosystem as food for many organisms. Direct predation of fungi on bacteria, however, was not considered until the 1980's.

Several studies published in the early 1980's demonstrated that fungi could survive using bacteria (already killed in this case) as the sole nutrient source (Fermor and Wood, 1981; Sparling et al., 1982). In particular, basidiomycetes, mushroom forming fungi, showed increased ability to grow on media of bacterial origin. Fermor and Wood (1981) established that the fungi being tested, *Agaricus sp*., secreted the enzymes necessary to degrade bacteria and utilize them as the sole source of carbon, nitrogen, and probably phosphorus. They also reported that, though the mycelial extension rate was lower than on standard media (almost half), the morphology of basidiomycetes was generally unaltered (growth rate was slower, but appeared similar physiologically). Other types of fungi such as *Aspergillus, Penicillium,* and *Trichoderma* were also able to degrade some dead bacterial media, but the growth rate and morphology of these groups was severely altered, indicating that basidiomycetes are best suited for utilization of bacteria as a nutrient source.

Fermor and Wood (1981) demonstrated the ability of fungi to use nutrients as they come available from dead and dying bacteria, however, they did not expand their experiments to include living bacteria. Barron (1988) established that live bacteria from the genera Psuedomonas and Agrobacterium could be used as a nutrient source for fungi. Over 100 fungal species were cultured on dextrose agar or malt extract agar and isolates were transferred to a water agar (WA) medium where they were allowed to grow for 3-7 days. Of the fungal species tested, four species, *Agaricus bisporus, Coprinus quadrifidus, Lepista nuda,* and

Pleurotus ostreatus exhibited a positive response by forming hyphae leading directly to the microcolonies.

> The presence of the bacteria stimulated the initiation and development of branches, from hyphae in the vicinity, that honed in on colonies. Even before the arrival of these directional hyphae at the site of attack, changes could be seen in physical appearance of the bacterial colonies, especially on the side of directional hyphae. Growth of the bacterial colony appeared to have stopped and the refraction changed giving the edge of the colony a dark outline… Directional hyphae remained unbranched until they penetrated the bacterial colonies, then branched repeatedly and irregularly and eventually formed a coralloid mass (Barron, 1988).

Only the four basidiomycete species were observed attacking bacteria in this manner (none of the 40 species belonging to Oomycota, Zygomycota, Ascomycota, or Deuteromycota shared this ability) (Barron, 1988).

For Pleurotus species, this method of bacterial predation is very similar to the sequence of events leading to predation of nematodes (Thorn and Barron, 1987). Barron (1988) noted that the nutrient content of a microcolony is similar to that of a small nematode. Other evidence suggests that the deliberate attack of surrounding microorganisms by fungi may not be driven by nutritional value *per se.* Data pertaining to fungal succession indicates that predation, while providing an intermediate nutrient source, may also be a method to colonize a more desirable, larger, or higher nutrient content food source (Gramss, 1987; Owens et al., 1994).

Gramss (1987) examined the colonization of timber by lignolytic basidiomycetes (e.g. *P. ostreatus*) as a dynamic interaction with microbial wood

substrate contaminants. He found that the initial intense and random colonization of wood substrate contaminants was greatly reduced when basidiomycetes colonized the substrate. Continued investigation demonstrated that the non-fungal microorganisms continued to decline to extinction or reached equilibrium consisting of few contaminants with the vast majority of biomass composed of wood-rotting fungi. Gramss (1987) noted that the fungi that are incapable of reducing the native microflora will fail to establish on the wood substrate at all, meaning that the ability to kill microorganisms is a necessary precursor to colonization of a woody substrate by lignolytic fungi.

Though *P. ostreatus* is not a species typically found in aquatic settings, antagonistic relationships between other aquatic fungi and bacteria have been evaluated. Gulis and Suberkropp (2003) demonstrated that aquatic fungal isolates exhibited inhibitory effects on bacteria in laboratory cultures. However, microcosm experiments indicate a bi-lateral antagonistic relationship (Gulis and Suberkropp, 2003; Mille-Lindblom and Tranvik, 2003), meaning that the presence of both aquatic fungi and bacteria significantly reduced the biomass produced by either organism. The likelihood of this relationship arising due to competition of resources is low, because antagonistic effects were recorded before nutrients became scarce (Mille-Lindblom and Tranvik, 2003).

The fact that inhibition of bacterial growth was demonstrated in culture experiments and not observed in microcosm experiments (Gulis and Suberkropp, 2003) points to the assumption that inhibitory fungal secretions become too diluted to be effective and/or wash away in aquatic environments, though this

relationship requires further clarification. Other terrestrial research indicates that while the presence of bacteria may reduce biomass productivity in *P. ostreatus*, it may also give *P. ostreatus* a competitive advantage over other fungi (Velazquez-Cedeno et al., 2004). Despite limited knowledge of fungi-bacteria antagonistic relationships in natural aquatic systems, the use of terrestrial fungi as a treatment for undesired bacteria has been/is being considered (Thomas, 2009; Stamets, 2001). Mycoremediation techniques have also been applied to an exceedingly vast array of environmental hazards. Major applications of mycoremediation techniques are explored below.

Mycoremediation Background

Mycoremediation is the use of a fungal treatment to reduce toxicity, carcinogenicity, pathogenicity, or other undesired effects of compounds, wastes, soils, and other environmental contamination. Most knowledge of mycoremediation comes from laboratory experiments, not field installations (Singh, 2006). Use of fungi as a bioremediation tool, however, has been applied to the treatment of a wide variety of waste, wastewaters, and hazardous/toxic compounds.

Yeasts and other fungi are used extensively to reduce the strength of a wide variety of food processing wastewaters with concomitant production of food or fodder fungi (Hang, 1980; Lemmel, 1979; Hang 1973). For example, *Candida utilis*, the most commonly used yeast for industrial bioremediation, can be used to reduce the biological oxygen demand (BOD), nitrogen, and phosphorus in

sauerkraut waste (Hang, 1973). *C. utilis* is also used in the assimilation (reduction of BOD) of lemonade processing wastewater (Hang, 1980). Starches may become concentrated in food processing waters associated with sago, vermicelli, and cassava. Combinations of yeasts and other fungi, such as *Aspergillus* and *Myceliophthora*, are used to reduce the starches, BOD, and chemical oxygen demand (COD) before these wastewaters enter natural systems (Okolo et al., 2006; Pazur and Ando, 1959). Furthermore, enzymes produced by fungi such as Aspergillus and Rhizopus can be used to convert starch-processing wastes into protein products (Yin et al., 1998) that may be used for mushroom cultivation or animal feed. Other food industries also produce significant waste that may be treated by mycoremediation.

Slaughterhouses produce large volumes of waste/wastewater that are high in protein, fat, and nitrogen (Masse and Masse, 2000; Ruiz et al., 1997). Fungi and yeasts are used to biodegrade these compounds into products that may be used for animal feed and methane production (Salminen and Rintala, 2002; Masse and Masse, 2000; Ruiz et al., 1997). Silage, another industrial waste, is a product of crops that are stored to ferment overwinter. Juices from fermenting crops, such as maize and other grasses, mix with groundwater to form silage, a highly acidic waste solution that is 300 to 1000 times more polluting than raw sewage and corrodes both concrete and steel (Snerik et al., 1977; National Rivers Authority, 1992). Arnold et al. (2000) demonstrated the feasibility of on-site treatment of silage effluent by the addition of fungal inoculum. Mycoremediation of silage has not received a great deal of attention, however, mycoremediation of other

pollutants, such as petroleum products, have been the target of considerable research efforts.

