WETLAND TREATMENT SYSTEMS FOR MUNICIPAL WASTEWATER AT A BOURBON DISTILLERY AND POTENTIAL VALUE OF INCORPORATING STILLAGE FOR WATER TREATMENT ENHANCEMENT

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biosystems and Agricultural Engineering in the Colleges of Agriculture and Engineering at the University of Kentucky

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ABSTRACT OF THESIS

WETLAND TREATMENT SYSTEMS FOR MUNICIPAL WASTEWATER AT A BOURBON DISTILLERY AND POTENTIAL VALUE OF INCORPORATING STILLAGE FOR WATER TREATMENT ENHANCEMENT

The use of constructed treatment wetlands, as a secondary treatment method for wastewater effluent from package treatment plants and distillery stillage has potential to be an innovative, sustainable method for improving water quality in the Central Kentucky region. However, the use of constructed wetlands to treat stillage and wastewater treatment plant effluent has been limited. Therefore, the objectives of this study were to: 1) quantify constructed wetland removal as a secondary treatment method for distillery's wastewater; 2) explore the potential to utilize constructed treatment wetlands to remove nutrients from bourbon stillage; and 3) optimize treatment design to meet wastewater effluent discharge limits. Four free water surface flow treatment wetland mesocosm experiments were completed during the summer of 2023. Denitrifying conditions were measured along with collection of water quality grab samples over the 10-day experiments. The constructed wetlands removed nitrate-N between 50 to 99%, *E. coli* 99%, and phosphate-P between 61 to 99%, depending on influent and period of the growing season. Bourbon stillage was found to enhance removal of nutrients when added to the wetlands in combination with the wastewater effluent. Findings support constructed treatment wetlands as a potential mechanism for secondary treatment for distillery wastewater and bourbon stillage.

KEYWORDS: Wetlands, Stillage, Wastewater, Mesocosms, Emerging contaminants

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To Mom, Dad, Jonathan, and Susan.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Low impact urban designs for best management practices (BMPs) have been a growing form of environmentally friendly innovations to manage and treat stormwater and wastewater. Constructed wetlands (CWs) are one such BMP that has been used over the past 50 years to treat nutrients and other pollutants such as agricultural runoff (Budd et al., 2011; Vymazal, 2010; Zhang, X., and Zhang, M., 2011), wastewater (Vymazal, 2007; Vymazal 2010), and stormwater (Vymazal, 2010). Stottmeister et al. (2003), emphasizes the numerous examples of CWs being suitable for treating a range of industrial and municipal wastewater. Stottmeister et al. (2003), go further to report an increasing interest in CWs for their ability to treat leachate, industrial effluent, and polluted groundwater. CWs mimic natural wetlands which are landscapes saturated by surface or groundwater, for a duration sufficient for vegetation found in saturated soil conditions, either seasonally or permanently (Messer et al., 2021). A CW is a type of engineered wetland that utilizes a natural wetlands process in soil, vegetation, and microbial activity to treat different kinds of wastewater (Vymazal, 2010).

An industry that could benefit from the use of CWs is the bourbon distilling industry. A portion of the municipal wastewater produced from distilleries contains water and spent grain (Mikucka and Zielińska, 2020). Stillage is the combination of water and spent grain and is a waste product that is processed using conventional types of wastewater treatments; however, some compounds are difficult to manage because they are non-biodegradable and need further decomposition before being discharged into streams

(Mikucka and Zielińska, 2020; Pant and Adholeya, 2007). CWs have worked for other parts of the spirits industry, such as wineries, where a CW was found to be the most environmentally friendly solution after a Life Cycle Assessment when compared to activated sludge (Flores et al., 2019; Vymazal et al., 2021). CWs are used in other parts of the world and could be utilized in the U.S. as secondary treatment process to further reduce stillage compounds for distilleries (Vymazal, 2014).

1.2 Constructed Wetlands

There are various types of constructed wetland (CW) designs. CWs can be classified into three main categories that are further divided into more specific groups as seen in Figure 1.1. The focus of this thesis will be on traditional CWs, specifically the free water surface flow CWs (FWS CWs). As seen in Figure 1.1, traditional CWs are subdivided into three categories of CWs: FWS CWs, subsurface flow (SSF CWs), and floating treatment wetlands (FTW CWs). FWS CWs have above ground surface flow, SSF CWs have below ground flow (Rousseau et al., 2008), and FTW CWs are where the plant roots are suspended in the water column with the use of a man-made floating material (McKercher et al., 2022). SSF CWs are broken down into two subcategories according to flow direction (horizontal and vertical).

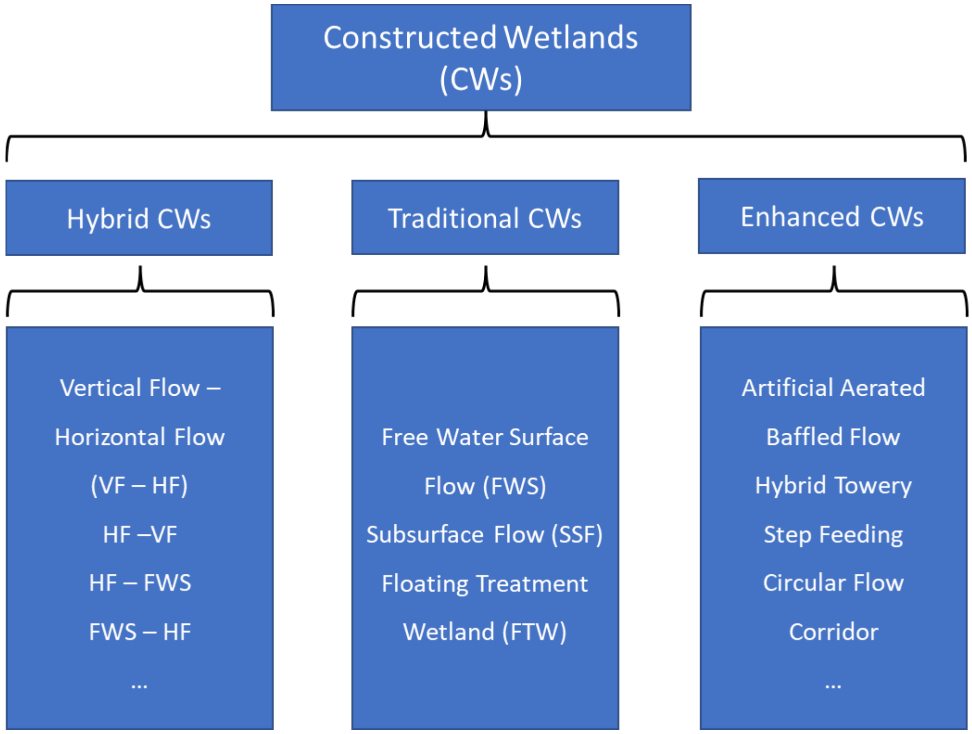

Figure 1.1 Diagram of the multiple designs of CWs for wastewater treatment (Adapted from Wu et al., 2015).

1.2.1 Benefits of Constructed Wetlands

There are a multitude of benefits that come from implementing CWs such as improved water quality (Messer et al., 2021; U.S. EPA, 2004), mitigation of climate change implications (e.g., extreme drought and/or floods) (Messer et al., 2021), and capability to adapt to fluctuating water levels (U.S. EPA, 2004). CWs are also becoming more popular due to their increased ecosystem services including water treatment, flood control, and wildlife habitat, (Vymazal, 2010). Additional benefits of CWs include their ability to work in most climate conditions, in most regions worldwide to treat municipal wastewater, agricultural and industrial effluent, mine drainage, urban and highway runoff, and polluted waterbodies (Wu, 2014). The adaptability of CWs to perform in most environments around the world results in an impactful area of study for sustainable BMP implementations. One such area is their ability to remove organic pollutants from wastewater and runoff thus improving the quality of discharge and benefitting the environment (Imfeld et al., 2019; Wu 2014). Additionally, CWs have proven to be dependable, successful, and cost efficient. Vymazal (2010) highlights these strengths by reporting all types of CWs often exhibit a high removal rate of most organics, which has led to their increased use in wastewater treatment for sewage, industrial, agricultural, and stormwater.

1.2.2 Setback of Constructed Wetlands

While CWs have many benefits for wastewater treatment, they are not without their limitations. For example, CWs often struggle to remove high percentages of phosphorus due to their anaerobic nature and have been found to release phosphorus during the first few years following construction (Vymazal, 2010). Additional setbacks for CWs include large area requirements (Boucher et al., 2011), variability of performance (Fitch 2014), and insufficient maintenance (Rousseau et al., 2008). Large area requirements are a setback because of the capital cost for the purchase of the land needed to build a CW (Boucher et al., 2011). There are also challenges with performance variability due to the complexity of environmental processes occurring within CWs, which can hinder their design and operation (Fitch, 2014). Such variability is due to the low volumetric activity that wetlands see making them sensitive to local conditions and creating "noise" within the system (Fitch, 2014). Insufficient maintenance can be one of the biggest struggles for CWs due to system clogging, uneven flow distribution, and overloading of the systems (Rousseau et al., 2008). These occurrences can lead to loss in functionality and system deterioration over time to a point where the CW no longer performs efficiently and would require dredging (Rousseau et al., 2008).

Therefore, daily maintenance schedules (e.g., monitoring flow level, water quality, and biological parameters) are imperative to keep CW systems operational (Vymazal, 2010). Vegetation management is an additional maintenance procedure that should be performed at quarterly intervals (Kadlec et al., 1996). This is not an extensive list of setbacks for CWs; however, it does highlight key issues for the implementation of CWs.

1.3 Nutrients of Concerns

Nutrients within a CW play a key role in the wastewater treatment process. Most CWs are built to mitigate a specific suite of nutrients-while also addressing other contaminants. In Kentucky, the two nutrients that are often targeted to be removed from an environment are nitrogen (N) and phosphorus (P). These specific nutrients are studied intensely in Kentucky because of their natural abundance from the limestone bedrock throughout the state and the addition of fertilizer for agricultural purposes. Carbon (C) is another nutrient to be considered because of its impact on climate change.

1.3.1 Nitrogen Challenges

There are many challenges when it comes to dealing with inorganic and organic forms of nitrogen (N). N is found in natural abundance in the state of Kentucky so any added N to the system has the potential to become a surplus. N is also one of the leading nutrients that enhance toxic algal blooms and hypoxia zones (Alexander et al., 2008). Areas prone to algal blooms and/or hypoxia zones are dangerous to aquatic life because they have little to no oxygen to use and have implications to human health (U.S. EPA, 2023b).

CWs are an effective method for removing N from an ecosystem or to continuously store N in its many forms (Tang et al., 2020). The three main transformation processes in the N cycle are ammonification, nitrification, and denitrification. One of the main forms of N entering municipal CWs is ammonia (NH_4) and a majority of CWs are designed to treat wastewater with high concentrations of NH_4 (Tang et al., 2020). Ammonia goes through the nitrification process to become nitrate (NO_3) which requires oxygen as the electron acceptor (Tang et al., 2020). This process is achieved with the use of microorganisms withing the CW (Tang et al., 2020). Ammonia can also be removed from the system by plant uptake because plants prefer ammonia to nitrate when both are present (Tang et al., 2020). The process then moves from nitrification to denitrification where NO_3 is transformed into harmless N_2 gas and leaves the system. Figure 1.2 depicts a simplified version of the nitrogen cycle where the three forms of nitrogen are stated at the end of each arrow, within each arrow is the process that is occurring, and necessary conditions for each process are listed beneath the arrow (Tang et at., 2020; Vymazal, 2007).

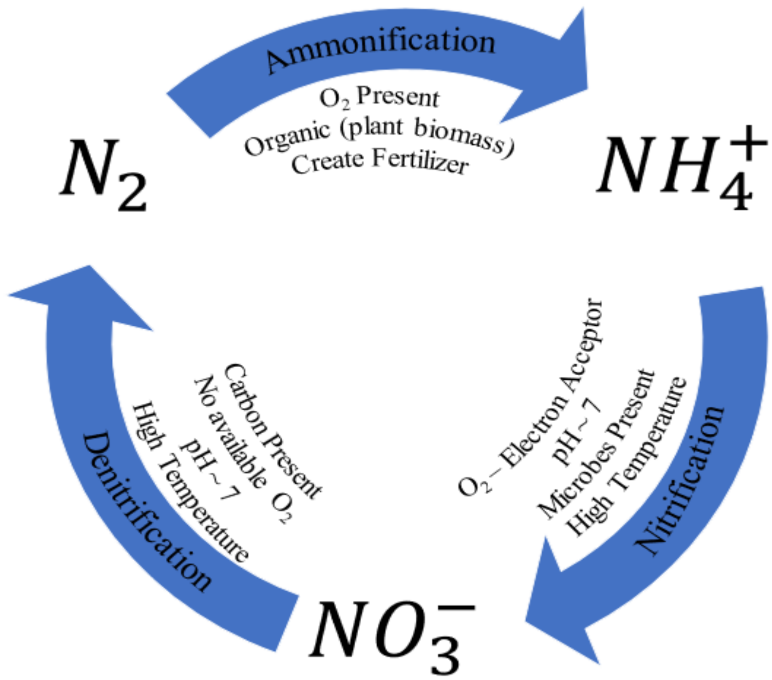

Figure 1.2 Simplified depiction of the nitrogen cycle (Tang et al., 2020).

1.3.2 Phosphorous Challenges

Phosphorus (P) is another nutrient that has the potential to promote algal blooms and hypoxia zones when it occurs in excess (Alexander et al., 2008; U.S. EPA, 2023b). P is known to attach to sediment and has a low removal rate in CWs because of the minimal mixing of soil particles and water (Vymazal 2010). However, if a CW is built to allow for soil accumulation, such that aerobic zones are present and in contact with soils with high cation exchange capacity (CECs), and the vegetation goes unharvested then there is the potential for wastewater to encounter the topsoil so that the CWs act as a long-term phosphorus sink (Richardson, 1985; Vymazal, 2010). In addition, orthophosphate is another important part of the P cycle especially within wetlands as it acts as the major link between organic and inorganic P (Vymazal, 2010).

1.3.3 Carbon Challenges

Carbon dioxide (CO_2) is one of the main greenhouse gases in discussion worldwide. CO_2 is formed from the combination of carbon (C) and oxygen, mainly from human activities (U.S. EPA, 2023a). CWs have proven to be a reliable sink for C emissions because they tend to store C in the soil instead of it being released into the atmosphere as CO_2 (Euliss et al., 2006; Messer, 2021; Zedler and Kercher, 2005). Additionally, the hydrophytic vegetation used in CWs can fix CO_2 through photosynthesis, removing it from the atmosphere before it has the chance to impact the environment (Maucieri et al, 2017; Vymazal, 2021).

Methane (CH_4) is another C related gas that plays an important role in Earth's atmosphere. CH_4 is considered an atmospheric tracer gas that absorbs infrared radiation more effectively than CO_2 (Bartlett and Harriss, 1993) making it the second most important greenhouse gas (Xu et al., 2019). A few major sources of CH_4 are livestock populations, rice paddies, gas lost during coal mining and oil production, and natural wetlands (Bartlett and Harriss, 1993; Cicerone and Oremland, 1998). Wetlands are sources of CH_4 because they provide an environment perfect for methanogenic bacteria which thrive in systems that have no oxygen and an abundant source of organic matter (Bartlett and Harriss, 1993).

1.4 Kentucky Bourbon Distilleries and Stillage

Alcohol distilleries have seen a worldwide increase in production due to advances in industrial applications (Mohana et al., 2009). One example of this was seen between 2005 – 2007 where ethanol production increased by 41% (Mikucka and Zielińska, 2020). Specifically for the bourbon industry there has been an increase in the number of distilleries being opened across the state of Kentucky. In 2009, there were 19 distilleries in the state, but that number spiked to 100 distilleries by 2023 (Coomes & Kronstein, 2022; Hockensmith, 2023). According to the Kentucky Distillers' Association (KDA) there were 2.7 million barrels of bourbon produced in 2022. Unfortunately, this large boom in the production of bourbon also has its own set of drawbacks. One such drawback is the large production of whole stillage, which is produced at 10x the quantity of bourbon. For example, in 2022 there were 2.4 million gallons of bourbon produced which means that there were 24 million gallons of stillage produced. The sheer amount of stillage being produced has been a long-time problem for the industry because of the expense to dispose of it (Hockensmith, 2023). Stillage can be disposed of in many ways with a few being to process it as an industrial wastewater product (Mikucka and Zielińska, 2020; Pant and Adholeya, 2007), sell it to farmers as a cattle supplement or fertilizer (Hockensmith, 2023), or drying it (Hockensmith, 2023). However, there is more supply than demand and currently there are no alternative ways to dispose of stillage that are cost effective for distilleries.

1.4.1 Kentucky Bourbon Industry Stillage Challenge

The biggest challenge for distilleries in Kentucky is that the disposal of stillage is not cost effective. In most cases, distilleries dry the whole stillage in dry houses which increases the stability of the stillage by removing a portion of its moisture content and lightening the product which makes for cheaper transportation costs (Hockensmith, 2023). In Kentucky, the distilleries are shipping this dry stillage to farmers for them to use as supplemental feed for their cattle or as fertilizer. However, as more distilleries are being built, the supply is now vastly outpacing the demand and the cost of drying and shipping is becoming an ever-increasing problem for distilleries. Not to mention that the distance to a farm may not be convenient or even possible for distilleries in urban environments to transport the dry stillage too.

1.4.2 Distillery Whole Stillage

Spent wash is produced during the distillation process where the fermented mass is put into a distillation column and the ethanol is separated from water and other volatile compounds (Hockensmith, 2023). Whole stillage is the combination of the water and spent wash that is removed from the bottom of the still. Bourbon whole stillage is thick and has a dark yellow pigment (Figure B2). Whole stillage is characterized by high chemical and biochemical oxygen demand (COD and BOD) (Beltran et al., 2001; Nataraj et al., 2006; Mikucka and Zielińska, 2020), acidic pH (3.5 – 4.5) (Hockensmith, 2023; Mikucka and Zielińska, 2020), high nutrient levels such as nitrogen, carbon, phosphorus, and potassium (Mikucka and Zielińska, 2020).

1.4.3 Potential Constructed Wetland Expenses for the Kentucky Bourbon Industry

The biggest obstacle for most low impact BMPs is trying to make them cost effective for the area they are being implemented in. This can be especially challenging for treating distillery wastewater because it can be difficult and expensive (Mikucka and Zielińska, 2020). There are also many factors to consider when looking at costs for a CW such as soil type, climate, terrain slope, groundwater table height, and discharge criteria (Rousseau et al., 2008). In addition, the larger the CW, the greater volume of wastewater it can treat per cubic meter (m^3) of wastewater making it cheaper and more cost effective assuming the same concentration of contaminants are entering the system (Rousseau et al., 2008). A convincing argument in favor of CWs is the low cost of maintenance, due to limited labor requirements such as site inspection, effluent sampling, cleaning of pumps, and weed control (Rousseau et al., 2008).

