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Fungi Perfecti, LLC

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MYCOFILTRATION BIOTECHNOLOGY FOR PATHOGEN MANAGEMENT

Mycofiltration technology uses the vegetative growth of bacteria-predating fungi to transform wood byproducts into an intricate and dynamic three-dimensional web of tube-like cells, called mycelium. This living microscopic net can strain, adsorb, and digest bacteria as a food source– reducing effluent bacteria concentration with a simple, small footprint intervention.

Comprehensive Assessment of Mycofiltration Biotechnology to Remove Pathogens from Urban Stormwater

Fungi Perfecti's EPA SBIR Phase I Research Results

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Researched & Reported by:	Paul Stamets, Marc Beutel, PhD., Alex Taylor, Alicia Flatt, Morgan Wolff, Katie Brownson

Executive Summary

Project Summary

This Small Business Innovative Research project developed the principle of *mycofiltration*—the use of fungal mycelium as a biologically active filter for removing contaminants from water. Since pollution from pathogens is the leading cause of critically impaired waters nationwide, with stormwater strongly linked to this contamination, this cutting edge research focused on removal of *E. coli* from water under runoff model flow conditions. Although there is substantial evidence that many fungi consume bacteria and secrete antibacterial metabolites, mycological research has remained largely isolated to ecological and pharmaceutical explorations. This mycofiltration research expanded knowledge of the application of fungal biotechnology in an innovative and interdisciplinary way by tying together the fields of public health, environmental engineering, and mycology.

The project identified physically durable and biologically resilient fungal species and low cost cultivation methods that can be implemented to produce a fungal biofilter, known as a MycoFilter^{TM}, that is capable of filtering *E. coli* from flowing water under laboratory conditions. Working with Washington State University, the research demonstrated the initial proof-of-concept that fungal mycelium can remove *E. coli* from flowing water, and that mycofilters can be developed that are not significantly impacted by excessive heat, cold, saturation, or dehydration.

Summary of Findings:

Fungal species that were expected to demonstrate antibacterial activity and resilient growth characteristics were grown on different substrate combinations to produce filtration media of various densities and pore sizes. Of the thirty batches of mycofilters initially produced, nineteen batches demonstrated the rate of growth needed to proceed to the resiliency testing portion of the project. Following resiliency testing, one species and substrate combination clearly stood out as far more resilient than the others.

When this lead-candidate mycofiltration media was analyzed for its ability to remove *E. coli* from flowing water, there was a statistically significant reduction compared with the controls. Further, there was no significant difference in performance between the filters that were produced under optimal conditions versus filters that had undergone harsh resiliency testing. Additionally, this bench scale test was conducted with the more difficult to remove "suspended" bacteria as opposed to the more common "sediment-bound" bacteria found in actual stormwater. Thus, this reduction clearly provided proof-of-concept evidence that this low-tech, low-cost, and versatile technology can fill a currently unmet need in the stormwater management community. Subsequent trials with influent containing both sediment and *E. coli* achieved additional reductions, in some instances approaching 100% removal.

In the course of this investigation, however, the research also demonstrated the analytical shortcomings of an EPA-approved and commercially available enzyme-linked chromogenic membrane filtration assay for the enumeration of *E. coli*. Third-party genetic testing indicated that this analytical method produced a number of false-positive results. These false-positives were identified as several non-pathogenic species including members of the genera Raoultella and Enterobacter. The presence of these false-positives was significant when straw was included in the mycofiltration media. The actual *E. coli* reductions that were achieved may therefore have been underestimated in some of the Phase I research trials that included straw in the media.

Conclusions:

Several conclusions may be drawn from the research results. The first is that there are fungal species that are appropriate candidates for the concept of mycofiltration. Of eight fungal strains that were tested over the course of the research, one clearly demonstrated resilience to harsh environmental conditions and a second showed promising characteristics. These species may therefore be considered as technically feasible for stormwater treatment applications. The second notable conclusion is that the permeability of mycofiltration media was generally in the range of 0.07 to 0.10 cm/sec—roughly equivalent to medium grain sand, which confirms applicability for field-relevant hydraulic loading. Additionally, mycofilters demonstrated a significant ability to remove suspended *E. coli* from flowing water. The final conclusion is that, as with other

stormwater BMPs, mycofiltration may be more effective against sediment-bound bacteria—in some cases approaching 100% *E. coli* removal.

The conclusion from the Phase I research on this innovative product is that specific fungal strains are resilient enough and biologically active enough to be considered for stormwater treatment applications against a variety of targets including pathogens, but that more research is needed to clearly define treatment design and operating parameters.

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Research Objectives

This Small Business Innovation Research project explored the development of *mycofiltration*—the use of fungal mycelium as a biologically active filter for removing pathogens from storm water. The research set out to identify which fungal species and substrate can filter *E. coli* from synthetic runoff while meeting the physical and temporal demands required for commercialization. Specifically, the research effort entailed two objectives:

- The first objective was to identify which fungal species and filter media combinations could maintain biological activity and appropriate permeability through the cycles of saturation, drying, heating, and freezing that will be encountered in mycofiltration applications.

- The second objective was to quantify the effects of mycofilters on bacteria. As a model for pathogen filtration, the *E. coli* removal capacity of the most viable fungal filter combinations identified in the first objective were evaluated using synthetic stormwater at an average coliform runoff concentration (~500-900 cfu/100mL) under high and moderate hydraulic loading conditions indicative of a 6-month storm (2-3 inch) and an average storm (1/2 inch).

Research Methods, Rationale, and Results

First Technical Objective

The first objective—to identify resilient and appropriately permeable fungal species and filter media combinations—constituted the majority of the work performed at Fungi Perfecti.

1 a) Growth Trial and Resiliency Testing- ***Methods***

Six fungal species expected to demonstrate antibacterial activity and resilient growth characteristics were grown on five different substrate combinations. Thirty batches of mycofilters were prepared, with each batch consisting of 17 filters: 13 inoculated mycofilters and four un-inoculated controls. Substrate components were prepared individually using a low energy input substrate preparation method which enables large scale mycelium production at a low cost. Batches of each substrate were prepared with mixtures of substrate material proportioned by volume. After each batch of substrate was proportioned, four un-inoculated controls were separated and refrigerated until further testing.

The remaining substrate was then inoculated with grain spawn (sterilized grain that was colonized by mycelium), and placed into burlap bags. Each bag was filled with a total of 10 Kg of inoculated material. The inoculated filters were incubated in a climate-controlled environment at 18-24 °C and their growth was periodically assessed. Following incubation, the mycofilters that demonstrated adequate growth were held in cold storage held at 1–2 °C for 3-4 weeks prior to the resiliency testing phase of the project.

Of the thirty batches of mycofilters initially produced, nineteen batches proceeded to the resiliency testing portion of the project. This consisted of cycles of saturation, drying, heating, and freezing. Before resiliency testing occurred, the mycofilters to be tested were removed from cold storage and allowed to acclimatize in a climate-controlled environment at 18-24 °C for 48 hours. For saturation testing, each mycofilter was submerged in water for 30 minutes, drained for two days at 15.5–19.5 °C and 78–87% relative humidity, re-submerged for 30 minutes, and refrigerated at 2.7–5.5 °C for two days. The mycofilters were then transported to a commercial freezer and stored at -20 °C for 24 hours.

Following freezing, the filters were returned to Fungi Perfecti and stored at 11.5–16 °C for seven days. This seven-day period was intended to dehydrate the mycofilters, however high relative humidity prevented complete dehydration, though substantial drought stress was achieved. The mycofilters were then subjected to a hot spell at 25.5–31.5 °C for 24 hours and then 32–40 °C for 22 hours. This was followed by a 20 minute submersion and six day recovery period at 16–17 °C and 78–83% relative humidity. Each batch of mycofilters was evaluated for vigor, percent colonization, and percent contamination at one mid-point during the resiliency testing (prior to the

heat stress test), and were evaluated again following the recovery period. The resiliency testing portion of the project significantly stressed each mycofilter batch; however, there were observable differences in recovery between the species.

1 b) Growth Trial and Resiliency Testing- ***Results***

The ability of various fungi to colonize mycofiltration substrate that was prepared using the commercial scale bulk cultivation techniques varied widely between species. These variations were documented photographically (Figure 1) and were assigned numerical ratings in three categories (vigor, percent colonization, percent contamination) based on qualitative assessments of growth according to Fungi Perfecti's standard observational metrics (Chart 1). At the end of the initial growth trial and resiliency testing period, Fungi Perfecti's *Stropharia* strain was clearly identified as the ideal candidate for mycofiltration applications; subsequent growth trials suggested that *Irpex* may also be a viable candidate.

