Summer 7-29-2022

# Studies on Air Injection in a Wastewater-Fed Subsurface Drip Irrigation System

Henry A. Gonzalez Hernandez
*University of Nebraska-Lincoln*, hgonzalezhernandez2@huskers.unl.edu

# STUDIES ON AIR INJECTION IN A WASTEWATER-FED SUBSURFACE DRIP IRRIGATION SYSTEM

by

Henry Alexander Gonzalez Hernandez

A THESIS

Presented to the Faculty of

The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Master of Science

Major: Mechanized Systems Management

Under the supervision of Professor Xin Qiao

Lincoln, Nebraska

July 2022

# STUDIES ON AIR INJECTION IN A WASTEWATER-FED SUBSURFACE DRIP IRRIGATION SYSTEM

Henry Alexander Gonzalez Hernandez, M.S.

University of Nebraska, 2022

Advisor: Xin Qiao.

In 2020, the Ogallala High Plains Aquifer supplied irrigation water for approximately 3.6 million hectares in Nebraska, making it the most irrigated state in the United States. Although Nebraska is highly dependent in groundwater, locations included in the Gering-Fort Laramie Irrigation District (border of Nebraska and Wyoming) rely on surface water resources. In addition to these conventional water sources, using non-conventional alternatives for crop production can be just as important during water shortage times such as the one that e occurred in 2019 with the collapse of the Goshen/Gering-Fort Laramie Canal. Feedlot runoff presents a great opportunity in Nebraska, but it can become a point source pollution of pharmaceutical and personal care products, which could be accumulated in plant tissues and soil.

In Chapter 1, we tested how passive air injection to the wastewater-fed SDI system could improve crop yield by increasing soil oxygen content at the root zone. Therefore, the objective of Chapter 1 was to evaluate the effect of air injection on corn and sugar beets yield when using feedlot runoff irrigation in SDI. Results have shown that passive air injection has been effective increasing soil oxygen content in shallow depth. Air injection did not affect sugar beet yield or sucrose content in any growing season, but significantly

increased corn yield in the 2021 growing season. In Chapter 2, we evaluated the effects of air injection into subsurface drip irrigation on the soil microbial diversity and the residual of antibiotic residues accumulated in soil pore water, soil, and plant matrices. The objective of Chapter 2 was to evaluate the effects of air injection on the reduction of antibiotic concentrations in the soil environment introduced by irrigation with feedlot wastewater. Results have shown that air injection was not effective in removing or treating antibiotics such as sulfamethoxazole or monensin Na. However, in air-injected sugar beet or corn plots, some microbes had a significantly higher abundance than in non-air-injected plots, revealing potential benefits for crop production.

# ACKNOWLEDGMENTS

I would like to thank God for providing this opportunity, for keeping me healthy and strong, and for all the blessings during my time at the Panhandle Research and Extension Center, University of Nebraska-Lincoln.

I would like to thank the Nebraska Environmental Trust (NET) for the funding support. I would like to thank my advisor, Dr. Xin Qiao, for his valuable support and patient guidance. Being part of a good academic program is as important as having a good advisor, and I have always believed that having an advisor who cares about the personal and professional development of the student is an advisor worth having. Thank you, Dr. Derek M. Heeren and Dr. Matteo D'Alessio for the assistance, encouragement, teaching, and constructive feedback to my work.

I would like to thank Dr. Tania Biswas and Dr. Tong Onanong for their guidance during my time at the Water Sciences Laboratory, University of Nebraska-Lincoln. I would also like to thank Dr. Clinton F. Williams and Mr. Allan Knopf for helping us analyze water and soil samples. Furthermore, I would like to thank Dr. Lisa Durso for helping us conduct the microbial diversity assay. An enormous thank you to Mr. Gene Kizzire, Mr. Les Kampbell, Mr. Logan Van Anne, and the members of the Irrigation and Digital Ag Lab for their help in conducting the field experiments. I would also like to thank Dr. Jeffrey Bradshaw for all his support since 2019.

I want to thank my family for supporting my dreams since I left my country in 2014. Finally, I thank my community of friends who have made my time in Nebraska an adventure of a lifetime.

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*Chapter 1 under review in the Journal of Irrigation and Drainage Engineering, American Society of Civil Engineers (ASCE)*

***Abstract***

As a state with the most irrigated agricultural land in the U.S., irrigation in Nebraska relies on freshwater resources such as groundwater and surface water. In addition to these conventional water sources, using non-conventional alternatives for crop production can be just as important during water shortage times. In this research, effluent from a feedlot was used as irrigation water source at a subsurface drip irrigation (SDI) system for corn (C) and sugar beet (SB) production in Western Nebraska during 2019 and 2021 growing seasons. We tested how passive air injection to the wastewater-fed SDI system could improve crop yield by increasing soil oxygen content at the root zone. Results indicate that air injection significantly increased soil oxygen content by 1% in SB and 1% in corn during the first year, and 15% in SB and 1% in corn during the second year. No significant difference was observed in sugar beet yield and sucrose yield between air injection (O) and non-injection (NO) treatments during both growing seasons. However, a significant difference was observed in sucrose rate in the second year favoring NO treatment. Although corn yield presented a positive response to air injection with a 5.50 % increase in O treatment compared to NO treatment in the first year, differences were not significant. In the second year, it was observed that O treatment significantly increased crop yield by 9.17% compared to NO treatment. Considering that no supplemental fertilizer was applied,

these results highlight that the presence of nutrients in feedlot runoff can enhance crop yields and reduce the use of synthetic fertilizers. In conclusion, air injection can significantly increase corn yield and improve the water use efficiency.

## 1.1 Introduction

The increase in population demands an increase in food production and water supply (Parris, 2010). In fact, food production doubled during the last three decades, and a 70% increase is needed by 2050 (Carvajal-Yepes et al. 2019). This increase, combined with the uneven global distribution of freshwater resources (Zhang and Xie, 2019) and freshwater shortages (De Angelis et al. 2021; Prajapati et al. 2021; Singh et al. 2019) presents a threat to food security. With agriculture accounting for 70% of available freshwater usage worldwide (FAO, 2016), the use of more efficient irrigation systems combined with the use of non-conventional water resources, such as greywater and recycled wastewater, represent suitable alternatives to freshwater usage (UNESCO, 2016; U.S. GAO, 2019).

The global irrigated area consists of 301 million hectares of production land (Caucci and Hettiarachchi, 2017). Although there is not a complete global inventory of wastewater usage for agricultural irrigation, it is estimated that untreated and partially treated wastewater was used for this purpose in at least 5 million hectares by 2010 (WWAP, 2017). By 2017, around 35.9 million hectares of irrigated croplands received urban wastewater, 86% of those were in countries such as China, India, Pakistan, Mexico, and Iran (Thebo et al. 2017). In Israel, 85% of its wastewater was treated and recycled for agricultural irrigation by 2015 (Avisar and Ronen-Eliraz, 2018; Reznik et al. 2017), contributing to the

reduction of freshwater demand for agricultural irrigation by 50% (Leonel and Tonetti, 2021).

In the United States, the use of recycled wastewater accounted for less than 1% of the total irrigation water used in 2015 (Dieter et al. 2018). California, Florida, Arizona, and Texas collectively produce 85% of the total recycled wastewater (Dery et al. 2019). First regulations for wastewater recycling and usage in the world were adopted in California as early as 1918, and it was estimated that around 31% of California's total recycled wastewater was used for agricultural irrigation in 2015 (Coate et al. 2019). In Florida, reclaimed wastewater was first used in Tallahassee for agricultural irrigation in the 1960s, and it was followed by the development of a reclaimed wastewater system for landscape irrigation in St. Petersburg during the 1970s. By 2007, 71% of Florida's reclaimed wastewater was used to supply landscape and agricultural irrigation (Toor and Rainey, 2009). In Arizona, reclaimed wastewater was used for power generation at the Grand Canyon Village in 1926. By 2010, 22% of the total recycled wastewater was used for agricultural irrigation (Midel et al. 2013) to grow crops such as cotton, wheat, and vegetables (Cusimano et al. 2015). Water reuse in Texas started with agricultural irrigation and has continued to sustain the production of cotton, wheat, and alfalfa in places like Lubbock and San Antonio (Ritter, 2021).

In Nebraska, the Ogallala High Plains Aquifer supplied irrigation water for approximately 3.6 million hectares in 2020, making it the most irrigated state and highly dependent on the aquifer for agricultural production (Upper Big Blue Natural Resource District, 2020). Nonetheless, pumping limits for groundwater applications are established by Nebraska Natural Resource Districts using a combination of the Rule of Reasonable

Use and the Correlative Rights Doctrine (Eisenhauer et al. 2021). Such pumping limits are given in terms of annual average pumping with no exceedance of a set value over three or five years (Yonts et al. 2018). Although groundwater is an important component, locations included in the Gering-Fort Laramie Irrigation District (border of Nebraska and Wyoming) rely on surface water resources (Goshen Irrigation District, 2022). In 2019, a water shortage event occurred in this region with the collapse of the Goshen/Gering-Fort Laramie Canal, affecting approximately 43,301 hectares of corn, sugar beets, dry edible beans, and alfalfa crops in western Nebraska (Nebraska Extension and University of Wyoming Extension, 2019).

Considering that Nebraska ranked second in cattle on feed inventory (2.82 million heads ) after Texas (2.93 million heads) by January 2022 (USDA-NASS, 2021), feedlot runoff may represent a potential alternative to either groundwater or surface water in Nebraska. However, wastewater and runoff coming from concentrated animal feeding operations might represent a point source for multiple pollutants such as antibiotics and heavy metals (Sun et al. 2017). Irrigation with untreated wastewater can lead to accumulations of heavy metals and pharmaceuticals and personal care products (PPCPs) in crops (Shahriar et al. 2021; Zhang and Shen, 2019). Different studies have been conducted to determine the accumulation of metals in plants. For example, accumulations of K, Mg, Ca, Na, and Fe were found from edible leaves to underground vegetables (Mahmood et al. 2020), translocation of Cd, Cr, Cu, Fe, Pb, and Zn was reported in all portions of the potato plant (Nzediegwu et al. 2019), and uptake and accumulation of PPCPs were observed in cucumber, tomato, carrot, radish, lettuce, spinach, and cabbage (Christou et al. 2017; Liu et al. 2020; Wu et al. 2013). In addition, according to Singh

(2021), increases in soil salinity are the most distinctive effects when wastewater is used for agricultural irrigation. Such effects might be enhanced in subsurface drip irrigation (SDI) systems, with the excessive accumulation of salts in the active root zone of the plant due to upward capillary movement (Oron et al. 1999; Zaman et al. 2018). SDI is a low-pressure, high-efficiency microirrigation system that delivers water and nutrients to the crop root zone through buried plastic drip tapes with embedded emitters (El-Nesr et al. 2014; Qiao 2018; Reich et al. 2014). The interest in SDI has increased due to its potential water savings and needs for water conservation, enhanced microbial activity in the rhizosphere, and potential crop yield increases (Camp et al. 2000; El-Nesr et al. 2014; Liu et al. 2021; Payero et al. 2005). However, microirrigation systems are point water source application to the rhizosphere and their use could lead to poor aeration in the root zone (Dhungel et al. 2012; Zhu et al. 2019). Low levels of oxygen in the root zone can prevent the diffusion of $CO_2$ and ethylene, reduce nitrogen fixation and consequently lead to hypoxia stress affecting crop yield (Yuan et al. 2016). These effects can be reduced with the injection of air into the water flow through the drip lines, which has been reported to increase soil oxygen content and crop yield (Cui et al. 2020; Du et al. 2020; Zhu et al. 2019). Air injection has resulted in the improvement of water use efficiency in SDI systems. For example, a 20% increase in seed yield was observed in chickpea (Pendergast et al. 2019), while an increase ranging between 14% and 28% and between 82% and 96% was observed in cotton lint yield and soybean fresh pod yield, respectively. Abuarab et al. (2013) observed an improvement in water use efficiency, irrigation water productivity, and yield ranging between 12.27% and 12.5% in corn. However, none of these studies used recycled wastewater for agricultural irrigation.

To the best of our knowledge, no other studies have been conducted on-farm using animal wastewater and air injection into SDI to grow crops. Similar studies have used air-injected SDI in greenhouse settings. It was reported that using aerated wastewater improved the aeration of clayey soils, reduced $N_2O$ production, and increased the aboveground biomass yield of lettuce (Baram et al. 2021; Baram et al. 2022). D'Alessio et al. (2020) investigated the impact of air-injected water containing PPCPs on plant uptake and observed an enhanced removal of PPCPs in the air injection treatment. Therefore, the objective of this study was to evaluate the effect of air injection on corn and sugar beets yield when using feedlot runoff irrigation in SDI. It is hypothesized that passive air injection could increase soil oxygen content in the root zone and improve the yield of corn and sugar beets.

## 1.2 Material and Method

### 1.2.1 Study site

The field experiment was conducted during the 2019 and 2021 growing seasons at the Mitchell Ag Laboratory, Panhandle Research and Extension Center (PHREC), University of Nebraska-Lincoln, Scottsbluff, NE (41.95 N, 103.70 W, elevation 1,186 meters above sea). The soil is classified as Tripp very fine sandy loam with a pH of 7.5, bulk density of 1.49 g $cm^{-3}$, and organic matter content of 0.89%. The field capacity of the soil is 22.4% and the wilting point is 10% (https://websoilsurvey.sc.egov.usda.gov/App/HomePage.htm). Weather data from 2010 to 2021 were obtained using an on-site weather station, which is part of the Automated Weather Data Network from the High Plains Regional Climate Center

(http://awdn.unl.edu/classic/home.cgi). An average annual precipitation and reference evapotranspiration ($ET_o$) of 292 mm and 1,746 mm was reported, respectively, including an average high daily temperature of 17.3°C and average low daily temperature of 1.8°C.

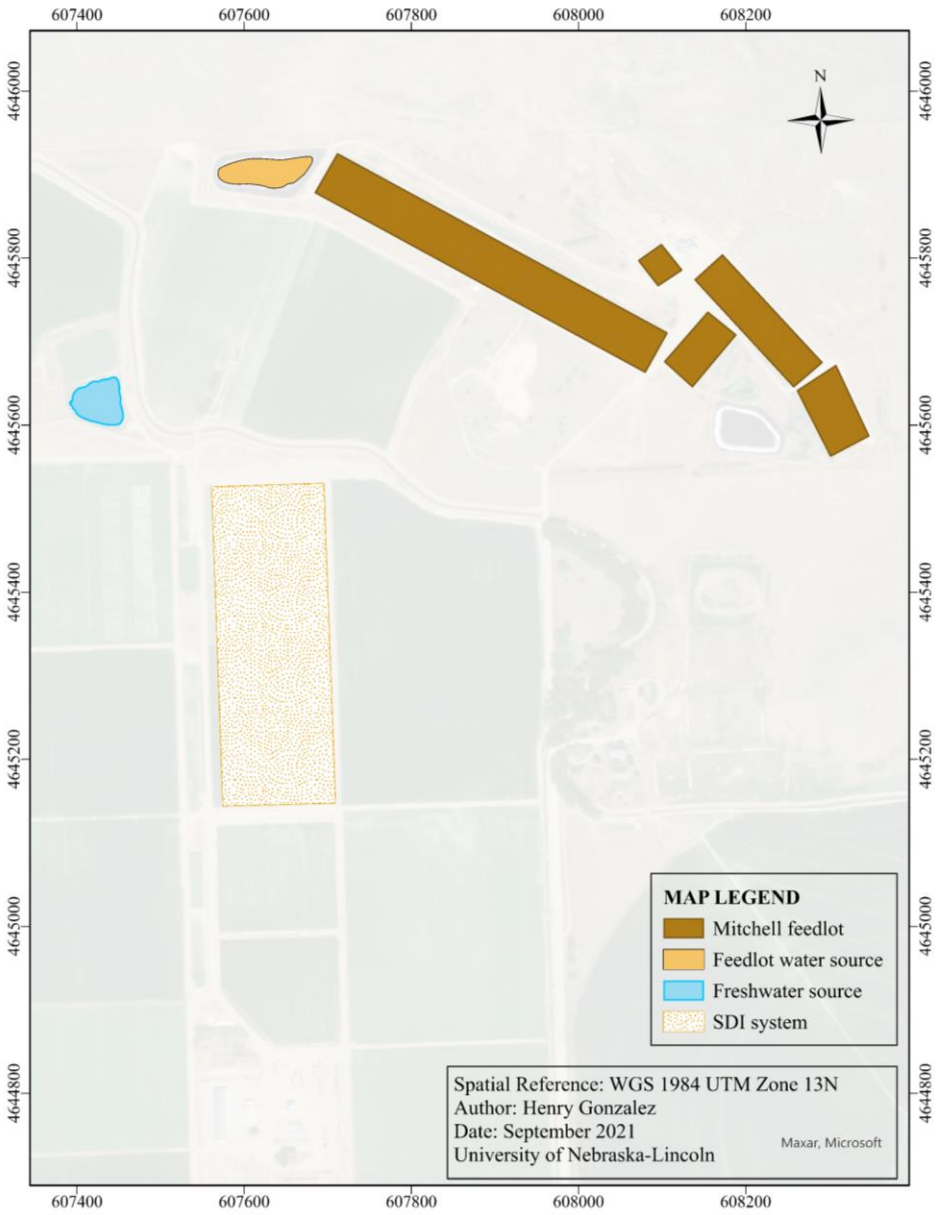


**Figure 1.1** Subsurface drip irrigation system at the Mitchell Ag Laboratory, Scottsbluff, Nebraska.

### 1.2.2 Subsurface drip irrigation system

The SDI was installed at a field with a total area of 5 ha. The SDI system has 40 management zones that can be individually and remotely controlled for start/stop irrigation events and apply specific irrigation amounts. Each management zone has a dimension of 184 m by 6.8 m, which can be planted with 12 rows of the crop at a row spacing of 56 cm (Figure 1.2). Drip tapes were installed at every other row spacing of 112 cm and were buried at depth of 25.4 cm. Emitters on the drip tapes (Rivulis, San Diego, California, USA) are spaced at 56 cm with an application rate of 0.6 liters per hour. Four consecutive Yardney sand media filters (Yardney Water Filtration Systems, Riverside, California, USA) are placed at the pumping station to clean irrigation water before entering the drip tape. Irrigation with feedlot runoff was applied weekly using a fixed rate (25 mm per irrigation event). Freshwater from a retention pond was used on weekdays during hot and dry days.