CWs are typically low in operation and maintenance costs because they utilize natural processes and do not need much effort to continue functioning (Hazra and Durso, 2022; Rahman et al., 2020; Vymazal, 2010; Wu et al., 2015). Maintaining a CW can help it to last for up to 20 years before it needs to be reassessed for functionality (Rousseau et al., 2008). Energy consumption for CWs is also low (Wu et al., 2014b) and is typically limited to pumping of the system when there are signs of clogging, which are not a frequent problem if maintenance is performed (Rousseau et al., 2008). CWs low cost of operation is in direct opposition to conventional wastewater treatment plants, which often require specialized services, are high in cost, and consume a fair amount of energy (Zhang et al., 2012; Wu et al., 2014; Wu et al., 2015).

1.5 Treatment Processes used in Other Parts of the World

In other parts of the world distillery stillage is treated as a waste product and processed through wastewater treatment facilities. There are a few ways to process stillage and the three ways that will be discussed in this section are: biological, physio-chemical, and thermal (Mikucka and Zielińska, 2020). These treatment types are often used in combination because use of an individual method would not be enough to break down the composition of the stillage (Mikucka and Zielińska, 2020). Distillery stillage is a difficult substance to properly dispose of due to its low pH and non-biodegradable compounds (Pant and Adholeya, 2007; Mikucka and Zielińska, 2020).

1.5.1 Biological Methods

The biological method for treating stillage includes aerobic and anaerobic processes (Mikucka and Zielińska, 2020). Typically, both treatments are used in combination because the individual treatments would not be able to manage the considerable amounts of organic pollutants that make-up stillage (Mikucka and Zielińska, 2020). Aerobic processes include microorganisms such as bacteria, cyanobacteria, yeast, and fungi and are used as a pre- and final treatment for stillage (Mikucka and Zielińska, 2020). The main disadvantage of aerobic treatment is the high energy consumption, but this treatment is often used due to its ease of use and effectiveness (Bolzonella et al., 2019; Mikucka and Zielińska, 2020). Anaerobic processes also include microorganisms; however, the key distinction is that there are different groups of microorganisms that function and interact with one another (Mikucka and Zielińska, 2020). The advantages to using anaerobic processes include the production of biogas (bioenergy) from the rich organic compounds in stillage, low energy cost, low nutrient load, and longer hydraulic retention time (Mikucka and Zielińska, 2020; Mohana et al., 2009). The combination of these two treatments can effectively remove the dark pigment and COD from stillage as opposed to using them individually (Mikucka and Zielińska, 2020).

1.5.2 Physio-chemical Methods

Physio-chemical methods include coagulation, electrocoagulation, absorption, advanced oxidation, and membrane filtration (Mikucka and Zielińska, 2020). The basic understanding of this method is that it is a combination of physical and chemical processes (Mikucka and Zielińska, 2020). The physical processes focus on the removal of suspended materials and the chemical processes eliminate soluble COD (Mikucka and

Zielińska, 2020). Inorganic coagulants like aluminum or copper make up a portion of the suspended particles in wastewater and can be treated with a coagulation processes (Mikucka and Zielińska, 2020; Sowmeyan and Swaminathan, 2008). Electrocoagulation can be used as an alternative to coagulation when dealing with wastewater that is rich in COD with the use of electrolytic dissolution (Mikucka and Zielińska, 2020). For the removal of color and specific organic pollutants, absorption on activated carbon is used (Mikucka and Zielińska, 2020). Advanced oxidation processes are Fenton, oxidation, ozonation, and wet oxidation which are typically used in combination to treat wastewater (Mikucka and Zielińska, 2020). The best membrane treatment for stillage is a pressure-driven filtration system that separates the influent based on size and shape (Mikucka and Zielińska, 2020).

1.5.3 Thermal Methods

The thermal method results in solid precipitates when the stillage is heated at high temperatures (Mikucka and Zielińska, 2020). Combustion is the main type of thermal method because it is an effective on-site disposal method (Mikucka and Zielińska, 2020). Combustion is a method of treatment that is gaining interest with distilleries because of its on-site use (Mikucka and Zielińska, 2020). The byproduct of stillage after going through a combustion process is potassium-rich ash that can be repurposed for land applications (Mane et al., 2006; Mikucka and Zielińska, 2020).

1.6 Project Goal, Objectives, and Hypothesis

The goal of this project was to determine the potential for wetlands to treat nutrients and other water quality concerns in wastewater effluent and to assess potential outcomes from the application of bourbon stillage to wetlands. Therefore, the objectives of this study were to:

1. Quantify constructed wetland removal potential as a secondary treatment method for distillery wastewater;
2. Optimize treatment design to meet wastewater effluent discharge limits:
3. Explore the potential to utilize constructed treatment wetlands to assess nutrients and water quality parameters from bourbon stillage.

Thus, we hypothesized wetland treatment systems would allow for municipal wastewater effluent to meet EPA discharge requirements and will reduce nutrients found within bourbon stillage.

CHAPTER 2. MATERIALS AND METHODS

2.1 FWS Mesocosm Setup

Four experiments were conducted over the summer of 2023 in greenhouses that were not temperature controlled and located at the University of Kentucky's North Farm. All fifteen mesocosms were constructed in late spring of 2023. The mesocosms were built in 100-gallon Rubbermaid® tanks with 30 cm of local topsoil (Bluegrass-Maury silt loam) and three species of native wetland plants. The wetland plants that were used were the cattail (*Typha latifolia*), soft-stem bulrush (*Schoenoplectus tabernaemontani*), and pickerel weed (*Pontederia cordata*) which are local to the Bluegrass Region (Figure 2.1).

Of the fifteen mesocosms that were constructed, three contained only soil (no plants) and were used as soil control mesocosms, while the remaining twelve were planted following the planting plan in Figure 2.2a. The planted mesocosms were divided into four treatments in replicates of three: Plant control, Effluent, Stillage 1, and Stillage 2 mesocosms. The Stillage 1, Stillage 2, and Effluent treatments were grouped together while the Soil and Plant Control treatments were spread out in the greenhouse (Figure 2.2b). Each mesocosm was outfitted with a meter stick that was utilized to account for water loss due to evapotranspiration. A mesh cloth was secured to the interior of the valves at the bottom of the Rubbermaid tanks which allowed for the Rubbermaid tanks to be drained between experiments without any soil loss. The use of potassium-nitrate (KNO_3) was used to enrich specific treatments during each experiment and can be seen in Table 2.1.

In early April of 2023 the mesocosms were established in the greenhouse, filled with soil, and planted. Beginning in May, the mesocosms were watered every 2 days until the plants were well established. After this, each mesocosms was inundated with 54 cm water until the start of the first experiment in early June 2023.

Table 2.1 KNO_3 enrichment concentrations per treatment per experiment.

Experiment	1	2	3	4
Soil Control	10 g	10 g	10 g	10 g
Plant Control	10 g	10 g	10 g	10 g
Stillage 1	10 g	10 g	-	-
Stillage 2	-	-	10 g	10 g
Effluent	10 g	10 g	-	10 g

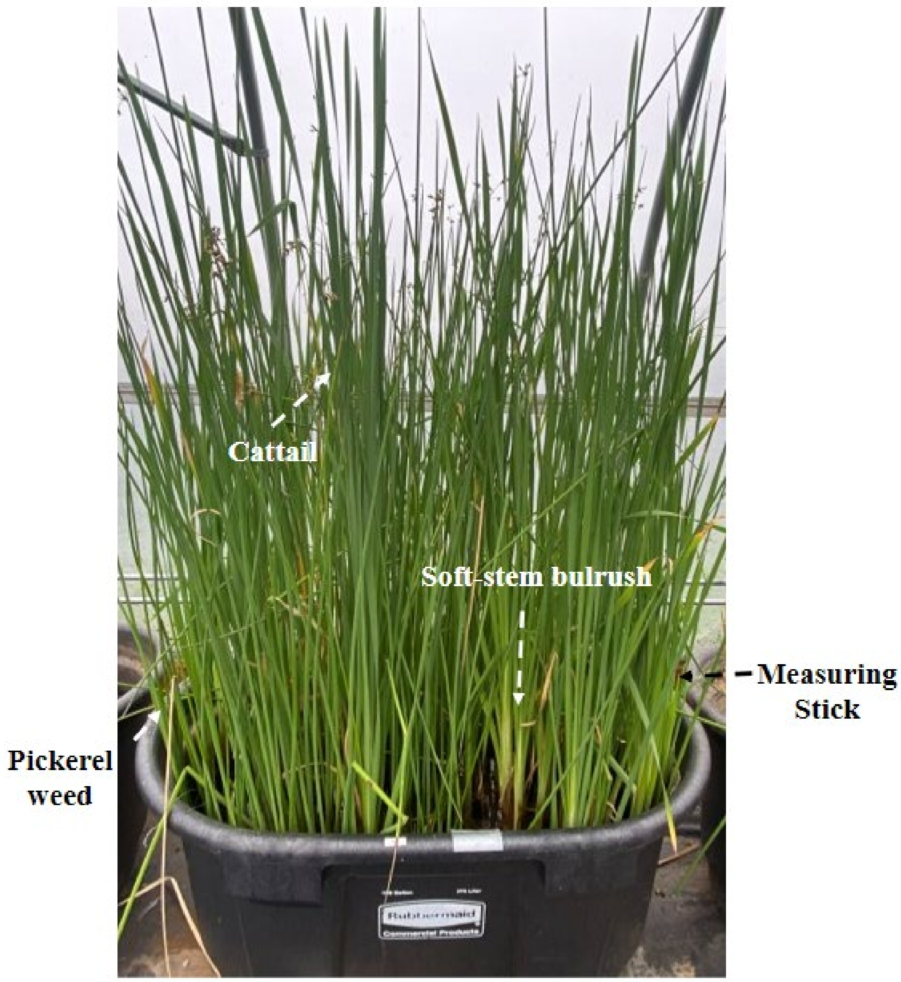

Figure 2.1 Detailed image that displays the three different types of common wetland plants used for the experiment and the location of the measuring stick.

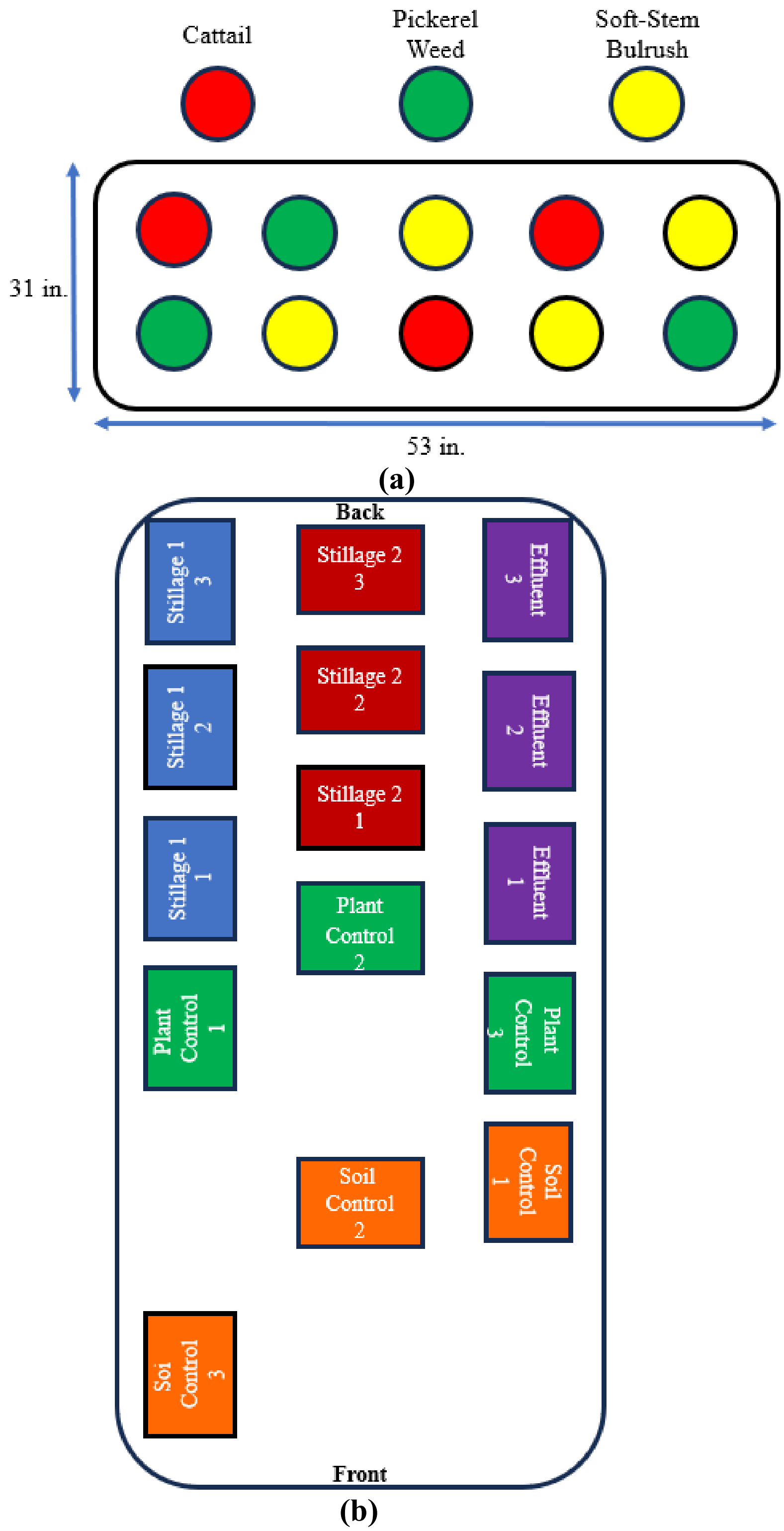

Figure 2.2 Rubbermaid tank dimensions and the pattern that the plants were planted within each mesocosms (a). The layout of the greenhouse which includes the position for each of the treatments and shows that the Stillage 1, Stillage 2, and Effluent treatments were grouped together (b).

2.2 Central Kentucky Distillery Wastewater Treatment Facility

The wastewater treatment plant for the central Kentucky distillery's bottling facility was a Besco Model BDP-100 precast concrete extended aeration sewage treatment plant. This treatment plant was designed with a maximum flow of 10,000 GPD and a 200 PPM 5-day BOD domestic sewage. This package plant included necessary vessels, weirs, and baffles. The plant included a 1000-gal sludge holding tank, one lot aeration/clarifier tank, two 3 hp main blower units with a variable frequency drive, one lot control for flow equalization, one lot air lift sludge return pump, one lot skimmer and internal piping, a UVIREX 230 UV disinfection unit, and one post aeration tank (Appendix B1).

2.3 Experimental Overview of FWS Municipal Effluent and Bourbon Stillage Experiments (Summer)

2.3.1 Experiment 1

Experiment 1 was conducted in early June and was used to gather baseline data for subsequent experiments. The Soil Control, Plant Control, Stillage 1, and Effluent treatment mesocosms were inundated with 145 L of tap water 24 hours before the start of Experiment 1 to allow for any chlorine in the tap water to evaporate. The tap water was measured using a flowmeter (P3 International, New York, N.Y.) attached to a hose. Laboratory grade potassium nitrate (KNO_3) (Fisher Scientific, Pittsburgh, P.A.) was used to enrich each mesocosm to a concentration of 10 mg L^{-1} of NO_3-N. At the conclusion of Experiment 1, every drain plug on the stock tanks was removed from each mesocosm to allow for water to drain out. This ensured that the water volume was consistent between experiments. A detailed description of each treatment for this project can be found in

Table 2.2 and the volume of tap water used for each treatment per experiment can be found in Table 2.3.

2.3.2 Experiment 2

Experiment 2 was conducted in mid-June, at least 7 days after the conclusion of Experiment 1 to allow for proper drainage of the mesocosms. Experiment 2 was where the stillage from the Central Kentucky bourbon distillery (Appendix B2) and municipal wastewater effluent from the bourbon distillery's bottling facility (Appendix B3) were introduced. Approximately, 57 L of stillage was added to the Stillage 1 mesocosms (30% of the total water volume), and 57 L of effluent was added to Effluent mesocosms (30% of total water volume added). The Stillage 1 and Effluent treatment mesocosms were filled with 128 L of tap water to reach the target liquid volume of 185 L. A summary of the amount of WWTP effluent and bourbon stillage used per treatment per experiment are detailed in Tables 2.4 and 2.5 The Soil Control, Plant Control, Stillage 1, and Effluent treatment mesocosms were enriched with 10 g of KNO_3. The Soil Control and Plant Control treatment mesocosms were inundated with 185 L of tap water. Every treatment mesocosm was filled with their respectable volumes 24 hours prior to the start of the experiment to allow for any chlorine in the tap water to evaporate prior to the start of the experiment. At the conclusion of Experiment 2, the drain plugs on the Soil Control, Plant Control, and Effluent mesocosm stock tanks was removed to allow for excess water to drain.

2.3.3 Experiment 3

Experiment 3 was conducted in late July at least 7 days after the conclusion of Experiment 2 to allow for proper drainage of the mesocosms. For Experiment 3 the volume of effluent being added to the Effluent treatment mesocosms was increased from 57 L to 76 L (41% of total water volume added). The Effluent mesocosms were then filled with 109 L of tap water to reach the target volume of 185 L. Unfortunately, at this time in the summer the distillery shut down their bourbon production, which is a common practice in Kentucky. Distilleries tend to shut down anywhere from 2 weeks to a few months to perform maintenance, cleaning, and upgrades. Therefore, new stillage was unavailable for this experiment. For Experiment 3, the three planted mesocosms that were not used during Experiments 1 and 2 were used during Experiment 3 and were labeled the Stillage 2 treatment mesocosms. The water/stillage mixture that remained in the Stillage 1 treatment mesocosms was pumped into the Stillage 2 treatment mesocosms to 145 L. Figure 2.3 displays an image of how the stillage/water mixture was pumped from the Stillage 1 treatment mesocosms into the Stillage 2 mesocosms. The Stillage 2 treatment mesocosms were then filled with 40 L of tap water to reach the target volume of 185 L. The Stillage 2 treatment mesocosms were outfitted with dual outlet aquarium electric air pumps attached to two high efficiency air stones (Haisen, China). The air stones had a flow rate of 1.5 L per minute and were placed at opposite ends of the stock tanks. The Stillage 2 treatment mesocosms were dosed with 200 mL of sodium hydroxide 10 N solution (VWR Life Science Solon, OH). The sodium hydroxide 10 N solution was used to increase the initial pH from an acidic state of 3.9 from Experiment 2 to a pH closer to 7. The pH adjustment was added to the Stillage 2 treatment mesocosms at 100 mL intervals on days 0 and 1 of Experiment 3. The Soil Control and Plant Control treatment mesocosms were inundated with 185 L of tap water. Every treatment mesocosm was filled with their respectable volumes 24 hours prior to the start of the experiment to allow for any chlorine in the tap water to evaporate prior to the start of the experiment. The Soil Control, Plant Control, and Stillage 2 treatment mesocosms were enriched with 10 g of KNO_3. The Effluent treatment mesocosms were not enriched with KNO_3 during this experiment due to the high concentrations of NO_3 -N that were observed during Experiment 2 (~40 mg L^{-1} on Day 0). At the conclusion of Experiment 3, every drain plug on the stock tanks was removed from each treatment mesocosm to allow for excess water to drain.