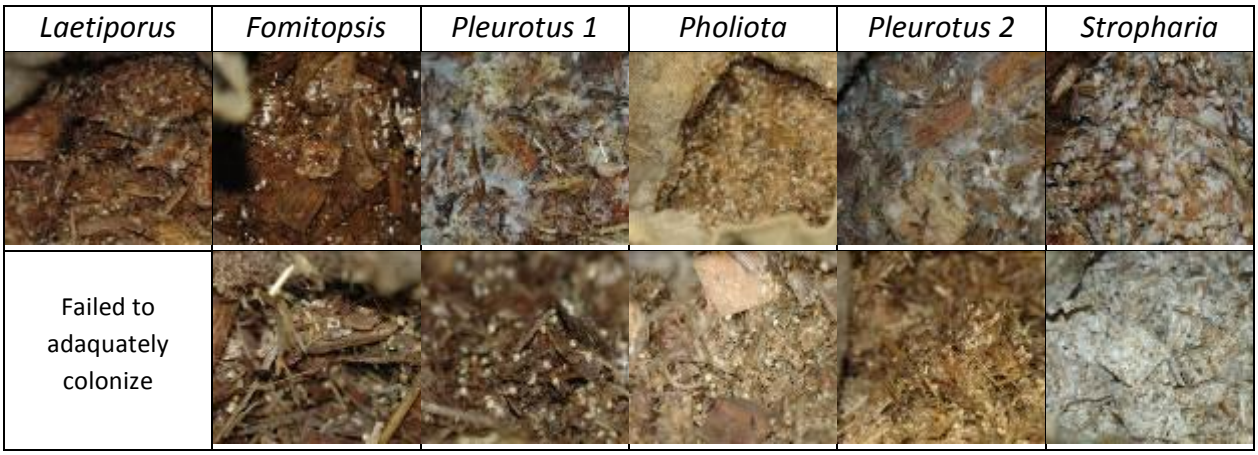

Figure 1: Visual representation of degree of colonization before resiliency testing (above) and after resiliency testing (below). Colonization can be seen as white mycelium spreading throughout the brown background of the substrate.

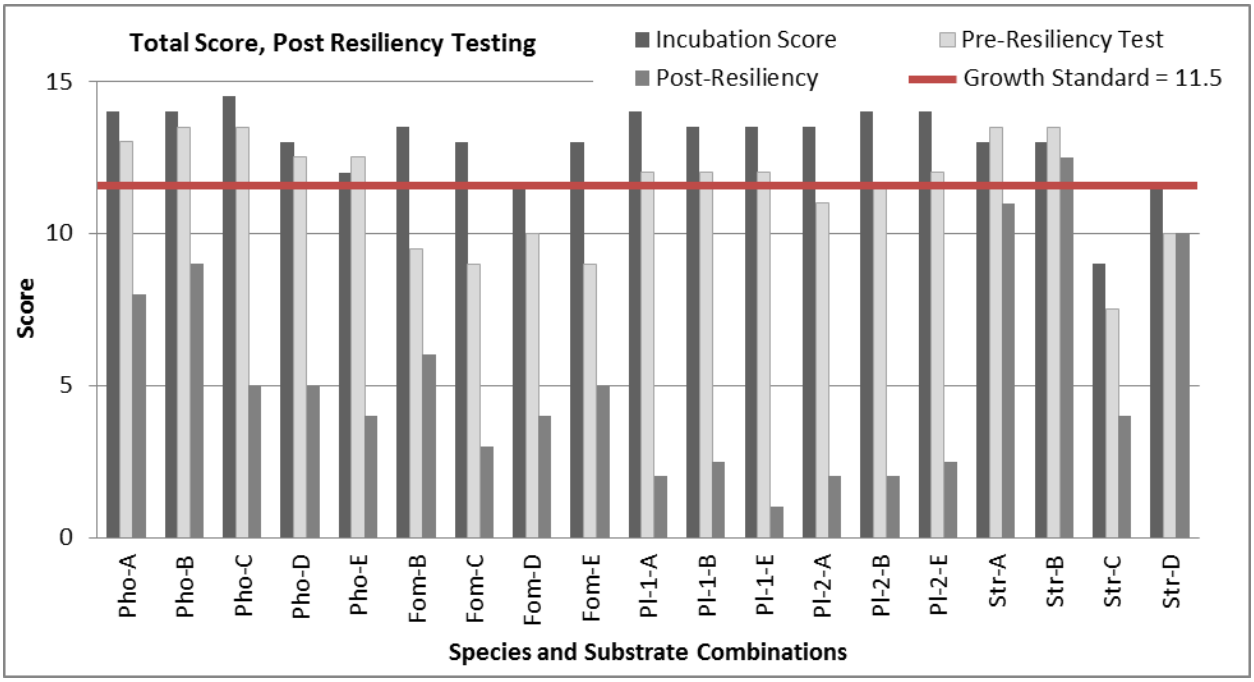

Chart 1: Growth assessment totals for mycofilters during incubation, before resiliency testing, and after resiliency testing. (Species codes: Pho- Pholiota spp.; Fom- Fomitopsis spp.; Pl-1- Pleurotus spp.; Pl-2- Pleurotus spp.; Str- Stropharia spp.; Substrate codes: A- 100% Chips; B- 50% Chips / 50% Sawdust; C- 25% Chips / 50% Straw / 25% Sawdust; D- 50% Chips / 25% Straw / 25% Sawdust; E- 25% Chips / 25% Straw / 50% Sawdust)

It was noted that the degree of initial colonization was not universally related to resilience under harsh environmental conditions (Chart 1). This seems to confirm the hypothesis that some species of fungi (*Stropharia* and *Pholiota*) are more resilient than others (*Pleurotus* and *Fomitopsis*) despite initial appearances of "vigorous" growth. The overall analysis clearly indicated that *Stropharia* mycelium was not substantially stressed by the resiliency testing protocol, in contrast to the other species.

To confirm these findings a second round of growth trials was undertaken with slight modifications to the cultivation methods. Additionally, thee mycofiltration media preparations that had not previously been tested were added to the candidate pool: a species of *Irpex* (Irp-F), and an additional preparation of *Pleurotus* (Pl-2-S), and an

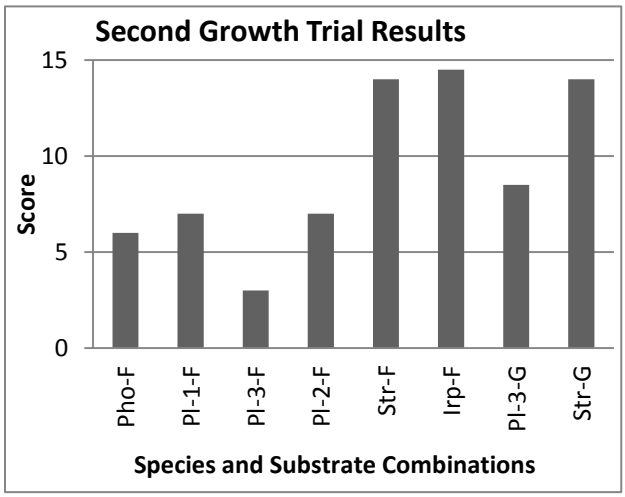

Chart 2: Total scores at the end of secondary mycofilter growth assessment.

additional strain of *Pleurotus* (Pl-3-G). These additional mycofilters were stored under the same controlled climatic conditions as the previous batch of burlap mycofilters, and incubated for 11 to 20 days, depending on the rate of growth. Upon full colonization or at the first sign of contamination by competing fungi, mycofilters were transferred to refrigeration at 4–5 °C. Each batch of mycofilters was qualitatively evaluated for vigor, percent colonization, and percent contamination at three points during incubation, assigned numerical ratings on a five point scale in these categories, and documented photographically. The results from this second round of cultivation tests generally confirmed the initial findings; the strongest candidates were *Irpex* and *Stropharia*.

Based on these results the two most viable candidates for mycofiltration applications were sent to WSU for bacteria removal analysis. The "Str-B" *Stopharia* media (resiliency tested, non-resiliency tested, and controls) was selected from the first growth trial, and the "Irp-F" *Irpex* (and corresponding controls) were sent from the second growth trial.

