Implementing Fungal Cultivation in Biofiltration Systems – The Past, Present, and Future of *Mycofiltration*

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Abstract: The intentional use of the vegetative growth of mushroom-forming fungi on wood mulch substrates as a biologically active filtration media, a process known as *mycofiltration*, is a promising new technology for enhancing biofiltration of stormwater, graywater, and agricultural runoff. Recent trials have documented that *Escherichia coli* can be selectively removed from contaminated water approximately 20% per cubic foot more effectively by mycofiltration than by wood mulch alone. This improvement in bacteria removal was consistent even after exposure of the mycofiltration media to harsh environmental conditions such as -15 to 40 °C (5 to 140 °F) temperature extremes. This article reviews the historical context, discusses the current state of research, describes best implementation practices, and highlights promising areas for future study to bring the cultivation of fungi in constructed ecosystems into common practice as a new ecological engineering tool for enhancing biological water treatment systems.

Key Words: ecological engineering, mushroom, mycelium, filtration, graywater, bacteria, sediment, stormwater, *E. coli*, fecal coliform

Introduction

Mushroom-forming fungi are primarily terrestrial, aerobic organisms whose vegetative growth takes the form of an intricate and dynamic three-dimensional web of tube-like cells called mycelium (figure 1). The use of the mycelium of select members of the kingdom of fungi for many applications in bioremediation (a process collectively called "fungal bioremediation" or "mycoremediation") has been well established (Gadd 2001; Singh 2006). Many mushroom-forming fungi of the phylum basidiomycota, which includes well known species such as the oyster mushroom (*Pleurotus ostreatus*) and turkey tail mushroom (*Trametes versicolor*) are further characterized as "white rot," an informal classification named for the white cellulose-rich material that is left behind as these organisms metabolize the lignin from their wood substrate. The powerful lignin-degrading enzymes produced by these white-rot basidiomycetes—most notably laccase, lignin peroxidase, and manganese peroxidase—are capable of co-degrading a diverse suite of recalcitrant chemical contaminants. Interestingly, several of these chemical degrading species are also known to predate bacteria, produce powerful antibiotic metabolites, and are widely grown commercially due to their ease of cultivation on a wide variety of substrate materials. The incorporation of these organisms into engineered water treatment ecosystems and biofiltration media have demonstrated improvements in bacteria reductions both in the laboratory and at scale. This documented application, among several others under investigation, can provide environmental engineers, water quality professionals and nursery managers with a new tool for enhancing biological water treatment systems.

Fundamental laboratory research supporting the use of wood and leaf litter degrading fungi for ecological services has been widely established in the broader context of mycoremediation. Interest in mycoremediation increased dramatically in the mid-1980s following the discovery of the enzyme lignin peroxidase in the white-rot basidiomycete *Phanerochaete chrysosporium* (Glenn and others 1983; Tien and Kirk 1983).