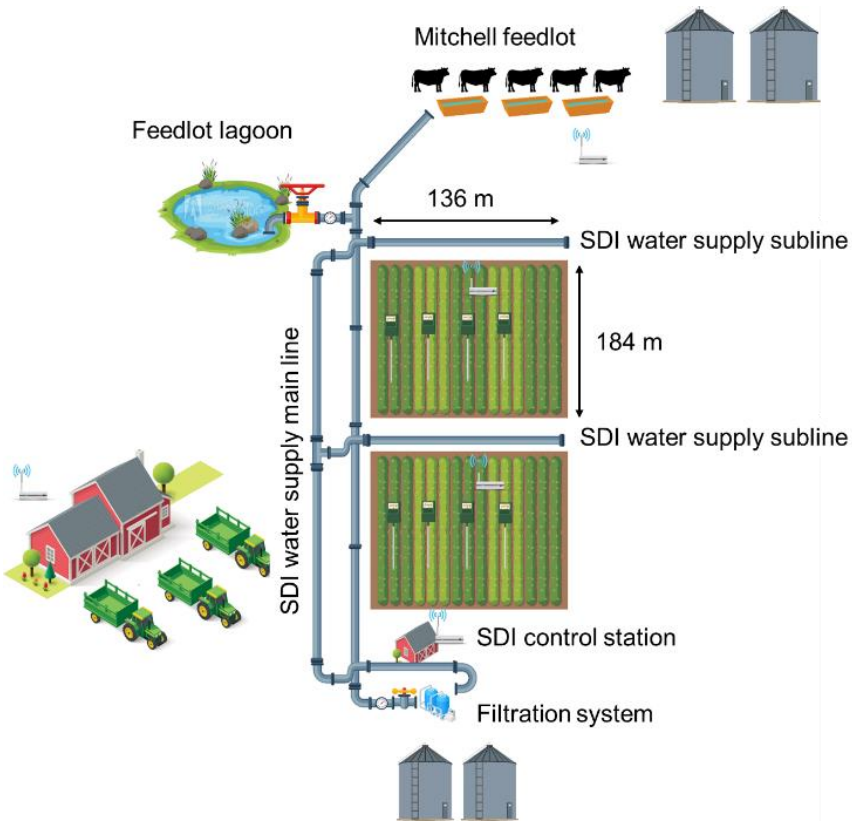


**Figure 1.2** Layout of the experimental field with subsurface drip irrigation system at the Mitchell Ag Laboratory, Scottsbluff, Nebraska.

### 1.2.3 Air injection treatments

The field experiment followed a complete randomized design. It consisted of two treatments, air injection (O) and non-air injection (NO). Each treatment was replicated five times in corn (C) and sugar beets (SB), respectively. Venturi-type air injectors (Mazzei Injector Company LLC, Bakersfield, California, USA) were installed to allow the air to be passively injected into the drip line and to the root zone. The air injectors work in the way that when the pressurized water enters the injector inlet, the change of water flow into a high-velocity stream in the injection chamber results in a decrease of the absolute pressure which creates a vacuum that draws air through the suction port. All injectors had an inlet

pressure of 206 kPa with an outlet pressure of 137 kPa. The average pressure loss was 69 kPa and created an air motive flow rate of 26 liters per minute, (Mazzei Injector Company LLC, 2006). For the O treatment, the injectors were left exposed to the air, while for NO treatments, the injectors were capped to prevent any air from getting into the drip line (Figure 1.3).


**Figure 1.3** Air is injected to water flow using venturi air injector and injector filter screens.

### 1.2.4 Measured parameters

For each crop, soil oxygen content and soil water content were continuously measured using Apogee soil oxygen sensors model 110 (Apogee Instruments, Logan, Utah, USA) and Sentek Drill and Drop probes (Sentek Sensor Technologies, Stepney South Australia, Australia) respectively. Sensors were installed at 2 replicates of each treatment for each crop in the middle of selected management zones and data was logged at 5 minutes

intervals using CR300 Campbell Scientific dataloggers (Campbell Scientific, Logan, Utah, USA) (Figure 1.4).

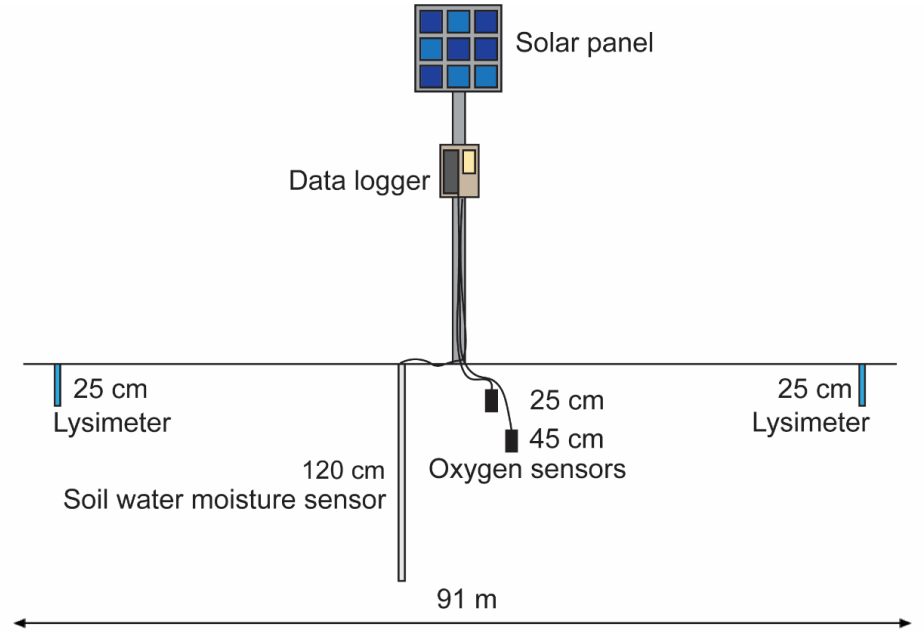


**Figure 1.4** Layout of sensors and lysimeters installation used during 2019 and 2021 growing seasons at the Mitchell Ag Laboratory, Scottsbluff, Nebraska.

At each sensing location, soil oxygen sensors were installed at 25 and 45 cm depth during both growing seasons. Oxygen sensors were calibrated under both zero-oxygen and atmospheric environments before installation (Figure 1.5). The zero-oxygen condition was achieved by injecting $N_2$ gas into a hermetically sealed container box until zero kPa was obtained for each oxygen sensor. Oxygen content values (kPa) for each sensor under zero-oxygen and atmospheric environments were recorded using a CR300 data logger (Campbell Scientific, Logan, Utah, USA) configured with a default calibration factor and offset value of 0.38 and 1.14, respectively. These readings were converted to measured voltage ($mV_M$) using derived Equation 1.1. The calibration factor for absolute oxygen readings was computed using Equation 1.2 using the barometric pressure at 1186 meters

above sea level, and the output voltage at atmospheric environments ($mV_c$) and zero oxygen ($mV_c$) previously computed in Equation 1.1. The offset value was derived by multiplying the calibration factor by the measured voltage output at zero-oxygen condition using Equation 1.3. These values were later used to compute calibrated readings once sensors were installed in the field (Apogee Instruments, Inc., 2021).


**Figure 1.5** Oxygen sensors calibration prior their installation in field.

$$O_2 = CF \times mV_M - offset \qquad \text{Eq. 1.1}$$

$$CF = \frac{0.2095\ P_B}{mV_c - mV_0} \qquad \text{Eq. 1.2}$$

$$Offset = (CF \times mV_0) \qquad \text{Eq. 1.3}$$

Where, $O_2$ is absolute oxygen, $CF$ is calibration factor, $mV_M$ is measured voltage output, $mV_c$ is measured voltage during calibration under atmospheric environments, $mV_0$ is measured voltage under zero oxygen condition, and $P_B$ is barometric pressure based on an elevation of 1186 meters above sea level.

Additionally, soil moisture sensors were installed at a depth of 120 cm (Figure 1.6) to measure volumetric water content (%) at 5, 10, 15, 35, 45, 55, 65, 75, 85, 95, 105, and 115 cm below the soil surface and obtained data was used to compute a soil water balance for seasonal evapotranspiration and root zone water depletion. Available water was computed on a weekly basis by subtracting the volumetric water content at wilting point from the readings of volumetric water content recorded in data loggers at each relative depth (Equation 1.4).

**Figure 1.6** A special auger was used to drill holes for soil moisture sensors at 120 cm depth.

Changes in the overall available water from 0 to 120 cm were used to compute the seasonal ET by conducting a soil water balance following Equation 1.5 (Eisenhauer et al. 2021). The root zone water depletion was computed using Equation 1.6. This approach allowed the tracking of soil water depletion, which has less error than available water, by

removing the uncertainty in $\Theta_{FC}$ because $\Theta_{FC,obs}$ was determined by the sensor, so $\Theta_v$ was subtracted from the $\Theta_{FC,obs}$. Moreover, it removed the sensor-to-sensor variation (Singh et al. 2020).

$$AW = (\Theta_v - \Theta_{wp}) \times R_d \qquad \text{Eq. 1.4}$$

$$ET = AW_b - AW_e + P + d_n \qquad \text{Eq. 1.5}$$

$$D = d_{rz} (\Theta_{FC,obs} - \Theta_v) \qquad \text{Eq. 1.6}$$

Where, $AW$ is available water, $\Theta_v$ is volumetric water content, $\Theta_{wp}$ is volumetric water content at wilting point (10%), $R_d$ is relative depth of each section in the sensor, $ET$ is the amount of evapotranspiration, $AW_b$ is total available water at the beginning of a period, $AW_b$ is total available water at the end of a period, $P$ is total precipitation during the period, and $d_n$ is net irrigation during the period (90% SDI system efficiency). $D$ is root zone soil water depletion (cm), $d_{rz}$ is root zone (cm), $\Theta_{FC,obs}$ is volumetric water content (%) associated with the field capacity obtained from each sensor, $\Theta_v$ is volumetric water content (%).

Water samples from the feedlot lagoon were collected into 250 mL amber glass jars before and after the sand filters during each irrigation event. In addition, pore water samples were collected using lysimeters (Irrometer Company Inc., Riverside, California, USA) after each irrigation event. Two lysimeters, 183 meters apart from one another, were installed 25 cm below the soil surface at 3 replicates of each treatment for each crop (Figure 1.7). Soil pore water samples were collected using a handheld vacuum pump at approximately 80 kPa vacuum at the lysimeters. Water samples were analyzed in terms of

pH, electrical conductivity (EC), and total dissolved solids (TDS) using a calibrated Oakton multiparameter probe (Oakton Instruments, Vernon Hills, Illinois, USA). Differences in pH, EC, and TDS were statistically analyzed using T-test in R (R Core Team, 2020).

### 1.2.5 Crop sampling

At the end of each growing season, crops were manually and mechanically harvested. As shown in Figure 1.8, two corn plants were randomly selected in each management zone from each treatment to evaluate the effect of air injection on the harvest index (%). Harvest index (HI) is defined as the ratio between the weight of dry grain yield and the total weight of dry matter above-ground dry biomass, which includes the weight of dry stover and dry grain (Pennington, 2013). American National Standards Institute (ANSI)/American Society of Agricultural and Biological Engineers (ASAE) S358.3 method was followed to dry out each plant in an oven at 55°C for 72 hours (ASAE, 1972).

Mechanical harvest was conducted in corn to evaluate the effect of air injection on corn yield (ton/ha) using a John Deere 9500 GPS-equipped combine with an 8-row corn header. For sugar beets, each zone was divided into four sampling blocks in which each block (1.1 m width, 15 meters length) consisted of two sugar beet rows. At each block, sugar beet was weighed using a customized weigh wagon (Figure 1.9). Additionally, subsamples were collected after weighing and were sent to Western Sugar Cooperative (Western Sugar, Scottsbluff, Nebraska, USA) for sucrose content analysis (%). Analysis of variance (ANOVA) with a significance level of $\alpha = 0.05$ was performed using R (R Core Team, 2020) on the crop yield.