2 a) Permeability Testing- ***Methods***

The final portion of the first technical objective was to evaluate mycofiltration species for appropriate permeability. This was undertaken because some fungal species can grow mats of mycelium that are too dense for effective filtration at typical stormwater runoff rates. The permeability testing component of the project was completed as a collaboration between WSU and Fungi Perfecti, using a permeameter cell located at Washington State University (WSU). In undertaking this testing, it was noted that the permeability of a given mycofilter would lie, at any point in its life cycle, between two extremes of permeability—uncolonized media (maximum permeability), and complete vigorous colonization (minimum permeability). Based on an initial analysis of the growth of the mycofilters, it was expected that the general permeability of all mycofilter batches could be adequately gauged by assessing the permeability of material representing these extremes.

To that end, testing was conducted on un-colonized media from all substrate combination batches and representative samples of a number of colonized substrates. Samples included: fully colonized samples of the *Stropharia* on three different media types (Str-A, Str-B, Str-E); *Pleurotus* mycelium on 100% straw, and *Pholiota* on media similar to PH-B.

The testing was conducted using a 4.5 inch constant head permeameter cell according to an adapted version of ASTM D2434-68(2006) "Standard Test Method for Permeability of Granular Solids (Constant Head)." Due to the presence of wood chips in the media, the mean particle size was significantly oversized relative to the permeameter cell diameter, and so the average hydraulic gradient ranged from 12-71% above the ASTM recommended range for coarse soils.

After the hydraulic gradient was minimized as much as possible for each sample, the head (*h*) and water temperature (*T*) were recorded and quantity of flow (*Q*) was measured in duplicate for three time intervals (*t*): 20, 40, and 60 seconds. The permeameter cell was reloaded and the procedure repeated three times for each type of mycofilter media analyzed. The distance between the manometer openings of the permeameter cell (*L*) and the cross-sectional area of the specimen (*A*) were recorded and the coefficient of permeability (*k*) was determined according to Darcey's law: $k = QL/Ath$ and corrected to 20 °C water by multiplying *k* by the appropriate viscosity of water correction ratio according to the standard method.

2 b) Permeability Testing- ***Results***

The permeability test results were variable due to the large particle size relative to the diameter of the permeameter cell (average coefficient of variation = 44.17%), however the coefficient of permeability was consistently in the range of 0.07 to 0.10 cm/sec—roughly equivalent to medium grain sand. This suggests that these species and substrate combinations will maintain adequate permeability for field-applicable hydraulic loading. Because infiltration rate is a function of surface area, for the purpose of clarity this data has been presented as a computed maximum infiltration rate for the surface area of the five gallon buckets that were used for the bench-scale bacteria removal testing (Chart 3).

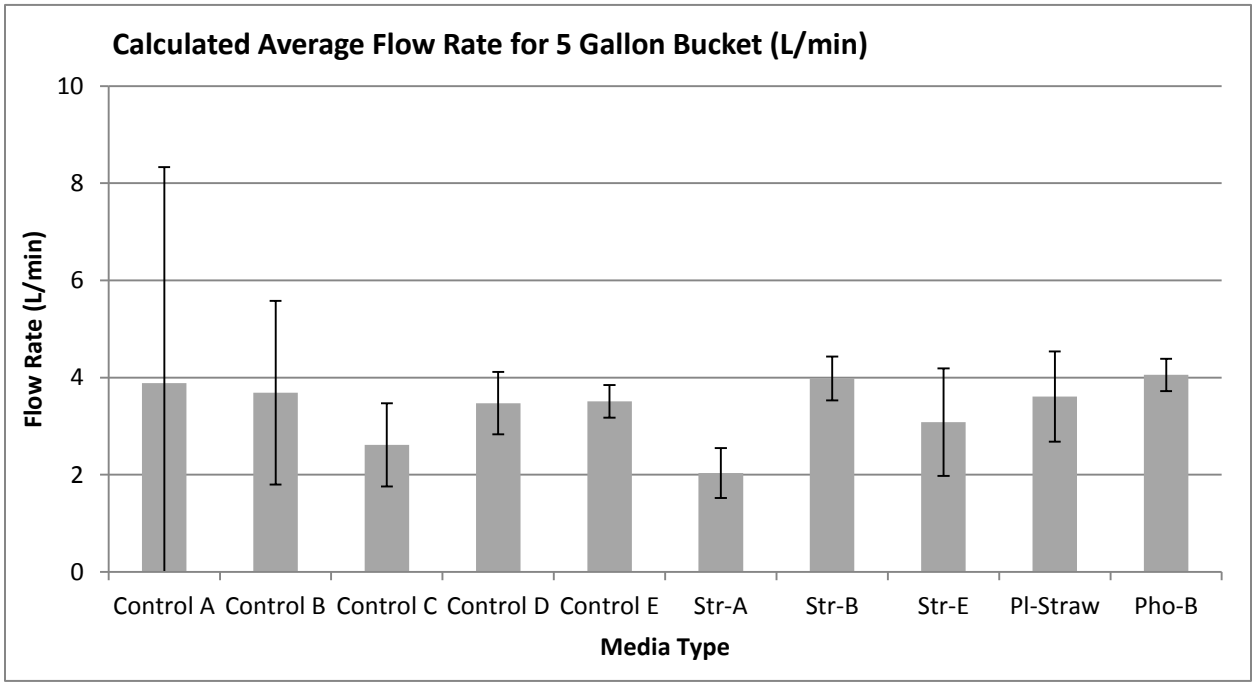

Chart 3: Permeability data representing expected low and high limits of infiltration for various colonized and un-colonized substrates

Second Technical Objective

The second objective—to quantify the effects of mycofilters on *E. coli*—constituted the work conducted at WSU. This objective was met through a series of bench-scale tests that compared the *E. coli* removal capacity of the most viable fungal filters identified by Fungi Perfecti, and in later trials evaluated the effect of sediment and increased media volume on filter performance.

1 a) Bacteria Removal Testing of Single Bucket Mycofilters- ***Methods***

Dr. Beutel, at WSU, tested the ability of an initial mycofilter batch, determined by Fungi Perfecti to be the most suited to field conditions, to remove *E. coli* from synthetic storm water at a typical bacterial concentration under two hydraulic loading rates. The filter batch was *Stropharia* mycelium from batch "Str-B" and consisted of nine experimental filters: (1) three inoculated and vigor-tested, (2) three inoculated (not vigor-tested), and (3) three un-inoculated control. The *E. coli* removal capacity of each mycofilter was assessed by trickle-feeding the mycofilter with a solution of known *E. coli* concentration (~500-900 cfu/100 mL) at two hydraulic loading rates (0.5 mL/min and 2.2 mL/min), and monitoring the effluent concentration of *E. coli* (Figure 2).

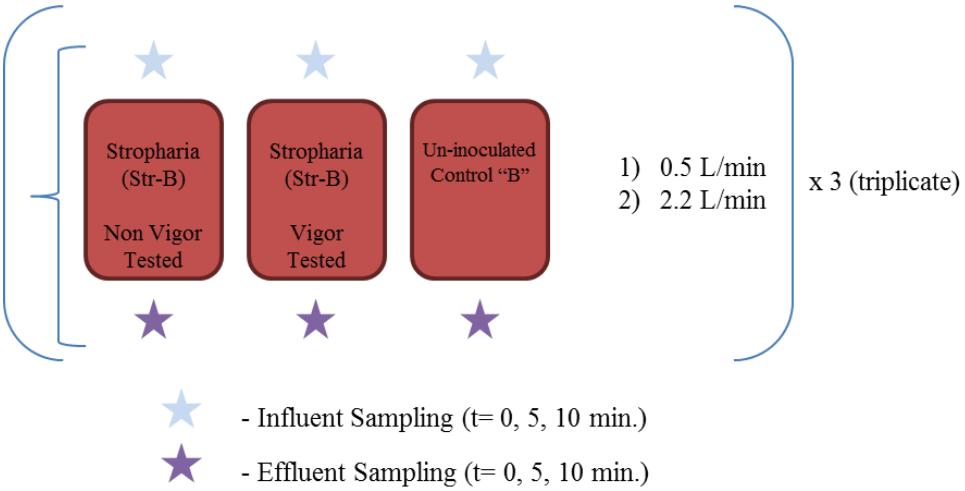

Figure 2: Experimental design for "Str-B" mycofiltration test

For bacteria removal analysis, the mycofilter media was gently transferred into a five gallon bucket with two rings of five 3/16-inch diameter holes in the center bottom of the bucket. Measured from the outside of the holes, the diameter of the inner ring was approximately 1 inch and the diameter of the outer ring was approximately 2 inches. To prevent the filter's substrate from clogging the holes, a 4 inch diameter wire mesh screen was placed over the holes on the inside of the bucket and tacked at four edges with silicon glue.

