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EXAMINING MYCOFILTRATION EFFICACY IN A FIRST ORDER STREAM

by

LACEY J. DAVIS

Under the Direction of Sarah H. Ledford, PhD

ABSTRACT

Bacterial contamination from sanitary and combined sewer overflows, leaking sewer infrastructure, and stormwater runoff decreases urban surface water quality. This research investigates a bioremediation technique, mycofiltration, to mitigate episodic bacterial contamination in first-order urban streams, which has previously been demonstrated to work in lab experiments. The objectives are: (1) establish the spatial distribution of *E. coli* in the Upper South River watershed, and (2) evaluate the potential for *Trametes versicolor* fungal spawn to decrease *E. coli* concentrations when accounting for short hydrologic retention and surface water-groundwater interactions inherent in streams via a stream table experiment. The *Trametes versicolor* mycofilter overall reduced concentrations of *E. coli*, but no more than was reduced by stream sediments alone. These findings suggest the usefulness of mycofiltration may be limited by decreased contact time or hyporheic flow paths that bypass the mycelium installation.

INDEX WORDS: Mycoremediation, Mycofiltration, Urban hydrology, South River, Water
quality, Bacterial contamination

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LACEY J. DAVIS

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DEDICATION

I dedicate this thesis to my grandparents, Sherry and Larry Binenfeld, for their unconditional love, and confidence in me. I would like to also dedicate this work to my mother, Laura Elaine Binenfeld Negelow Davis Aven, and my sister, Laina Rachel Davis, who are my greatest teachers and unwavering touchstones.

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1   INTRODUCTION

Urbanization poses threats to the integrity of water quality throughout the United States primarily due to increased impervious surfaces and associated stormwater runoff, decrease in groundwater recharge, stream bank destabilization, and increased contaminant transmission (Alder, 2013; Burns et al., 2005; Gaffield et al., 2003). Various research has shown that impervious surfaces not only increase flood peaks and runoff volume, but also transmit bacterial and chemical pollutants into surface waters at rapid rates (Fig. 1; DeWalle and Swistock, 2000; Gaffield et al., 2003).

Coupling climate change issues with continued population growth exacerbates already mounting urban water quality issues through the United States, and in particular, in the rapidly expanding metro Atlanta area (DeWalle and Swistock, 2000; EPD, 2008; Gaffield et al., 2003). By 2050, population growth in the city of Atlanta is anticipated to almost double (Jeong et al. 2018). Dekalb County, in southeastern metro Atlanta is expecting a population increase of 22% by the year 2040 (ARC, 2015). This escalated population growth will add strain to an already aging water infrastructure system and be exacerbated by increased frequency and intensity of precipitation events likely contributing to increased stormwater runoff, sanitary sewer overflows (SSOs) and decreased water quality in the coming years (EPD, 2008). Conservative climate change predictions expect an overall increase of precipitation in certain areas across the United States (Jeong et al., 2018). Evidence is already being seen, with Dekalb County experiencing its the third wettest year in 30 years in 2020, with over 60 inches of precipitation (Champion, 2020).

More than 6,000 streams across Georgia are considered impaired and do not meet current water quality standards (EPD, 2008). Numerous contaminants ranging from heavy metals, increased sediment loads, and pesticides and fertilizers threaten water quality in the Atlanta area,

but bacterial contamination in surface water is the largest water quality issue to manage in Georgia (EPA, 2004; GAAS, 2014). According to the Georgia Environmental Protection Division, over half of stream impairments in Georgia's surface waters are caused by bacterial contamination caused by *Escherichia coli* and Total Coliform (EPD, 2008). These bacteria are a naturally occurring part of human and animal digestive systems and found in both human and animal waste (EPA, 2004). They enter urban waterways through leaking sewer infrastructure, SSOs, combined sewer overflows (CSOs), and stormwater runoff (EPA, 2004; GAAS, 2014). Although the consequences of bacterial contamination on water quality are well researched, they remain a significant source of water quality degradation throughout the United States and the metro Atlanta area and continue to pose numerous human health risks (EPA, 2016; Walsh et al., 2005). Understanding ways to remove bacterial contamination from urban streams is required to improve stream health and access to recreation for citizens. This thesis presents the results of an investigation into the effectiveness of mycofiltration for *E. coli* removal in a first-order Piedmont stream as well as the past and current *E. coli* contamination in the Upper South River.
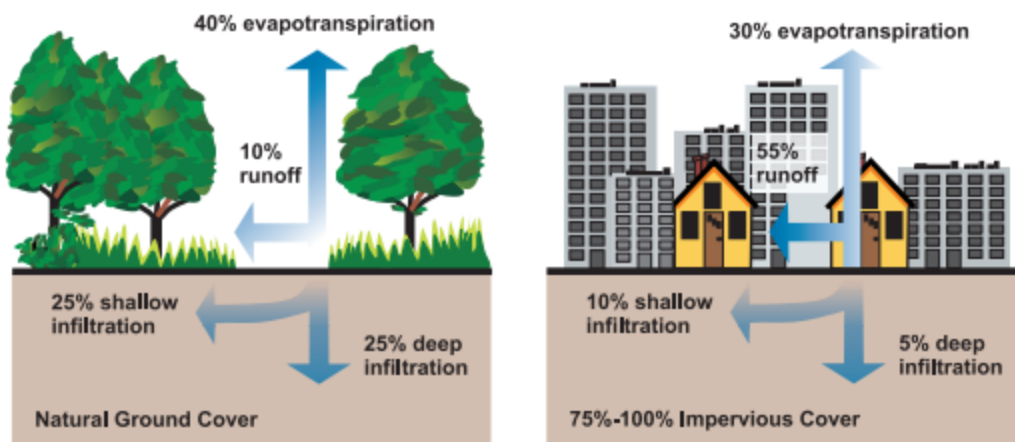
Figure 1 Impervious surfaces and stormwater runoff (EPA, 2003).

1.1   South River Watershed

The South River is located on Muscogee Creek Indigenous Peoples land (NLD, 2020) and is part of the Upper Ocmulgee River Basin. Today, the South River provides drinking water supplies to Clayton, Henry and Rockdale Counties (SRWA, 2020; USGS, 2020). It originates in highly urbanized South Atlanta (Figure 2) and flows southeast through DeKalb, Fulton, Jasper, Newton, Rockdale, and Clayton counties until it converges with the Alcovy River and the Yellow River draining into Lake Jackson (ARK, 2020; SRWA, 2020; USGS, 2020). The outlet of Lake Jackson marks the beginning of the Ocmulgee River which continues to flow southeast until it merges into the Oconee River (ARK, 2020; SRWA, 2020; USGS, 2020). Finally, the Oconee River joins the Altamaha River which continues eastward ultimately draining into the Atlantic Ocean (ARK, 2020; SRWA, 2020; USGS, 2020). Overall, the South River watershed contains about 60 tributaries and is comprised of land use ranging from agricultural, residential, commercial, and industrial (Scott, 2014; USGS, 2020). The Upper South River has an extensive history and continued issues with bacterial contamination as a result of failed regulatory enforcement and failing infrastructure (Mitchell, 2019; SRWA, 2020; USACE, 2012). This section aims to (1) provide an overview of Combined Sewer Overflows (CSOs) and Sanitary Sewer Overflows (SSOs) in Dekalb County, and (2) frame the following mycofiltration lab research as a scalable prototype for future field deployment.

Figure 2. Headwaters of the Upper South River are a milky white color due to leachate from a historical cotton processing plant and is designated a class 1 hazardous waste site.

1.1.1 Overview of SSOs and CSOs in the Upper South River

In urban areas, storm sewers and sanitary sewers are the primary categories of sewer systems (EPA, 2004; EPA, 2016). Storm sewer systems carry stormwater runoff directly into surface waters, while sanitary sewer systems are responsible for transporting untreated sewage (domestic, commercial and industrial) to wastewater treatment facilities for treatment (EPA, 2004; EPA, 2016; USGS, 2020). Sanitary sewers can be further categorized as separate or combined. The City of Atlanta has historically operated with numerous combined sewer systems (CSS) which- unlike separate sewer systems (SSS) are constructed to carry stormwater runoff and sewage in one combined piping system, and are designed to overflow during the highest precipitation events as a preventative measure against pipe breakage (Borden, 2015; SRWA,

2020). During high precipitation events (and subsequent high stormwater collection) CSS can result in CSOs and subsequent high concentration of *E. coli* may enter the surface water (EPA, 2004). Due to the 1,925 CSOs that occurred between 1988-1991 in the South River and Chattahoochee River, the City of Atlanta was required to eliminate CSOs through the Georgia Water Control Act (Borden, 2015). However, the Custer Ave CSO facility and the Intrenchment Creek Water Quality Control Facility remain on the South River and continue to threaten water quality (Scott, 2014; SRWA, 2020).

In contrast, Sanitary Sewer Overflows (SSOs) generally result from fats, oil, and grease clogs, aging Wastewater Collection Transmission System (WCTS) and resulting high stormwater intrusion (the infiltration of precipitation through damaged sewer infrastructure), root intrusion, lack of maintenance, and population growth (USDC, 2010; CRK, 2014; DWM, 2015; EPA, 2016). Unlike the City of Atlanta, Dekalb County only has SSS and therefore only experiences SSOs. Between the years 2006 and 2010, Dekalb County experienced at least 2,846 SSOs (USDC, 2010) and 836 raw sewage spills directly into Snapfinger Creek, and other tributaries to the Upper Ocmulgee (USDC, 2010).

Both CSOs and SSOs result in the discharge of untreated sewage, wastewater, and stormwater directly into surface water (Borden, 2015; CRK, 2014; EPA, 2004; EPA, 2016). Because of this, CSOs and SSOs pose risks to public health and water quality, and both are considered point source discharges regulated by the EPA and EPD through the Clean Water Act (CWA) and associated National Pollutant Discharge Elimination Permits (NPDES) permits (EPA, 2004; EPA, 2016). SSOs and CSOs cause numerous and varied environmental impacts such as decreases in dissolved oxygen and resulting decrease in aquatic abundance and diversity,

excessive nutrient loads and resulting eutrophic conditions, and entanglement or digestion of floatables by wildlife (EPA, 2004).