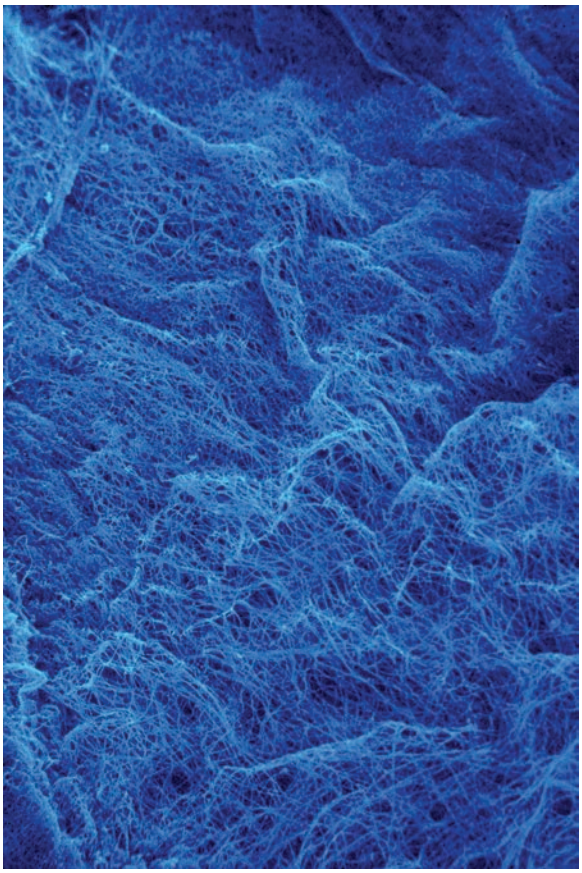

Figure 1. Scanning Electron Micrograph of mycelium viewed at approximately 100x. Credit: Paul Stamets.

Subsequent findings pioneered by Dr. John A. Bumpus and Dr. Steven D. Aust at Utah State University found that lignin peroxidase and other fungal enzymes could efficiently co-degrade persistent chemical toxins (Aust 1990; Bumpus and others 1985). A significant body of research throughout the next several decades documented many applications for white-rot basidiomycete fungi in bioremediation such as the degradation of polycyclic aromatic hydrocarbons (Leonardi and others 2007; Steffen and others 2007); polychlorinated biphenyls (Ruiz-Aguilar and others 2002); and diverse pesticides such as diuron, chlorpyrifos, and atrazine (Bending 2002). Many additional applications and promising bioremediation candidate species including *Pleurotus ostreatus, Irpex lacteus, Stropharia rugoso-annulata,* and *Trametes versicolor* have been thoroughly reported and reviewed (Singh 2006). By the early 20th century, *P. chrysosporium* was widely recognized as a "model biotechnology fungus" and became the first member of the basidiomycete phylum to be sequenced (Martinez 2004).

Around the same time as the early research on *P. chrysosporium* for chemical degradation, new research was uncovering facets of bacterial-fungal microbial ecology that would later critically inform mycofiltration research. As early as 1961, researchers working to advance the button mushroom industry had discovered that certain bacteria including *Pseudomonas putida, Bacillus megaterium,* and *Azotobacter vinelandii* fulfill essential roles in button mushroom cultivation including triggering the formation of mushrooms (Curto and Favelli 1972). Femor and Wood (1981) further documented that several species of wood degrading fungi, particularly basidiomycete fungi, could even be grown using killed bacteria as the sole nutrient source. Several years later, Dr. George L. Barron at the University of Guelph found that some common and even culinary basidiomycetes such as the button mushroom (*Agaricus brunnescens = A. bisporus*), oyster mushroom (*Pleurotus ostreatus*), blewit (*Lepista nuda*), and the scaly ink cap (*Coprinus quadrifidus*) are capable of seeking out and predating living colonies of bacteria (*Agrobacterium tumefaciens* and *Pseudomonas putida*) as sources of nutrition. This work complemented Barron's previous study documenting that *Pleurotus ostreatus* can also paralyze and consume nematodes (Barron and Thorn 1987). This bacteria-predating feature was unique to basidiomycetes and occurred in only four of the roughly 100 phylogenetically diverse fungal cultures that were screened (Barron 1988).

The term *mycofiltration*—defined as the use of intentionally cultivated networks of fungal mycelium to facilitate water quality improvements in engineered ecosystems—first appears in the literature in 1993 (Stamets 1993). Related concepts such as the use of fungal bioreactors were investigated as early as 1969 for decolorizing Kraft bleach plant effluent (Marton and others 1969), and throughout the 1980s using gel-immobilized fractionated mycelium for wastewater treatment (Livernoche and others 1981). The incorporation of fungi into outdoor biofiltration systems, however, began when a serendipitously placed 'garden giant' (*Stropharia rugoso-annulata*) mushroom bed reduced bacteria runoff from upland pasture (Stamets 2005).