When not being tested, mycofilters were stored in a walk-in cooler at 4 ^{o}C. To assure that testing was controlled for temperature, each mycofilter was acclimated in the laboratory at room temperature (~20 ^{o}C) for 24 hours before testing. The mycofilter was placed on a drainage basin held 8½ inches above the lab bench by two stacked bricks on either side of the bucket. The bricks also supported the edges of a 5½ inch diameter plastic funnel with a ½ inch diameter, 2-foot long plastic tube attached to neck of the funnel. During testing, the holes in the bottom of the five gallon bucket were aligned with the top of the funnel for effluent collection. A Masterflex 7523-20 peristaltic pump with a 7018-52 head and fitted with Masterflex L/S-18 tubing was used to pump the influent water from a feed tank into the mycofilter. Flow was distributed over the top of the mycofilter through a coiled discharge line placed on top of the mycofilter material. The line consisted of a coiled, ½ inch soft-walled tube with small holes every 2-4 inches along the tube. Material at the top of the mycofilter was also gently formed into a conical shape on the top of the filter to promote drainage into the center of the mycofilter.

A standard methodology was developed to minimize physical variability of the filter media and the biological variability of the influent. Each mycofilter was initially submerged in de-chlorinated tap water with no *E. coli* to achieve a uniform level of saturation, and then allowed to drain for 15 minutes prior to testing. The mycofilter was then loaded with synthetic storm water. Individual batches of 30 L of influent were prepared prior to testing each mycofilter.

To prepare the influent, a large, clean plastic container was filled with 30 L of tap water de-chlorinated with 0.75 g of sodium thiosulfate and allowed to mix for 15 min using an aquarium air pump with air stones. A 5 mL stock solution of *E. coli* ATCC 11775 inoculum was prepared by incubation in Trypticase Soy Broth at 250 rpm and 37 ^{o}C for 16-18 hours until the culture reached stationary phase, as determined by consistent cell densities on several drop-plate serial dilutions. The stock solution was then used to prepare a 1 mL diluted solution with a concentration of approximately 2 x 10^{7} cfu/100 mL that was used to inoculate the influent to produce a final volume of 30 L with a target *E. coli* concentration of around 800 cfu/100 mL. This percolation solution preparation was repeated for each mycofilter percolation test. All of the mycofilters were tested with an *E. coli* solution inoculated from the same stock culture plate.

Replicate samples were collected at multiple time points for two hydraulic loading rates. After the initial submerge and drain period, synthetic storm water was percolated through the mycofilter at a rate of 0.5 L/min with samples being collected at 0 (when outflow starts), 5, and 10 minutes. The mycofilters were allowed to drain for 15 minutes, and then loaded with 2.2 L/min of percolation solution. Again, samples were collected at 0, 5, and 10 minutes. Inflow samples were also collected at the beginning of each filter run. To confirm system cleanliness, water samples were also collected during the initial submersion period. Samples include the dechlorinated water used to submerge the mycofilter and the drain water from the mycofilter. For each filter test, a total of 10 water samples were collected (2 samples during submersion period;

2 inflow samples; 3 outflow samples during the 0.5 L/min test; 3 outflow samples during the 2.2 L/min test). All samples were collected in sterile sample bottles and stored at 4 °C. Samples were tested for bacteria within 6 hours of collection.

Each sample was simultaneously monitored for *E. coli* and fecal coliform using the Colisan C MF method, a U.S. Environmental Protection Agency (EPA) approved method distributed by Micrology Laboratories (http://www.micrologylabs.com/Home). Fecal coliform was measured to assess the potential for false positives due to presence of Klebsiella species bacteria that are commonly found on decaying wood. To analyze for *E. coli* and fecal coliform with Colisan C MF kit, a diluted water sample is poured through a filter. An agar-based medium is then added to a Petri plate and the filter is placed on the plate. The Petri plate is then covered, inverted, and incubated at 35 ^{o}C for 24 hrs. Colonies are then counted. Blue/purple colonies indicate the presence of *E. coli* and pink colonies indicate the presence of non-*E. coli* thermotolerant "fecal" coliforms.

Values are represented in the conventional colony forming units (CFU) per 100 mL of water. Each water sample was evaluated in duplicate at a dilution of 1:20 or at dilutions of (1:10 and 1:20), with the final value in the sample being the average of all values that were within the acceptable count range per filter (less than a total of ~100 CFU per filter). Method blanks were also run approximately every tenth sample. Measurements of *E. coli* and thermotolerant "fecal" coliform bacteria levels in effluent from the filtration experiments (inoculated and vigor-tested, inoculated but untested, and control) for each loading rate and each mycofilter type were tabulated and evaluated for statistical differences as presented in Table 1, and as discussed below.

1 b) Bacteria Removal Testing of Single Bucket Mycofilters- ***Results***

The first set of mycofilters tested for bacteria removal capacity at WSU were the *Stropharia* mycofilters grown on media containing a 50/50 mix of large and small wood chips, "Str-B." This initial test demonstrated a reduction of *E. coli* concentration by roughly 20% at a flow rate of 0.5 L/min ($p<0.05$).

The summary of this trial is illustrated in Chart 4 below and detailed results are presented on the following page in Table 1. Notably, this reduction was demonstrated for both the vigor tested and non-vigor tested material. Further, the vigor tested material was able to achieve a reduction of roughly 14% at the high flow rate of 2.2 L/min ($p<0.01$). Significantly, these reductions were achieved by a relatively small quantity of mycelium—a volume of around 15 L. Additionally, there was a very low probability of false-positives from non-*E. coli* bacteria in this data set because insignificant quantities of bacteria were found in the "pre-flush effluent."

As described in the methods section above, this "pre-flush" sample was taken to assess the presence of bacteria that were resident in the filter. As insignificant quantities of blue-staining bacteria were found in this pre-flush effluent, it is unlikely that false positives were observed in this data set. As discussed in detail in the following section, later research evaluated the effects of sediment and increased media depth on *E. coli* removal. The interpretation of these later trials, however, was somewhat complicated by the presence of false positives, as a result of incorporating straw into the filter media (discussed under *Additional Research Results*).

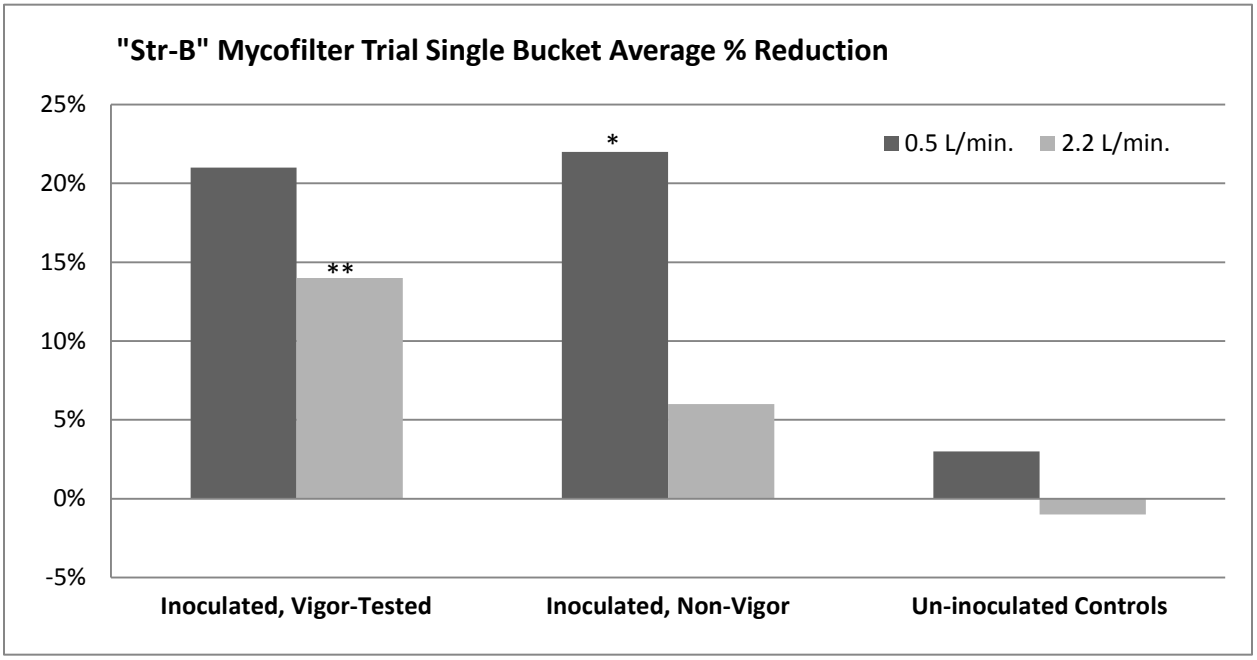

Chart 4: *E. coli* removal capacity of *Stropharia* mycelium compared with un-inoculated controls of media type *B* (large and small wood chips). * $p \leq 0.05$, ** $p \leq 0.01$ - significantly different from controls based on two-tail Student's T-test.

