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# Evaluating the effects of pine and miscanthus biochar on water activity and Escherichia coli populations in commercial broiler litter

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The decrease in subtherapeutic antibiotic administration in poultry has increased the need to address production challenges caused by pathogens, such as *E. coli*. One potential way to improve bird health and reduce bacterial infection is through the addition of litter amendments that absorb moisture. Biochar (BC) has previously been shown to increase water holding capacity in poultry litter, but its effects on *E. coli* mitigation are unknown. The objectives of this research were to 1) evaluate water activity of poultry litter amended with pine and miscanthus BC, and 2) determine the effects of different BC inclusion rates on litter *E. coli* populations. The studies found that BC increased water activity when mixed with broiler litter, and pine BC resulted in lower *E. coli* counts over time than miscanthus BC. An inclusion rate of 30% by weight of pine BC was most effective at reducing *E. coli* populations in broiler litter.

# DEDICATION

I dedicate this work to my family and friends who always supported me when I needed it most, especially my parents Keith and Anna Marty. Their love and support gave me the strength to continue and complete my degree. I would like to thank my saving grace, Dr. Maryam Mohammadi-Aragh, for joining at the most crucial point to steer us in the right direction and for her guidance throughout the rest of my degree. I would also like to thank my fellow graduate students Dru Carey and Matt Rowland for lending a hand whenever needed. I apologize for the baked poultry litter smell everyone on the third floor had to endure multiple times during the year.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

# LITERATURE REVIEW

## 1.1 Overview

Poultry is the second most consumed meat worldwide and global consumption continues to rise in developing countries because it is a low-cost form of high-quality protein (Chai et al., 2017). To meet the growing demand, global poultry production is forecasted to increase 1% in 2021, mostly driven by increased consumption in China and Brazil (USDA, 2021). In the U.S alone, per capita broiler meat consumption in 2020 was 96.2 pounds, up 14 pounds from 2010 (NCC, 2021). Currently, the U.S. and Brazil are the top broiler producers with an estimated 33.5 million metric tons of broiler meat produced in 2019 (Shahbandeh, 2019).

According to the National Chicken Council, there are approximately 30 commercial companies in the U.S. involved in raising, processing, and marketing broiler chickens. Approximately 95% of broilers are raised on family-owned farms that have contracts with broiler companies. The top broiler producing states are Alabama, Arkansas, Georgia, Mississippi, and North Carolina. The U.S. poultry industry employs over 400,000 employees directly and 1.6 million indirectly through other services to the industry. Production value in the U.S. is nearly 21.7 million dollars with Mississippi contributing nearly 1.7 million dollars (NCC, 2021).

While the U.S. poultry industry provides a high-quality, cost-efficient source of protein to consumers, mitigating the presence of pathogens during production is a challenge. In fact, contaminated poultry has been documented as the leading cause of food-borne illness by

microorganisms in humans (FDA, 2016). Traditionally, prophylactic antibiotics administered in the feed and water have been the primary means of controlling production losses in poultry. However, the current trend to reduce the use of antibiotics in broiler production has made pathogen control more challenging and has necessitated the need for alternative strategies.

Chickens transmit diseases by horizontal and vertical transmission through hatcheries and broiler houses (Poulsen et al., 2017). Horizontal transmission usually occurs via contaminated materials, such as litter, water, and feed. Pathogens may be vertically transmitted following contamination of the egg or eggshell through infections in the oviduct (Poulsen et al., 2017). Avian pathogenic *Escherichia coli* (APEC) and its associated diseases can be transmitted horizontally or vertically and are associated with significant economic losses to the poultry industry. Diseases associated with APEC can attack many different organs of a bird and are frequently associated with local or systemic infections (Matter et al., 2011; De Carli et al., 2015). The most common infections associated with APEC usually begin with airsacculitis, a localized infection of the air sacs that can spread to other internal organs, creating a systemic infection. Research has discovered that some virulence genes and phylogenic groups are similar to a few specific human and avian extraintestinal pathogenic *E. coli* (ExPEC) strains (Manges and, Johnson 2012). In APEC strain O1:K1:H7, researchers identified that the genomes of human uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) were very similar (Johnson et al., 2007). APEC and human ExPEC diseases with closely related genomes can potentially be a risk to bird health and a concern for human food supplies.

Creating a pathogen-free environment in all stages of poultry production is important to bird health and welfare and to human health (de Lange, 2016). Antibiotic-free (ABF) and no antibiotics ever (NAE) poultry production is becoming necessary as the day-to-day consumer is

becoming more interested in food production (Salois et al., 2016; Fancher et al., 2020). Salois et al. (2016) calculated the difference in conventional and 100% ABF systems and found that ABF systems, in general, are more expensive and resource-intensive to operate.

## 1.2 Alternative Strategies to Mitigate *E. coli*

In Brazil, control of Enterobacteriaceae pathogens between flocks is done using a combination of quicklime (CaO) and canvas tarping (Lopes et al., 2015). The addition of quicklime to the litter helps control pathogens by reducing of water activity ($A_w$) while also increasing the pH (Lopes et al., 2013). Ferreira et al. (2004) and Ahn et al (2008) speculated that the CaO reacting with water to become $Ca(OH)_2$ prompted a reduction of moisture content, $A_w$, and overall presence of bacteria. Lopes et al. (2015) found that the addition of quicklime and tarping greatly reduced Enterobacteriaceae CFUs compared to only tarping the litter.

Experimental trials were conducted by Pope and Cherry (2000) to test whether Poultry Litter Treatment (PLT®) had any effect on pathogens in the litter. Secondary testing in the processing plant was done to determine if the effects in the litter followed the birds. *E. coli* populations in the litter were significantly lower in the PLT treated houses compared to the control but they were not statistically different when tested in the processing facility. Pope and Cherry (2000) concluded that PLT® could potentially suppress survival of *E. coli* in the litter, but it cannot completely eliminate bacterial presence, although it may reduce the ubiquity of pathogens entering the processing plant.

## 1.3 Biochar as an Alternative Method to Reduce *E. coli* Populations in Poultry Litter

Biochar is a carbonaceous material that is comprised of charred organic matter residues produced by chemical and thermal conversion in oxygen-limited environments (Jones et al.,

1997; Masiello, 2004). Pyrolysis of biomass to produce biochar can be done at a range of temperatures and times to affect the physio-chemical properties of the biochar. Advanced thermal conversion processes can range from 200˚C to greater than 800˚C and vary in pyrolysis times of less than a second during flash pyrolysis to hours during slow pyrolysis. High pyrolysis temperatures of 400 – 800˚C cause a distribution of condensed aromatic structures (Keiluweit et al., 2010). These structures have lower oxygen to carbon ratios that are resistant to microbial degradation (Glaser et al., 2002; Kimetu and Lehmann, 2010; Zimmerman, 2010).

Biochar has the potential to alter microbial communities. Hill et al. (2019) experimented with RPMI 1640 medium to grow *E. coli* overnight for 12 hours and determine the effect of activated carbon (AC) and biochar *E. coli* growth when compared to an untreated media. Hill et al. (2019) used biochar that originated from Douglas fir wood chips and was gasified at 900 – 1000˚C for 1 – 10 seconds. They detected that AC and biochar absorbed a substantial number of metabolites and amino acids upon being introduced to the media. The treated RPMI media altered the growth of *E. coli* compared to untreated media, which they attributed to the AC and biochar absorbing nutrients. AC and biochar both reduced *E. coli* growth when compared to untreated media.

Composting of poultry litter is a way to reduce pathogens like *E. coli* and *Salmonella* to be used as a safe soil amendment and reduce odor (Bolan et al., 2010). The downside of composting is that up to 88% of total nitrogen can be lost through $NH_3$ during composting (Ogunwande et al., 2008). In a composting study conducted by Vandecasteele et al. (2016), 10% Green Waste Biochar (GWB) was the optimal inclusion rate for breaking down organic matter during composting. Compared to the control (no biochar), GWB addition resulted in both a decrease of total N and $NH_3$ by over 50%. The addition of GWB has potential to retain nitrogen

for a better soil amendment while the composting aids the properties of the litter to be used as fertilizer.

Amendments to litter have been widely used in commercial broiler production houses to control ammonia volatilization and overall environment quality (Linhoss et al., 2019). Even though high moisture is related to increased incidence of foot pad dermatitis and leads to increased ammonia levels, little research has been conducted on using litter amendments to reduce moisture levels. The birds raised on biochar demonstrated increased body weight, body weight gain, and feed intake with lower feed conversion ratios, mortality, and footpad scores (Linhoss et al., 2019). These results were comparable to peanut hull, acidified pine, and coconut husk char done in a study by Ritz et al. (2011). A negative effect of biochar was particle dust suspension but after a few days of being saturated through bird excreta it was no longer a problem. After the trial was conducted, necropsies were performed and found biochar in the nares and frontal sinuses but none in the lungs or air sacs indicating the BC did not affect the birds. The recommended commercial application rate was determined to be less than 20% and a coarse particle size (greater than 0.853 mm) for the highest absorption and least dust particle suspension.

Pyrolysis of biochar at different temperatures has the potential to impact *E. coli* transport through sandy soils. Microorganisms can be transported through the soil by multiple factors, such as ionic strength and configuration of the carrier fluids, pH, and organic matter composition (Scholl et al., 1990; Johnson and Logan, 1996; Bolster et al., 2001; Walker et al., 2004; Bolster et al., 2006; Kim et al., 2009; Harvey et al., 2011). An important factor of biochar is that pyrolysis temperature impacts the chemical properties (Downie et al., 2009). Both low (350˚C) and high (700˚C) temperature biochar's increased the pH and total carbon content of the soil.

Afrooz & Boehm (2016) experimented with biochar-modified filters against traditional sand filters to investigate whether the presence of biofilm altered the efficiency of removing *E. coli* from stormwater runoff. Biofilm is a thin film of bacterial cells that adhere to each other and surfaces, such as sand or biochar, producing a slimy extracellular matrix. Overall, the use of biochar-modified filters increased the removal of contaminants from stormwater runoff comparatively to the sand only biofilter. However, high levels of organic matter and biofilm growth in the biofilter was shown to reduce the efficiency of filters modified with biochar.

Leisenring et al. (2012) examined the effect of various biochar feedstock and pyrolysis temperatures on the *E. coli* removal from stormwater. Low temperature (LT) biochar was produced at 350˚C and high temperature (HT) was produced at 700˚C. Biochar-sand columns removed more *E. coli* from the stormwater compared to the sand only column, but pyrolysis temperature was shown to effect *E. coli* removal. The LT biochar removed significantly more *E. coli* than the other two biochar's with no organic matter present.

Biochar may also influence water activity ($A_w$), which is a thermodynamic property that determines the availability of water in a sample and its inclination to escape (Dunlop et al., 2016). Specifically, $A_w$ is the ratio of the partial vapor pressure of water in a material to the standard state partial vapor pressure of water. Dunlop et al. (2016) found that new bedding material had the highest $A_w$ and decreased throughout the grow-out due to break down of organic material. METER Food (2020) determined that microbial activity begins at an $A_w$ of 0.87, while *E. coli* begins to grow at an $A_w$ of 0.95. The cohesiveness of litter is correlated with both moisture content and $A_w$ (Bernhart and Fasina 2009). The litter becomes more cohesive when moisture increases from 18 to 22% (0.75 to 0.85 $A_w$, respectively) while critical hydration levels of poultry litter are reached between 0.75 and 0.90 $A_w$ (Bernhart and Fasina 2009). Dunlop et al.

(2016) determined that poultry litter $A_w$ should remain below the critical hydration level to ensure a free-flowing litter to transfer moisture from fresh excreta into the litter, promoting a reduction of overall $A_w$ and microbial growth.

## 1.4 Objectives

Mitigating *E. coli* in poultry litter can improve bird performance, welfare, and potentially reduce the spread of diseases to humans. The goal of this research is to examine biochar as a possible litter treatment to reduce transmission and to reduce colonization of *E. coli* in poultry litter and limit further transmission to susceptible poultry and associated disease. Specific objectives of this research are the following:

- Evaluate the effects of biochar inclusion rates on water activity in broiler litter.
- Evaluate the effects of litter amendment application rate on *E. coli* populations in broiler litter.

# CHAPTER II

# EFFECT OF VARYING INCLUSION RATES OF PINE AND MISCANTHUS ON THE WATER ACTIVITY OF USED BROILER LITTER

## 2.1 Introduction

Biochar (BC) originates from the pyrolysis of renewable organic waste products, and it is often used for agricultural or environmental purposes. The physio-chemical characteristics of biochar are heavily influenced by the temperature at which it is produced and the time that is pyrolyzed. Past studies have investigated the use of biochar for plant growth and water treatment and filtration, but few have investigated BC as a potential poultry litter amendment. Linhoss et al. (2019) conducted a study to determine the effects on broiler performance while using BC as a litter amendment. They determined that the addition of BC to the pine shavings showed no adverse effects to bird welfare and that it significantly increased water holding capacity. There is a potential for BC to affect bacterial growth in poultry litter due to its ability to absorb moisture, leaving less available for microbial activity.

Water activity ($A_w$) is an important factor in the food science industry as it directly affects the ability for bacterial growth. Dunlop et al. (2016) describes $A_w$ as a thermodynamic property in relation to the availability of water in a sample and its inclination to escape. Dunlop et al. (2016) found that fresh materials used for bedding had the highest $A_w$ and that it decreased throughout the grow-out due to the breakdown of organic material. Previous grow-out litter that has already broken down the organic materials would provide a lower $A_w$ base material for the

introduction of a new flock, potentially benefiting its ability to remediate bacterial growth (Dunlop et al., 2016). Microbial activity begins with an $A_w$ of 0.61 for yeast and molds while bacterial growth begins at 0.87 (METER Food 2020). BC can affect water activity ($A_w$) depending upon the type of material, time of pyrolysis, and temperature that affect the chemical properties of BC (Ippolito et al., 2020).

The goals of this research were to 1) examine the effects of poultry litter and pine and miscanthus BC combinations on $A_w$ at different inclusion rates and different starting moisture contents, and 2) characterize some of the physical and chemical properties of pine and miscanthus BC.

## 2.2 Litter and Treatments

Pine-based poultry litter was collected from a commercial broiler house located on the campus of Mississippi State University. Pine BC was obtained from a commercial lumbermill in Mississippi and was produced at pyrolysis temperatures between 700 and 1000˚C. Miscanthus BC was made by researchers in Sustainable Bioproducts at Mississippi State University. Miscanthus was milled to 3 mm prior to being placed in the pyrolysis chamber at a temperature of 450˚C for 1 minute. Pine BC, miscanthus BC, and poultry litter were sieved to uniform sizes between 850 µm – 1400 µm (Figure 2.1).


Figure 2.1 Sieved poultry litter (A), pine BC (B), and miscanthus BC (C) used in this experiment.

Treatments were arranged in a 2 × 7 × 7 factorial design with main effects being BC type, BC inclusion rate, starting moisture content. Miscanthus and pine BC were mixed with litter at inclusion rates of 0, 10, 20, 30, 40, 50, and 100% by weight (Table 2.1) at six initial moisture contents (10, 15, 20, 25, 30, 40, 50%). Total sample weight for all pine BC inclusions was 175 g. Miscanthus BC has a much lower bulk density than pine BC and at higher BC inclusion rates it would not fit in the sample containers. Therefore, as the miscanthus BC inclusion rate increased, the overall sample weight was reduced. Mixing of litter and biochar combinations were performed using a mechanical sieve shaker (AS 200, Retsch, Hann, Germany) for five minutes at 80% amplitude.