The Dawn of Mycofiltration for Pathogen Management

Field trials to replicate the runoff management technique discovered by Stamets in 1993 were conducted intermittently at sites throughout Mason County, Washington, over a ten year period with assistance from the Mason Conservation District, Mason County Public Works and Health Departments, and the Squaxin Island Tribe. The principal reason for interest in this treatment application is that pathogens are the leading cause of water quality impairment in the United States, accounting for over 10,000 Total Maximum Daily Load allocations (TMDLs) nationwide—24% more than the next leading pollutant (National Summary 2012). Urban stormwater and agricultural runoff contribute significantly to this problem (National Research Council 2008; Mishra and others 2008), and leading treatment options are not consistently effective (Clary and others 2010; Center for Watershed Protection 1999). Researchers with the Vermont Association of Conservation Districts and the University of Illinois found that even an integrated runoff management approach incorporating a variety of best management practice (BMP) techniques was not able to consistently remove *E. coli* from dairy runoff (Kominami and Lovell 2012). The burden of bacteria pollution is particularly evident in Washington State, where the shellfish industry is valued at $80 million annually, and where a single pathogen related closure of a shellfish harvest area can total over $3 million in losses (Booth and others 2006).

From 2007 to 2008, two mycofiltration treatment studies in northwestern Washington documented bacteria removal from agricultural runoff under widely different loading and design parameters. In an experimental treatment conducted by the Mason County Public Works department, a significant though relatively short-term 38% reduction ($p<0.01$) in fecal coliform bacteria was achieved in a shallow suburban creek (Kenny 2008). Although the high hydraulic loading rate eventually led to anaerobic conditions and dieback of the mycofiltraton media, this installation suggested that bacteria reductions could be achieved, even at a flow rate several orders of magnitude larger than typical comparable BMP loading.

These results were corroborated in 2009 by a study conducted by Pacific Northwest National Laboratory under contract to the Jamestown S'Klallam Tribe. This study evaluated the performance differences in rain gardens (planted bioretention basins) in an agricultural region of the Dungeness watershed of Washington State. Two mirror-image rain gardens were constructed to compare the performance differences between a garden inoculated with *Pleurotus ostreatus* and *Stropharia rugoso-annulata* mycelium and a rain garden without fungal inoculum in the wood mulch layer. The mycelium-enhanced rain garden (figure 2) removed 24% more fecal coliform from runoff at the low influent concentration of 30 colony forming units (CFU)/100 ml than the control rain garden without mycelium. When the experimental treatment cells were spiked with dairy lagoon waste (259,000 CFU/100 ml), the control rain garden had a short-term export of bacteria (376 CFU/100 ml) one hour after the influent spike, while the mycelium-enhanced rain garden resisted coliform export with effluent concentrations remaining below 10 CFU/100 ml over the same period (Thomas and others 2009).

Mesocosm Tests Confirm Treatment Potential

In 2012, a mesocosm-scale study jointly conducted by Fungi Perfecti, LLC and Washington State University (WSU) confirmed the potential of mycofiltration media to remove *E. coli* from synthetic stormwater under laboratory conditions (Beutel and others 2014). The first objective was to identify which fungal species and filter media combinations could maintain biological activity under stressful environmental conditions. The second objective was to quantify the effects of this mycofiltration media on bacteria at different flow rates. Eight fungal strains were grown on five different substrate combinations and were exposed to periods of saturation, drying, heating, and freezing to assess the potential for survival under field-relevant conditions. The ability of mycofiltration media to remove *E. coli* was determined through a series of bench-scale tests conducted independently at the WSU Department of Civil and Environmental Engineering that compared the *E. coli* removal capacity of the most resilient fungal filters identified by Fungi Perfecti, LLC.

Mycofiltration media treatments consisted of 25 L (6.6 gal) containers with dense but permeable fungal mycelium growth on wood chips or a combination of wood chips and straw. Although several previous field trials documented bacteria removal with *Pleurotus ostreatus*, this species failed to demonstrate resilience to adverse environmental conditions. Notably, *Stropharia rugoso-annulata* and *Irpex lacteus* demonstrated exceptional promise for field applications and were identified as lead candidates based on this criteria. Replicate biofilters were loaded with sediment-free dechlorinated tap water spiked with ~700 CFU/100 ml of *E. coli* at low (0.5 L/min; 0.43 $m^3/m^2 \cdot d$) and high (2.2 L/min; 1.9 $m^3/m^2 \cdot d$) hydraulic loading. Influent and effluent samples were monitored over time for fecal coliform and *E. coli* using the Coliscan membrane filter chromogenic method.