2.3.4 Experiment 4

Experiment 4 was conducted in mid-August, at least 7 days after the conclusion of Experiment 2 to allow for proper drainage of the mesocosms. For experiment 4 the volume of effluent was increased from 76 L to 151 L (81.6% of total water volume) for the Effluent treatment mesocosms. The Effluent treatment mesocosms were then filled with 34 L of tap water to reach the target volume of 185 L. No additional stillage or water from treating previous wetlands with stillage was added to the Stillage 2 treatment mesocosms. Instead, the Stillage 2 treatment mesocosms received 76 L of effluent (41% of the total volume) and were filled with 109 L of tap water to reach the target volume of 185 L. The air stones were removed for this experiment and no sodium hydroxide 10 N was used. The Soil Control and Plant Control treatment mesocosms were inundated with 185 L of tap water. Every treatment mesocosm was filled with their respectable volumes 24 hours prior to the start of the experiment to allow for any chlorine in the tap water to evaporate prior to the start of the experiment. The Soil Control, Plant Control, Stillage 2, and Effluent treatment mesocosms were enriched with 10 g of KNO_3. At the conclusion of Experiment 4, plant, root, and soil samples were collected from the Soil Control, Plant Control, Stillage 1, Stillage 2, and Effluent treatment mesocosms.

Table 2.2 Detailed description of the five different treatments used for each experiment.

Treatment	Description
Soil Control	Three treatments containing 30 cm of local topsoil and not planted
Plant Control	Six constructed wetlands containing 30 cm of local topsoil and planted with three species of native wetlands plants
Stillage 1	Three constructed wetlands containing 30 cm of local topsoil, planted with three species of native wetland plants, and receiving the application of raw bourbon stillage at varied amounts per experiment
Stillage 2	Three constructed wetlands containing 30 cm of local topsoil, planted with three species of native wetland plants, and receiving the stillage/water mixture from Stillage 1 treatments in Experiment 3 and effluent in Experiment 4
Effluent	Three constructed wetlands containing 30 cm of local topsoil, planted with three species of native wetland plants, and receiving treated wastewater effluent at varying amounts per experiment

Table 2.3 Volume of tap water added to each of the treatment mesocosms per experiment.

Experiment	1	2	3	4
Soil Control	145 L	185 L	185 L	185 L
Plant Control	145 L	185 L	185 L	185 L
Stillage 1	145 L	128 L	-	-
Stillage 2	145 L		40 L	185 L
Effluent	145 L	128 L	109 L	34 L

Table 2.4 Volume of WWTP effluent that was applied to the respective treatments per experiment.

Experiment	1	2	3	4
Stillage 2	0 L	0 L	0 L	76 L
Effluent	0 L	57 L	76 L	151 L

Table 2.5 Volume of raw bourbon stillage that was applied to the respective treatments per experiment.

Experiment	1	2	3	4
Stillage1	0 L	57 L	0 L	0 L
Stillage 2	0 L	0 L	76 L	0 L

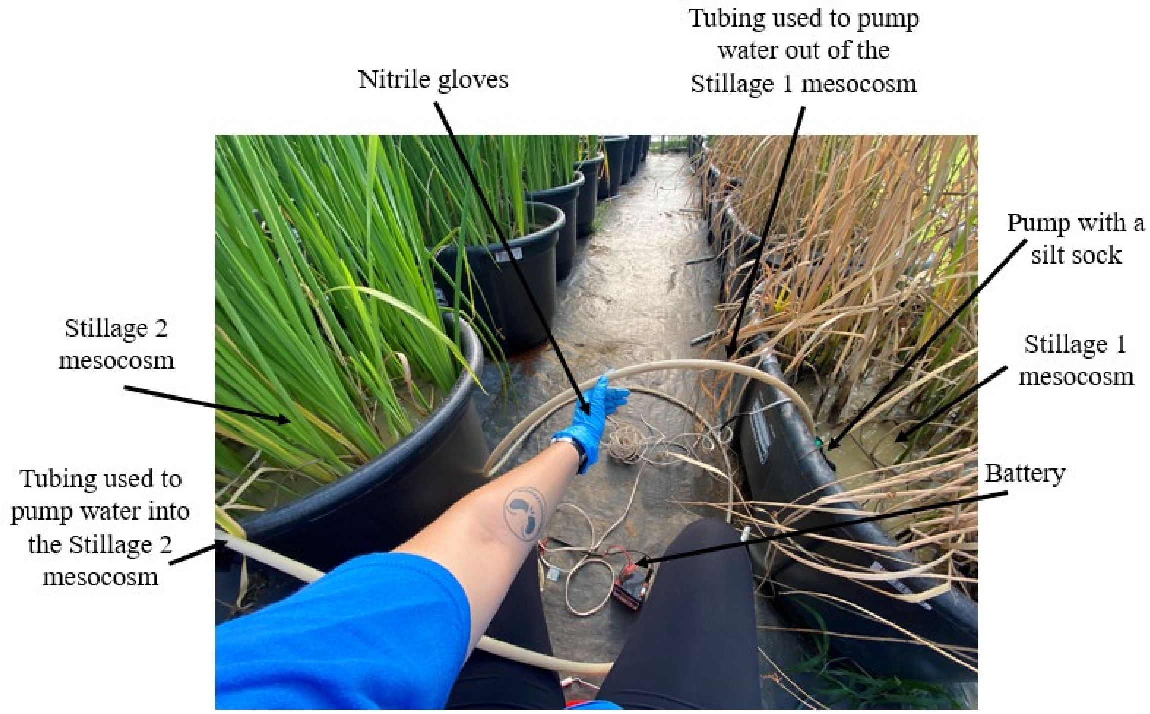

Figure 2.3 A detailed image showing how the stillage/tap water mix was pumped from the old stillage mesocosms into the new stillage mesocosms.

2.4 Sampling Procedure

The duration of each experiment was 10 days starting on day 0 and ending on day 10. Nitrate-N (NO_3-N), phosphate-P (PO_4-P), ammonium-N (NH_4-N), and dissolved organic carbon (DOC) were sampled during all four experiments. Grab samples for NO_3-N, PO_4-P, and NH_4-N were taken on days 0, 1, 2, 3, 5, 7, and 10, while DOC was sampled on days 1, 5, and 10. All grab samples were collected 15-cm below the air-water interface after each mesocosm was stirred with a PVC stick for 1 minute. Total Kjeldahl nitrogen (TKN) concentrations were measured for in Experiment 2 and were sampled on days 1, 5, and 10. Biochemical oxygen demand (BOD) was measured in Experiment 3 and was sampled on days 1, 5, and 10. *E. Coli* concentrations were measured in Experiment 4 and were sampled on days 0, 1, 5, and 10.

NO_3-N, PO_4-P, and NH_4-N samples were collected using a 50 mL syringe that was filled with mesocosm water and filtered through a GF/F filter into two 20 mL scintillation vials. The NH_4-N samples were acidified using sulfuric acid. DOC was collected by filling the syringe with water, filtering it through a 0.45 μm filter into a 40 mL glass vial, and acidifying it with sulfuric acid. TKN was collected in 125 mL plastic vial and preserved with sulfuric acid. BOD was collected using a 300 mL wide-mouthed glass bottles and capped with a glass stopper. Finally, *E. Coli* was collected using a 100 mL plastic bottle. After samples were collected for each treatment, they were stored in a cooler on ice and transported to a walk-in refrigerator that held a stable temperature of 36˚F in the C.E. Barnhart Building until they were analyzed.

2.4.1 Physiochemical Parameters

Physiochemical parameters were also measured throughout the four experiments using a handheld YSI ProQuatro multiparameter meter (YSI Inc., Yellow Springs, Ohio) that was outfitted with water temperature, dissolved oxygen (DO), specific conductivity (SPC), total dissolved solids (TDS), pH, and oxidation-reduction potential (ORP) sensors. Physiochemical parameters were recorded on sampling days 0, 1, 2, 3, 5, 7, and 10. A comprehensive summary of the sampling for each nutrient, contaminants of emerging concern (CEC), and water quality parameters for each experiment can be found in Table 2.6.

Table 2.6 Sampling schedule for all nutrients, contaminants of emerging concern, and water quality parameters for each experiment.

Sample	Bottle size	Storage	0	1	2	3	5	7	10
NO_3-N / PO_4-P	20 mL Filtered 0.7 µm	Fridge 2 days	X	X	X	X	X	X	X
NH_4-N	20 mL Filtered 0.7 µm 1 drop of Sulfuric Acid	Fridge 28 Days	X	X	X	X	X	X	X
TOC	40 mL Filtered 0.45 µm 1 drop of Sulfuric Acid	Fridge 28 days		X			X		X
TKN	125 mL Unfiltered 1 drop of Sulfuric Acid	Fridge 28 Days		X			X		X
BOD_5	300 mL Unfiltered	Incubator 20 °C 5 Days		X			X		X
E. coli	120 mL Unfiltered	Incubator 35 °C 24 Hours	X	X			X		X
Handheld YSI			X	X	X	X	X	X	X

2.5 Nutrient Analysis

2.5.1 Water Characteristics

All sample analyses were performed in the *meso*Lab (Lexington, KY). The approved EPA methods used included: NO_3-N (EPA-126-C Rev. 2), PO_4-P (EPA-145-C Rev. 1), NH_4-N (EPA-129-C Rev. 3), and TKN (EPA-111-C Rev. 1) to either Seal Analytical AQ400 Discrete Analyzer (Seal Analytical, Mequon, WI). DOC water samples were analyzed on a Shimadzu TOC-L Total Organic Carbon Analyzer (Shimadzu Scientific Instruments, Kyoto, Japan) using EPA Method 9060A (U.S. EPA, 2004b). BOD was measured using the YSI meter coupled with DO probe using EPA

Method BOD 5.2. *E. coli* was measured using the IDEXX (IDEXX Laboratories, Inc, Westbrook, ME) Colilert-18 Method.

Samples for DOC were indicated by analyzing the Non-Purgeable Organic Carbon (DOC) using a Shimadzu TOC-L Total Organic Carbon Analyzer using the EPA Method 9060A (U.S. EPA, 2004b). Unfortunately, the Stillage 1 mesocosms were not able to be sampled for DOC due to the inability of the water to be passed through the required filter.

2.5.2 Soil and Plant Samples

Plant, soil, and root samples were collected after the completion of Experiment 4 to determine any changes in soil nutrients at the end of all four experiments. Soil samples were collected from all five treatments, while plant and root samples were collected from the Plant Control, Stillage 1, Stillage 2, and Effluent treatments. A 10% destruction was completed for these samples from each mesocosm. A sample population of the plants from each mesocosm, except for the Soil Control mesocosms, was collected by separating biomass from the top, middle, and base of the plants. A composite sample of the roots from the four planted treatments was collected as well and were rinsed to remove sediment prior to being placed in Ziplock® bags. Soil samples were taken from the same area as the root and plant samples. After each sample was collected it was placed into a Ziplock® bag and into a cooler for transportation. The soil samples were dried and weighed at the *meso*Lab and sent to the University of Kentucky Regulatory Services' Soil Testing Lab (Lexington, K.Y.) to tested for TN, TP, bulk density, CEC, TC, and pH based on the Soil Analysis Handbook of Reference Methods (2018). Dried biomass samples of above and below surface samples were shipped to Ward Laboratory

(Kearney, NE) to analyze for %N, %P, %C, nitrogen, phosphorus, calcium, magnesium, sulfur, zinc, manganese, copper, boron, molybdenum, and carbon using the Dumas Dry Combustion method for TN and TC content (Bertsch and Ostinelli, 2019; Plank 1991)

2.5.3 Removal Rates

NO_3-N, PO_4-P, BOD, DOC, and *E. coli* removal rates were calculated for every mesocosm at the end of each experiment. The first-order removal rate equation was used to solve for k based on reasonable fit of degradation. The first-order removal rate equation was used (Keilhauer, M.G., 2019):

$$C_T = C_O e^{-kt}$$

where,

C_T = final NO_3-N concentration (mg L^{-1})

C_o = initial NO_3-N concentration (mg L^{-1})

t = time (d)

k = removal rate (d^{-1})

The overall percentage of removal for NO_3-N, PO_4-P, BOD, DOC, and *E. coli* was also calculated using the Day 1 concentration in each mesocosm (C_o) and the last day before the minimum detection limit was measured (C_T):

$$Removal\ (\%) = \frac{C_O - C_T}{C_O} x100$$

2.6 Statistical Analysis

All data for NO_3-N, PO_4-P, DOC, and *E. coli* were normalized before being statistically analyzed. Comparisons were made between the five treatments and time using multiple least square regression. To account for variability in starting concentrations for each treatment between the three replicates was used to normalize concentrations:

$$y'(t) = \frac{y(0)}{y(t)}$$

where, y′(t) = ratio of NO_3-N concentrations at time t, y(t) = NO_3-N concentration at time t, and y(0) = initial NO_3-N concentration. The Tukey's honest significant difference (HSD) test was used to determine the significance of the effects of mesocosm treatment and experiment. All statistical comparisons used a statistical significance of $\alpha = 0.05$. Physicochemical characteristics (pH, temperature, specific conductivity, and dissolved oxygen) and BOD concentrations did not require normalization. All statistical analyses were performed using Statistical Analysis Software (SAS Institute Inc, N.C., USA).

Multivariate statistical analysis was completed for each of the four experiments to determine any differences in NO_3-N reductions between the two mesocosm treatments and the controls over time using linear mixed model in SAS glimmax ® (SAS Institute, Cary, N.C.):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \varepsilon_{ijk}$$

where, μ is the overall mean response, α_i is the fixed effects, β_j are the random effects, $\alpha\beta_{ij}$ are the random effects due to interactions, and ε_{ijk} is the error unaccounted for by the effects. *i* was the treatment type (Soil Control, Plant Control, Stillage 1, Stillage 2, Effluent), *j* was time, and *k* was the replication (1, 2, 3).

CHAPTER 3. Results and Discussion

3.1 Nutrient Assessment

3.1.1 NO_3-N Removal Rate

NO_3-N concentrations varied between experiments and treatments, with removal varying between 50 to 100% depending on treatment and experiment (Table 3.1).

During Experiment 1, all treatments were enriched with only NO_3-N with a target of 10 mg L^{-1} to ensure all treatments were functionally similar. Significant differences were observed between treatments during sampling days ($\alpha = 0.05$; Table A1); however, the treatments responded similarly, until Day 7 when NO_3-N approached non-detectable values (Figure 3.1). Significant differences were observed within treatments between sampling day(s) with NO_3-N decreasing with time ($\alpha = 0.05$; Table A2).

During Experiment 2, all treatments were enriched to a target concentration of 10 mg L^{-1} of NO_3-N. However, WWTP effluent and whole stillage were also added to the Effluent and Stillage 1 treatments. Significant differences were observed between treatments starting on day 5 ($\alpha = 0.05$; Table A3). With exception to the Stillage 1 treatment, all other treatments responded similarly until approaching non-detectable values of NO_3-N (Figure 3.2) and decreased through time ($\alpha = 0.05$; Table 3.1). Significant differences were observed within treatments starting on day 3 for the Stillage 1 and Effluent treatments ($\alpha = 0.05$; Table A4). This was likely due to the extremely low pH impacting both plant and microbial removal in the Stillage 1 treatment and the exceedingly high starting NO_3-N concentration in the Effluent treatments, which were 3X higher than all other treatments ($\alpha = 0.05$; Table 3.1).

During Experiment 3, all treatments, except for the Effluent treatment, were once again enriched to a target concentration of 10 mg L^{-1} of NO_3-N and the Stillage 2 treatment was introduced into the experiment. Significant differences were observed between treatments starting on day 3 ($\alpha = 0.05$; Table A5), where the Stillage 2 treatment had lower NO_3-N concentrations and exhibited a higher removal rate compared to all other treatments (Figure 3.3). Significant differences between sampling day(s) within each of the four treatments was once again evaluated ($\alpha = 0.05$), where several significant differences were observed for all treatments through time during the experiment but did not exhibit a trend other than decreasing with time (Table A6).

During Experiment 4, all treatments were once again enriched to a target concentration of 10 mg L^{-1} of NO_3-N. However, WWTP effluent was added to the Effluent and Stillage 2 treatments. Significant differences were observed on days 2 and 3 between all treatments (Figure 3.4), where the Stillage 2 treatment exhibited higher NO_3-N removal rates compared to other treatments ($\alpha = 0.05$; Table A7). Significant differences between sampling day(s) within each of the four treatments was once again evaluated ($\alpha = 0.05$), where several significant differences were observed for all treatments throughout time during the experiment (Table A8).

Average NO_3-N removal rates varied between treatments (Table 3.1), where removal rates increased throughout the growing season. Effluent treatment had overall similar removal rates to the Soil and Plant Controls. However, the Stillage 1 Treatment that had not been adjusted for pH had significantly lower removal rates compared to other treatments (0.66 ± 0.32 day^{-1}), while the Stillage 2 treatment with adjusted pH and the stillage routed from the Stillage 1 treatment had enhanced NO_3-N removal rates ($2.41 \pm$

1.72 day^{-1}) compared to all other treatments in the study, with NO_3-N being below detection within 3 days of enrichment (Table 3.1).

Low removal rates seen in all treatments during Experiment 1 were similar to observations of Keilhauer et al., (2019), which observed low removal rates of 0.02 ± 0.01 d^{-1} during the beginning of the growing season shortly after plants were added. The Effluent treatment in Experiments 2, 3, and 4 had the highest percent of NO_3-N removed from the system, 93.3%, 99.3%, and 99.7%, respectively. This was similar to Jasper et al. (2019), who observed a 95% removal of NO_3-N from a wetland receiving wastewater during a summer period with an initial inlet NO_3-N concentration of 20.7± 0.7 mg L^{-1}.