Table 2.1 Sample weights for pine and miscanthus BC inclusion rates.

| Inclusion Rate (%) | Litter (g) | BC (g) | Total Sample Weight (g) |
|---|---|---|---|
| **Pine** | | | |
| 0 | 175 | 0 | 175 |
| 10 | 157.5 | 17.5 | 175 |
| 20 | 140 | 35 | 175 |
| 30 | 112.5 | 52.5 | 175 |
| 40 | 105 | 70 | 175 |
| 50 | 87.5 | 87.5 | 175 |
| 100 | 0 | 175 | 175 |
| **Miscanthus** | | | |
| 0 | 175 | 0 | 175 |
| 10 | 157.5 | 17.5 | 175 |
| 20 | 96 | 24 | 120 |
| 30 | 77 | 33 | 110 |
| 40 | 54 | 36 | 90 |
| 50 | 40 | 40 | 80 |
| 100 | 57 | 0 | 57 |

After mixing, samples were dried in an oven at 103˚C for 24 hours and weighed. Samples were moistened with distilled water to target moisture contents of 10, 15, 20, 25, 30, 40, and 50% (Table 2.2). Samples were covered and allowed to condition for 24 hours. After conditioning, wet-basis moisture content (MC) was determined on three replicates per BC inclusion rate (39 total) to compare target and actual MC values (Table 2.3). Wet-basis MC was determined using the methods detailed in ANSI/ASAE S358.3. Equation 2.1 was used to calculate wet-basis MC.

$$MC_{wb}(\%) = \frac{Water\ Wt.}{(Dry\ Sample\ Wt. + Water\ Wt.)} \times 100 \qquad (2.1)$$

Table 2.2 Weight of components used to reach target moisture contents for pine and miscanthus BC.

| Target $MC_{wb}$ (%) | Litter + BC (g) | Water Added (g) | Total Sample Wt. Litter + BC +Water (g) |
|---|---|---|---|
| **Pine** | | | |
| 10 | 22.7 | 2.5 | 25.2 |
| 15 | 22.7 | 4.0 | 26.7 |
| 20 | 22.7 | 5.7 | 28.3 |
| 25 | 22.7 | 7.6 | 30.2 |
| 30 | 22.7 | 9.7 | 32.4 |
| 40 | 22.7 | 15.1 | 37.8 |
| 50 | 22.7 | 22.7 | 45.3 |
| **Miscanthus** | | | |
| 10 | 15.0 | 1.7 | 16.7 |
| 15 | 15.0 | 2.7 | 17.7 |
| 20 | 15.0 | 3.8 | 18.8 |
| 25 | 15.0 | 5.0 | 20.0 |
| 30 | 15.0 | 6.4 | 21.4 |
| 40 | 15.0 | 10.0 | 25.0 |
| 50 | 15.0 | 15.0 | 30.0 |

Table 2.3 Target and actual MC values for all treatment combinations.

| BC Inclusion Rate (%) | Target MC % | Pine Mean Measured MC (%) | Miscanthus Mean Measured MC (%) |
|---|---|---|---|
| 0 | 10 | 10.2 ± 0.2 | * |
| | 15 | 14.3 ± 0.3 | * |
| | 20 | 18.9 ± 0.1 | * |
| | 25 | 24.6 ± 0.3 | * |
| | 30 | 28.5 ± 0.3 | * |
| | 40 | 39.0 ± 0.4 | * |
| | 50 | 48.8 ± 0.7 | * |
| 10 | 10 | 9.5 ± 0.1 | 10.4 ± 0.2 |
| | 15 | 14.0 ± 0.2 | 15.1 ± 0.1 |
| | 20 | 19.4 ± 0.3 | 20.8 ± 0.2 |
| | 25 | 23.9 ± 0.2 | 25.2 ± 0.3 |
| | 30 | 28.5 ± 0.2 | 29.9 ± 0.8 |
| | 40 | 38.9 ± 0.1 | 39.6 ± 0.9 |
| | 50 | 48.8 ± 0.1 | 50.6 ± 0.2 |

Table 2.3 (continued)

| BC Inclusion Rate (%) | Target MC % | Pine Mean Measured MC (%) | Miscanthus Mean Measured MC (%) |
|---|---|---|---|
| 20 | 10 | 10.3 ± 0.3 | 10.5 ± 0.3 |
| | 15 | 14.5 ± 0.2 | 14.7 ± 0.2 |
| | 20 | 19.4 ± 0.2 | 20.1 ± 0.1 |
| | 25 | 24.4 ± 0.0 | 25.0 ± 0.4 |
| | 30 | 30.0 ± 0.4 | 29.8 ± 0.5 |
| | 40 | 39.4 ± 0.3 | 41.2 ± 0.3 |
| | 50 | 49.1 ± 0.3 | 49.7 ± 0.3 |
| 30 | 10 | 10.3 ± 0.1 | 10.6 ± 0.5 |
| | 15 | 14.7 ± 0.1 | 15.0 ± 0.4 |
| | 20 | 19.9 ± 0.1 | 19.5 ± 0.6 |
| | 25 | 24.7 ± 0.5 | 24.4 ± 0.6 |
| | 30 | 30.0 ± 0.2 | 29.1 ± 0.4 |
| | 40 | 40.4 ± 0.9 | 39.8 ± 0.1 |
| | 50 | 49.4 ± 0.6 | 48.7 ± 0.9 |
| 40 | 10 | 9.1 ± 0.1 | 10.0 ± 0.6 |
| | 15 | 14.6 ± 0.3 | 14.2 ± 0.1 |
| | 20 | 19.4 ± 0.2 | 19.4 ± 0.3 |
| | 25 | 24.2 ± 0.2 | 24.2 ± 0.7 |
| | 30 | 29.6 ± 0.1 | 29.6 ± 0.4 |
| | 40 | 36.9 ± 0.2 | 39.4 ± 0.3 |
| | 50 | 49.7 ± 0.3 | 49.8 ± 0.7 |
| 50 | 10 | 9.4 ± 0.2 | 8.4 ± 0.5 |
| | 15 | 14.1 ± 0.4 | 13.2 ± 0.1 |
| | 20 | 19.0 ± 0.2 | 18.0 ± 0.2 |
| | 25 | 24.4 ± 0.2 | 23.0 ± 0.7 |
| | 30 | 29.7 ± 0.2 | 28.9 ± 0.7 |
| | 40 | 39.4 ± 0.3 | 40.1 ± 0.2 |
| | 50 | 49.5 ± 0.1 | 48.8 ± 0.1 |
| 100 | 10 | 9.4 ± 0.2 | 7.0 ± 0.5 |
| | 15 | 13.9 ± 0.5 | 11.5 ± 0.2 |
| | 20 | 19.4 ± 0.7 | 17.1 ± 0.1 |
| | 25 | 24.5 ± 0.3 | 23.5 ± 0.3 |
| | 30 | 30.0 ± 0.3 | 27.9 ± 0.3 |
| | 40 | 39.8 ± 0.4 | 36.3 ± 0.6 |
| | 50 | 49.8 ± 0.2 | 47.2 ± 0.7 |

* Same as pine.

## 2.3 Sampling and Assessment

### 2.3.1 Water Activity

Water activity ($A_w$) is a ratio of the vapor pressure of water in a substance to the vapor pressure of pure water at a temperature equilibrium. It is a useful metric for determining the amount of moisture in a material that is available for microbial growth. $A_w$ is potentially a better water measuring system due to its relationship with biological, chemical, and physical characteristics (van der Hoeven-Hangoor et al., 2014). $A_w$ was measured using a dewpoint soil water potential meter (WP4C, Meter, Pullman, WA). Equation 2.2 was used to convert water potential to water activity. Prior to data collection, the meter was calibrated using a 0.50 mol/kg KCl salt solution. Calibration readings were within ± 0.05 MPa of the correct reading for the KCl standard at 25°C. The water meter generated readings in two modes: precise (20 minutes) and fast (5 – 7 minutes). Readings were similar in both modes, so the fast mode was used to reduce testing time.

$$A_W = EXP\frac{(MPa \cdot 18.02)}{(8.3143 \cdot (T℃ + 273.15))} \quad (2.2)$$

Where:

MPa = megapascals

T˚C = temperature (Celsius)

### 2.3.2 Brunauer-Emmett-Teller (BET) Analysis

Specific surface area, total pore volume, and pore diameter were determined for miscanthus and pine BC via Brunauer-Emmett-Teller (BET) analysis. The analysis was done

using $CO_2$ and $N_2$ adsorption gases at 273 and 77K, respectively. BET was performed by the MSU Chemistry Department using a high throughput surface area and porosity analyzer (Micrometrics, Tristar II Plus, Norcross, GA).

### 2.3.3 Point of Zero Charge

Point of zero charge (PZC) was analyzed to determine the pH at which poultry litter, miscanthus, and pine BC surface charges were equal to zero. Adsorption of ions on the surface of BC has shown to be pH dependent. Seven pH levels (1, 3, 5, 7, 9, 11, 13, and 15) were used to sample PZC. 50 µg of pine BC, miscanthus BC, and poultry litter were added to three replicate sample tubes containing 25 mL of a NaCl solution. The pH was increased with NaOH or decreased with HCl to reach desired pH levels. Sample tubes were capped, placed into Ziploc bags, and mixed in a mechanical shaker (Orbital Shaker, Thermo Forma, Waltham, MA) for 24 hours. Solid material was filtered (Grade 1 – 110 mm, Whatman, Buckinghamshire, UK) and pH was determined for the resulting supernatant using a pH meter (Hanna, HI 3221, Woonsocket, RI).

### 2.3.4 Ultimate Analysis

Ultimate analysis of poultry litter, pine BC, and miscanthus BC was performed using an organic elemental analyzer (Elementar, Unicube, Langenselbold, Hesse, Germany) that provides carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) concentrations. The instrument does not have the ability to determine oxygen (O), but it was calculated by subtracting the total C, H, N, and S concentrations from 100%. 5 mg samples of BC and litter were loaded into the organic elemental analyzer and analyzed. Results were generated in roughly 15 minutes per sample, including necessary calibrations prior to running the samples. Ash content was taken by

weighing the samples prior to being placed in an oven at 600˚C for 24 hours, the resulting ash leftover was weighed to determine ash content.

### 2.3.5 Scanning Electron Microscope

Images were taken of pine BC and miscanthus BC using a scanning electron microscope (JEOL, JSM-6500F, Peabody, MA). The samples were sputter coated with 15 nm of silver by a sputter coater (Electron Microscopy Sciences, EMS150T ES, Hatfield, PA) to produce a better image quality for carbon-based materials. Magnification of two versions were used to see the broader spectrum of the materials and in depth of the pores in the BC. The images provide a visual representation of the materials pore structure and overall surface morphology.

## 2.4 Statistical Analysis

Collected data for $A_w$ was analyzed using PROC Mixed in SAS Version 9.4. Significant differences were made at $P < 0.05$ and means were separated by Fisher's LSD via PDMIX800 (Saxon, 1998). Treatments were arranged in a 2 x 7 x 7 factorial design with main effects being type of BC, BC inclusion rate, and starting moisture content. All statistical data can be found in Appendix A for more information on water activity results.

## 2.5 Results

Table 2.4 shows that type 3 tests of fixed effects for biochar type, MC, BC inclusion rate, and all possible interactions were significant ($P < 0.0001$). $A_w$ for pine miscanthus was significantly higher than miscanthus (Table 2.5). Tables 2.6 and 2.7 show that $A_w$ tended to increase with increasing moisture content and BC inclusion rate.

Table 2.4 Type 3 tests of fixed effects.

| Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| Type | 1 | 196 | 149.51 | <.0001 |
| MC | 6 | 196 | 11408.6 | <.0001 |
| BC | 6 | 196 | 291.03 | <.0001 |
| Type*MC | 6 | 196 | 64.82 | <.0001 |
| Type*BC | 6 | 196 | 102.31 | <.0001 |
| BC*MC | 36 | 196 | 64.21 | <.0001 |
| Type*BC*MC | 36 | 196 | 16.24 | <.0001 |

Table 2.5 $A_w$ comparison by BC type.

| Type | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|
| p | 0.8608 | 0.000655 | A |
| m | 0.8495 | 0.000655 | B |

Table 2.6 $A_w$ comparison by moisture content.

| MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|
| 50 | 0.9727 | 0.001226 | A |
| 40 | 0.9550 | 0.001226 | B |
| 30 | 0.9263 | 0.001226 | C |
| 25 | 0.8960 | 0.001226 | D |
| 20 | 0.8533 | 0.001226 | E |
| 15 | 0.7880 | 0.001226 | F |
| 10 | 0.5947 | 0.001226 | G |

Table 2.7 $A_w$ comparison by BC inclusion rate.

| BC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|
| 100 | 0.8945 | 0.001226 | A |
| 40 | 0.8598 | 0.001226 | B |
| 50 | 0.8592 | 0.001226 | B |
| 30 | 0.8569 | 0.001226 | B |
| 20 | 0.8494 | 0.001226 | C |
| 10 | 0.8366 | 0.001226 | D |
| 0 | 0.8296 | 0.001226 | E |

Figure 2.2 shows that $A_w$ increased with moisture content for all pine BC inclusion rates. In addition, at each initial moisture content level, $A_w$ tended to increase with higher levels of BC inclusion. $A_w$ followed the same general increasing trend with moisture content for all BC inclusion rates, except 100%. At 10% MC, $A_w$ was the lowest for the 100% BC inclusion rate, but at 15% MC it increased by 0.49, well above the roughly 0.15 – 0.20 increase experienced by the other BC inclusion rates. $A_w$ at 15% MC and 100% BC inclusion was retested and the results were similar.

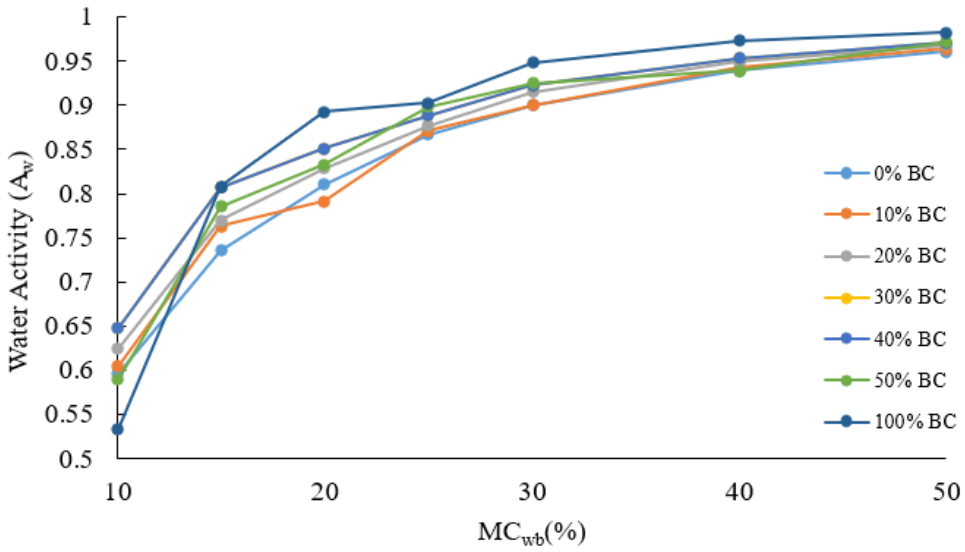


Figure 2.2 Moisture content ($MC_{wb}$) by water activity ($A_w$) for different pine BC inclusion rates.