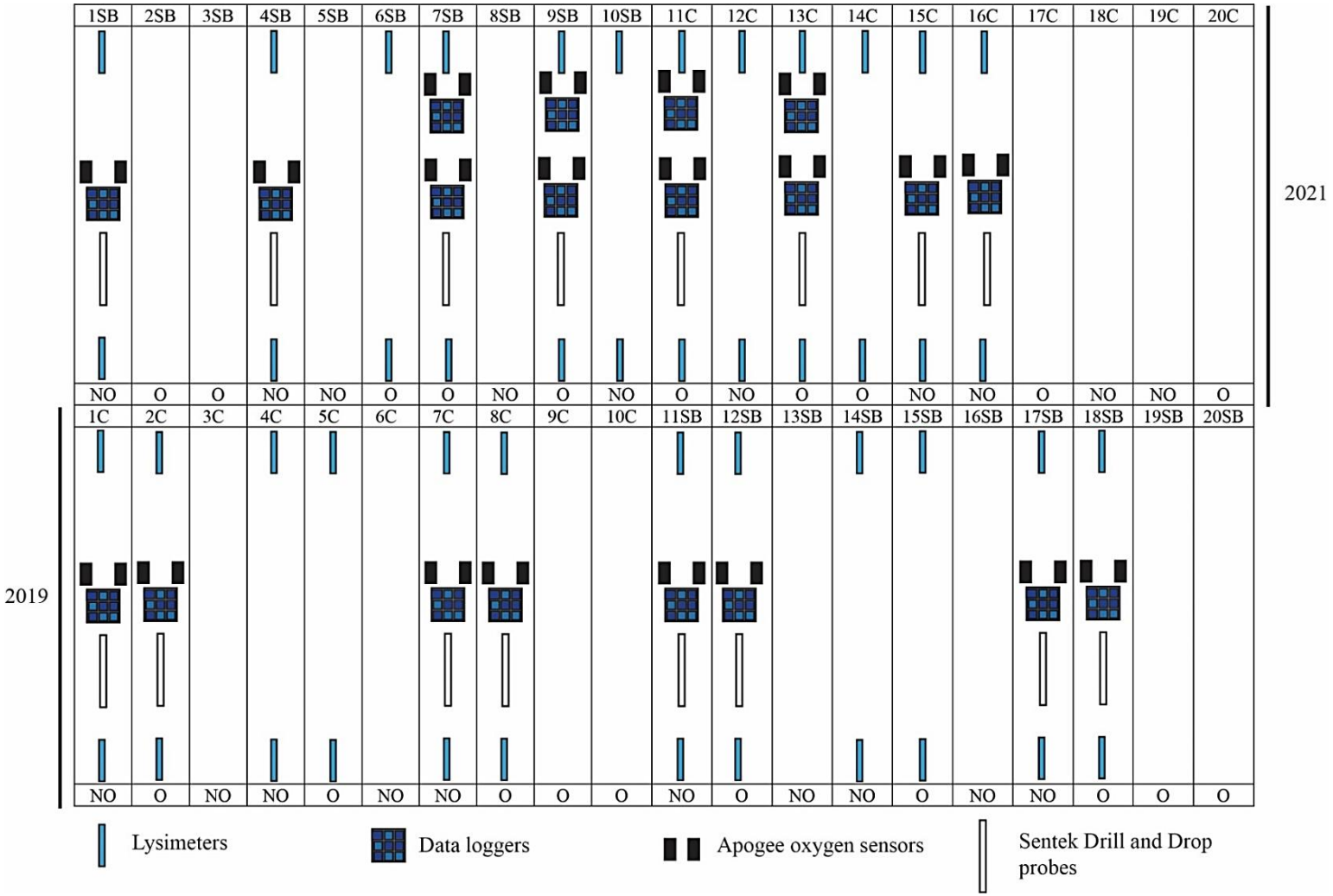


**Figure 1.7** Sensors installation layout of the experimental field at the Mitchell Ag Laboratory.


**Figure 1.8** Manual harvest layout of corn field at the Mitchell Ag Laboratory.

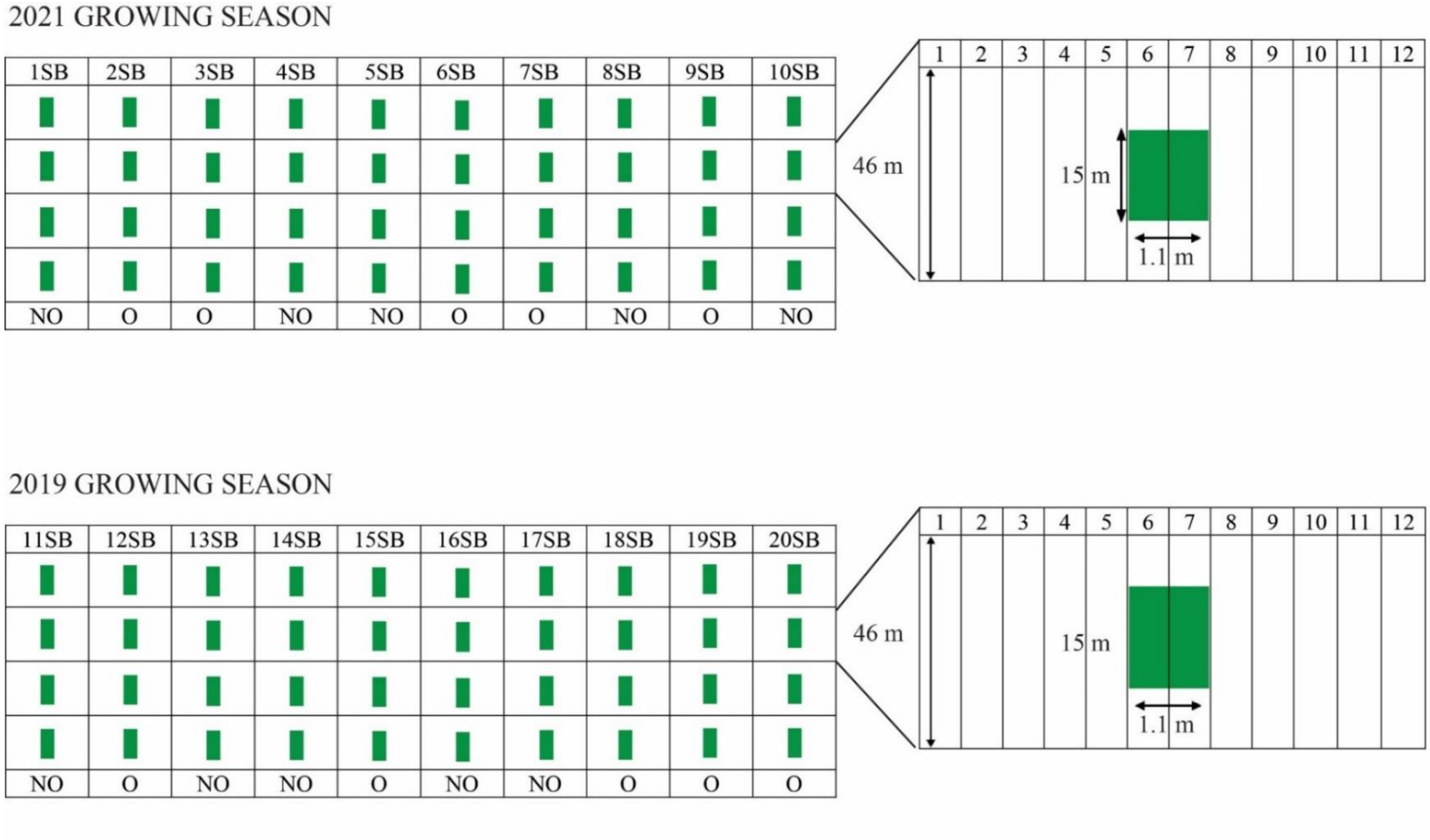


**Figure 1.9** Mechanical harvest layout of sugar beets field at the Mitchell Ag Laboratory.

## 1.3 Results and Discussion

### 1.3.1 Irrigation water quality

Table 1.1 summarizes the characteristics of feedlot runoff before and after the sand filters of SDI that was used for irrigation during the 2021 growing season. Before filtering at the SDI system, it was observed that the average pH (9.15 ± 0.73), EC (0.88 ± 0.05 dS $m^{-1}$), and TDS (623.89 ± 32.27 mg $L^{-1}$) from the feedlot lagoon were above the EPA recommended range of pH (6.5 to 8.4), EC (0.7 dS $m^{-1}$), and TDS (450 mg $L^{-1}$) for agricultural irrigation (EPA, 2012). Although the lagoon water had consistently high pH (9.15 ± 0.73), EC (0.88 ± 0.05 dS $m^{-1}$), and TDS (623.89 ± 32.27 mg $L^{-1}$) values throughout the study, significantly low pH (7.94 ± 0.30), EC (0.66 ± 0.37 dS $m^{-1}$) ($p < 0.01$), and TDS (475.40 ± 29.63 mg $L^{-1}$) ($p < 0.01$) values were reported after the filtration system (EPA, 2012).

**Table 1.1.** Main chemical characteristics of the feedlot runoff used in this study and EPA guidelines for agricultural irrigation (EPA, 2012).

| Parameters | Feedlot runoff | After filtration | EPA guideline[d] Restriction for agricultural irrigation | | |
|---|---|---|---|---|---|
| | | | None | Slight to moderate | Severe |
| pH | 9.15 ± 0.73 | 7.94 ± 0.30 | Normal range between 6.5 and 8.4 | | |
| EC[a], dS $m^{-1}$ | 0.88 ± 0.05 | 0.66 ± 0.37 | < 0.7 | 0.7 – 3.0 | > 3.0 |
| TDS[b], mg $L^{-1}$ | 623.89 ± 32.27 | 475.40 ± 29.63 | < 450 | 450 - 2000 | > 2000 |

[a] Electrical conductivity.
[b] Total dissolved solids.
[c] Water samples collected after filtration with sand filters.
[d] Degree of restriction for agricultural irrigation.

Table 1.2 summarizes the characteristics of the soil pore water collected from the lysimeters. The presence of injected air had not statistically impact on pH (NO: 7.96 ± 0.24, O: 7.94 ± 0.27) ($p = 0.75$), EC (NO: 1.03 ± 0.11 dS $m^{-1}$, O: 1.00 ± 0.34 dS $m^{-1}$) ($p =$

0.70), and TDS (NO: 738.62 ± 141.10 mg $L^{-1}$, O: 987.56 ± 613.16 mg $L^{-1}$) ($p$ = 0.07) values among the water samples collected in the corn field. Similar results were observed among the water samples collected in the sugar beets field in terms of EC (NO: 0.94 ± 0.12 dS $m^{-1}$, O: 0.99 ± 0.13 dS $m^{-1}$) ($p$ = 0.06) and TDS (NO: 679.16 ± 76.20 mg $L^{-1}$, O: 700.81 ± 93.96 mg $L^{-1}$) ($p$ = 0.26); however, the impact of injected air was statistically different in terms of pH (NO: 7.95 ± 0.14, O: 7.88 ± 0.17) ($p$ < 0.01). A comparison of these parameters for samples collected from lysimeters indicated that the lowest values of pH (7.68 ± 0.21) and EC (0.94 ± 0.24 dS $m^{-1}$) were observed during the first irrigation event in all crops and treatments, with increases in each parameter among irrigation events which might suggest potential accumulation of salts. Increases in EC values might potentially lead to adverse effects on the overall yield of crops if the threshold for each crop is exceeded. However, that was not the case with these crops since corn and sugar beets have a salt tolerance threshold of 1.7 dS $m^{-1}$ and 7.0 dS $m^{-1}$, respectively (Maas and Hoffman, 1977).

**Table 1.2.** Chemical characteristics of water samples collected from the lysimeters during the different irrigation events.

| Irrigation | Treatment | pH | EC (dS $m^{-1}$) | TDS (mg $L^{-1}$) |
|---|---|---|---|---|
| Corn | NO | 7.96 ± 0.24 | 1.03 ± 0.11 | 738.62 ± 141.10 |
| | O | 7.94 ± 0.27 | 1.00 ± 0.34 | 987.56 ± 613.16 |
| Sugar beets | NO | 7.95 ± 0.14 | 0.94 ± 0.12 | 679.16 ± 76.20 |
| | O | 7.88 ± 0.17 | 0.99 ± 0.13 | 700.81 ± 93.96 |

### 1.3.2 Soil water content and soil oxygen content

In the 2019 growing season, total seasonal rainfall was 310 mm. Additionally, a total of 216 mm of irrigation was applied among ten irrigation events, with 84% feedlot runoff (181 mm) and 16% freshwater (35 mm). In the 2021 growing season, total seasonal rainfall was 121 mm. The 2021 growing season was drier and warmer season than the 2019

growing season. In the 2021 growing season, a total of 371 mm of irrigation was applied among fourteen irrigation events, with 46% feedlot runoff (168 mm) and 54% freshwater (203 mm). Since feedlot runoff was only available during the weekend, freshwater was used on weekdays during hot and dry days. Hence more freshwater was applied during the 2021 growing season to meet the higher evaporative demand. Figure 1.10 shows root zone soil water depletion (D) in the 120 cm soil profile during the two growing seasons. Adequate irrigation was supplied so MAD was not exceeded. In both years, no significant difference was observed in D among treatments for each crop. However, different initial D was observed at some treatments in which D was recovered later in the season (Figure 1.10B and 1.10C).

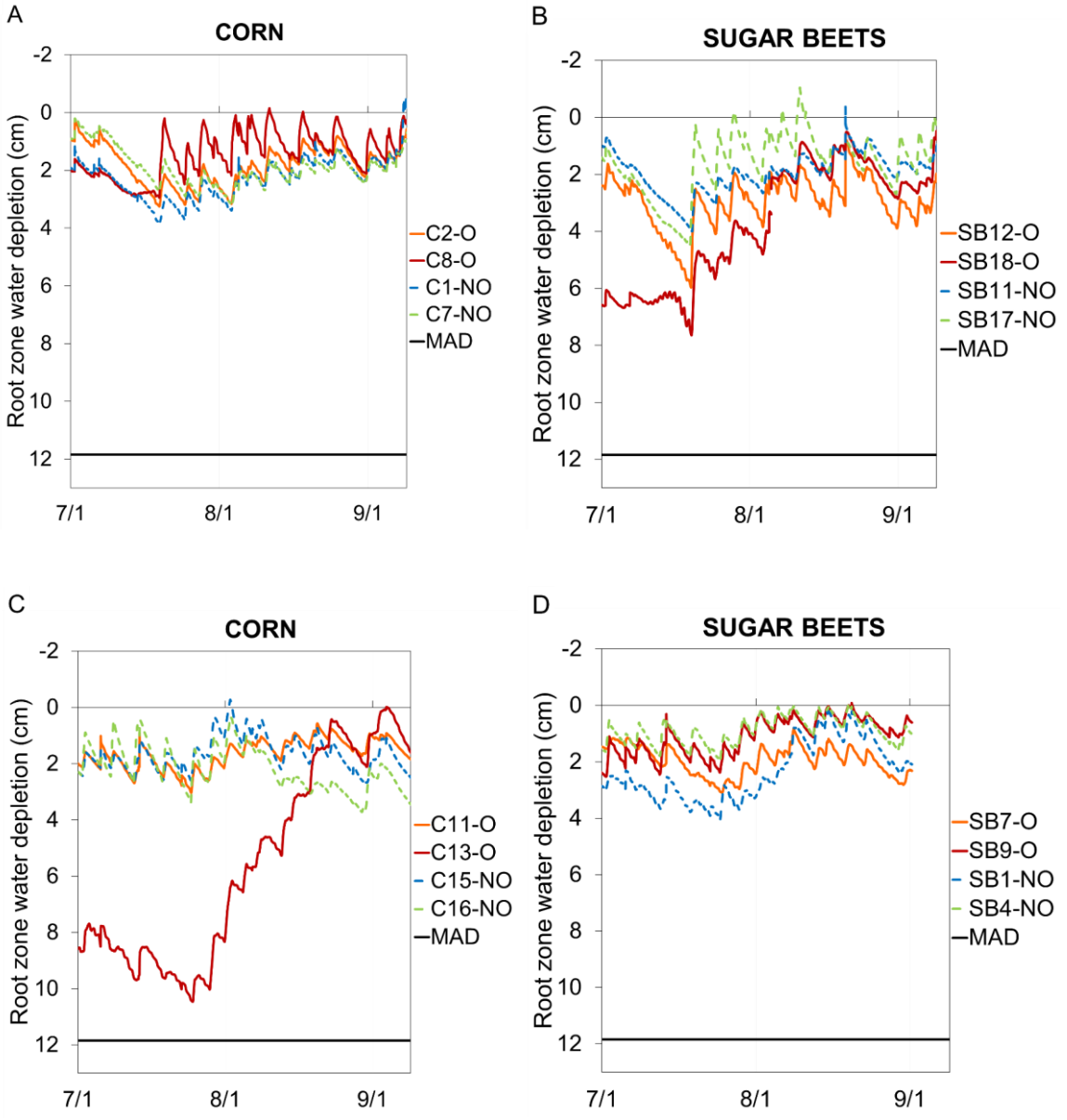


**Figure 1.10** Changes in the root zone water depletion (cm) in corn (A) and sugar beets (B) during the 2019, and corn (C) and sugar beets (D) during the 2021 growing season. In legend, C represents corn, SB represents sugar beet; number follow C/SB is management zone number; NO means non-air injection treatment and O means air injection treatment.

Figure 1.11 shows a 3-day (7/12/2021 – 7/15/2021) change of soil oxygen content in O and NO treatment following a 25 mm irrigation event (7/12) and a 20 mm rainfall event (7/13) during the 2021 growing season. It can be seen that soil oxygen content in O treatment was higher than in NO treatment in either corn or sugar beet plots. The higher soil oxygen content in O was probably due to the displacement of water by injected air. In addition, soil oxygen content decreased either after irrigation or rainfall, but it decreased more steeply after rainfall than after irrigation. This was possibly due to water being applied differently. In SDI, the 25 mm of irrigation was applied over a period of 24 hours by dripping down through emitters. Whereas the rainfall of 20 mm on 7/13 happened in 1 hour. As irrigation or rainfall stopped, soil oxygen content gradually increased (recovered). According to Chen (2018), soil drying and wetting processes regulate the displacement of air or water from soil void spaces; soil water content increases and soil oxygen content decreases as the wetting stage begins, and opposite trends are seen during the drying stage (Chen, 2018). Furthermore, a diurnal change of soil oxygen content was observed, where soil oxygen increased in the morning and reached to peak around noon, then decreased in the afternoon. This was possibly due to increased soil respiration due to rising soil temperature and possibly higher microbial activities in the morning (Chen et al. 2011).

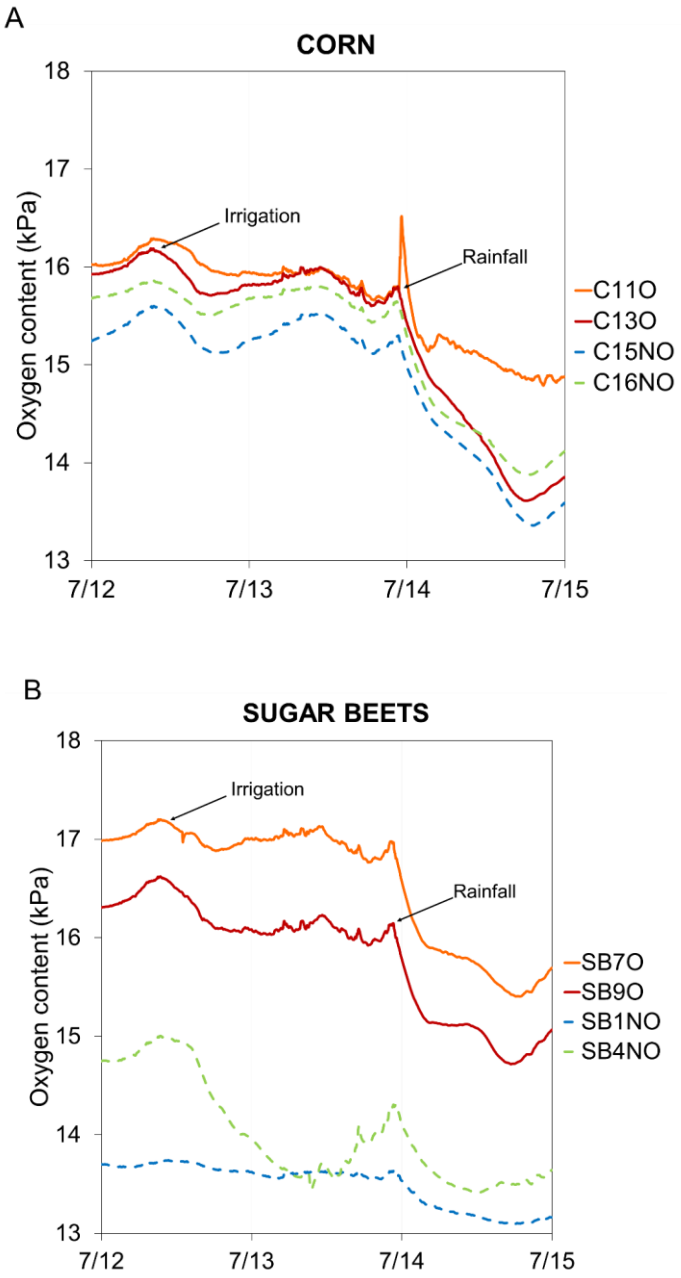


**Figure 1.11** Three-day soil oxygen content at 25 cm depth following a 25 mm irrigation event and 20 mm rainfall during 2021 growing season in corn (A) and sugar beets (B). In legend, C represents corn, SB represents sugar beet; number follow C/SB is management zone number; NO means non-air injection treatment and O means air injection treatment.