Hydrocarbons, chemicals composed mainly of hydrogen and carbon, are components of petroleum that can be highly toxic. Hydrocarbons are broken down in nature by bacteria, yeasts, and other fungi. Bacteria and yeasts have been demonstrated to degrade 2, 3, 4 (Narro, 1992) and 5 (Warshawsky, 1995) aromatic ring chains, though 4 and 5 ring chains are degraded inefficiently (Singh, 2006). However, filamentous fungi, such as *P. ostreatus*, are known to degrade chains of 2, 3, 4, (Novotny et al., 1999) and 5 (In Der Wiesche, Martens, and Zandrazil, 2003) fused benzene rings efficiently and without preference for smaller rings. Hydrocarbons are similar in chemical bond structure to lignin, a major component of wood. Therefore, fungi that decompose wood, known as white rot fungi, produce enzymes that can also break down hydrocarbons. Despite the growing wealth of knowledge, use of fungi to break down hydrocarbons into less toxic molecules has remained experimental in application.

Polychlorinated byphenyls (PCB's) are compounds that were widely used in the 20th century for their chemical stability and electrical insulating properties (EPA, 1979). These compounds were phased out worldwide in the 1970's due to their carcinogenic, mutagenic, teratogenic (malformation of embryo or fetus), and other toxic properties (EPA, 1979). These compounds have been incinerated, buried in approved landfills, chemically dechlorinated, and physically separated as means of decontamination and/or containment (EPA, 2012). Persistence of PCB's in both air and soil has lead to the evaluation of other remediation

methods. It has been demonstrated that PCB's are broken down by bacteria (Bedard, 1990), phytoplankton (Biggs et al., 1980), and fungi (Cloete and Celliers, 1999). Experimentation on this application of mycoremediation is still in the laboratory trial phase.

Another chemical widely used into the 1970's is dichlorodiphenyltrichloroethane (DDT). DDT was used as a pesticide until toxic effects to humans and animals were detected, leading industrial nations to ban DDT use in 1972. Bumpus and Aust (1987) demonstrated that the white rot fungi *Phanerochaete chrysosporium, P. ostreatus, Phellinus weirii,* and *Polyporus versicolor* all mineralized DDT. The chemical pathway of this remediation is not completely known, but production of toxic metabolites have been observed. Many of these metabolites can be further degraded by soil fungi (Foght et al., 2001). A wide variety of other insecticides and herbicides are also known to be degraded by fungi (Mougin et al., 2000; Jolivalt et al., 2000; Hirai et al., 2004). Specific chemicals and remediation rates are listed by Singh (2006) and exhibit a wide range of species and effectiveness, reaching 100% (Jolivalt et al., 2000) in some cases. The majority of knowledge on degradation of pesticides by microorganisms is derived from cultural studies involving individual organisms acting on individual pesticides.

Heavy metals are another group of toxins of environmental concern with a possible solution arising from fungal treatments. It is reported that 389 of the 703 National Priority List sites in the USA contain toxic metal contamination and at least 100,000 sites are estimated in Europe (Singh, 2006). The remediation of

sites contaminated with heavy metals using fungi is termed mycosorption or mycoaccumulation (also termed bioabsorption or bioaccumulation, which includes all organisms used in the absorption process, such as bacteria). This type of remediation is different from the previously described methods because metals are toxic in their elemental state and cannot be broken down further into a less toxic substance. Some bacteria and fungi have the ability to absorb high concentrations of heavy metals (Michelot et al., 1998; Taboski et al., 2005). Toxic metals are contained in the fungal biomass. This biomass may be harvested for recycling of the metals (Galun et al., 1983) or it must be disposed of. Mycosorption may be applicable in remediation of other chemicals that are difficult to break down such as herbicides, phenols, and dyes. Another application of mycoremediation that may have widespread implications for human and ecosystem health is the treatment of waterways contaminated with FCB.

Applications of Mycoremediation to Reduce Fecal Coliform Pollution

In 2007 and 2008 a study was conducted in the Dungeness River watershed which was intended to demonstrate the ability of fungi-treated bioretention cells to remediate FCB and nutrients from a water stream (Thomas et al., 2009). The experimental design for this project consisted of an inflow pipe that was split equally into two diverging paths. Each path directed the inflow stream into a large bio-retention cell (constructed wetland) planted with native vegetation. The bioretention cells were dug to the dimensions 3 m wide by 9 m long by 1.5 m deep. A perforated drain pipe was placed at the bottom of the

bioretention cells and covered by 15 cm of gravel. Permeable landscape fabric was placed on top of the gravel and covered with 75 m^3 of soil. On top of the soil, a layer of Alder (*Alnus Rubra*) mulch, measuring 14 m^3 in volume, was added. The treatment cell also received 14 m^3 of mulch, but 3 m^3 of that mulch was inoculated with *Pleurotus ostreatus*, *Pleurotus ulmarius*, and *Stropharia rugoso-annulata* prior to installation. Native vegetation was then planted in both bioretention cells and an impermeable wall was built between the two cells to ensure that the water in the treatment and control cells did not mix.

This study was conducted in three phases: 1) a fecal coliform and nutrient study, 2) a fluorescein dye study, and 3) a dairy lagoon waste loading experiment. In the first phase, coliform bacteria and nutrients were simply monitored at the inflow, control outflow, and treatment outflow on a monthly basis. The second phase introduced a dye into the system to determine the water retention time. The third phase introduced higher levels of FCB on a one time basis with monitoring occurring at irregular intervals (many at first with longer periods between each subsequent test) (Thomas et al., 2009).

Phase one lasted 6 months, performing data collection monthly. The results of the study from phase one demonstrated effective removal of FCB in both the control and treatment cells. The control cell reduced FCB by 66% from an average 30 colony forming units per 100ml (CFU/100ml) to 10 CFU/100ml. The treatment cell reduced FCB by 90% from an average 30 CFU/100ml to 3 CFU/100ml. It should also be noted that significant water loss occurred from inflow to outflow due to soil absorption, evaporation, evapotranspiration, and

incorporation into biomass. The inflow averaged 11 L/min (in both cells) while the outflow rate averaged 3.6 L/min, a 68% reduction in flow rate.

The results of phase two demonstrated that the fungal treatment cell had a much longer retention time. Substantially less total dye was recovered from the treatment cell, possibly indicating that the fungi altered its composition. It is also important to note that though the retention times varied between the two cells, the outflow was similar for both the control and treatment cells.

In phase three, a total of 194,250 CFU were added to the inflow over a 15 minute period. The control outflow increased up to 376 CFU/100 ml after 1 h. There was a significant difference in fecal coliform bacteria between the control and treatment groups up to the 28 h sampling point. The treatment outflow coliform counts remained constant for the duration of the experiment with an average of 5 CFU/100 ml. The results of this experiment demonstrate a significant impact on fecal coliform density in a water stream when treated with fungal inoculate. This study did not consider a treatment consisting solely of mycelium. If mycelium alone can perform as well as with plant installations, it would be a far cheaper option to only install inoculated wood chips and allow flora to establish naturally.

The above study is one of two unpublished scientific documents on mycoremediation of fecal coliform bacteria. It is the most recent of the two, citing the other, so it is assumed that this is the current state of knowledge. The other document, “Water quality research project using mushroom mycelia for

mycofiltration of fecal bacteria," by Hayward and Stamets (1998), was submitted to the Washington State Conservation Commission (WSCC). Currently, neither the authors nor the WSCC is able to locate a copy of this report. It is worth noting that Stamets (2001) also published an anecdotal recounting of mycoremediation of FCB in a field installation.