For all miscanthus BC inclusion rates, water activity increased with moisture content (Figure 2.3). For most of the MC levels, $A_w$ increased with higher BC inclusion rates. Interestingly, the trend of increasing $A_w$ with moisture content is like that of pine BC, however, the 100% inclusion rate did not experience the same drastic increase in $A_w$ between 10 and 15% MC.

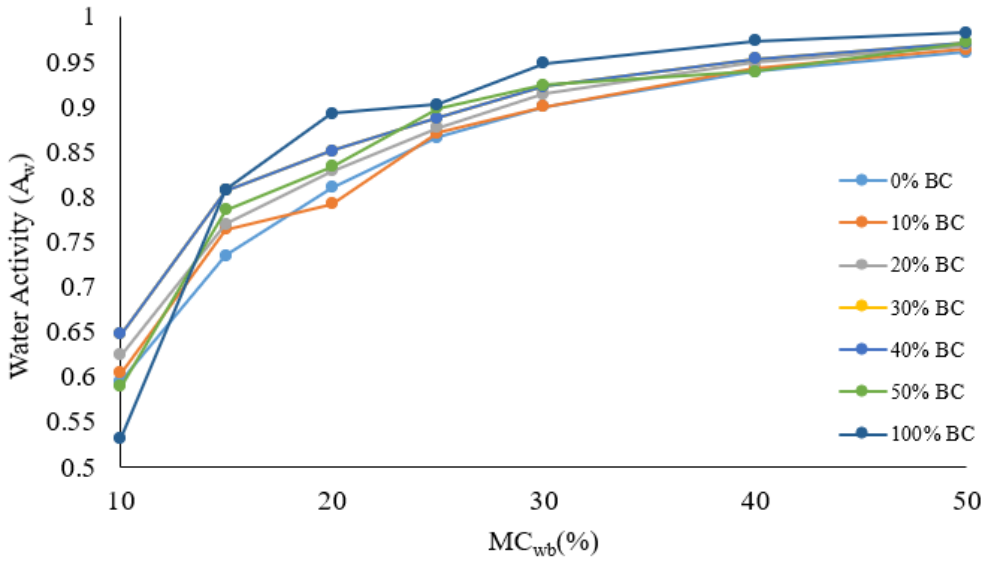


Figure 2.3 Moisture content ($MC_{wb}$) by water activity ($A_w$) for different miscanthus inclusion rates.

As mentioned in the previous paragraphs, $A_w$ increased with increasing initial MC for all BC inclusions rates. In addition, Figure 2.4 shows that for all initial MC levels, except 10%, pine BC had a higher mean $A_w$ than miscanthus BC. Figure 2.5 shows that $A_w$ increased slightly with increasing rates of BC, but the trend was not nearly as pronounced as with initial MC.

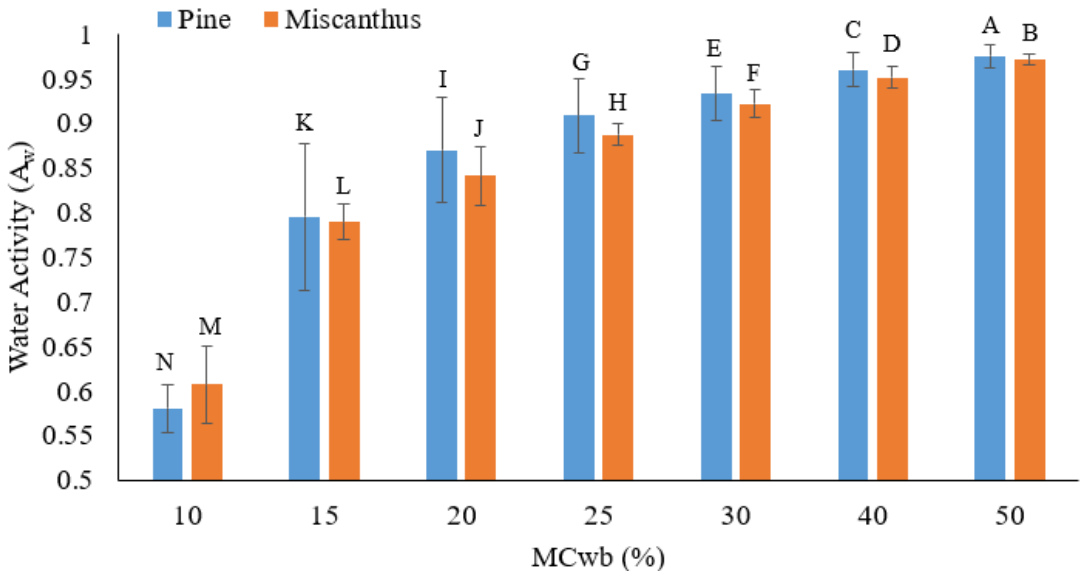


Figure 2.4 Mean water activity ($A_w$) and standard deviation for pine and miscanthus BC as influenced by moisture content ($MC_{wb}$) by type.

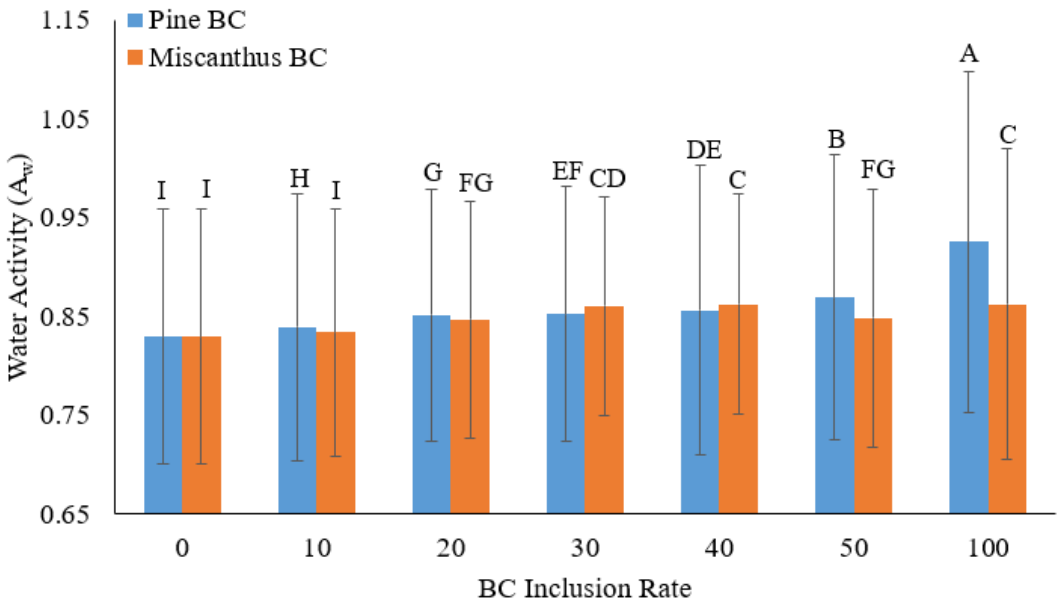


Figure 2.5 Mean water activity ($A_w$) and standard deviation for pine and miscanthus BC as influenced by BC inclusion rate.

### 2.5.1 BET Analysis

BET analysis was performed to help characterize biochar morphology using $N_2$ and $CO_2$ sorption gases. Determining surface morphology using the physical adsorption of gases is common in materials research of porous solids. However, as shown in Tables 2.8 and 2.9, results are not always consistent between adsorption gases. Diffusion of $N_2$ into carbon micropores is a very slow process at low temperatures and may lead to non-equilibrium data being recorded during the adsorption measurement and to a less accurate measurement of microporosity (Garrido et al., 1987). The advantage of $CO_2$ is that it can access smaller pores than $N_2$ and is less restricted by diffusion limitations. In a comprehensive review of the physical and structural properties of biochar, Chia et al. (2015) asserted that $CO_2$ is a better adsorption gas than N2 and provides a more accurate measurement of surface area.

BET analysis with $N_2$ gas resulted in a specific surface area (SSA) and pore volume for pine BC that were over 30 times higher than miscanthus BC. However, BET analysis with $CO_2$ gas resulted in SSA and pore volume values that were comparable for both pine and miscanthus BC. Discrepancies in the results could be attributed to the behavior of the adsorption gases during testing, or to difference in BC morphology.

Table 2.8 BET surface area, pore volume, and pore diameter of pine and miscanthus BC as measured with $N_2$ adsorption.

| Sample ID | $N_2$ BET Specific Surface Area ($m^2/g$) | Pore Volume ($cm^3/g$) | Pore Diameter (Å) |
|---|---|---|---|
| Miscanthus BC | 1.1491 ± 0.0122 | 0.000338 | 11.7696 |
| Pine BC | 34.6549 ± 0.1106 | 0.009472 | 10.9329 |

Table 2.9 BET surface area, pore volume, and pore diameter of pine and miscanthus BC as measured with $CO_2$ adsorption.

| Sample ID | $CO_2$ BET Specific Surface Area ($m^2/g$) | Pore Volume ($cm^3/g$) | Pore Diameter (Å) |
|---|---|---|---|
| Miscanthus BC | 109.7775 ± 0.5564 | 0.021135 | 7.7012 |
| Pine BC | 123.3280 ± 0.8612 | 0.024763 | 8.0315 |

### 2.5.2 Point of Zero Charge

Figures 2.6 - 2.8 show point of zero charge for pine BC, miscanthus BC, and poultry litter. Pine BC (Fig. 2.6) and poultry litter (Fig. 2.8) shared a net neutral charge at a pH of approximately 8, indicating a positive charge at solution pH values less than 8 and a negative charge at solution pH values greater than 8. Miscanthus (Fig. 2.7) exhibited a net neutral charge at a pH of approximately 7, and will, therefore, have a positive charge at solution pH values less than 7 and negative charge at solution pH values greater than 8. The differences in PZC between

pine (Fig. 2.6) and miscanthus (Fig. 2.7) BC indicate that the materials have differing capacities for the adsorption of substances.

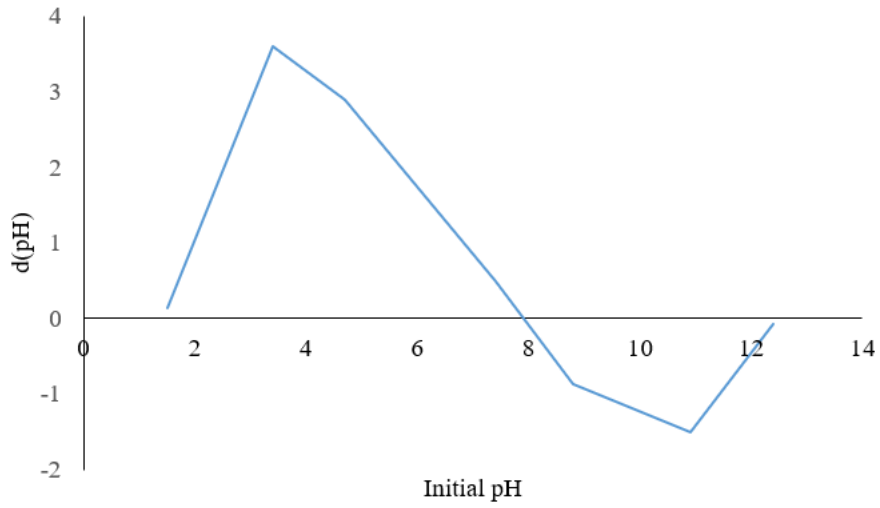


Figure 2.6 Point of zero charge for pine BC.

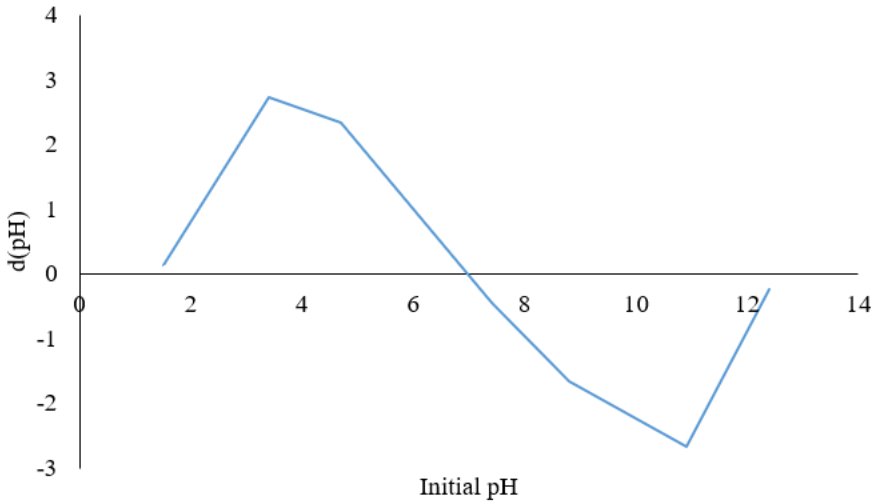


Figure 2.7 Point of zero charge for miscanthus BC.

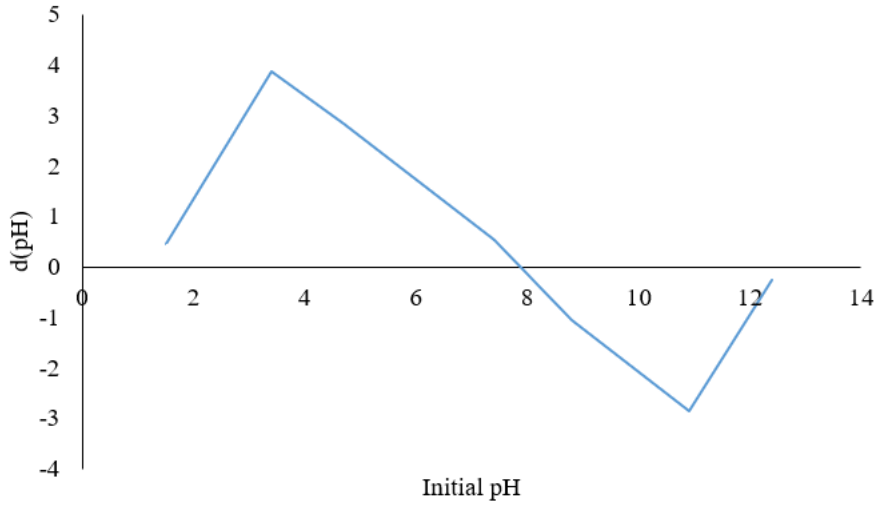


Figure 2.8 Point of zero charge for poultry litter.