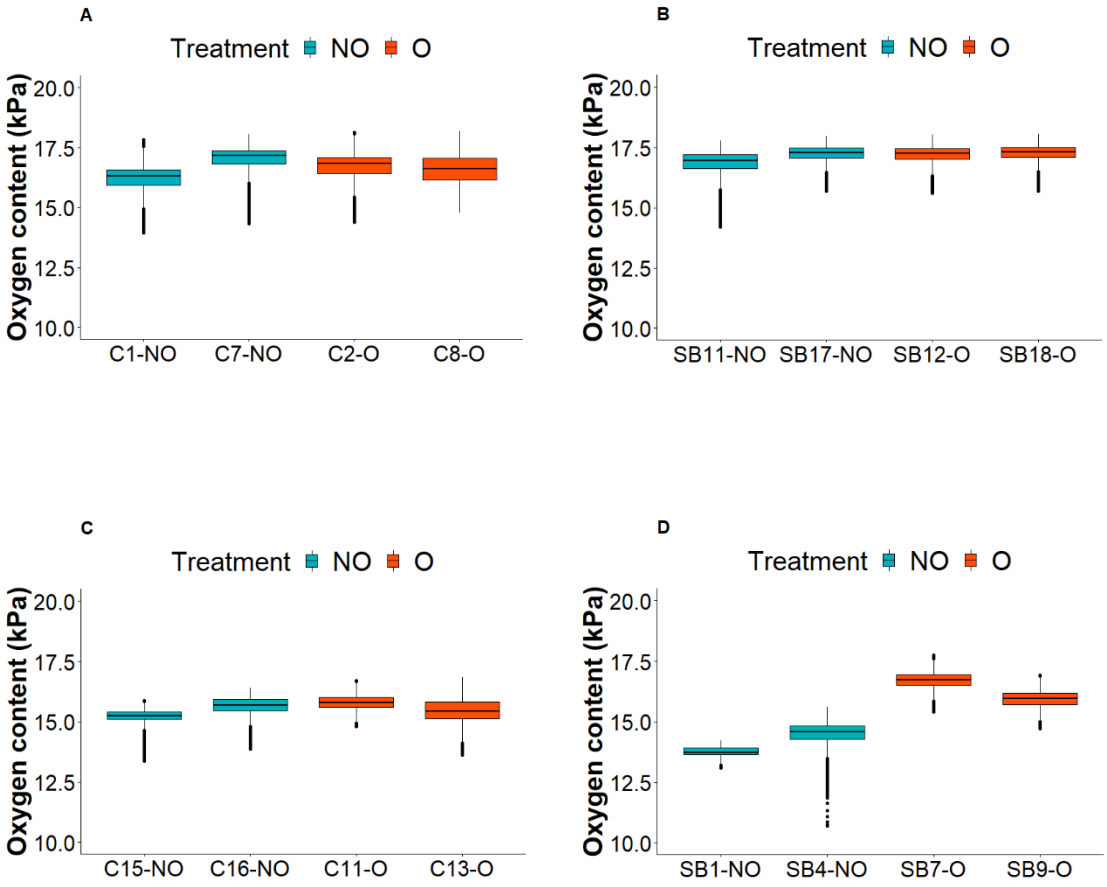


**Figure 1.12** Average soil oxygen content (kPA) at 25 cm depth for O and NO treatments during 2019 (5A) and 2021 (5C) growing seasons in corn (C); 2019 (5B) and 2021 (5D) growing seasons in sugar beets (SB). Numbers after each acronym refer to management zones where sensors were installed.

Figure 1.12 shows overall distribution of soil oxygen content in NO and O treatments in corn during 2019 (Figure 5A) and 2021 (Figure 5C) growing season, and sugar beets during 2019 (Figure 5B) and 2021 (Figure 5D) growing season. Air injection significantly increased soil oxygen content in sugar beets (NO:17.0 ± 0.54 kPa vs O:17.2 ± 0.41 kPa, $p < 0.01$) and corn (NO: 16.6 ± 0.75 kPa vs O: 16.7 ± 0.70 kPa, $p < 0.01$) during the 2019 growing season, and in SB (NO:14.2 ± 0.52 kPa vs O: 16.3 ± 0.54 kPa, $p < 0.01$) and corn (NO: 15.4 ± 0.43 kPa vs O: 15.6 ± 0.55 kPa, $p < 0.01$) during the 2021 growing

season. Similar results in subsurface drip irrigation have been reported by Baram et al. (2021) where soil oxygen concentration increased significantly in O treatment (19.2%) compared to NO treatment (18.2%), Baram et al. (2022) where the average soil oxygen concentrations significantly increased in O treatment (19.2%) compared to NO treatment (18.9%) in sandy soils, and O treatment (19%) compared to NO treatment (18%) in clayey soils.

### 1.3.3 Crop yield

The effects of air injection on sugar beet yield, sucrose content, sucrose yield, and WUE are shown in Table 1.3. During the 2019 growing season, sugar beet yield in O treatment (48.94 ± 15.08 ton $ha^{-1}$) was outperformed by NO treatment (50.30 ton $ha^{-1}$). Sucrose yield was relatively higher in O treatment (7.82 ± 1.61 ton $ha^{-1}$) than in NO treatment (7.24 ± 1.72 ton $ha^{-1}$). However, differences in sugar beet yield ($p$ = 0.76), sucrose content ($p$ = 0.72), and sucrose yield ($p$ = 0.79) were not statistically significant between the two treatments in the 2019 growing season. While seasonal evapotranspiration varied from 270 mm to 287 mm in O treatment, and 320 mm to 333 mm in NO treatment, resulting in significant differences ($p$ = 0.02) in WUE of NO treatment (0.15 kg $ha^{-1}$ $mm^{-1}$) and O treatment (0.19 kg $ha^{-1}$ $mm^{-1}$). During the 2021 growing season, differences in sugar beet yield ($p$ = 0.36) and sucrose yield ($p$ = 0.20) were not significant, but sucrose content was significantly higher in NO treatment ($p$ = 0.001). The seasonal evapotranspiration varied from 455 mm to 461 mm in O treatment, and 453 mm to 461 mm in NO treatment, resulting in a WUE of 0.11 kg $ha^{-1}$ $mm^{-1}$ in NO treatment and 0.10 kg $ha^{-1}$ $mm^{-1}$ in O treatment ($p$ = 0.35). In a similar study by Vyrlas et al. (2014), sugar beet yield, sucrose content, and sucrose yield were not significantly different between air

injection and non-air injection treatments. However, crop yield and sucrose content in this study were lower than crop yield (75.11 ton $ha^{-1}$) and sucrose content (17.56%) reported in Nebraska during the last five years (USDA-NASS, 2021).

**Table 1.3.** Sugar beet yield (ton $ha^{-1}$), seasonal irrigation (I: mm), seasonal precipitation (P: mm), seasonal evapotranspiration (ET: mm), sucrose content (%), sucrose yield (ton $ha^{-1}$), and water use efficiency (WUE: kg $ha^{-1}$ $mm^{-1}$) data for the 2019 and 2021 growing seasons.

| Year | Trt | I, mm | P, mm | ETc, mm | Yield, ton $ha^{-1}$ | Sucrose content, % | Sucrose yield, ton $ha^{-1}$ | WUE, kg $ha^{-1}$ $mm^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| 2019* | NO | 216 | 310 | 327.10 ± 9.14 | 50.30 ± 12.13[a] | 14.34 ± 0.58[a] | 7.22 ± 1.80[a] | 0.15[a] |
| | O | | | 257.45 ± 11.94 | 48.94 ± 15.08[a] | 14.41 ± 0.62[a] | 7.05 ± 2.17[a] | 0.19[b] |
| 2021 | NO | 371 | 121 | 457.48 ± 5.05 | 49.58 ± 19.61[c] | 18.28 ± 0.36[c] | 9.07 ± 3.60[c] | 0.11[c] |
| | O | | | 458.30 ± 4.27 | 45.03 ± 17.96[c] | 17.72 ± 0.69[c] | 7.93 ± 3.05[d] | 0.10[c] |

[a] and [b] were used for 2019 growing season while [c] and [d] were used for 2021 growing season. Groups with different letter were significant ($\alpha = 0.05$). *Crops suffered hail damage in 2019 growing season and caused lower yield than regional average.

The effects of air injection on corn yield are shown in Table 1.4. Even though crop yield presented a positive response to air injection with a 5.50 % increase in O treatment (7.70 ± 0.90 ton $ha^{-1}$) compared to NO treatment (7.30 ± 1.00 ton $ha^{-1}$) in the 2019 growing season, differences were not significant ($p = 0.11$). The seasonal evapotranspiration varied from 313 mm to 327 mm in O treatment, and 307 mm to 326 mm in NO treatment, resulting in a WUE of 24.05 kg $ha^{-1}$ $mm^{-1}$ and 23.05 kg $ha^{-1}$ $mm^{-1}$ in O and NO treatments, respectively. However, differences were not significant ($p = 0.11$). Crop yield from the 2019 growing season was lower than the overall crop yield (11.66 ± 0.41ton $ha^{-1}$) reported in Nebraska during the last five years (USDA-NASS, 2021). Multiple hailstorms throughout this growing season potentially had a negative effect on crop yield. During the 2021 growing season, O treatment significantly ($p = 0.04$) increased crop yield by 9.17% (12.14 ± 2.23 ton $ha^{-1}$) when compared to NO treatment (11.12 ± 2.88 ton $ha^{-1}$). Similar results were reported by Abuarab et al. (2013), with a significant increase in corn yield

under subsurface drip irrigation with air injection (12.60 – 12.85 ton $ha^{-1}$) compared to subsurface drip irrigation (11.22 – 11.42 ton $ha^{-1}$) and surface drip irrigation (9.14 – 9.28 ton $ha^{-1}$) without air injection. The seasonal evapotranspiration varied from 459 mm to 507 mm in O treatment, and 502 mm to 519 mm in NO treatment, resulting in significant ($p$ = 0.001) lower WUE of NO treatment (21.78 kg $ha^{-1}$ $mm^{-1}$) than O treatment (25.12 kg $ha^{-1}$ $mm^{-1}$). Crop yield from the 2021 growing season was similar to the average crop yield reported in Nebraska (11.66 ton $ha^{-1}$) in the last five years (USDA-NASS, 2021).

**Table 1.4.** Corn yield (ton $ha^{-1}$), seasonal irrigation (I: mm), seasonal precipitation (P: mm), seasonal evapotranspiration (ET: mm), water use efficiency (WUE: kg $ha^{-1}$ $mm^{-1}$), and harvest index data for the 2019 and 2021 growing seasons.

| Year | Trt | I, mm | P, mm | ETc, mm | Yield, ton $ha^{-1}$ | WUE, kg $ha^{-1}$ $mm^{-1}$ | Harvest index, % |
|---|---|---|---|---|---|---|---|
| 2019* | NO | 216 | 310 | 316.75 ± 13.68 | 7.30 ± 1.00[a] | 23.05[a] | NA |
| | O | | | 320.22 ± 9.24 | 7.70 ± 0.90[a] | 24.05[a] | NA |
| 2021 | NO | 371 | 121 | 510.78 ± 12.20 | 11.12 ± 2.88[c] | 21.78[c] | 62.43 ± 7.17[c] |
| | O | | | 483.38 ± 33.18 | 12.14 ± 2.23 | 25.12[d] | 65.58 ± 6.78[c] |

[a] was used for 2019 growing season while [c] and [d] were used for 2021 growing season. Groups with different letter were significant ($\alpha = 0.05$). *Crops suffered hail damage in 2019 growing season and caused lower yield than regional average.

## 1.4 Conclusion

This study evaluated the effect of air injection into a feedlot runoff fed subsurface drip irrigation system on corn and sugar beets production in western Nebraska in U.S. Results indicated that air injection can significantly increase soil oxygen content in shallow soil depth. No significant difference was observed in terms of sugar beet yield or sucrose yield between O and NO treatments during both growing seasons. Air injection seemed to have a positive effect on corn yield by having a higher yield in the 2019 growing season (5.5% increase, not significant) and a significantly higher yield in the 2021 growing season (9.17% increase, $p$ = 0.04). Air injection was also able to increase WUE, indicated by

significantly higher ($p$ = 0.02) WUE in O (0.19 kg $ha^{-1}$ $mm^{-1}$) than NO (0.15 kg $ha^{-1}$ $mm^{-1}$) in sugar beet and significantly higher ($p$ = 0.001) WUE in O (25.12 kg $ha^{-1}$ $mm^{-1}$) than NO (21.78 kg $ha^{-1}$ $mm^{-1}$) in corn. Similarly, this study has also demonstrated the potential of using feedlot runoff as an irrigation source to produce crops. Without the application of fertilizers, corn at air injected plots yielded like the average corn yield in Nebraska. The presence of nutrients in feedlot runoff could potentially be beneficial for crop yields and reduce the use of synthetic fertilizers. Considering the low cost of air injectors and its beneficial effects on crop yield, we suggest this approach to farmers already owning a SDI system.

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# CHAPTER 2. EFFECT OF AIR INJECTION INTO SUBSURFACE DRIP IRRIGATION (SDI) ON THE SOIL MICROBIAL DIVERSITY AND THE REMOVAL OF ANTIBIOTIC RESIDUES IN SOIL PORE WATER, SOIL AND PLANT MATRICES.


## *Abstract*

The use of non-conventional water alternatives, such as feedlot runoff, can be used to meet the irrigation demands of corn (C) and sugar beets (SB) in Western Nebraska. However, feedlot runoff might contain antibiotics so their introduction to the soil environment and potentially, their accumulation, can result in the contamination of groundwater and the conferral of antibiotic resistance characteristics to the soil microbiome. In this research, we tested how passive air injection to the wastewater-fed subsurface drip irrigation (SDI) system could benefit the reduction of antibiotic concentrations in the soil environment. Soil pore water and soil samples were collected during each irrigation event and analyzed for antibiotics. Additionally, we evaluated the effects of air injection in the soil microbiome.

Groups of antibiotics detected in the feedlot lagoon at the 0.01 µg/L reporting limit include macrolide (azithromycin, erythromycin, and tylosin), fluoroquinolone (ciprofloxacin), phenicol (florfenicol), sulfonamide combinations (ormetoprim and trimethoprim), ionophore (Monensin Na) and sulfonamide (sulfadimethoxine, sulfamerazine, and sulfamethoxazole). At the soil environment level, results from the antibiotic analysis conducted in soil pore water samples indicated that the impact of air injection on the concentrations of sulfamethoxazole was not significant in sugar beets ($p$ =

0.33) and corn ($p$ = 0.25) throughout the 2021 growing season. Moreover, the impact of air injection on the concentrations of monensin Na was not significant in sugar beets ($p$ = 0.18) and corn ($p$ = 0.84). However, the results from the microbial diversity assay revealed that the abundance of *Alphaproteobacteria* ($p$ = 0.05) in sugar beets, and *Acidimicrobiia* ($p$ = 0.04) increased in time under O treatment.

## 2.1 Introduction

As discussed in Chapter 1, the increase in population and the uneven global distribution of freshwater resources represent a threat to food security. The use of wastewater for agricultural irrigation is presented as an alternative to meet these increasing demands. In 2017, approximately 22.1 million out of 35.9 million hectares of wastewater irrigated croplands worldwide received untreated wastewater (Thebo et al., 2017). In the United States, the Cleaning Water Act (CWA) established, as a minimum requirement, the implementation of secondary wastewater treatments to meet the treated wastewater disposal parameters, which include Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), Total Nitrogen (N total), Total Phosphorus (P total), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) (EPA, 2004). However, treatments at this level only guarantee the degradation of the organic matter fraction, potentially resulting in the disposal complex compounds such as pharmaceuticals and personal care products (PPCPs) to the environment.

The accumulation of these components does not only represent a contamination source to soils (Chen et al., 2014), which movement to deeper soil layers could contaminate groundwater sources (Huang et al., 2013), but they could also have effects on the soil

microbial communities by conferring antibiotic resistance characteristics if antibiotics were present in wastewater (Chen et al., 2014; Qiao et al., 2018). These characteristics, which are conferred by antibiotic resistance genes (ARGs) on transferable plasmids, are considered emerging pollutants with public health implications because of their potential introduction to human pathogens through the food production chain (Allen et al., 2010; Wang et al., 2006). (Pan and Chu, 2018) observed that irrigation with wastewater was the major source of antibiotics that entered the soil environment and reported a relationship between the concentrations of these antibiotics in the soil to their concentrations in wastewater.