The experiment presented in Chapter 2 and analyzed in Chapter 3 adds a laboratory microcosm experiment to the current knowledge of mycoremediation of FCB. The purpose of this experiment is to demonstrate *P. ostreatus* mycelia as a causal agent in the reduction of FCB from a solution. This experiment also removes many of the variables observed in field experiments, such as weather, water accounting, plants, animals, and other microorganisms. A complete description of the methodology for this experiment is presented in Chapter 2.

Chapter 2 Materials and Methods

Overview

This study consisted of an experiment under laboratory conditions to test the effectiveness of *P. ostreatus* in reducing the abundance of viable (colony forming) *E. coli* bacteria in a water stream or solution. In this experiment, an *E. coli* solution was pumped into glass tubes containing sawdust (control) and sawdust inoculated with *P. ostreatus* (treatment). The *E. coli* solution was pumped to the top of tubes and filtered by gravity into a collection beaker below. The abundance of colony forming bacteria was measured in the inflow and outflow solution using standard microbiological methods including serial dilution and the spread plate method (Koch, 1994). Using this method, bacteria is transferred to a culture plate containing a maximum growth media (TSA in this case) as a sample of the inflow or outflow solution. Viable *E. coli* grows rapidly on the nutrient rich media allowing enumeration by the naked eye after 24-48 h incubation.

Determining whether a cell is alive or dead is difficult because there are many characteristics of a live, metabolically active cell and several ways to measure these characteristics. For example, viability of a bacterial cell has been defined as the ability of a cell to multiply after incubation under standard microbiological conditions (Postgate et al., 1961). This increase in cell abundance can be measured by plate counts of cell cultures, however, not all species of bacteria are culturable. Furthermore, bacteria may enter a physiological state described as viable but not culturable (VBNC or VNC)

(Roszac and Colwell, 1987). A number of methods have been described using criteria other than cell division to determine cell viability. Among these are 1) CTC – uses reduction of tetrazolium salts as an indicator of active electron transport (Rodriguez et al., 1992), 2) FDA – detection of esterase on a fluorogenic substrate as an indicator of metabolic activity (Bogaerts et al., 1998), 3) detection of nutrient uptake from substrate (Rahman, et al., 1994, Shahamat et al., 1992), 4) NA inhibition – naladixic acid is used to inhibit cell division causing growing cells to elongate (elongated cells are considered viable) (Goss et al., 1965) and 5) membrane permeability – uses flourogenic compounds to stain permeable and impermeable membranes different colors (Leuko et al., 2004). Due to limited resources and time, enumeration was conducted using cell division in culture (spread plate method).

The experiment considered a treatment group and a control. Both groups consisted of 5 g (dry weight) autoclave-sterilized, wetted sawdust contained in open-ended glass tubes. The treatment group was inoculated with *P. ostreatus* while the control group was not inoculated. This design ensures equivalent masses in the treatment and control groups, however, does not control for the increased surface area created by the mycelium. A working solution of *E. coli* (approximately 100,000 CFU/ml) was passed through treatment and control tubes with samples being taken at the inflow and outflow after 100 ml of the working solution had been filtered. These samples were analyzed for bacterial abundance by serial dilution and spread plate techniques. The experimental design is illustrated in Figure 1.

Substrate Tube Preparation

Open ended borosilicate glass tubing (dia=20mm X length=200mm) was selected for its low coefficient of thermal expansion that results in resistance to thermal shock, making it ideal for heat/pressure sterilization. Borosilicate glass is also resistant to chemical reactivity. The sawdust used as a substrate for fungal growth was 100% *Alnus rubra*, a hardwood species that commonly acts as a substrate for *P. ostreatus* in nature and edible mushroom cultivation. This sawdust was donated by Fungi Perfecti (http://www.fungi.com/), a company specializing in mycology supplies outside of Olympia, WA.

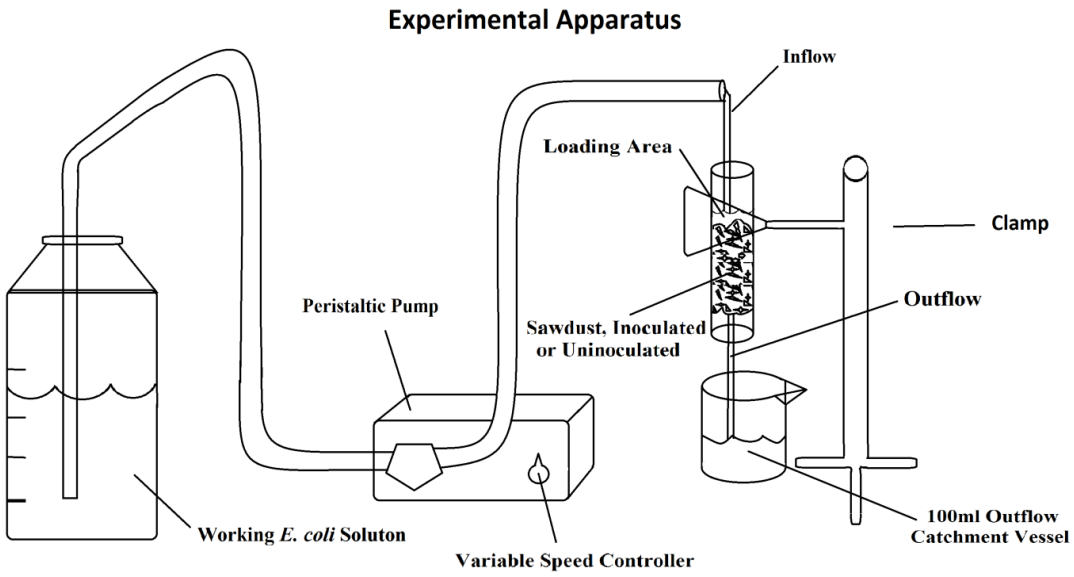

Figure 1: Experimental apparatus showing the path of the working solution. Starting from the left, the working *E. coli* solution is pumped by a peristaltic pump into the culture tube where it is filtered by gravity into the 100 ml catchment beaker below.

Sawdust was oven dried maintaining temperatures between 37 °C and 65 °C. Once completely dry, it was sieved so that the sawdust particle size fell between .355mm and 3.962 mm. 5 g of dry sawdust was required to fill each tube, which was determined empirically. 300 g of sawdust (5 g per tube X 60 tubes = 300 g) was weighed and wetted to saturation. Excess water was expelled from the sawdust by squeezing within a fist. When these steps had been completed, equivalent amounts of sawdust were inserted into the glass tubes. The purpose of drying the sawdust was to ensure that equal masses of sawdust were placed into each tube. The purpose of sifting sawdust was to maintain consistent particle sizes across individual tubes.

A spruce dowel rod (20 mm diameter) matching the inside diameter of the glass tubes was used to collect sawdust from the inside of the glass and pack it into a cylinder within the tube. On one end of each tube, a stainless steel mesh screen was inserted to keep particles from collecting in the outflow beaker. The packed cylinder measured 10 mm in radius and about 140 mm in height (± 2 mm from tube to tube). 60 tubes were prepared in this manner and capped with Kimble® Kim Kaps for culture tubes. The tubes were then heat/pressure sterilized in an autoclave for 1h at 121°C and 15 psi. These tubes were allowed to cool overnight in preparation for inoculation.

Experimental Treatments

The open-ended culture tubes for the control group remained capped with polypropylene Kimble® Kim Kaps for culture tubes until needed. Kim Kaps allow for limited air exchange, thus reducing the likelihood of contamination from airborne microorganisms. 30 treatment culture tubes were inoculated with .5 g of *P. ostreatus* spawn (donated by Fungi Perfecti) under an ethanol-sterilized laminar flow hood to reduce the likelihood of contamination. Inoculation was achieved using a gloved hand to weigh .5 g of spawn on an ethanol sterilized laboratory scale and place it on the top (opposite end from the stainless steel mesh screen) edge of the substrate. Once inoculated, treatment tubes were immediately re-capped and placed inside an ethanol-sterilized cooler (used as an incubator) until hyphae (filamentous extensions of mycelium) reached the bottom of the tube. Heat produced by the metabolic activity of the growing fungus within the cooler was sufficient for incubation at room temperature. When hyphae reached the bottom of the tube, both the culture and treatment tubes were determined to be ready for experimentation.