Water quality in the United States is primarily regulated by the CWA (Borden, 2015; EPA, 2016; Pennington and Cech, 2010). The CWA was signed into law in 1972 to provide a regulation of pollutant discharges in surface and groundwater throughout the United States (Borden, 2015; Pennington and Cech, 2010). Essentially, the CWA states that a point source pollutant can only be discharged into a waterway with the use of permit, and this is enforced by the EPA and EPD through the use of NPDES permits (Borden, 2015; Doyle, 2012). Although NPDES permits specify the quantity and type of pollutants allowed for discharge in surface and groundwater, (Doyle, 2012; Martinez, 2016; Pennington and Cech, 2010), Dekalb County has continuously failed to meet these requirements and thus violated the CWA (USDC, 2010).

As a result of years of noncompliance with the federal CWA and the GA Water Quality Control Act, primarily due to excessive sewage spills, the EPA filed a lawsuit against Dekalb County in 2010 (USDC, 2010). This lawsuit resulted in the agreement of a settlement by way of a consent decree between Dekalb County, the EPA, and EPD (EPA, 2015; USDC, 2010). Specific violations charged to Dekalb County include the "illegal discharges of untreated sewage" and failure "to operate and maintain the collection and transmission systems of its treatment works" (EPA, 2017). The resulting 2011 consent decree demanded that Dekalb County repair and update its WCTS in the South River, Snapfinger Creek and Peachtree Creek in order to reduce its annual SSOs and CSOs within nine years as well as pay almost $900,000 in civil penalties (USDC, 2010).

According to the consent decree, 64% of the Dekalb County's WCTS infrastructure is between 25-50 years old and in need of replacement (USCD, 2010). Dekalb County Department

of Watershed Management (DWM) has developed a rehabilitation plan to limit SSOs (DWM, 2015). Intended rehabilitation methods include techniques such as "cured-in-place line pipe, pipe bursting, manhole lining, manhole replacement, manhole height adjustment, manhole ring and cover replacement, manhole frame sealing, open cut pipe replacement, point repairs, manhole raising, manhole ring and cover replacement, and service lateral rehabilitation" (DWM, 2015). These rehabilitation methods are intended to reduce pollutants as required by the consent decree yearly as follows: "Total Suspended Solids (TSS) reduced by 9,743 pounds, Biological Oxygen Demand (BOD) reduced by 9,424 pounds, Chemical Oxygen Demand (COD) reduced by 22,133 pounds, Total Nitrogen reduced by 1,437 pounds, Total Phosphorous reduced by 272 pounds" (EPA, 2015). These improvements to Dekalb County's WCTS are estimated to cost over $1 billion (News Release, 2020). Although the consent decree requires repairment of one-third of Dekalb County's sewer infrastructure, it does stipulate repair for the remaining sections despite continued SSOs throughout the entire system (Mindock, 2020).

The consent decree expired on June 20th, 2020; however, Dekalb County had failed to meet the requirements of the consent decree by this date (SRWA, 2020). In fact, in 2017, 6.4 million gallons of untreated sewage spilled into Snapfinger Creek, the largest SSO in 11 years, and within the first 9 months of 2020, Dekalb County SSOs had already resulted in over 2 million gallons of untreated sewage pouring into the Upper South River (DWM, 2020; Niesse, 2017). Due to an inability to meet the initial deadline, in October 2020, the EPA, EPD, and US Department of Justice extended the consent decree (News Release, 2020). The new, extended consent decree prioritizes about 100 locations in the WCTS, requires Dekalb County to increase its reporting to the EPD and EPA, and to pay over $1 million in civil penalties to the United States and the State of Georgia (News Release, 2020). In response to this inability to make

substantial change in decreasing SSOs since 2011, the SRWA filed a citizen lawsuit stating that Dekalb County remains in violation of the CWA and NPDES permits (Mindock, 2020). However, the lawsuit was dismissed in court by U.S. District Judge Steven D. Grimberg because according to the court, the EPA and EPD extension of the consent decree settles the case (Mindock, 2020).

1.1.2 Current E. coli contamination in the Upper South River

Although the Georgia Environmental Protection Division (EPD) has monitoring locations on the Upper South River and its tributaries (Figure 3), consistent Total Coliform monitoring is lacking. The most recent report (2014) on the Upper South River highlights segments that do not meet Total Coliform bacteria total maximum daily load (TMDL). TMDL is defined as the amount of pollutant a body of water can legally contain according to the CWA and is calculated using the formula below:

$$TMDL = \sum WLAs + \sum LAs + MOS$$

where WLAs are the total amount of point source waste allocated, LAs are the total amount of nonpoint source waste load allocated, and MOS refers to the margin of safety (Scott, 2014). Table 1 lists stream segments in the Upper South River that exceed the TMDL and thus are considered impaired.
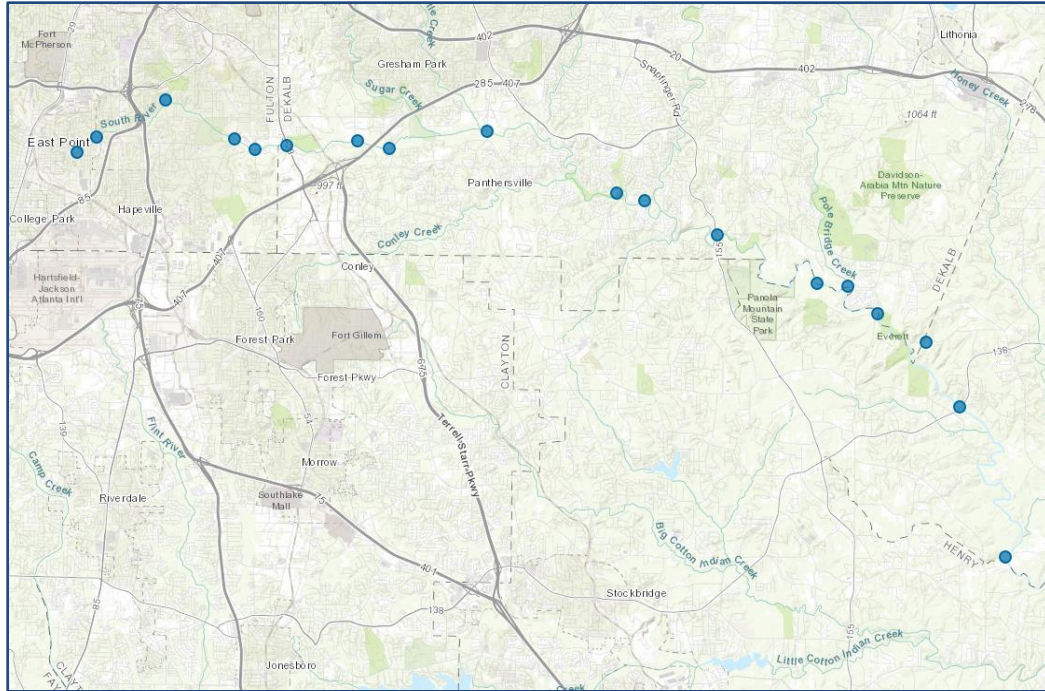
Figure 3 GA EPD's Watershed Protections Branch Monitoring Stations on the Upper South River (EPD, 2021)

Table 1 Impaired Stream Segments in HUC 0301070301 (Scott, 2014).

Stream Segment	Location	Stream Length (miles)	Area (acres)
Cobbs Creek	Headwaters to Shoal Creek	7	6,398
Conley Creek	Headwaters to South River	9	9,857
Doless Creek	Headwaters to Doolittle Creek	2	1,242
Doolittle Creek	Headwaters to South River	5	4,776
Honey Creek	Headwaters to South River	13	18,050
Intrenchment Creek	Headwaters to South River	6	7,241
McClain Branch (McClane Creek)	Headwaters to Honey Creek	2	2,622
Shoal Creek	Headwaters to South River	7	5,324
Snapfinger Creek	DeKalb County	18	24,622
McDaniel Branch (North Fork - South River)	Atlanta (Fulton County)	3	3,666
South River	Atlanta to Flakes Mill Road	16	65,108
South River	Flakes Mill Road to Pole Bridge Creek	9	116,867
South River	Pole Bridge Creek to Hwy 20	15	159,229
Sugar Creek	u/s Memorial Driver to South River	6	5,673

Specific causes of stream impairment in these areas are attributed to stormwater runoff, animal waste, SSOs and CSOs, Wastewater Treatment Facilities, and septic tank failures (Scott, 2014). Unfortunately, the steps towards improving water quality in the South River by the Metro North Georgia Water Planning District (MNGWPD) such as the creation of a Stormwater Management Plan (SWMP), public education programs, streambank stabilization and restoration, and sewer infrastructure repair, have not been sufficient and current SSOs and CSOs remain a significant and escalating problem for the South River (MNGWPD, 2009; Scott, 2014; SRWA, 2020). To supplement these findings and provide a more current picture of the issue, the South River Watershed Alliance (SRWA) began a water quality monitoring program in 2019 with the objective of obtaining consistent water quality data such as Total Coliform and *E. coli*.

According to the EPA, *E. coli* concentrations are used as standards for determining water quality in regard to bacterial contamination and to protect the public from contact with harmful bacteria during recreation and/or swimming (EPA, 2016; GAAS, 2014). *E. coli* standards for Recreational Waters indicate that a designated swimming area must contain <235 (cfu/100mL), <298 (cfu/100mL) in a moderate swimming area, <410 (cfu/100mL) in a light swimming area, and <576 (cfu/100mL) in an infrequent swimming area (GAAS, 2014). Because the South River is not designated for recreational use, the CWA does not necessitate the above *E. coli* concentration standards to be met. The South River's designated use classification is currently "Fishing" which is the lowest classification for surface water and affords the lowest protection in terms of pollutant levels (SRWA, 2020). The SRWA has been working to shift the South River's designation to "Recreation" in order to force the EPD to implement improved water quality standards (SRWA, 2020). And although, the state of Georgia has recognized that the need to redraft the categories of surface water designated uses stating that "the designations for fishing

are not stringent enough for certain sensitive ecosystems" (EPD, 2004) no progress has been made to execute these changes. Because the South River is unofficially utilized by residents for recreation and fishing, higher water quality is imperative to minimize public health threats.