Antibiotics are used in human medicine for infectious diseases, and animal farming for growth promotion and disease prevention (Ben et al., 2019; Naderi Beni et al., 2020; Pan and Chu, 2018; Qiao et al., 2018). In veterinary, their use can be classified as therapeutical and non-therapeutical (Pan and Chu, 2018; Peak et al., 2007). The first category includes all the components that are used to control a clinical disease and to prevent its spread to healthy animals, while the second category includes all those that contributes to prevent a clinical disease and to promotes growth (Peak et al., 2007). Tetracyclines and sulfonamides are the most common used groups of antibiotics in veterinary medicine (Pan and Chu, 2018). However, they are not completely metabolized, and a major amount is excreted in urines and feces (Chen et al., 2014). In an assessment for veterinary medicinal products, Montforts et al. (1999) reported that antibiotics such as chlortetracycline was metabolized as low as 25% of the total amount applied to young bull. Although antibiotics can accumulate in soils, their degradation in the soil environment can be affected by soil properties such as pH and organic matter (Yang et al., 2009). Moreover, their persistence

has been attributed to the affinity of antibiotics to organic particles and sorption processes (Pikkemaat et al., 2016). These factors determine the mobility of antibiotics across soil profile to watersheds, bioavailability to microorganisms for their degradation and plant uptake (Hu et al., 2010; Pikkemaat et al., 2016).

Tetracyclines are characterized by their stability in acid media, and salt formation in both acid and basic values (Pikkemaat et al., 2016). Biological factors have been found to be the main drivers in the degradation of this compound but depending on the soil pH, soil moisture is another abiotic factor that promotes its degradation via hydrolysis (Yang et al., 2009). Pouliquen et al. (2007) reported an increase in the hydrolysis of tetracyclines when pH ranged between 7.0 and 8.4. Moreover, when tetracyclines are present in unsaturated soils, an increase in the degradation rate is reported due to a higher availability of soil oxygen. On the other hand, when this compound is in saturated soils, a decrease in the degradation rate is reported (Ingerslev et al., 2001). Despite their susceptibility to photodegradation, it has been reported a decrease in this characteristic at the topsoil due to its fixation to soil porous (Hamscher et al., 2002). However, this fixation can be as beneficial since it reduces its mobility throughout soil profile.

In sulfonamides, biological degradation is the major driver in their degradation, but it can also be correlated to soil depth, soil pH, soil temperature, and soil oxygen content (Chen and Xie, 2018). Differences in the degradation rate of sulfonamides were reported by Srinivasan and Sarmah (2014), who observed a faster degradation rate in the topsoil compared to the subsoil. Moreover, the removal of sulfonamides was enhanced in the topsoil when the temperature was increased. On the other hand, the increase in the laccase activity, which also contributes to the removal of sulfonamides, was reported in the pH

values between 3 and 6 (Ding et al., 2016). Additionally, it was reported that sorption of sulfonamides increased when exposed to acidic soils (Engelhardt et al., 2015). Liu et al. (2010) reported that the half-life of sulfonamides, which is the time it takes to sulfonamides to be reduced by half their initial concentration, was longer (up to 16 days) under anoxic conditions compared to aerobic conditions (up to 8 days). This finding suggests that the presence of oxygen can benefit the degradation of sulfonamides.

Organic carbon content has been reported to have a strong influence on the sorption, which includes adsorption and absorption, and desorption processes of antibiotics in the group of sulfonamides and tetracyclines (Chen et al., 2020; Conde-Cid et al., 2020). However, the diversity of antibiotic types in wastewater could result in the competence for the adsorption sites in soils and a lower retention capacity caused by saturation (Conde-Cid et al., 2020; Conkle et al., 2010). Additionally, interactions between antibiotics and metals in soils have been reported beneficial and competitive ways (Bao et al., 2013; Zhao et al., 2013). A large group of pharmaceuticals are known for containing electron-donor characteristics which positively interacts with metal ions (Zhao et al., 2013). Zhao et al. (2013) reported that adsorption of tetracyclines was benefited by the presence of Cu (II) and Pb (II). However, different results are possible considering the soil pH values. Because of the high complexation ability between tetracyclines and Cu (II), the adsorption process can be enhanced in both tetracyclines and Cu (II) when soil pH value is less than 7. Nevertheless, when soil pH is higher than 7, it results in the release of tetracyclines and Cu (II) from soils. A beneficial interaction between tetracyclines and Pb (II) was reported by the adsorption of tetracyclines in the presence of Pb (II) in alkaline soils. Bao et al. (2013) observed a competitive effect of oxytetracycline and $Cd^{2+}$ for soil adsorption sites. It was

reported that adsorption and desorption processes of $Cd^{2+}$ were affected when adsorption of oxytetracycline first occurred. Similarly, adsorption and desorption processes of oxytetracycline was affected when adsorption of $Cd^{2+}$ first occurred. However, a lower adsorption rate was observed when concentrations of both pollutants were increased.

The objective of this study was to evaluate the effects of air injection on the reduction of antibiotic concentrations in the soil environment introduced by irrigation with feedlot wastewater. It is expected that air injection will increase the soil oxygen content, and this increase together with soil properties and biological factors may contribute to the degradation of these compounds. This evaluation will consist in the chemical and microbiological analysis of soil pore water and soil samples collected after irrigation events with feedlot wastewater, and corn and sugar beet plants collected during harvesting.

## 2.2 Material and Method

### 2.2.1 Study site and experimental design

The field experiment was conducted in the 2021 growing season at the Mitchell Ag Laboratory, Panhandle Research and Extension Center (PHREC), University of Nebraska-Lincoln, Scottsbluff, NE (41.95 N, 103.70 W, elevation 1,186 meters above sea). For information regarding soil characteristics, weather data, irrigation events, and treatment layout used in the study site, please refer to the “study site” section in Chapter 1.

### 2.2.2 Analysis of pharmaceuticals in water matrices

As described in the “measured parameters” section in Chapter 1, water samples were collected from the feedlot lagoon, which served as an irrigation water source, and

lysimeters (Irrometer Company Inc., Riverside, California, USA) after each irrigation event for their analysis of pharmaceuticals at the Irrigation and Digital Ag Lab, Panhandle Research and Extension Center, University of Nebraska-Lincoln, Scottsbluff, Nebraska, USA. Before their installation, lysimeters were soaked in water for 8 hours. Then, a 2.5 cm diameter auger was used to dig in 28 cm depth to allow for 2.5 cm of silica flour slurry. Near the surface, a 2.5 cm layer of bentonite was used to seal the hole and prevent water to move across the tube (Figure 2.1).

**Figure 2.1** Installation and testing using a vacuum pump of the lysimeters used in this experiment.

Reagents used in water samples include analytical grade chemicals (Fisher Scientific, Waltham, MA, USA) such as methanol, acetonitrile, and disodium hydrogen phosphate; citric acid monohydrate was purchased at Acros Organics, Geel, Belgium, and ethylenediaminetetraacetic acid (EDTA) disodium salt (Sigma-Aldrich, St. Louis, MO, USA). Targeted analytes for this experiment are included in the Group 1 of the EPA 1694 method and were extracted under acidic conditions. A citrate phosphate buffer (McIlvain buffer) with ethylenediaminetetraacetic acid (EDTA) disodium salt was used to keep the pH of the water matrices around 4.0. To prepare the McIlvain-EDTA buffer, 500 mL of 0.1 M citric acid and 280 mL of 0.2 M disodium hydrogen phosphate were added to 1.0 L

of distilled de-ionized (DDI) water. Then, the pH was adjusted to 4.0 using citric acid or di-sodium hydrogen phosphate solution. This solution was transferred to a 2 L flask and filled up with DDI water up to the mark, the resulting solution is the McIlvain buffer (Jansen et al., 2019). Finally, 37.124 g of EDTA disodium salt were added for each 1.0 L of McIlvain buffer. To prepare the 0.1 M citric acid solution, 9.606 g of citric acid monohydrate were used for each 500 mL of DDI water. The 0.2 M disodium hydrogen phosphate was prepared using 14.196 g of disodium hydrogen phosphate for each 500 mL of DDI water.

Analyte Spike, Surrogate Spike, and Internal Standard Spike were used throughout the experiment. It was used a 0.1 ng/µL Analyte Spike containing the 35 targeted analytes. Analytes include Ampicillin, Azitromycin, Ceftiofur Sodium, Florfenicol (with MeOH for better solubility), Monensin Na Hydrate (with MeOH), Novobiocin Sodium (with MeOH), Penicillin G Potassium, Penicillin V Potassium, Sulfadiazine, Tiamulin (with MeOH), and Trimethoprim (Honeywell International Inc., Charlotte, NC, USA), Ciprofloxacin HCl $H_2O$, Erythromycin, Lincomycin HCl, Oxolinic Acid, Penillic Acid, Roxithromycin, Sulfamerazine, Thiabendazole, and Virginiamycin M1 (Sigma-Aldrich, St. Louis, MO, USA), Sulfachloropyridazine, Sulfadimethoxine, Sufamethizole, Sulfamethoxazole, and Sulfathiazole (MP Biomedicals, Irvine, CA, USA), are Carbadox, Cefotaxime (with DMSO for better solubility), and Danofloxacin (Santa Cruz Biotech, Dallas, TX, USA), Clarithromycin, Ormetoprim, Oxacillin Na $H_2O$. Other remaining analytes (TCl America, Portland, OR, USA), DCCD + 5% Ceftiofur (Toronto Research Chamicals Inc, North York, Ontario, Canada), Sulfamethazine (Acros Organics, Geel, Belgium), Sulfanilamide

(Fisher Scientific, Waltham, MA, USA), and Tylosin Tartrate (ICN Biomedicals, Costa Mesa, CA, USA).

To track those analytes, a 0.1 ng/µL Surrogate Spike containing 0.1 ng/µL of $^{13}$C3-DEA (Cambridge Isotope Laboratories Inc, Tewksbury, MA, USA), Orphenadrine HCl (Sigma-Aldrich, St. Louis, MO, USA) and Oleandomycin (ICN Biomedicals, Costa Mesa, CA, USA) in acetonitrile was used. To quantify these analytes, it was used a 0.1 ng/µL Internal Standard containing 0.1 ng/µL of $^{13}$C6-Sulfamethazine, $^{13}$C6-Sulfamethoxazole, $^{13}$C6-Thiabendazole, $^{13}$C-N-Methylerythromycin, $^{13}$C3-Trimethoprim, and $^{13}$C3,15N-Ciprofloxacin purchased at Cambridge Isotope Laboratories Inc, Tewksbury, Massachusetts, USA, DCCD-d3 (Toronto Research Chamicals Inc, North York, Ontario, Canada), and Salinomycin (Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile..

#### 2.2.2.1 Method detection limit

EPA 1694 method (EPA, 2007) was used to determine the occurrence of antibiotics in the feedlot runoff as well as in the lysimeter samples. Briefly, liquid samples, initially concentrated in OASIS HLB solid phase extraction cartridges (Waters Corporation, Milford, MA, USA) were eluted, and analyzed using a liquid chromatography combined with tandem mass spectrometry (LC/MS/MS). A method detection limit (MDL) for the EPA 1694 G1 procedure for water matrices was conducted at the Water Sciences Laboratory, University of Nebraska-Lincoln, Lincoln, Nebraska, USA. As defined by (EPA, 2016), the MDL procedure provides the minimum concentration of an analyte in each matrix that can be measured and reported with 99% confidence that such concentration is greater than zero. Since the MDL procedure requires at least seven

samples, and the EPA 1694 method is often used at the Water Sciences Laboratory making method blanks available for its use, eight samples (MDL samples) and one method blank (Laboratory Reagent Blank) were processed.

As shown in Figure 2.2, labeled amber jars were used to weight out 50 grams of distilled de-ionized (DDI) water. Similarly, the Laboratory Reagent Blank (LRB) was prepared using 50 mL of DDI water. Then, 10 mL of McIlvain-EDTA buffer solution was added to all 50 mL amber jars. All MDL samples were spiked with 100 µL of 0.02 ng/ µL Analyte Spike for a total of 2 ng. LRB and MDL samples were then spiked with 100 µL of 0.1 ng/µL Surrogate Spike. LRB and MDL samples were extracted onto OASIS HLB 200mg/6cc solid phase extraction cartridges (Waters Corporation, Milford, Massachusetts, USA) preconditioned with 6 mL of acetonitrile and 6 mL of DDI water, by aspirating the samples at no more than 1 mL per minute (Figure 2.3).

As shown in Figure 2.4, SPE cartridges were slowly eluted in labeled glass test tubes with 10 mL of acetonitrile at 1 mL/min using a Visiprep$^{TM}$ SPE Vacuum Manifold (Sigma-Aldrich Co. LLC, Darmstadt, Germany). Although acetonitrile was used in this method, methanol is also used to elute OASIS HLB 200mg/6cc solid phase extraction cartridges. A Visidry$^{TM}$ Drying Attachement with Visiprep 12-port model (Sigma-Aldrich Co. LLC, Darmstadt, Germany) was used to evaporate these samples under nitrogen at 35°C to 500 µL (Figure 2.5). Then, all samples were spiked with 100 µL of 0.1 ng/µL Internal Standard. Samples were evaporated for a second time under nitrogen at 35°C to 40 µL. As the final step, 160 µL of DDI water was added to each glass test tube and each sample was transferred to a labeled glass insert using disposable pipettes and vortexed (Figure 2.6).

**Figure 2.2** Amber jars containing 50 grams (around 50 mL) of DDI water, 10 mL of McIlvain-EDTA buffer solution and Analyte/Surrogate Spikes.

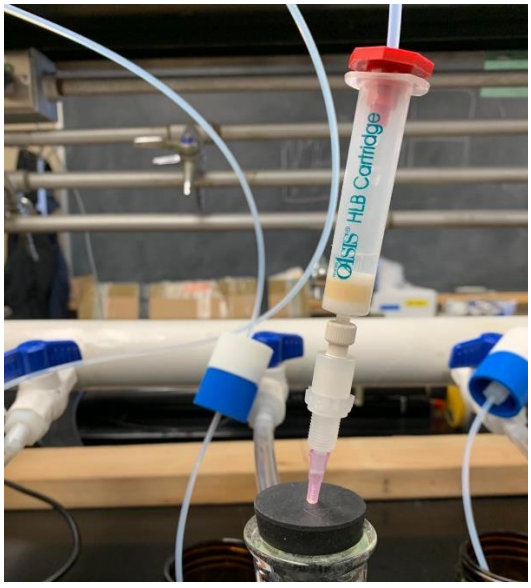


**Figure 2.3** Sample extraction process using OASIS HLB 200mg/6cc solid phase extraction cartridges.

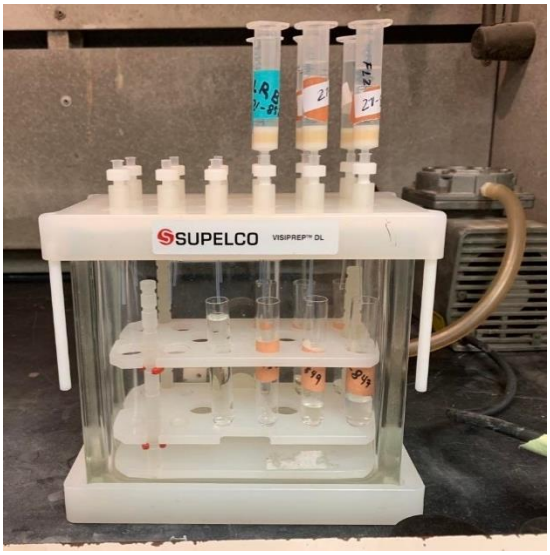


**Figure 2.4** Visiprep$^{TM}$ SPE Vacuum Manifold holding the MDL and LRB samples during the elution process.


**Figure 2.5** The Visidry$^{TM}$ Drying Attachement with the Visiprep 12-port model during the sample evaporation process.

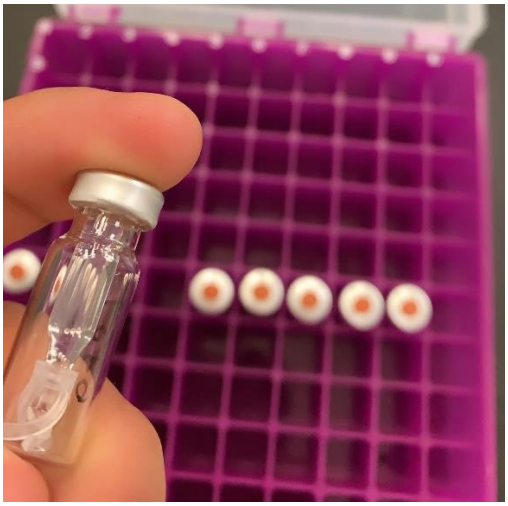

**Figure 2.6** Glass inserts containing 40 µL of sample and 160 µL of DDI water.

These samples were analyzed by the WSL personnel using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) by Xevo LC/MS with UniSpray source (Waters Corporation, Milford, Massachusetts, USA). Data reduction was conducted at the WSL using the MassLynx v4.2 software (Waters Corporation, Milford, Massachusetts, USA).

