DEGRADATION OF HARMFUL BACTERIA IN SIMULATED WASTEWATER AND STORMWATER RUNOFF BY THE WHITE ROT FUNGUS *PLEUROTUS OSTREATUS*

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Bachelors of Science in Plant Science with Distinction

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(From left to right, Anastasia Chirnside, John Paul Harris, Nancy Gregory)

Many thanks from our research team!

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ABSTRACT

Microbial pollutants from non-point sources are a common problem in many watersheds all over the world. Current methods for dealing with these pollutants are limited and recent literature suggests that one of the most common solutions, vegetative or riparian buffers, while initially effective in removing harmful bacteria from runoff, have been found to accumulate coliform bacteria over time. Due to the fact that within those systems there is no intentional mode of bacterial degradation, over time these bacteria subsequently get released back into the environment. However, research in an unrelated field has shown that certain fungi such as *Pleurotus ostreatus* will actively seek out and degrade bacteria *in situ*.

Capitalizing on that fact, the white rot fungus *Pleurotus ostreatus* was analyzed as a potential biocontrol agent used to reduce the amount of coliform bacteria in simulated wastewater and storm water runoff. On water agar plates, *Pleurotus ostreatus* was seen to actively search out bacterial colonies, invade them and consume them within 72 hours. Based on this principle, biocell reactors (BCR) were used to determine the effectiveness of spent mushroom compost (SMC) containing *Pleurotus ostreatus* to reduce the concentration of *Escherichia coli* in simulated wastewater and stormwater runoff. Loading was based on a 2" rainfall event over 24 hours at a fixed contamination level (bacterial concentration of 1×10^4 cells/mL). Overall *E.coli* concentrations in the effluent of the reactors containing live compost showed higher *E.coli* concentrations compared to the dead controls during the first 24 hours. After the first 12 hours however, the overall concentrations in the live reactors began to decrease while the concentration in the dead control began to increase. After allowing the reactors to rest for 24 hours and simulating a subsequent uncontaminated rain event, the *E.coli*

concentration in the dead controls increased exponentially while the overall concentrations in the live reactors continued to decrease. The simulated wastewater effluent treatments, while having the lowest total concentration of *E.coli*, did not decrease over time. This suggests that the presence of the live fungus kills the adsorbed *E.coli* and that nutrient concentrations may play a significant role in the level of predation observed.

Chapter 1

INTRODUCTION

1.1 Background Information

In 2010 the Environmental Protection Agency (EPA) proposed a revision to the Total Coliform Rule (TCR), a national primary drinking water regulation, that would "require public water systems that are vulnerable to microbial contamination to identify and fix problems, and establish criteria for systems to qualify for and stay on reduced monitoring, thereby providing incentives for improved water system operation." It is not surprising that these regulations would affect the largest estuary in the United States, the Chesapeake Bay.

With approximately one-third of the Delmarva Peninsula's land being used for agriculture, the EPA has quickly focused on local farmers and, in some cases, even residential areas as point sources for coliform contamination. If individuals are unable to meet the demands to reduce their total coliform emissions, they can face further mandates, litigation and fines. While there are current methods for meeting these specifications today, many can be considered expensive, unsightly or invasive by those being forced to implement them. Of the methods proposed, vegetative buffers or filter strips are one of the most common ways of preventing contaminated runoff from reaching surface waters. Primarily, these vegetative or riparian buffers are used to reduce the amount of sediment and nutrients entering the watershed. While the literature suggests that these buffers can be effective in reducing the total amount of

coliform found in runoff, recent studies have shown that these vegetative buffers can reach a maximum loading point that, once reached, converts the buffer system into a breeding ground for coliform bacteria and other potential pathogens (Fox 2011).

Given the limitations on current best management practices (BMPs), this study investigated the potential of a relatively new biotechnology which may be used improve the current standards. More efficient, cheaper and greener options of water treatment are not only better received by the consumers, but are also more likely to be implemented. In this investigation, the white rot fungus *Pleurotus ostreatus* was analyzed as a potential biocontrol agent used to reduce the amount of coliform bacteria in simulated wastewater and storm water runoff.

1.1.1 *Pleurotus ostreatus* and its current applications

More commonly referred to as the oyster mushroom, *P. ostreatus* is grown in large quantities across the globe in commercial mushroom farms as a culinary crop. In nature, these fungi are found all over the world as decomposers affecting felled trees and detritus on the forest floor using a set of ligninolytic extracellular enzymes to break down the lignin found in these decaying plant tissues. It is because of these enzymes that *P. ostreatus* and the other white rot fungi have gained recent praise in wastewater treatment for their ability to degrade petroleum and aromatic hydrocarbons such as pyrene, anthracene, and phenanthrene (Aggelis *et al*., 2003). While the mechanisms are not fully understood, this cocktail of enzymes cleaves recalcitrant aromatic rings presumably through the production of epoxide rings to facilitate a more complete decomposition of the compound in question. However, there is also evidence to support that these fungi and the genus *Pleurotus* in particular, may be able to further improve water quality by capturing and degrading harmful bacteria.

1.1.2 *Pleurotus* predation of nematodes and bacteria

While screening a wide variety of wood rotting fungi, Barron *et al* (1992) discovered that *P. ostreatus* was capable of killing and digesting nematodes. Additionally, they also discovered that bacteria shed from these nematodes and the resulting colonies were also being targeted and consumed. Bacteria were then artificially streaked across a water agar plate pre-inoculated with *P. ostreatus* and similar results were observed. Under low nutrient conditions very fine directional hyphae of *P. ostreatus* have been seen to actively search out and enter nearby micro-colonies of bacteria. These hyphae ramify within the colony and form haustoria-like branches of specialized cells that secrete lytic and other enzymes capable of destroying and digesting the bacteria (Barron, 2003). Predations of specific species of bacteria have not been described and the potential for *P. ostreatus* to degrade some of the more problematic bacteria such as *Escherichia coli* has yet to be explored until now.

Chapter 2

METHODS

2.1 Culture maintenance

The *Pleurotus ostreatus* strain HK35 obtained from Phillips Mushroom Farms (Kennet Square, PA) was maintained on Millers 2% malt agar plates and transferred as needed. An unregistered laboratory strain of *Escherichia coli* donated and verified by the University of Delaware Microbiology Laboratory was obtained on both Trypticase soy agar and Eosin methylene blue agar. These cultures were maintained on Miller's Luria Broth agar plates.

2.2 Predation study

A flame sterilized 7-mm cork bore was used to aseptically transfer mycelium plugs from 2% malt agar plates onto water agar plates for the purposes of the predation study. These water agar plates were then allowed to incubate at room temperature for 72 hours. After the 72 hour incubation time, 200uL of a $1x10^4$ cells/mL suspension of *E.coli* in sterile deionized distilled water (DDW) was then spread over top of the previously inoculated water agar plates. These were allowed to incubate for 72 hours and were then imaged using a Zeiss LSM 510 VIS confocal microscope. Images were taken with the C- Apochomat 40x/1.2 W corr UV-VIS-IR objective without fluorescence. The configuration consisted of a plane scan with a stack size of 1024x1024x1, and an HFT 405/488/561 primary dichroic at a wavelength of 488nm and power of 4.2%. These images were taken using only transmitted light.

2.3 Packed-bed reactor (PBR)

2.3.1 Media preparation and inoculation

Polystyrene foam was cut into cut into 1cm^3 cubes (density= 264.08 kg/ m^3) and the resulting cubes were then weighed into eight-1.5g aliquots. These aliquots were then placed in sterile 250mL Erlenmeyer flasks filled with 50mL of 2% malt broth. The cubes were then aseptically depressed with a sterile glass rod to fill the pores of the cubes with broth. Half of these aliquots were then inoculated with *P. ostreatus* using a flame sterilized 7 mm cork borer. These flasks were then incubated in an unlit incubator at room temperature for 7 days and were gently shaken by hand every 24 hours.

Several suspensions of *E.coli* were made in 20 mL of sterile DDW and the concentration was counted using a hemocytometer. Cell concentrations of the suspensions were recorded and aliquots from these suspensions were combined with sterile DDW to create a 4 L stock solution with concentration of 1 x 10^4 cells/mL. This stock solution was then used as the primary influent for the packed-bed reactor.

2.3.2 Reactor Setup

Two glass, jacketed column bioreactors (PBR) as described in Chirnside *et al.* (2011) were used in this experiment (Figure 1). The length of the PBR measured 210 mm with an inside diameter (ID) of 44 mm. A small aquarium pump complete with a 0.2 micron filter was connected to an inlet in the bottom of the PBR. Two 4 L Erlenmeyer flasks served as influent reservoirs and were connected to a second inlet at the bottom of the reactor. At the top of the PBR another port was connected to an overflow chamber that maintained a constant working volume of 200 mL and provided a reservoir for the recycle flow. This recycled effluent was pumped back into

the PBR at twice the influent rate (2:1 recycle ratio). Adjacent to that a small piece of Tygon® tubing was connected to the remaining port for sample collection.

Influent was pumped into the reactor at a rate of 0.347 ml min^{-1} using a low flow Masterflex® C/L dual channel, variable speed peristaltic pump (Model 77120-30). Recycle flow was also pumped into the PBR using a second low flow Masterflex® C/L dual channel, variable speed peristaltic pump (Model 77120-30) at a rate of 0.694 ml min^{-1}. This produced a hydraulic retention time of approximately 8 hours. One-way flow valves were used on all tubing (microbore tubing - 2.79 mm ID) to avoid backflow. Sterilized, flexible, clear plastic, laboratory tubing (Tygon® R-3606) was used to connect the aquarium pump to the reactor. The entire apparatus was autoclaved before placement of the foam cubes. After loading the PBR, heated water was pumped through the 10 mm thick outer jacket to maintain a constant temperature of 37º C.

