Use of the Fungus *Stropharia rugosoannulata* in Mycoremediation of Wetland Water Contaminated by Crow Feces

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Abstract

Mycoremediation uses live fungus to clean-up contaminated sites. In this study, water from a wetland polluted by crow feces and known to contain antibiotic resistant (AR) bacteria was subjected to mycoremediation. Two different mushroom species were investigated initially to see if counts of the indicator bacteria *E. coli* changed when wetland water (WW) was passed through 12" columns containing mycelium-inoculated wood chips. Initial run through trials with King Stropharia (*Stropharia rugosoannulata)* mycelium demonstrated 2 hours of residence of water in the columns resulted in 30-50% reductions (n=2), while 24 hours residence resulted in 50- 100% reduction (n=2). Blue oyster (*Pleurotus ostreatus)* mycelium reduced *E. coli* counts (by 50%) only in the 24 hour residence time trial. We, therefore, continued studies with King Strophoria. To quantify the effect of mycelium on *E. coli* and other bacteria, in the absence of other factors present in WW, dechlorinated tap water was spiked with either enumerated *E. coli, Salmonella,* or *Campylobacter*. Spiked water (70 CFU/100 ml) was added to the *S. rugosoannulata* inoculated woodchip column and 10 ml was withdrawn after retention for 3, 6, or 9 hrs in the column. *Salmonella* and *E. coli* showed 100% reduction in CFUs in 6 hrs. At 3 hours *E. coli* showed no change, and Salmonella a 22% reduction in CFUs. Campylobacter was quantified by extracting total DNA from water samples after 3, 6, and 9 hrs, and subjecting to quantitative PCR for determination of gene copy numbers (GCN), when a 32% reduction was observed after 6 hrs. When *S. rugosoannulata* inoculated woodchips were placed in a 55 gallon barrel out in the wetland roost area, and surface runoff was directed through the barrel with no attempt at retention, 0-30 % reduction was observed in *E. coli* CFUs in 3 different trials while Campylobacter showed 41%-47% (n=3) reduction in GCN. Extracted DNA when tested by qPCR for AR genes, showed reduction in ESBL gene, *bla* $_{CTX-M}$,(96-98%), *tetM* (19-78%), *tetA* (5%-30%) , *tet B* (15-46%) and *strB* (39-78%) genes, indicating bacteria other than *E. coli* that contained these genes were affected. These results demonstrate that mycoremediation has the potential to be effective in removal of certain bacteria. Methods to increase residence time are necessary to achieve an effective reduction. Notably, *E. coli* was not a good indicator for this removal.

Introduction and Goals

A large amount of crow feces is deposited throughout the year on the UW Bothell/Cascadia College Campus, especially in the campus wetland which serves as a roost to over 15,000 crows in the peak winter season. We reported earlier that the wetland contains a variety of antibiotic resistant bacteria, with many of them being multidrug resistant (1). Muti-locus sequence typing of the *E. coli* and *Campylobacter* strains indicated wild birds as a primary source for the bacteria.

Many species of fungi have demonstrated the ability to reduce the toxicity of substrates and degrade recalcitrant pollutants. In addition, several species are predators of bacteria and nematodes and it has been documented that they can live using bacteria as their sole nutrient source (2) . *Agaricus bisporus*, an edible mushroom has been shown to degrade both Gram-positive and Gram-negative bacteria by their natural release of several enzymes as well as antibiotic metabolites (3). Use of fungal mushroom for remediation of contaminated sites is called mycoremediation. Our goal was to test whether mycoremediation could reduce the bacterial load and antibiotic resistant genes in the wetlands, a method that was recently reported to be effective for reducing pathogen loads in water (4). The mushroom species King Stropharia , *Stropharia rugosoannulata* , was tested under laboratory conditions and in the field. Spiked water as well as polluted wetland water was examined.

Methods

1. **Preparation of biofilter material:** Sterile red Alder Sawdust /wood chip mix were inoculated with *Pleurotus columbinus or Stropharia rugosoannulata* fungus and allowed to grow at room temperature for at least two months.
2. **Preparation of biofiltration columns:** PVC plastic pipes, 1.5 inch diameter and 1-foot long, were filled with 750 grams of either mycelium-inoculated, or uninoculated alderwood sawdust/woodchip mix (**Figure 3**).
3. **Preparation of spiked synthetic stormwater:** Tap water dechlorinated with sodium thiosulfate (25 mg/L) was spiked overnight with a culture of *E. coli* and Salmonella or C. jejuni (grown for 36-48 hr) at a concentration of 70 CFU/100 ml.
4. **Collection of wetland water (WW):** 500 ml of water was collected in sterile bottles, before and after passage through biofilters **(Figure 2)**
5. **Biofiltration in the lab:** Water was added evenly over the top surface of the biofilter column and allowed to percolate. Inflow samples, 100 ml, were collected at the beginning of each analysis. Outflow water samples (10 ml) were collected in triplicate at various time intervals.
6. **Enumeration of *E. coli* and Other Coliform bacteria in Colony Forming Units (CFU):** Appropriately diluted water samples were filtered onto 0.45 um filter pads. Filter pads were placed onto a petri dish containing an absorbent pad soaked with Coliscan MF medium (Hach) and incubated at 35^0 C for 24 hrs following EPA Method 10029. Blue (*E. coli*) and red (Other Coliform) colonies were counted.
7. **Enumeration of Salmonella:** Appropriately diluted water samples were filtered onto 0.45 um filter pads. Filters were rinsed and the rinsate was placed on Xylose Lysine Deoxycholate (XLD) Agar. Black Colonies were counted.
8. **Enumeration in Gene Copy Numbers (GCN):** GCN was measured in the water samples to investigate reduction in Campylobacter and antibiotic resistance genes. Total DNA were extracted directly from 0.45 μM filters using the Water Master kit (Epicenter). qPCR was performed using the primers and probes listed in **Table 1** for Campylobacter genus, antibiotic resistance genes for tetracycline: tetA, tet B, tetM, streptomycin resistant genes strA, strB, and beta-lactamase genes bla$_{CMY}$ and bla-$_{CTX}$. Controls and standards were generated for quantitative measurements of each of the genes, using PCR amplicons that were cloned into *E. coli* Cells (Topo ® TA Cloning kit, Invitrogen) or used directly.