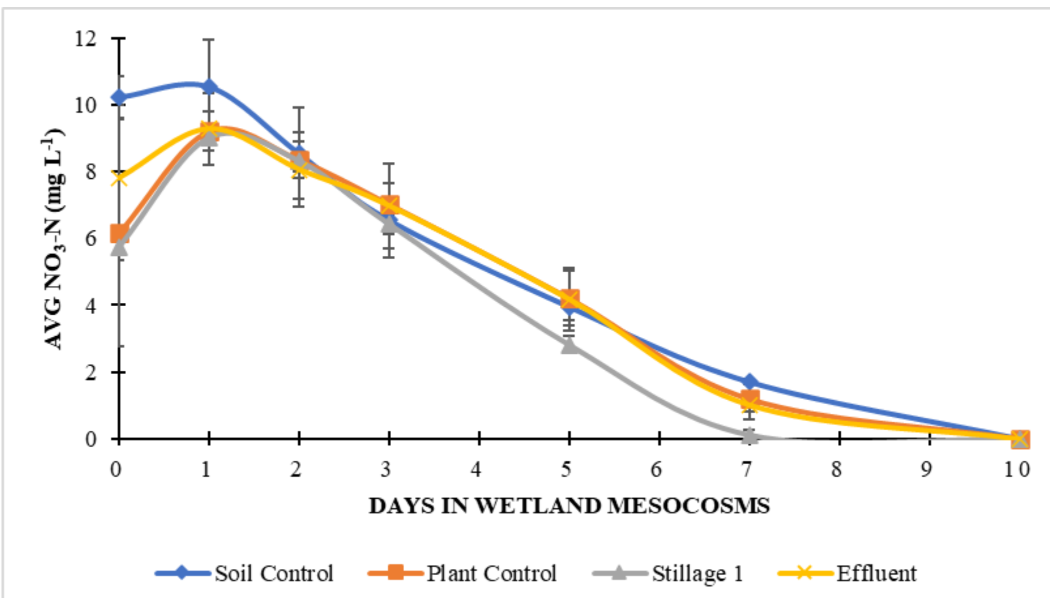

Figure 3.1 NO_3-N concentrations for the Soil Control, Plant Control, Stillage 1, and Effluent treatments during Experiment 1.

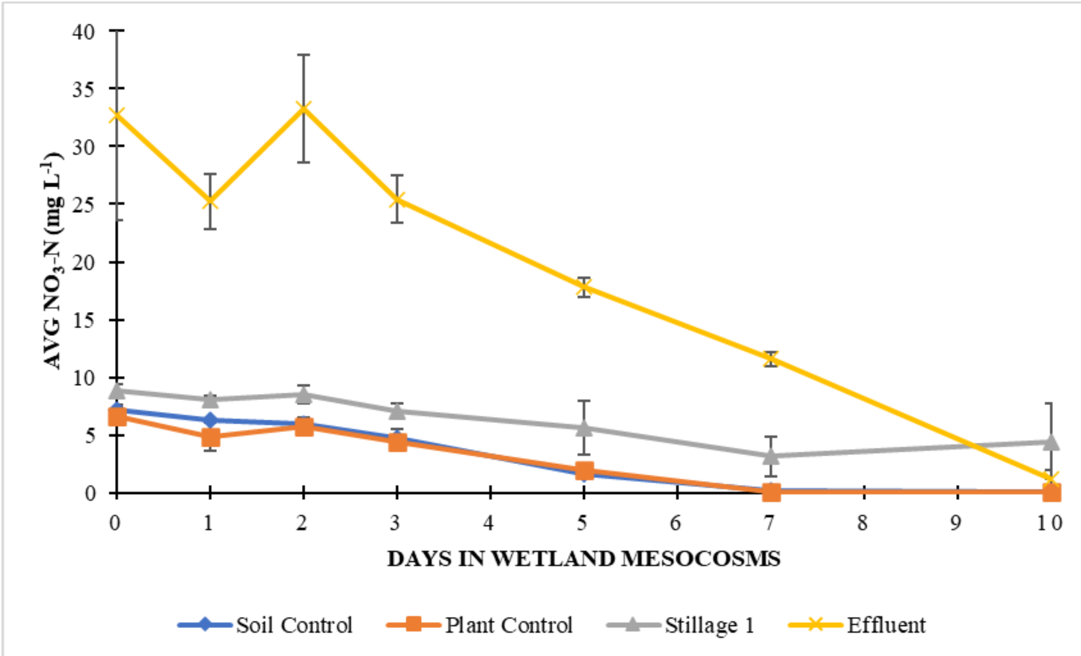

Figure 3.2 NO_3-N concentrations for the Soil Control, Plant Control, Stillage 1, and Effluent treatments during Experiment 2.

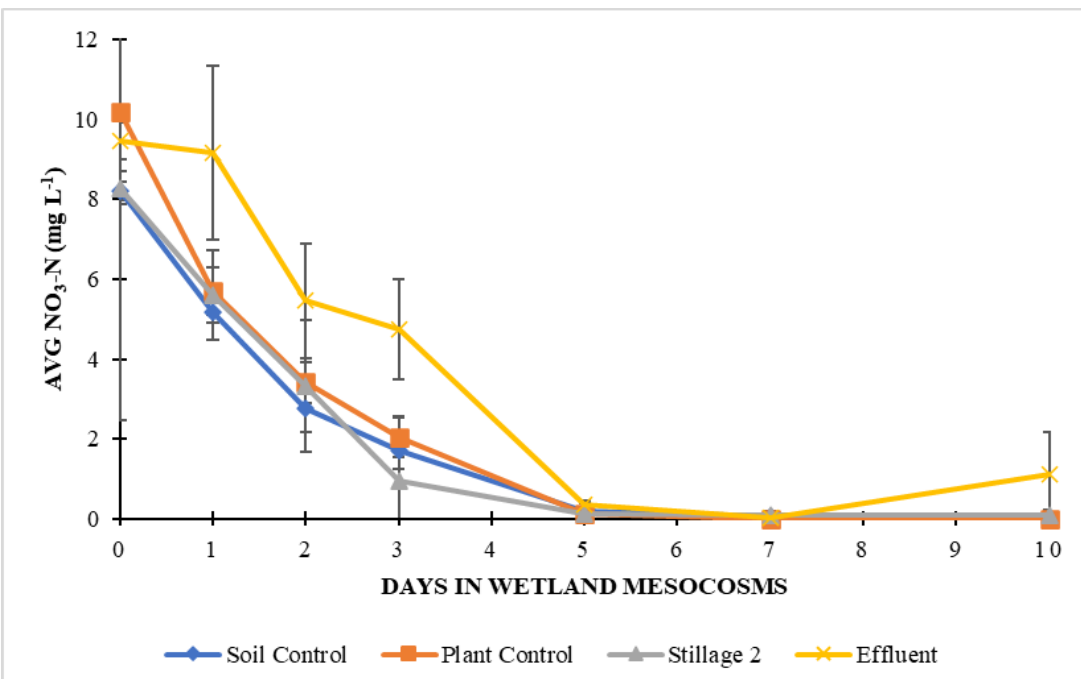

Figure 3.3 NO_3-N concentrations for the Soil Control, Plant Control, Stillage 2, and Effluent treatments during Experiment 3.

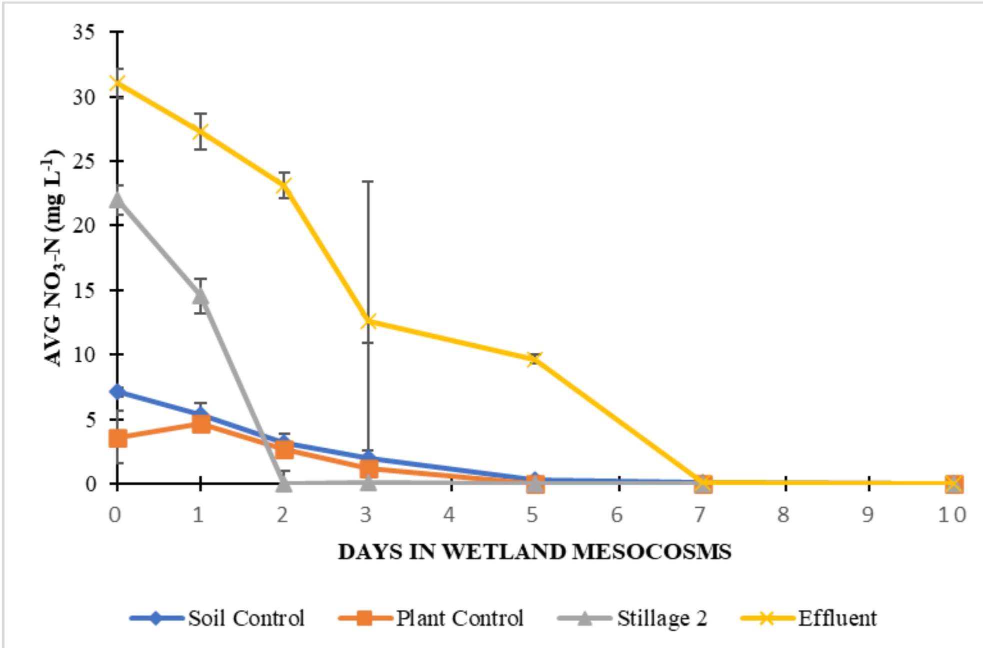

Figure 3.4 NO3-N concentrations for the Soil Control, Plant Control, Stillage 2, and Effluent treatments during Experiment 4.

Table 3.1 Average removal rates (k, d^{-1}) and standard deviations in mesocosms for all five treatments after the conclusion of all experiments. Removal rates were determined using initial NO_3-N concentrations and NO_3-N concentrations prior to reaching the minimal detection limit detection limit (0.20 mg L^{-1}). "N/A" indicates data was unable to be collected during experiment.

	Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**	
Treatments	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**
Soil Control	0.18 ± 0.03	99.7 ± 1.0	0.26 ± 0.03	99.0 ± 0.34	0.47 ± 0.15	99.3 ± 1.0	0.44 ± 0.08	99.7 ± 1.0
Plant Control	0.15 ± 0.03	99.7 ± 1.0	0.08 ± 0.07	98.6 ± 0.08	0.46 ± 0.04	99.3 ± 1.0	0.37 ± 0.07	99.7 ± 1.0
Stillage 1	0.24 ± 0.04	99.7 ± 1.0	0.09 ± 0.06	46.7 ± 38.6	N/A	N/A	N/A	N/A
Stillage 2	N/A	N/A	N/A	N/A	0.54 ± 0.39	99.3 ± 1.0	2.41 ± 1.72	99.7 ± 1.0
Effluent	0.16 ± 0.02	99.7 ± 1.0	0.15 ± 0.04	96.3 ± 4.9	0.37 ± 0.04	99.3 ± 1.0	0.42 ± 0391	99.7 ± 1.0

Table 3.2 Denitrification parameter ranges that include pH, DO, temperature, and mean DOC (with standard deviation), during Experiments 1, 2, and 3. "N/A" indicates data was unable to be collected during experiment.

	Experiment 1				**Experiment 2**				**Experiment 3**			
Denitrification Factors	**Soil Control**	**Plant Control**	**Stillage 1**	**Effluent**	**Soil Control**	**Plant Control**	**Stillage 1**	**Effluent**	**Soil Control**	**Plant Control**	**Stillage 2**	**Effluent**
pH	7.7 – 9.2	7.2 – 8.2	7.2 – 8.0	7.2 – 7.9	7.3 – 8.1	7.0 – 7.5	3.5 – 3.8	6.2 – 7.2	7.21 – 8.28	6.74 – 7.58	5.17 – 5.98	6.48 – 7.33
DO (mg O L^{-1})	5.5 – 11.8	2.4 – 5.7	2.7 – 5.2	3.7 – 6.6	1.7 – 6.0	2.4 – 5.2	1.0 – 2.9	2.5 – 4.4	1.37 ± 3.13	1.46 – 2.61	0.05 – 0.17	0.73 – 3.84
Temperature (°C)	18.9 – 22.9	18.8 – 22.4	18.4 – 21.8	18.7 – 22.5	20.6 – 25.5	20.4 – 24.6	21.0 – 24.9	20.7 – 24.4	24.5 – 28.8	23.3 – 27.8	24.7 – 28.7	16.7 – 27.6
DOC (mg C L-^{1})	10.0 ± 1.61	5.10 ± 0.49	5.13 ± 0.52	4.69 ± 0.45	9.20 ± 0.80	6.20 ± 0.86	N/A	12.1 ± 0.41	9.25 ± 0.71	3.94 ± 0.27	1654 ± 46.9	10.4 ± 1.09

Table 3.2 (continued) Denitrification parameter ranges that include pH, DO, temperature, and mean DOC (with standard deviation), during Experiment 4. “N/A” indicates data was unable to be collected during experiment.

	Experiment 4			
Denitrification Factors	**Soil Control**	**Plant Control**	**Stillage 2**	**Effluent**
pH	7.6 – 8.4	7.1 – 7.3	7.2 – 7.6	6.7 – 7.1
DO (mg O L^{-1})	2.0 – 5.2	0.9 – 3.0	0.0 – 1.2	0.6 – 1.9
Temperature (°C)	22.1 – 26.7	21.4 – 25.9	21.7 – 26.7	21.0 - 25.6
DOC (mg C L^{-1})	10.2 ± 1.14	4.27 ± 0.27	79.4 ± 25.5	11.7 ± 0.20

3.1.2 Indicators of Denitrification

Denitrifying conditions were measured throughout the study. The primary conditions needed to promote denitrification are moderate pH (6 to 8), moderate to warm water temperatures (15 °C to 24 °C), NO_3- N available in the water, usable organic matter (carbon) to act as a carbon donor, and anaerobic conditions (DO concentrations < 2 mg L^{-1}) (Kielhauer et. al., 2019; Messer et al., 2017a; Vymazal, 2007).

All parameters were in the range of denitrifying conditions, with the exception of DO levels during Experiment 1 for all treatments and pH in the Stillage 1 treatment in Experiment 2 (Table 3.2). Significant differences were observed throughout time in each experiment ($\alpha = 0.05$; Tables A9 – 41). One of the most notable differences between treatments was DOC being higher and DO being lower in the Stillage 1 and Stillage 2 treatments, indicating likely high microbial activity. These observations were also consistent with the higher NO_3- N removal rates observed in the Stillage 2 treatments in Experiments 3 and 4.

In a review focused on nitrogen removal in constructed wetlands multiple researchers have observed the need for a sufficient and biologically available carbon source for NO_3^- reduction in wastewater wetlands (Lee et al., 2009). This aligns with observations of this study, where the Stillage 2 treatment during Experiment 4, where high DOC concentrations resulted in higher NO_3-N removal rates compared to the other treatments with lower DOC concentrations. Ultimately, the stillage likely acted as an available carbon source, with higher concentrations enhancing denitrification conditions.

Anaerobic conditions were also a critical requirement for denitrification to occur. In the absence of O_2 facultative anaerobic bacteria instead will use available NO_3- to create ATP (Nichols, D.S. 1983). The lowest DO recorded during this study was in the Stillage 2 treatment during Experiment 4, which had the highest removal rate of NO_3-N. Further, the differences observed between the Stillage 2 treatment in Experiments 2 and 3, coincides with implications of acidic pH drastically decreasing denitrification in a system (Nichols, D.S. 1983). In Experiment 3, the pH was adjusted after the stillage was added to the system to an average of 5.7. DOC concentrations typically increased, while DO concentrations decreased through time. Unfortunately, the adjusted pH did not improve the NO_3-N removal rate (0.54 ± 0.39 day $^{-1}$). During Experiment 4, the pH did not need to be readjusted as no new stillage was added and had an average reading of 7.4, which was within the preferred range for denitrification to occur (~7). At the same time DOC remained high and DO remained low; however, the removal rate for NO_3-N improved significantly (2.41 ± 1.72 day^{-1}).

3.1.3 PO_4-P

PO_4-P varied between experiments and treatments, with removal varying between 50 to 90% depending on treatment and experiment, and some treatments were observed to have negative removal rates (Table 3.3).

During Experiment 1, significant differences were observed between treatments ($\alpha = 0.05$; Table A42) where all treatments responded similarly until day 10 when PO_4-P approached non-detectable limits (Figure 3.5). Significant differences were also observed between sampling day(s) within each of the four treatments where PO_4-P typically decreased with time ($\alpha = 0.05$; Table A43).

During Experiment 2, WWTP effluent and whole stillage were added to the Effluent and Stillage 1 treatments. Significant differences were observed in all treatments, except for the Stillage 1 treatment, and all other treatments responded similarly until approaching non-detectable values of PO_4-P and decreased through time ($\alpha = 0.05$; Table A44). Significant differences were observed for the Stillage 1 and Effluent treatments starting on day 3 ($\alpha = 0.05$) where the Effluent treatment experienced higher PO_4-P concentrations compared to the two control treatments, likely due to the higher concentrations of PO_4-P that was already in the effluent compared to the PO_4-P concentrations in the tap water that the controls received. The differences in the Stillage 1 treatment to other treatments were due to the exceedingly high starting PO_4-P concentrations in the stillage, which were roughly 57X higher than the control treatments and 7X higher than the Effluent treatment ($\alpha = 0.05$; Figure 3.6). PO_4-P concentrations in the Stillage 1 treatment also Increased through time during the experiment. Significant differences were also observed between sampling day(s) within the Soil Control and Effluent treatments but did not exhibit a trend other than decreasing with time ($\alpha = 0.05$; Table A45).

During Experiment 3, WWTP effluent and whole stillage were added to the Effluent and Stillage 2 treatments. Significant differences were observed for the Stillage 2 and Effluent treatments starting on day 2 ($\alpha = 0.05$; Table A46). Similar to the Stillage 1 treatment in Experiment 2, the Stillage 2 treatment had an exceedingly high PO_4-P starting concentration, which was 100X higher compared to the control treatments and 13X higher compared to the Effluent treatment ($\alpha = 0.05$; Figure 3.7). In contrast to the Stillage 1 treatment in the previous experiment, the Stillage 2 treatment observed a decrease in PO_4-P through time during the experiment. Once again, the Effluent treatment had a higher starting PO_4-P concentration compared to the control treatments, likely due to a high concentration of PO_4-P was present in the applied effluent. Significant differences were also observed between sampling day(s) within each of the four treatments but did not exhibit a trend other than decreasing with time ($\alpha = 0.05$; Table A47).