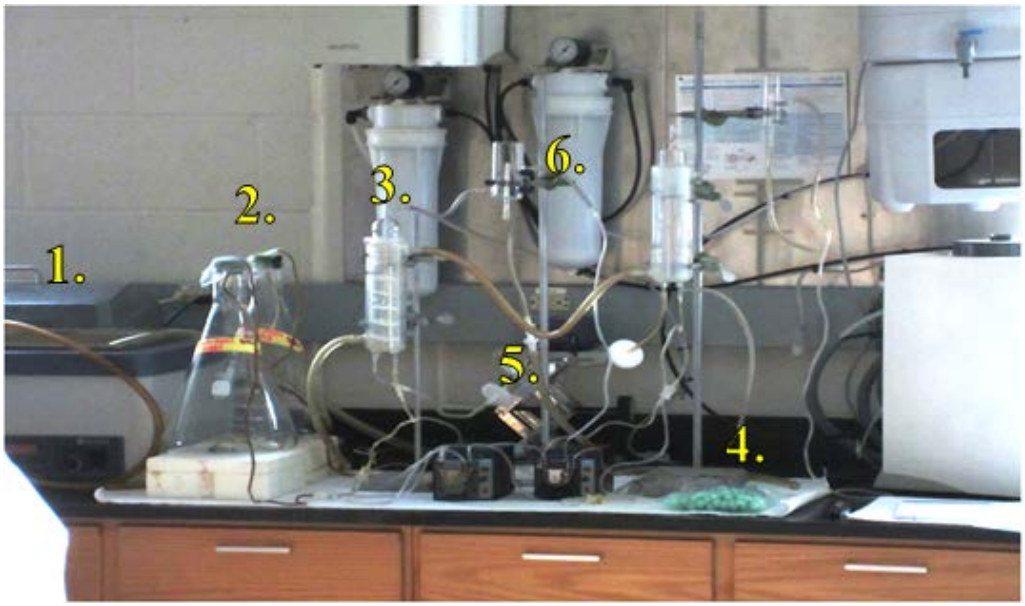

Figure 1. Packed-bed bioreactor setup. A heated water bath (1) warms the reactor chamber (3). This chamber is then filled with inoculated media, in this case foam cubes (4) and the influent is pumped into it through the reservoir tanks (2).The solution is simultaneously aerated by small aquarium pumps fitted with .2 μm filters. Two peristaltic (5) pumps provide the influent and effluent flow. The column on the far right (6) allows for recycle flow.

2.3.3 Reactor loading and operation

The reactor chambers were first loaded with a small mesh plastic disk (radius 21.5 cm) connected to a thin nylon line. On top of this plastic mesh disk, 1 aliquot of foam cubes was applied. The control run for this experiment contained foam cubes that had been soaked in nutrient solution, but were without fungus. A secondary run was made with the inoculated foam cubes. Following the first addition of foam cubes, a 1cm high polystyrene foam disk with a radius of 22 cm was threaded onto the nylon line. A final aliquot of foam cubes was applied on top of this foam disk and another foam disk was threaded onto the end of the nylon line.

Afterwards, the glass lid was connected to the top of the reactor chamber and pumping was initiated. Pumps were left to fill the entire reactor chamber with influent over the course of about 8 hours. Influent and effluent *E.coli* concentrations were monitored by both hemocytometer count and plate dilutions over the course of 5 days.

A second 5-day run was completed with the exact same parameters with the exception of eliminating the *P. ostreatus* colonized polystyrene cubes and instead using the cubes that had not been inoculated. This served as an inert control.

2.3.4 Sampling Protocol

Effluent samples and stock samples (20 mL) were collected every 24 hours and were immediately processed. Dilutions of 10^2, 10^3, 10^4 and 10^5 were made of each sample and 100 μL of each dilution were pipetted onto LB agar plates. These were incubated at room temperature for 72 hours and the remainder of the undiluted samples were then stored in the refrigerator for nutrient analysis.

2.4 Biocell Reactors (BCR)

2.4.1 Reactor set up and operation

Biocell reactors were made using eight-64 oz plastic Tupperware containers. One hole was drilled in the bottom of each container proximal to one end to allow for effluent collection. A plastic fitting was then attached with 100% silicon adhesive to allow for the attachment of a length of tubing (Tygon® R-3606). Another hole was drilled in the side opposite the previous hole towards the top of the container allowing the attachment of the tubing that provided the influent. The BCRs were then filled with filter media consisting of either live or killed (autoclaved) mushroom compost.

Influent was provided by the same peristaltic pumps as mentioned in the methods for the packed-bed reactor. Each peristaltic pump was responsible for supplying influent to two different reactors at any given time. Flow rates modeled a 2" rain event over the course of 24 hours providing a flow rate of 0.794 mL min^{-1}. Containers were then placed underneath the BCR to catch the effluent (Figure 2).

Figure 2. Biocell reactor setup. Reactor chambers R1-R6 (left to right) are filled with *P. ostreatus* inoculated mushroom compost. Influent is being supplied via three peristaltic pumps from the large Erlenmeyer flasks behind the reactors. Effluent is being collected in the bottles towards the bottom of the picture.

Pumps were connected to a given stock solution, switched on at t=0 hours (h), and allowed to run for 24 hours. At t=24 h the pumps were turned off and the reactors were allowed to "rest" for another 24 hours. During this time the stock solution containers were washed, autoclaved, filled with sterile DDW and re-connected to the reactors. At t=48 h, 24 hours after the first run, the pumps were switched on to simulate another 24 hour rain event without the *E.coli* load.

2.4.2 Media and stock preparation

2.4.2.1 Experiment 1: Live media and dead control

E.coli stock solutions were made using the methods previously described, but instead of making the of 1 x 10^4 cells/mL stock solution with sterile DDW, it was

made using a 0.1 M solution KH_2PO_4, $NaHPO_4$ (pH 6.7). A separate 3 L stock solution was made for each peristaltic pump which in turn supplied bacterial suspension to two (2) BCR.

Spent mushroom compost (SMC) was obtained from Phillips Mushroom Farms containing *P. ostreatus* strain HK35. After homogenizing the media, 500 g of live spent mushroom compost were loaded into three of the 6 BCR. This set of reactors (R1-R3) represented 3 replicates of Treatment 1 (T1) consisting of a 24 hour 2" rain event containing buffered *E.coli* solution draining through the live SMC. The other three BCR were filled with 500 g of mushroom compost that had been autoclaved at 121° C for 20 minutes to kill any native microorganisms including *P. ostreatus*. This set of BCR (R4-R6) represented 3 replicates of Treatment 2 (T2) consisting of a 24 hour 2" rain event containing buffered *E.coli* solution draining through the dead control (autoclaved SMC).

After the first rain event, all PBR were allowed to "rest" for 24 hours. Following the rest period, all PBR were treated with another 24 hour 2" rain event containing sterile DDW draining through both T1 and T2 BCR.

Another subset of SMC was used to measure both percent moisture (gravimetric; Klute, 1996) and pH (Klute, 1996). To determine the nutrient composition of the compost, five solid samples were sent to the University of Delaware Soil Testing Lab for analysis.

2.4.2.2 Experiment 2: Nutrient control and simulated wastewater effluent

All six BCR were filled with 500 g of live mushroom compost. The first three BCR (R1-R3) represented Treatment 3 (T3), consisting of a 24 hour 2" rain event containing sterile DDW draining through the live SMC serving as a live control for the

overall system. The other three BCR (R4-R6) represented Treatment 4 (T4), consisting of a 24 hour 2" rain event containing a simulated wastewater effluent spiked with *E. coli* at a concentration of 5.19 x 10^3 cells/mL draining through live SMC. The simulated wastewater used was that of Koh *et al*. 2012 consisting the following (per L): 121.7mg NH_4Cl, 9 mg KH_2PO_4, 1368.4 mg CH_3COONa and 1 mL of a trace element stock containing 10 g/L $CaCl_2*H_2O$, 8 g/L $FeCl_3*6H_2O$, 5g/L $MgSO_4*7H_2O$, 2g/L $CoCl_2*6H_2O$, 2g/L Thiamine HCl, 1g/L $NaSiO_3*9H_2O$, 0.55g/L $Al_2(SO_4)*16H_2O$,. 0.05g/L $MnCl_2*2H_2O$, 0.001g/L $(NH_4)_6Mo_7O_{24}*6H_2O$, 0.001g/L $ZnSO_4*7H_2O$, 0.001g/L H_3BO_4 and 0.001g/L $CuSO_4*5H_2O$

2.4.3 Sampling Protocol

Samples were taken from the stock solution at t=0 h and every 12 hours (h) afterwards except during the "rest" period. Effluent sampling began at t=12 h and also continued every 12 hours thereafter with the exception of the "rest" period. Dilutions were immediately made from these samples and *E. coli* concentrations were determined by the membrane filter technique (SMWW 9222D; Standard Methods for the Examination of Water and Wastewater [Greenberg *et al*., 1992]). The diluted samples were filtered using a 0.02 µm filter. These filters were then plated onto Millipore brand M-FC broth with rosolic acid and allowed to incubate at 44C° for 24 hours. Plates were then stored for no more than 24 hours at 4C° before viable cell counts were made.

Every 24 hours, the total daily effluent from each reactor was collected and stored at 4° C for up to seven days before being processed by the University of Delaware Soil Testing Lab. Parameters tested included pH, electrical conductivity, ammonium, nitrates and trace elements. Additionally, the samples were analyzed for

Total Kjeldahl Nitrogen (TKN) by the Bioresources Engineering Soil and Water Quality Lab (Semi-Micro-Kjeldahl Method 4500-Norg C; SMWW, 1992). This protocol was utilized for both Experiment 1 and Experiment 2.

Chapter 3

RESULTS

3.1 Predation study

Microcolonies of *E.coli* formed over the inoculation site between 24 and 48 hours after inoculation of the water agar plates containing *P.ostreatus*. After 72 hours, the plates showed fine lateral hyphae of *P.ostreatus* branching off from the larger hyphae and ramifying into tight masses of finely branched nodular coralloid hyphae around bacterial colonies (Figure 3). These structures resemble haustoria and can be composed of one or more of these fine directional hyphae. The observed *E.coli* colonies were completely degraded within 72 hours leaving only these haustoria-like predation structures. After this degradation, new hyphae were not produced from the predation site. The hyphae that composed the predation structure did not continue to grow, branch out, or produce additional directional hyphae after the degredation of the bacterial colony. However, the hyphae that produced the directional hyphae forming the predation structure did continue to grow. Size and quantity of these predation sites correlated to the size and number of the *E.coli* colonies in the area (Figure 4).