Table 1 - Summary of Results for "Str-B" Single Mycofilter Tests

		Low Flow (0.5 L/min)		High Flow (2.2 L/min)	
Replicate	Influent[a]	Effluent[b]	Percent Removal[c]	Effluent[b]	Percent Removal[c]
		Un-inoculated 'B' Controls			
1	759 ± 114	726 ± 78	4	828 ± 120	-9
2	721 ± 80	741 ± 123	-3	740 ± 69	-3
3	601 ± 105	551 ± 104	8	556 ± 58	7
Average ± Standard Error			3 ± 3		-1 ± 5
		***Stropharia* 'Str-B' Mycofilters (not vigor tested)**			
1	725 ± 161	530 ± 155	27	625 ± 74	14
2	679 ± 57	544 ± 68	20	601 ± 92	11
3	701 ± 112	574 ± 106	18	758 ± 69	-8
Average ± Standard Error			22 ± 3*		6 ± 7
		***Stropharia* 'Str-B' Mycofilters (vigor tested)**			
1	933 ± 139	559 ± 155	40	756 ± 60	19
2	660 ± 130	644 ± 115	2	548 ± 78	17
3	781 ± 102	575 ± 164	26	704 ± 167	10
Average ± Standard Error			21 ± 13		14 ± 3**

[a]Influent values are average plus/minus one standard deviation of quadruplicate bacteriological analyses conducted on two samples collected at the start of each run (low flow and high flow).

[b]Effluent values are average plus/minus one standard deviation of quadruplicate bacteriological analyses conducted on samples collected after 5 and 10 minutes.

[c]Percent removal is calculated as $(C_{in} - C_{out}) / C_{in} \times 100$.

*$p \leq 0.05$, **$p \leq 0.01$; significantly different from controls based on two-tail Student's T-test.

The filter batch from the second growth trial consisted of *Irpex* mycelium on a 25/50/25 mix of large chips, small chips, and straw (Irp-F) and consisted of six experimental filters: three inoculated, and three un-inoculated controls. The filters were grown in five gallon buckets during the second growth assessment, and so the set included only the three un-inoculated controls and three inoculated filters that did not undergo vigor testing. Due to time constraints involved with the growth of mycofilters, a resiliency test of this species was planned, pending promising bacteria removal results. As illustrated in Figure 3, bacteria removal testing was analogous to the experimental design used to test the "Str-B" *Stropharia* mycofilters, and the research was conducted according to the same methods as previously described.

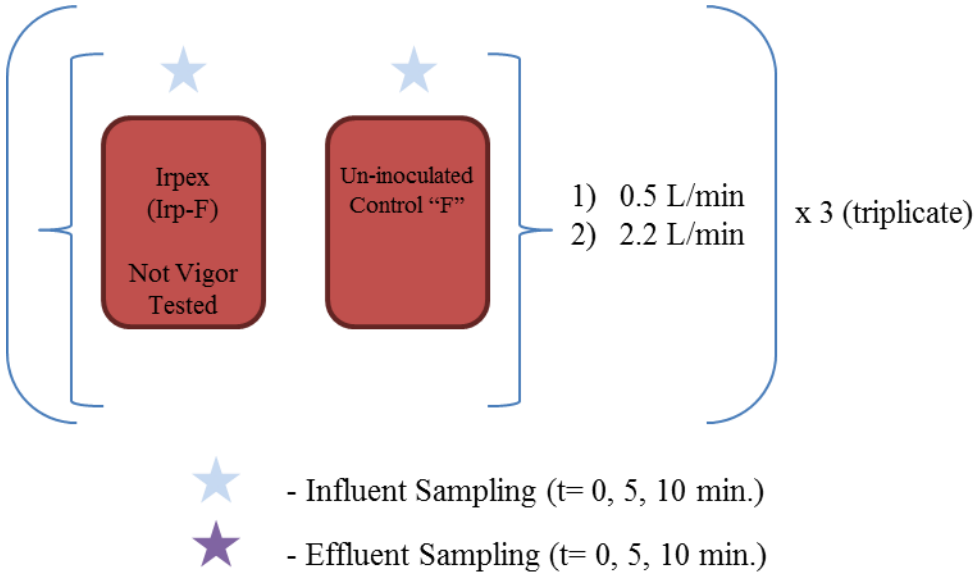

Figure 3: Experimental design for "Irp-F" mycofiltration test

As presented in Table 2, the *Irpex* filters failed to show a consistent removal of *E. coli*, though overall the inoculated mycofilters removed some bacteria and exported far fewer bacteria than the un-inoculated controls. Notably, the concentration of bacteria in the effluent of the control media was significantly higher than the concentration of the bacteria entering the media. This trend was not observed in the previous test. The difference between this un-inoculated control media and that which was previously tested was the presence of straw. As described in the *Additional Research Results* section, subsequent tests confirmed the hypothesis that the presence of straw in the media contributed to a net export of bacteria that gave a false-positive result as *E. coli* using the Coliscan MF method. Overall, the comparison between the *Irpex* and the *Stropharia* data sets offers some confirmation of the hypothesis that different fungal species, as well as different growth substrates have differing abilities to filter *E. coli* from flowing water, though the difference is uncertain due to the cofounding influence of false positives in the *Irpex* data set (see *Additional Research Results*).

Table 2 - Summary of Results for "Irp-F" Single Mycofilter Tests

		Low Flow (0.5 L/min)		High Flow (2.2 L/min)	
Replicate	Influent[a]	Effluent[b]	Percent Removal[c]	Effluent[b]	Percent Removal[c]
		Un-inoculated Controls			
1	533 ± 248	1385 ± 502	-145	738 ± 249	-47
2	628 ± 263	TNTC	N/A	1312 ± 244	-108
3	507 ± 209	1167 ± 295	-132	827 ± 321	-61
Average ± Standard Error			-139 ± 7		-72 ± 18
		***Irpex* Mycofilters (not vigor tested)**			
1	483 ± 186	290 ± 138	40	547 ± 186	-12
2	523 ± 190	637 ± 255	-11	534 ± 184	-13
3	515 ± 187	452 ± 173	6	600 ± 165	-10
Average ± Standard Error			12 ± 15*		-12 ± 1*

[a]Influent values are average plus/minus one standard deviation of quadruplicate bacteriological analyses conducted on two samples collected at the start of each run (low flow and high flow).

[b]Effluent values are average plus/minus one standard deviation of quadruplicate bacteriological analyses conducted on samples collected after 5 and 10 minutes.

[c]Percent removal is calculated as $(C_{in} - C_{out}) / C_{in} \times 100$.

*$p \leq 0.10$; significance relative to controls based on two-tail Student's T-test.

2 a) Volume-dependent analysis of E. coli removal under sediment-spiked conditions by Pleurotus spp. - ***Methods***

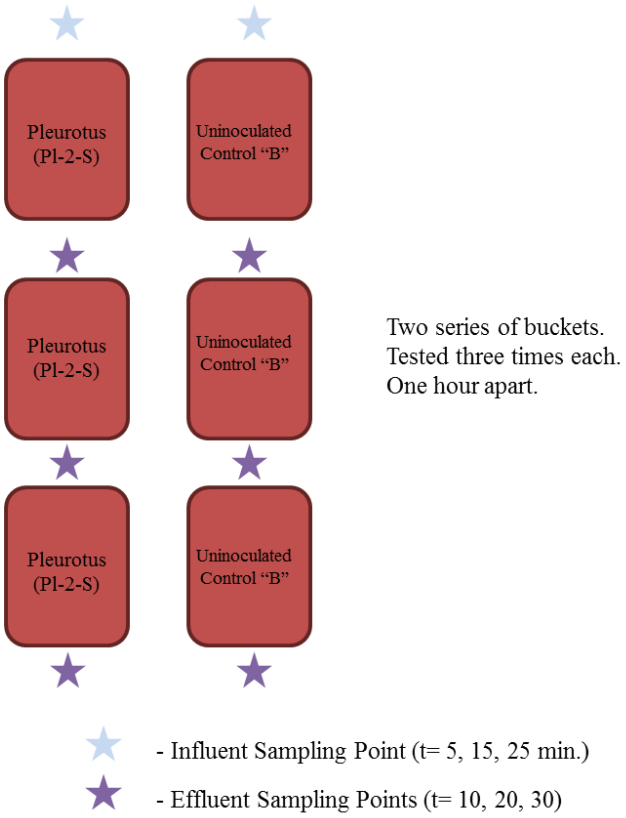

Figure 4: Experimental design for "Pl-2-S" mycofiltration tests. This design was first tested with sediment free influent (*E. coli* only), followed by a test where influent was spiked with both sediment and *E. coli*.