### 2.5.3 Ultimate Analysis

Table 2.10 shows that pine was 97.25% carbon, while miscanthus was 69.07%. In a metanalysis of feedstock on biochar characteristics, Ippolito et al. (2020) reported higher carbon content for BC derived from woody materials than grasses, but not as much of a difference as reported here. The higher temperatures at which the pine BC was produced (700 – 1,000 ℃) most likely resulted in the elevated carbon levels and reduced hydrogen and oxygen levels. Production of biochar at high temperatures has been shown to increase carbon content, while reducing hydrogen and oxygen content (Ippolito et al., 2020). Grasses were higher in oxygen levels while wood-based biochar was greater in carbon. Ash contents of the materials were determined in Table 2.11, pine having the lowest content of the materials.

Table 2.10 Ultimate analysis results for pine BC, miscanthus BC, and poultry litter.

| Element | Pine (%) | Miscanthus (%) | Poultry Litter (%) |
|---|---|---|---|
| C | 97.25 | 69.07 | 33.97 |
| H | 0.704 | 3.544 | 4.964 |
| N | 0.41 | 0.50 | 3.80 |
| O | 1.59 | 26.801 | 24.131 |
| S | 0.046 | 0.085 | 1.075 |

Table 2.11 Ash content for pine BC, miscanthus BC, and poultry litter.

| | Pine | Miscanthus | Poultry Litter |
|---|---|---|---|
| **Initial Wt. (g)** | 0.103 | 0.102 | 0.105 |
| **Ash Wt. (g)** | 0.008 | 0.02 | 0.04 |

### 2.5.4 Scanning Electron Microscope

The physical structure of BCs is heavily influenced by the structural characteristics of the original biomass feedstock, the pyrolysis temperature, and the rate at which the feedstock is heated during the pyrolysis process. Pine BC has higher amount of C than miscanthus. Pine BC was also produced at a higher temperature and shows increasing degree of microporosity. Highest treatment temperature (HTT) is regarded to have the most significant effect on biochar structure. Zabaniotou et al. (2008) found that as HTT increased, so did the surface area of the final product for activated carbon. However, Brown et al. (2006) found that as HTT increased for pine BCs, the surface area decreased significantly. HTT-influenced reactions will vary significantly with different feedstocks. Pore sizes within the micropore range make the greatest contribution to the overall surface area. Micropore volume increased with increasing HTT. In our study, it would be useful to have a breakdown of the pore sizes. Both BCs in Figures 2.9 and 2.10 are scaled at 100 µm and differ in magnifications specified in the figure description.

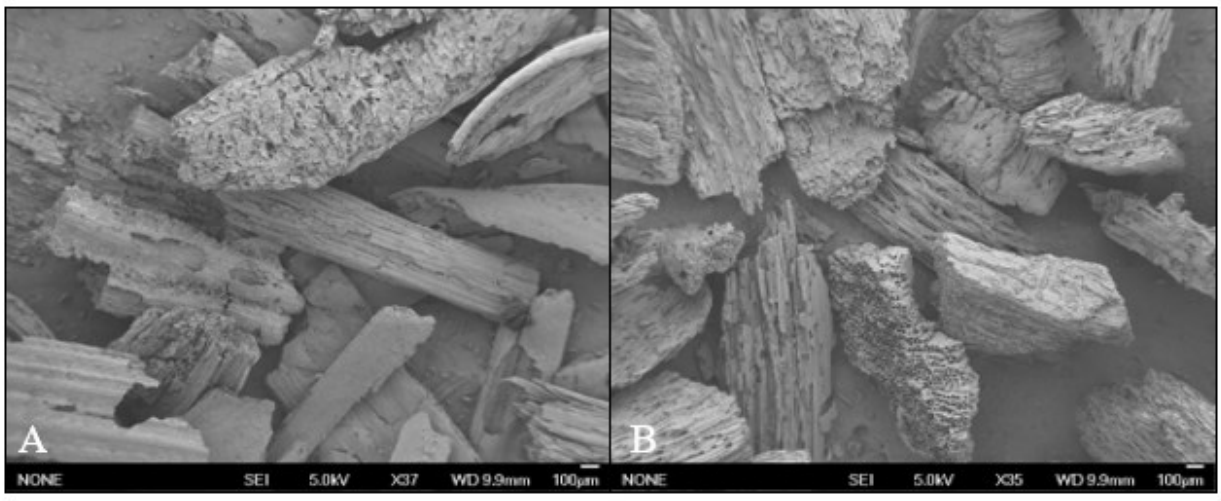


Figure 2.9 Miscanthus BC magnified x37 (A) and pine BC magnified x35 (B).

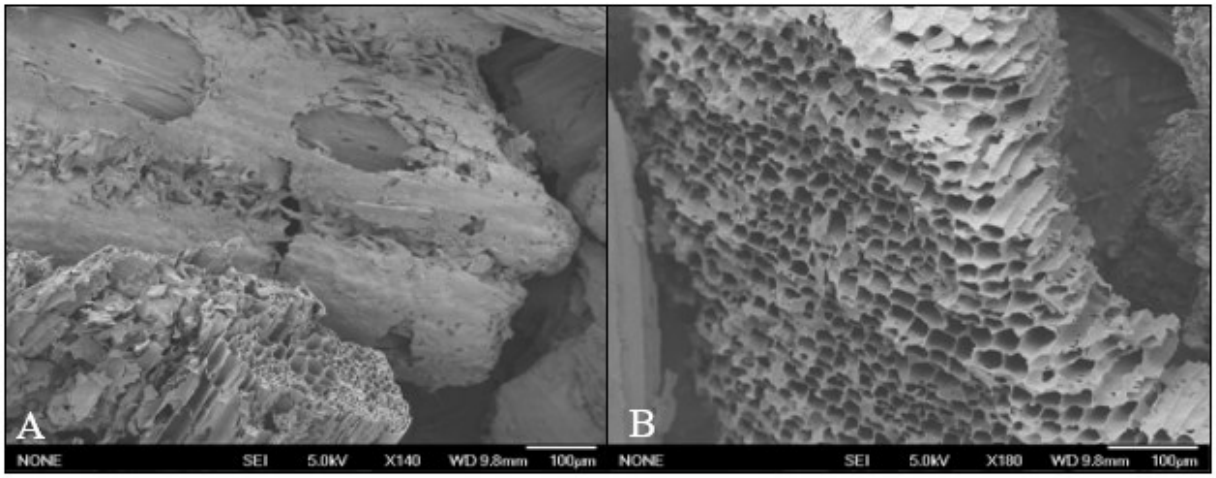


Figure 2.10 Miscanthus BC magnified x140 (A) and pine BC magnified x180 (B).

## 2.6 Discussion

Broilers are grown on litter and therefore encounter excreta during grow-out. In fact, they commonly ingest small quantities of litter, so the microbial makeup of the litter can influence gut microbiology. Since the quality of the litter can influence overall bird health and performance, it important that it is managed so that it is not detrimental to production. Litter also serves to absorb water from excreta and drinker line spillage until it can be removed by the ventilation system. Litter moisture can affect several different production parameters, including microbial activity (Dunlop et al., 2016). However, $A_w$ may be a more appropriate measure of the potential for microbial activity in poultry litter. $A_w$ is a measure of the partial vapor pressure of water in a solution divided by the partial vapor pressure of water and is a good indicator of how much water is available for microbial growth. Microorganisms commonly found in poultry house environments, such *Aspergillus* spp., *Staphylococcus* spp., *Salmonella* spp., *E. coli*, and *Clostridium* spp., grow in an $A_w$ between 0.75 and 0.98 (Lu et al., 2003; Fontana 2007; Taoukis

and Richardson, 2007; Singh et al., 2014). Dunlop et al. (2016) noted that a reduction of $A_w$ could decrease growth of negative microbial populations within the litter.

Increasing diffusion of water into the litter is important to reduce $A_w$ because moisture in the excreta that does not absorb to the litter relies on ventilation to evaporate and slows the drying processes, maintaining a high $A_w$ (Dunlop et al., 2016). Therefore, moisture transfer from excreta to litter could quicken moisture evaporation, reduce $A_w$, and ultimately lower microbial loads (Dunlop et al., 2016).

One factor that impacts the $A_w$ of poultry litter is friability (ease of crumbling), where higher friability may lead to lower $A_w$ and consequently microbial load reductions (Dunlop et al., 2016). During the broiler's life span in a house, the litter is being turned and manipulated by broiler activity, where the birds are constantly mixing the litter and breaking it down into smaller pieces (Dunlop et al., 2016). This continuous mixing allows for wet and dry materials to come into contact and facilitate the diffusion of water from wet to dry materials, thereby reducing $A_w$ (Dunlop et al., 2016). However, if litter is caked, it creates a compacted area of high moisture, decreasing the ability for moisture to evaporate. This increases $A_w$ and creates an environment favorable for microbial growth (Dunlop et al., 2016). BC has been reported to reduce compaction in poultry litter. In a study conducted by Mohammadi-Aragh et al. (2021), loblolly pine BC was added to poultry litter with inclusion rates of 0, 5, 10 and 20% and was left open to aerate in a testing block over time. Increasing the BC inclusion rate resulted in observable differences in poultry litter compaction. Treatments without BC developed into a thick, compacted sludge with intense odors while treatments with 20% BC were fine-textured and easily crumbled (Mohammadi-Aragh et al., 2021). Although the study did not measure friability, the results show

noticeable differences in the degree of compaction among BC inclusion rates. Therefore, adding rigid, porous materials, such as BC, to the litter may be useful to prevent caking and lower $A_w$.

Dunlop et al. (2016) conducted a study to examine the relationship of water activity of poultry litter to moisture content during a grow-out. They showed that the relationship between $A_w$ and moisture content changes during grow-out and follows a standard exponential curve. Interestingly, they also showed that $A_w$ decreased over the course of a flock with the addition of excreta and the breakdown of organic bedding material. Bernhart and Fasina (2009) also showed a similar relationship between $A_w$ and moisture content in poultry litter. Data presented here also shows that $A_w$ increased with moisture content. Although, these studies show the same general trends, there are discrepancies between $A_w$ values and corresponding moisture contents. For example, at 10% moisture content, our study showed a range of $A_w$ values between 0.53 and 0.63 for pine and miscanthus BC at all inclusion rates. Bernhart and Fasina (2009) and Dunlop et al. (2016) reported $A_w$ values of approximately 0.5 and 0.72, respectively, for poultry litter at 10% moisture content, suggesting that differences in bedding materials may alter the results for $A_w$. $A_w$ is closely related to the microbial, chemical, and physical properties of materials and since the materials represented in these studies are not identical, it is expected that they would have different $A_w$. Different materials at the same moisture content will usually have different $A_w$ (and vice versa). Therefore, making direct comparisons between different materials is difficult.

Biochar has a higher $A_w$ so water will tend to diffuse from biochar to litter. $A_w$ of excreta is 0.96 – 0.99, therefore $A_w$ of the litter needs to be less than this for there to be a thermodynamic gradient to drive the diffusion of water from the excreta into the litter, which we have for both biochar and the litter and 0 and 100% inclusion rates.

It was initially estimated that increasing biochar inclusion rate would lead to lower $A_w$. However, for both miscanthus and pine BC, increasing BC inclusion rate led to slight increases in $A_w$. There was an 11% and 4% increase between a 0% inclusion rate and a 100% inclusion rate for pine and miscanthus BC, respectively. In fact, since $A_w$ was lower at 0% inclusion rate than 100%, the thermodynamic gradient when BC and poultry litter are mixed would drive water from biochar into the poultry litter. Even though BC addition slightly increased $A_w$, its potential to reduce caking and promote friability may promote faster drying, which is important for pathogen reduction (Dunlop et al., 2016). Van der Hoeven-Hangoor et al. (2014) reported the $A_w$ of poultry litter to be 0.96-0.99. When averaged across BC inclusion rates, $A_w$ for pine and miscanthus BC at 40% MC are 0.96 and 0.95, respectively. At 30% MC, the average $A_w$ was 0.93 and 0.92 for pine and miscanthus BC, respectively. Although 30% is on the upper end for MC found in commercial broiler houses, our data showed that there may still be a thermodynamic gradient present to drive moisture from excreta into the litter. However, at moisture contents 40% and above, poultry litter loses its ability to absorb any excess water in excreta due to having similar $A_w$ values. The point of zero charge (PZC) data indicates that the materials have differing capacities for the adsorption of substances. To reduce the potential for microbial growth in litter, Dunlop et al. (2016) recommend maintaining $A_w$ around 0.85 – 0.91. Our results showed that poultry litter (without BC inclusion) at 30% MC had an $A_w$ of 0.90. While 30% MC is within the range recommended by Dunlop et al. (2016), 25% MC in poultry litter had an $A_w$ of 0.87 and serves as a more conservative target for maximum MC during a flock. BC inclusion rates can be as high as 40% in litter with a MC of 25% and still have an $A_w$ below 0.91.

The higher $A_w$ for raw BC than poultry litter may be due to morphological features. Larger macropores (>10 μm) are more clearly visible in the SEM images of miscanthus and pine BC. Macropores dominate the pore volume of most BC (Vanek et al., 2016), but it is the micropores (0.2 μm) that contribute most to the overall surface area (Chia et al., 2015). No data on the influence of pore size or surface area on water activity has been published on BC, but studies on the influence of water retention curves and pore size distribution in soils may help provide insight into why $A_w$ at a given moisture content is higher for BC than poultry litter. When water content decreases in soils, the large pores tend to empty first because large capillaries hold water less tightly than small capillaries (Wolf et al., 2013). Since BC is dominated by macropores, $A_w$ may be slightly elevated over poultry litter because the macropores simply are not binding the water as tightly.

# CHAPTER III

# EFFECTS OF VARYING INCLUSION RATES OF PINE AND MISCANTHUS BIOCHAR ON *ESCHERICHIA COLI* POPULATIONS IN USED BROILER LITTER

## 3.1 Introduction

Poultry litter in broiler houses is a naturally diverse microbiological habitat for *E. coli* and other pathogens that may cause adverse effects to bird welfare as well as humans (Arief Ismail et al., 2016). Litter absorbs moisture from bird excreta and drinking water spillage. Common moisture contents in commercial broiler litter during a grow-out is 20 - 30%. Water and excreta moisture is retained in the litter until it evaporates and is ventilated out of the house. Excess moisture can lead to decreased welfare due to footpad dermatitis and high ammonia levels, which can reduce bird performance (Linhoss et al., 2019).

Traditionally used amendments in the poultry industry are litter acidifiers, biological treatments, and water absorbents. Litter acidifiers, such as Poultry Litter Treatment® (PLT), are the most commonly used in the industry and have been shown to consistently reduce $NH_3$ production (Cook et al., 2011). PLT® is an example of a widely used acidifier that was employed to compare the effects of BC on *E. coli* reduction in this study. Biological treatments can alter microbial growth positively or negatively, depending upon their intended purpose. They can be used to prevent pathogen growth, fungal growth, or ammonia volatilization (Cook et al., 2011). Absorbents, such as BC, are used to absorb moisture and differ from acidifiers because they do not lower the pH.