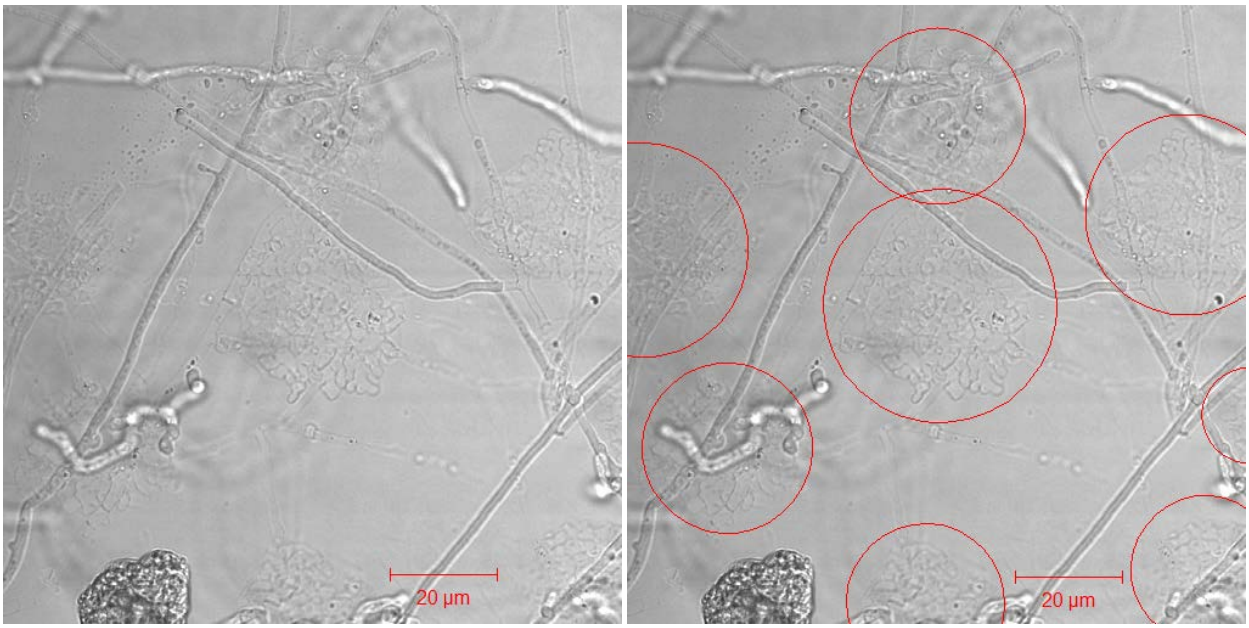

Figure 3. *Pleurotus ostreatus* predation structures. These two identical photos show predation structures left behind by *P. ostreatus* after degrading colonies of *E.coli*. The predation structures are circled in red. Images were taken at 400x with a 2x digital zoom.

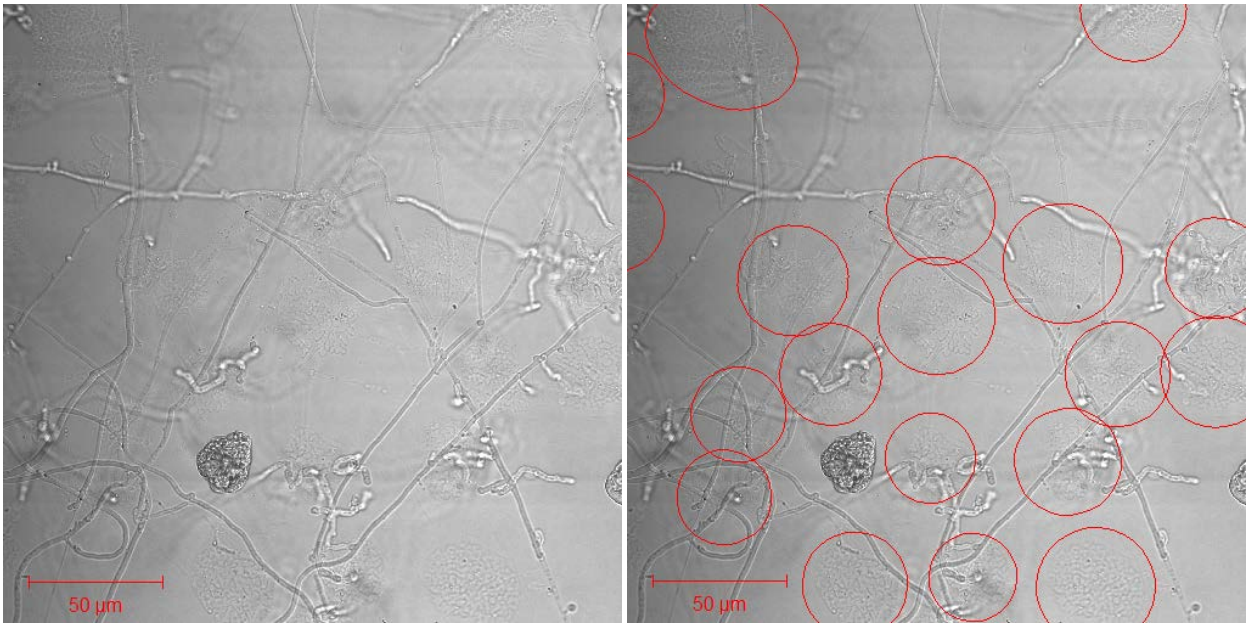

Figure 4. *Pleurotus ostreatus* predation structures. These identical images show the density of the predation structures at 400x and a digital zoom of 1. Field of view in these images is 5.39 cm^2 (230 µm x 230 µm).

3.2 Packed-bed Reactor

The influent reservoirs of the packed bed reactors (PBR) did not maintain a constant or predictable concentration of *E.coli*. This affected the reactor loading and produced inconsistent results across both reactors during both trials. The initial control run containing foam cubes soaked in 2% malt broth (no fungus) faced several issues including stock solution die off and a mechanical failure within the first few hours. After starting the PBR, the influent intake breached the surface of the Reservoir 1, causing air to be pumped into the PBR instead of *E.coli* solution.

Despite inoculating the PBR to a concentration of 1.0×10^4 cells/mL, after 24 hours no *E.coli* was detectable in the reservoir. Inoculation did occur as the effluent showed nearly a ten thousand fold increase from the initial inoculation concentration. The water within the reactor became turbid and stayed turbid for 24 hours following the initial reservoir inoculation. After that, however, *E.coli* were no longer seen in the effluent (Table 1.).

Table 1. Packed-bed reactor control– E.coli concentrations (CFU). Foam cube control containing un-inoculated foam cubes soaked in 2% malt broth. E.coli counts measured in colony forming units (CFU) from LB agar plates every 24 hours.

	Packed-bed reactor 1		Packed-bed reactor 2	
	Influent *E.coli* (CFU)	Effluent *E.coli* (CFU)	Influent *E.coli* (CFU)	Effluent *E.coli* (CFU)
Day 1	0.00E+00	9.26E+07	0.00E+00	9.04E+07
Day 2	0.00E+00	8.91E+07	0.00E+00	4.50E+07
Day 3	0.00E+00	0.00E+00	0.00E+00	0.00E+00
Day 4	0.00E+00	0.00E+00	0.00E+00	0.00E+00

After being autoclaved and packed with fresh inoculated cubes the reactors were run again. Reservoir 1 did not show any *E.coli* present via hemocytometer count or viable cell count while plate counts from Reservoir 2 did show *E.coli* in the stock solution after 24 hours. The concentration in Reservoir 2 had elevated to over ten times the original inoculation concentration, reaching a peak concentration of 1.0×10^6 on day 3. The effluent from both PBR showed numbers slightly lower than those of the control (no fungal inoculant) but still much higher cell counts than that of the stock solution and inoculant. (Table 2).

Table 2. Packed-bed reactor – Inoculated cubes. Foam cubes soaked in 2% malt broth were inoculated with *P. ostreatus*, allowed to grow for 7 days and loaded into the reactor. *E.coli* counts measured in colony forming units (CFU) from LB agar plates every 24 hours.

	Packed-bed reactor 1		Packed-bed reactor 2	
	Influent *E.coli* (CFU)	Effluent *E.coli* (CFU)	Influent *E.coli* (CFU)	Effluent *E.coli* (CFU)
Day 1	0.00E+00	4.40E+07	0.00E+00	6.60E+06
Day 2	0.00E+00	7.22E+07	2.00E+05	2.01E+07
Day 3	0.00E+00	1.14E+08	1.00E+06	2.60E+07
Day 4	0.00E+00	7.70E+07	1.20E+05	1.25E+08

3.3 Biocell Reactors

3.3.1 *E.coli* concentrations

Using the buffer solution instead of sterile DDW dramatically increased the stability of the *E.coli* concentrations within the stock solution. By t=12 h, the *E.coli*

concentration equilibrated at approximately 3.25 x 10^3 CFU for the remainder of the experiment.

For Experiment 1, *E.coli* concentrations in T1 effluent (Live BCR, R1-R3) with the exception of R2 showed higher *E.coli* concentrations compared to T2 (dead controls, R4-R6) during the first 24 hours (1.18 x 10^7 vs. 5.89 x 10^3 ave CFU). After the first 12 hours, however, with the exception of R2, the concentration in T1 effluent began to decrease while the concentration in T2 increased (Figure 5). Following the 24 h dry period and subsequent sterile DDW rain event, the *E.coli* concentration in T2 increased exponentially from 5.26 x 10^5 to 2.5 x 10^7 ave CFU. However, the concentration in T1 BCR, with the exception of R3, continued to decrease from 1.18 x 10^7 to 1.81 x 10^6 ave CFU.

For Experiment 2, the *E.coli* concentrations in T4 effluent (Live BCR, *E.coli* in SSW) rose after the first 24 hours and continued to rise (5.17 x 10^3 to 3.79 x 10^6 Ave CFU/ mL; Figure 6) . Like Treatments 1 and 2, the *E.coli* concentrations in the T4 effluent decreased after 60 h, with the exception of reactor 1 (Figure 6**).** There was no *E. coli* found in any of the samples from T3 in which sterile DDW was loaded through live SMC.