Preparation of Media for *E. coli* Cultures

Both liquid and solid media were used to support growth of *E. coli* in this experiment. The nutrient content of both media were supplied from dehydrated tryptic soy broth (TSB). Tryptic soy broth is a casein digest product of soybean commonly used for the cultivation of a wide range of fastidious organisms in culture.

Liquid media was prepared according to the manufacturer's guidelines and commonly used microbiological recipes. The "maximum growth media" recipe was modified by reducing the nutrient concentration by ½. This recipe alteration allowed for the conservation of powdered ingredients, while providing sufficient bacterial growth for the experiment. 7.5 g of dehydrated TSB media was weighed on a balancing laboratory scale and added to a 1 L Duran bottle with 500 ml H_2O (original recipe calls for 15 g TSB/500ml H_2O). The Duran bottle containing liquid media was shaken until thorough mixing was visually evident. 5 ml of this liquid media was added to each of 10 test tubes and capped with an autoclavable screw cap. The large Duran bottle of liquid solution and the 10 smaller liquid media test tubes were momentarily set aside while solid media was prepared, at which point all, media was autoclaved together.

Solid tryptic soy agar (TSA) media for culture plate preparation was mixed in a similar process. Solid media was prepared using a full strength (1X) concentration TSA media recipe. This concentration was used to ensure the highest level of recovery of viable bacteria. Culturable bacteria that come into contact with this nutrient rich media grow and produce colonies quickly. 15 g of dehydrated TSB was weighed with a balancing laboratory scale and added to a 1 L Duran bottle containing 500 ml H_2O. 6 g dehydrated agarose crystals for microbiological use was also added to the Duran bottle. The bottle and its contents were shaken until thorough mixing was evident.

Both the solid and liquid media were autoclaved at 121 °C and 15 psi for 1 h. Liquid media was allowed to cool at room temperature overnight. Solid media

was allowed to cool for about 15m, at which time it was still warm enough to pour and cool enough to ensure that Petri dishes would not melt or warp. Solid media was poured until it covered the surface of the Petri dish (at this point, referred to as an agar plate or culture plate). Petri lids were placed on top of agar plates and plates were allowed to solidify overnight. After allowing agar plates to solidify and incubate for 2 days, the culture plates were checked visually for growth. If no growth was evident, the plate was considered uncontaminated, wrapped with laboratory Parafilm, and placed, inverted, into a refrigerator until needed. Contaminated plates were disposed of. This procedure was repeated as needed to produce culture plates on which to detect and enumerate *E. coli*.

Preparation of *E. coli* Solutions

E. coli strain 12099 was obtained from the T-4 phage laboratory at The Evergreen State College on a streak culture plate. Cells from at least three *E. coli* colonies were collected on a flame-sterilized microbiological transfer loop. A 5 ml culture tube was uncapped and the transfer loop was inserted into the liquid TSB media and agitated to ensure sufficient transfer of bacteria into the liquid media. The culture tube was placed into an agitating incubator (Lab Line Instruments model 4645R) set to 145 RPM and 37°C for 48 h. After 48 h, the culture had reached log phase which was confirmed by consistent cell densities determined by serial dilution and plating on TSA media. These 5 ml *E. coli* cultures would be used for up to 5 days, at which time, a new culture was produced using the same protocols. This process ensured that the bacteria solution used for experimentation contained only fresh and vigorous *E. coli*.

Preparation of a working *E. coli* solution was as follows. 100 µL of a 5 ml *E. coli* culture was transferred using an auto-micropipetter (10 µL- 200 µL) into a 1 L Duran bottle containing 1 L of de-ionized H_2O. De-ionized H_2O was used to reduce the chance of introducing bacteria or other microorganisms occurring in tap water. This dilution (100 µL *E. coli* solution in 1 L de-ionized H_2O) consistently produced *E. coli* densities of 100,000 CFU/ml (10^7 CFU/100ml) determined by serial dilution and spread plating. A working solution produced in this manner was used in all experimental trials.

This concentration of *E. coli* is far greater than the acceptable contamination rate for recreational fresh water in Washington state under WAC 173-201A-260, which is .5-2 colony forming (viable) cells/ml for recreational waters depending on the classification of the water use (extraordinary contact recreation, primary contact recreation, and secondary contact recreation) and .1 CFU/ml for drinking water (Department of Ecology, 2003). The high concentration used in this experiment provided the multi-faceted benefits of ease (both for creating dilutions and for mathematical calculations), ability to detect bacteria within the countable range for the spread plate method, and ability to detect changes in the abundance of colony forming bacteria. This concentration of bacteria is also consistent with FCB densities in raw sewage, which is typically 10^4-10^6 CFU/ml (10^6-10^8 CFU/100 ml) (Rose et al., 1996; Audic, 1990; Miescer and Cabelli, 1982).

Conventional water quality testing differs from the method used in this study slightly. In the most common procedure, a known volume of H_2O is

filtered via vacuum pump onto a MF. The filter is then transferred to a culture plate containing selective growth media for *E. coli*, incubated for 24-48 h, and colonies are counted. The MF technique is labor intensive and fails to count VBNC cells. Because, in this study, the bacterial solution contains only *E. coli* cells, a selective media was not required.

Experimental Design

The working *E. coli* solution was pumped into the top of the open ended culture tubes (See Figure 1) at flow rates ranging from 2 ml/min (.033 ml/s) to 20 ml/min (.333 ml/s) sustained until 100 ml of the working solution had been filtered though the column. Determination of flow rate took into consideration the flow rate of other bioremediation and mycoremediation of FCB experiments, which produced a range of flow rates from 10 ml/min ft^2 (.1 ml/min cm^2) (Davis et al., 2001) to 2 L/min ft^2 (2.15 ml/min cm^2) (Seth Book, Mason County Conservation District, Personal Communication). Ultimately, however, the flow rate was determined by the rate at which filtration could be achieved through treatment tubes as they became densely packed with mycelium.

The top of the experimental columns contained an area of 314 mm^2 (3.14cm^2) for a flow rate to area ratio ranging from .637 ml/min cm^2 to 6.369 ml/min cm^2. Though this experiment was designed on a small scale, flow rate to area ratios are 3-6 times higher in this study than in other bioremediation experiments (Davis et al., 2001; Seth Book, Mason County Conservation District, Personal Communication). As previously mentioned, achieving flow through a

column densely colonized with mycelium was difficult in many treatment tubes. In some cases, a hydrophobic mycelial mass formed on the top layer of the treatment tubes. In cases where flow could not be achieved, it was necessary to pierce the top layer of mycelium to a depth of 1 cm. This piercing of mycelium was achieved using a pipette tip (200 μL). In some samples, however, piercing the top layer of mycelium still did not produce flow of the working solution through the tubes, which reduced the number of treatment trials to 16.

Inflow rates to the top of the tubes were managed using a peristaltic pump (Master Flex 7518-02, .6-460 ml/min). Using this pump, flow rate could be adjusted in real time. Flow rates were determined by the rate at which the working solution filtered through the pump by gravity (which varied between individual trials). The top of the culture tubes contained an open area not consumed by substrate/mycelium to a depth of 5 cm. Using the peristaltic pump, this zone was used as a loading area (see Figure 1) where the working solution was allowed to collect to a depth of 2-3 cm. Control tubes generally filtered more quickly than treatment tubes. To account for this discrepancy, the peristaltic pump was controlled to produce a flow rate consistent with the flow rate for the corresponding treatment trial. Treatment and control trials were alternated to maintain similar flow rates and to ensure that the working solution entering the tubes was as consistent as possible.