To compute the SPE recovery, the amount of each analyte recovered from each OASIS HLB 200mg/6cc SPE cartridge (Table 2.1) is divided by the amount of each analyte added to each sample (presented as MDL spike in Table 2.2) and multiplied by 100. The value is expressed as a percentage (%). To account for outliers in the recovery rate, a Maximum Normed Residual Test (Grubbs' test) was conducted at 5% level of significance for 8 samples (2.032). Equation 2.1 was used to obtain the minimum Grubb's test statistic value for each analyte. Equation 2.2 was used to obtain the maximum Grubb's test statistic value for each analyte. If the Grubb's test statistic value was higher than the Grubb's test

value (2.032), the recovered analyte was classified as an outlier and the sample was not considered in the SPE recovery rate for that specific analyte.

$$\text{Grubb's test} = \frac{\text{Mean (ng)} - \text{Minimum (ng)}}{\text{Standard deviation (ng)}} \qquad \text{Eq. 2.1}$$

$$\text{Grubb's test} = \frac{\text{Maximum (ng)} - \text{Mean (ng)}}{\text{Standard deviation (ng)}} \qquad \text{Eq. 2.2}$$

#### 2.2.2.2 EPA 1694 G1 procedure for water matrices

At the Irrigation and Digital Ag Lab, water samples were processed following the EPA 1694 G1 procedure for water matrices (EPA, 2007). Labeled clean amber jars were used to weight out 50 grams of water collected in lysimeters and feedlot lagoon when volume collected allowed. Otherwise, the total volume of each sample was weighed out and values were recorded. A Laboratory Fortified Matrix (LFM), Laboratory Fortified Blank (LFB), and Laboratory Reagent Blank (LRB) samples were prepared. The LFM sample was used to verify that the sample matrix did not influence the analytical results (EPA, 2017), so one LFM sample was included in each batch of samples processed. To prepare this sample, 50 grams of water collected in field were weighted out in a labeled amber jar, in this case, samples from the feedlot lagoon were used given its availability. A LFB sample was included in each batch of samples to test the ability of the laboratory to measure accurately and precisely, and to determine whether the methodology is controlled (EPA, 2017). A LRB was included in each batch of samples to determine if inferences, such as method analytes, were present in the laboratory environment, reagents or equipment used throughout the method (EPA, 2017). To prepare the LFB and LRB

samples, 50 grams of DDI water were weighted out in labeled amber jars. In addition, 10 mL of McIlvain-EDTA buffer solution was added to all 50 grams samples. In those cases where weight of samples were less than 50 grams, McIlvain-EDTA buffer solution was added in proportion to their weight. All samples were then spiked with 100 µL of 0.1 ng/µL Surrogate Spike. LFM and LFB samples were additionally spiked with 100 µL of 0.1 ng/µL Analyte Spike. Samples were extracted onto labeled OASIS HLB 200mg/6cc solid phase extraction cartridges (Waters Corporation, Milford, Massachusetts, USA) preconditioned with 6 mL of acetonitrile and 6 mL of DDI water, by aspirating the samples at no more than 1 mL per minute. Then, SPE cartridges were shipped to the USDA Arid Land Agricultural Research Center (USDA-ALARC), Maricopa, Arizona, USA.

At the Arid Land Agricultural Research Center, SPE cartridges were slowly eluted in labeled glass test tubes with 3 mL of methanol at 1 mL/min, and 3 mL of acetonitrile in case some compounds elute more complete with a less polar solvent. A CentriVap Centrifugal Concentrator (Labconco Corporation, Kansas City, Missouri, USA) was used to evaporate these samples at 35°C to 40 µL. Then, all samples were reconstituted using 160 µL of 15% acetonitrile solution containing 100 µL of 0.1 ng/µL Internal Standard. As the final step, each sample was transferred to a labeled glass insert using disposable pipettes and vortexed. These samples were analyzed by the USDA-ALARC personnel using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS). In each irrigation event, the concentration of every antibiotic detected in soil pore water was normalized (C/C0) by dividing that concentration (C) to the concentration detected in the feedlot runoff (C0). Differences in these normalized concentrations were statistically analyzed using T-test in R (R Core Team, 2020).

### 2.2.3 Microbial diversity assay

Soil samples were collected after each irrigation event at locations where lysimeters were installed. In total, 24 soil samples were collected in individual labeled Ziploc bags using a 25-cm long auger and stored in a cooler filled with ice packs. At the Irrigation and Digital Ag Lab, one subset was obtained from each soil sample to conduct the analysis of microbial communities. Sterilized spatulas were individually used to compose a subset of each sample, and gloves were cleaned up using isopropyl alcohol before and after the manipulation of each sample. Each subset consisted of 5 mL sterilized vials filled up with soil samples to around ¾ of their capacity or 4 mL mark, then the cap of each vial was tightened and sealed using parafilm. These samples were stored in a frost-free freezer and later delivered for their analysis at the Agroecosystem Management Research Unit, Agricultural Research Service (USDA-ARS), Lincoln, Nebraska, USA.

Reagents were provided in the DNeasy PowerSoil Pro Kit. It contained a Solution CD1, which is a cell lysis buffer composed of chaotropic salts. A second generation Inhibitor Removal Technology (IRT) contained in the Solution CD2 was used to precipitate non-DNA organic and inorganic material, contributing to preserve the DNA purity of the matrices. Solution CD3, which is a highly concentrated salt solution, was used to allow binding of DNA and prevent binding non-DNA organic or inorganic material. It is also included in the kit a Solution EA, which is a wash buffer used to remove non-aqueous materials from the MB Spin Column filter membrane that might contaminate the matrices. A Solution C5, which is an ethanol-based solution, is additionally included to clean the DNA by removing residual salts and other contaminants. Solution C6 (10 mM Tris), a

solution that lacks salts, was used to release the DNA from the MB Spin Colum filter membrane at the end of the procedure.

#### 2.2.3.1 DNeasy PowerSoil Pro Kit Procedure

At the USDA-ARS, soil samples were prepared for a diversity assay 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) Long Read following the DNeasy PowerSoil Pro Kit Procedure (QIAGEN, 2018). PowerBead Pro Tubes, which contain a buffer solution that essentially prevent nucleic acids to degrade and dissolve humic acid were spun to allow for the beads to settle at the bottom of the tubes. Each tube was filled up with 250 mg of soil sample followed by 800 µL of Solution CD1, and vortexed for 5 seconds. PowerBead Pro Tubes were secured horizontally in a Vortex Genie 2 (Daigger, Buffalo Grove, Illinois, USA) and vortexed for 10 minutes, then they were centrifuged using a Heraeus Pico 21 centrifuge (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 15,000 *x g* for 1 minute. The supernatant from each PowerBead Pro Tubes was transferred to clean 2 mL Microcentrifuge Tubes and 200 µL of Solution CD2 was added. These Microcentrifuge Tubes were then vortexed for 5 seconds and centrifuged at 15,000 *x g* for 1 minute. The supernatant from each Microcentrifuge Tube was transferred to clean 2 mL Microcentrifuge Tubes, in which 600 µL of Solution CD3 was added and vortexed for 5 seconds. Then, 650 µL of the lysate was transferred to MB Spin Columns and centrifuged at 15,000 *x g* for 1 minute. The next step was to discard the flow-through obtained at each MB Spin Column, transfer the remaining lysate to the same MB Spin Columns and centrifuge at 15,000 *x g* for 1 minute. Each MB Spin Column was placed into clean 2 mL Collection Tubes, in which 500 µL of Solution EA was added and

centrifuged at 15,000 *x g* for 1 minute. After discarding the flow-through from the MB Spin Columns and placing them back into the same 2 mL Collection Tubes, 500 µL of Solution C5 was added and centrifuged at 15,000 *x g* for 1 minute. Each MB Spin Column was placed into clean 2 mL Collection Tubes and centrifuged at 16,000 *x g* for 2 minutes. Then, each MB Spin Column was placed into 1.5 mL Elution Tubes and 100 µL of Solution C6 was added to the center of the white filter membrane. These tubes were centrifuged at 15,000 *x g* for 1 minute. As the final step, the flow-through (DNA) contained in each Elution Tube was transferred to 0.5 Eppendorf Tubes. These tubes were then shipped to the Molecular Research Laboratory, Shallowater, Texas, USA to conduct the requested assay. Results were statistically analyzed at the Class level using T-test in GraphPad Prism 9 (GraphPad Software, LLC, 2022).

The DNeasy PowerSoil Pro Kit Procedure is graphically described in Figure 2.7.

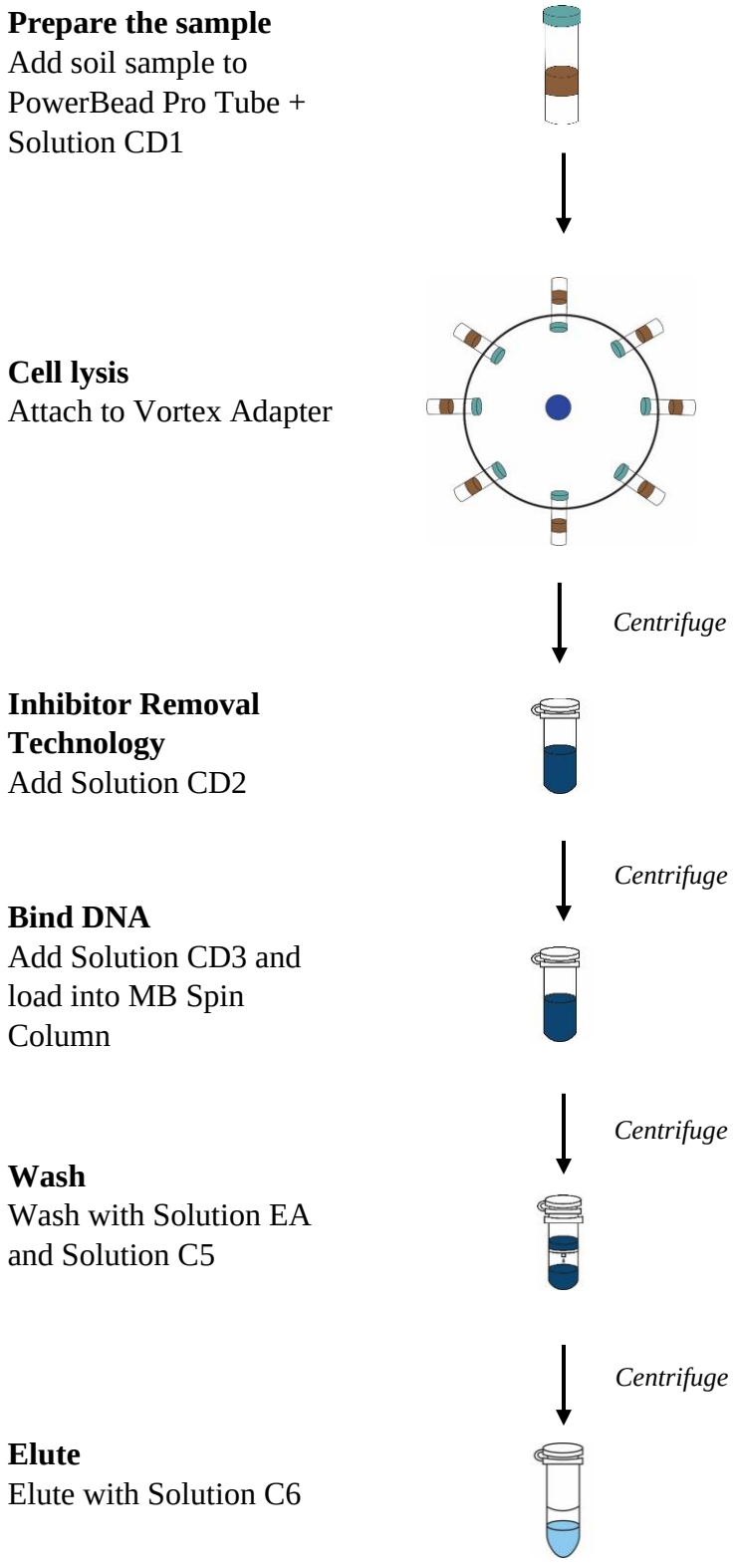


**Figure 2.7** DNeasy PowerSoil Pro Kit Procedure (QIAGEN, 2018)

## 2.3 Results and Discussion

### 2.3.1 Antibiotics estimation in water matrices

The Method Detection Limit (MDL) provided information regarding the solid phase extraction (SPE) recovery for each analyte. As shown in Table 2.1, some samples contained a higher amount of a specific analyte compared to other samples with the same analyte (highlighted in red). Outliers were not detected in the minimum values in any sample but were detected in the maximum values of MDL #8 for Ceftiofur and Virginiamycin, and MDL #2 for Sulfamethoxazole (Table 2.2). As observed in Figure 2.8, Novobiocin recovery was not determined because of low, or none amounts of analyte were detected (1.1 ± 0.1 ng). These results indicate that this method is not suitable for the extraction of Novobiocin.

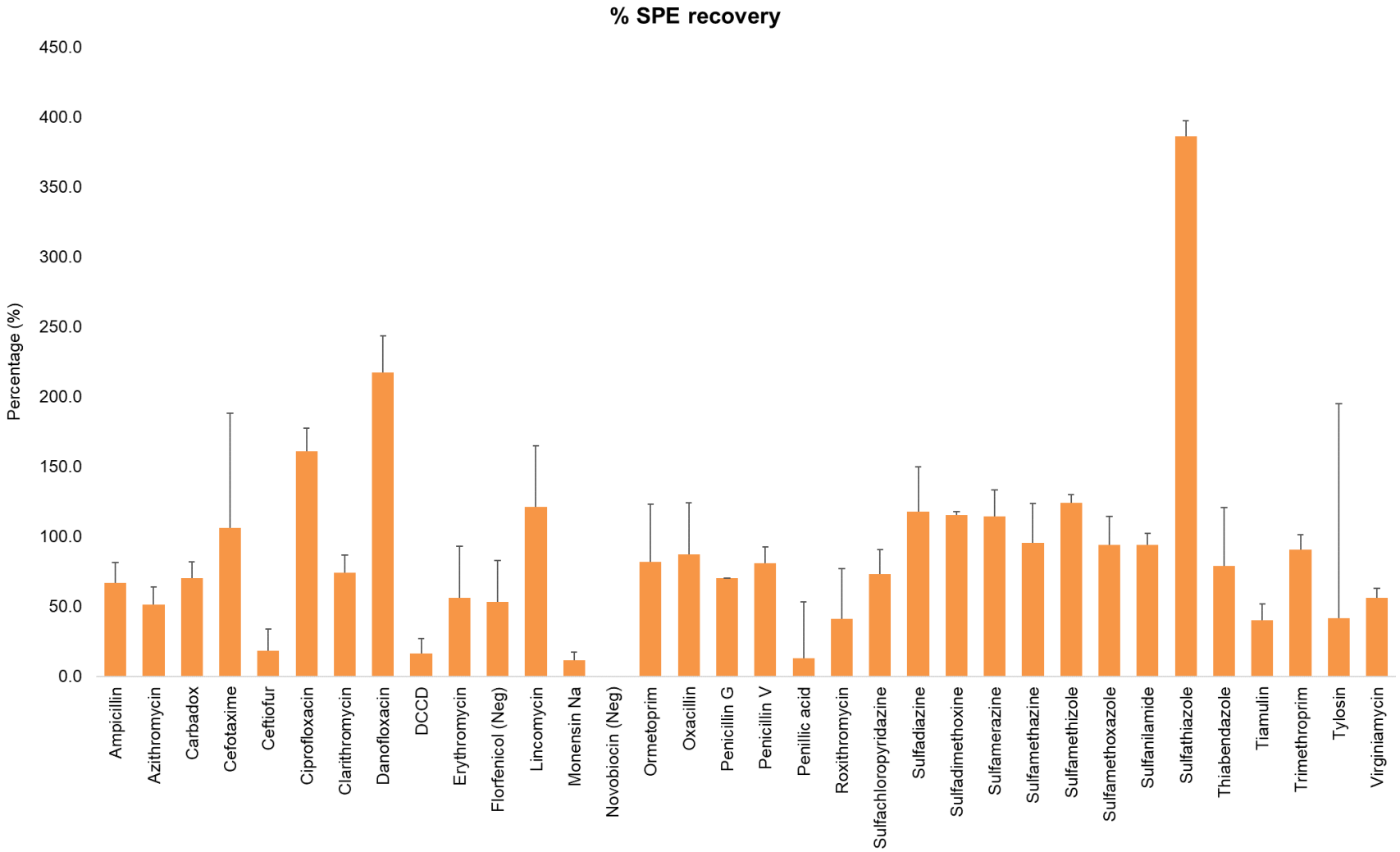


**Figure 2.8** SPE recovery (%) obtained from the Method Detection Limit for water samples.

**Table 2.1.** Amount of Analyte Spike applied to the Method Detection Limit (MDL) for veterinary use subgroup in water samples.