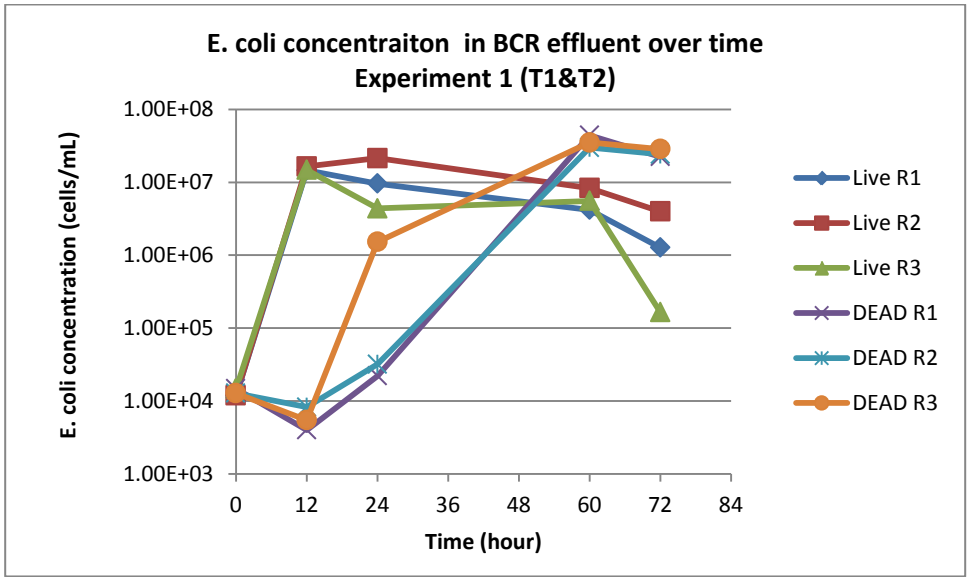

Figure 5. Change in *E coli* concentration in BCR over time for Experiment 1 (semi-log plot). Initial concentration at t=0 is that of the influent reservoir stock solution (1.0 x 10^4 cells/mL). The gap in sampling points between 24 and 60 h denotes the "rest" period when no runoff was applied to the BCR.

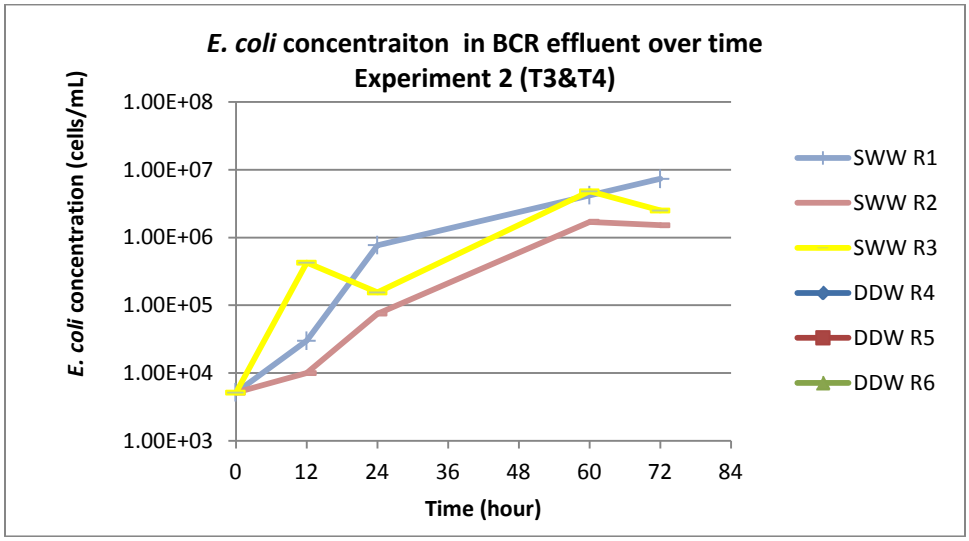

Figure 6. Change in *E.coli* concentration in BCR effluent over time for Experiment 2 (semi-log plot). Initial concentration at t=0 is that of the influent reservoir stock solution (5.16 x 10^3 cells/mL). The gap in sampling points between 24 and 60 h corresponds to the "rest" period when no runoff was applied to the BCR.

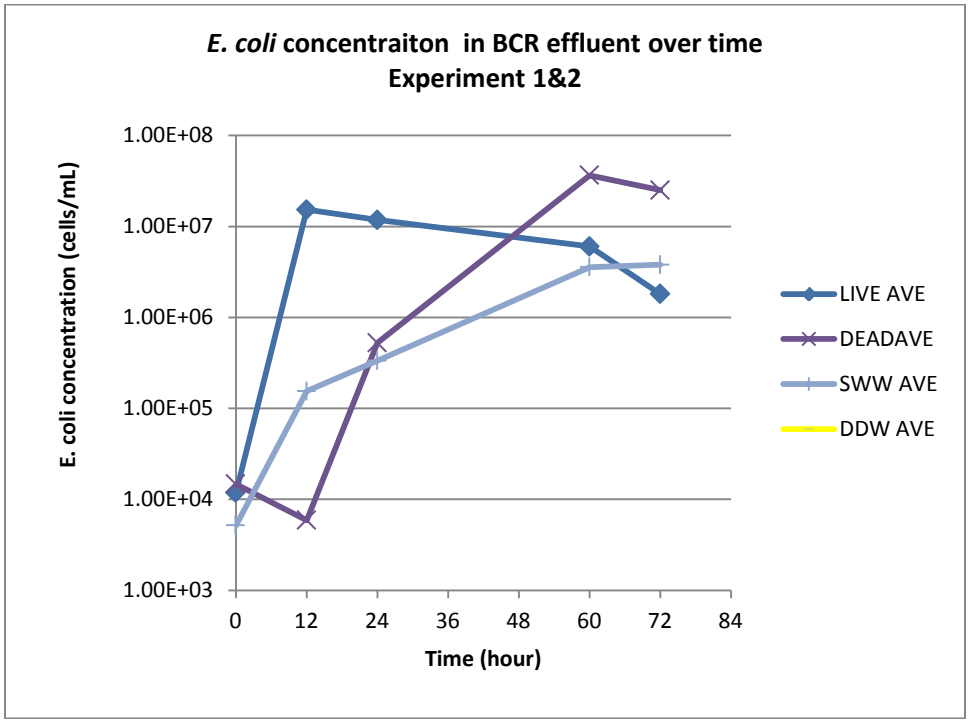

Figure 7. Change in *E coli* concentration in BCR effluent over time for Experiment 1 & 2 (semi-log plot). Initial concentration at t=0 is that of the influent reservoir stock solution. The gap in sampling points between 24 and 60 h corresponds to the "rest" period when no runoff was applied to the BCR. No *E. coli* was found in T3 (DDW AVE) effluent.

3.3.2 pH and nutrient analysis of effluent

3.3.2.1 pH in the effluent

The pH increased over time in the effluent for all treatments (Figure 3.6). However, values did not increase above the pH measured in the simulated wastewater. The electrical conductivity (mmhos/ml) decreased over time in the effluent of all treatments (Figure 9). The EC measured 0.79 mmhos/mL in the effluent of the live control treatment (T3) where DDW was flushed through the BCR. For all other treatments, the EC within the effluent decrease to values close to T3 within 72 h.

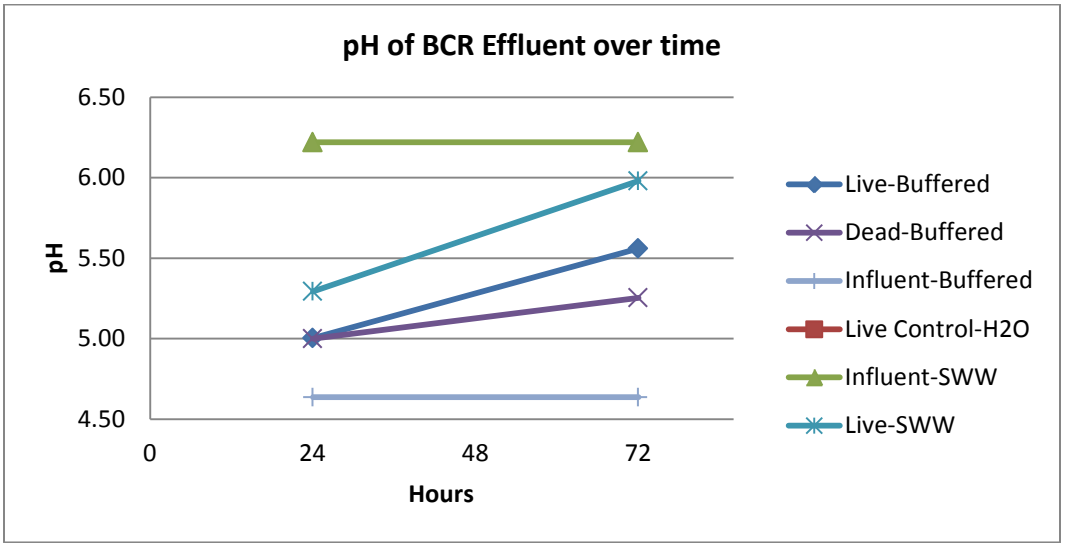

Figure 8. Change in pH in BCR effluent over time. Data points represent average of 3 replications per treatment.

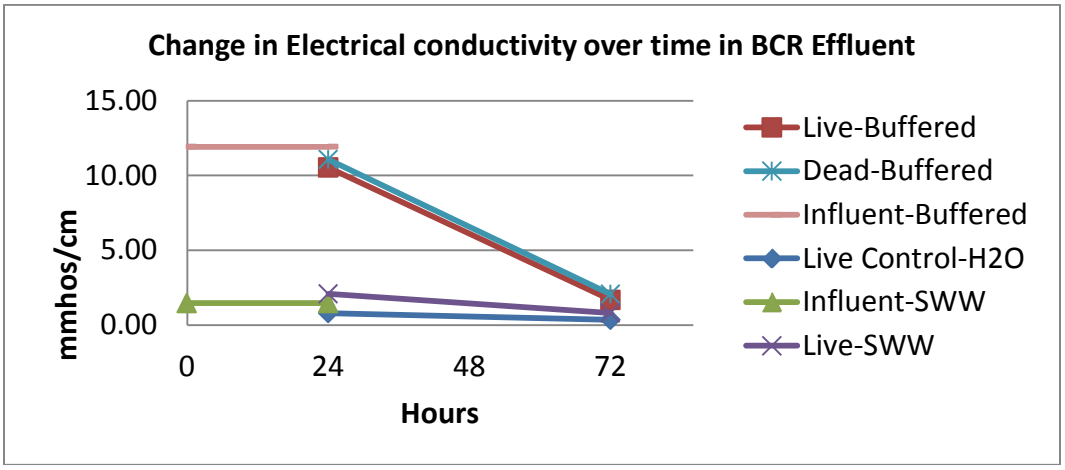

Figure 9. Change in Electrical conductivity in BCR effluent over time. Data points represent average of 3 replications per treatment.