Removal of *E. coli* by mycofiltration biofilters was evaluated using media that had been exposed to simulated field conditions. Media that had been exposed to harsh environmental conditions such as -15 to 40 °C (5 to 140 °F) temperature extremes and periods of saturation were termed "Vigor-Tested" biofilters. This media was evaluated in relation to mycofiltration biofilters that had been grown and stored under moderate environmental conditions (Non-Vigor-Tested biofilters) as well as un-inoculated wood chips (Control Filters). *Stropharia rugoso-annulata* grown on wood chips yielded a 20% improvement in *E. coli* removal relative to the wood chip Control Filters (figure 3) at the hydraulic loading rate of 0.5 L/min (0.43 $m^3/m^2 \cdot d$). The removal of *E. coli* was similar between Vigor-Tested and Non-Vigor-Tested media, although the Non-Vigor-Tested media had lower variability ($p<0.05$). Additional testing suggested that *E. coli* removal improved when sediment was incorporated into the synthetic stormwater. This result is consistent with other stormwater management research that has documented a correlation between sediment and bacteria removal due to sorption of bacteria onto sediment surfaces and removal by physical mechanisms such as particulate settling or physical straining (Davies and Bavor 2000).

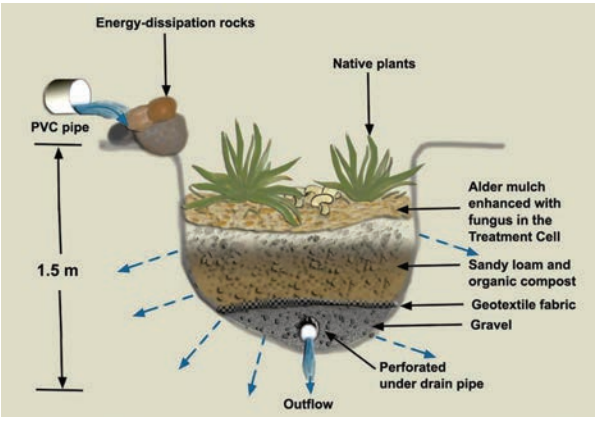

Figure 2. Cross-section schematic of a biofiltration treatment cell containing sand/organic material fill over perforated drainage pipe with native plants and a mycelium enhanced mulch layer (not to scale). Reproduced with permission, courtesy of Pacific Northwest National Laboratory—Report #PNWD-4054-1 (Thomas and others 2009).

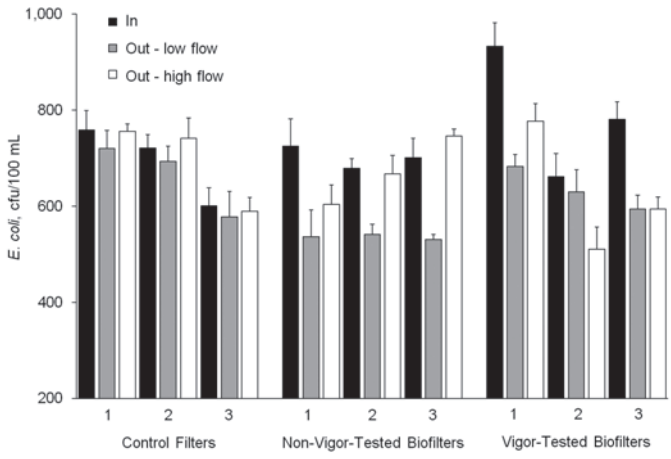

Figure 3. *E. coli* concentration in inflow and outflow from *Stropharia rugoso-annulata* biofilters. Three treatments are shown: Control Filters; Non-Vigor-Tested biofilters; and Vigor-Tested biofilters. Filters were tested under low flow (0.5 L/min) and high flow (2.2 L/min) conditions. Bars are average values and error bars are standard deviation of replicate *E. coli* analyses (n = 2-4). Figure and data from Beutel and others (2014).