1.1.3   Human health impacts

Although policies such as the CWA coupled with modern water treatment technologies have enabled the majority of the U.S. population access to pathogen free water for consumption and food production, Total Coliform and *E. coli* concentrations in surface water regularly exceed safe standards (Gaffield et al., 2003). CSOs and SSOs in particular threaten public health by exposure to untreated sewage and subsequent intestinal parasites, viruses and bacteria (EPA, 2004). Additionally, CSO and SSO consequences are exacerbated during precipitation events by an influx of other pollutants via stormwater runoff into surface water (EPA, 2004). As discussed preciously, bacterial contamination is the leading cause of stream impairment in the United States and impaired segments may pose significant public health concerns (EPA, 2004).

Research indicates that nonpoint source pollution, and stormwater runoff in particular, increases the public's exposure to bacterial contamination and propensity for acute or chronic illness and are directly related to increased concentrations of *E. coli*, Total Coliform, *Giardia*, and *Cryptospondium* pathogens in surface water (Gaffield et al., 2003). *E. coli* in particular poses problematic public health risks due to antibiotic resistance of numerous strains (Pini and Geddes, 2020). Accurate data regarding the quantity of illness related to waterborne diseases remains elusive due to the fact that the majority of cases are unreported due to the difficulty in accurate diagnosis; however, acute cases of waterborne illness caused by the recreational use of bacterially contaminated water include "ear and eye discharges, skin rashes, and gastrointestinal problems" (Gaffield et al., 2003). Immunocompromised people, children, people who are

pregnant, and elderly people remain the most vulnerable to serious illness as a result of such

pathogens (Gaffield et al., 2003). Additionally, the increased temperatures and increased

precipitation rates associated with climate change are likely to increase surface waterborne

illnesses which result from the recreational use of bacterially contaminated water (Gaffield et al.,

2003).

1.1.4   What is mycofiltration?

Mycofiltration is a specific type of mycoremediation and refers primarily to the use of

fungi for contaminant removal from water. Mycoremediation is a technique used to remove a

range of pollutants from soil and/or water (Cotter, 2014; Kulshreshtha et al., 2014). The prefix

'myco' refers to fungi and '-remediation', in general, refers to the process of cleaning or

correcting (Cotter, 2014). Research has shown mycelium are capable of removing a variety of

chemical, heavy metal, and bacterial contaminants from terrestrial and aquatic environments

(Taylor and Stamets, 2014). Although it is a relatively new area of research, mycofiltration has

been used for remediation of polluted water due to industrial agricultural practices, animal

husbandry, and certain industrial manufacturing sites, as well as from stormwater runoff and

failing sewer systems (Cotter, 2014; Pini and Geddes, 2020; Singh, 2006; Stamets et al., 2013;

Taylor et al., 2015). Mycofiltration installations vary in scale, medium, and contaminant removal

and although mycofiltration is typically used post contamination, it can be used preventatively in

areas where pollutant contamination is inevitable (Cotter, 2014).

Best management practices (BMP) and green infrastructure may reduce the quantity of

stormwater pollutants such as *E. coli* after precipitation events by increasing stormwater

infiltration and groundwater recharge; however, they may not completely remove the

contamination (Gaffield et al., 2003; Martinez, 2016). BMPs range in scale from high impact

infrastructures such as sand filtration, wetlands, and retention ponds, to low impact practices such as household rainwater collection units, swales, and green roofs (DWM, 2018). Mycoremediation may be a useful addition to the above BMPs due to its relative low cost and minimal impact. Additionally, research has estimated that mycoremediation may be a more affordable alternative to current wastewater treatment operations that utilize bacteria and/or sterilization because mycoremediation (excluding biosorption) does not create byproducts (Molla and Fakhru'l-Razi, 2012). Coupling various green infrastructure techniques, stormwater management programs, and mycofiltration installations may be key towards successful management of non-point source pollution.

1.2   Study objectives

Mycofiltration may be a useful addition to innovative community led responses in lieu of failed government regulatory enforcement and failure to improve Dekalb County's sewer infrastructure, but there has been limited research that considers the impact of hydrology on its effect. As has been well observed in remediation of nutrient contamination by streams (Craig et al., 2008; Hall et al., 2009; Bernhardt et al., 2017), the time-scale of interaction between the remediation media and contaminants may be key in understanding the efficacy of the approach. However, when this is accounted for, mycofiltration may be an appropriate approach for small order urban streams. Small scale stream restoration techniques founded in environmental equity such as mycofiltration may provide an affordable alternative while mitigating environmental injustice-the unequal distribution of environmental amenities or hazards (Gould and Lewis, 2016). Because environmental restoration can lead to environmental injustice, in particular green gentrification which results in increased property values and subsequent displacement or exclusion of economically vulnerable and marginalized residents (Gould and Lewis, 2016), it is

imperative that mycoremediation research balances attainable restoration goals with community needs while centering community involvement (Doyle and Shields, 2012; Walsh et al., 2005). In particular, mycoremediation may contribute to increased visibility of the water quality issues facing the Upper South River and bolster the SRWA's argument for improved water quality designation.

This research will specifically answer the questions (1) How effective *is Escherichia coli* (*E. coli*) removal from surface water when interacting with *Trametes vericolor* fungal spawn for short periods of time in a laboratory representation of a first-order stream? and (2) Will statistically significant decreases in *E. coli* concentrations occur when accounting for hydrologic interactions? Initially, I hypothesized that the presence of *Trametes vericolor* would reduce *E. coli* contamination linearly, even when the natural flowpaths and retention times of streams were considered.

This research consisted of a lab-based experiment and field based research which combined aim to provide an understanding of the current distribution of *E. coli* contamination in the Upper South River and evaluate the feasibility of a prototype for future mycofiltration installations within the Upper South River Watershed. The lab-based experiment utilized a stream table filled with sediment from Ripplewater Creek (a tributary of the Upper South River) to simulate a flowing first-order stream and deployed a mycofilter containing *Trametes versicolor* mycelium to mitigate *E. coli* concentrations in the surface water. Naturally occurring *E. coli* from Ripplewater Creek was isolated, cultured, and routed through a flow rate variable pump with deionized water at a constant rate through the stream table for 5 hours. A mycofilter of *Trametes vericolor* fungal spawn and *Quercus alba* sawdust trimmings were deployed at one section across the stream table, and samples were taken and analyzed with an IDEXX Quanti-

Tray System hourly. These results were compared to experiments where the *E. coli*-laced water was run without the mycofilter. Additionally, I analyzed stream samples along the Upper South River with the IDEXX Quanti-Tray System in order to determine spatial and temporal distribution of *E. coli* concentrations. Prototypes are especially imperative for mycofiltration installations because they help determine the best suited fungal species and substrate for the contaminant and location, the quantity of mycofilters necessary for substantial contaminant removal rates in relation to rate of discharge (Cotter, 2014). This study aims to provide data applicable for future mycofiltration installations within in the Upper South River.

2   BACKGROUND

2.1   Evidence of mycofiltration efficacy

Bioremediation is an umbrella term which describes the use of microorganisms to reduce contaminants levels (Kapahi and Sachdeva, 2017; Rhodes, 2014); mycoremediation and mycofiltration are methods of bioremediation that involve fungi. Bioremediation can be accomplished through a variety of mechanisms such as biosorption, where heavy metals get adsorbed on the surface of the biosorbent, bioaccumulation, wherein microorganisms bind to heavy metals and concentrate them (Velásquez and Dussan, 2009), and biodegradation, the transformation/breaking down of inorganic compounds into usable organic compounds (Kulshreshtha et al., 2014). It is important to note, that although biosorption (also known as hyperacuumulation) has been shown to be an effective bioremediation tool, it does not degrade the contaminant but merely transfers the contaminate to the fungi which then needs to be properly disposed (Cotter, 2014; Kulshreshtha et. al, 2014). Research demonstrates that mycelium are capable of biosorption, bioaccumulation, and biodegradation. However, biodegradation is the proposed mechanism for *E. coli* reduction through mycofiltration and this

is particularly helpful because biodegradation specifically transforms the contaminant into a benign substance and does not produce contaminated waste products (Kulshreshtha et al., 2014). Additionally, although this research focuses on the use of mycelium to remove contamination, past research has shown that live or dead mushrooms (the fruiting body of fungi) and even the spent mushroom substrate can be used as for bioremediation (Kapahi and Sachdeva, 2017; Kulshreshtha et al., 2014).

In a mycoremediation installation, the mycelium removes contamination in a variety of ways (Bhadouria et al., 2019; Cotter, 2014; Stamets, 2005). Mycelium have shown the ability to interrupt cellular replication and degrade cell membranes (Cotter, 2014). Interestingly, they can also change the pH of the surrounding environment thus making the area inhospitable to microorganisms (Bhadouria et al., 2019; Cotter, 2014; Singh, 2006). Various chemical reactions such as oxidation, reduction, biological degradation, and co-metabolic reactions are also pathways towards degradation of contaminants (Bhadouria et al., 2019). Lastly, they may also limit microbial growth by reducing nutrient availability in surrounding areas through species exclusion, the taking up of physical space thus preventing growth of other organisms (Cotter, 2014; Singh, 2006). Mycelium are able to do all of the above through enzyme secretion, and they can be highly effective because (unlike bacteria) they are not bound by cells and therefore they do not require direct contact "with a compound in order to begin to degrade it" (Cotter, 2014). This may be particularly useful in mycofiltration projects because the mycelium need only to be in close (not direct) contact with the pathogen in order to digest it (Cotter, 2014).

2.1.1   *Mycelium behavior*

Mycelium are the rooting body of fungi and are decomposers by their very nature (Cotter, 2014; Singh, 2006; Kulshreshtha, et al., 2008; Stamets, 2005). In natural systems, they secrete

enzymes which metabolize materials for consumption (Singh, 2006; Stamets, 2005). Specifically, they manufacture peroxidase, cellulose, ligninase, and laccase enzymes which break down the lignins and cellulose in woody material (Cotter, 2014; Singh, 2006; Rhodes, 2014; Stamets, 2005). This decomposition results in humus which is a material full of more accessible nutrients for other organisms to consume and provides an imperative role in the nutrient cycling (Rhodes, 2013).