| Compound | MDL #1 | MDL #2 | MDL #3 | MDL #4 | MDL #5 | MDL #6 | MDL #7 | MDL #8 |
|---|---|---|---|---|---|---|---|---|
| Ampicillin | 1.2 | 1.0 | 1.4 | 1.6 | 1.9 | 1.1 | 1.4 | 1.2 |
| Azithromycin | 0.9 | 0.6 | 1.2 | 1.0 | 1.5 | 1.3 | 1.2 | 0.6 |
| Carbadox | 1.1 | 1.0 | 1.6 | 1.4 | 1.8 | 1.5 | 1.3 | 1.4 |
| Cefotaxime | 28.4 | 17.8 | 23.5 | 28.8 | 14.4 | 17.8 | 19.3 | 20.3 |
| Ceftiofur | 1.6 | 1.5 | 3.6 | 5.6 | 5.0 | 6.6 | 1.7 | 13.2 |
| Ciprofloxacin | 3.2 | 2.6 | 4.1 | 2.9 | 4.1 | 2.0 | 3.1 | 3.8 |
| Clarithromycin | 1.1 | 0.6 | 1.5 | 1.1 | 2.0 | 1.6 | 1.3 | 2.6 |
| Danofloxacin | 4.4 | 3.3 | 5.8 | 3.4 | 5.3 | 4.1 | 4.6 | 4.0 |
| DCCD | 1.6 | 2.2 | 4.7 | 2.8 | 4.9 | 3.2 | 3.1 | 4.1 |
| Erythromycin | 1.2 | 1.1 | 1.2 | 1.1 | 1.2 | 1.1 | 1.2 | 1.1 |
| Florfenicol (neg) | 1.9 | 3.2 | 10.6 | 6.0 | 23.4 | 13.8 | 4.6 | 21.6 |
| Lincomycin | 3.3 | 3.5 | 1.9 | 2.9 | 2.1 | 1.9 | 2.4 | 1.5 |
| Monensin Na | 0.7 | 0.3 | 1.7 | 5.6 | 6.3 | 2.4 | 1.6 | 0.3 |
| Novobiocin (neg) | | | | | | | | |
| Ormetoprim | 1.4 | 1.3 | 1.8 | 1.8 | 1.8 | 1.6 | 1.5 | 2.0 |
| Oxacillin | 10.2 | 10.2 | 25.2 | 14.1 | 30.3 | 22.9 | 8.1 | 18.8 |
| Oxolinic acid | 0.3 | 0.3 | 0.9 | 0.5 | 2.1 | 1.2 | 0.5 | 2.0 |
| Penicillin G | 13.5 | 8.9 | 14.7 | 12.6 | 19.9 | 17.9 | 12.0 | 12.6 |
| Penicillin V | 11.8 | 7.6 | 21.5 | 14.8 | 25.5 | 21.0 | 9.0 | 18.6 |
| Penillic acid | 2.8 | 2.7 | 2.4 | 3.1 | 3.1 | 2.2 | 1.6 | 3.0 |
| Roxithromycin | 0.6 | 0.2 | 0.9 | 0.6 | 1.5 | 0.9 | 0.7 | 1.1 |
| Sulfachloropyridazine | 0.5 | 0.9 | 1.7 | 1.7 | 2.1 | 1.5 | 1.2 | 2.0 |
| Sulfadiazine | 2.3 | 2.3 | 2.4 | 2.5 | 2.2 | 2.5 | 2.3 | 2.3 |
| Sulfadimethoxine | 2.0 | 2.8 | 1.9 | 2.9 | 2.0 | 2.3 | 2.5 | 2.0 |
| Sulfamerazine | 2.0 | 2.3 | 2.5 | 2.2 | 2.2 | 2.5 | 2.3 | 2.4 |
| Sulfamethazine | 1.5 | 2.1 | 2.2 | 1.8 | 1.9 | 2.1 | 1.8 | 2.0 |
| Sulfamethizole | 3.6 | 3.5 | 3.0 | 2.4 | 1.4 | 2.2 | 2.1 | 1.5 |
| Sulfamethoxazole | 2.2 | 3.1 | 1.4 | 2.1 | 1.8 | 1.9 | 1.9 | 1.9 |
| Sulfanilamide | 20.8 | 20.7 | 14.7 | 20.1 | 19.1 | 17.7 | 20.3 | 17.3 |
| Sulfathiazole | 12.2 | 10.9 | 7.3 | 9.1 | 3.8 | 5.8 | 8.6 | 4.0 |
| Thiabendazole | 1.5 | 1.4 | 1.7 | 1.7 | 1.7 | 1.4 | 1.6 | 1.5 |
| Tiamulin | 0.4 | 0.3 | 0.8 | 0.5 | 1.7 | 1.3 | 0.5 | 0.9 |
| Trimethoprim | 1.6 | 1.7 | 2.0 | 1.8 | 2.3 | 1.8 | 1.5 | 1.9 |
| Tylosin | 1.1 | 0.6 | 0.7 | 0.8 | 1.0 | 0.7 | 1.2 | 0.5 |
| Virginiamycin | 4.7 | 3.4 | 11.5 | 7.1 | 25.4 | 20.1 | 6.6 | 64.9 |

**Table 2.2.** Maximum Normed Residual Test (Grubb's test) for the detection of outliers in the analyte amounts.

| Compound | Mean | STD | MDL spike (ng) | Min | Grubb's test | Grubb's value (5%) | Result | Max | Grubb's test | Grubb's value (5%) | Result |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ampicillin | 8.1 | 1.5 | 2.0 | 1.0 | 1.3 | 2.0 | na | 1.9 | 1.8 | 2.0 | na |
| Azithromycin | 12.2 | 1.3 | 2.0 | 0.6 | 1.4 | 2.0 | na | 1.5 | 1.4 | 2.0 | na |
| Carbadox | 6.2 | 1.2 | 2.0 | 1.0 | 1.4 | 2.0 | na | 1.8 | 1.7 | 2.0 | na |
| Cefotaxime | 17.5 | 8.2 | 20.0 | 14.4 | 1.3 | 2.0 | na | 28.8 | 1.5 | 2.0 | na |
| Ceftiofur | 1.3 | 0.3 | 20.0 | 1.5 | 0.9 | 2.0 | na | 13.2 | 2.1 | 2.0 | Reject MDL # 8 |
| Ciprofloxacin | 1.0 | 0.3 | 2.0 | 2.0 | 1.7 | 2.0 | na | 4.1 | 1.2 | 2.0 | na |
| Clarithromycin | 1.4 | 0.3 | 2.0 | 0.6 | 1.4 | 2.0 | na | 2.6 | 1.8 | 2.0 | na |
| Danofloxacin | 21.3 | 5.2 | 2.0 | 3.3 | 1.2 | 2.0 | na | 5.8 | 1.6 | 2.0 | na |
| DCCD | 4.9 | 3.9 | 20.0 | 1.6 | 1.5 | 2.0 | na | 4.9 | 1.3 | 2.0 | na |
| Erythromycin | 3.2 | 0.7 | 2.0 | 1.1 | 1.2 | 2.0 | na | 1.2 | 1.2 | 2.0 | na |
| Florfenicol (neg) | 1.5 | 0.6 | 20.0 | 1.9 | 1.1 | 2.0 | na | 23.4 | 1.5 | 2.0 | na |
| Lincomycin | 4.4 | 0.9 | 2.0 | 1.5 | 1.3 | 2.0 | na | 3.5 | 1.5 | 2.0 | na |
| Monensin Na | 3.3 | 1.2 | 20.0 | 0.3 | 0.9 | 2.0 | na | 6.3 | 1.7 | 2.0 | na |
| Novobiocin (neg) | 1.1 | 0.1 | | | | | | | | | |
| Ormetoprim | 10.6 | 8.3 | 2.0 | 1.3 | 1.5 | 2.0 | na | 2.0 | 1.5 | 2.0 | na |
| Oxacillin | 2.4 | 0.7 | 20.0 | 8.1 | 1.2 | 2.0 | na | 30.3 | 1.6 | 2.0 | na |
| Oxolinic acid | 2.4 | 2.3 | 2.0 | 0.3 | 0.9 | 2.0 | na | 2.1 | 1.6 | 2.0 | na |
| Penicillin G | | | 20.0 | 8.9 | 1.5 | 2.0 | na | 19.9 | 1.7 | 2.0 | na |
| Penicillin V | 1.6 | 0.2 | 20.0 | 7.6 | 1.3 | 2.0 | na | 25.5 | 1.4 | 2.0 | na |
| Penillic acid | 17.5 | 8.1 | 20.0 | 1.6 | 1.9 | 2.0 | na | 3.1 | 0.9 | 2.0 | na |
| Roxithromycin | 1.0 | 0.7 | 2.0 | 0.2 | 1.6 | 2.0 | na | 1.5 | 1.7 | 2.0 | na |
| Sulfachloropyridazine | 14.0 | 3.5 | 2.0 | 0.5 | 1.7 | 2.0 | na | 2.1 | 1.2 | 2.0 | na |
| Sulfadiazine | 16.2 | 6.4 | 2.0 | 2.2 | 1.3 | 2.0 | na | 2.5 | 1.6 | 2.0 | na |
| Sulfadimethoxine | 2.6 | 0.5 | 2.0 | 1.9 | 0.9 | 2.0 | na | 2.9 | 1.6 | 2.0 | na |
| Sulfamerazine | 0.8 | 0.4 | 2.0 | 2.0 | 2.0 | 2.0 | na | 2.5 | 1.1 | 2.0 | na |
| Sulfamethazine | 1.5 | 0.6 | 2.0 | 1.5 | 1.9 | 2.0 | na | 2.2 | 1.3 | 2.0 | na |
| Sulfamethizole | 2.4 | 0.1 | 2.0 | 1.4 | 1.3 | 2.0 | na | 3.6 | 1.4 | 2.0 | na |
| Sulfamethoxazole | 2.3 | 0.4 | 2.0 | 1.4 | 1.2 | 2.0 | na | 3.1 | 2.2 | 2.0 | Reject MDL # 2 |
| Sulfanilamide | 2.3 | 0.2 | 20.0 | 14.7 | 1.9 | 2.0 | na | 20.8 | 0.9 | 2.0 | na |
| Sulfathiazole | 1.9 | 0.2 | 2.0 | 3.8 | 1.3 | 2.0 | na | 12.2 | 1.5 | 2.0 | na |
| Thiabendazole | 2.5 | 0.8 | 2.0 | 1.4 | 1.2 | 2.0 | na | 1.7 | 1.2 | 2.0 | na |
| Tiamulin | 2.0 | 0.5 | 2.0 | 0.3 | 1.1 | 2.0 | na | 1.7 | 1.9 | 2.0 | na |
| Trimethoprim | 18.8 | 2.1 | 2.0 | 1.5 | 1.2 | 2.0 | na | 2.3 | 1.9 | 2.0 | na |
| Tylosin | 7.7 | 3.1 | 2.0 | 0.5 | 1.2 | 2.0 | na | 1.2 | 1.6 | 2.0 | na |
| Virginiamycin | 1.6 | 0.1 | 20.0 | 3.4 | 0.7 | 2.0 | na | 64.9 | 2.3 | 2.0 | Reject MDL # 8 |

Table 2.3 summarizes the analytes detected in the samples collected from the feedlot lagoon at the 0.01 µg/L reporting limit. The groups reported in the feedlot lagoon include macrolide (azithromycin, erythromycin and tylosin), fluoroquinolone (ciprofloxacin), phenicol (florfenicol), sulfonamide combinations (ormetoprim and trimethoprim), ionophore (Monensin Na) and sulfonamide (sulfadimethoxine, sulfamerazine and sulfamethoxazole).

**Table 2.3.** Main analytes reported in the feedlot lagoon used in this study detected at the 0.01 µg/L reporting limit.

| Analytes | Irrigation | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Azithromycin, µg/L | | | | | | 0.08 | |
| Ciprofloxacin, µg/L | | 0.10 | 0.02 | 0.61 | 0.07 | | |
| Erythromycin, µg/L | 1.90 | | | | | | |
| Florfenicol, µg/L | | 0.17 | | | | | |
| Monensin Na, µg/L | 0.79 | 0.87 | 16.10 | 13.38 | 10.23 | 14.10 | 12.34 |
| Ormetoprim, µg/L | | 0.25 | | 0.02 | | | |
| Sulfadimethoxine, µg/L | 0.05 | | | | 0.06 | 0.01 | |
| Sulfamerazine, µg/L | | | | | | 0.01 | 0.10 |
| Sulfamethoxazole, µg/L | 0.28 | 0.07 | 0.05 | 1.48 | 0.12 | 0.10 | 0.10 |
| Trimethoprim, µg/L | | | 0.02 | 0.02 | 0.05 | | |
| Tylosin, µg/L | | | 1.04 | 0.26 | 0.23 | 0.18 | 0.27 |

The macrolide antibiotics detected in this study were azithromycin and erythromycin, which are used in human and veterinary medicine, and tylosin, which is an antibiotic strictly used in veterinary medicine (Schlüsener and Bester, 2006). Macrolides are eliminated through hepatic metabolism, but the metabolic ratio may vary across macrolide types (Anadón and Reeve-Johnson, 1999). Excretion of macrolides can happen in both urines and feces, and at different ratios. Between 2% and 5% of orally administered erythromycin is excreted in its active form in urines, while 6.5% of orally administered azithromycin is excreted in its active form in urines (Anadón and Reeve-Johnson, 1999).

However, it has been also reported that azithromycin has a metabolite ratio of 50% in mammals (Mitchell, 2005) which suggest that excretion in feces might be representative. In this experiment, azithromycin was detected in the water sample collected from the feedlot lagoon during Irrigation 6 (0.08 µg/L), while erythromycin was detected only in water samples collected during Irrigation 1 (1.90 µg/L). Moreover, tylosin was detected in Irrigation 3 (1.04 µg/L), Irrigation 4 (0.26 µg/L), Irrigation 5 (0.23 µg/L), Irrigation 6 (0.18 µg/L) and Irrigation 7 (0.27 µg/L). Biological degradation is the main driver of the degradation of these compounds, where mineralization of the N-methyl position with the proliferation of biodegrading microorganisms in erythromycin has been reported. Similarly, biodegradation of azithromycin has been reported with the removal of the azalide ring (Topp et al., 2016).

The only fluoroquinolone detected in this experiment was ciprofloxacin, which is an antibiotic used in human and veterinary medicine. Fluoroquinolones are known to have a low metabolization rate which results in the excretion, in the active form, of 40% to 90% of the oral administration in the urines and feces (Liu et al., 2018). Moreover, it was reported that ciprofloxacin, in its active form, can be largely excreted in urines (44.7%) and in less matter, feces (25.0%) (MEDSAFE, 2012). Although the degradation of ciprofloxacin in soils is challenging due to its physiochemical properties (Liu et al., 2018), it was observed that the biodegradation of this compound increased when it was exposed to a increase in temperature, reporting removal rates of 21% at 5°C, 89.7% at 25°C and 92.2% at 45°C (Liao et al., 2016). In this experiment, ciprofloxacin was detected in water samples collected from the feedlot lagoon during Irrigation 2 (0.10 µg/L), Irrigation 3 (0.02 µg/L), Irrigation 4 (0.61 µg/L) and Irrigation 5 (0.07 µg/L).

Florfenicol, a phenicol antibiotic used in veterinary medicine, was detected in this experiment in samples collected from the feedlot lagoon during Irrigation 2 (0.17 µg/L). It has been reported that most of the dosed florfenicol is excreted in urines in its active form (Liu et al., 2003). In calves, around 64% of the excreted florfenicol is in its active form (Varma et al., 1986). In pigs, between 45% and 60% of the excreted florfenicol is in its active form and additionally, between 11.2% to 17% is excreted as florfenicol amine and less than 10% is excrete as florfenicol amine (Park et al., 2007). Similar to the other groups of antibiotics, microbial communities play a major role in the biodegradation of florfenicol (Qiu et al., 2021; Xu et al., 2015). Moreover, hydrolyzation of florfenicol to florfenicol amine was reported at pH 4 (Qiu et al., 2021).

Monensin Na, an ionophore antibiotic used as a feed additive in the beef and dairy cattle industries, was consistently detected in samples collected from the feedlot lagoon during Irrigation 1 (0.79 µg/L), Irrigation 2 (0.87 µg/L), Irrigation 3 (16.10 µg/L), Irrigation 4 (13.38 µg/L), Irrigation 5 (10.23 µg/L), Irrigation 6 (14.10 µg/L) and Irrigation 7 (12.34 µg/L). It has been reported that around 80% of the dosed monensin is excreted in feces (Li et al., 2020). Given its high mobility in soil (Li et al., 2020), biotic and abiotic factors play an essential role in the sorption and degradation processes occurring in the soil environment. Yoshida et al. (2010) reported that soil microbial communities, soil water content, and soil organic matter affects the degradation of monensin. Increases in the soil water content, when no other parameter changes, results in the increase of the degradation of monensin. Like soil water content, increases in the soil organic content also results in the increase of the degradation of this compound.

Sulfonamides were also detected. The sulfonamide combination trimethoprim was reported at very low concentrations during Irrigation 3 (0.02 µg/L), Irrigation 4 (0.02 µg/L) and Irrigation 5 (0.05 µg/L) while ormetoprim was reported during Irrigation 2 (0.25 µg/L) and Irrigation 4 (0.02 µg/L). Sulfadimethoxine was reported during Irrigation 1 (0.05 µg/L), Irrigation 5 (0.06 µg/L) and Irrigation 6 (0.01 µg/L) meanwhile sulfamerazine was only reported in Irrigation 6 (0.01 µg/L) and Irrigation 7 (0.10 µg/L). Finally, the second compound that was consistently detected throughout the growing season is sulfamethoxazole, which reported concentrations as follows: Irrigation 1 (0.28 µg/L), Irrigation 2 (0.07 µg/L), Irrigation 3 (0.05 µg/L), Irrigation 4 (1.48 µg/L), Irrigation 5 (0.12 µg/L), Irrigation 6 (0.10 µg/L) and Irrigation 7 (0.10 µg/L). It is known that the main driver for the degradation of these compounds is microbiological activity (Chen and Xie, 2018).

In water samples collected from each lysimeter, sulfamethoxazole and monensin Na were detected in all irrigation events in both crops. These results are presented as a normalized concentration (C/C0); in this case, C0 is the concentration for each antibiotic detected in the water samples collected from the feedlot lagoon during each irrigation event while C is the concentration of each antibiotic detected in those samples collected in lysimeters during each irrigation event. As shown in Figure 2.9 and Fig 2.10, the impact of air injection on the concentrations of sulfamethoxazole was not significant in sugar beets ($p = 0.33$) and corn ($p = 0.25$) throughout the growing season.