Inflow and Outflow Sampling

When 100 ml of the working solution had been filtered through a tube, samples of the inflow and outflow were collected in separate ethanol sterilized vials and the total time of filtration was recorded. Filtration of 100 ml was determined visually in the collection vessel (a beaker collecting the filtered working solution) marked with volume measurements (see Figure 1 above). At this point, a sterile vial was used to collect 2 ml of the solution currently being pumped from the end of the hose. The hose was removed from the top of the culture tube and placed into the collection vial. At the same time, a separate sterile vial was used to collect 2 ml of the outflow dripping from the bottom of the culture tube.

100 μL aliquots of both the inflow and outflow samples were transferred to test tubes for serial dilution using an auto micropipetter (200 μL). 900 μL of de-ionized H_2O was added to each test tube containing sample aliquots using an auto-micropipetter. These tubes were agitated (shaken by hand) to ensure complete mixing. This process was repeated using a 100 μL aliquot from the diluted samples in test tubes, giving a final concentration that was 1/100th of the original working solution. This method of serial dilution is common in microbiological practices.

Detection and Enumeration of FCB

The bacterial density of the second serial dilution was enumerated using the spread plate method (Koch, 1994). 100 μL of the 1/100th concentration samples were added to culture plates containing TSA media. An ethanol sterilized glass spreader (bent glass rod) was used to spread the liquid over the surface of the media until all liquid had been absorbed by the media or evaporated. Liquid was determined to be absorbed or evaporated when the glass rod was no longer lubricated by the liquid and exhibited extreme friction or stickiness as the rod swept across the field.

Once excess liquid had been evaporated or absorbed, Petri lids were placed on top of the culture plates and plates were sealed using laboratory Parafilm. Plates were then incubated, inverted, at room temperature (26°C). Inverting the culture plates reduces the possibility of any air-borne contaminants trapped inside of the culture dish landing on the media and contaminating the sample.

After 24 h of incubation, *E. coli* colonies were counted using a marker to place a dot on the outside of the dish just over the colony. This process ensured that colonies were not counted twice. The number of colonies was recorded and culture plates were again allowed to incubate, inverted, for 24 h. After the second incubation, culture plates were re-examined for colonies that were not visible after the first incubation period. This method allowed for differentiation of colonies in close proximity to one another that may have been indistinguishable as separate

colonies after 48 h, while giving sufficient time for cells to recover and produce colonies large enough to see with the naked eye. Total colony counts after the second incubation period were recorded for the inflow and outflow of each individual trial. Some plates were not inoculated to provide a positive control. Also, multiple dilution series were plated for select trials to confirm that dilutions resulted in bacterial abundance that differed by a factor of 10. Both multiple plating and positive controls confirmed the acceptability of this methodology.

Chapter 3 Results and Discussion

Presentation of Data

The mean concentration of *E. coli* bacteria in the inflow was 114,200 CFU/ml (1.1 X 10^8 CFU/100 ml) in the treatment group and 87,000 CFU/ml (8.7 X 10^7 CFU/100ml) in the control group. The mean concentration of *E. coli* in the outflow was 89,930 CFU/ml (8.9 X 10^7 CFU/100ml) in the treatment group and 100,500 CFU/ml (1 X 10^8 CFU/100ml) in the control group. The variance for the treatment inflow was 76,000 and 93,900 in the treatment outflow. The variance was 75,800 in the control inflow and 138,700 in the control outflow. The sample size was 14 for the treatment group and 11 in the control group. Different sample sizes arose due to removal of extreme outliers and contamination of some samples that resulted in no data. All data collected in this experiment, including values not considered in statistical analysis, are presented in Table 1.

All data and statistical analyses are representative of the sample data with two outliers removed. Both outliers presented data points in which the bacterial abundance increased by several hundred percent (both in the control group) and they are presented along with all other data in Table 1. Contamination of samples was determined by visual examination. A contaminated culture plate appeared cloudy and/or exhibited visible streaks where the glass rod spreader was used, indicating the presence of other bacteria. This appearance differed from that of a "clean" culture plate which contained colonies of a light yellow-milky color that

had distinct edges. Figure 2 shows the visual difference between a counted culture plate and one that was omitted due to contamination.

Experimental Data					
Treatment Group					
#	Time (mins)	Inflow Count	Outflow Count	Cells Reduced	Percent Change
1	10	115	85	30	-26.09
2	9	159	137	22	-13.8
3	16	156	90	66	-42.3
4	7	123	60	63	-51.2
5	15	151	164	-13	8.6
6	35	93	90	3	-3.2
7	4	93	95	-2	2.1
8*	No Data – Contamination				
9	5.5	83	101	-18	2.2
10	45	123	53	70	-56.9
11	13	107	57	50	-46.7
12	45	94	72	22	-23.4
13	16	134	105	29	-21.6
14*	No Data – Contamination				
15	14	90	76	14	-15.6
16	6	78	74	4	-5.13
Mean	17.2	114.2	89.9	24.3	-19.5
Control Group					
#	Time (mins)	Inflow Count	Outflow Count	Cells Reduced	Percent Change
1	50	75	63	12	-16.0
2	7	50	96	-46	92.0
3*	7.5	34	192	-158	465.0
4*	5	76	280	-204	268.4
5	30	75	57	18	-24.0
6	30	133	126	7	-5.26
7	6	85	117	-32	37.6
8*	No Data – Contamination				
9	6	107	71	36	-33.6
10	14	74	141	-67	90.5
11	7	80	84	-4	5.0
12*	No Data – Contamination				
13	7	72	120	-48	66.7
14	17	138	170	-32	23.2
15	4	68	60	8	-11.8

16*	No Data – Contamination				
Mean	16.2	87	100.5	-13.5	20.4

Table 1 : Data collected in the 100 ml *E. coli* solution filtration experiment. Time is the time required to filter 100 ml of *E. coli* solution. Inflow and outflow counts are the number of *E. coli* colonies cultured after two serial dilutions (1/100th concentration). Cells reduced and percent reduced are arithmetic calculations of the original data presented in the table. * Indicates data omitted from statistical analysis

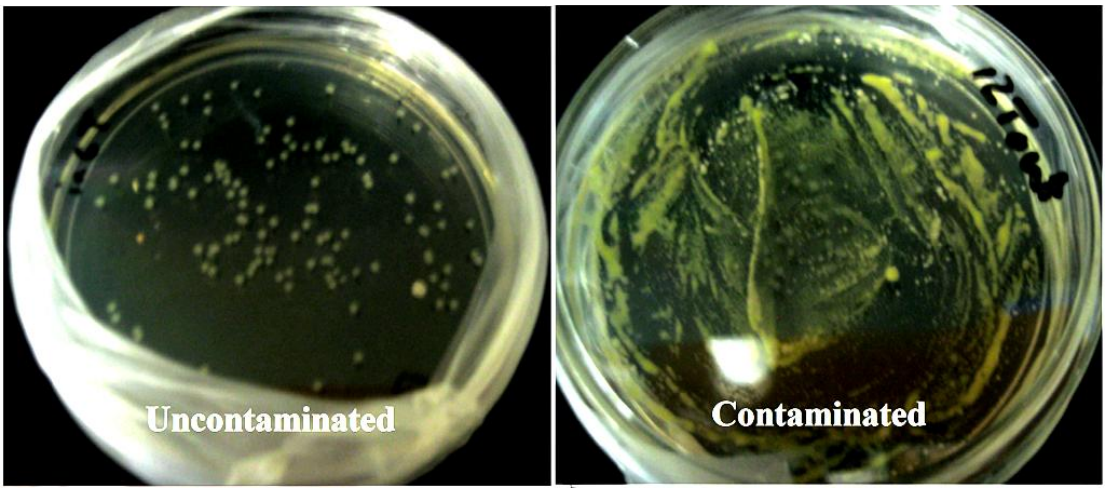

Figure 2: Demonstrates the visual difference between a contaminated and uncontaminated plate culture.