In South America, poultry producers control bacterial pathogens during downtimes between flocks by utilizing quicklime (CaO) and tarping of the litter (Lopes et al., 2015). They found that quicklime increases pH and reduces $A_w$, enabling the reduction of pathogens. They also determined that the use of quicklime alone or quicklime and tarping is more effective at reducing pathogens than tarping only. Hill et al. (2019) found that BC has the potential to alter microbial communities in other medias. *E. coli* was grown for 12 hours in RPMI 1640 medium to determine whether activated carbon (AC) or BC could alter *E. coli* growth compared to an untreated control (Hill et al., 2019). They used fir wood chips pyrolyzed at temperatures between 900 – 1000˚C for 1 – 10 seconds. Hill et al. (2019) noted that the treatments adsorbed a significant number of amino acids and metabolites significantly reduce *E. coli* populations in the medium when compared to the control (Hill et al., 2019).

Past studies have shown that BC has the ability to influence microbial growth. Therefore, the goal of this study was to assess the effects of various inclusion rates of miscanthus and pine BC on *E. coli* populations in poultry litter.

## 3.2 Methods

Poultry litter was collected from a commercial broiler house located on the campus of Mississippi State University. Samples were collected from under the drinker line with a current flock of 27d old birds. The experiment was set up in a 2 x 6 factorial arrangement of treatments with main effects of pine and miscanthus litter amendment, with PLT® and inclusion rate. Pine and miscanthus BC was mixed with the poultry litter at inclusion rates of 0, 5, 10, 20, 25, and 30%. The 0% BC treatment served as the control for pine and miscanthus. Poultry Litter Treatment (PLT®, Jones-Hamilton) was also included as a comparison and surface applied to the litter at a rate 150 lbs. per 1000 $ft^2$. Three replicates were used for the control, BC-litter

treatments, the BC-PLT treatment (36 total samples). For day 0 (before adding BC or PLT), 12 samples were collected from treatments to assess baseline *E. coli* numbers. Therefore, 48 total samples were analyzed for *E. coli*.

Poultry litter was collected the morning of testing and sieved to particle sizes between 850 µm – 1,400 µm. 30 grams of litter were added to 250 mL bottles (Nalgene, Rochester, NY) and placed in an incubator at 26˚C for 48 hours (Figure 3.1) to establish microbial microcosms. Viable *E. coli* was found in previously collected litter samples, so *E. coli* inoculation was unnecessary. The opening of each bottle was covered with six layers of cheesecloth to allow air flow and prevent cross-contamination while in the incubator. BC was sieved to the same particle size as litter and then mixed into the bottles at rates of 5, 10, 20, 25, and 30% by weight (g) of amendment. To standardize the mixing procedure, bottles were vortexed for 30 s each. PLT® was surface applied (not mixed) to the litter at 2.13 g per 0.0314 $ft^2$, a rate that is commonly used in the commercial broiler industry. After adding the litter amendments, the samples were incubated for seven days. On day 2 and day 7 samples were collected for *E. coli*, and 2 g from each treatment rep were pooled for nutrient analysis and stored at -80°C.

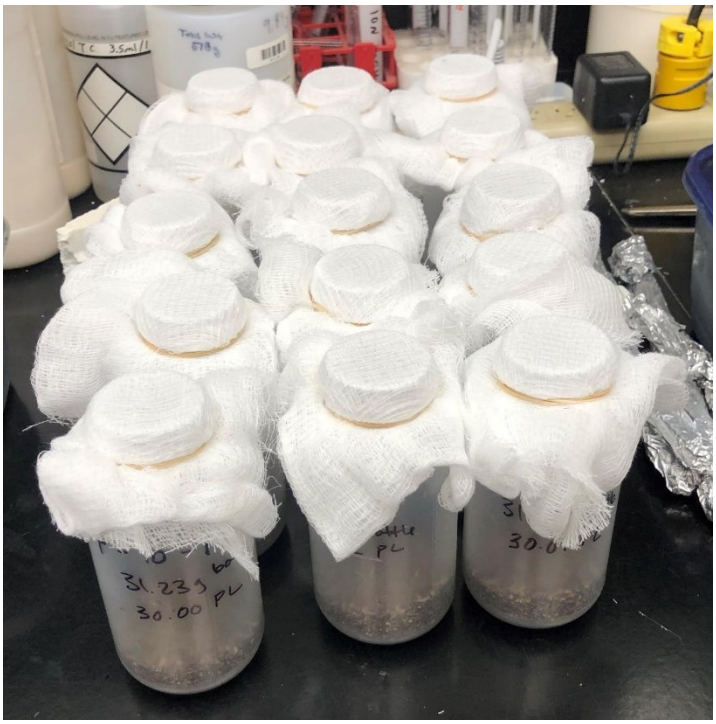

Figure 3.1 Microcosms covered in cheesecloth to allow airflow but minimize cross-contamination during incubation.

### 3.3 Microbial Sampling

For each BC-litter and PLT-litter replicate, a subsample of 5 grams was taken and placed in 50 mL Falcon® tubes (Corning, Corning, New York). The tubes were filled with 30 mL of sterile phosphate-buffered saline (PBS) (Fisher Bioreagents, Pittsburgh, PA) and vortexed for 1 minute. Large debris was removed by low-speed centrifugation (50 x g, 15 min, 4˚C). The supernatant was poured into new 50 mL Falcon® tubes and centrifuged at high-speed (3,650 x g, 15 min, 4˚C) to pellet microbial cells. The supernatant was removed, and the pellet was resuspended in 1 mL of PBS, vortexed for 1 minute, and serially diluted in PBS to $10^{-2}$, $10^{-4}$, $10^{-5}$, $10^{-6}$, $10^{-7}$, and $10^{-8}$.

*E. coli* was enumerated using Chromocult® agar plates (Merck KGaA, Darmstadt,

Germany). Eosin Methylene Blue (EMB) (Becton, Dickson and Co., Sparks, MD) selective media were used to validate *E. coli* growth. Chromocult® plates were enumerated after 48 hours incubation at 37°C. *E. coli* colonies grew on the agar in a blue/violet color. Chromocult® colonies were expressed in colony forming units per 1 g (CFU/g), which is a standard representation for microbial enumerations. In Figure 3.2, the *E. coli* are colonized on Chromocult®. Chromocult® dilutions of $10^{-5}$ and $10^{-6}$ were used for counting colonies as they were within range of detection (20 – 200 counts).

Samples identified in Figures 3.8 to 3.12 were stored in the freezer and sent off to the Mississippi State chemistry lab to be analyzed for nitrogen, phosphorus, potassium, pH, and moisture content. For sampling on day 0, 2 g from three random samples were pooled, and on days 2 and 7, 2 g from each treatment replicate were pooled and stored at -80°C until ready for analysis at the Mississippi State Chemical Laboratory (n= 25).

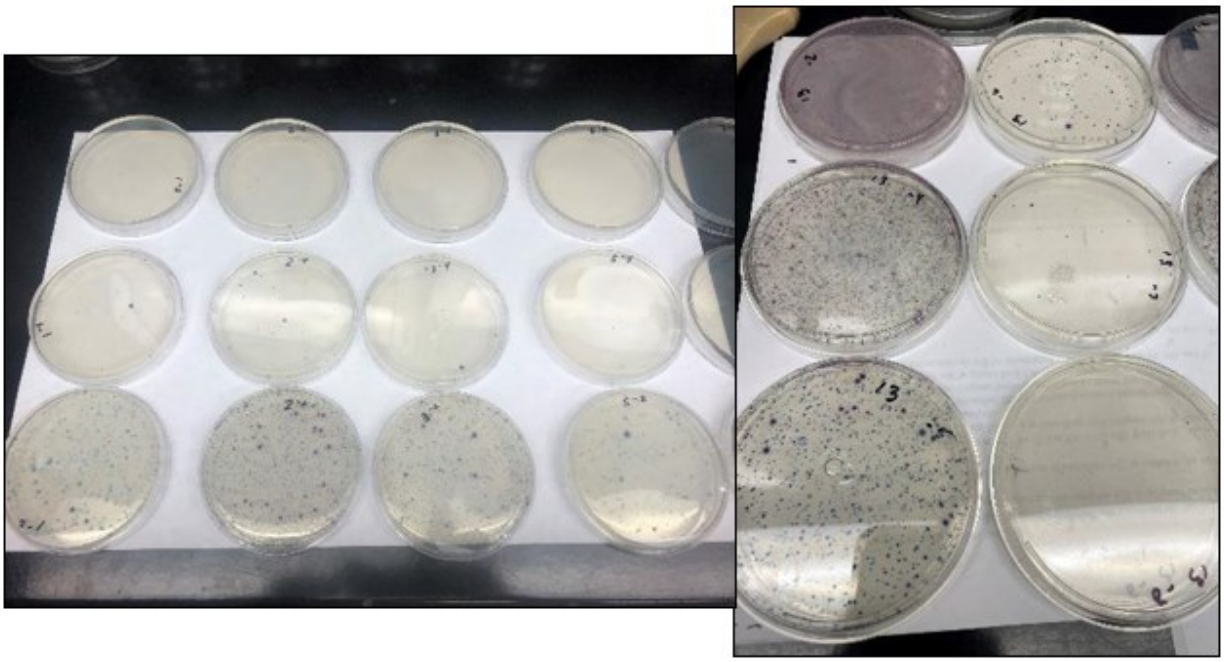

Figure 3.2 Chromocult® agar plates with *E. coli* growth.

### 3.4 Statistical Analysis

*E. coli* data was analyzed using PROC Mixed in SAS Version 9.4 and means were separated by Fisher's LSD via PDMIX800 (Saxon, 1998). Significant differences were made at $P < 0.05$. The experiment was set up in a 2 x 6 factorial arrangement of treatments with main effects of pine and miscanthus litter amendment, with PLT®, and inclusion rate. All statistical data can be found in Appendix B for more information.

### 3.5 Results

Fixed effects for *E. coli* are shown in Table 3.1, everything tested had a significant value ($P < 0.05$). Litter and BC mixture was sampled on day 2 and 7 to be analyze microbial activity of *E. coli*. Miscanthus BC resulted in a significantly higher *E. coli* growth than pine BC (Figure 3.3). The pine biochar characteristics in production proved to be a better amendment for the control of *E. coli* over miscanthus.

Table 3.1 Type 3 Tests of Fixed Effects.

| Effect | Num DF | Den DF | F Value | Pr > F |
|---|---|---|---|---|
| **BC** | 6 | 82 | 27.28 | <.0001 |
| **Type** | 1 | 82 | 57.34 | <.0001 |
| **Day** | 2 | 82 | 1124.94 | <.0001 |
| **BC x Day** | 12 | 82 | 13.59 | <.0001 |
| **BC x Type** | 6 | 82 | 6.30 | <.0001 |
| **Day x Type** | 2 | 82 | 13.66 | <.0001 |
| **BC x Day x Type** | 12 | 82 | 2.39 | 0.0108 |

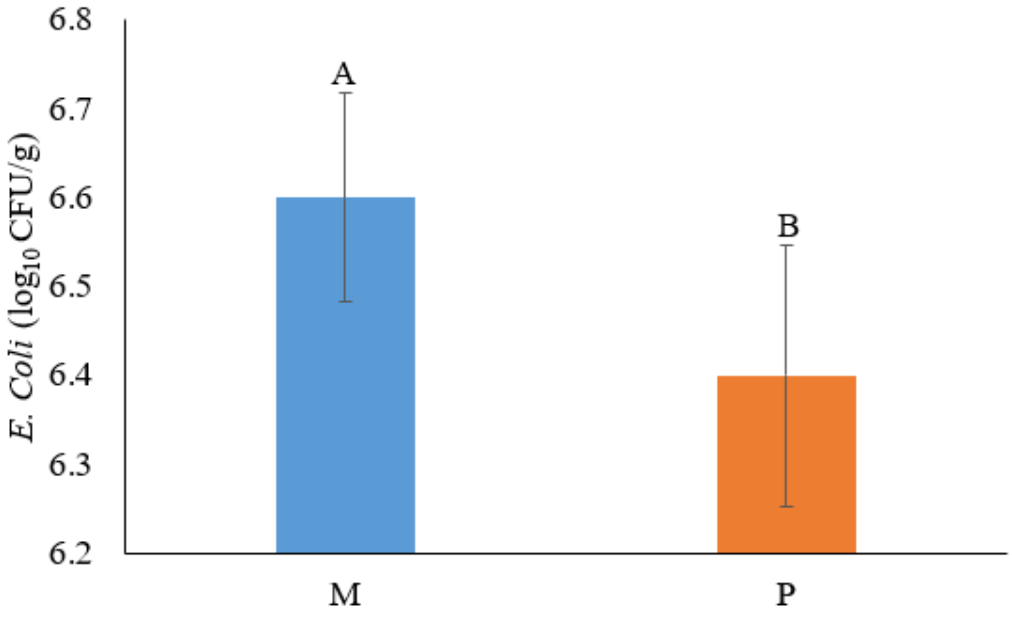


Figure 3.3 Comparison of *E. coli* growth in microcosms amended with pine and miscanthus BC.

In Figure 3.4, the *E. coli* counts decreased per day resulting in a lower CFU. The reduction of *E. coli* counts could be influenced by a closed system, no nutrients were added during the experiment. Miscanthus and pine BC inclusion rates are compared together in Figure

3.5, and there are opposite trends between pine and miscanthus *E. coli* counts. Pine trends downward while miscanthus has an upward trend for *E. coli* growth.

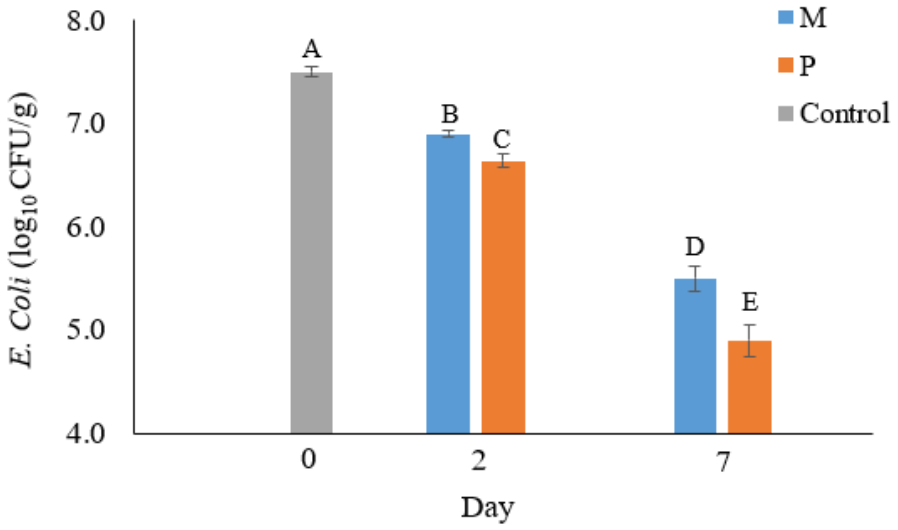


Figure 3.4 Comparison of *E. coli* growth by day in microcosms amended with pine and miscanthus BC.