3.3.2.2 Nitrogen in the effluent

The Total Kjeldahl Nitrogen (TKN) in the effluent at t= 24 h and t=72 h for the BCR treated with only sterile DDW was 43.37±5.58 and 29.92±10.54 mg/L, respectively. This represented a 31% decrease in total nitrogen concentration between the 24 and 72 hour sampling. The amount of TKN in the effluent of T1 (Live-

Buffered) BCR was slightly lower than that found in the effluent of the Live-Control (T3). The amount of TKN found in the effluent from the Dead-Buffered control (T2) showed an overall increase in leachable nitrogen. These differences however were not found to be significant. The amount of TKN in the effluent from the BCR treated with simulated wastewater (T4) did not differ significantly from the other treatments; however, there was great variability among the replications that may be due to the large amounts of particulate matter found in the effluent (Table 3).

Table 3.Total Kjeldahl Nitrogen (TKN) levels in BCR effluent: The total N value in R4(Dead-Buffered) at the 72 hour mark and R3(Live-SWW) at the 24 and 72 hour mark were found to be statistical outlier due to the particulate matter contributing to the nitrogen reading.

	24 Hours		72 Hours	
	TKN (ppm):	Particulate	TKN (ppm):	Particulate
R1 (Live-Buffered)	24.78	No	18.59	No
R2 (Live-Buffered)	34.08	No	23.23	No
R3 (Live-Buffered)	40.72	No	37.18	No
R4 (Dead-Buffered)	52.66	No	209.11	**Yes**
R5 (Dead-Buffered)	47.46	No	37.18	No
R6 (Dead-Buffered)	86.74	No	43.37	No
R1 (Live-Control)	44.92	No	26.33	No
R2 (Live-Control)	37.18	No	21.69	No
R3 (Live-Control)	48.02	No	41.82	No
R1 (Live-SWW)	34.077	No	15.49	No
R2 (Live-SWW)	46.469	No	54.21	No
R3 (Live-SWW)	972.753	**Yes**	784.23	**Yes**
Live-Buffered Average:	33.19 ± 8.01		26.33 ± 9.68	
Dead-Buffered Average:	62.29 ± 21.34		96.55 ± 4.38	
Live-Control Average:	43.37 ± 5.58		29.95 ± 10.54	
Live-SWW Average:	40.273 ± 8.762		34.850 ± 27.379	

Inorganic nitrogen concentrations found in the effluent of all treatments were low compared to TKN concentrations indicating that organic nitrogen was the major form of nitrogen found in the effluent. Even though the SWW influent solution contained elevated NH_4-N concentrations (43.38 mg/L), the live PBR was able to reduce the amount by 35% during the first 24 hours (28.13mg/L).

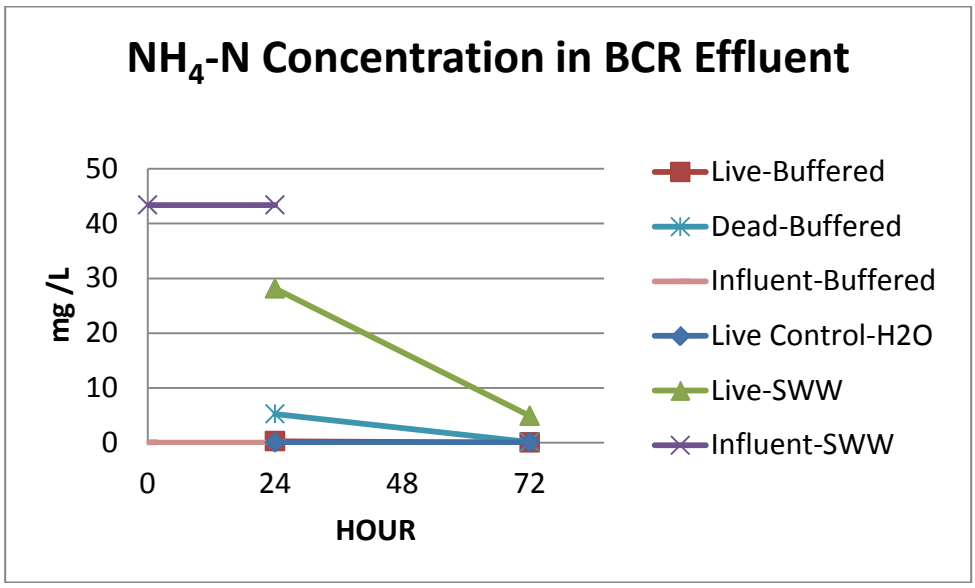

Figure 10. Change in NH_4-N concentration in BCR effluent over time. Data points represent average of 3 replications per treatment.

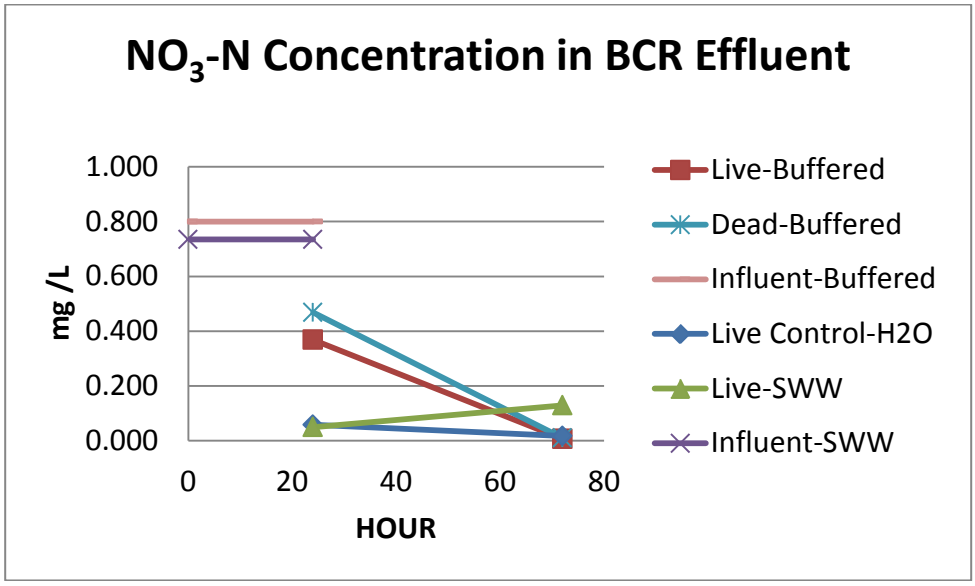

Figure 11. Change in NO_3-N concentration in BCR effluent over time. Data points represent average of 3 replications per treatment.

3.3.2.3 Micronutrients in effluent

Within the first 24 hours of a non-contaminated rain event (T3, Exp.2), the spent mushroom compost (SMC) leached a variety of nutrients into the effluent listed below in table .

The total amount of nutrients leached from each of the reactors is listed below in Table 4.

Table 4. Total amount of nutrients leached from BCRs over 72 hours. Units were rounded to the nearest hundredth.

Sample	Al	As	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Pb	S	Zn	NH4-N	NO3-N
ID	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
Live buffered R1	0.32	0.04	0.31	68.95	0.01	0.00	0.48	3621.21	58.20	0.57	2450.10	6148.80	0.00	18.60	0.12	0.75	0.04
Live buffered R2	0.10	0.04	0.41	87.68	0.01	0.01	0.40	3828.75	68.44	0.75	2544.90	6471.40	0.00	20.87	0.10	0.00	0.31
Live buffered R3	0.31	0.05	0.33	66.91	0.01	0.01	0.37	4049.94	54.94	0.51	2408.00	6464.10	0.00	21.19	0.14	0.00	0.44
Dead buffered R1	0.32	0.09	0.78	105.86	0.01	0.01	0.59	4381.40	113.72	1.13	2398.70	6629.50	0.00	41.67	0.25	6.98	0.36
Dead buffered R2	0.04	0.09	0.46	58.33	0.01	0.00	0.39	4423.17	52.11	0.54	2431.60	6829.30	0.00	23.03	0.17	2.07	0.54
Dead buffered R3	0.41	0.07	0.73	105.98	0.01	0.00	0.38	4384.41	111.61	1.02	2203.40	6607.50	0.00	41.40	0.22	6.96	0.51
Live Control R1	0.24	0.07	0.28	15.78	0.01	0.01	0.23	313.76	12.65	0.19	12.63	3.00	0.00	19.12	0.04	0.00	0.01
Live Control R2	0.01	0.09	0.49	28.92	0.01	0.02	0.28	506.00	23.45	0.33	14.20	4.33	0.00	31.16	0.07	0.16	0.08
Live Control R3	0.37	0.08	0.42	25.57	0.01	0.01	0.33	479.58	21.65	0.37	13.44	2.68	0.00	35.82	0.08	0.00	0.12
SWW R1	0.06	0.11	0.56	41.12	0.01	0.02	0.48	659.22	39.42	0.38	248.77	19.88	0.00	39.63	0.11	26.25	0.08
SWW R2	0.12	0.15	0.65	48.61	0.01	0.02	0.59	800.31	45.83	0.41	281.30	51.02	0.00	45.67	0.12	36.06	0.27
SWW R3	0.49	0.11	0.48	32.28	0.01	0.02	0.57	550.48	32.47	0.32	275.31	46.81	0.00	34.45	0.09	36.75	0.18

There was no input of both K and Na into the effluent of T3 from the SMC. However in T1 and T2, where influent salt concentrations were high, the concentrations of Na and K were reduced to control levels within 72 h.