Mycelium can be broken down into two main groups: white rot and brown rot (Cotter, 2014; Stamets, 2005). The majority of literature regarding mycoremediation uses white rot fungi and this is mainly due to their predication towards consuming lignin whereas brown rot fungi generally break down cellulose (Cotter, 2014; Rhodes, 2014). White rot fungi use a variety of enzymes and thus are able to break down and/or transform a variety of organic molecules. This non-specificity garners them an advantage in the field of mycoremediation (Mir-Tutusaus et al., 2014). Notable white rot fungal species include *Pleurotus spp, Trametes versicolor, Ganoderma lucidum* (Cotter, 2014; Mir-Tutusaus et al., 2014). Certain fungi species have affinities with particular contaminants and species vary in their ability to bioremediate. Additionally, bioremediation effectiveness is highly dependent on numerous factors such as contact time, temperature, substrate, biomass, and pH (Cotter, 2014; Kapahi and Sachdeva, 2017; Kulshreshtha et al., 2014).

2.2   Heavy metal and chemical mycoremediation

Various mushroom species and genera have been shown to sequester (biosorb) high concentration of numerous heavy metals, in their mycelium as well as their fruiting bodies while others have shown the ability to biodegrade heavy metals, hydrocarbons, and other chemical pollutants (Kapahi and Sachdeva, 2017; Kulshreshtha et al., 2014). For example, the

extracellular enzymes that *Pleurotus spp.* (oyster mushroom) secrete have shown the ability to degrade polymers (da Luz et al, 2013), crude oil (Olusola and Anslem, 2010) and sorb heavy metals such as cadmium, copper, zinc, iron, lead, and nickel (Lamrood and Ralegankar, 2013; Oyetayo et al., 2012; Tay et al., 2011). More specifically, certain species within the genus show an affinity for certain heavy metal biosorption; for example, in previous research higher cadmium uptake was exhibited in *P. ostreatus* versus higher mercury uptake by *P. sajor-caju* (Kapahi and Sachdeva, 2017). A variety of other fungi species such as *Lentinula edodes, Corolus versicolor, Agaricus bisporus, Lactarius piperatus*, *Trametes versicolor* and *Fomes fasciatus* have shown degradation and biosorption of a variety of complex pollutants ranging from Polycyclic aromatic hydrocarbons (PAHs) to a variety of heavy metals (Manna and Amutha, 2017**;** Kulshreshtha et al., 2014).

Recent research has indicated that white rot fungi may be able to remediate agrochemicals, organochlorine pesticides, organophosphonate agrochemicals such as dichlorodiphenyltrichlor oethane (DDT) and pentachlorophenol (PCP) in soil and water (Bhadouria et al., 2019; Hu et al., 2020). *Trametes versicolor* in particular was shown to degrade agrochemicals ranging from carbofuran, oxytetracyclin, imiprothrin, and cypermethrin (Mir-Tutusaus et al., 2014). Additional research has shown that *Trametes versicolor* and other white rot fungi have the ability to remediate certain micro pollutants which are not removed by current WWTP operations (Álamo et al., 2018; Beltrán-Flores et al., 2020; Mir-Tutusaus et al, 2018). Micro pollutants including pharmaceutically active compounds (such as acetaminophen, ibuprofen, naproxen, salicylic acid), various antibiotics, psychiatric drugs, and endocrine disruptors have been effectively removed or transformed from wastewater using *T. versicolor* (Mir-Tutusaus et al., 2018). However, numerous limitations remain which prevent the use of

mycofiltration for wastewater treatment such as the need for nutrient addition to the system, microorganism competition, and changing pH to optimize growth of fungi (Mir-Tutusaus et al., 2018).

2.3   Bacterial mycoremediation

Research on mycoremediation for bacterial contamination ranges from use in wastewater treatment plants, agricultural runoff, and stormwater retention ponds and runoff. Regardless of the scale of the project, it is imperative to utilize the best fungal species for the contamination (Cotter, 2014). Numerous species of fungi have shown antimicrobial properties; specifically, *Trametes versicolor* (Cotter, 2014), *Calvatia spp*, *Ganoderma spp*., and *Pleurotus spp* (Cotter, 2014; Singh, 2006). Because this field of study is burgeoning, research remains limited; however, field and lab studies which are foundational to the study of mycofiltration and show promise for future research will be explored below.

Stamets (2005) details field and lab mycoremediation research in his book *Mycelium Running*. He specifically used mycofiltration as a best management practice (BMP) for controlling bacteria contamination in surface water from agricultural runoff (Stamets, 2005). In his field experiment, he inoculated *Storpharia rugoso annulata* mycelium into woodchip swales downstream from his livestock pen and documented a 100% decrease in Total Coliform concentrations in the effluent after one year despite an increase in livestock population (Stamets, 2005). His subsequent laboratory research (Stamets et al., 2013) with Washington State University identified fungal species for mycofiltration, cultivation methods such as the MycoFilter^{TM}, and demonstrated the potential effectiveness of *E. coli* removal through mycofiltration. This research identified *Storpharia spp.* as the preferred mycelium for mycofiltration due to its rate of initial colonization and resilience under various environmental

stressors such as temperature and dehydration, and propensity at *E. coli* concentration reduction (Stamets et al., 2013). Stamets et al. performed a series of single bucket tests wherein an *E. coli* concentration of about 800 cfu/100mL per 30 L bucket, was percolated through a *Storpharia spp.* mycelium mycofilter at a flow rate of 0.5L/min and 2.2 L/min (Stamets et al., 2013). The mycofilters were filled with a 1:1 ratio of mycelium to large and small wood chips. Their results indicated an *E. coli* reduction by about 20% at 0.5L/min flow rate and about a 14% reduction of *E. coli* at the 2.2 L/min rate (Stamets et al., 2013) indicating that longer contact time between *E. coli* and mycelium is necessary for efficacy. Overall, this research concluded that mycofiltration (unlike other stormwater BMP) are capable of removing free-floating (not sediment-bound) bacteria (Stamets et al., 2013). It is important to note, that while this research did percolate water through the filter, it did not account for natural hydrologic conditions including hyporheic flow through the streambed.

Similarly, Martinez (2016) utilized synthetic stormwater in a pond setting to investigate the efficacy of *Pleurotus ostreaus* mycelium for *E. coli* reduction. Their research showed a 98% reduction in *E. coli* concentrations after a three-week period, and importantly, indicated that reduction increased after each subsequent week (Martinez, 2016). Thomas et al. (2015) also used *Storpharia* rugoso annulata coupled with *Pleurotus ostreatus*, and *Pleurotus ulmarius* mycelium inoculated alder mulch in bioretention cells in a human made wetland and compared those bioretention cells with ones that only had alder mulch without mycelium inoculation. After a six-month period, the control cells showed an *E. coli* reduction of 66%, and the mycelia-inoculated cells produced an *E. coli* reduction of 90% (Thomas et al., 2015). The most recent research on mycofiltration for bacterial removal focused on contact time related to *E. coli* removal and found that mycelium are capable of *E. coli* removal up to 96 h after inoculation in a sterile lab

environment (Pini and Geddes, 2020). However, they note, that the efficacy of short term contact and longer than 96 h contact is unknown (Pini and Geddes, 2020). Again, this research did not account for hydrology; the lab inoculated and natural stream water were contained in receptacles and did not flow as would occur in a natural stream environment (Pini and Geddes, 2020).

Despite multiple studies that purport the effectiveness of *Storpharia spp* in mycofiltration, Cotter (2014) found that while *Storpharia spp* may initially reduce bacterial concentrations, long-term it may ultimately increase the bacterial concertation (Cotter, 2014). Additionally, although numerous studies have focused on *Pleurotus spp* usefulness in mycoremediation in soil, Gulis and Suberkropp (2003)'s research has indicated that the enzymes secreted by *Pleurotus spp* may be diluted in aquatic environments and could have limited efficacy in mycofiltration applications. *Trametes vericolor* mycelia have been shown to have antimicrobial activity in isolated laboratory studies against various bacteria including *E. coli, Staphylococcus aureaus, Pseudomonas aeruginosa,* and *Methicillin-Resistant Staphylococcus aureus (MRSA)* (Gebreyohannes et al., 2019; Hleba et al., 2014).

3 METHODS

3.1 South River Watershed Alliance sampling procedures

To understand the potential applicability of this restoration technique, samples were collected to quantify the extent of *E. coli* contamination in the Upper South River. Sampling locations on the Upper South River headwater, tributaries, and main stem stream segments were identified, and all samples were obtained utilizing the Adopt a Stream Quality Assurance/Quality Control (QA/QC) chemical and biological protocols. Each stream sample was taken at base flow, at roughly the same time of day, and at the exact same location. Samples were analyzed at the Chattahoochee Riverkeeper's Neighborhood Water Watch water quality lab (CRK, 2014). Nine

total sample sites were located on the main stem of the Upper South River and its tributaries

(Figure 11).
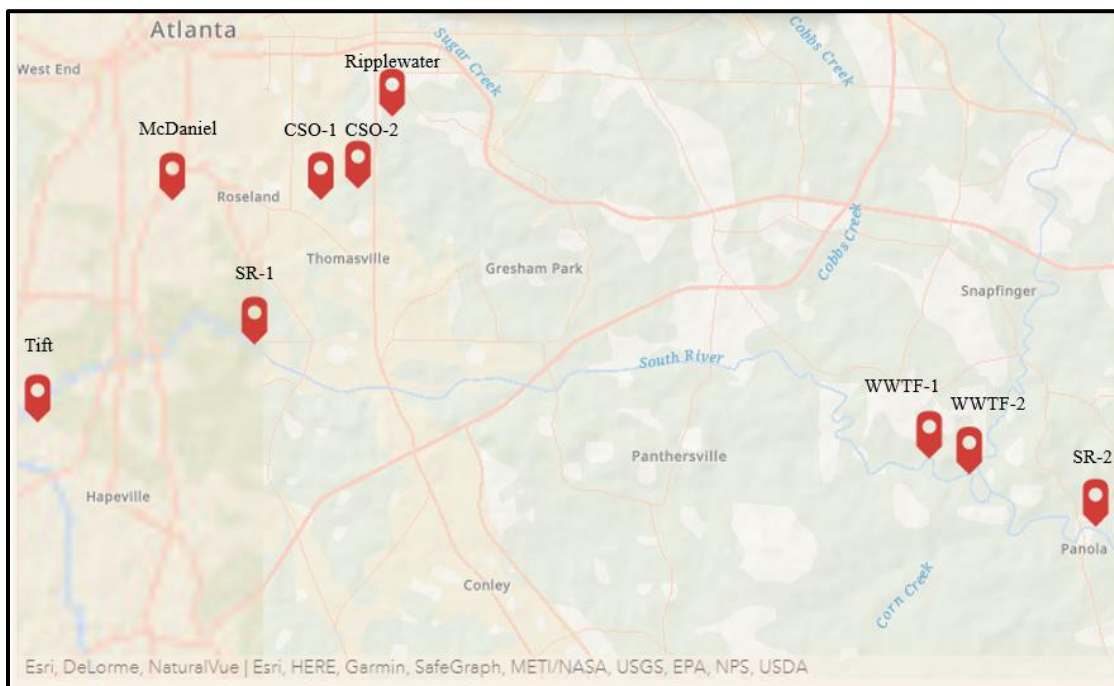
Figure 4 Sample sites along the Upper South River

Each site was chosen due to accessibility and relation to CSO and WWTP facilities. The

Tift site is located at the southern headwaters of the Upper South River. McDaniel is a tributary

to the South River and is located below the McDaniel Branch constructed wetlands near Arthur

Langford Park in SW Atlanta. SR-1 is located on the main stem of the Upper South River

adjacent to the Brownsmill Golf Course. CSO-1 is a tributary to the Upper South River and is

located upstream from Custer Combined Sewer Overflow Facility. CSO-2 is a tributary of the

Upper South River and is located downstream from Custer Combined Sewer Outflow Facility.