Setting and Field Experiment Apparatus

Figure 1. Map of the campus and wetland of UW Bothell and Cascadia College. The red dot indicates the location of the sampling site for wetland water used in laboratory experiments as well as where runoff was directed into field mycoremediation systems (see Figure 3). Blue arrows indicate direction of water flow to and from the site. Dashed arrows indicate flow in pipes. The crow roost boundary fluctuates year to year, though the southern portion is relatively stable. Aerial photo from Google.

Figure 2. Photographs of the field mycoremediation systems.
A) The 55-gallon barrel system was half full of woodchips inoculated with King Stropharia mycelium. An inlet and outlet port allowed contaminated water to flow freely through the system. A pipe was used to convey water at a controlled rate and disperse it evenly over the myco-filter media. Results from the 11/18/17 trial are shown in Figure 4. ~7,500 liters of contaminated runoff ran through the barrel over a 24 hour period. B) The gutter system worked in much the same way but with smaller volumes of inoculated woodchips. Results from trials using this system are shared in Figure 5 and Tables 4 and 5.

Laboratory Set-up

Figure 3: Four filter columns on ring stands with the control containing uninoculated wood chips. Wetland water samples, as well as dechlorinated tap water that was spiked with one of three different bacteria species, were run through columns like these. Water was retained in the columns for different lengths of time (see Table 3) before subsamples were collected. Duplicate samples were collected at each time point.

Results from Field Mycoremediation Experiments

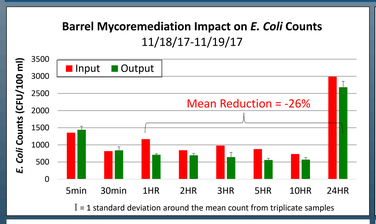

Figure 4. Differences in *E. coli* counts for runoff water before (input) and after (output) flowing through the barrel mycoremediation system at set sampling times.

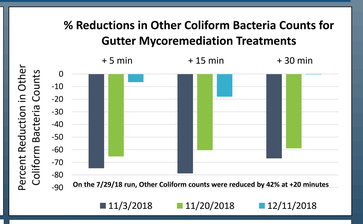

Figure 5. Unlike the barrel system, the gutter system experiments fostered a decrease in Other Coliform bacteria counts instead of *E. coli*.

Primers and Probes

strA	5'- TCAATCCCGACTTCTTACCG	
	5'-CACCATGGCAAACAACATA	
	Hex-TGCTCGACCAAGAGCGGC-BHQ-1	
strB	5' ATCGCTTTGCAGCTTTGTTT	
	5' ATGATGCAGATCGCCATGTA	
	FAM-ATGCCTCGGAACTGCGT-BHQ-2	
tetA	5'-GCTACATCCTGCTTGCCTTTC	SYBR
	5'-CATAGATCGCCGTGAAGAGG	
tetB	5'-TTGGTTAGGGGCAAGTTTTG	
	5'GTAATGGGCCAATAACACCG	SYBR
bla-CMY-2-F	5'CAGACGCGTCCTGCAACCATTAAA	
bla-CMY-2-R	5'TACGTAGCTGCCAAATCCACCAGT	SYBR
	5'GGTTTCTCTTGGATACTTAAATCAATCR	
tetM	5'CCAACCATAYAATCCTTGTTCRC 5' FAM-ATGCAGTTATGGAARGGGATACGCTATGGY-BHQ-1	
CTX-M consensus primer -F	5'-ATGTGCAGYACCAGTAARGTKATGGC	
CTX-M consensus primer-R	5'-ATCACKCGGRTCGCCXGG RAT-3'	
CTX-M-1 group probe	5'FAM-CAGGTGCTTATCGCTCTCGCTCTGTT-BHQ-3'	
CTX-M probe for all groups W/O CTX-M-1	5'-VIC-CGACAATACNGCCATGAA-MGB-NFQ-3'	
Salmonella (invA)		
invA_176F	5'-CAACGTTTCCTGCGGTACTGT	
invA_291R	5'-CCCGAACGTGGCGATAATT	
invA-Tx_208	FAM-CTCTTTCGTCTGGCATTATCGATCAGTACCA-TAMRA	
Campylobacter spp. (16S rRNA)		
Camp-F2	5'-CACGTGCTACAATGGCATAT	
CampR2	5-GGCTTCATGCTCTCGAGTT	
Camp-Probe	5-FAM-CAG AGA ACA ATC CGA ACT GGG ACA BHQ1	

Table 1.