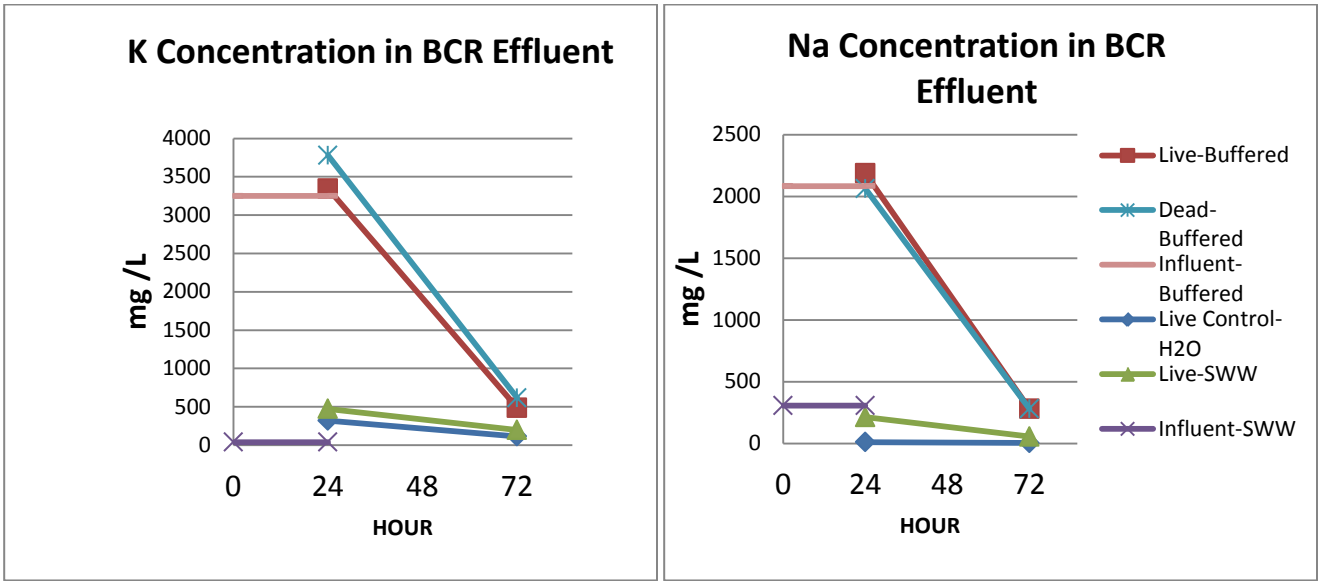

Figure 12. K and Na concentrations in the BCR Effluent. The high K and Na concentrations in present in the buffered solutions decreased close to those of the control runs after 72 hours.

For most of the nutrients measured in the effluent, the amount of nutrients leached from the SMC after 72 h decreased by over 50% of their original values measured at 24 h (Appendix A2 – A4) many of which fell below detection limits. The only element that did not was aluminum which actually increased over time.

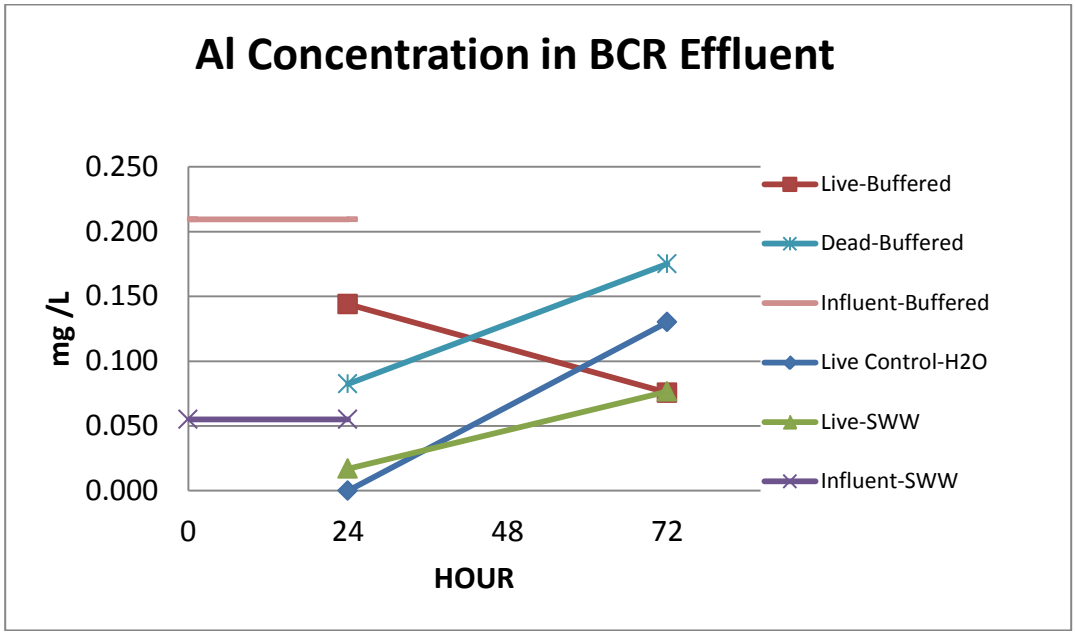

Figure 13. Aluminum concentration in BCR Effluent over time. Aluminum was the only element to consistently increase (with the exception of the live-buffered treatment) over time.

Chapter 4

DISCUSSION

4.1 *Pleurotus ostreatus* as a predator of bacteria.

The early work of Barron (1993) established that *Pleurotus ostreatus* did in fact predate on both nematodes and bacteria colonies, but this had only been confirmed under low nutrient conditions. It was speculated that this behavior evolved from the fungi's need for supplementary nitrogen due to its ligninolytic diet being so rich in carbon. Based on this information Barron suggested that many fungi were most likely not simply saprobes, but were instead facultative parasites. Using these findings as a guide, we sought to replicate Barron's experiments and investigate these biological processes in hopes of employing them in as a novel method of water treatment.

The structures observed in the predation study were almost identical to the images of *Pleurotus ostreatus* predation sites photographed by Barron et al. This suggested that the strain we had chosen as a model organism did in fact predate on the *E.coli* within the allotted 72 hours as suggested by Barron et al. Predation was thought to begin by at least 48 hours after inoculation, but because of the differences between the unknown strain in the original literature and *P.ostreatus* strain HK35 the length of time required for *P.ostreatus* HK35 to begin predation is unknown.

4.2 Effectiveness of the Packed-bed Reactor

The primary issues with the packed-bed reactor were the lack of nutrients or buffering salts in the stock solution and the residual nutrients contained in the polystyrene cubes used to grow the fungus. The stock solution was unable to provide a consistent concentration of *E.coli* into the reactor over the course of the experiment. Those bacteria that did successfully load into the reactor then were able to feed on the residual malt broth that was absorbed by the foam cubes causing a bacterial bloom that overwhelmed any predatory functions *P.ostreatus* HK35 may have had.

Due to time constraints, there was not sufficient time for *P. ostrestus* to acclimate to the PBR environment. In previous experiments utilizing a different white rot fungus, the fungus was introduced to the reactor and then feed a complete nutrient solution for 7 to 10 days. This acclimation period allowed the fungus to thoroughly colonized the foam cubes within the PBR (Chirnside, 2011). The trials may have been more successful if an acclimation period occurred before loading the *E. coli* into the reactors.

4.3 Effectiveness of the biocell reactor

The overall effectiveness of the biocell reactors was mainly due to being able to overcome the issues inherent in the experimental design of using the packed-bed reactors. Buffer concentrations were optimized to maintain a given concentration of *E.coli* over the course of 72 hours and the nutrient levels of the media were fairly low.

During the first 24 hours of treatment, the reactors with live compost being treated with the *E.coli* buffer showed an increase in the concentration of *E.coli* compared to the dead control. This growth of *E. coli* is most likely due to the availability of nutrients found in the media and to the nutrients made available through

the breakdown of media constituents by the extracellular enzymes of *P.ostreatus*. At first it was theorized that autoclaving the spent mushroom compost may have changed the availability of some of the nutrients within the media, but the quantity of nutrients available in the effluent of the two treatments did not vary significantly from one another. Similarly, the pH and EC also showed no significant variation between treatments.

After the 24 hour resting period (*i.e.*, no input of water), the reactors were then loaded with sterile water to simulate another 24 hour 2" rain event without *E.coli* loading soon after the initial contaminated load. The results indicated that the concentrations of *E.coli* in the dead control climbed exponentially whereas the concentration in the live treatment fell. Yet again, there was no significant change in the nutrient composition, pH or EC of the effluent to warrant this change suggesting that the result had to be based on the predation ability of the fungi. These results also match fairly well with the water agar predation study timeline in the sense that after the resting period, the concentration of *E.coli* dropped dramatically in the live test as this was the time Barron *et al* suggested predation would occur.

The simulated waste water effluent did not sustain the *E.coli* long enough or consistently enough to provide a steady loading. After 24 hours, the E. coli concentration in the influent had dropped to less than 10 cells/mL. This could explain the lower concentrations of E. coli found in the effluent of Experiment 2 treatments compared to Experiment 1 treatments (10^4 and 10^5 CFU in Experiment 2 vs. 10^7 and 10^8 CFU in Experiment 1). This trend between experiments was only seen for the first 24 h of the study. Growth of *E. coli* in the SWW treatment effluent increased during the dry period and began to level off after 72 h, while E. coli concentration in

Treatment 1 (buffered influent) live SMC decreased. The availability of the nutrients in the SWW may have reduced the predation behavior of *P. ostreatus*. or that the *E.coli* concentrations were too low to provoke a predation response.

The nutrients leached from the media remained low and decreased over the lifespan of the reactor. However, once the media began breaking down, particulate matter began entering the effluent causing increased nutrient readings. The mushroom compost media does represent a finite load of nutrients that can eventually make its way into the watershed. Once determining that load and determining the lifespan of a given reactor, better estimations can be made in terms of total nutrient output over time.

Overall it is clear that *P.ostreatus* does predate on bacterial colonies including *E.coli* and that under certain conditions it can be effective in reducing the amount of *E.coli* found in simulated runoff. These results suggest that spent mushroom compost containing *P. ostreatus* could potentially be used to reduce *E.coli* in surface runoff with minimal environmental impact. With more research and a better understanding of the mechanisms behind the predation and a more concrete timeline in which predation occurs, a standalone *P.ostreatus* treatment system could be developed. With our current knowledge however, coupling this treatment with a vegetative buffer may increase its effectiveness and prevent the buildup of *E.coli* within the vegetative buffer system as described in Fox *et al*.

FUTURE PLANS

Establishing a timeline of when and how predation occurs would be useful in the construction of a better optimized reactor. The mechanism by which the *E.coli* are killed and lysed is still unknown. The use of time lapse microscopy and live cell imaging could shed some light onto both of these processes in the future. The addition of an *E.coli* strain expressing green fluorescent protein (GFP) would also be helpful as it could help quantify predation in an area over time. Combining these methods with adjustments in the source of nutrients and their concentration could also provide reference to the environment in which predation occurs.