Mycelium of *Pleurotus spp.* (Pl-2-S) was grown under sterile laboratory conditions and delivered to WSU to be evaluated for its bacteria removal potential. Due to a sample mix-up at WSU, the sterilized *Pleurotus* mycelium was evaluated against a control that was intended for a different data set. The un-inoculated control that was tested was therefore the "B" control media from the first *Stropharia* test. While this material lacked the straw that was present in the *Pleurotus* "Pl-2-S" media, it does offer some comparison between mycelium-infused and un-colonized media filtration.

Prior to testing with *E. coli*, each bucket was submerged in clean (*E. coli* free) de-chlorinated tap water and allowed to drain for 15 minutes. The soak water and the water that drained off one of these saturated mycofilters were sampled for bacteria to validate the cleanliness of the un-spiked influent water source and to assess the presence of bacteria resident in the filter media.

Following this saturation and draining period, the three buckets of a given filter media were stacked in a vertical series. Influent was prepared using the methods previously described and was loaded into the top mycofilter at a low loading rate of approximately 300 mL/min. Effluent from the top filter ran into second mycofilter, and then effluent from the second filter ran into the third mycofilter. A "run" consisted of collecting influent samples at 5, 15, and 25 minute time points, and collecting effluent samples at time 10, 20, and 30 minute time points from all three buckets in an experimental unit. The series of filters was then allowed to drain for one hour followed by a second 30 minute loading, allowed to drain for a second one hour period, and then loaded a third and final time for 30 minutes. Thus, each of three mycofilter media types was "run" three times (1 hour apart), with samples collected from three post-filtration points at three time intervals.

Batches of influent were prepared by spiking the synthetic stormwater with model sediment consisting of fine diameter ground silica (U.S. Silica Sil-Co-Sil 125, effective diameter of 125 microns). After spiking the synthetic stormwater with bacteria as previously described, influent levels were spiked with sediment to a concentration of around 20 mg/L and kept in suspension by bubbling vigorously with air during the experiment. Influent and effluent samples were collected and analyzed for bacteria as previously described. The tests were designed to assess the effect of sediment on bacteria removal. This was an important consideration because a correlation has been demonstrated between sediment removal and bacteria mitigation in other stormwater BMPs (Davies and Bavor, 2000). The premise is that bacteria preferentially adhere to sediment particles in stormwater rather than existing in a "free-floating" state. If, as is the case with other stormwater BMPs, mycofiltration can effectively remove sediment, then actual field-applicable bacteria reductions may be more appropriately gauged by this modified method.

2 b) Volume-dependent analysis of E. coli removal under sediment-spiked conditions by Pleurotus spp.- ***Results***

The buckets from two different media types (sterilized mycofiltration media "Pl-2-S," and non-sterile un-inoculated control media "B"), were stacked vertically with effluent trickling from the bottom of each bucket into the top of the next. These effluent samples are tabulated below for each of three "runs." As illustrated in Chart 5 and detailed in Table 3. Statistical analysis using a simple t-test (unpaired t-tail assuming unequal variances) illustrated that the *Pleurotus* removal rates (100%, 100%, 100%) were significantly higher than the controls ($p < 0.01$).

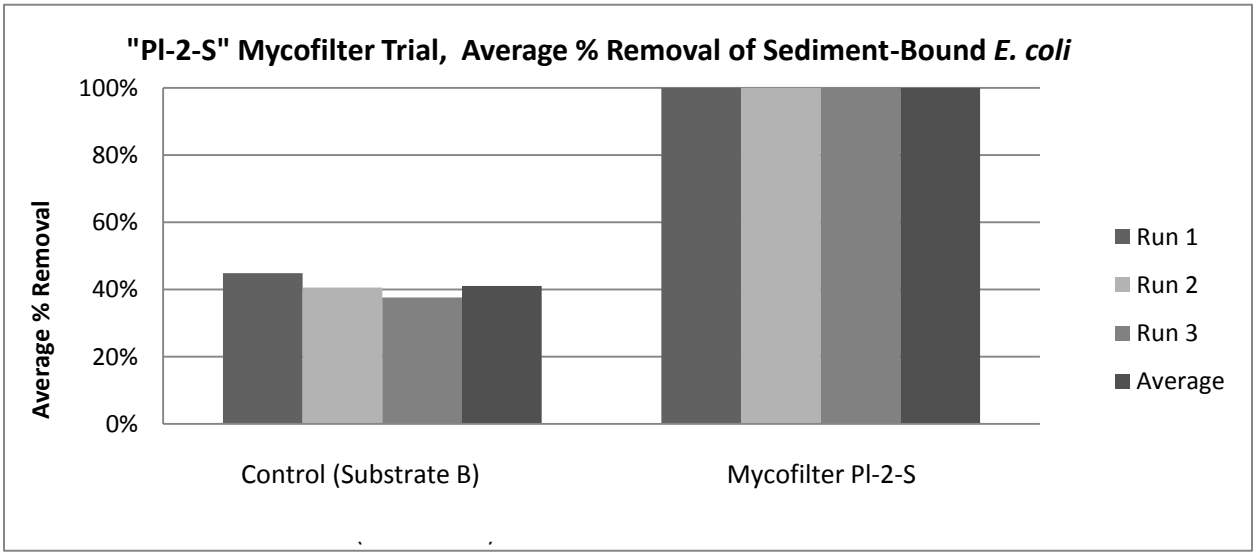

Chart 5: *Pleurotus* filter series test removal averages for *E. coli* when influent also contained silica sediment.

Table 3 - Summary of Results for *Pleurotus* Mycofilter Sediment & Bacteria Series Tests

	Run 1			Run 2			Run 3		
	Average	Stdev	% Conc Removal	Average	Stdev	% Conc Removal	Average	Stdev	% Conc Removal
Un-inoculated Control (50% WHOLE CHIPS, 50% FINE CHIPS)									
Influent	877	67		853	116		833	29	
Mycofilter 1 Effluent	690	130	21%	587	92	31%	563	67	32%
Mycofilter 2 Effluent	597	176	14%	507	55	14%	477	59	15%
Mycofilter 3 Effluent	483	42	19%	507	49	0%	520	245	-9%
Average Removal			18%			15%			13%
Standard Error of Removal			2%			9%			12%
Overall Removal			45%			41%			38%
Sterilized *Pleurotus* "Pl-2-S" (25% WHOLE CHIPS, 50% FINE CHIPS, 25% STRAW)									
Influent	917	101		940	62		1010	108	
Mycofilter 1 Effluent	750	75	18%	570	503	39%	930	87	8%
Mycofilter 2 Effluent	77	133	90%	0*		100%	0*		100%
Mycofilter 3 Effluent	0*		100%	0*		100%	0*		100%
Average Removal			69%			80%			69%
Standard Error of Removal			26%			20%			31%
Overall Removal			100%			100%			100%

0* - Blue colonies were observed but tested negative as E. coli.

While these removal rates do show promise, there were some limitations of the methods for this trial that are relevant to the accurate interpretation of this data. Much of the effluent from the mycofilters was assessed to be low or free in *E. coli*; however the Coliscan plates used to enumerate the bacteria in the effluent were somewhat difficult to interpret.

The influent plates had clear, small blue colonies indicating the presence of *E. coli*. A "confirmatory reagent" known as Kovac's solution was used as well to confirm the identity of these bacteria as *E. coli*. Kovac's solution detects the presence of indole—a molecule produced by *E. coli*, but not produced by many other bacteria—by producing a magenta zone (confirmatory reaction) or a clear or yellow zone around the colony (negative reaction). These influent sample colonies tested positive (turned red) when stained with Kovac's solution.