Results from Laboratory Mycoremediation Experiments

Change in *E. coli* and Salmonella Species in CFU/100 ml for Laboratory Mycoremediation Experiments

		E. coli			Salmonella		
	Time (hrs)	Input (CFU)	Output (CFU)	% Removal	Input (CFU)	Output (CFU)	% Removal
Trial 1	0	170			170		
	0.25	170	350	0	170	170	0
	1	270	350	0	150	315	0
	2	275	245	10.9	165	255	0
	16	295	65	77.9	220	15	93.2
		E . coli			Salmonella		
	Time (hrs)	Input (CFU)	Output (CFU)	% Removal	Input (CFU)	Output (CFU)	% Removal
Trial 2	0	445	445	0	435	435	0
	3	360	415	0	225	175	22.2
	6	505	0	100	213	0	100

Table 2. Time indicates how long water resided in sample bottles (input) or the columns (output) before sampling. Longer residence time in the columns results in better reduction of pathogens.

Change in Campylobacter Species in Gene Copy Numbers (GCN) for Laboratory Mycoremediation Experiments

Time (hrs)	Input (GCN)	Output (GCN)	% Removal
0	ND	ND	
3	78	285	0
6	2518.8	1715	31.9
9	29	36.5	0

Table 3. Time indicates how long water resided in the columns before sampling. ND is not detected.

Change in Campylobacter and Salmonella Species in Gene Copy Numbers (GCN) for Field Mycoremediation Experiments

	Trial 1 (7/29/18)			Trial 2 (11/20/18)			Trial3 (12/11/18)		
	Input (GCN)	Output (GCN)	% Removal	Input (GCN)	Output (GCN)	% Removal	Input (GCN)	Output (GCN)	% Removal
Campylobacter (16S rRNA)	4,576.4	2,451.8	46.4	526.6	52.6	47.4	464.0	184.6	39.8
Salmonella (invA)	ND	ND		ND	ND		ND	ND	

Table 4. Input = water sampled from the runoff pipe at RS2 (see Figures 1 and 2). Output = water sampled from the gutter in Figure 2B after passing through the mycoremediation wattle. Water was collected at + 20 mins in the experiment on 7/29/18 and at +15 minutes for the 11/20/18 and 12/11/18 runs.

Change in Antibiotic Resistance Genes in Gene Copy Numbers (GCN) for Field Mycoremediation Experiments

Antibiotic Resistance Gene	Trial 1 (7/29/18)			Trial 2 (11/20/18)			Trial 3 (12/11/18)		
	Input (GCN)	Output (GCN)	% Removal	Input (GCN)	Output (GCN)	% Removal	Input (GCN)	Output (GCN)	% Removal
tet(A)	27,685.3	2,500	91	>1,000	0	100	509,993.1	347,320.2	31.9
te(B)	3,319.4	10.76	100	147,205.9	125,274	14.9	369,778.8	252,070.5	31.8
tet(M)	7,132.6	5,892.2	17.3	407	90.2	77.8	2,567	2,148.7	16.3
strA	ND	ND		ND	ND		ND	ND	
strB	11,197.5	2,464.4	78	17,464.4	10,708.8	39	8,865.7	153.19	98.3
bla-CMY	>1000	0	100	ND	ND	ND	55,505	0	100
blaCTX	439,6094	172,395.3	96	1.5E +08	907,925	100	ND	ND	ND

Table 5. Total DNA extracted from filters was tested with primers in Table 1 using qPCR. All detected antibiotic resistance genes were significantly reduced in number by passage through the mycoremediation system.

Conclusions

1. For *E. coli* and *Salmonella*, a 6 hr retention time was most effective in reducing counts in laboratory experiments.
2. Control trials, where water was passed through woodchips without mycelium, showed increases of *E. coli* counts with increased residence time in lab experiments.
3. For Campylobacter, counts went down overnight even in the spiked water kept on bench top. Laboratory mycoremediation was effective only after 6 hrs of retention.
4. The first field experiment showed fairly consistent reduction (average of 26%) in *E. coli* CFU in water passing through the mycoremediation media between 1 and 24 hrs into the experiment. Subsequent field experiments, using smaller volumes of mycoremediation media and briefer contact times for water passing through the filter, showed reductions in Other Coliform bacteria.
5. AR genes bla-ctx, strB, and tet (B) were consistently reduced following passage through the mycoremediation media, indicating those bacteria that possessed these gene were affected by the biofilter. Free floating genes from degraded bacteria may have also affected the final removal numbers.
6. Both bioretention and biosorption may have been a factor in reductions of bacteria counts in water after passing through mycelium inoculated media.

References

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