With the information gained from working with the biocell reactors, the packed bed reactors need to be revisited. These reactors can be further optimized by allowing for greater fungal biomass to develop on the filter media, providing some kind of buffer to sustain the *E.coli* in the stock solution, and by reducing the amount of nutrients present in the reactor chamber with the filter media. These factors are necessary to ensure that the reactor operates with consistency. After that can be established, the knowledge gained from the nutrient assays and the work with the Biocell reactor can be applied to further optimize the system.

The effectiveness of the biocell reactors was demonstrated over a short period of time in the live treatment, but the longevity of the reactor and its effectiveness over time remains unknown. Extending these treatments past 72 hours and varying the load and rest cycles would provide a more comprehensive investigation of the biocell reactor setup. Coupling this with an exhaustive nutrient analysis would not only allow for a better estimation of the useful lifetime of a reactor, but also allow for better estimates of nutrient leaching from the reactor over time. This data could then be

compared to the treatment to see if different loading patterns increase or decrease the lifetime of the reactor setup.

The media used in the biocell reactors, although plentiful as a waste product from mushroom farms, is not always locally available to a person or company looking to reduce their *E.coli* loading. Research into different kinds of waste materials including spoiled hay, wood chips, bamboo chips, disaster debris, and sawdust for example could improve the versatility of the system and allow for integrated waste management. Using the spent mushroom compost as an inoculant, these media could then be colonized by the fungus and used in place of the mushroom compost, thus reducing the possible nutrient load from the reactor to the environment. Additionally, synthetic media may also prove useful in the future. If predation is in fact based on the carbon: nitrogen ratio as is hypothesized, then growing the fungi on a carbon rich substrate could yield better results and allow for an increased nitrogen load in the influent. Substrates such as porous carbon foam could provide the structural support, mechanical filtration and high carbon environment necessary for the fungus to optimize predation.

Due to the difficulty of maintaining a constant concentration of *E.coli* in the stock solutions, a better more reliable means of doing so should be investigated. The incorporation of a chemostat into the design of the experiment may help provide that stability in the future. The need for this artificial stock solution may not be necessary if the reactors could be placed *in situ*. Monitoring these reactors in locations where they may one day be common place could provide better insight into what the actual bacterial and nutrient loads would be on a system as well as the size of the system necessary to treat a given amount of acreage. Similarly, actual wastewater or

wastewater effluent could be obtained from a local wastewater treatment plant and run through establish reactors to eliminate the need for an artificial stock solution.

Finally, the scope of this project should not be limited to just *Pleurotus sp*. as many other fungi, many of which are white rot fungi, have been shown to prey on bacteria as a supplemental food source. Quantifying the extent to which these fungi prey on bacteria will most likely reveal more effective predators that can be incorporated into these man-made biofilters. Additional qualities such as nutrient utilization, heavy metal hyper accumulation and anti-bacterial byproducts may also be worthwhile to investigate to provide a more comprehensive and effective filter.

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Appendix

A.1 Moisture Data

Day1				Day 2			
Tin #	Tin wt.	Wet wt.	Wet SMC Wt.	Dry Wt.	Dry SMC Wt.	Water Wt.	% Moisture Dry Wt. Basis
1	13.69	26.74	13.05	19.78	6.09	6.96	114.29
2	13.31	31.76	18.45	21.9	8.59	9.86	114.79
3	13.39	21.6	8.21	17.13	3.74	4.47	119.52
4	13.5	33.72	20.22	22.85	9.35	10.87	116.26
5	13.45	32.1	18.65	22.29	8.84	9.81	110.97
6	13.32	32.05	18.73	22.34	9.02	9.71	107.65
7	13.53	31.34	17.81	22.18	8.65	9.16	105.90
8	13.34	30.31	16.97	21.5	8.16	8.81	107.97
9	13.28	30.69	17.41	21.73	8.45	8.96	106.04
10	13.46	30.44	16.98	21.84	8.38	8.6	102.63
11	13.56	30.47	16.91	21.43	7.87	9.04	114.87
12	13.78	31.35	17.57	22.04	8.26	9.31	112.71
13	13.66	30.78	17.12	21.68	8.02	9.1	113.47
14	13.54	28.46	14.92	20.62	7.08	7.84	110.73
15	13.66	28.35	14.69	20.54	6.88	7.81	113.52
16	13.47	32.42	18.95	22.57	9.1	9.85	108.24
17	13.22	29.54	16.32	20.88	7.66	8.66	113.06
18	13.27	33.32	20.05	22.56	9.29	10.76	115.82
19	13.6	29.76	16.16	21.51	7.91	8.25	104.30
20	13.49	34.13	20.64	23.61	10.12	10.52	103.95
21	13.42	38.5	25.08	25.37	11.95	13.13	109.88
22	13.48	34.52	21.04	23.54	10.06	10.98	109.15
23	13.36	32.02	18.66	22.35	8.99	9.67	107.56
24	13.72	36.54	22.82	24.68	10.96	11.86	108.21
25	13.29	37.9	24.61	25.16	11.87	12.74	107.33
26	13.44	39.32	25.88	25.77	12.33	13.55	109.90
27	13.38	39.34	25.96	25.56	12.18	13.78	113.14
				Average % Moisture:		110.44±4.21	

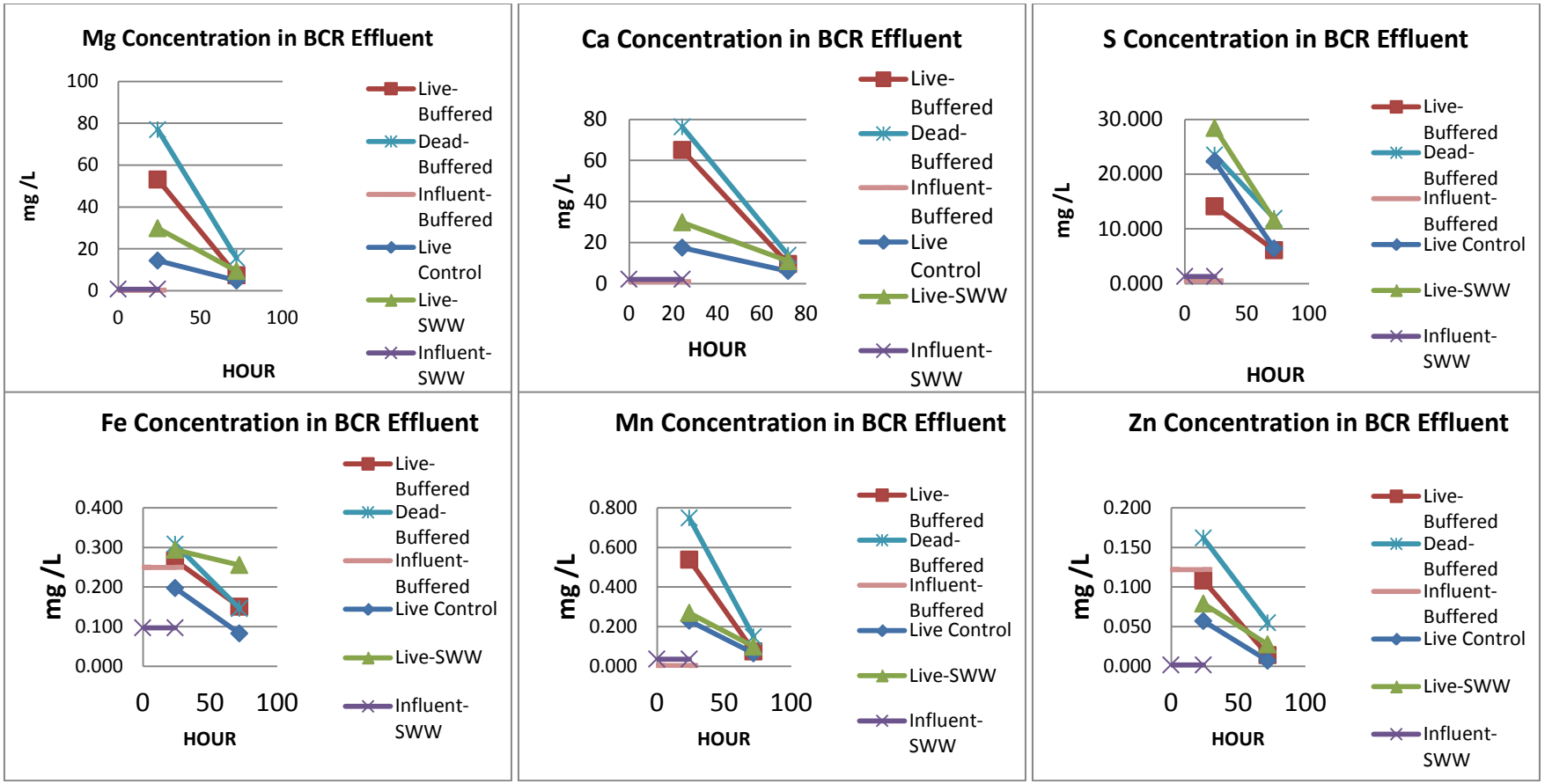
A.2 Micronutrient Data (Experiment 1)