During Experiment 4, WWTP effluent was added to the Effluent and Stillage 2 treatments. Significant differences were observed starting on day 2 for the Stillage 2 treatment and on day 3 for all other treatments ($\alpha = 0.05$; Table A48). The Stillage 2 treatment had a higher starting PO_4-P concentration compared to the control treatments, similar to the previous two experiments; however, PO_4-P concentrations were only 50X higher than the control treatments and had lower starting concentrations compared to the Effluent treatment ($\alpha = 0.05$; Figure 3.8). Similar to the Stillage 1 treatment in experiment 2, the Stillage 2 treatment exhibited a negative removal rate because PO_4-P increased through time during the experiment ($\alpha = 0.05$). The Soil Control treatment also observed a negative removal rate, which was likely due to absence of plants in this treatment ($\alpha = 0.05$). The Effluent treatment observed the highest starting PO_4-P concentration during this experiment, which resulted in the highest percent removal compared to all other treatments ($\alpha = 0.05$). Significant differences were also observed between sampling day(s) within each of the treatments, apart from the Stillage 2 treatment ($\alpha = 0.05$; Table A49).

Average PO_4-P removal rates varied between treatments (Table 3.3), where removal rates increased and decreased throughout the growing season. The Effluent treatment had overall similar removal rates compared to the Soil and Plant Controls, apart from the Soil Control treatment in Experiment 4. The Stillage 1 treatment had a similar removal rate compared to all other treatments during Experiments 1 and 3. However, during Experiments 2 and 4 the removal rates became negative -0.08 ± 0.10 day^{-1} and -0.06 ± 0.03 day^{-1}, respectively.

A trend emerged from the Stillage 1 and 2 treatments during Experiments 2 and 4, where PO_4-P increased through time during each experiment. Patrick and Khalid (1974) observed anaerobic conditions promoted a larger release of PO_4-P from soils than aerobic conditions. The negative removal rates for PO_4-P, during those two experiments, were likely impacted by the low to almost nonexistent DO observed for those treatments. The Stillage 2 treatment, in Experiment 3, was different than the Stillage 1 and Stillage 2 treatments in Experiments 2 and 4 because the addition of air stones increased DO in the treatment, thus playing a part in the positive removal rate during Experiment 3 (0.013 ± 0.05 day^{-1}). The Effluent treatment had higher removal rates for Experiments 2, 3, and 4 compared to Gale et al (1994), which compared the removal rates of PO_4-P between constructed wetlands (CW) and natural wetlands (NW) that were inundated with treated wastewater in Florida. Gale et al. (1994), observed removal rates of 0.035 to 0.072 day^{-1} between two CW and two NW, which were all consistently lower than the PO_4-P removal rates observed during Experiments 2, 3, and 4 (0.09 day^{-1}, 0.19 day^{-1}, and 0.09 day^{-1}).

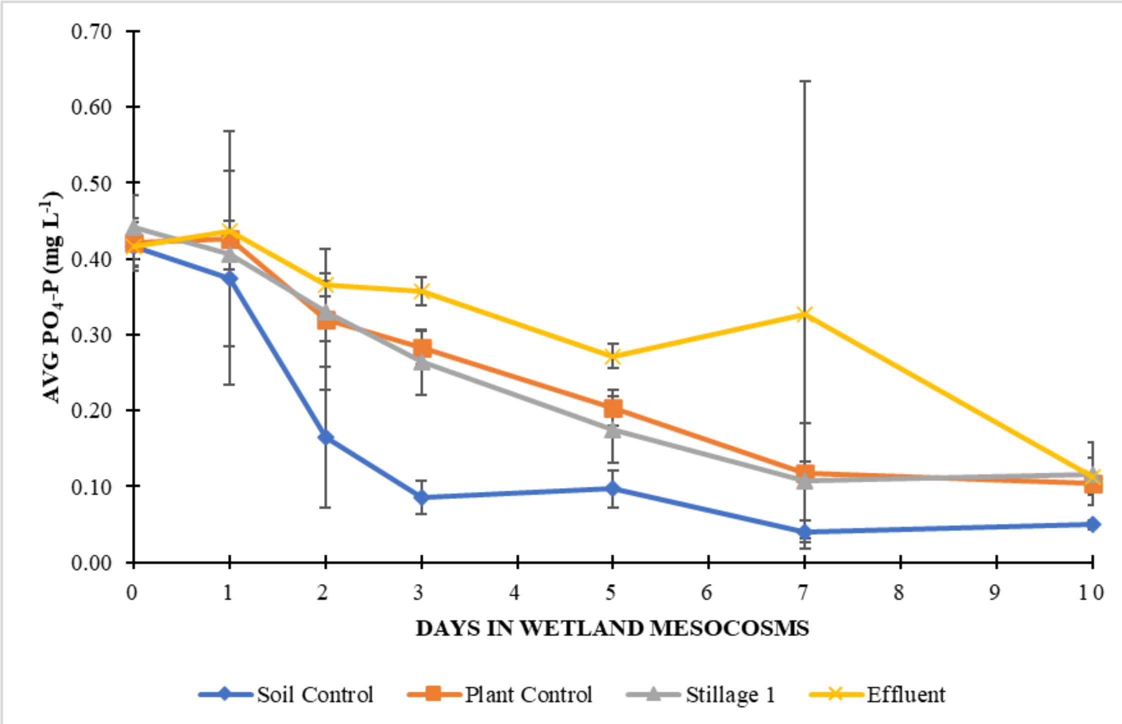

Figure 3.5 PO_4-P concentrations for the Soil Control, Plant Control, Stillage 1, and Effluent treatments during Experiment 1.

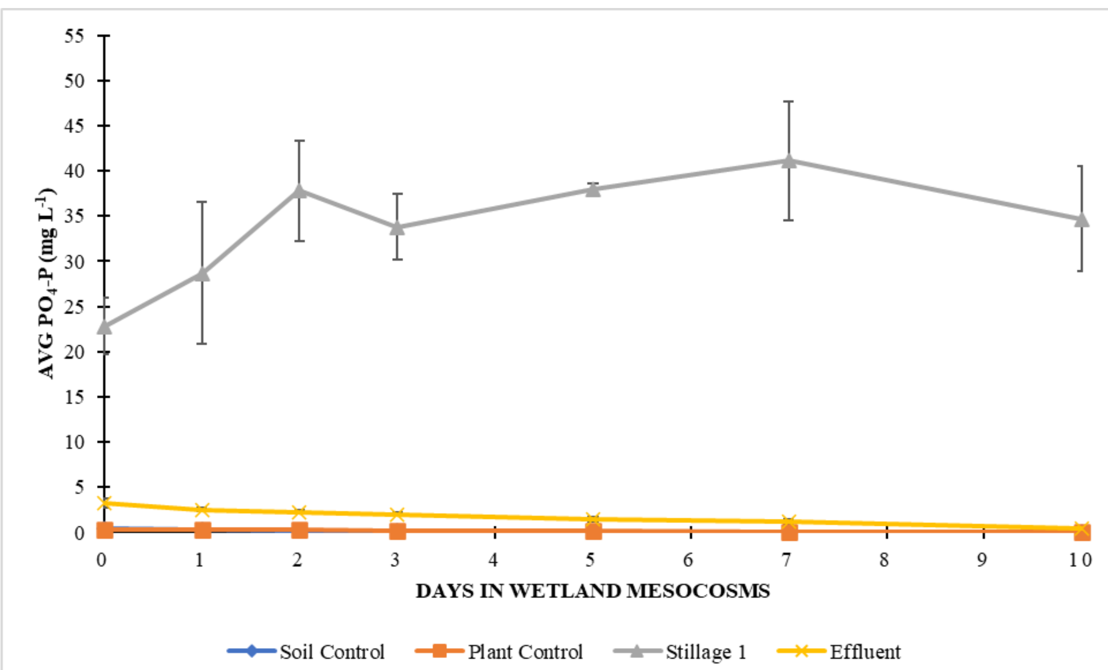

Figure 3.6 PO_4-P concentrations for the Soil Control, Plant Control, Stillage 1, and Effluent treatments during Experiment 2.

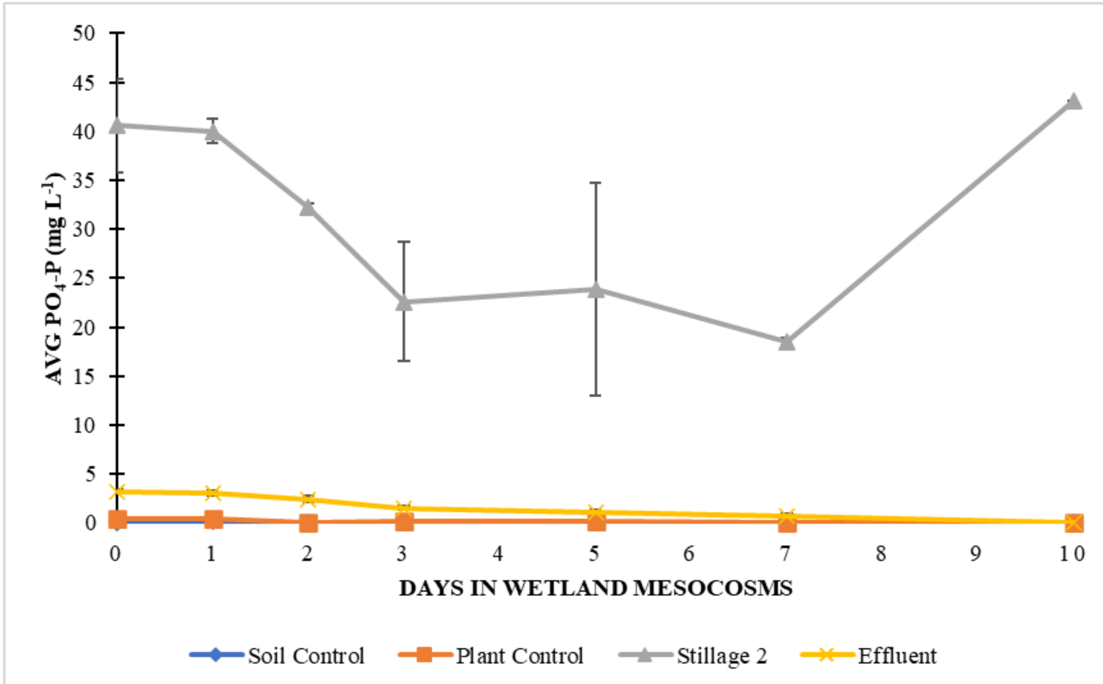

Figure 3.7 PO_4-P concentrations for the Soil Control, Plant Control, Stillage 2, and Effluent treatments during Experiment 3.

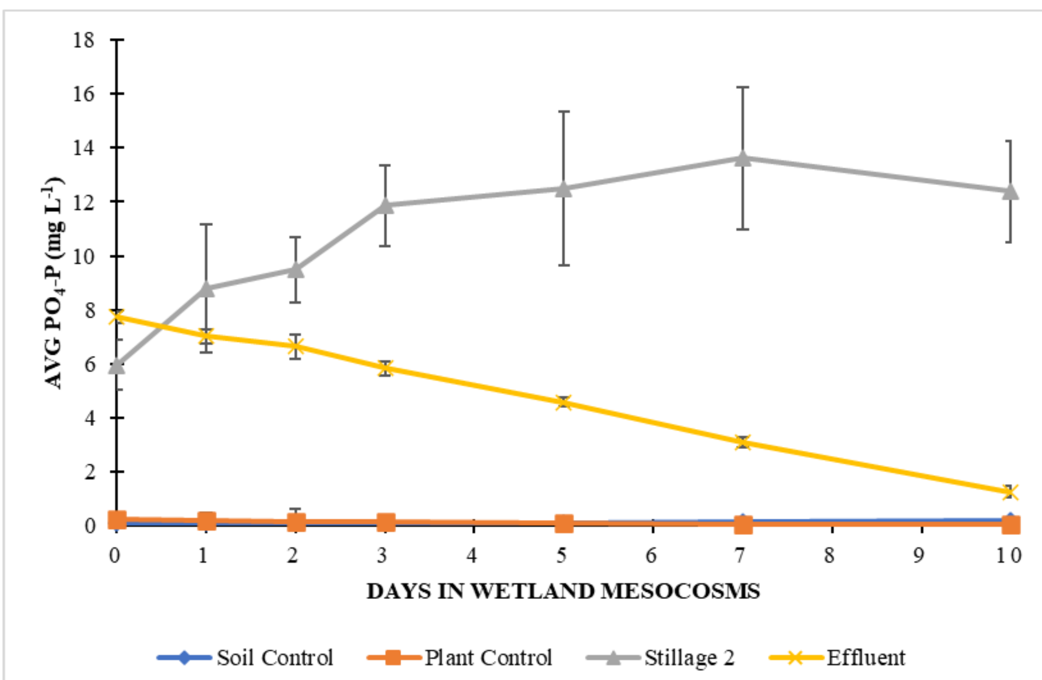

Figure 3.8 PO_4-P concentrations for the Soil Control, Plant Control, Stillage 2, and Effluent treatments during Experiment 4.

Table 3.3 Average removal rates (k, d^{-1}) and standard deviations in mesocosms for all five treatments after the conclusion of all experiments. Removal rates were determined using initial PO_4-P concentrations and PO_4-P concentrations prior to reaching the minimal limit of detection (0.20 mg L^{-1}).

	Experiment 1		**Experiment 2**		**Experiment 3**		**Experiment 4**	
Treatments	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**	**k (day^{-1})**	**Removal (%)**
Soil Control	0.38 ± 0.12	87.4 ± 0.02	0.20 ± 0.09	85.9 ± 0.05	0.44 ± 0.04	74.2 ± 0.15	-0.02 ± 0.05	-69.2 ± 0.57
Plant Control	0.16 ± 0.07	70.8 ± 0.04	0.09 ± 0.04	82.9 ± 0.03	0.44 ± 0.15	88.3 ± 0.01	0.15 ± 0.08	61.8 ± 0.07
Stillage 1	0.15 ± 0.06	66.3 ± 0.14	-0.08 ± 0.10	-66.0 ± 0.20				
Stillage 2					0.13 ± 0.05	53.7 ± 0.02	-0.06 ± 0.03	-124 ± 0.13
Effluent	0.09 ± 0.04	67.7 ± 0.08	0.09 ± 0.02	78.1 ± 0.05	0.19 ± 0.02	70.2 ± 0.07	0.09 ± 0.01	75.1 ± 0.02

3.1.4 E. *coli*

Significant differences in *E. coli* concentrations were observed between treatments through time during the 10-day experiment in Experiment 4 ($\alpha = 0.05$; Table A50), with the Stillage 2 treatment resulting in higher concentrations compared to the Effluent treatments throughout the entirety of Experiment 4. The EPA has set the maximum allowable limit of *E. coli* to 235 cfu per 100 mL for primary contact recreators (United States Environmental Agency 2012). Significant differences were observed on day 10, where the Effluent treatment had fallen below the maximum limit thus achieving the initial goal of reducing *E. coli* to below EPA standards. The Effluent treatment resulted in a 99% reduction during Experiment 4, which is similar to previous studies that observed a 50 to 99% reduction in CW wastewater treatment systems (Coleman et al., 2001; Jillson et al., 2001; Tanner et al., 1998). However, the Stillage 2 treatment stayed above the allowable limit for secondary freshwater throughout the entirety of Experiment 4 (980.4 to 2419.6 cfu per 100 mL) (Figure 3.9). Significant differences were also observed between sampling day(s) within the Effluent treatment ($\alpha = 0.05$; Table A51) but did not exhibit a trend other than decreasing with time.

Slower *E. coli* reduction in the Stillage 2 treatment was likely due to more limited UV penetration due to the higher dissolved organic matter (DOM) and suspended solids in the water column. O'Green and Bianchi (2015) observed that the ability of a wetland to reduce *E. coli* can be negatively impacted by an excess of vegetation resulting in shading. Further, several studies have reported delayed *E. coli* removal with increased DOM likely from reduced UV penetration into the water column (O'Green and Bianchi 2015; MacIntyre et al., 2006; Boutilier et al., 2009). Further, Kollu & Ormeci (2014) observed regrowth of *E. coli* when inactivated cells and nutrients were present in UV treated wastewater.

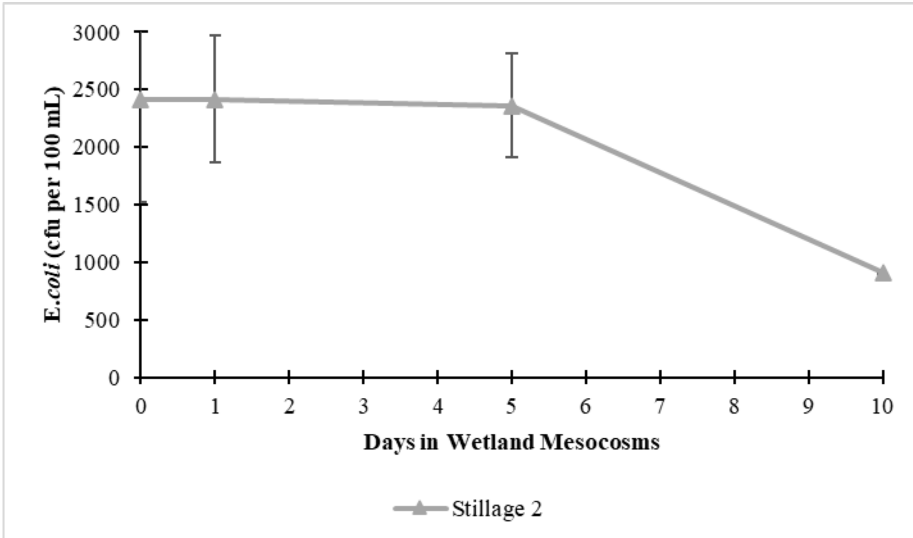

Figure 3.9 *E. coli* concentrations for the Stillage 2 treatment during Experiment 4.

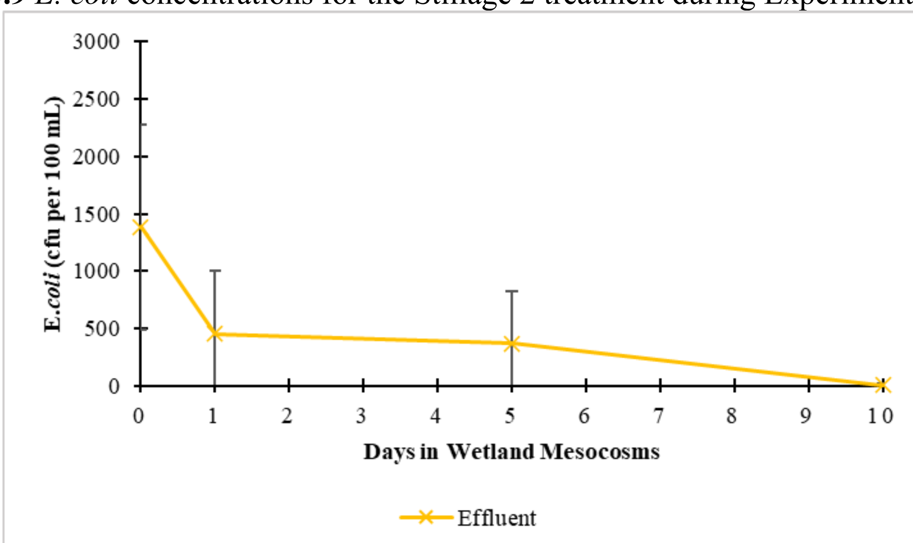

Figure 3.10 *E. coli* concentrations for the Effluent treatment during Experiment 4.