WWTF-1 is located on the main stem of the Upper South River upstream from the Snapfinger

Wastewater Treatment Facility. WWTF-2 is located on the main stem of the Upper South River

downstream from Snapfinger Treatment Facility. SR-2 is on the main stem of the Upper South

River as well at the Panola Shoals Trailhead. Lastly, Ripplewater is a tributary to the Upper

South River and is located south of Brownwood Park.

3.2   Experiment design

Overall, three experiments were performed in order to determine the efficacy of *T. versicolor* mycelium in *E. coli* removal from a first-order stream. A 2.5' X 6' stream table was

utilized to simulate a flowing stream. Sediment from Ripplewater Creek (tributary to the Upper

South River) was collected, placed in the bottom of the stream table at a 2-inch depth, and

bleached. A Geotech Geopump™ Peristaltic DC pump was used to pump water at a constant rate

(5.2 +/- 0.28 mL/s) through the stream table. The first experiment was performed with only

deionized (DI) water to determine the baseline *E. coli* concentrations after sediment bleaching.

The second experiment ran DI water containing 615.85 +/- 154.72 MPN/100 mL *E. coli*. The

final experiment was performed with DI water containing 642.08 +/- 211.31 MPN/100mL *E. coli*

and one cross vein deployment of a mycofilter containing *Trametes versicolor (T. versicolor)*

mycelium. Each experimental setup was run three times and the system was sterilized with 10%

chlorinated DI water between runs to remove residual *E. coli* and subsequently flushed with 24 L

DI water to remove residual chlorine.

3.2.1   Stream table design and sediment installation

A 2.5' X 6' stream table was sterilized and placed on a worktable in Dr. Sarah H.

Ledford's urban hydrology laboratory. A hydroponic filter was installed at the outflow end of the

stream table covered with landscaping fabric to mitigate sediment build up and prevent clogging

of the outflow. Ripplewater Creek sediment was collected from the streambed at 33°73'62.6" N,

-84°34'66.1" W and placed inside the steam table at a depth of 2 inches (Figure 5). Sediment was

typical of an urban piedmont streambed and consisted of a mixture of fine clay, sand, and silt

sediment, as well as gravel and pebbles. Pieces of broken glass, trash, and small decomposing invertebrates were also present in the sediment. A 5-gallon bucket was placed at the head of the stream table; a steady flow of DI water was pumped into the stream table with a Geotech Geopump^{TM} Peristaltic DC pump and Masterflex^{TM} ¼ inch ID tubing. A second 5-gallon bucket was placed at the tail of the stream table to collect the outflow water (Figure 6). With time, a channel formed in the sediment between the inflow and outflow points.

Sediment sterilization was achieved before each test by adding 10% bleach to 22L of DI water and running through the system. This procedure was repeated until an initial sediment sterilization was obtained and outflow water contained 0-3 MPN/100mL *E. coli*. After each experiment, the sediment was re-sterilized until *E. coli* concentrations returned to low concentrations (below 10 MPN/100mL).

Figure 5 Stream table filled with Ripplewater Creek sediment.

Figure 6 Stream table effluent

3.2.2 In-situ sample collection

Samples for *E. coli* isolation from Ripplewater Creek were collected in-situ and stored according to Georgia Adopt-A-Stream QA/QC Bacterial and Chemical Monitoring Certification procedures. Procedures included 1) labeling the Nasco Whirl-Pak sample bag with the current time, date, and experiment, 2) wearing unpowered latex disposable gloves and taking samples without touching the inside of the sample bag, 3) obtaining a minimum sample volume of 100mL, 4) placing the sample immediately in a refrigerator, 5) collecting the samples at mid-stream, and 6) analyzing the sample within 6 hours of collection while maintaining a stable sample temperature between 1-4°C (GAAS, 2014).

3.2.3 E. coli isolation and growth

E. coli from the sample water was isolated and cultured at GSU's School of Public Health in Dr. Lisa Casanova's laboratory (Table 2, 3, 4, 5; Figure 7, 8). Cultured *E. coli* was stored at -

20 degrees C. After use, the *E. coli* contaminated water was neutralized with a 10% bleach solution, and then disposed.

Table 2 Bacterial culture tracking sheet.

	Bacterial culture tracking sheet
	Organism: *Escherichia coli*
	Source: Ripplewater Creek, Atlanta, Georgia
	Purpose: Grow purified stock pool of wild type isolate
	Titer: 2.8×10^9 CFU/mL
Date	**Done**
6/28/20	Water sample collected from Ripplewater Creek
7/2/20	Analyzed by membrane filtration on BioRad Rapid *E. coli* 2 agar 100 mL, 10 mL, 1 mL Incubation 44.5°C 24 hr
7/3/20	Single isolated *E. coli* colonies picked from 10 mL plates streaked for isolation on BioRad Rapid *E. coli* 2 Incubation 37°C 24 hr
7/4/20	Purified by sequential isolation streak on tryptic soy agar Incubation 37°C 24 hr
7/5/20	Purified by sequential isolation streak on tryptic soy agar Incubation 37°C 24 hr
7/6/20	Purified by sequential isolation streak on tryptic soy agar Incubation 37°C 24 hr
7/7/20	2 isolated colonies picked Inoculated into 150 mL tryptic soy broth each Incubation shaking 100 rpm 37°C 24 hr
7/8/20	300 mL culture combined 20% glycerol added Frozen at -80°C Titer by spread plate assay on TSA
7/9/20	Titer read

Table 3 Determining E.coli titer.

Determining *E. coli* titer
Date: 7/8/20
Organism: *Escherichia coli*
Source: Ripplewater Creek, Atlanta, Georgia
Purpose: Titer of purified stock pool of wild type isolate
Method: spread plate   Media: TSA   Diluent: TSB
Incubation: 37°C 24 hr
Read date: 7/9/20
10=undiluted stock
9=100µL in 900µL TSB
Plated: 10, 9, 8, 7, 6, 5, 4, 3, 2, 1
100 µL per plate 2 plates per dilution

Table 4 Determining E. coli titer continued.

Dilution	10	10	9	9	8	8	7	7	6	6
Colonies	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC

Dilution	5	5	4	4	3	3	2	2	1	1
Colonies	TNTC	TNTC	143	193	22	21	0	1	0	0

Table 5 Calculations for determining E. coli titer.

dilution	volume of original stock plated	plate 1	plate 2	total colonies	total volume	titer (CFU/100 mL)
10	1.00E-01	TNTC	TNTC			
9	1.00E-02	TNTC	TNTC			
8	1.00E-03	TNTC	TNTC			
7	1.00E-04	TNTC	TNTC			
6	1.00E-05	TNTC	TNTC			
5	1.00E-06	TNTC	TNTC			
4	1.00E-07	143	193	379	2.2E-07	**1.72E+09**
3	1.00E-08	21	22			
2	1.00E-09	1	0			
1	1.00E-10	0	0			
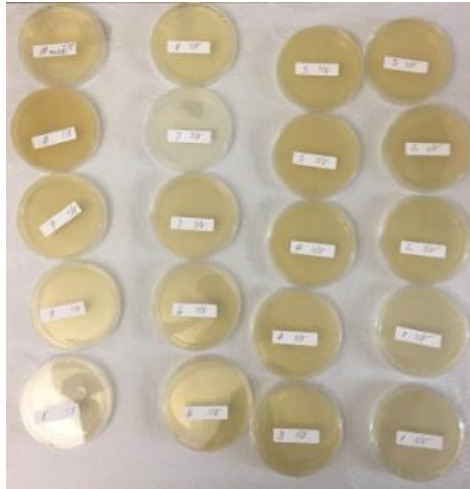
Figure 7 E. coli bacterial titer.

Figure 8 Isolated wild E. coli type.

3.2.4   Mycelium cultivation and deployment

As discussed earlier, other species of mycelium such as *Pleurotus spp.* and *Stropharia rugoso-annulata* have shown to be bacteraphagues (Cotter, 2014; Singh, 2006); however, *T. versicolor* mycelium was chosen for the experiments due to its ability to survive amid high discharge rates (Cotter, 2014), which may be useful for in future field deployment. *T. versicolor*

cultivation is relatively simple because it colonizes on a variety of hardwood mediums and has shown the ability to outcompete other fungi in its growth rate (Cotter, 2014). Untreated *Quercus alba* sawdust trimmings were obtained from a local cabinet shop and stored in a cool, dry place until needed. *T. versicolor* sawdust spawn (Cotter, 2014) was purchased from Mushroom Mountain and kept refrigerated (38°F) until needed for incubation. The spawn is naturally white in color (Figure 9) and has an acidic odor; no changes in coloration or odor were observed throughout the experiment.