Mycofiltration with *Irpex lacteus* appeared less effective; however, the presence of straw in *Irpex* media may have negatively influenced bacteria removal. Mycofiltration and control media that contained straw commonly exported bacteria that tested positive for fecal coliform that was later identified as *Raoultella planticola* (=*Klebsiella planticola*) (Beutel and others 2014; Drancourt and others 2001). This finding corroborated results by Caplenas and Kanarek (1984), when they documented that Klebsiella bacteria species grow on woody material yet test positive as "fecal coliform" and confound water quality assays for fecal contamination. While some species of Klebsiella such as *Klebsiella pneumoniae* can be pathogens in hospital settings, non-fecal-source members of the genus *Klebsiella* are ubiquitous in the environment. Furthermore, current epidemiological analyses have not identified a correlation between Klebsiella bacteria in recreational waters and health risk from fecal-borne pathogens (U.S. EPA 2009). These results highlight the limitation of using the fecal coliform test to assess pathogen removal in biologically rich wood-based ecotechnologies like mycofiltration.

The cultivation of saprophytic basidiomycete fungi into wood mulch as a stand-alone biofilter or as an amendment to a bioretention system has demonstrated the ability to enhance bacteria reductions in water treatment applications under both controlled and uncontrolled environmental conditions (Beutel and others 2014; Thomas and others 2009). Fungal species that demonstrate exceptional resiliency to field-relevant conditions have been identified. Preliminary mesocosm data to optimize flow rate to allow for appropriate sizing of treatment systems has been reported (Beutel and others 2014; Stamets and others 2013). This fundamental delivery system research provides a critical foundation for future research, although key deployment questions such as effective treatment life and relevance of indicator removal to disease risk reduction remain to be answered.

Future Research and Potential Applications

While a number of potential uses of fungal mycelium as a complementary environmental engineering tool are numerous and have been reviewed (Singh 2006; Stamets 2005), recent research continues to highlight new treatment approaches and applications. Studies evaluating fungal cultivation for improving the particulate trapping ability of mycelium-enhanced mulches, fungal enzyme catalyzed sediment-bound pollutant degradation, and the synergistic microbial treatment of chemical and biological pollutants warrant special attention as topics for future research.

Organic materials, such as the straw and woodchip matrix used in the production of mycofiltration media, are commonly used in bioretention systems to help reduce total suspended solids (TSS) by promoting the localized settling of particulates. Given that the surface area of mycelium in the upper 10 cm (3.95 in) of soils has been reported to range from 3 to 90 m^2 per m^2 (1.2 to 107.6 yd^2 per yd^2) of ground surface area, it is likely that enhancing mulch with saprophytic soil-interfacing fungi such as *Stropharia rugoso-annulata* and *Irpex lacteus* can improve the TSS removal capacity of these organic materials (Leaky and others 2004). Further, the dense growth habit of some fungi can trap soil particles between their cells (hyphae), effectively forming microaggregates (Gadd and others 2011). Physical straining of particulates may be further increased by mucilaginous fungal excretions, which can contribute to biofilm development (Caesar-Tonthat 2002). As illustrated in figure 4, these properties may improve the physical characteristics of mulch to improve sediment capture, prevent re-suspension of pollutants during high-flow events, and stabilize slopes after wildfires or the decommissioning of logging roads (Stamets and Summerlin 2011). An added benefit of this approach may also be the reduction of colored effluent from heavily mulched landscapes (figure 5). The removal of tannins, lignin, and related byproducts from pulp effluent has been well researched (Pellinen and Joyce 1990), and this research may translate to reduced chemical oxygen demand exports by mulches that are colonized by white-rot fungi.

Figure 4. Mycelium of the rhizomorph-forming mushroom *Stropharia rugoso-annulata* can dramatically alter the physical and chemical properties of wood mulch for added stabilization and sediment retention, among other applications. Photo credit: Paul Stamets.