3.1.5 Biochemical Oxygen Demand

NPDES Permit Writers' Manual (2010) has set the standards for BOD removal at an 85% minimum, 30-day average of 30 mg L^{-1} and 45 mg L^{-1} for a 7-day average (U.S. EPA, 2019), thus a single day allowable limit would be ~6.42 mg L^{-1}. Li & Lie (2019), summarized that a moderately polluted river will have a BOD range between 2 and 8 mg L^{-1} and anything below 2 mg L^{-1} is considered pristine while anything above 8 mg L^{-1} is considered severely polluted. It is important to note that BOD has an inverse relationship with DO. When testing BOD in wastewater treatment plants a low BOD means that there is less oxygen being removed from the water by bacteria and microorganisms. This results in high DO levels for more advanced forms of aquatic life to use. Therefore, low BOD and high DO results in a healthy ecosystem for streams.

Unfortunately, not enough BOD data was collected during this project to report accurately. Concentrations ranged from 0.27 to 1.07 mg L^{-1} during Experiment 3 and only one usable concentration was calculated during Experiment 4 due to limited dilutions to have values above 0 mg L^{-1} (1.14 mg L^{-1}).

3.2 Nutrient Uptake in Plants

Plant uptake is often a major nutrient removal pathway in wetland treatment systems (Messer et la., 2017; Lee et al., 2009; U. S. EPA 1988). Biomass was found to be greatly degraded following the application of the non-adjusted pH stillage for Experiment 2 (Figure 3.11-3.13). Similar implications have been seen in biomass following the addition of invasive species (Magee & Kentula, 2005), oxygen depletion (Brix & Sorrell 1996), and pharmaceuticals (Carvalho et al., 2014). Experiment 2 was the first addition of distillery stillage to the experimental mesocosms. At the end of Experiment 2, almost all plants within the Stillage 1 mesocosms had turned yellow and wilted. This was likely due to low available oxygen and the acidic pH in the water.

The total nitrogen (TN), total phosphorus (TP), and total carbon (TC) content was assessed at the end of the study in the plant roots and above ground biomass to compare differences between treatments (Table 3.4). The average TN content in the above ground biomass at the end of the experiments was 8.28 ± 0.46 g m^{-2} for the Plant Control treatment, 11.35 ± 0.56 g m^{-2} for the Stillage 1 treatment, 8.49 ± 1.07 g m^{-2} for the Stillage 2 treatment, and 9.22 ± 0.77 g m^{-2} for the Effluent treatment. The TN content observed at the end of the four experiments were much lower compared to a study investigating floating treatment wetlands amended with spent coffee and not containing soil, which observed TN concentrations ranging from 26.5 to 43.8 g m^{-2} (Keilhauer et al., 2019).

The average TP content in the above ground biomass at the end of the experiments was 1.51 ± 0.01 g m^{-2} for the Plant Control treatment, 1.59 ± 0.01 g m^{-2} for the Stillage 1 treatment, 8.49 ± 0.02 g m^{-2} for the Stillage 2 treatment, and 9.22 ± 0.01 g m^{-2} for the Effluent treatment. These concentrations were much lower compared to a study on wetland treatments for agricultural drainage water which observed TP to range between 18 to 57 g m^{-3} (Messer et al., 2017). The large difference in TP was likely due to Messer et al., (2017) study having using nutrient rich soils and a monoculture of soft-stem bulrush that had been established over 3 years. Notably, the Effluent treatment in this study had significantly higher uptake of TP compared to all other treatments. This was likely due to high concentrations of phosphorus in the effluent upon collection.

The average TC content in the above ground biomass at the end of the experiments was 386.2 ± 0.2 g m^{-2} for the Plant Control treatment, 184.0 ± 0.1 g m^{-2} for the Stillage 1 treatment, 347.7 ± 0.1 g m^{-2} for the Stillage 2 treatment, and 487.4 ± 0.1 g m^{-2} for the Effluent treatment. This is considerably lower compared to Burke et al. (2011), who determined that a wastewater treatment wetland in California observed a TC concentration of 3,317.6 g m^{-2} for their average total live biomass. The vast difference in observed concentrations is understandable considering that Burke et al. (2011) conducted their study on a 0.8 ha (1.9 acre) CW that received its inflow from an oxidation pond. Notably, the Effluent treatment had significantly higher uptake of TC compared to all other treatments. This was likely due to high concentrations of available carbon in the effluent upon collection.

Figure 3.11 Stillage 1 mesocosm after Experiment 1 and before distillery stillage was added.

Figure 3.12 Stillage 1 mesocosm after the addition of stillage during Experiment 2.

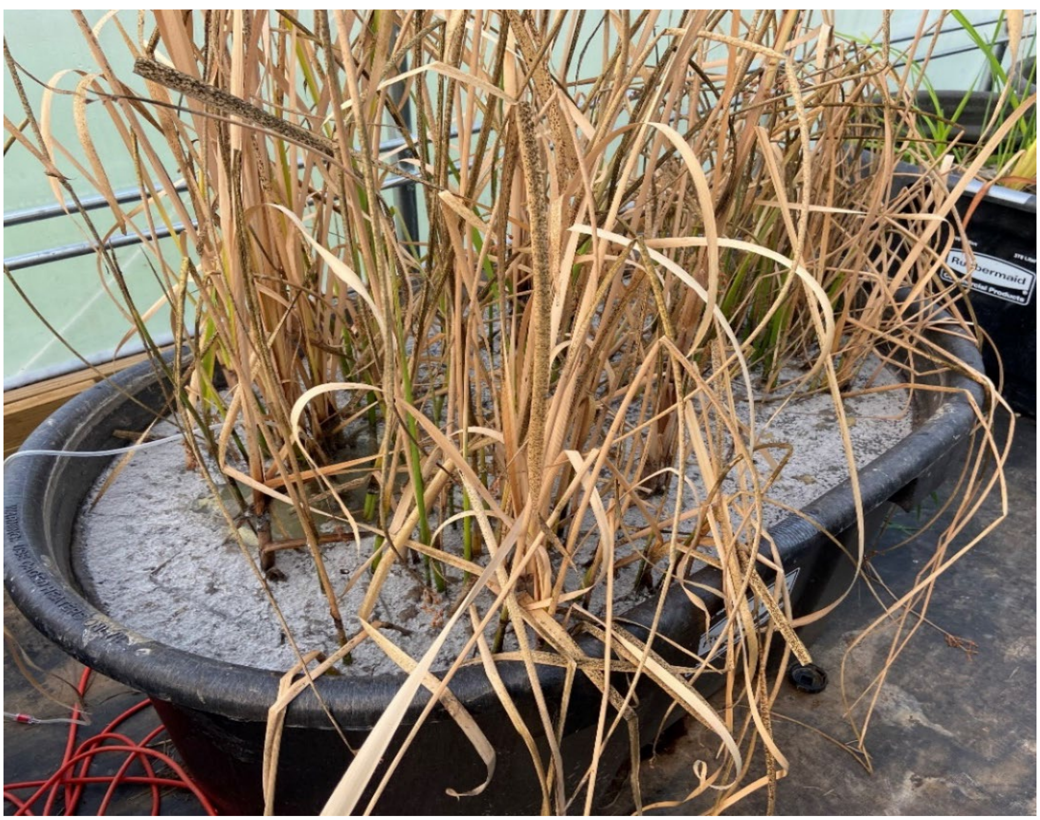

Figure 3.13 Stillage 1 mesocosm at the completion of Experiment 2.

Table 3.4 Total Nitrogen (TN), Total Phosphorus (TP), and Total Carbon (TC) above and below concentrations (g m^{-2}) for all planted treatments at the conclusion of the experiments.

	TN		TP		TC	
Treatments	**Above**	**Below**	**Above**	**Below**	**Above**	**Below**
Plant Control	8.28 ± 0.46	0.22 ± 0.02	1.51 ± 0.01	0.37 ± 0.02	386.2 ± 0.20	39.8 ± 1.17
Stillage 1	11.35 ± 0.56	-	1.59 ± 0.01	-	184.0 ± 0.10	-
Stillage 2	8.49 ± 1.07	0.17 ± 0.01	8.49 ± 0.02	0.27 ± 0.01	347.7 ± 0.10	17.5 ± 0.60
Effluent	9.22 ± 0.77	0.59 ± 0.02	9.22 ± 0.01	1.40 ± 0.03	487.4 ± 0.10	49.6 ± 0.34

CHAPTER 4. CONCLUSIONS

4.1 Conclusions

Constructed wetlands were found to provide high nutrient and *E. Coli* removal potential. The Effluent treatment was observed to remove 64 to 99% of NO_3-N, 61 to 99% of PO_4-P, and 99% removal of *E. coli*, while the Stillage 1 and 2 treatments were observed to have 50 to 100% removal of NO_3-N. However, the Stillage 1 and 2 treatments had an overall increase in PO_4-P over time. Further, the addition of stillage and effluent to the wetland enhances NO_3-N removal rates. Overall, wastewater treatment discharge limits were met for NO_3-N and *E. coli* for all treatments, with exception to *E. coli* in the stillage treatments. Findings from this project are anticipated to be utilized to size and implement a secondary wastewater treatment wetland for a distillery in Central Kentucky.

4.2 Future Work

Future studies are needed to assess the wetland design implications for treating municipal wastewater by studying the biochemical oxygen demand (BOD), as it is often used as an indicator of water quality health. Further, this study only assessed the potential capability of these systems in their first year of establishment during the growing seasons. Therefore, further assessment of seasonal treatment potentials for NO_3-N, PO_4-P. and *E. coli* are needed to adequately size the wetland to treat contaminants in municipal wastewater effluent year-round prior to being discharged.

APPENDICES

APPENDIX A

Supplemental statistical data for all nutrients and contaminants collected for all four experiments.

Table A1 Statistical differences between treatments on specific sampling days for NO_3-N during Experiment 1 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
7	Soil Control	Stillage 1	<.0001
7	Plant Control	Stillage 1	<.0001
7	Effluent	Stillage 1	<.0001
10	Soil Control	Effluent	<.0001
10	Soil Control	Stillage 1	<.0001
10	Soil Control	Plant Control	0.0043

Table A2 Statistical differences within treatments on specific samplings days for NO_3-N during Experiment 1 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	0.0176
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	7	0.0002
Soil Control	2	10	<.0001
Soil Control	3	7	0.0016
Soil Control	3	10	<.0001
Soil Control	5	7	0.0408
Soil Control	5	10	<.0001
Soil Control	7	10	<.0001
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	7	<.0001
Plant Control	3	10	<.0001
Plant Control	5	7	0.0016
Plant Control	5	10	<.0001
Plant Control	7	10	<.0001
Stillage 1	1	5	0.0051
Stillage 1	1	7	<.0001
Stillage 1	1	10	<.0001
Stillage 1	2	5	0.0089
Stillage 1	2	7	<.0001
Stillage 1	2	10	<.0001
Stillage 1	3	5	0.0429
Stillage 1	3	7	<.0001
Stillage 1	3	10	<.0001
Stillage 1	5	7	<.0001
Stillage 1	5	10	<.0001
Stillage 1	7	10	<.0001
Effluent	1	5	0.0476
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	7	<.0001
Effluent	3	10	<.0001
Effluent	5	7	0.0009
Effluent	5	7	<.0001
Effluent	5	10	<.0001

Table A3 Statistical differences between treatments on specific sampling days for NO_3-N during Experiment 2 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Stillage 1	0.0128
5	Soil Control	Effluent	0.0158
7	Soil Control	Stillage 1	<.0001
7	Soil Control	Effluent	<.0001
7	Stillage 1	Plant Control	<.0001
7	Effluent	Plant Control	< .0001
10	Soil Control	Plant Control	0.0001
10	Soil Control	Stillage 1	<.0001
10	Soil Control	Effluent	<.0001
10	Stillage 1	Plant Control	<.0001
10	Effluent	Stillage 1	0.0003

Table A4 Statistical differences within treatments on specific samplings days for NO_3-N during Experiment 2 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	1	0.0156
Soil Control	0	3	<.0001
Soil Control	0	5	<.0001
Soil Control	0	7	<.0001
Soil Control	0	10	<.0001
Soil Control	1	3	<.0001
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	3	<.0001
Soil Control	2	5	<.0001
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	<.0001
Soil Control	3	7	0.0011
Soil Control	3	10	<.0001
Soil Control	5	7	<.0001
Soil Control	5	10	0.0482
Soil Control	7	10	0.0008
Plant Control	0	1	0.0022
Plant Control	0	3	<0.0001
Plant Control	0	5	<0.0001
Plant Control	0	7	<0.0001
Plant Control	0	10	<0.0001
Plant Control	1	3	<0.0001
Plant Control	1	5	<0.0001
Plant Control	1	7	<0.0001
Plant Control	1	10	<0.0001
Plant Control	2	3	<0.0001
Plant Control	2	5	<0.0001
Plant Control	2	7	<0.0001
Plant Control	2	10	<0.0001
Plant Control	3	5	<0.0001
Plant Control	3	10	<0.0001
Plant Control	5	7	<0.0001
Plant Control	7	10	0.0001
Stillage 1	0	3	<.0001
Stillage 1	0	5	<.0001

Stillage 1	0	7	<.0001
Stillage 1	0	10	<.0001
Stillage 1	1	3	<.0001
Stillage 1	1	5	<.0001
Stillage 1	1	7	<.0001
Stillage 1	1	10	<.0001
Stillage 1	2	3	<.0001
Stillage 1	2	5	<.0001
Stillage 1	2	7	<.0001
Stillage 1	2	10	<.0001
Stillage 1	3	5	<.0001
Stillage 1	3	7	<.0001
Stillage 1	3	10	<.0001
Effluent	0	3	<.0001
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	3	<.0001
Effluent	1	5	<.0001
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	3	<.0001
Effluent	2	5	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	10	0.0001
Effluent	5	7	<.0001
Effluent	7	10	0.0005

Table A5 Significant differences between treatments on specific Samplings Days for NO_3-N during Experiment 3 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
3	Soil Control	Stillage 2	0.0330
3	Effluent	Stillage 2	0.0089
3	Stillage 2	Plant Control	0.0262
10	Soil Control	Effluent	0.0046
10	Effluent	Stillage 2	0.0153
10	Effluent	Plant Control	0.0009

Table A6 Statistical differences within treatments on specific samplings days for NO_3-N during Experiment 3 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	0.0002
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	5	0.0018
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	0.0081
Soil Control	3	7	<.0001
Soil Control	3	10	<.0001
Soil Control	5	10	0.0102
Plant Control	1	5	<.0001
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	5	0.0004
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	5	0.0025
Plant Control	3	7	<.0001
Plant Control	3	10	<.0001
Plant Control	5	10	0.0055
Stillage 2	1	3	0.0011
Stillage 2	1	5	<.0001
Stillage 2	1	7	<.0001
Stillage 2	1	10	<.0001
Stillage 2	2	3	0.0078
Stillage 2	2	5	0.0005
Stillage 2	2	7	<.0001
Stillage 2	2	10	<.0001
Stillage 2	3	10	0.0076
Effluent	1	5	0.0007
Effluent	1	7	<.0001
Effluent	1	10	0.0005
Effluent	2	5	0.0038
Effluent	2	7	<.0001
Effluent	2	10	0.0028
Effluent	3	5	0.0058
Effluent	3	7	<.0001
Effluent	3	10	0.0043
Effluent	5	7	0.0020
Effluent	7	10	0.0027

Table A7 Significant differences between treatments on specific samplings days for NO_3-N during Experiment 4 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
2	Soil Control	Stillage 2	0.0003
2	Effluent	Stillage 2	<.0001
2	Stillage 2	Plant Control	0.0003
3	Soil Control	Stillage 2	0.0037
3	Effluent	Stillage 2	0.0008
3	Stillage 2	Plant Control	0.0086
5	Effluent	Stillage 2	0.0057
5	Effluent	Plant Control	0.0011
10	Effluent	Stillage 2	0.0183

Table A8 Statistical differences within treatments on specific samplings days for NO3-N during Experiment 4 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	0.0135
Soil Control	1	7	0.0009
Soil Control	1	10	<.0001
Soil Control	2	5	0.0383
Soil Control	2	7	0.0031
Soil Control	2	10	<.0001
Soil Control	3	7	0.0095
Soil Control	3	10	<.0001
Soil Control	5	10	0.0096
Plant Control	1	5	<.0001
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	5	0.0003
Plant Control	2	7	0.0001
Plant Control	2	10	<.0001
Plant Control	3	5	0.0025
Plant Control	3	7	0.0010
Plant Control	3	10	<.0001
Stillage 2	1	2	<.0001
Stillage 2	1	3	0.0003
Stillage 2	1	5	0.0005
Stillage 2	1	7	0.0006
Stillage 2	1	10	<.0001
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	7	<.0001
Effluent	3	10	<.0001
Effluent	5	7	0.0002
Effluent	5	10	<.0001
Effluent	7	10	0.0241

Table A9 Significant differences between treatments on specific samplings days for DOC during Experiment 1 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Effluent	0.0088
5	Soil Control	Stillage 1	0.0127
5	Soil Control	Plant Control	0.0009
10	Soil Control	Effluent	<.0001
10	Soil Control	Stillage 1	0.0001
10	Soil Control	Plant Control	<.0001

Table A10 Statistical differences within treatments on specific samplings days for DOC during Experiment 1 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	<.0001
Soil Control	1	10	<.0001
Soil Control	5	10	<.0001
Plant Control	1	5	<.0001
Plant Control	1	10	<.0001
Plant Control	5	10	<.0001
Stillage 1	1	5	<.0001
Stillage 1	1	10	<.0001
Stillage 1	5	10	0.0001
Effluent	1	5	<.0001
Effluent	1	10	<.0001
Effluent	5	10	0.0004

Table A11 Significant differences between treatments on specific samplings days for DOC during Experiment 2 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Effluent	<.0001
5	Soil Control	Plant Control	0.0010
5	Effluent	Plant Control	<.0001
10	Soil Control	Effluent	<.0001
10	Soil Control	Plant Control	<.0001
10	Effluent	Plant Control	<.0001