Untreated burlap mycofilters were constructed (2" W X 14" L) based on Stamets' (2005) mycofilter design (Figure 10). The mycofilters were initially sterilized for 10 minutes in de-chlorinated boiling water and then filled with a 2:1 ratio containing *T. vericolor* sawdust spawn and sterilized *Quercus alba* sawdust weighing a total of 330.0 grams. Sawdust sterilization was obtained by first vacuum sealing the sawdust and then placing in a 1100 W Sous Vide cooker at 178°F (Figure 11). The mycelium was then incubated at 75-85°F for 2-5 weeks in a sterilized 18-gallon plastic bin. A heating lamp was used to guarantee consistent temperature and the inoculated burlap mycofilters were placed in a lattice formation to allow for adequate air circulation. Suggested incubation time for optimal mycelia efficacy is 2-5 weeks (Cotter, 2014; Pini and Geddes, 2020). Daily misting with de-chlorinated water prevented the mycelium from drying out. All equipment was sterilized beforehand with 190 proof Everclear. Latex gloves and a face mask were worn while handling the mycelium, sawdust, and mycofilters.
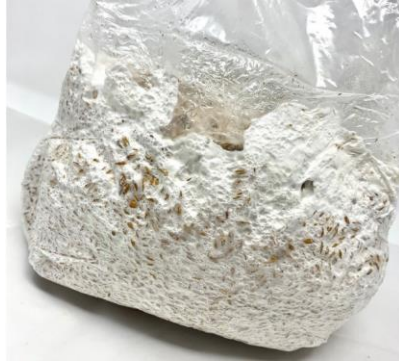
Figure 9 Trametes versicolor mycelium.

Figure 10 Trametes versicolor burlap mycofilter deployed in stream table.

Figure 11 Sterilized Quercus alba sawdust.

3.3   Dilution calculations and procedure

A "spike" of *E. coli* was put into a bucket containing 12L DI water and stirred for 5 minutes to fully incorporate the *E. coli*, with a goal concentration of 1000MPN/100 mL. The water in the bucket was then pumped through the stream table with the flow rate variable pump for 5 hours, requiring 8 input buckets. Details of the dilution process and ratios displayed in Table 6 and a sample was taken from each bucket to measure inflow *E. coli* concertation. Sample water was pumped through the table at a rate of 5.2 +/- 0.28 mL/s, with the pump rate measured at the beginning of each experiment. Water continuously flowed into 5 gallon buckets at the end of the stream table. A sample was collected from outflow of the stream table before it entered the lower buckets each hour for the length of the experiment. Buckets were sterilized with a 10% bleach solution after use.

Table 6 Procedure for E. coli dilution.

Procedure:
Prepare 1 50 mL tube for each 12 L bucket you plan to use for an experiment
Label these "spike"
Place 19.5 mL of DI water in each of these tubes
Set aside
Prepare 3 50 mL tubes labeled 1, 2, 3
Place 19 ml DI water in 1, 2, and 3
Defrost 1 mL of stock
Place 1 mL stock in tube #1 and shake well
Place 1 mL tube #1 in tube #2 and shake well
Place 1 mL tube #2 in tube #3 and shake well
Place 0.5 mL from tube #3 in EACH of the tubes labeled "spike"
Spike tubes already had DI, so shake well
Pour 1 spike tube into each bucket and mix well.

3.3.1   Analyzing E. coli concentrations

Samples of the outflow were collected in Nasco Whirl-Pak and immediately placed in a refrigerator at 4°C. Samples were prepared for the IDEXX Colilert-18 Quanti-tray system analyzation by transferring 100mL of the sample into a 120mL sample vessels using a pipette. Next, the IDEXX Colilert reagent powder was added to each sample and dissolved. This mixture was then poured into Quanti-Tray/2000 and bubbles were allowed to settle. Finally, the Quanti-Tray was sealed with the Quanti-Tray Sealer. Each sample was labeled with the date, sample name, and dilution (no dilution was used). The sealed samples were then placed in an incubator at 35°C for 18-22 hours. After incubation, the samples were removed and individually inspected in natural light and using a UV-light. Results were read according to the IDEXX Coliler-18 test Procedure (2017) and recorded on the back of the Quanti-Tray, in the lab notebook, and ultimately onto a Microsoft Excel spreadsheet. The IDEXX Coliler-18 test is approved by the EPA to monitor drinking water, surface water, ground water, and wastewater (CRK 2014, IDEXX, 2017). The IDEXX Quanti-Tray using the Most Probable Number (MPN) statistical method to determine bacterial concentrations (IDEXX, 2017). Disinfection was prioritized throughout the experiment. Sterile latex gloves were worn at all times. Disinfecting wipes were used to wipe down all surfaces. All materials that were used were disinfected with bleach and wastewater from the experiment and sterilized with a 10% bleach solution before transmitting it into the sewer system.

An ANOVA (analysis of variance) statistical analysis was used to determine the statistical significance of the results on the null hypothesis that the means of the data sets *(E. coli concentrations at the outflow with and without the T. versicolor)* are the same and the alternative hypothesis that they are different. If $p<0.05$ then the null hypothesis is rejected in favor of the

alternative, while if p>0.05, the null hypothesis is accepted (Helsel et al., 2020). These results were calculated in Matlab using the anova1() function. In addition, the slope of the linear regression of outflow *E. coli* concentrations through each experiment was tested for significance using ANOVA in Excel. This was to test if *E. coli* concentrations decreased during each experiment. Percent retention was calculated utilizing the following equation:

$$\frac{\text{Average Inflow- Average Outflow}}{\text{Average Inflow}}$$

4   RESULTS

4.1   Upper South River *E. coli* concentrations

Significantly high *E. Coli* levels were found in all sample sites, with samples collected on nine dates. On average, 6.4 sites of the nine sampled were over the EPA limit for swimming of 235 cfu/100mL on any one sampling date. The McDaniel, CSO-2, and the Ripplewater sites seem to have chronic contamination problems due to their consistently high *E. coli* concentrations (Figure 12; Table 7). The highest concentrations were seen across all sites on 3/5/2020, where it had rained 1.52 in over 24 hours before sampling and 3.07 inches over 72 hours. This clearly indicates SSO spills and CSOs were likely prevalent during this large rain event. Atlanta receives approximately 50 inches of rain per year, so this event represents 6% of annual rain in three days. Interestingly, on this date, CSO-2, below the Custer Avenue CSO, was the only site below the EPA limit likely due to primary treatment. This impact of stormflow on *E. coli* contamination is supported by the samples from 10/17/19, which was after 0.52 inches of rain over the prior 72 hours. On this date, all sites but CSO-1 were above the limit. CSO-1 is the only site that routinely fell below the EPA limits. Even at baseflow, when 72-hour antecedent

precipitation was 0 in, any sites were above the standards but there was wide variability in the
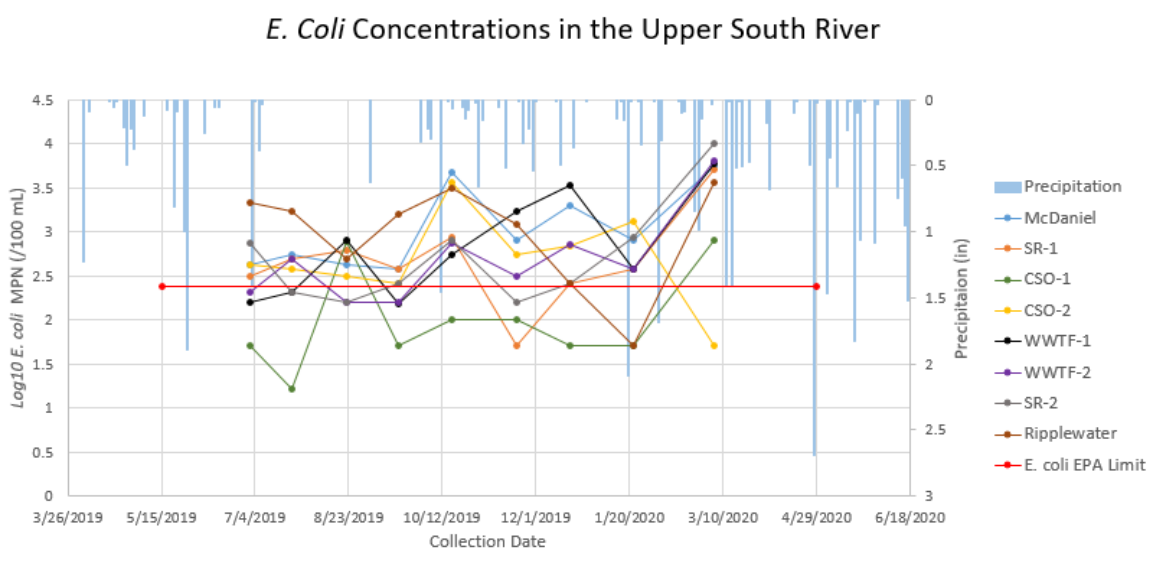
absolute concentrations of *E. coli* at baseflow.

Figure 12 E. coli concentrations in the Upper South River (Note: Because the Tift Site was below the detection limit, it is not included in this figure.)

Table 7 E. coli and Total Coliform data for each sample site.

Collection Date	Site Name	Total Coliform (MPN/100mL)	*E.coli* (MPN/100mL)	Above *E. Coli* Limit
7/1/2019	Tift	1360	<50	
7/1/2019	McDaniel	7825	420	X
7/1/2019	SR-1	38505	315	X
7/1/2019	CSO-1	16275	50	
7/1/2019	CSO-2	28970	420	X
7/1/2019	WWTF-1	11235	155	
7/1/2019	WWTF-2	23055	205	
7/1/2019	SR-2	20530	740	X
7/1/2019	Ripplewater	86645	2140	X
7/18/2019	Tift	2600	<50	
7/18/2019	McDaniel	>120980	120980	X
7/18/2019	SR-1	>120980	10060	X
7/18/2019	CSO-1	19365	<50	
7/18/2019	CSO-2	46040	2085	X
7/18/2019	WWTF-1	86645	2070	X
7/18/2019	WWTF-2	70680	1390	X
7/18/2019	SR-2	>120980	1795	X
7/18/2019	Ripplewater	120980	6505	X
8/22/2019	Tift	1065	<50	
8/22/2019	McDaniel	7610	420	X
8/22/2019	SR-1	28970	610	X
8/22/2019	CSO-1	28970	780	X
8/22/2019	CSO-2	27375	315	X
8/22/2019	WWTF-1	9945	805	X
8/22/2019	WWTF-2	7575	155	
8/22/2019	SR-2	6770	155	
8/22/2019	Ripplewater	70680	490	X
9/19/2019	Tift	420	<50	
9/19/2019	McDaniel	9210	375	X
9/19/2019	SR-1	>120980	370	X
9/19/2019	CSO-1	7645	50	
9/19/2019	CSO-2	24420	260	X
9/19/2019	WWTF-1	9945	150	
9/19/2019	WWTF-2	9675	155	
9/19/2019	SR-2	7500	260	X
9/19/2019	Ripplewater	34550	1590	X