Figure 5. Comparison of effluent clarity between Alder (*Alnus rubra*) wood chips colonized by *Stropharia rugoso-annulata* (left) and un-colonized wood chips (right). Media of each was saturated with clean water for four minutes and drained (unpublished data). Photo credit: Alex Taylor.

Mycofiltration research to date has also documented that complex microbial populations can exist in media that is macroscopically dominated by a single species of saprophytic fungus (Flatt 2013). While the microbiome of these systems is certainly complex, export of *Klebsiella* spp. bacteria from straw-containing media both with and without the presence of saprophytic fungi has been documented in mycofiltration trials (Beutel and others 2014). Notably, *Klebsiella* spp. bacteria have been found to degrade a diverse suite of lower molecular weight petroleum hydrocarbons including toluene, xylene, naphthalene, and nonane (Rodrigues and others 2009). There is a large body of laboratory research demonstrating the ability of white-rot fungi to remediate high molecular weight polycyclic aromatic hydrocarbons in soil (Bhatt and others 2002; Leonardi and others 2007). Notably, the most promising fungal species identified for mycofiltration, *Stropharia rugoso-annulata,* has also been identified as one of the most efficient degraders of polycyclic aromatic hydrocarbons (PAHs) among the litter-decomposing fungi, with reductions of up to 70%, 86% and 84% of benzo(a)anthracene, benzo(a)pyrene, and dibenzo(a,h)anthracene, respectively (Steffen and others 2007). Future field trials and controlled mesocosm studies should seek to determine the extent to which the bench-scale removal of aromatic hydrocarbons by fungi can translate to treatment of petroleum contaminated runoff, the prevention of sediment contamination in bioretention cells, and the possibility of synergistic degradation of PAHs by saprophytic fungi and commensal *Klebsiella* spp. bacterial populations.

Synergistic microbial action by mycofiltration media or mycelium-enhanced mulches may also demonstrate promise as future biocontrol agents in nursery applications. Several species of the "imperfect" (non-sexually reproducing) fungus *Trichoderma*, have been used as biocontrol agents against a variety of plant pathogens including species of *Pythium* and *Phytophthora* (Howell 2003). Following biocontrol screening methods described by Elliott and others (2009), a preliminary investigation of *Trichoderma* species for biocontrol against virulent isolates of the forest and nursery pathogen *Phytophthora ramorum* has shown promising results as illustrated in table 1 (Elliott 2013 personal communication). Notably, several species of *Trichoderma* are also common competitor molds in commercial mushroom production since one of the growth characteristics of this organism is an ability to parasitize other fungi. A possible future application of mycofiltration may therefore be to increase *Trichoderma* populations and longevity in soil or biofiltration media by providing host fungi to support the long-term presence of select biocontrol species of *Trichoderma*. Additionally, saprophytic fungi may also act alone as biocontrol agents against nematodes (Barron 1977; Barron and Thorn 1986; Hong and others 2006). The use of mycofiltration media, alone or in combination with other biocontrols, presents a unique opportunity for research in the rapidly growing field of applied microbial ecology for control of plant pathogens.

Table 1. Preliminary investigation for biocontrol against virulent isolates of the forest and nursery pathogen *Phytophthora ramorum.*

Species	% Inhibition of *P. ramorum* regrowth	% Inhibition of *P. ramorum* growth
Gliocladium virens	71%	100%
Gliocladium virens	76%	100%
Gliocladium virens	62%	100%
Gliocladium virens	53%	100%
T. atroviride	44%	100%
T. atroviride	56%	100%
T. pseudoharzianum	62%	0%
T. pseudoharzianum	35%	13%

Concluding Remarks

The intentional application of fungi in the environment for ecological services that support human needs and remediate previous human impacts has been well established as an ecologically rational approach. While much work remains to be done in determining best application practices and defining treatment parameters, networking knowledge and skill sets between mushroom cultivators, environmental scientists and policy makers sets the stage for widespread implementation of mycofiltration methods in the near future. As this important body of research advances, the intentional incorporation of fungi in environmental engineering design may one day become as commonplace as the planting of cattails in constructed wetlands is today.

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