Statistical Analysis

The distribution of the inflow and outflow count data for the treatment and control groups reflect the properties of a normal distribution (Figure 3). The distribution of percent bacteria reduced from the inflow to the outflow in the treatment group exhibits a normal but skewed distribution (Figure 4). The distribution of the percent bacteria reduced in the control group, however, is non-normally distributed (Figure 4). Because of the range of normally and non-normally distributed data, both parametric and non-parametric statistics were used to analyze data.

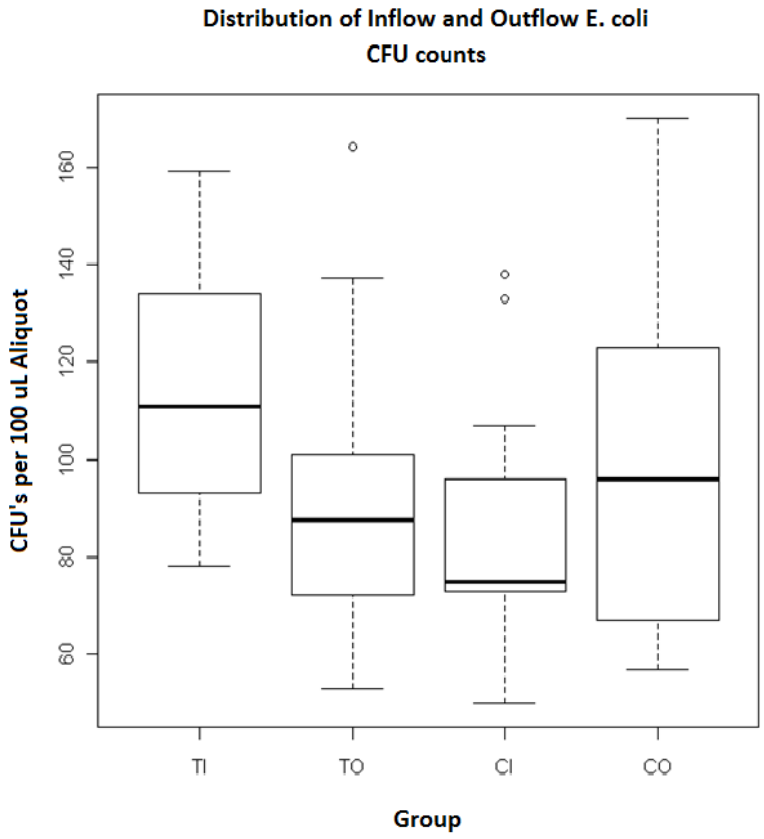

Figure 3: Distribution of inflow and outflow *E. coli* counts. TI = Treatment inflow, TO = Treatment outflow, CI = Control inflow, Co = Control outflow. This data represents the number of CFU's of *E. coli* in a 100 µL aliquot of a sample after 1/100 dilution. To convert to CFU's/ml, multiply by 1,000. To convert to CFU's/100 ml, multiply by 100,000.

Statistical analysis was performed in R, a robust, open source, statistical package used for a wide variety of statistical analyses. For parametric analysis, t-tests were used to determine if there was a significant difference between the means of the treatment inflow and outflow, the means of the control inflow and outflow, and the means of percent bacteria reduced between the control and treatment groups. Alpha was set at .05. 1-tailed paired t-tests were performed. Bacterial abundance within the solution was significantly reduced between the

treatment inflow and outflow groups (p=.025, paired t-test, n=14). Bacterial abundance was not significantly altered between the control inflow and outflow (p=.192, paired t-test, n=11).

The primary objective of this experiment was to test if fungal treatment effectively reduced the abundance of bacteria in solution as it was filtered through the experimental apparatus. A t-test of independent means comparing the mean percent bacteria reduced in treatment vs. control groups was preformed. The treatment group significantly reduced bacterial abundance within the solution compared to the control group ($p=4.14 \times 10^{-5}$, t-test independent means, n=14,11). The treatment group *reduced* bacterial abundance by an average of 19.5% while bacterial abundance within the control group *increased* by 20.4% on average. (Table 1 and Figure 4).

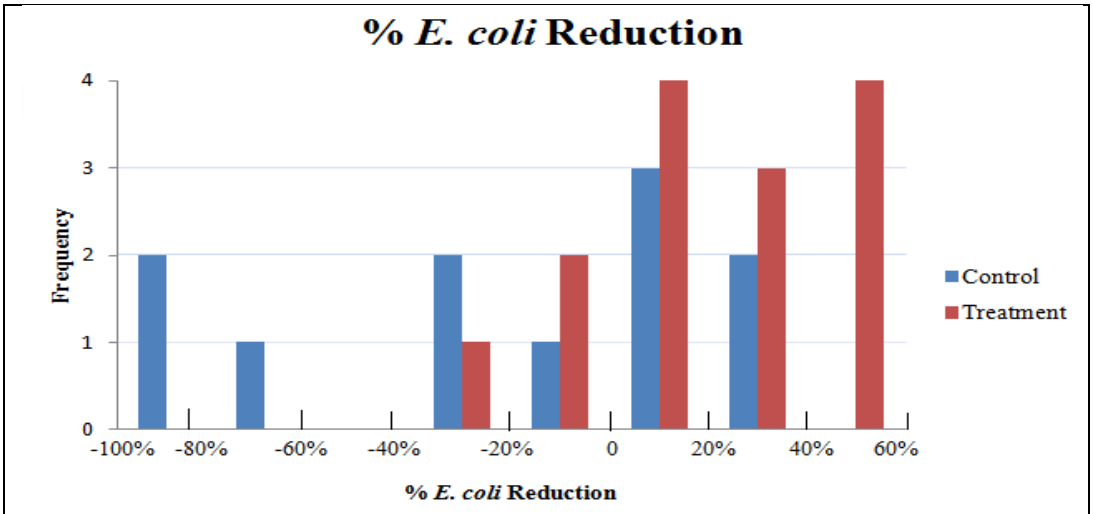

Figure 4: Distribution of percent bacteria reduction from inflow to outflow in the control and treatment groups.

Because the control group did not exhibit the characteristics of a normal distribution, non-parametric tests were also used to describe the data. A Wilcoxon paired ranked sum (also called MWW, Mann-Whitney, and Mann-Whitney-Wilcoxon) test was performed using R. The results of this analysis confirm the results of the parametric tests. Bacterial abundance was significantly reduced in the treatment group ($p = .01$, MWW, $n = 14$). Conversely, bacterial abundance was not significantly altered in the control group ($p = .195$, MWW, $n=11$). Due to unequal sample sizes, a MWW test for the percent reduction of bacterial abundance between treatment and control groups was not possible. It is possible that high variance resulted in a statistical error, however, replication and use of both parametric and non-parametric statistics indicate correct statistical analysis.

Retention Time Analysis

How does flow rate (i.e., contact time, retention time) affect bacterial abundance? This is one major question presented by this data set. The time required to filter 100 ml of the bacterial solution was recorded along with the bacterial count data (Table 1). The relationship between time required to filter 100 ml and reduction in bacterial abundance was examined (Figure 5).