The effluent plates from the first mycofilter bucket generally had a light pink hazy background, possibly from overcrowding from non-*E. coli* thermotolerant "fecal" coliforms. However, it is important to note that there were no thermotolerant coliforms in the influent, and there was no fecal matter in the mycofilters. It was hypothesized that these pink colonies were Klebsiella spp., a coliform that can be present in woody material (Caplenas and Kanarek, 1984). This theory was confirmed with subsequent testing, as described under *Additional Research Results*. Effluent

plates from the first mycofilter bucket also had some distinct blue colonies growing on top of the pink haze. However they tested non-positive for *E. coli* with Kovac's solution. The effluent plates from the second and third mycofilter buckets both had a pink/magenta haze, again possibly due to overcrowding with Klebsiella. There may have been blue colonies underneath the pink layer, however if present they could not be distinguished. A test with Kovac's solution on these plates may have given a positive result, which would have been indicated by the colony turning red; however, it was difficult to distinguish whether or not the indicator was turning red due to the magenta haze in the background. In the final analysis, the weight of evidence indicates that the visible colonies on the Coliscan plates for this experimental run were not *E. coli*, and that the mycofilters were effective in removing *E. coli* from water spiked with *E. coli* and sediments.

As discussed in detail under *Additional Research Results*, the reliability of the Coliscan MF method as well as the 'confirmatory' reaction elicited by Kovac's reagent is questionable. However, while the combination of Coliscan and Kovac testing certainly produced false-positive results, this method did not produce false-negative results. Namely, there were no instances that could be found where *E. coli* was present (on a genetic test) but failed to appear on the Coliscan media.

Therefore, the exports of bacteria seen in trials of media that contained straw (whether inoculated or controls) is not indicative that the media harbored or exported *E. coli*, but rather that a native non-pathogenic bacterial community exists in straw-containing media and elicits false-positive reactions as detailed in Table 4, below. As these bacterial exports were not seen in the *Stropharia* "Str-B" trial, presumably the straw in the media is responsible for the majority of these organisms. Therefore, the reported and statistically significant *E. coli* removal rate of approximately 20% per 0.6 ft^3 by the "Str-B" *Stropharia* mycofilters ($p<0.05$) is a reliable indicator of the potential of this species, and greater reductions are likely to be seen a field setting due to the occurrence of sediment-bacteria binding in these situations.

Additional Research Results

In a series of efforts aimed at a comprehensive interpretation of the data obtained during the *Second Technical Objective*, additional research was conducted, at significant extra cost to Fungi Perfecti LLC, which fell outside of the EPA-funded scope of the two proposed technical objectives.

1) *Genetic identification of pink-staining thermotolerant "fecal" coliform bacteria resident within un-inoculated controls and mycofiltration media-* ***Methods, Results and Discussion***

The first component of this additional research was an evaluation of the presence of other coliform bacteria present within the filter media. This research was undertaken because it has been previously documented that bacteria of the genus Klebsiella grow naturally on decaying wood, and may contribute to false positives in water quality analysis when the outmoded "fecal coliform" test is used (Caplenas and Kanarek, 1984). The Coliscan MF media allows for the simultaneous monitoring of thermotolerant coliform bacteria as well as *E. coli*. When using this media, the non-*E. coli* thermotolerant "fecal" coliforms turn pink (based on the production of the galactosidase enzyme) while *E. coli* turns blue (based on the production of both galactosidase and glucuronidase enzymes). Each water sample that was collected during each phase of the *Second Technical Objective* was therefore evaluated for both types of bacteria using the methods previously described. The overall conclusion from this monitoring is that woody media (whether colonized by fungi or not) tends to export these non-*E. coli* thermotolerant "fecal" coliforms. Additionally, it appears that the addition of straw to the media increases the quantities of thermotolerant coliforms that are exported–both from the controls and from the inoculated mycofilters (Chart 6).

Chart 6: Summary of available data for non-*E. coli* fecal coliform colonies in mycofiltration media and control media effluent. Note: SR-B and Control B contained no straw.

Chart 6: Summary of available data for non-*E. coli* fecal coliform colonies in mycofiltration media and control media effluent. Note: SR-B and Control B contained no straw.

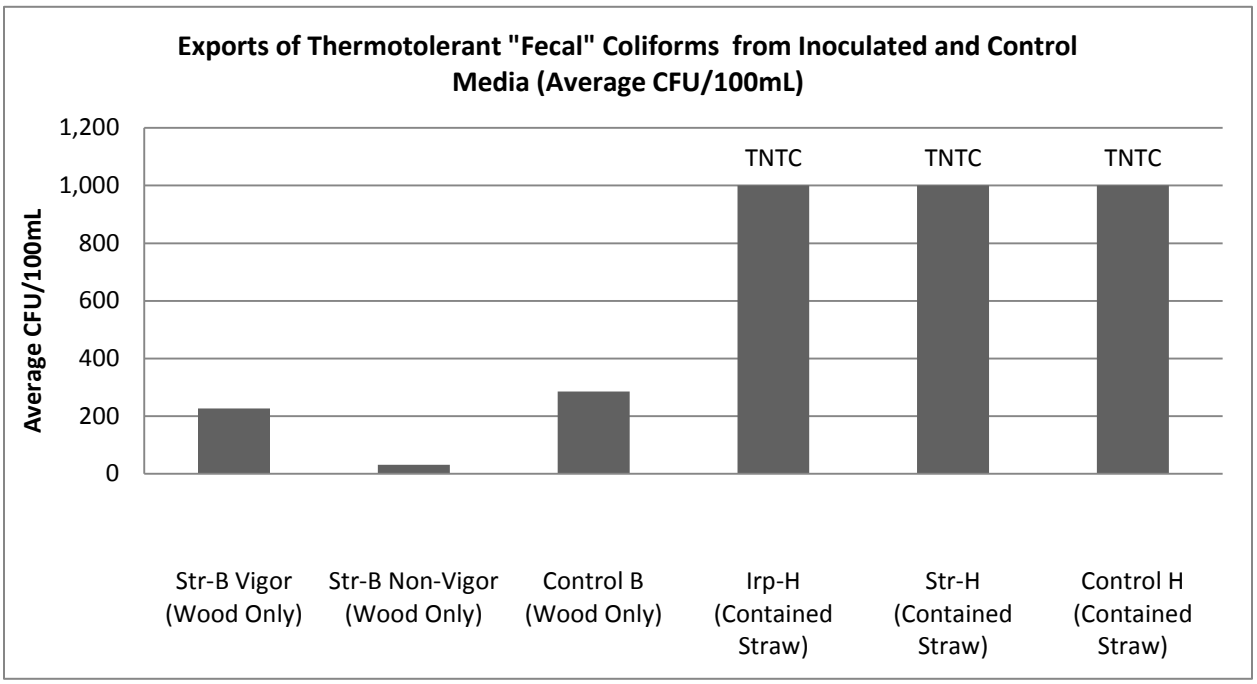

Chart 6: Summary of available data for non-*E. coli* fecal coliform colonies in mycofiltration media and control media effluent. Note: SR-B and Control B contained no straw.

As negligible quantities of fecal coliform bacteria were contained in the synthetic influent, and as the mycofilters contained no fecal matter, it is likely that these pink colonies were members of the genus Klebsiella, as demonstrated by Caplenas and Kanarek (1984). In an effort to confirm this hypothesis, a small sub-study was conducted and several samples were sent to "Microcheck"—an independent bacteriology identification laboratory (Northfield, VT). In the first test, clean de-chlorinated tap water was passed through the *Pleurotus spp.* "Pl-2-S" media that had previously been tested for *E. coli* filtration. Effluent was collected, diluted, filtered through the Coliscan MF membrane, and incubated. The Coliscan membrane was then sent to Microcheck for identification. After sub-culturing the Coliscan filter plate to separate CFUs, the bacterium *Raoultella planticola* ATCC 33558 was identified. Significantly, this bacterial species was formerly grouped into the genus Klebsiella until it was reclassified into the "new" genus Raoultella in 2001 (Drancourt, 2001). As previously mentioned, Klebsiella species are known to grow on wood and to confound public health water quality assays.

Subsequently, the effluent of *Stropharia* Str-H mycofilters (contained wood and straw) were also evaluated using genetic techniques. In this experiment, clean de-chlorinated, bacteria-free tap water was passed through three *Stropharia* Str-H mycofilters for 10 minutes, effluent was collected, diluted 1:100 to clearly separate CFUs, and plated using the Coliscan MF method. One pink-staining colony was subcultured from each of two separate *Stropharia* mycofilters. These pure

subcultures were sent to Microcheck for genetic identification. Once again, both of these pink-staining bacteria were identified as *Raoultella planticola* ATCC 33558.

These results are significant because they indicate with reproducibility that the pink-staining "coliforms" that are resident within both the mycofilters and un-inoculated controls are actually non-fecal thermotolerant coliforms that have not been associated with primary contact public health risks. As noted in Caplenas and Kanarek (1984):

> "Since Klebsiella is not exclusively or specifically associated with fecal waste, the study findings suggest that highly specific indicators of fecal waste contamination, i.e., thermotolerant fecal coliforms as measured by *E. Coli*, be used as the standard fecal coliform guideline. This would promote a more specific and accurate indicator-to-pathogen ratio and subsequently eliminate the interfering response of Klebsiella from a non-fecal source."