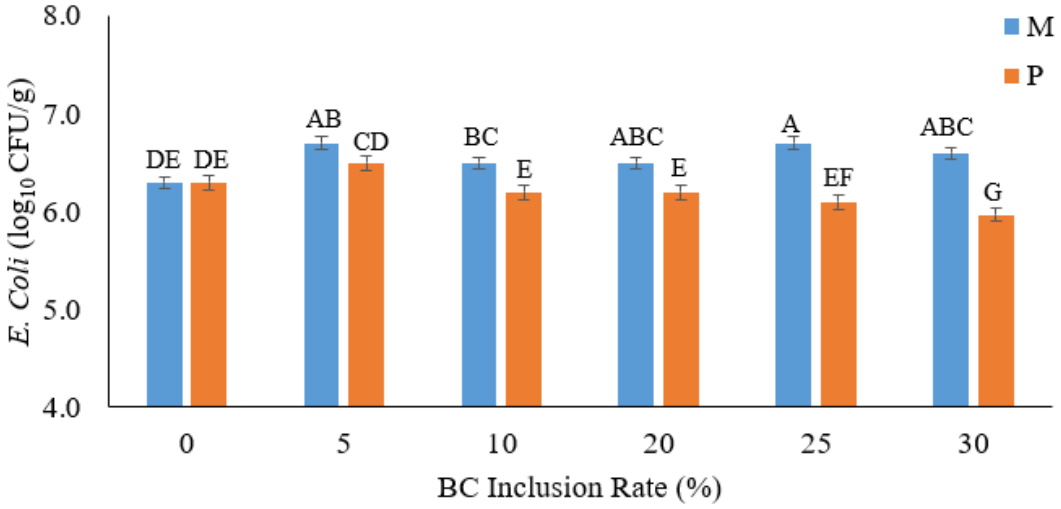


Figure 3.5 Comparison of *E. coli* growth by BC inclusion rate in microcosms amended with pine and miscanthus BC.

Overall, as the sampling progresses from day 2 to day 7 the *E. coli* reduces for pine (Figure 3.6). On day 2, *E. coli* growth decreases as pine BC inclusion rate increases. *E. coli* was greatly reduced on day 7, a pine BC inclusion rate of 20% BC had the largest decrease in *E. coli*. PLT remained with the highest CFUs on day 7. In Figure 3.7, miscanthus had not changed very much from inclusion rates on day 2 with PLT being the highest. On day 7, the *E. coli* decreased but not nearly the amount that pine had made it decrease. The control was lower than the rest of the results for miscanthus, PLT still being the highest overall.

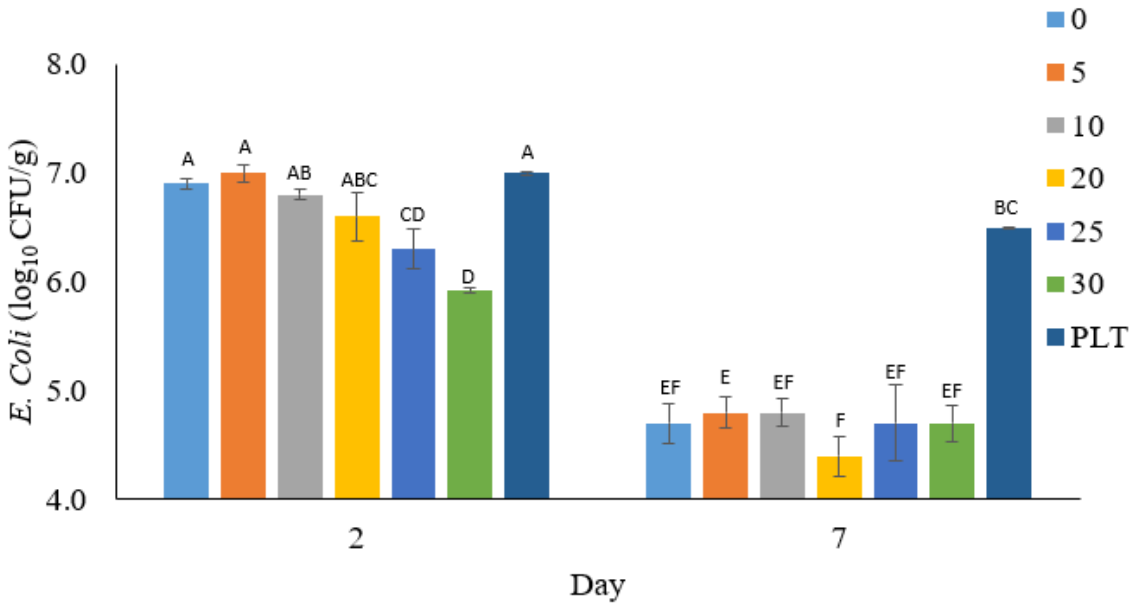


Figure 3.6 *E. coli* count by day and BC inclusion rate for pine.

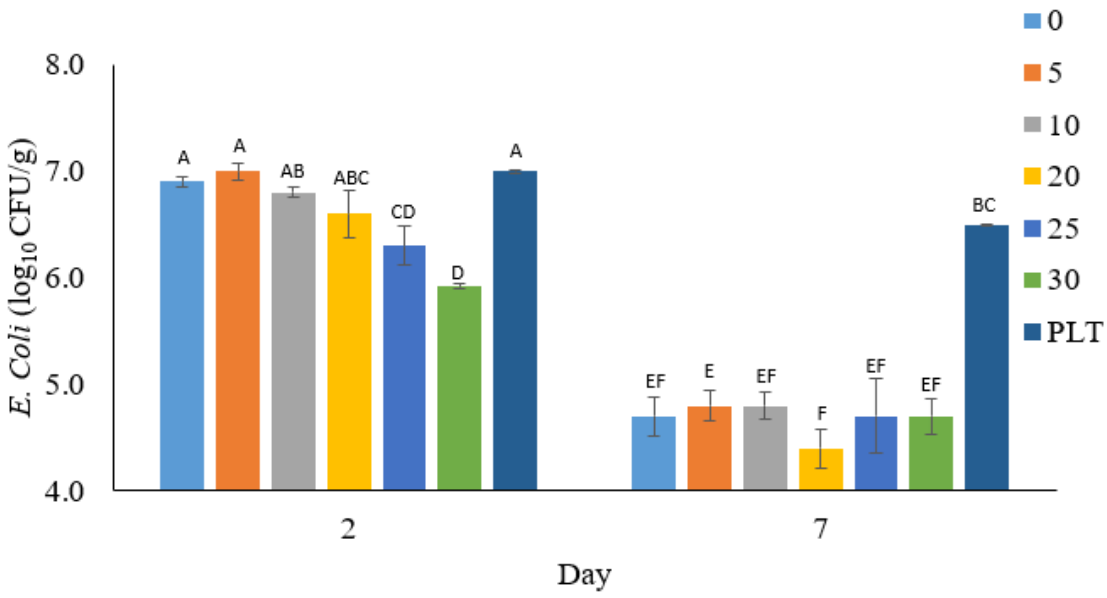


Figure 3.7 *E. coli* count by day and BC inclusion rate for miscanthus.

Figures 3.8 through 3.12 show the N, P, K, pH, and moisture values from all samples, the data was collected on day 2 and day 7 of the experiment. Figure 3.8 shows a decrease in nitrogen as the inclusion rate increases. Miscanthus contains more nitrogen on day 2 than pine does but decreases on day 7. Phosphorus varies with both treatments having some increasing with inclusion rates over sampling days (Figure 3.9).

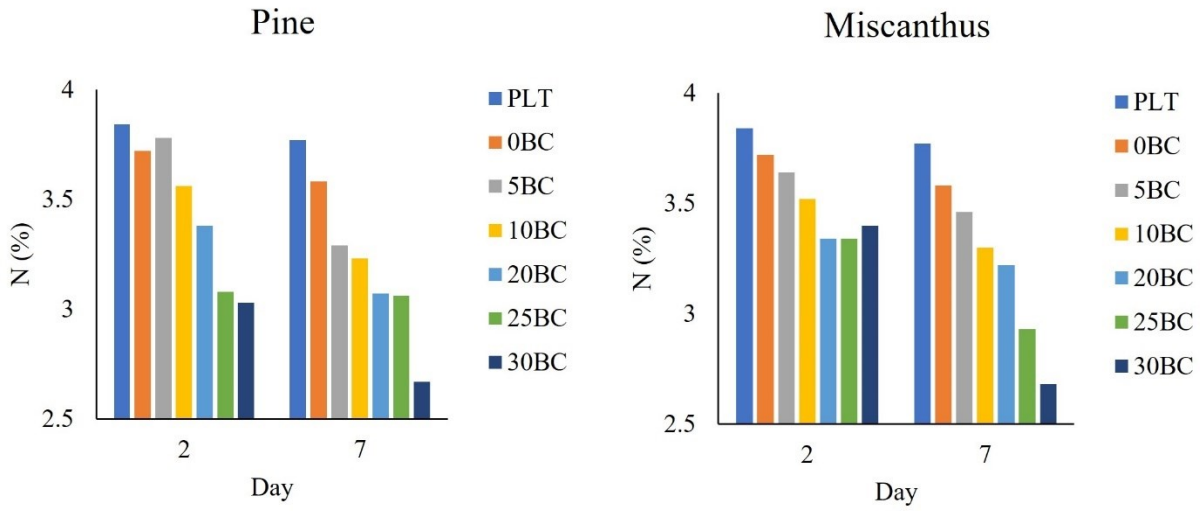


Figure 3.8 Nitrogen (%) by day for microcosms amended with varying rates of pine and miscanthus BC.

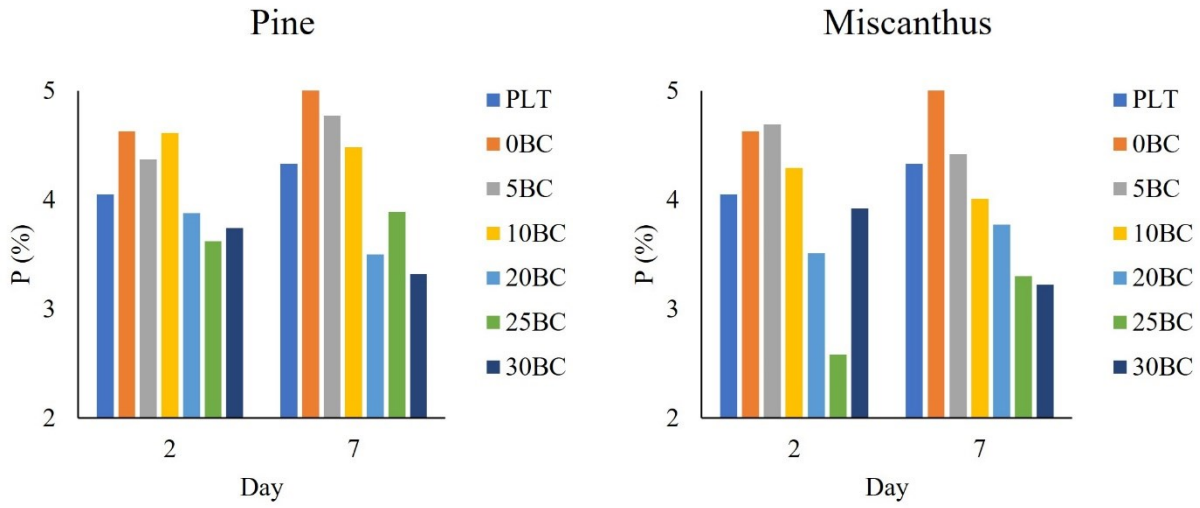


Figure 3.9 Phosphorus (%) by day for microcosms amended with varying rates of pine and miscanthus BC.

In Figure 3.10 potassium in both BC relates to potassium as some inclusion rates increase from day 2 to 7. In Figure 3.11, ranges of pH were roughly the same for both BC, but miscanthus had an increased pH for 30% inclusion rate on day 7. PLT, being an acidifier, is the lowest of about 5.75 pH while the BC were around 7 pH. In Figure 3.12, MC decreases per BC inclusion rate of both treatments from day 2 to day 7. PLT® had a lower starting than 0% BC, following a decreased MC on day 7.

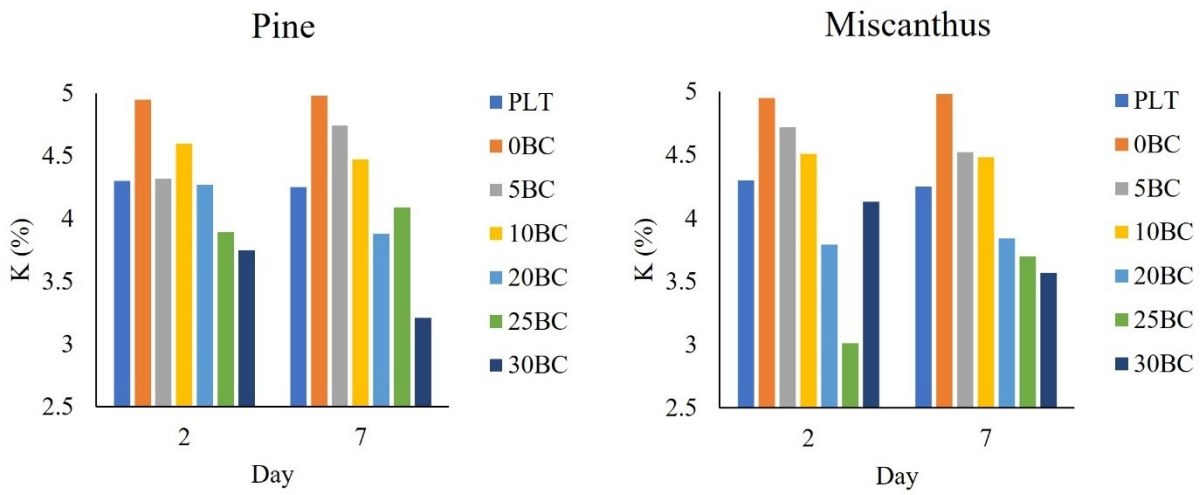


Figure 3.10 Potassium (%) by day for microcosms amended with varying rates of pine and miscanthus BC.

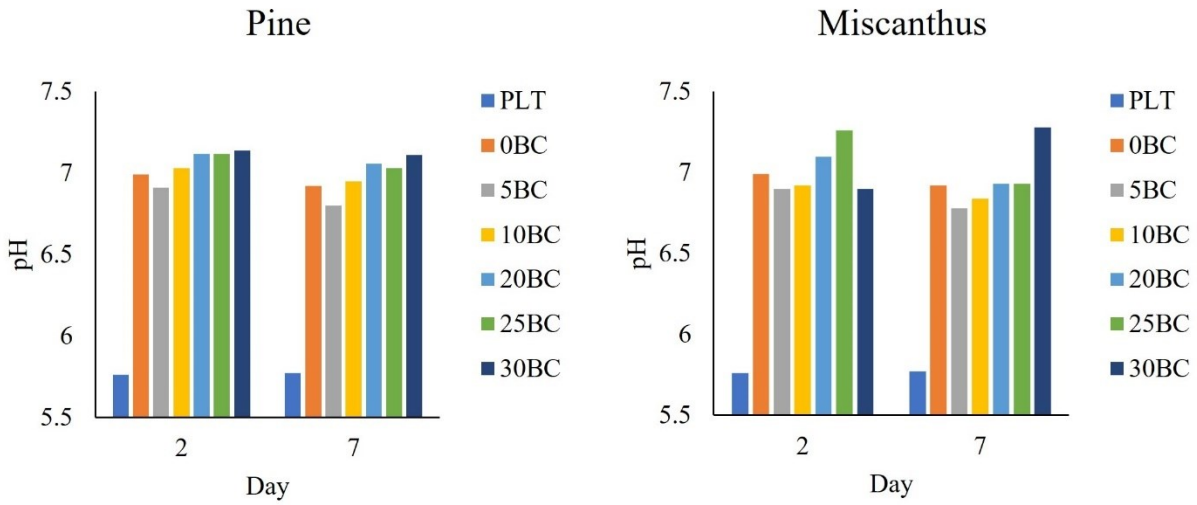


Figure 3.11 pH by day for microcosm amended with varying rates of pine and miscanthus BC.