Linear regressions for the percent bacteria reduced are displayed for both the treatment and control group along with the R^2 value in Figure 5. The low R^2 values, .1053 for the treatment and .1443 for the control, indicate that retention time/contact time is not a good explanatory variable for percent bacteria reduced. This does not mean that retention time has no effect on the ability of either group

to reduce the abundance of *E. coli*, but only that retention time is not a good *predictor* of bacterial abundance. The poor R^2 values are a result of high variability between data points. This high variability has been noted in previous mycoremediation experiments dealing with FCB (Seth Book, Mason County Conservation District, Personal communication; Thomas et al., 2009).

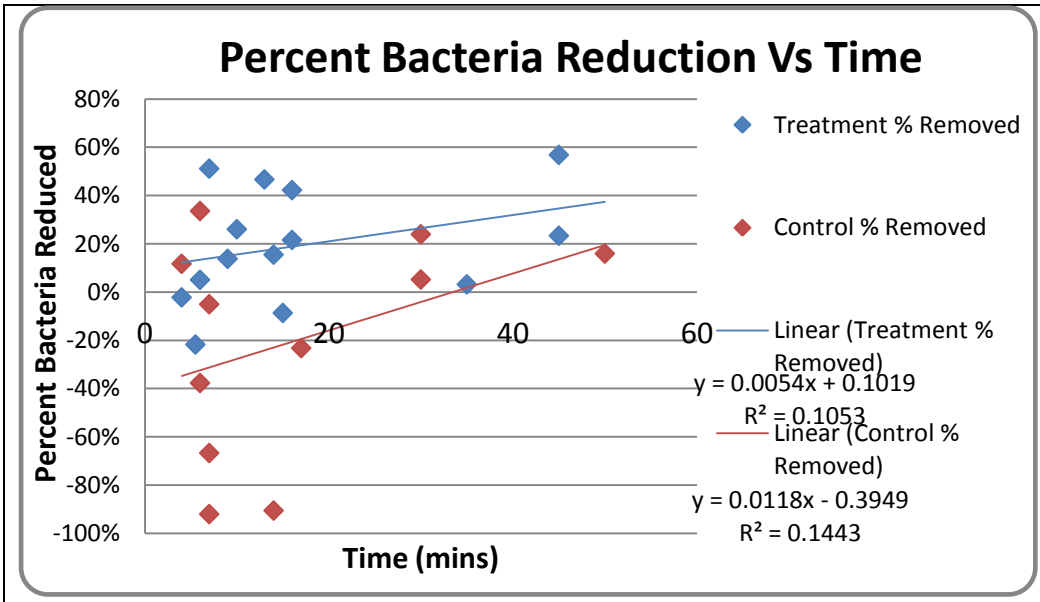

Figure 5: Percent bacteria reduced from inflow to outflow in treatment and control groups plotted against the time (in minutes) required to filter 100 ml of *E. coli* solution. Linear regression lines for treatment and control are drawn and the corresponding formula and R^2 values are presented in the legend.

A peristaltic pump with a variable speed controller was used to maintain similar flow rates/retention times between treatment and control groups. However, due to contamination and outliers, some of these data points were not used in the final statistical analysis. The final distribution of retention times for treatment and control groups is shown numerically in Table 1 and graphically in

Figure 6. Despite some data points being omitted, the relative distributions are similar.

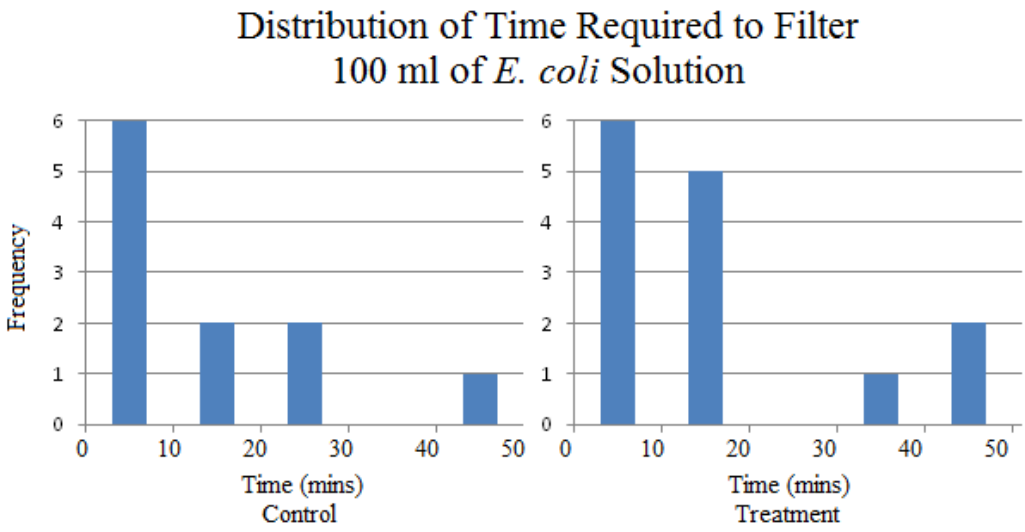

Figure 6: Distribution of the time required to filter 100 ml of the working *E. coli* solution in the treatment and control groups.

Summary of Findings

The results of this experiment demonstrate a significant reduction of bacteria held in solution when filtered through a column of sawdust and mycelium. The sterile sawdust control did not significantly alter the abundance of bacteria in solution. These results were confirmed using both parametric and non-parametric statistics. This study did not attempt to determine the mechanism by which *E. coli* bacteria are reduced. Previous evidence supports both active, physical predation (Barron, 1992; Fermor and Wood, 1981; Barron and Thorn, 1987) and enzymatic mechanisms (Barron and Thorn, 1987).

An unexpected finding in this study was the substantial increase in bacterial abundance from inflow to outflow in some trials. Bacterial abundance in individual trials rose as much as 21% in the treatment group and 92% in the control group (and even higher in omitted outlier data). These data do not seem to indicate an error in dilution rates. Errors in serial dilutions would be expected to yield data that differed by an order of magnitude. Serial dilution methodology was also confirmed by plating multiple dilution series from a single sample, which did produce counts differing by a factor of 10. One explanation is that the bacteria became concentrated within the treatment and control tubes until a maximum concentration threshold was reached, at which time, excess bacteria began to be flushed out with the rest of the solution. Rigorous testing of this hypothesis was outside of the scope and resources of this project. A one-time preliminary test in which inflow and outflow samples were taken continuously did seem to follow this trend. Resources did not permit the use of multiple samples to produce an average inflow and outflow for each trial, but, preliminary sampling indicated small variations (± about 10 to 15 colonies/plate or 10,000 to 15,000 colonies/ml).

Another possible explanation is that bacteria became concentrated at the surface micro-layer interface, the place where standing water meets air. If bacteria was concentrated in this zone, it would have filtered through the tubes when the pump was turned off at the end of a trial. This is the point when outflow samples were taken. Concentration of bacteria at this interface has been observed (Bezdek and Carlucci, 1972). Yet another possibility is that bacterial aggregation

resulted in inconsistent bacterial loading. Aggregation of bacteria has been observed on glass (treatment tubes, working solution vessel), plastic (peristaltic pump tubing), other bacteria (*E. coli* in solution) (Vaca et al., 2011) and as a response to stress (Tsimring and Levine, 1995). Further investigation into this hypothesis and the sources of variation is required to better understand these relationships.

It should be noted that several preliminary experiments were performed in preparation for the experiment presented in this thesis. These experiments did not meet the standards of scientific rigor, but, the preliminary results may be helpful to further investigations. It is expected that the use of de-ionized H_2O may alter the biological processes of both fungi and bacteria and could influence the relationship between them. Initial tests using tap water were extremely variable. Conclusions from these data are difficult to make and are probably inaccurate.