10/17/2019	Tift	610	<50	
10/17/2019	McDaniel	>120980	4670	X
10/17/2019	SR-1	77655	875	X
10/17/2019	CSO-1	8910	100	
10/17/2019	CSO-2	46040	3585	X
10/17/2019	WWTF-1	23055	545	X
10/17/2019	WWTF-2	21760	740	X
10/17/2019	SR-2	23055	790	X
10/17/2019	Ripplewater	>120980	3065	X
11/21/2019	Tift	50	50	
11/21/2019	McDaniel	2285	790	X
11/21/2019	SR-1	1870	50	
11/21/2019	CSO-1	1680	100	
11/21/2019	CSO-2	4940	550	X
11/21/2019	WWTF-1	19365	1680	X
11/21/2019	WWTF-2	4180	315	X
11/21/2019	SR-2	3155	155	
11/21/2019	Ripplewater	11120	1205	X
12/19/2019	Tift	150	50	
12/19/2019	McDaniel	4545	1940	X
12/19/2019	SR-1	3190	260	X
12/19/2019	CSO-1	1080	50	
12/19/2019	CSO-2	6670	675	X
12/19/2019	WWTF-1	30655	3385	X
12/19/2019	WWTF-2	12445	710	X
12/19/2019	SR-2	4665	260	X
12/19/2019	Ripplewater	5845	260	X
1/22/2020	Tift	480	260	X
1/22/2020	McDaniel	5110	790	X
1/22/2020	SR-1	7825	375	X
1/22/2020	CSO-1	1230	50	
1/22/2020	CSO-2	7765	1295	X
1/22/2020	WWTF-1	8080	375	X
1/22/2020	WWTF-2	4665	375	X
1/22/2020	SR-2	13615	865	X
1/22/2020	Ripplewater	4400	50	

3/5/2020	Tift	120980	6005	X
3/5/2020	McDaniel	52310	5475	X
3/5/2020	SR-1	49020	5085	X
3/5/2020	CSO-1	30655	790	X
3/5/2020	CSO-2	100	50	
3/5/2020	WWTF-1	120980	5955	X
3/5/2020	WWTF-2	55995	6480	X
3/5/2020	SR-2	120980	9840	X
3/5/2020	Ripplewater	120980	3560	X

4.2 Lab experiments

The initial series of experiments (WOEC-1,2,3) determined base line concentrations of *E. coli* present in the Ripplewater creek sediment after sterilization of the sediment. As shown in Figure 13 and 14, temporal variance occurred throughout each experiment (WOEC-1, WOEC-2, and WOEC-3), but there were no statistically significant temporal trends. During this experiment, three samples were taken at each timestep to calculate variability.
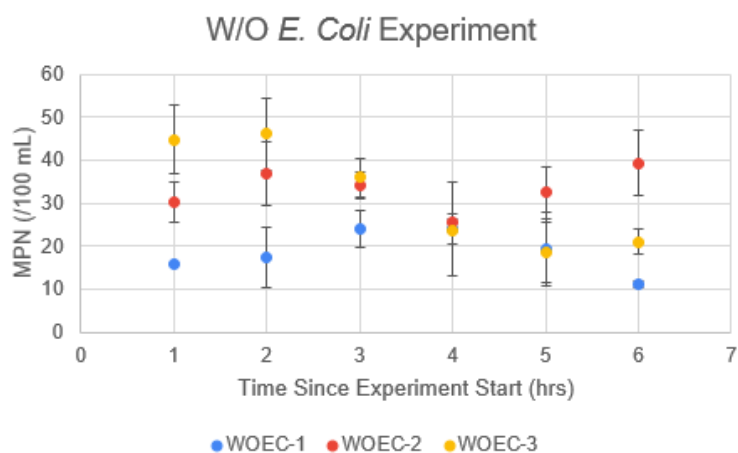

Figure 13 E. coli concentrations every hour for the experiment with bleached sediment and E. coli-free inflow. Error bars indicate the standard deviation of three outflow samples taken at each timestep.
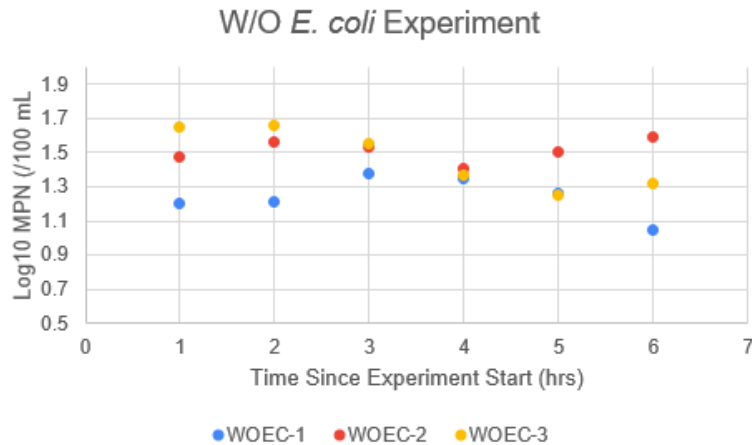

Figure 14 E. coli concentrations every hour for the experiment with bleached sediment and E. coli-free inflow on a Log10 scale.

The second set of experiments (WEC-1,2,3), determined the input concentrations of *E. coli* as well as observed natural changes in the *E. coli* concentrations due to metabolic processes in the sediment alone, despite sediment bleaching. Figures 15 and 16 displays the results of the second set of experiments wherein inputs of DI water spiked with *E. coli* (MPNs ranging from (307.6 to 866.4) were pumped into the stream table system after which hourly samples were obtained from the stream table output. Temporal variance was also observed throughout each experiment in this subset. However, despite temporal variance, *E. coli* concentrations nominally decreased compared to input concentrations by the end of each experiment. Variations through time in *E. coli* concentrations here may be due in part by human error and/or IDEXX error. Possible human error could have occurred during the determination of the titer concentration, in handling the *E. coli* during transport and storage, and/or during the performance of the *E. coli* dilutions.
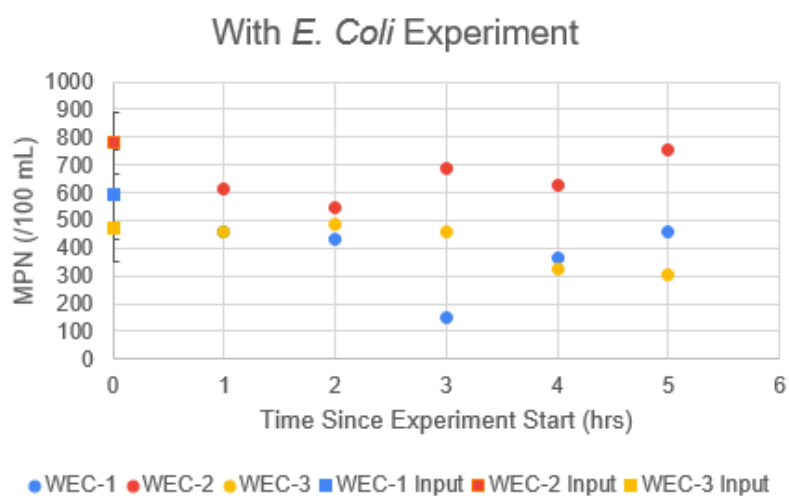

Figure 15 E. coli concentrations every hour during the experiment with E. coli-spiked input water but no mycelium. Triplicate samples were collected of the initial inflow concentrations, which is shown at time 0 for each experiment.
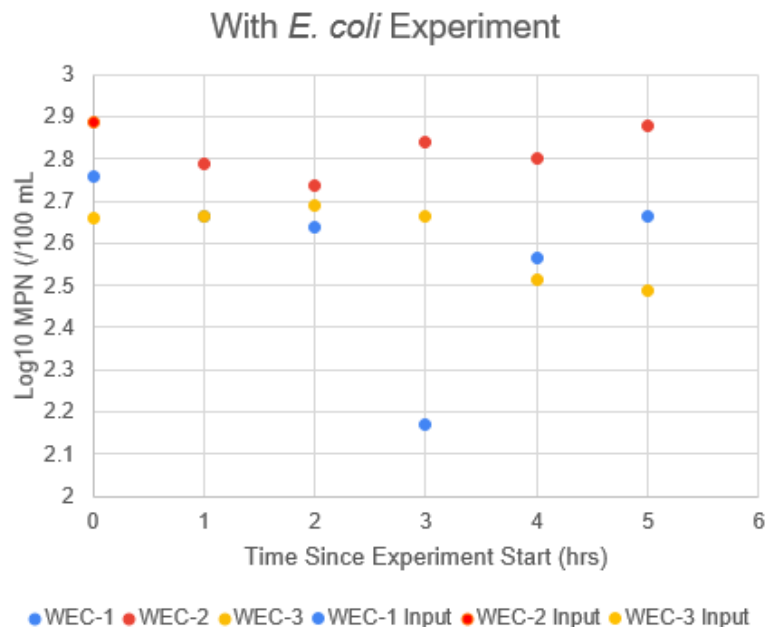
Figure 16 E. coli concentrations every hour during the experiment with E. coli-spiked input water but no mycelium on a Log10 scale. Triplicate samples were collected of the initial inflow concentrations, which is shown at time 0 for each experiment.