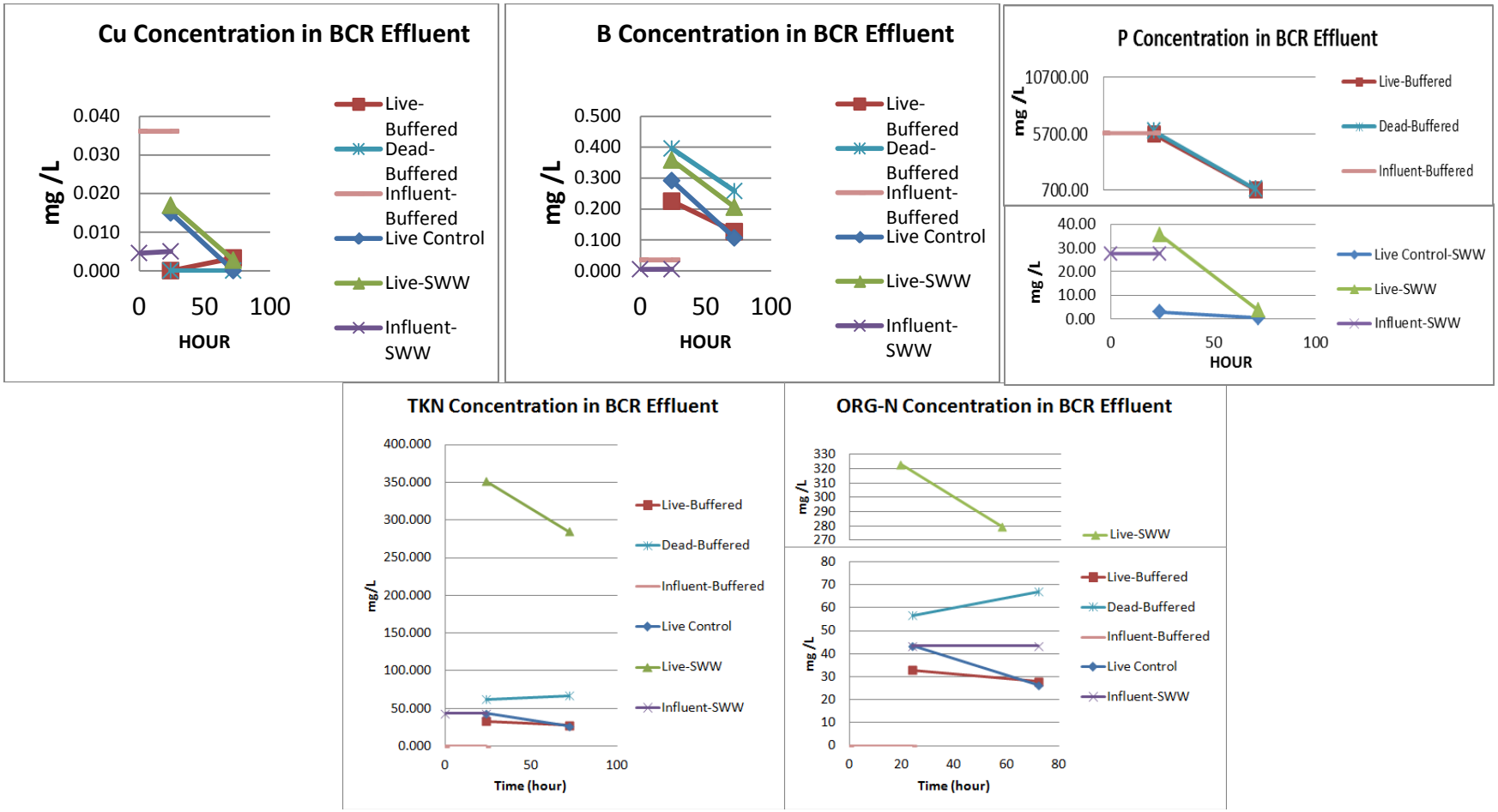
Sample ID	pH	EC (mmhos/cm)	Al (mg/l)	As (mg/l)	B (mg/l)	Ca (mg/l)	Cr (mg/l)	Cu (mg/l)	Fe (mg/l)	K (mg/l)	Mg (mg/l)	Mn (mg/l)	Na (mg/l)	P (mg/l)	Pb (mg/l)	S (mg/l)	Zn (mg/l)	NH4-N (mg/l)	NO3-N (mg/l)
Live R1T24	4.94	10.89	0.141	0.016	0.204	61.056	0.008	-0.012	0.295	3225.800	51.600	0.503	2225.000	5575.0	-0.039	13.590	0.110	0.74	0.36
Live R1T72	5.58	1.33	0.176	0.026	0.110	7.892	0.002	0.002	0.183	395.410	6.596	0.068	225.100	573.8	-0.032	5.013	0.011	0.01	-0.13
Live R2T24	5.03	10.10	0.098	0.029	0.263	76.402	0.012	0.009	0.235	3260.100	60.670	0.658	2199.000	5614.0	-0.037	14.650	0.088	-0.45	0.31
Live R2T72	5.53	2.03	-0.071	0.013	0.146	11.278	0.001	0.000	0.169	568.650	7.772	0.097	345.900	857.4	-0.070	6.217	0.014	-0.18	0.00
Live R3T24	5.04	10.59	0.193	0.024	0.209	57.775	0.008	-0.009	0.271	3550.200	47.120	0.451	2140.000	5744.0	-0.036	14.147	0.127	-0.43	0.44
Live R3T72	5.57	1.68	0.121	0.028	0.125	9.134	-0.002	0.009	0.098	499.740	7.823	0.059	268.000	720.1	-0.007	7.042	0.017	-0.14	-0.13
Dead R4T24	5.07	10.62	0.151	0.062	0.500	90.167	0.013	-0.010	0.395	3732.600	96.070	0.913	2086.000	5792.0	-0.072	29.280	0.189	6.98	0.36
Dead R4T72	5.32	2.17	0.173	0.032	0.276	15.693	-0.002	0.006	0.194	648.800	17.650	0.212	312.700	837.5	-0.045	12.390	0.062	-0.10	-0.17
Dead R5T24	4.92	11.21	-0.071	0.039	0.203	44.531	0.010	-0.017	0.254	3708.400	37.470	0.417	2104.000	5917.0	-0.039	10.720	0.119	2.07	0.54
Dead R5T72	5.16	2.42	0.111	0.048	0.253	13.796	-0.003	-0.015	0.141	714.770	14.640	0.120	327.600	912.3	-0.078	12.310	0.053	-0.13	-0.15
Dead R6T24	5.01	11.37	0.167	0.052	0.485	94.387	0.011	0.000	0.275	3895.400	97.250	0.912	2004.000	6038.0	-0.044	30.440	0.176	6.66	0.51
Dead R6T72	5.28	1.57	0.241	0.022	0.245	11.591	0.000	0.000	0.101	489.010	14.360	0.111	199.400	569.5	-0.021	10.964	0.049	0.30	-0.17
E.coli Buffer Stock 1	4.65	11.33	0.344	0.016	0.103	0.846	0.009	0.078	0.257	3058.000	0.178	0.005	2195.000	5729.0	-0.046	0.481	0.115	-0.35	0.79
E.coli Buffer Stock 2	4.65	12.18	0.181	0.014	0.006	1.605	0.012	0.026	0.337	3310.500	0.369	0.006	2072.000	5815.0	-0.050	0.806	0.178	-0.30	0.83
E.coli Buffer Stock 3	4.61	12.24	0.103	0.025	-0.004	0.114	0.006	0.005	0.154	3383.100	0.030	0.000	1984.000	5723.0	-0.038	0.151	0.073	-0.36	0.79
Sterile H20	4.93	0.00	-0.090	-0.005	-0.002	0.036	0.006	-0.004	0.078	2.270	0.012	-0.002	3.787	0.89	-0.032	0.130	0.000	-0.07	0.15

A.3 Micronutrient Data (Experiment 2)

Sample		EC	Al	As	B	Ca	Cr	Cu	Fe	K	Mg	Mn	Na	P	Pb	S	Zn	NH4-N	NO3-N
ID	pH	(mmhos/cm)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)	(mg/l)
Live Control R1T24	5.75	0.62	-0.11	0.04	0.22	12.68	0.00	0.01	0.16	240.81	10.25	0.16	9.19	2.74	-0.03	15.45	0.04	-0.05	-0.04
Live Control R1T72	6.37	0.23	0.24	0.02	0.06	3.10	0.00	-0.01	0.07	72.95	2.40	0.03	3.44	0.26	-0.06	3.67	0.00	-0.04	0.01
Live Control R2T24	5.67	0.81	-0.15	0.05	0.31	18.69	0.01	0.02	0.19	331.07	14.97	0.22	9.58	3.57	-0.03	21.70	0.06	-0.03	0.05
Live Control R2T72	5.94	0.52	0.01	0.04	0.18	10.23	0.00	0.00	0.09	174.93	8.48	0.11	4.62	0.76	-0.06	9.46	0.01	0.16	0.03
Live Control R3T24	5.84	0.95	0.23	0.04	0.35	20.97	0.00	0.01	0.24	387.16	17.97	0.31	10.19	2.29	-0.06	29.81	0.07	-0.06	0.11
Live Control R3T72	5.83	0.30	0.14	0.04	0.08	4.60	0.00	-0.01	0.08	92.42	3.68	0.06	3.25	0.39	-0.05	6.01	0.01	-0.06	0.01
SWW R1T24	5.34	2.02	0.06	0.06	0.37	32.01	0.00	0.02	0.28	497.58	31.53	0.28	197.40	18.37	-0.04	30.26	0.09	22.93	-0.01
SWW R1T72	6.10	0.67	-0.12	0.05	0.18	9.11	0.01	0.00	0.20	161.64	7.89	0.10	51.37	1.51	-0.03	9.37	0.02	3.32	0.08
SWW R2T24	5.20	2.24	-0.27	0.08	0.41	35.13	0.01	0.02	0.34	554.27	34.29	0.29	226.80	45.33	-0.04	31.99	0.09	30.05	0.10
SWW R2T72	5.84	0.93	0.12	0.07	0.24	13.48	0.01	0.00	0.25	246.04	11.54	0.12	54.50	5.69	-0.04	13.68	0.03	6.01	0.17
SWW R3T24	5.34	2.00	0.26	0.05	0.29	22.13	0.00	0.01	0.26	365.25	23.76	0.24	214.60	43.10	-0.05	22.96	0.06	31.41	0.04
SWW R3T72	6.00	0.81	0.23	0.06	0.19	10.16	0.01	0.00	0.31	185.23	8.71	0.08	60.71	3.71	-0.04	11.49	0.03	5.34	0.14
Sterile H20	4.93	0.00	-0.09	-0.01	0.00	0.04	0.01	0.00	0.08	2.27	0.01	0.00	3.79	0.89	-0.03	0.13	0.00	-0.07	0.15
SWW Stock 1	6.12	1.52	0.04	0.01	0.00	1.92	0.01	-0.01	0.11	53.75	0.64	0.04	311.20	40.73	-0.04	1.20	0.00	45.02	0.75
SWW Stock 2	6.32	1.42	0.07	0.00	0.01	2.13	0.01	0.01	0.09	22.77	0.76	0.04	300.90	14.61	0.02	1.37	0.00	41.74	0.73

A.4 Nutrient fluctuations overtime





A.4. Biocell reactor nutrient data. These graphs represent the effluent collected over two 24 hour periods (t=0-24, t=48-72). Concentrations are reflective of the full 24 hour period. Those nutrients not shown in graph form either did not significantly differ from the control or were below the detection limits.