Another preliminary experiment used the final experimental design with the exception of an ice bath surrounding the working solution. The ice bath was intended to slow the growth of bacteria during the course of the experiment. Preliminary results exhibited increases in bacterial abundance in both the treatment and control groups. These data indicate that at cooler temperatures, *E. coli* may gain a competitive advantage over *P. ostreatus*. A possible explanation for this result is that the mechanism of bacterial reduction may be inactivated at cooler temperatures. Because *E. coli* counts in natural systems tend to be highest in warm months, this phenomenon may not alter the effectiveness of

mycoremediation of FCB in field applications, however, further study is needed to clarify this relationship.

Conclusions and Further Research

FCB and pathogenic organisms associated with fecal contamination pose a threat to human health. Efforts to mitigate negative health effects of human and animal waste without degrading a site's biotic activity, productivity, or available habitat have led to examinations of bioremediation techniques. A simple technique that has been proposed is filtration of contaminated waters through a network of mycelium. Paul Stamets (2001) reports great (anecdotal) success using mycofiltration to reduce the abundance of FCB in a water stream.

Thomas et al. (2009) compared fecal coliform contamination in a stream filtered through a constructed wetland bioretention cell with and without the presence of mycelium. The experimental results supported the hypothesis that filtration through a bioretention cell with mycelium increased the bacterial reduction over an un-inoculated cell. This study is the only documented analysis of mycoremediation of FCB available at this time.

In continuation of that field research, this experiment was designed for laboratory investigation. The study conducted by Thomas et al. involved a large *in situ* installation containing plants, an open air environment, and porous boundaries. Because many variables are introduced by the presence of an open system containing many organisms and extreme lack of water accounting (68% of

water entering the system did not exit the system), a laboratory study was appropriate to further examine the effect fungi has on bacterial abundance.

The experiment presented in this thesis is an effort to test mycoremediation of FCB under controlled conditions. This experiment accounts for all water entering and exiting the system and rapidly (only seconds apart) measures bacterial abundance in the inflow and outflow streams. Thus, there is certainty that the water entering the system (inflow measurement) is the same water exiting the system (outflow measurement). Because the experimental design consists of sterile sawdust (control) compared to sterile sawdust inoculated with *P. ostreatus* (treatment), it can be concluded that any true difference observed between the two groups is due to the presence of the fungi either directly or indirectly.

The results of this experiment indicate that the presence or absence of *Pleurotus ostreatus* mycelium on sawdust has an influence on bacterial abundance within a solution as it is filtered through the sawdust/mycelium. Though the data exhibited high variation, statistical analysis indicates that the presence of *P. ostreatus* mycelium causes a *reduction* in bacterial abundance within the solution. These conclusions are supported by both parametric and non-parametric statistics. The results of this study support the continuation of research into mycoremediation techniques involving mycofiltration to mitigate or remediate fecal-contaminated waterways.

One area of further research would involve demonstration of the mechanisms affecting filtration. Though predation of bacteria by *P. ostreatus* has been described (Barron, 1988), this process has never been demonstrated in an aqueous solution (Barron's experiment consisted of predation in laboratory culture on a limited nutrient media). A possible alternative theory is that surface area is related to reduction in bacterial abundance. This could be tested by repeating the experiment presented in this paper with equal surface areas across the treatment and control groups rather than equal masses. A stronger way to test this hypothesis would be to kill or inactivate the fungi without altering its physical or chemical makeup. However, at this point, a process that would kill the fungi without changing physical and chemical properties has not been identified.

Another area in which research could continue involves determining the temporal and nutritional characteristics for optimal remediation of FCB. It is unknown whether the stage of colonization (actively growing, fully colonized, or depleted substrate) affects the fungi's ability to remediate FCB. It would be interesting to see how mycofiltration changes from inoculation to a state where the original substrate has been broken down completely. Previous research indicates that the predation of *P. ostreatus* on microorganisms is related to the relative abundance of nutrients (Blackburn and Hays, 1966), however, this theory never been tested in solution.

A relationship that is not well understood is how bacterial concentration affects mycofiltration. In the only publication on this topic, Thomas et al. (2009) used low bacterial concentrations filtered through large volumes of mycelium. In

this experiment, high bacterial concentrations were filtered through small volumes of mycelium. The upper bound and curve of effectiveness cannot be calculated given the small amount of data available. The data presented in this paper represents the highest ratio of bacterial concentration to fungal biomass used in mycofiltration of FCB. The efficiency of bacterial reduction was lower than the Thomas et al. (2009) study (97% compared to 19.5%), however, the total bacterial reduction is the highest on record.

An experiment that could help to shed light on the relationship between inoculum (amount of mycelium), bacterial concentration, and reduction in bacterial abundance could follow the same experimental design used in this thesis. One way to determine this relationship would be to run this experiment in series, where the outflow from one tube would be sampled as it becomes the inflow to another tube. If filtration is consistent, a bacterial reduction rate of 20% (similar to the findings of this report) would reduce bacterial abundance by 87% after filtration through 10 treatment tubes. Another way to test the relationship between concentration and fungal biomass would be to test tubes of varying sizes with varying concentrations of *E. coli* solution.

For applications to human health and safety, an important continuation of the research presented in this paper would be to extend this study to the pathogenic organisms for which *E. coli* is an indicator, such as *Klebsiella* and *Giardia*. *E. coli* is rarely pathogenic, so the ability of *P. ostreatus* to remove *E. coli* may be insignificant to human health applications if the other pathogenic organisms have the ability to pass through unaltered. In fact, mycofiltration may

well reduce the ability to detect fecal contamination further down the line where truly pathogenic organisms are present, because water quality sampling does not typically test for the actual pathogenic organisms. This possible limitation of mycofiltration is similar to documented limitations of chlorine treatment (see Chapter 1), in which indicator bacteria are killed, but, pathogenic protazoans and viruses are not. The use of *E. coli* as the sole indicator of fecal contamination has been evaluated and is currently supported (Baudisova, 1997). A test detecting pathogenic organisms is the most important area to continue this line of research because it targets the organisms of true health concern.

It is also possible that bacterial reductions due to the mycofiltration treatment are exaggerated by the detection methodology used in this experiment. It is possible that the reductions in bacterial abundance are due to the fungal treatment causing *E. coli* to enter a VBNC state. Other treatments, such as UV irradiation, have been demonstrated to cause this same discrepancy (George, 2002; Said et al., 2010). Though there is no evidence to suggest that mycofiltration causes *E. coli* to enter a VBNC state, there is also no evidence that refutes the possibility. If mycofiltration continues to gain acceptance as a treatment method, VBNC bacteria must be evaluated.

For a field demonstration of mycoremediation of FCB, a rigorous study could attempt to account for all water in the system minus a small amount lost to evaporation. In this type of experiment, the mycelium would be contained in an impermeable reservoir so that none of the solution would be lost to infiltration into the soil. In this manner, one could determine that groundwater was not

entering/exiting the system. This way, barring rain, there is no external source of water to dilute samples and very little of the solution is lost before exiting the system.

In conclusion, mycoremediation is a tool that deserves increased consideration. Both field (Thomas, 2009) and laboratory (presented here) experiments using rigorous statistical analysis provide evidence that mycofiltration may be used effectively as a means of remediation of fecal-polluted water streams. The mechanism by which mycofiltration reduces FCB in solution is not well understood and deserves further research in both laboratory and field conditions. While the results are encouraging, further investigation is required to determine the source of variation when using mycofiltration techniques. The mycofiltration method of remediation merits consideration by scientists and policy-makers alike.

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