In the final series of experiments (WTT-1,2,3), the Turkey Tail (*T. versicolor*) mycelium was deployed in one cross section across the stream table (Figures 17, 18). Although overall the output samples resulted in lower *E. coli* concentrations than the input, there was not a statistically significant linear decline in concentration during any experiments. Calculating the overall decrease in *E. coli* concentration from input to the end of the experiment shows no difference in retention with the presence of the mycelium (Table 8, p=0.65). Although *E. coli* removal was observed throughout various experiments overall, the removal of *E. coli* cannot be contributed to the *T. versicolor* fungal spawn. However due to budget and time constraints, the experiment was not performed more than 6 times total, thus limiting the statistical strengths of these tests.
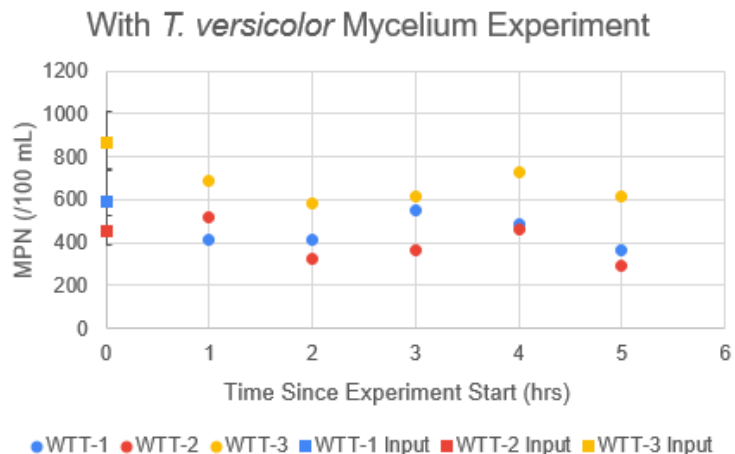
Figure 17 With Turkey Tail (Trametes versicolor) mycelium. Triplicates were collected of the input concentrations, shown at time 0, with the standard deviation shown in the error bars.
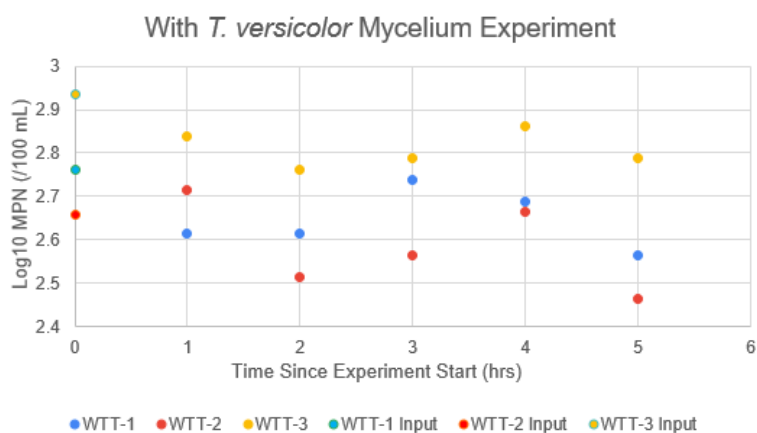
Figure 18 With Turkey Tail (Trametes versicolor) mycelium on a Log10 scale. Triplicates were collected of the input concentrations, shown at time 0.

Table 8 Concentration decrease of E. coli for each experiment.

Experiment	Without Mycelium Initial Inflow Concentration	Without Mycelium Final Outflow Concentration	Without Mycelium % Reduction	With Mycelium Initial Inflow Concentration	With Mycelium Final Outflow Concentration	With Mycelium % Reduction
1	594.5	374.2	37.0477	593.95	444.5	33.6221
2	780.2	647.0	20.5826	459.0	392.0	17.0827
3	473.0	408.74	15.7093	873.3	643.9	35.6351
Average	615.9	476.6	24.4474	642.1	493.5	28.7799

*ANOVA analysis does not indicate a difference between average retention with mycelium and average retention without mycelium. P=0.6

5   DISCUSSION AND CONCLUSION

5.1   Discussion

The first objective of this study was to explore the effectiveness of mycofiltration in decreasing *E. coli* concentrations during a short span of time (5 hours). Previous research demonstrated *E. coli* removal success varying from 99.25% to 99.74% from *Pleurotus ostreatus* (oyster mushroom) mycelium deployment for 48 to 96 hour durations (Pini and Geddes, 2020); however, that research occurred on a shaker table where mycelium and contaminants could interact continuously. In contrast, for actual field applications and to understand real-world implications, hydrologic flow must be considered. Data produced from this research and accounting for hyporheic flow paths, suggests that 4.5% more *E. coli* was removed with the *T. versicolor* mycofilter than without the mycofilter.

While these results are discouraging, they do not negate the potential for mycofiltration efforts in longer term projects but merely suggest the limitations of mycofiltration for rapid response solutions to bacterial contamination in surface water. Previous research demonstrates the efficacy of mycoremediation in field and lab applications (Kapahi and Sachdeva, 2017; Kulshreshtha et al., 2014; Pini and Geddes, 2020; Stamets, 2005; Singh, 2006) and specifically the use of *T. versicolor* as a good fit for mycoremediation (Álamo et al., 2018; Beltrán-Flores et al., 2020; Cotter, 2014; Gebreyohannes et al., 2019; Hleba et al., 2014; Mir-Tutusaus et al., 2014). Furthermore, this study was limited by the use of only one species of mycelium, only one row of mycelium installation, and limited trials due to budget and time concerns. Additionally, due to MPN statistical calculation methodology, the IDEXX Colilert-18 presents a potentially large margin of error when considering short term *E. coli* reductions.

The next objective of this study was to investigate effects of hydrology, specifically discharge rate, on mycofiltration effectiveness and determine the significance of contact time between *E. coli* contaminated water and the mycofilter. Research regarding contact time is limited but thus far, overall data suggests that mycofiltration efficacy increases with an increase in contact time. For example, *E. coli* removal increased after each subsequent week to 98% after a total of three weeks immersed in a lab based pond setting (Martinez, 2016); however, additional research has indicated a lower rate of reduction after 96 h immersed in a shaker table with the highest rates at 48 h (Pini and Geddes, 2020). Lastly, increased discharge rate (likely due to decreased contact time) has been shown to reduce mycofiltration efficacy (Stamets et al., 2013). For example, research which did not account for hyporheic flow (but instead forced water through the mycofilter) found a 20% reduction at 8.3 mL/s flow rate and a 14% reduction of *E. coli* at the 36.7 mL/s rate with the use of *Storpharia spp.* mycofilter (Stamets et al., 2013). This experiment follows that trend, showing a 28.8% reduction of *E. coli* at 5.2mL/s, although we cannot differentiate the removal from natural sediment metabolism compared to the removal from mycofiltration.

In a hypothetical field environment of a small order urban Piedmont stream, water could be passing through the mycofilters at around 23 L/s discharge (average daily baseflow discharge at USGS gage 02336030, North Fork of Peachtree, from July 1, 2019 to April 1, 2020) and after high precipitation events the discharge rate, particularly in urban areas, could increase to 1773 L/s (highest mean daily discharge at that same gage for the same period). These considerations are imperative to investigate in the laboratory before deployment in the field, and remain in contrast to previous laboratory research which does not account for discharge (Pini and Geddes, 2020). While *E. coli* concentrations did decrease throughout the experiment when interacting

with *T. versicolor* mycelium at an average discharge rate of 5.2 +/- 0.28 mL/s, the results of this study do not show statistically significant decreases that can be attributed to the mycofilter at much lower discharges than would be seen in first-order urban streams.

Differences in the response seen in these lab experiments to other published work highlight multiple issues to be considered and addressed in future work. First, this experiment allowed for hyporheic flow paths to form around the mycelium structure, a hydrologic flow path that is well-known but has not been considered by other experiments. These hyporheic flow paths could allow for water to move around the mycelium structure, depending on how they system is installed, and decrease the interactions between contaminant and mycelium, especially considering highly permeable systems. While hyporheic flow is well known for removing contaminants, such as nitrogen, by driving interactions between streambed microbes, anoxic zones, and increased residence times (Passeport et al.,2013), without the presences of mycelium in the hyporheic zone, there is no benefit for *E. coli* remediation. Consideration of how to address this issue will greatly improve the potential impact of future restoration approaches. This includes considerations such as the potential effects of increasing the burlap barrier width or including multiple burlap barriers along the stream table as well as the potential use of different species of fungal spawn in future research. Secondly, mycelium deployment duration also plays a pivotal role in *E. coli* reduction (Cotter, 2014). Highest rates of *E. coli* reduction have been shown within a 48-hour period, but significant reduction rates continue up to 96 hours after deployment (Pini and Geddes, 2020. This research stands in contrast to previous research because the time frame of each experiment was significantly shorter (5 hours total). This research has demonstrated that although *E. coli* reduction does begin within shorter time frames, mycelium efficacy may not be significant for episodic bacterial contamination. While contact

time has been explored in past research, variation in *E. coli* concentrations remains largely

unexamined; however, *E. coli* input concentrations may also affect the efficacy of mycofiltration.

Of the six experiments during this research, *E. coli* inputs ranged in average from 472.95 to

873.3 MPN/100mL, other research utilized 800 cfu/100mL (Stamets et al., 2013). Additionally,

this research's field data shows that *E. coli* concentrations in the Upper South River are

significantly higher and often exceed 1000 MPN/100mL (with a maximum concentration of

120,980 MPN/100mL observed) which may affect future field applications. Research utilizing

extremely high *E. coli* concentrations has yet to be explored, but may provide insight into future

mycofiltration efficacy.

5.2   Conclusion

This research examined the efficacy of *T. versicolor* mycelium in reducing concentrations

of *E. coli* in flowing waters during short periods of time laboratory-scaled environment. The

findings of this study are that the *T. versicolor* mycofilter reduced concentrations of *E. coli* with

temporal variance throughout the experiments; however, results do not indicate any more

removal of *E. coli* from the water column compared to flow without the mycelium. These

findings suggest the usefulness of mycofiltration as a water quality restoration technique in

streams may be limited by decreased contact time between contaminant and mycelium or

hyporheic flow paths that bypass the mycelium installation. According to this research, more

laboratory and field-scale research is needed to determine the effectiveness of *Trametes*

versicolor mycelium as well as to determine contact time. However, it must be noted that

mycoremediation is a burgeoning remediation method and has proven effective for heavy metal,

chemical, and bacterial removal from soils (Kapahi and Sachdeva, 2017; Kulshreshtha et al.,

2014; Singh, 2006). Additionally, it shows promise as a viable method for remediation of water

reservoirs, waste water treatment plants, and small scale surface water remediation installations (Cotter, 2014; Martinez, 2016; Molla and Fakhru'l-Razi, 2012; Pini and Geddes, 2020; Singh, 2006). The usefulness of this technique once refined may be helpful in mitigating high bacterial concentrations in small order urban streams resulting from increased precipitation events due to climate change and continued coverage of impervious surfaces. This is important in light of the lack of policy change and implementation to protect small order streams from consistent degradation and limit human health exposure to high bacterial loads (Walsh and Ward, 2019).

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