Indeed the legitimacy of "fecal coliform" as a water quality indicator has been repeatedly called into question since it was initially proposed by The National Technical Advisory Committee (NATC) of the Department of Interior in 1968. In the EPA's newly released 2012 Recreational Water Quality Criteria guidance, *E. coli* and *Enterococci* are identified as the best reliable indicators of health risk due to fecal pollution. The pink "fecal coliform" exported in the media used in this research must therefore be considered within the appropriate context; the public health significance of these bacteria is highly questionable.

2) *An evaluation of the reliability of the Coliscan MF media and Kovac's reagent to selectively detect the presence of E. coli*- ***Methods, Results and Discussion***

The second component of the additional research was to assess the potential for false positive results for *E. coli* measurements using the Coliscan MF media and Kovac's reagent. When using the Coliscan MF method, the Coliscan media presumably identifies *E. coli* by the simultaneous reactions of the enzymes glucuronidase and galactosidase with dyes in the media to stain *E. coli* colonies a blue/purple color. As previously discussed, a "confirmatory reagent" known as Kovac's solution was used as well. Kovac's solution detects the presence of indole—a molecule produced by *E. coli*, but not produced by many other bacteria—by producing a magenta zone (confirmatory reaction) or a clear or yellow zone around the colony (negative reaction). Previous trials of filter media produced Coliscan filter plates with blue colonies that were both indole-positive and indole-negative. This discrepancy suggested that "Coliscan +" but "indole -" blue bacterial colonies were false positives.

Based on this suspicion of false positives from the "Coliscan +" but "indole -" bacteria found on the filters, a small trial was conducted to assess the reliability of the Coliscan MF media using genetic techniques. In this sub-study two *Stropharia* Str-H buckets (contained wood and straw) and two corresponding un-inoculated control buckets (neither of which had been previously tested with *E. coli*) were flushed de-chlorinated tap water that was not spiked with *E. coli*. Water

samples of the effluent were collected and serial dilutions were prepared. These dilutions were analyzed with the Coliscan MF method as previously described. Each plate was assessed for the presence of blue colonies, which are "identified" as *E. coli* according to the Coliscan MF method. These blue colonies were then labeled alphabetically. Each of these labeled blue colonies was then sub-cultured onto Brain Heart Infusion agar plates, which were labeled correspondingly.

The original blue colony on the filter plate was then treated with Kovac's solution, thereby identifying each subculture plate as either indole positive or indole negative. The indole positive identifications were, however, less certain than the indole negative identifications due to the nature of the Kovac testing protocol. These subcultures were then sent to Microcheck for identification. As outlined in Table 4, subcultures were sent from seven CFUs in total, and represented indole positive and indole negative colonies that stained blue on the Coliscan media. These colonies were isolated from the effluent from *Stropharia* (Str-H) media as well as un-inoculated "H" media controls. Six of these plates were analyzed in duplicate by Microcheck as a control for Microcheck's methods and for the purity of the sub-culture. As a positive control for Microcheck's methods, the stock culture of *E. coli* ATCC 11775 was also sent for genetic identification, and was confirmed.

Table 4: Genetic identification of bacterial species that tested positive as *E. coli* using the Coliscan MF method, and either positive or negative using the 'confirmatory' Kovac's reagent test.

Bucket Code	Color on Coliscan MF Plate	Reaction to Kovac's Reagent (Presence of indole)	Replication of Genetic Testing	Identification	Confidence	% Match
SR-H-21	Blue	K-	Singleton	*Staphylococcus hominis hominis* ATCC=27844	Species	100.0
SR-H-33	Blue	K-	Duplicate	*Enterobacter aerogenes* (duplicate confirmed)	Species	99.88
SR-H-33	Blue	K-	Duplicate	*Enterobacter aerogenes* (duplicate confirmed)	Species	99.88
SR-H-33	Blue	K+	Duplicate	*Raoultella planticola* ATCC=33558 (duplicate confirmed)	Species	99.92
SR-H-33	Blue	K+	Duplicate	*Enterobacter aerogenes* (duplicate confirmed)	Species	99.88
Control 7	Blue	K+	Duplicate	*Enterobacter hormaechei* or	Genus	96.64
				Enterobacter pyrinus	Genus	96.70
Control 3	Blue	K+	Duplicate	*Raoultella planticola* ATCC=33558 (duplicate confirmed)	Species	99.82

The genetic analysis did not identify any of the blue bacteria as E. coli. According to other published reviews of these methods, the false positive rate for chromogenic membrane filtration media such as the Coliscan MF method (an EPA approved water quality test) should be around 5% (McLain et al., 2011). Therefore, approximately 95% of these blue CFUs should have been

confirmed as E. coli. To the contrary, these results indicate that neither the Coliscan media nor the secondary test using Kovac's solution were specific in their ability to detect E. coli.

These results are significant because they indicate that there was a potential for false positive results (though not false negative) using the Coliscan C MF method to evaluate the efficacy of mycofiltration. The reliability of membrane filter techniques using a chromogenic media is limited to relatively clean samples with low bacterial diversity (McLain et al., 2011). High false positive rates have also been correlated to crowded plates, which was a common occurrence in this study due to the *Raoultella* (*Klebsiella*) bacteria (Olstadt et al. 2007; Pitkänen et al., 2006). Olstadt et al. (2007) looked at the ability of different USEPA approved *E. coli* tests to suppress high levels of *Aeromonas spp.*, in an effort to mimic real-world conditions where there are numerous bacteria present in a given water sample. In that study, Coliscan was unable to suppress some strains of *Aeromonas spp.,* even at levels as low as 10 cells meaning that the Coliscan test could be less reliable when using highly populated bacterial samples.

The Coliscan MF method uses the detection of enzymes galactosidase and glucuronidase to identify fecal coliforms and *E. coli*. *Enterobacter aerogenes* and *Klebsiella pneumonia* (now *Raoultella planticola*) are known to produce both of these enzymes under certain laboratory conditions (Kämpfer et al., 1991; Geissler et al. 2000). A study by Alonso et al. (1999) found that some strains of *Enterobacter* and *Klebsiella* produced the glucuronidase enzyme, which was assumed to be exclusively produced by *E. coli* in the Coliscan MF method. Furthermore, *Enterobacter* and *Klebsiella* have been shown to ferment lactose and produce indole in a laboratory study, meaning that the confirmatory reagent used in this study could have elicited a double false positive (Bernasconi et al., 2006).

There is therefore a probability that the previously reported *E. coli* removal rates are better than this study was able to detect. It is likely that improved methodology would have eliminated some CFU counts form the straw-containing controls as well. However, the initial "Str-B" trial indicated that in the absence of a probability of false positives, the *Stropharia* mycelium removed more bacteria than un-inoculated controls. As previously discussed, this data set was unlikely to have false-positives because insignificant levels of bacteria were found in the "pre-flush effluent." This indicated that there was not a native population of false-positive producing bacteria growing on the straw-free media, and thus the bacteria examined during filtration testing truly represented the experimental *E. coli* used in this study.

Research Conclusions

Overall, several conclusions may be drawn from the research results. The first is that there are fungal species that are appropriate candidates for the concept of mycofiltration. Of eight fungal strains that were tested over the course of the research, *Stropharia* clearly demonstrated resiliency to cycles of saturation, drying, heating, and freezing that represent the conditions that would be seen in the field. Furthermore, after undergoing these biological stresses, statistically significant reductions in *E. coli* concentrations were observed, with no significant difference between the resiliency tested and the non-resiliency tested filters (Table 1).

The second conclusion is that *Stropharia* demonstrated a significant capability to remove freely suspended *E. coli* from flowing water. This is significant, as many BMPs achieve bacteria reductions simply by removing sediment-bound pathogens. That the mycofilters demonstrated a capacity for treating free-floating bacteria is novel. Additionally, as with other stormwater BMPs, mycofiltration may be more effective against sediment-bound bacteria, and could possibly achieve 100% *E. coli* removal.

The final conclusion is that a significant number of false-positives were documented when using the Coliscan MF method, and that the presence of straw in the filter media seems to be correlated with these false-positives. The result of these false-positives is that the actual *E. coli* reductions that were achieved are probably higher than reductions documented for trials of straw-containing media evaluated in the Phase I research. The conclusion from this Phase I project is that specific fungal strains are resilient enough to be considered for stormwater treatment applications against a variety of targets including pathogens, but that more research is needed to clearly define treatment design and operating parameters.

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