Table A12 Statistical differences within treatments on specific samplings days for DOC during Experiment 2 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	<.0001
Soil Control	1	10	0.0004
Soil Control	5	10	0.0116
Plant Control	1	5	0.0050
Plant Control	1	10	0.0007
Plant Control	5	10	<.0001
Effluent	1	5	0.0023
Effluent	1	10	<.0001
Effluent	5	10	<.0001

Table A13 Significant differences between treatments on specific samplings days for DOC during Experiment 3 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Plant Control	0.0060
5	Soil Control	Stillage 2	<.0001
5	Soil Control	Effluent	<.0001
5	Stillage 2	Plant Control	<.0001
5	Effluent	Stillage 2	<.0001
5	Effluent	Plant Control	<.0001
10	Soil Control	Plant Control	<.0001
10	Soil Control	Stillage 2	<.0001
10	Soil Control	Effluent	<.0001
10	Stillage 2	Plant Control	<.0001
10	Effluent	Stillage 2	<.0001
10	Effluent	Plant Control	<.0001

Table A14 Statistical differences within treatments on specific samplings days for DOC during Experiment 3 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	<.0001
Soil Control	1	10	<.0001
Soil Control	5	10	0.0002
Plant Control	1	5	0.0298
Plant Control	1	10	0.0132
Plant Control	5	10	<.0001
Stillage 2	1	5	<.0001
Stillage 2	1	10	<.0001
Stillage 2	5	10	0.0083
Effluent	1	5	0.0006
Effluent	1	10	<.0001
Effluent	5	10	<.0001

Table A15 Significant differences between treatments on specific sampling days for DOC during Experiment 4 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Stillage 2	<.0001
5	Plant Control	Stillage 2	<.0001
5	Effluent	Stillage 2	<.0001
10	Soil Control	Stillage 2	<.0001
10	Soil Control	Effluent	<.0001
10	Plant Control	Stillage 2	<.0001
10	Effluent	Stillage 2	<.0001

Table A16 Statistical differences within treatments on specific samplings days for DOC during Experiment 4 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	10	0.0384
Stillage 2	1	5	<.0001
Stillage 2	1	10	<.0001
Effluent	1	10	0.0024

Table A17 Significant differences between treatments on specific sampling days for temperature during Experiment 1 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
3	Soil Control	Stillage 1	0.0009
3	Effluent	Stillage 1	0.0298
3	Plant Control	Stillage 1	0.0373
10	Soil Control	Effluent	0.0186
10	Soil Control	Stillage 1	0.0040

Table A18 Statistical differences within treatments on specific samplings days for temperature during Experiment 1 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	2	0.0007
Soil Control	0	3	<.0001
Soil Control	0	7	<.0001
Soil Control	0	10	<.0001
Soil Control	1	3	<.0001
Soil Control	1	5	0.0030
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	3	<.0001
Soil Control	2	5	<.0001
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	<.0001
Soil Control	5	7	<.0001
Soil Control	5	10	<.0001
Plant Control	0	2	0.0052
Plant Control	0	3	<.0001
Plant Control	0	7	<.0001
Plant Control	0	10	<.0001
Plant Control	1	3	<.0001
Plant Control	1	5	0.0007
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	3	<.0001
Plant Control	2	5	<.0001
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	5	<.0001
Plant Control	5	7	<.0001
Plant Control	5	10	<.0001
Stillage 1	0	2	0.0023
Stillage 1	0	5	0.0373
Stillage 1	0	7	<.0001
Stillage 1	0	10	<.0001
Stillage 1	1	2	0.0373
Stillage 1	1	3	<.0001
Stillage 1	1	5	0.0023
Stillage 1	1	7	<.0001

Stillage 1	1	10	<.0001
Stillage 1	2	3	<.0001
Stillage 1	2	5	<.0001
Stillage 1	2	7	<.0001
Stillage 1	2	10	0.0040
Stillage 1	3	5	<.0001
Stillage 1	5	7	<.0001
Stillage 1	5	10	<.0001
Effluent	0	2	0.0114
Effluent	0	3	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	2	0.0236
Effluent	1	3	<.0001
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	3	<.0001
Effluent	2	5	0.0001
Effluent	2	7	<.0001
Effluent	2	10	0.0003
Effluent	3	5	<.0001
Effluent	3	10	0.0052
Effluent	5	7	<.0001
Effluent	5	10	<.0001
Effluent	7	10	0.0236

Table A19 Significant differences between treatments on specific sampling days for temperature during Experiment 2 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Effluent	0.0279
0	Soil Control	Stillage 1	0.0031
0	Plant Control	Effluent	0.0044
0	Plant Control	Stillage 1	0.0004
5	Soil Control	Effluent	0.0001
5	Soil Control	Stillage 1	0.0279
5	Soil Control	Plant Control	0.0011
7	Soil Control	Effluent	0.0008
7	Soi Control	Stillage 1	0.0369
7	Soil Control	Plant Control	0.0279
7	Effluent	Stillage 1	<.0001
7	Plant Control	Stillage 1	<.0001
10	Soil Control	Effluent	0.0011
10	Effluent	Stillage 1	0.0022

Table A20 Statistical differences within treatments on specific samplings days for temperature during Experiment 2 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	1	0.0156
Soil Control	0	3	<.0001
Soil Control	0	5	<.0001
Soil Control	0	7	<.0001
Soil Control	0	10	<.0001
Soil Control	1	3	<.0001
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	3	<.0001
Soil Control	2	5	<.0001
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	<.0001
Soil Control	3	7	0.0011
Soil Control	3	10	<.0001
Soil Control	5	7	<.0001
Soil Control	5	10	0.0482
Soil Control	7	10	0.0008
Plant Control	0	1	0.0022
Plant Control	0	3	<.0001
Plant Control	0	5	<.0001
Plant Control	0	7	<.0001
Plant Control	0	10	<.0001
Plant Control	1	3	<.0001
Plant Control	1	5	<.0001
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	3	<.0001
Plant Control	2	5	<.0001
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	5	<.0001
Plant Control	3	10	<.0001
Plant Control	5	7	<.0001
Plant Control	7	10	0.0001
Stillage 1	0	3	<.0001
Stillage 1	0	5	<.0001
Stillage 1	0	7	<.0001
Stillage 1	0	10	<.0001
Stillage 1	1	3	<.0001
Stillage 1	1	5	<.0001
Stillage 1	1	7	<.0001
Stillage 1	1	10	<.0001
Stillage 1	2	3	<.0001

Stillage 1	2	5	<.0001
Stillage 1	2	7	<.0001
Stillage 1	2	10	<.0001
Stillage 1	3	5	<.0001
Stillage 1	3	7	<.0001
Stillage 1	3	10	<.0001
Effluent	0	3	<.0001
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	3	<.0001
Effluent	1	5	<.0001
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	3	<.0001
Effluent	2	5	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	5	<.0001
Effluent	3	10	0.0001
Effluent	5	7	<.0001
Effluent	7	10	0.0005

Table A21 Significant differences between treatments on specific sampling days for temperature during Experiment 3 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Effluent	<.0001
0	Soil Control	Stillage 2	0.0031
0	Soil Control	Plant Control	<.0001
0	Plant Control	Effluent	<.0001
0	Effluent	Stillage 2	<.0001
1	Soil Control	Effluent	<.0001
1	Soil Control	Plant Control	0.0080
1	Plant Control	Effluent	0.0001
1	Effluent	Stillage 2	<.0001
2	Soil Control	Effluent	<.0001
2	Soil Control	Plant Control	0.0008
2	Effluent	Stillage 2	0.0002
2	Plant Control	Stillage 2	0.0338
3	Soil Control	Effluent	<.0001
3	Soil Control	Plant Control	0.0008
3	Effluent	Stillage 2	0.0031
5	Soil Control	Effluent	0.0003
5	Soil Control	Plant Control	0.0043
5	Effluent	Stillage 2	0.0003
5	Plant Control	Stillage 2	0.0043
7	Soil Control	Effluent	<.0001
7	Soil Control	Plant Control	0.0002
7	Effluent	Stillage 2	<.0001
7	Plant Control	Stillage 2	<.0001
10	Soil Control	Effluent	<.0001
10	Soil Control	Plant Control	0.0002
10	Effluent	Stillage 2	<.0001
10	Plant Control	Stillage 2	<.0001

Table A22 Statistical differences within treatments on specific samplings days for temperature during Experiment 3 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	1	0.0043
Soil Control	0	2	<.0001
Soil Control	0	3	<.0001
Soil Control	0	5	<.0001
Soil Control	0	10	<.0001
Soil Control	1	2	<.0001
Soil Control	1	3	<.0001
Soil Control	1	5	<.0001
Soil Control	1	7	0.0031
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	7	<.0001
Soil Control	3	10	<.0001
Soil Control	5	7	<.0001
Soil Control	5	10	<.0001
Soil Control	7	10	<.0001
Plant Control	0	2	<.0001
Plant Control	0	3	<.0001
Plant Control	0	5	<.0001
Plant Control	0	10	<.0001
Plant Control	1	2	<.0001
Plant Control	1	3	<.0001
Plant Control	1	5	<.0001
Plant Control	1	10	0.0043
Plant Control	2	5	0.0439
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	7	<.0001
Plant Control	3	10	<.0001
Plant Control	5	7	<.0001
Plant Control	5	10	<.0001
Plant Control	7	10	<.0001
Stillage 2	0	2	<.0001
Stillage 2	0	3	<.0001
Stillage 2	0	5	<.0001
Stillage 2	0	7	0.0002
Stillage 2	1	2	<.0001
Stillage 2	1	3	<.0001

Stillage 2	1	5	<.0001
Stillage 2	2	5	0.0059
Stillage 2	2	7	<.0001
Stillage 2	2	10	<.0001
Stillage 2	3	7	<.0001
Stillage 2	3	10	<.0001
Stillage 2	5	7	<.0001
Stillage 2	5	10	<.0001
Stillage 2	7	10	<.0001
Effluent	0	1	<.0001
Effluent	0	2	<.0001
Effluent	0	3	<.0001
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	2	<.0001
Effluent	1	3	<.0001
Effluent	1	5	<.0001
Effluent	1	7	<.0001
Effluent	2	3	0.0146
Effluent	2	5	0.0043
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	7	<.0001
Effluent	3	10	<.0001
Effluent	5	7	<.0001
Effluent	5	10	<.0001
Effluent	7	10	<.0001

Table A23 Significant differences between treatments on specific sampling days for temperature during Experiment 4 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Effluent	0.0368
0	Soil Control	Stillage 2	0.0029
2	Soil Control	Effluent	0.0124
3	Soil Control	Effluent	0.0077
3	Effluent	Stillage 2	0.0048
5	Soil Control	Effluent	0.0013
5	Soil Control	Plant Control	0.0195
5	Effluent	Stillage 2	0.0022
5	Plant Control	Stillage 2	0.0299
7	Soil Control	Effluent	0.0002
7	Soil Control	Plant Control	0.0368
7	Effluent	Stillage 2	0.0003
7	Plant Control	Stillage 2	0.0451
10	Soil Control	Effluent	0.0098
10	Soil Control	Plant Control	0.0156
10	Effluent	Stillage 2	0.0037
10	Plant Control	Stillage 2	0.0061

Table A24 Statistical differences within treatments on specific samplings days for temperature during Experiment 4 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	3	0.0299
Soil Control	0	5	<.0001
Soil Control	0	7	<.0001
Soil Control	0	10	<.0001
Soil Control	1	2	0.0048
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	3	0.0017
Soil Control	2	5	<.0001
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	<.0001
Soil Control	3	7	<.0001
Soil Control	3	10	<.0001
Plant Control	0	3	0.0156
Plant Control	0	5	<.0001
Plant Control	0	7	<.0001
Plant Control	0	10	<.0001
Plant Control	1	5	<.0001
Plant Control	1	7	<.0001
Plant Control	1	10	<.0001
Plant Control	2	3	0.0017
Plant Control	2	5	<.0001
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	5	<.0001
Plant Control	3	7	<.0001
Plant Control	3	10	<.0001
Stillage 2	0	2	0.0008
Stillage 2	0	5	<.0001
Stillage 2	0	7	<.0001
Stillage 2	0	10	<.0001
Stillage 2	1	2	0.0368
Stillage 2	1	5	<.0001
Stillage 2	1	7	<.0001
Stillage 2	1	10	<.0001
Stillage 2	2	3	0.0156

Stillage 2	2	5	<.0001
Stillage 2	2	7	<.0001
Stillage 2	2	10	<.0001
Stillage 2	3	5	<.0001
Stillage 2	3	7	<.0001
Stillage 2	3	10	<.0001
Effluent	0	3	0.0061
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	5	<.0001
Effluent	1	7	<.0001
Effluent	1	10	<.0001
Effluent	2	3	0.0010
Effluent	2	5	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	5	<.0001
Effluent	3	7	<.0001
Effluent	3	10	<.0001

Table A25 Significant differences between treatments on specific sampling days for pH during Experiment 1 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Plant Control	0.0069
0	Soil Control	Stillage 1	0.0069
0	Soil Control	Effluent	0.0128
1	Soil Control	Plant Control	0.0002
1	Soil Control	Stillage 1	0.0006
1	Soil Control	Effluent	0.0020
2	Soil Control	Plant Control	<.0001
2	Soil Control	Stillage 1	<.0001
2	Soil Control	Effluent	<.0001
3	Soil Control	Plant Control	<.0001
3	Soil Control	Stillage 1	<.0001
3	Soil Control	Effluent	<.0001
3	Plant Control	Effluent	0.0031
3	Plant Control	Stillage 1	0.0331
5	Soil Control	Plant Control	<.0001
5	Soil Control	Stillage 1	<.0001
5	Soil Control	Effluent	<.0001
5	Plant Control	Effluent	0.0028
7	Soil Control	Plant Control	<.0001
7	Soil Control	Stillage 1	<.0001
7	Soil Control	Effluent	<.0001
7	Plant Control	Stillage 1	0.0288
10	Soil Control	Plant Control	<.0001
10	Soil Control	Stillage 1	<.0001
10	Soil Control	Effluent	<.0001

Table A26 Statistical differences within treatments on specific samplings days for pH during Experiment 1 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	1	0.0189
Soil Control	0	2	<.0001
Soil Control	0	3	<.0001
Soil Control	0	5	<.0001
Soil Control	0	7	<.0001
Soil Control	0	10	<.0001
Soil Control	1	2	0.0275
Soil Control	1	3	<.0001
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	3	0.0053
Soil Control	2	5	0.0001
Soil Control	2	7	0.0090
Soil Control	2	10	0.0056
Plant Control	0	2	0.0164
Plant Control	0	3	<.0001
Plant Control	0	5	<.0001
Plant Control	0	7	<.0001
Plant Control	0	10	0.0110
Plant Control	1	3	0.0038
Plant Control	1	5	0.0004
Plant Control	1	7	0.0004
Plant Control	2	3	0.0263
Plant Control	2	5	0.0038
Plant Control	2	7	0.0041
Plant Control	3	10	0.0378
Plant Control	5	10	0.0059
Plant Control	7	10	0.0062
Stillage 1	0	2	0.0251
Stillage 1	0	3	0.0069
Stillage 1	0	5	<.0001
Stillage 1	0	7	0.0010
Stillage 1	1	5	0.0059
Stillage 1	2	5	0.0346
Stillage 1	5	10	0.0016
Stillage 1	7	10	0.0240
Effluent	0	5	0.0122
Effluent	0	7	0.0002
Effluent	0	10	0.0470
Effluent	1	7	0.0099
Effluent	2	7	0.0105
Effluent	3	7	0.0431

Table A27 Significant differences between treatments on specific sampling days for pH during Experiment 2 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Effluent	0.0008
0	Soil Control	Stillage 1	<.0001
0	Effluent	Stillage 1	<.0001
0	Plant Control	Effluent	0.0175
0	Plant Control	Stillage 1	<.0001
1	Soil Control	Effluent	0.0034
1	Soil Control	Stillage 1	<.0001
1	Soil Control	Plant Control	0.0435
1	Effluent	Stillage 1	<.0001
1	Plant Control	Stillage 1	<.0001
2	Soil Control	Effluent	<.0001
2	Soil Control	Stillage 1	<.0001
2	Soil Control	Plant Control	0.0243
2	Effluent	Stillage 1	<.0001
2	Plant Control	Stillage 1	<.0001
3	Soil Control	Effluent	<.0001
3	Soil Control	Stillage 1	<.0001
3	Soil Control	Plant Control	0.0003
3	Effluent	Stillage 1	<.0001
3	Plant Control	Effluent	<.0001
3	Plant Control	Stillage 1	<.0001
5	Soil Control	Effluent	<.0001
5	Soil Control	Stillage 1	<.0001
5	Soil Control	Plant Control	<.0001
5	Effluent	Stillage 1	<.0001
5	Plant Control	Stillage 1	<.0001
7	Soil Control	Effluent	<.0001
7	Soil Control	Stillage 1	<.0001
7	Soil Control	Plant Control	0.0024
7	Effluent	Stillage 1	<.0001
7	Plant Control	Stillage 1	<.0001
10	Soil Control	Stillage 1	<.0001
10	Effluent	Stillage 1	<.0001
10	Plant Control	Stillage 1	<.0001

Table A28 Statistical differences within treatments on specific samplings days for pH during Experiment 2 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	3	0.0159
Soil Control	0	5	0.0068
Soil Control	0	7	0.0243
Soil Control	1	2	0.0144
Soil Control	1	3	0.0002
Soil Control	1	5	<.0001
Soil Control	1	7	0.0003
Soil Control	3	10	0.0017
Soil Control	5	10	0.0006
Soil Control	7	10	0.0029
Plant Control	0	1	0.0137
Plant Control	0	5	0.0435
Plant Control	1	2	0.0267
Plant Control	1	3	0.0255
Plant Control	1	7	0.0075
Plant Control	1	10	0.0584
Plant Control	5	7	0.0255
Stillage 1	0	10	0.0320
Stillage 1	1	10	0.0232
Stillage 1	2	10	0.0399
Stillage 1	3	10	0.0144
Stillage 1	5	10	0.0279
Effluent	0	3	<.0001
Effluent	1	3	0.0022
Effluent	1	7	0.0232
Effluent	1	10	0.0243
Effluent	2	3	<.0001
Effluent	3	5	0.0013
Effluent	3	7	<.0001
Effluent	3	10	<.0001
Effluent	5	7	0.0349
Effluent	5	10	0.0365