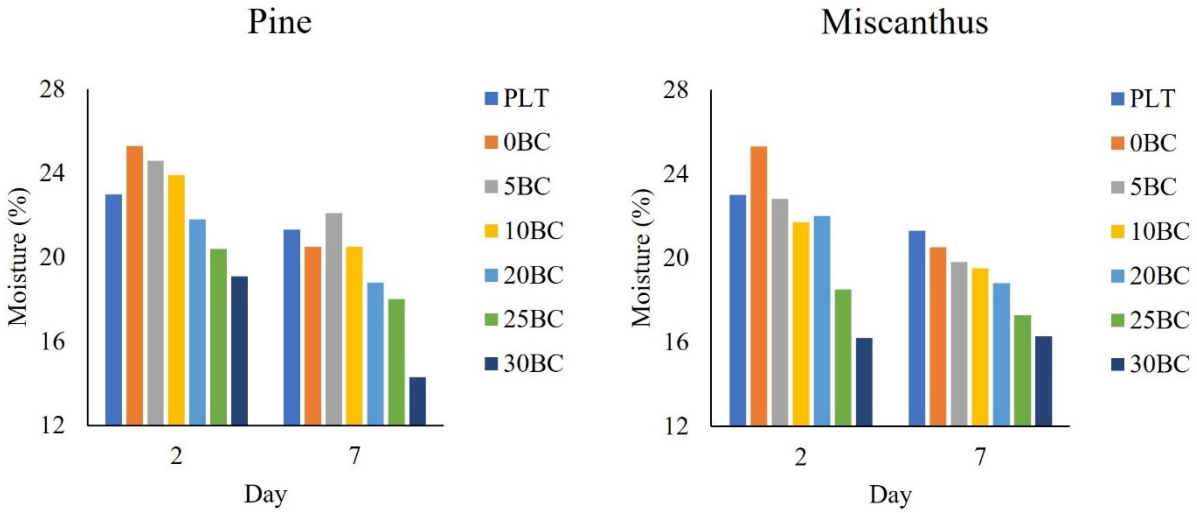


Figure 3.12 Moisture (%) for microbial samples by day for microcosm amended with varying rates of pine and miscanthus BC.

## 3.6 Discussion

While miscanthus resulted in higher *E. coli* growth in this study, $A_w$ was lower for miscanthus in the first experiment. Where water activity is higher, typically, bacterial growth will follow. The characteristics of the BC influenced the growth of the microbial populations by choice of material, pyrolysis temperature and time of pyrolysis (Ippolito et al., 2020). In future studies, $A_w$ should be taken when each sample was taken on day 2 and day 7 for more information on growth of *E. coli* while it is being assessed.

BC type had greatly influenced how microbial growth was affected. Pine BC had significantly less *E. coli* CFUs than miscanthus. Inclusion rates for pine did not have much affect but miscanthus had higher rates with increasing BC inclusion. Pyrolysis temperature and length of pyrolysis could have been a factor making the BC characteristics differ, in accordance with the findings from Ippolito et al. (2020). Pine had high pyrolysis heat (700 - 1000˚C) compared to miscanthus at 450˚C. Ippolito et al. (2020) stated that with higher pyrolysis temperatures, the resulting higher SSA retains more nutrients and contaminants, and an increased pore volume is associated with water availability being affected.

In addition, BC's highly stable aromatic structure confers resistance to microbial degradation, as microorganisms cannot easily utilize the carbon as an energy source (Lehmann et al., 2015). Feedstock choice and pyrolysis temperature have an impact on microorganism's ability to catabolize BC, with higher pyrolysis temperature increasing microbial resistance (Baldock and Smernik, 2002; Singh et al., 2012). Because miscanthus BC was produced at lower temperatures than pine, the carbon may have been more bioavailable to *E. coli*. This is reflected in the ultimate analysis, where miscanthus BC has a lower carbon content and higher oxygen and hydrogen content compared to pine, which is a result of less volatilization.

BC derived from softwood tree species has previously demonstrated an ability to reduce *E. coli* in bench-scale studies. Hill et al. (2019) conducted an experiment on *E. coli* growth in RPMI 1640 Medium using a similar BC from Biochar Supreme LLC (Everson, WA). The BC used in this study was derived from Douglas fir, another softwood species. However, the Douglas Fir BC was produced more precisely with a pyrolysis temperature of 900 - 1000˚C for 1 – 10 seconds. Hill et al. (2019) found that BC adsorbed more nutrients than the untreated media and reduced *E. coli* growth overall. Douglas fir BC causing a reduction in *E. coli* is consistent with the study that observed lower *E. coli* counts for pine BC.

Comparing inclusion rates to each other, the highest *E. coli* abundance was PLT®. The litter acidifier is used primarily to help with ammonia control, in terms of microbial control concept is the lower the pH level, there could be a lower bacterial load (Hardin and Roney 1989). The BC inclusion rates reflected close to the same CFUs amongst each other. Pope and Cherry (2000) conducted a study comparing the presence of *E. coli* in commercial broiler litter that was treated with PLT® and a control of untreated litter. Pope and Cherry (2000) applied PLT® at a rate of 5 pounds per 100 square feet, exactly 3 times less than the application rate used in our study. Their research found that the use of PLT® reduced presence of *E. coli* by nearly half the CFUs compared to untreated litter (Pope and Cherry 2000). Our research contradicted this finding, on day 2 the control and PLT® were similar levels but on day 7 the untreated litter had lower *E. coli* counts than that of the PLT®-treated litter on day 7.

Because the study was done by a closed system, no nutrients were added once the experiment units (Nalgene bottles) were incubated. This results in a decrease in nutrients as the bacteria proliferate, and eventually the bacteria will die off as a part of natural growth cycle and

nutrient restriction. In future research, a live trial could be done with pens of broilers to see the effects of the BC over a period with nutrients being continuously replenished by the birds.

Although BC effects on microbial populations has been previously studied, there is less information on the effects of pine and miscanthus BC on bacterial abundance in poultry litter. A study conducted by Mohammadi-Aragh et al. (2021) found that increasing levels of BC inclusion significantly reduced bacterial abundance in poultry litter. BC addition to soils and composts has been studied extensively, and BC's effect on pathogenic bacteria endemic to poultry litter has been evaluated. Soil can become populated with *E. coli*, causing possible contamination throughout the food system. Kolton et al. (2011) reported that BC addition to soils decreased the overall proportion of Proteobacteria, such as *Salmonella*, *E. coli* 0157:H7, *Shigella*, and *Vibrio*, by 24%. Gurtler et al. (2014) studied the effect on *E. coli* by adding both fast and slow pyrolysis BC to the soil and discovered that *E. coli* was reduced by 2.8 log CFU/g after BC addition compared to the control (no BC). In another study, willow BC (600˚C pyrolysis temp) was added to sewage sludge, another high bacterial-load material, and composted (Kopeć et al., 2017). After 140 days of composting, the authors found that willow BC addition reduced *E. coli* and *Salmonella* but resulted in an overall increased abundance in total bacteria (Kopeć et al., 2017). This finding demonstrated that wood BC's may selectively reduce pathogenic bacteria and is consistent with the findings in our study that observed a significant decrease in *E. coli* with wood BC addition at a similar pyrolysis temperature. Trupiano et al. (2017) used a commercial BC (500˚C pyrolysis temperature) derived from orchard-pruning's, another wood feedstock, to study its effects on composting and lettuce growth. The study found that BC addition to compost decreased cultivatable microorganisms but showed increases in enzymes involved in phosphorus, nitrogen, and carbon cycling (Trupiano et al., 2017). An increase in pH and other nutrients were

also found. It is possible that BC could hold $NH_4^+$ in the soil, adding to total N, and improving conditions for positive plant growth (Trupiano et al., 2017).

BC has been linked to many other studies for successfully modifying soil microbiota and nutrients. Wang et al. (2020) added a wheat straw BC, pyrolyzed at 550˚C, to the soil that enhanced the bacterial richness and diversity to boost plant growth. Wang et al. (2020) noted that soil increased in pH, P, and K while in our study pH increased but P and K decreased per increasing inclusion rate of BC.

Poultry litter can be an excellent, inexpensive fertilizer for many farmers once the litter is cleaned out of the broiler house (Arief Ismail et al., 2016). It is typically composted, typically losing nutrients to volatilization. Agyarko-Mintah et al. (2017) studied the effect of green waste BC (GWBC) and poultry litter BC (PLB) had on retaining nutrients and lowering greenhouse emissions from composting poultry litter. The GWBC was made from forest wastes like tree branches, bark, and leaves. Agyarko-Mintah et al. (2017) results suggested that both BC were effective with 10% addition for lowering $N_2O$ and $CH_4$. With the addition of BC into the litter, there is greater possibilities for increasing the retention of nutrients in the poultry litter.

# CHAPTER IV

# CONCLUSIONS

Feedstock choice, pyrolysis temperatures and times, and chemical composition can produce BC with different physical and chemical characteristics (Ippolito et al., 2020). Differences in the final products are evident in the properties of pine and miscanthus BC discussed in this thesis. For example, wood-based materials have higher amounts of carbon but are lacking in other elements, such as N, S, P, K, Ca, and P (Ippolito et al., 2020). BC produced from grasses, such as miscanthus, have higher concentrations of potassium and calcium and lower amounts of carbon. Future studies could examine the influence of different pyrolysis conditions on water activity, morphological characteristics of pine and miscanthus BC, and pathogen mitigation in broiler litter.

The decrease in subtherapeutic antibiotic administration in broiler production has increased the need to address production challenges caused by pathogens, such as *E. coli*. One potential way to improve bird health and reduce bacterial infection is through the addition of litter amendments that absorb moisture and reduce water activity. The objectives of this research were to 1) evaluate water activity of poultry litter amended with various rates of pine and miscanthus BC, and 2) determine the effects of different BC inclusion rates on litter *E. coli* populations. Results indicated that water activity of poultry litter increased with increasing rates of BC. However, elevated water activities at higher BC inclusion rates did not necessarily lead to increased *E. coli* growth in broiler litter. In fact, higher inclusion rates of pine BC decreased *E.*

*coli* growth, while higher miscanthus BC inclusion rates increased growth. Pine was pyrolyzed at a higher temperature (700 – 1,000º C) than miscanthus, which may have created BC more recalcitrant to decomposition and overall microbial growth. Results from these studies show that while BC may show promise as broiler litter amendment, production parameters (i.e., pyrolysis temperature, pyrolysis time, and parent material) of BC affect its chemical and physical structure and should be considered when researching BC for specific applications.

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# APPENDIX A

# WATER ACTIVTY (CHAPTER II) – SUPPLEMENTAL TABLES

Table A.1 Interaction effects of type x MC.

| Type | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|
| p | 50 | 0.9756 | 0.001734 | A |
| m | 50 | 0.9697 | 0.001734 | B |
| p | 40 | 0.9607 | 0.001734 | C |
| m | 40 | 0.9493 | 0.001734 | D |
| p | 30 | 0.9338 | 0.001734 | E |
| m | 30 | 0.9188 | 0.001734 | F |
| p | 25 | 0.9088 | 0.001734 | G |
| m | 25 | 0.8832 | 0.001734 | H |
| p | 20 | 0.8705 | 0.001734 | I |
| m | 20 | 0.8362 | 0.001734 | J |
| p | 15 | 0.7957 | 0.001734 | K |
| m | 15 | 0.7804 | 0.001734 | L |
| m | 10 | 0.6087 | 0.001734 | M |
| p | 10 | 0.5806 | 0.001734 | N |

Table A.2 Interaction effects of type x BC.

| Type | BC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|
| p | 100 | 0.9262 | 0.001734 | A |
| p | 50 | 0.8696 | 0.001734 | B |
| m | 100 | 0.8629 | 0.001734 | C |
| m | 40 | 0.8629 | 0.001734 | C |
| m | 30 | 0.8610 | 0.001734 | CD |
| p | 40 | 0.8567 | 0.001734 | DE |
| p | 30 | 0.8528 | 0.001734 | EF |
| p | 20 | 0.8515 | 0.001734 | FG |
| m | 50 | 0.8488 | 0.001734 | FG |
| m | 20 | 0.8473 | 0.001734 | G |

Table A.2 (continued)

| Type | BC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|
| p | 10 | 0.8393 | 0.001734 | H |
| m | 10 | 0.8339 | 0.001734 | I |
| p | 0 | 0.8296 | 0.001734 | I |
| m | 0 | 0.8296 | 0.001734 | I |

Table A.3 Interaction effects of BC x MC.

| BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|
| 100 | 50 | 0.9897 | 0.003244 | A |
| 100 | 40 | 0.9849 | 0.003244 | AB |
| 50 | 50 | 0.9776 | 0.003244 | BC |
| 40 | 50 | 0.9765 | 0.003244 | BC |
| 100 | 30 | 0.9714 | 0.003244 | CD |
| 20 | 50 | 0.9701 | 0.003244 | CD |
| 30 | 50 | 0.9694 | 0.003244 | CDE |
| 10 | 50 | 0.9647 | 0.003244 | DEF |
| 0 | 50 | 0.9607 | 0.003244 | EF |
| 40 | 40 | 0.9572 | 0.003244 | FG |
| 50 | 40 | 0.9564 | 0.003244 | FG |
| 20 | 40 | 0.9511 | 0.003244 | GH |
| 30 | 40 | 0.9490 | 0.003244 | GH |
| 100 | 25 | 0.9483 | 0.003244 | GH |
| 10 | 40 | 0.9471 | 0.003244 | HI |
| 100 | 20 | 0.9422 | 0.003244 | HIJ |
| 0 | 40 | 0.9391 | 0.003244 | IJ |
| 50 | 30 | 0.9360 | 0.003244 | JK |
| 40 | 30 | 0.9272 | 0.003244 | KL |
| 30 | 30 | 0.9258 | 0.003244 | L |

Table A.3 (continued)

| BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|
| 20 | 30 | 0.9163 | 0.003244 | M |
| 50 | 25 | 0.9092 | 0.003244 | MN |
| 10 | 30 | 0.9069 | 0.003244 | N |
| 0 | 30 | 0.9004 | 0.003244 | NO |
| 40 | 25 | 0.8973 | 0.003244 | OP |
| 100 | 15 | 0.8916 | 0.003244 | OPQ |
| 30 | 25 | 0.8904 | 0.003244 | PQ |
| 20 | 25 | 0.8856 | 0.003244 | Q |
| 10 | 25 | 0.8752 | 0.003244 | R |
| 0 | 25 | 0.8662 | 0.003244 | RS |
| 40 | 20 | 0.8625 | 0.003244 | S |
| 50 | 20 | 0.8619 | 0.003244 | S |
| 30 | 20 | 0.8442 | 0.003244 | T |
| 20 | 20 | 0.8397 | 0.003244 | T |
| 10 | 20 | 0.8121 | 0.003244 | U |
| 0 | 20 | 0.8107 | 0.003244 | U |
| 40 | 15 | 0.7950 | 0.003244 | V |
| 50 | 15 | 0.7906 | 0.003244 | VW |
| 30 | 15 | 0.7843 | 0.003244 | W |
| 20 | 15 | 0.7656 | 0.003244 | X |
| 10 | 15 | 0.7546 | 0.003244 | Y |
| 0 | 15 | 0.7345 | 0.003244 | Z |
| 30 | 10 | 0.6353 | 0.003244 | (2)A |
| 20 | 10 | 0.6172 | 0.003244 | (2)B |
| 40 | 10 | 0.6029 | 0.003244 | (2)C |
| 0 | 10 | 0.5955 | 0.003244 | (2)C |
| 10 | 10 | 0.5954 | 0.003244 | (2)C |
| 50 | 10 | 0.5826 | 0.003244 | (2)D |
| 100 | 10 | 0.5336 | 0.003244 | (2)E |

Table A.4 Interaction effects of Type x MC x BC.

| Type | BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|---|
| p | 100 | 50 | 0.9969 | 0.004587 | A |
| p | 100 | 40 | 0.9963 | 0.004587 | A |
| p | 100 | 30 | 0.9949 | 0.004587 | AB |
| p | 100 | 25 | 0.9936 | 0.004587 | AB |
| p | 100 | 20 | 0.9914 | 0.004587 | AB |
| p | 50 | 50 | 0.9835 | 0.004587 | BC |
| m | 100 | 50 | 0.9825 | 0.004587 | BCD |
| p | 40 | 50 | 0.9821 | 0.004587 | BCD |
| p | 100 | 15 | 0.9753 | 0.004587 | CDE |
| p | 50 | 40 | 0.9741 | 0.004587 | CDE |
| m | 100 | 40 | 0.9736 | 0.004587 | CDEF |
| p | 20 | 50 | 0.9720 | 0.004587 | CDEFG |
| m | 50 | 50 | 0.9717 | 0.004587 | CDEFG |
| m | 40 | 50 | 0.9708 | 0.004587 | CDEFG |
| m | 30 | 50 | 0.9702 | 0.004587 | DEFG |
| p | 30 | 50 | 0.9687 | 0.004587 | EFG |
| m | 20 | 50 | 0.9683 | 0.004587 | EFG |
| p | 10 | 50 | 0.9656 | 0.004587 | EFGH |
| m | 10 | 50 | 0.9637 | 0.004587 | EFGHI |
| p | 40 | 40 | 0.9611 | 0.004587 | FGHIJ |
| p | 0 | 50 | 0.9607 | 0.004587 | GHIJK |
| m | 0 | 50 | 0.9607 | 0.004587 | GHIJK |
| m | 40 | 40 | 0.9533 | 0.004587 | HIJKL |
| p | 20 | 40 | 0.9533 | 0.004587 | HIJKL |

Table A.4 (continued)

| Type | BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|---|
| p | 10 | 40 | 0.9519 | 0.004587 | IJKL |
| m | 30 | 40 | 0.9491 | 0.004587 | JKLM |
| m | 20 | 40 | 0.9490 | 0.004587 | JKLM |
| p | 30 | 40 | 0.9489 | 0.004587 | JKLM |
| m | 100 | 30 | 0.9480 | 0.004587 | KLM |
| p | 50 | 30 | 0.9476 | 0.004587 | LM |
| m | 10 | 40 | 0.9423 | 0.004587 | LMN |
| m | 0 | 40 | 0.9391 | 0.004587 | MNO |
| p | 0 | 40 | 0.9391 | 0.004587 | MNO |
| m | 50 | 40 | 0.9388 | 0.004587 | MNO |
| p | 40 | 30 | 0.9317 | 0.004587 | NOP |
| p | 30 | 30 | 0.9295 | 0.004587 | OPQ |
| m | 50 | 30 | 0.9245 | 0.004587 | PQR |
| m | 40 | 30 | 0.9228 | 0.004587 | PQR |
| m | 30 | 30 | 0.9222 | 0.004587 | PQR |
| p | 50 | 25 | 0.9202 | 0.004587 | PQR |
| p | 20 | 30 | 0.9185 | 0.004587 | QRS |
| m | 20 | 30 | 0.9141 | 0.004587 | RST |
| p | 10 | 30 | 0.9138 | 0.004587 | RST |
| p | 40 | 25 | 0.9066 | 0.004587 | STU |
| m | 100 | 25 | 0.9030 | 0.004587 | TUV |
| p | 30 | 25 | 0.9010 | 0.004587 | UV |
| m | 0 | 30 | 0.9004 | 0.004587 | UVW |
| p | 0 | 30 | 0.9004 | 0.004587 | UVW |
| m | 10 | 30 | 0.8999 | 0.004587 | UVW |
| m | 50 | 25 | 0.8982 | 0.004587 | UVW |
| p | 20 | 25 | 0.8945 | 0.004587 | UVW |

Table A.4 (continued)

| Type | BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|---|
| m | 100 | 20 | 0.8930 | 0.004587 | VW |
| p | 50 | 20 | 0.8902 | 0.004587 | VWX |
| m | 40 | 25 | 0.8880 | 0.004587 | WXY |
| m | 30 | 25 | 0.8799 | 0.004587 | XYZ |
| p | 10 | 25 | 0.8797 | 0.004587 | XYZ |
| m | 20 | 25 | 0.8768 | 0.004587 | YZ(2)A |
| p | 40 | 20 | 0.8741 | 0.004587 | Z(2)A |
| m | 10 | 25 | 0.8707 | 0.004587 | Z(2)A |
| p | 0 | 25 | 0.8662 | 0.004587 | (2)A |
| m | 0 | 25 | 0.8662 | 0.004587 | (2)A |
| m | 40 | 20 | 0.8509 | 0.004587 | (2)B |
| p | 20 | 20 | 0.8504 | 0.004587 | (2)B |
| m | 30 | 20 | 0.8445 | 0.004587 | (2)BC |
| p | 30 | 20 | 0.8439 | 0.004587 | (2)BC |
| m | 50 | 20 | 0.8336 | 0.004587 | (2)CD |
| p | 10 | 20 | 0.8324 | 0.004587 | (2)CD |
| m | 20 | 20 | 0.8290 | 0.004587 | (2)D |
| m | 0 | 20 | 0.8107 | 0.004587 | (2)E |
| p | 0 | 20 | 0.8107 | 0.004587 | (2)E |
| m | 100 | 15 | 0.8080 | 0.004587 | (2)EF |
| m | 40 | 15 | 0.8067 | 0.004587 | (2)EFG |
| p | 50 | 15 | 0.7960 | 0.004587 | (2)FGH |
| m | 30 | 15 | 0.7947 | 0.004587 | (2)GH |
| m | 10 | 20 | 0.7919 | 0.004587 | (2)H |
| m | 50 | 15 | 0.7852 | 0.004587 | (2)HI |
| p | 40 | 15 | 0.7833 | 0.004587 | (2)HI |
| p | 30 | 15 | 0.7739 | 0.004587 | (2)IJ |

Table A.4 (continued)

| Type | BC | MC | $A_w$ | Standard Error | Letter Group |
|---|---|---|---|---|---|
| m | 20 | 15 | 0.7696 | 0.004587 | (2)J |
| m | 10 | 15 | 0.7640 | 0.004587 | (2)J |
| p | 20 | 15 | 0.7617 | 0.004587 | (2)J |
| p | 10 | 15 | 0.7453 | 0.004587 | (2)K |
| p | 0 | 15 | 0.7345 | 0.004587 | (2)K |
| m | 0 | 15 | 0.7345 | 0.004587 | (2)K |
| m | 30 | 10 | 0.6667 | 0.004587 | (2)L |
| m | 40 | 10 | 0.6476 | 0.004587 | (2)M |
| m | 20 | 10 | 0.6245 | 0.004587 | (2)N |
| p | 20 | 10 | 0.6099 | 0.004587 | (2)O |
| m | 10 | 10 | 0.6047 | 0.004587 | (2)OP |
| p | 30 | 10 | 0.6039 | 0.004587 | (2)OP |
| p | 0 | 10 | 0.5955 | 0.004587 | (2)PQ |
| m | 0 | 10 | 0.5955 | 0.004587 | (2)PQ |
| m | 50 | 10 | 0.5895 | 0.004587 | (2)Q |
| p | 10 | 10 | 0.5862 | 0.004587 | (2)QR |
| p | 50 | 10 | 0.5757 | 0.004587 | (2)R |
| p | 40 | 10 | 0.5582 | 0.004587 | (2)S |
| p | 100 | 10 | 0.5349 | 0.004587 | (2)T |
| m | 100 | 10 | 0.5323 | 0.004587 | (2)T |

# APPENDIX B

## *E. COLI* (CHAPTER III) – SUPPLEMENTAL TABLES

Table B.1 BC inclusion rate by day on *E. coli* populations.

| BC | Day | *E. coli* CFU/g | Standard Error | Letter Group |
|---|---|---|---|---|
| 99 | 0 | 7.6533 | 0.08916 | A |
| 5 | 0 | 7.6133 | 0.08916 | AB |
| 20 | 0 | 7.5500 | 0.1261 | AB |
| 25 | 0 | 7.5217 | 0.08916 | AB |
| 30 | 0 | 7.4400 | 0.08916 | AB |
| 10 | 0 | 7.3717 | 0.08916 | BC |
| 0 | 0 | 7.1800 | 0.08916 | CD |
| 99 | 2 | 7.0167 | 0.08916 | DE |
| 0 | 2 | 6.9333 | 0.08916 | DEF |
| 5 | 2 | 6.8617 | 0.08916 | EF |
| 10 | 2 | 6.7867 | 0.08916 | EF |
| 20 | 2 | 6.7550 | 0.08916 | FG |
| 25 | 2 | 6.5300 | 0.08916 | GH |
| 99 | 7 | 6.4500 | 0.08916 | H |
| 30 | 2 | 6.3250 | 0.08916 | H |
| 25 | 7 | 5.2700 | 0.08916 | I |
| 5 | 7 | 5.2433 | 0.08916 | I |
| 30 | 7 | 5.0950 | 0.08916 | IJ |
| 10 | 7 | 4.9750 | 0.08916 | JK |
| 20 | 7 | 4.7450 | 0.08916 | KL |
| 0 | 7 | 4.6600 | 0.08916 | L |

Table B.2 BC inclusion rate by type on *E. coli* populations.

| BC | Type | *E. coli* CFU/g | Standard Error | Letter Group |
|---|---|---|---|---|
| 99 | p | 7.0400 | 0.07280 | A |
| 99 | m | 7.0400 | 0.07280 | A |
| 25 | m | 6.7489 | 0.07280 | B |
| 5 | m | 6.6944 | 0.07280 | BC |
| 30 | m | 6.6056 | 0.07280 | BCD |
| 20 | m | 6.5244 | 0.09398 | BCD |
| 10 | m | 6.5100 | 0.07280 | CD |
| 5 | p | 6.4511 | 0.07280 | DE |
| 0 | p | 6.2578 | 0.07280 | EF |
| 0 | m | 6.2578 | 0.07280 | EF |
| 10 | p | 6.2456 | 0.07280 | F |
| 20 | p | 6.1756 | 0.07280 | F |
| 25 | p | 6.1322 | 0.07280 | FG |
| 30 | p | 5.9678 | 0.07280 | G |

Table B.3 Day by type on *E. coli* populations.

| Day | Type | *E. coli* CFU/g | Standard Error | Letter Group |
|---|---|---|---|---|
| 0 | m | 7.5271 | 0.05404 | A |
| 0 | p | 7.4243 | 0.04766 | A |
| 2 | m | 6.8510 | 0.04766 | B |
| 2 | p | 6.6371 | 0.04766 | C |
| 7 | m | 5.4995 | 0.04766 | D |
| 7 | p | 4.9114 | 0.04766 | E |

Table B.4 BC inclusion rate by day by type on *E. coli* populations.

| BC | Day | Type | *E. coli* CFU/g | Standard Error | Letter Group |
|---|---|---|---|---|---|
| 99 | 0 | m | 7.6533 | 0.1261 | A |
| 5 | 0 | m | 7.6533 | 0.1261 | A |
| 99 | 0 | p | 7.6533 | 0.1261 | A |
| 25 | 0 | m | 7.6033 | 0.1261 | AB |
| 20 | 0 | p | 7.6000 | 0.1261 | AB |
| 30 | 0 | m | 7.5833 | 0.1261 | AB |
| 5 | 0 | p | 7.5733 | 0.1261 | ABC |
| 10 | 0 | m | 7.5167 | 0.1261 | ABCD |
| 20 | 0 | m | 7.5000 | 0.2184 | ABCDE |
| 25 | 0 | p | 7.4400 | 0.1261 | ABCD |
| 30 | 0 | p | 7.2967 | 0.1261 | BCDEF |
| 10 | 0 | p | 7.2267 | 0.1261 | CDEFG |
| 0 | 0 | m | 7.1800 | 0.1261 | DEFG |
| 0 | 0 | p | 7.1800 | 0.1261 | DEFG |
| 99 | 2 | m | 7.0167 | 0.1261 | EFGH |
| 99 | 2 | p | 7.0167 | 0.1261 | EFGH |
| 5 | 2 | p | 6.9667 | 0.1261 | FGH |
| 20 | 2 | m | 6.9333 | 0.1261 | GH |
| 0 | 2 | p | 6.9333 | 0.1261 | GH |
| 0 | 2 | m | 6.9333 | 0.1261 | GH |
| 10 | 2 | m | 6.8200 | 0.1261 | HI |
| 25 | 2 | m | 6.7633 | 0.1261 | HIJ |
| 5 | 2 | m | 6.7567 | 0.1261 | HIJ |
| 10 | 2 | p | 6.7533 | 0.1261 | HIJ |
| 30 | 2 | m | 6.7333 | 0.1261 | HIJ |
| 20 | 2 | p | 6.5767 | 0.1261 | IJK |

Table B.4 (continued)

| BC | Day | Type | *E. coli* CFU/g | Standard Error | Letter Group |
|---|---|---|---|---|---|
| 99 | 7 | p | 6.4500 | 0.1261 | JK |
| 99 | 7 | m | 6.4500 | 0.1261 | JK |
| 25 | 2 | p | 6.2967 | 0.1261 | K |
| 30 | 2 | p | 5.9167 | 0.1261 | L |
| 25 | 7 | m | 5.8800 | 0.1261 | L |
| 5 | 7 | m | 5.6733 | 0.1261 | LM |
| 30 | 7 | m | 5.5000 | 0.1261 | MN |
| 10 | 7 | m | 5.1933 | 0.1261 | NO |
| 20 | 7 | m | 5.1400 | 0.1261 | OP |
| 5 | 7 | p | 4.8133 | 0.1261 | PQ |
| 10 | 7 | p | 4.7567 | 0.1261 | Q |
| 30 | 7 | p | 4.6900 | 0.1261 | QR |
| 25 | 7 | p | 4.6600 | 0.1261 | QR |
| 0 | 7 | p | 4.6600 | 0.1261 | QR |
| 0 | 7 | m | 4.6600 | 0.1261 | QR |
| 20 | 7 | p | 4.3500 | 0.1261 | R |