Table A29 Significant differences between treatments on specific sampling days for pH during Experiment 3 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Stillage 2	<.0001
0	Effluent	Stillage 2	<.0001
0	Plant Control	Stillage 2	<.0001
1	Soil Control	Effluent	<.0001
1	Soil Control	Stillage 2	<.0001
1	Soil Control	Plant Control	<.0001
1	Effluent	Stillage 2	<.0001
1	Plant Control	Effluent	0.0429
1	Plant Control	Stillage 2	<.0001
2	Soil Control	Effluent	<.0001
2	Soil Control	Stillage 2	<.0001
2	Soil Control	Plant Control	<.0001
2	Effluent	Stillage 2	<.0001
2	Plant Control	Effluent	0.0001
2	Plant Control	Stillage 2	<.0001
3	Soil Control	Effluent	<.0001
3	Soil Control	Stillage 2	<.0001
3	Soil Control	Plant Control	0.0001
3	Effluent	Stillage 2	<.0001
3	Plant Control	Effluent	0.0339
3	Plant Control	Stillage 2	<.0001
5	Soil Control	Effluent	<.0001
5	Soil Control	Stillage 2	<.0001
5	Soil Control	Plant Control	<.0001
5	Effluent	Stillage 2	<.0001
5	Plant Control	Effluent	0.0019
5	Plant Control	Stillage 2	<.0001
7	Soil Control	Effluent	<.0001
7	Soil Control	Stillage 2	<.0001
7	Soil Control	Plant Control	0.0028
7	Effluent	Stillage 2	<.0001
7	Plant Control	Effluent	0.0028
7	Plant Control	Stillage 2	<.0001
10	Soil Control	Effluent	<.0001
10	Soil Control	Stillage 2	<.0001
10	Soil Control	Plant Control	<.0001
10	Effluent	Stillage 2	<.0001
10	Plant Control	Stillage 2	<.0001

Table A30 Statistical differences within treatments on specific samplings days for pH during Experiment 3 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	2	0.0005
Soil Control	0	3	0.0087
Soil Control	0	5	<.0001
Soil Control	0	10	<.0001
Soil Control	1	2	0.0234
Soil Control	1	5	0.0007
Soil Control	1	10	<.0001
Soil Control	2	7	0.0004
Soil Control	2	10	<.0001
Soil Control	3	5	0.0282
Soil Control	3	7	0.0071
Soil Control	3	10	<.0001
Soil Control	5	7	<.0001
Soil Control	5	10	0.0022
Soil Control	7	10	<.0001
Plant Control	0	1	0.0081
Plant Control	0	7	0.0030
Plant Control	0	10	0.0004
Plant Control	1	2	0.0220
Plant Control	1	5	0.0009
Plant Control	1	10	<.0001
Plant Control	2	7	0.0100
Plant Control	2	10	0.0016
Plant Control	3	10	0.0001
Plant Control	5	7	0.0043
Plant Control	5	10	0.0040
Plant Control	7	10	<.0001
Stillage 2	0	1	0.0249
Stillage 2	0	2	<.0001
Stillage 2	0	3	<.0001
Stillage 2	0	5	<.0001
Stillage 2	0	7	0.0031
Stillage 2	0	10	0.0004
Stillage 2	1	2	0.0114
Stillage 2	1	3	0.0002
Stillage 2	1	5	0.0005
Stillage 2	3	7	0.0021
Stillage 2	3	10	0.0131

Stillage 2	5	7	0.0054
Stillage 2	5	10	0.0300
Effluent	0	1	<.0001
Effluent	0	2	0.0002
Effluent	0	3	0.0061
Effluent	0	5	0.0071
Effluent	0	7	<.0001
Effluent	1	3	0.0282
Effluent	1	5	0.0249
Effluent	1	10	<.0001
Effluent	2	10	<.0001
Effluent	3	10	<.0001
Effluent	5	10	<.0001
Effluent	7	10	<.0001

Table A31 Significant differences between treatments on specific sampling days for pH during Experiment 4 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Effluent	0.0395
1	Soil Control	Effluent	0.0002
1	Soil Control	Plant Control	0.0116
1	Effluent	Stillage 2	0.0083
2	Soil Control	Effluent	<.0001
2	Soil Control	Stillage 2	0.0169
2	Soil Control	Plant Control	0.0027
2	Effluent	Stillage 2	0.0083
2	Plant Control	Effluent	0.0449
3	Soil Control	Effluent	<.0001
3	Soil Control	Stillage 2	0.0011
3	Soil Control	Plant Control	0.0011
3	Effluent	Stillage 2	0.0212
5	Soil Control	Effluent	<.0001
5	Soil Control	Stillage 2	0.0063
5	Soil Control	Plant Control	0.0012
7	Soil Control	Effluent	0.0001
7	Soil Control	Stillage 2	0.0089
7	Soil Control	Plant Control	0.0012
10	Soil Control	Effluent	0.0002
10	Soil Control	Stillage 2	0.0120
10	Soil Control	Plant Control	0.0006

Table A32 Statistical differences within treatments on specific samplings days for pH during Experiment 4 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	3	0.0097
Soil Control	0	7	0.0473
Soil Control	0	10	0.0218

Table A33 Significant differences between treatments on specific sampling days for DO during Experiment 1 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
1	Soil Control	Effluent	0.0205
1	Soil Control	Stillage 1	0.0396
2	Soil Control	Effluent	0.0005
2	Soil Control	Stillage 1	0.0007
2	Soil Control	Plant Control	0.0009
3	Soil Control	Effluent	<.0001
3	Soil Control	Stillage 1	<.0001
3	Soil Control	Plant Control	<.0001
5	Soil Control	Effluent	<.0001
5	Soil Control	Stillage 1	<.0001
5	Soil Control	Plant Control	<.0001
7	Soil Control	Effluent	0.0001
7	Soil Control	Stillage 1	<.0001
7	Soil Control	Plant Control	<.0001
7	Effluent	Stillage 1	0.0473
10	Soil Control	Effluent	0.0037
10	Soil Control	Stillage 1	0.0002
10	Soil Control	Plant Control	<.0001

Table A34 Statistical differences within treatments on specific samplings days for DO during Experiment 1 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	3	0.0145
Soil Control	0	5	<.0001
Soil Control	0	7	<.0001
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	2	5	<.0001
Soil Control	2	7	0.0020
Soil Control	3	5	0.0006
Soil Control	3	7	0.0101
Soil Control	5	10	<.0001
Soil Control	7	10	0.0003
Plant Control	0	10	0.0060
Plant Control	1	10	0.0109
Plant Control	5	10	0.0020
Plant Control	7	10	0.0121
Stillage 1	0	10	0.0157
Stillage 1	5	10	0.0183
Effluent	1	5	0.0354
Effluent	1	7	0.0097
Effluent	2	5	0.0241
Effluent	2	7	0.0063
Effluent	3	5	0.0111
Effluent	3	7	0.0026
Effluent	5	10	0.0248
Effluent	7	10	0.0075

Table A35 Significant differences between treatments on specific sampling days for DO during Experiment 2 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Stillage 1	0.0154
0	Plant Control	Stillage 1	0.0253
2	Soil Control	Stillage 1	0.0055
3	Soil Control	Stillage 1	0.0024
5	Soil Control	Stillage 1	<.0001
5	Soil Control	Plant Control	0.0110
5	Effluent	Stillage 1	0.0261
7	Soil Control	Stillage 1	0.0004
7	Effluent	Stillage 1	0.0023
7	Plant Control	Stillage 1	0.0033
10	Plant Control	Stillage 1	0.0183

Table A36 Statistical differences within treatments on specific samplings days for DO during Experiment 2 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	0	10	0.0044
Soil Control	2	10	0.0168
Soil Control	3	10	0.0331
Soil Control	5	10	0.0013
Soil Control	7	10	0.0180
Plant Control	0	1	0.0327
Plant Control	0	2	0.0390
Plant Control	0	3	0.0415
Plant Control	0	5	0.0498
Stillage 1	1	7	0.0291
Stillage 1	1	10	0.0304

Table A37 Significant differences between treatments on specific sampling days for DO during Experiment 3 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Stillage 2	<.0001
0	Effluent	Stillage 2	<.0001
0	Plant Control	Effluent	0.0094
0	Plant Control	Stillage 2	<.0001
1	Soil Control	Effluent	<.0001
1	Soil Control	Stillage 2	<.0001
1	Soil Control	Plant Control	0.0340
1	Plant Control	Effluent	0.0034
1	Plant Control	Stillage 2	<.0001
2	Soil Control	Stillage 2	<.0001
2	Effluent	Stillage 2	0.0105
2	Plant Control	Stillage 2	<.0001
3	Soil Control	Effluent	0.0249
3	Soil Control	Stillage 2	<.0001
3	Plant Control	Stillage 2	0.0028
5	Soil Control	Stillage 2	0.0080
5	Plant Control	Stillage 2	0.0045
7	Soil Control	Effluent	0.0302
7	Soil Control	Stillage 2	<.0001
7	Effluent	Stillage 2	0.0177
7	Plant Control	Stillage 2	<.0001
10	Soil Control	Stillage 2	0.0020
10	Effluent	Stillage 2	0.0005
10	Plant Control	Stillage 2	<.0001

Table A38 Statistical differences within treatments on specific samplings days for DO during Experiment 3 (α = 0.05).

Treatment	**Sampling Day**	**Sampling Day**	**P-Value**
Soil Control	0	2	0.0364
Soil Control	0	3	0.0267
Soil Control	0	5	0.0006
Soil Control	0	10	0.0024
Soil Control	1	2	0.0232
Soil Control	1	3	0.0168
Soil Control	1	5	0.0003
Soil Control	1	10	0.0014
Soil Control	5	7	0.0458
Plant Control	0	3	0.0165
Plant Control	0	5	0.0153
Plant Control	3	10	0.0422
Plant Control	5	10	0.0395
Effluent	0	1	<.0001
Effluent	0	2	<.0001
Effluent	0	3	<.0001
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	10	0.0245

Table A39 Statistical differences within treatments on specific samplings days for DO during Experiment 3 (α = 0.05).

Treatment	**Sampling Day**	**Sampling Day**	**P-Value**
Soil Control	0	2	0.0364
Soil Control	0	2	0.0267
Soil Control	0	5	0.0006
Soil Control	0	10	0.0024
Soil Control	1	2	0.0232
Soil Control	1	3	0.0168
Soil Control	1	5	0.0003
Soil Control	1	10	0.0014
Soil Control	5	7	0.0458
Plant Control	0	3	0.0165
Plant Control	0	5	0.0153
Plant Control	3	10	0.0422
Plant Control	5	10	0.0395
Effluent	0	1	<.0001
Effluent	0	2	<.0001
Effluent	0	3	<.0001
Effluent	0	5	<.0001
Effluent	0	7	<.0001
Effluent	0	10	<.0001
Effluent	1	10	0.0245

Table A40 Significant differences between treatments on specific sampling days for DO during Experiment 4 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
0	Soil Control	Stillage 2	0.0254
1	Soil Control	Effluent	0.0082
1	Soil Control	Stillage 2	0.0004
1	Plant Control	Stillage 2	0.0268
2	Soil Control	Effluent	0.0006
2	Soil Control	Stillage 2	<.0001
2	Soil Control	Plant Control	0.0063
2	Plant Control	Stillage 2	0.0391
3	Soil Control	Effluent	0.0012
3	Soil Control	Stillage 2	<.0001
3	Soil Control	Plant Control	0.0134
3	Plant Control	Stillage 2	0.0305
5	Soil Control	Stillage 2	0.0120
10	Soil Control	Effluent	0.0403
10	Soil Control	Stillage 2	0.0103

Table A41 Statistical differences within treatments on specific samplings days for DO during Experiment 4 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	2	5	0.0218
Soil Control	2	7	0.0032
Soil Control	2	10	0.0249
Soil Control	3	5	0.0324
Soil Control	3	7	0.0051
Soil Control	3	10	0.0367

Table A42 Significant differences between treatments on specific sampling days for PO_4-P during Experiment 1 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
5	Soil Control	Effluent	0.0183
7	Soil Control	Effluent	<.0001
7	Soil Control	Stillage 1	0.0447
7	Soil Control	Plant Control	0.0201
7	Effluent	Stillage 1	0.0102
7	Plant Control	Effluent	0.0240
10	Soil Control	Stillage 1	0.0341

Table A43 Statistical differences within treatments on specific samplings days for PO_4-P during Experiment 1 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	2	<.0001
Soil Control	1	3	<.0001
Soil Control	1	5	0.0004
Soil Control	1	7	<.0001
Soil Control	1	10	<.0001
Soil Control	2	5	<.0001
Soil Control	2	7	<.0001
Soil Control	2	10	<.0001
Soil Control	3	5	<.0001
Soil Control	3	7	<.0001
Soil Control	3	10	<.0001
Soil Control	5	7	0.0130
Soil Control	5	10	0.0405
Plant Control	1	2	<.0001
Plant Control	1	3	<.0001
Plant Control	1	5	0.0193
Plant Control	1	7	0.0002
Plant Control	1	10	0.0001
Plant Control	2	5	<.0001
Plant Control	2	7	<.0001
Plant Control	2	10	<.0001
Plant Control	3	5	<.0001
Plant Control	3	7	<.0001
Plant Control	3	10	<.0001
Stillage 1	1	2	<.0001
Stillage 1	1	3	<.0001
Stillage 1	1	5	0.0154
Stillage 1	1	7	<.0001
Stillage 1	1	10	0.0005
Stillage 1	2	5	<.0001
Stillage 1	2	7	<.0001
Stillage 1	2	10	<.0001
Stillage 1	3	5	<.0001
Stillage 1	3	7	<.0001
Stillage 1	3	10	<.0001
Effluent	1	2	<.0001
Effluent	1	3	<.0001
Effluent	1	10	0.0002

Effluent	2	5	<.0001
Effluent	2	7	<.0001
Effluent	2	10	<.0001
Effluent	3	5	<.0001
Effluent	3	7	<.0001
Effluent	3	10	<.0001
Effluent	5	10	0.0129
Effluent	7	10	0.0260

Table A44 Significant differences between treatments on specific sampling days for PO_4-P during Experiment 2 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
3	Soil Control	Effluent	0.0373
3	Soil Control	Stillage 2	0.0009
5	Soil Control	Effluent	0.0130
5	Soil Control	Stillage 2	<.0001
5	Soil Control	Plant Control	0.0043
5	Effluent	Stillage 2	0.0037
5	Plant Control	Stillage 2	0.0114
7	Soil Control	Effluent	<.0001
7	Soil Control	Stillage 2	<.0001
7	Soil Control	Plant Control	<.0001
7	Effluent	Stillage 2	0.0004
7	Plant Control	Stillage 2	0.0021

Table A45 Statistical differences within treatments on specific samplings days for PO_4-P during Experiment 2 (α = 0.05).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	3	0.0054
Soil Control	1	5	<.0001
Soil Control	1	7	<.0001
Soil Control	2	3	0.0192
Soil Control	2	5	0.0002
Soil Control	2	7	<.0001
Soil Control	3	7	0.0002
Soil Control	5	7	0.0183
Effluent	1	7	0.0149
Effluent	2	7	0.0332

Table A46 Significant differences between treatments on specific sampling days for PO_4-P during Experiment 3 (α = 0.05).

Sampling Day	Treatment	Treatment	P-value
2	Soil Control	Effluent	0.0370
2	Soil Control	Stillage 2	0.0268
2	Soil Control	Plant Control	0.0307
10	Soil Control	Effluent	0.0035
10	Soil Control	Stillage 2	0.0312
10	Plant Control	Effluent	0.0391

Table A47 Statistical differences within treatments on specific samplings days for PO_4-P during Experiment 3 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	2	0.0441
Soil Control	1	5	0.0365
Soil Control	1	7	0.0395
Soil Control	1	10	0.0004
Soil Control	2	3	0.0002
Soil Control	2	5	0.0001
Soil Control	2	7	0.0001
Soil Control	2	10	<.0001
Plant Control	1	10	<.0001
Plant Control	2	10	<.0001
Plant Control	3	10	0.0004
Plant Control	5	10	0.0008
Plant Control	7	10	0.0020
Stillage 2	1	10	<.0001
Stillage 2	2	10	<.0001
Stillage 2	3	10	<.0001
Stillage 2	5	10	<.0001
Stillage 2	7	10	<.0001
Effluent	1	10	<.0001
Effluent	2	10	<.0001
Effluent	3	10	<.0001
Effluent	5	10	<.0001
Effluent	7	10	<.0001

Table A48 Significant differences between treatments on specific sampling days for PO_4-P during Experiment 4 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
3	Plant Control	Stillage 2	0.0138
5	Soil Control	Stillage 2	0.0002
5	Effluent	Stillage 2	0.0186
5	Plant Control	Effluent	0.0682
5	Plant Control	Stillage 2	<.0001
7	Soil Control	Effluent	0.0001
7	Soil Control	Plant Control	<.0001
7	Effluent	Stillage 2	0.0003
7	Plant Control	Effluent	0.0068
7	Plant Control	Stillage 2	<.0001
10	Soil Control	Effluent	<.0001
10	Soil Control	Plant Control	<.0001
10	Effluent	Stillage 2	<.0001
10	Plant Control	Stillage 2	<.0001

Table A49 Statistical differences within treatments on specific samplings days for PO_4-P during Experiment 4 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Soil Control	1	5	0.0062
Soil Control	1	10	0.0323
Soil Control	2	7	0.0065
Soil Control	2	10	0.0015
Soil Control	3	5	0.0093
Soil Control	3	10	0.0226
Soil Control	5	7	<.0001
Soil Control	5	10	<.0001
Plant Control	1	5	0.0025
Plant Control	1	7	<.0001
Plant Control	1	10	0.0004
Plant Control	2	5	0.0280
Plant Control	2	7	<.0001
Plant Control	2	10	0.0057
Plant Control	3	7	0.0004
Plant Control	3	10	0.0292
Plant Control	5	7	0.0336
Effluent	1	7	0.0141
Effluent	1	10	<.0001
Effluent	2	7	0.0216
Effluent	2	10	<.0001
Effluent	3	10	<.0001
Effluent	5	10	0.0002
Effluent	7	10	0.0068

Table A50 Significant differences between treatments on specific sampling days for *E. coli* during Experiment 4 ($\alpha = 0.05$).

Sampling Day	Treatment	Treatment	P-value
10	Effluent	Stillage 2	0.0135

Table A51 Statistical differences within treatments on specific samplings days for *E. coli* during Experiment 4 ($\alpha = 0.05$).

Treatment	Sampling Day	Sampling Day	P-Value
Effluent	1	10	0.0007
Effluent	5	10	0.0079

APPENDIX B

Pictures of the wastewater treatment plant where the effluent was collected, a 5-gallon bucket of effluent, and a 5-gallon bucket of the whole stillage that was collected from the distillery for the experiments.

Figure B 1 Schematic of package wastewater treatment plant at the Central Kentucky Distillery's Bottling Facility.

Figure B 2 Whole stillage collected from the Central Kentucky Distillery.

Figure B 3 Wastewater effluent collected from the Central Kentucky Distillery's bottling plant.

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