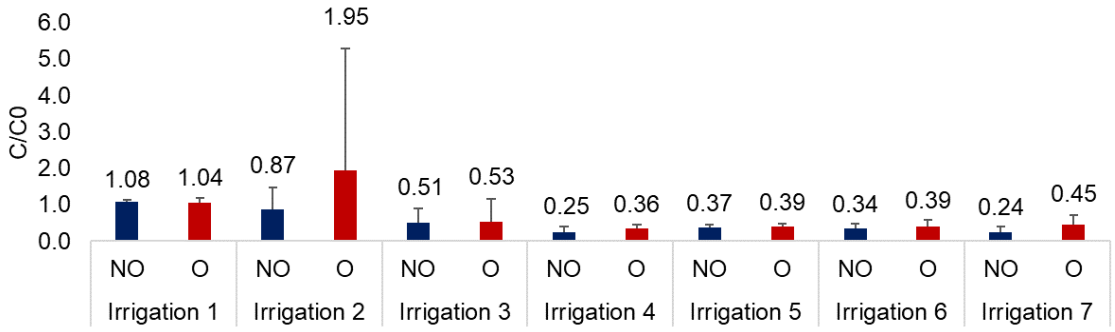


**Figure 2.9** Average detection, with standard deviation, of sulfamethoxazole in the samples collected from lysimeters in sugar beets. NO means non-air injection treatment and O means air injection treatment.

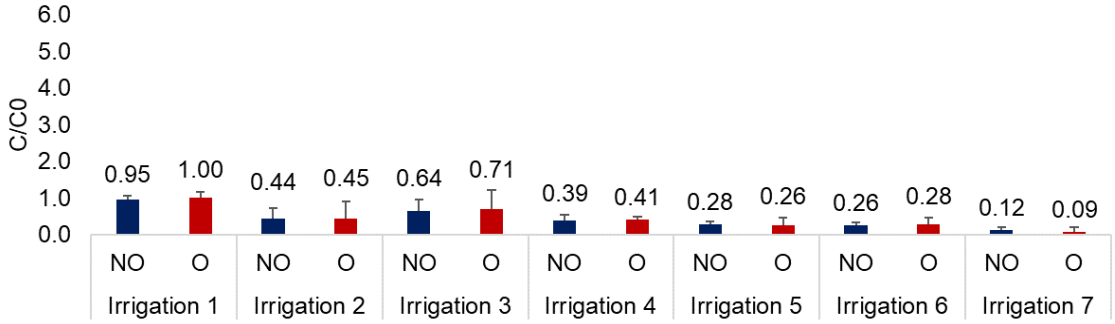


**Figure 2.10** Average detection, with standard deviation, of sulfamethoxazole in the samples collected from lysimeters in corn. NO means non-air injection treatment and O means air injection treatment.

As shown in Figure 2.11 and Fig 2.12, the impact of air injection on the concentrations of monensin Na was not significant in sugar beets ($p$ = 0.18) and corn ($p$ = 0.84) throughout the growing season. It can be seen that the concentration of monensin Na in the feedlot lagoon was high from Irrigation 3 to Irrigation 7, and lower normalized concentrations were observed in the same irrigation events. Given the high mobility of monensin Na in the soil environment (Li et al., 2020), the sorption processes of this compound seemed to

be low compared to sulfamethoxazole. However, samples collected from lysimeters provide a limited picture of the overall environmental interactions.

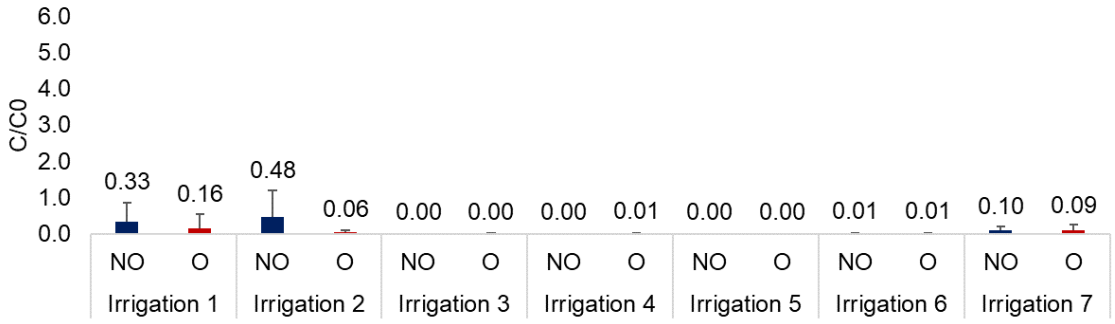


**Figure 2.11** Average detection, with standard deviation, of monensin Na in the samples collected from lysimeters in sugar beets. NO means non-air injection treatment and O means air injection treatment.

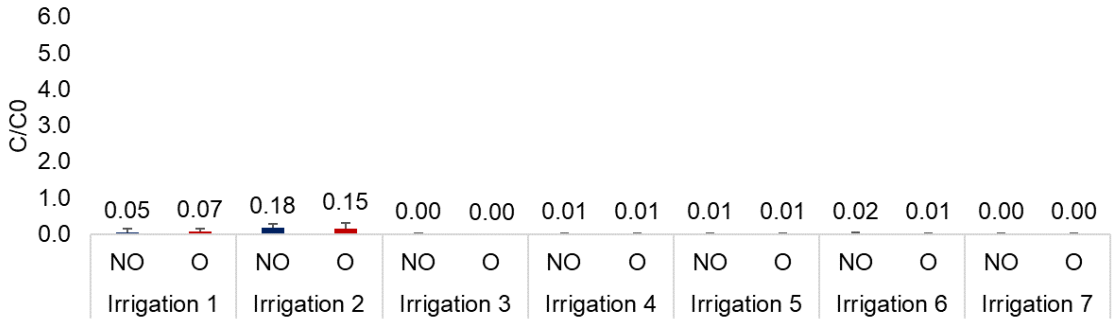


**Figure 2.12** Average detection, with standard deviation, of monensin Na in the samples collected from lysimeters corn. NO means non-air injection treatment and O means air injection treatment.

### 2.3.2 Microbial communities in soil

The results from the diversity assay 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) Long Read were analyzed at the Class taxonomic

level. The samples analyzed include those collected during Irrigation 3 and Irrigation 7, which corresponds to the most complete set of soil samples collected early in the season and the last irrigation of the season respectively. In Figure 2.13 and Figure 2.14, all Classes with more than 1% of the total population are shown individually, therefore, Classes with less than 1% of the total population were grouped as "other". It was observed that the five most abundant Classes in both crops under O and NO treatments were *Bacilli*, *Alphaproteobacteria*, *Planctomycetia*, *Deltaproteobacteria*, and *Holophagae*.

To evaluate the effect of air injection and the possible effects of the antibiotics detected in the irrigation water, the results from Irrigation 3 were compared to the results obtained from Irrigation 7 for each crop. In sugar beets, a significant increase in the abundance of *Planctomycetia* (Irrigation 3: 979 vs Irrigation 7: 1141, $p = 0.007$) and *Acidobacteriia* (Irrigation 3: 233 vs Irrigation 7: 302, $p = 0.01$) was observed in NO treatment while a significant increase in the abundance of *Alphaproteobacteria* (Irrigation 3: 826 vs Irrigation 7: 1136, $p = 0.05$) was observed in O treatment. In corn, air injection significantly increased the abundance of *Acidimicrobiia* (Irrigation 3: 119 vs Irrigation 7: 173, $p = 0.04$) but it resulted in the decrease of the abundance of *Chitinophagia* (Irrigation 3: 303 vs Irrigation 7: 239, $p = 0.02$).

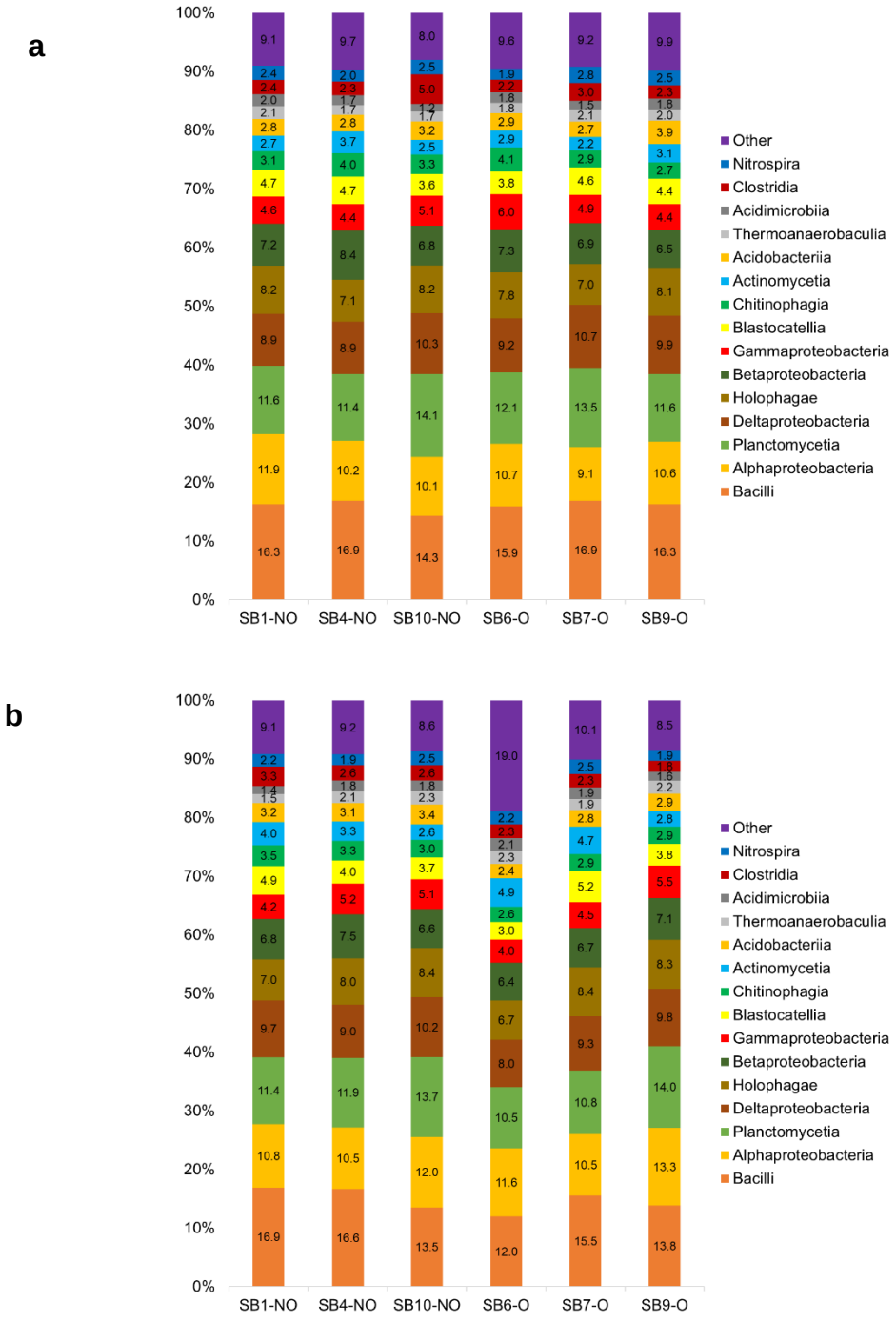


**Figure 2.13** Class level relative abundance of bacterial communities in samples collected in sugar beets field during Irrigation 3 (a) and Irrigation 7 (b). NO means non-air injection treatment and O means air injection treatment.

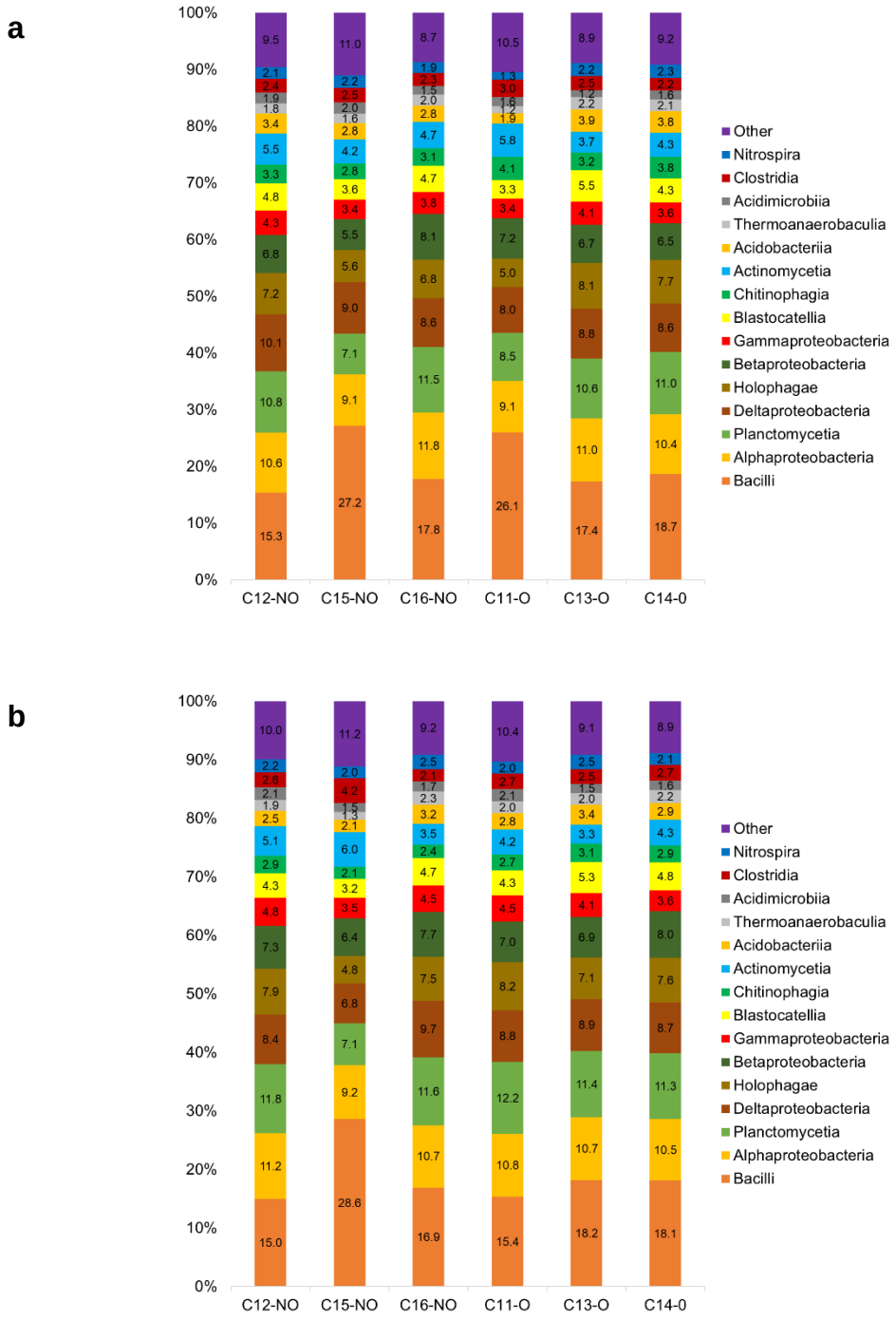


**Figure 2.14** Class level relative abundance of bacterial communities in samples collected in corn field during Irrigation 3 (a) and Irrigation 7 (b). NO means non-air injection treatment and O means air injection treatment.

## 2.4 Conclusion

The first objective of this chapter was to evaluate the effects of air injection into a subsurface drip irrigation system on the reduction of antibiotic concentrations in the soil environment introduced by irrigation with feedlot wastewater. Results indicated the presence of sulfamethoxazole and monensin Na in soil pore water collected from sugar beets and corn fields. In summary, it was observed that the impact of air injection on the concentrations of sulfamethoxazole was not significant in sugar beets ($p = 0.33$) and corn ($p = 0.25$) throughout the growing season. Moreover, the impact of air injection on the concentrations of monensin Na was not significant in sugar beets ($p = 0.18$) and corn ($p = 0.84$) throughout the growing season. However, conclusions cannot be made based on these results since soil pore water samples were used to obtain a partial picture of the overall environmental interactions.

The second objective of this chapter was to evaluate the response of microbial communities to air injection. The results from the diversity assay 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) Long Read indicated that the abundance of *Alphaproteobacteria* ($p = 0.05$) in sugar beets and *Acidimicrobiia* ($p = 0.04$) in corn, increased in time under O treatment. However, it also resulted in the decrease in the and *Chitinophagia* (Irrigation 3: 910 vs Irrigation 7: 795, $p = 0.03$) in corn. Moreover, a significant increase in the abundance of *Planctomycetia* ($p = 0.007$) and *Acidobacteriia* ($p = 0.01$) was observed in NO treatment.

## 2.5 References

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## 2.6 Appendix

In this section, methods for the analysis of pharmaceuticals in soil and plant matrices are presented. Due to COVID-19, we were delayed significantly on plant tissue and soil sample analysis since labs were shut down. Yet plant tissue and soil samples are being prepared at the Irrigation and Digital Ag Lab and will be analyzed following the termination of this project. Results will be shared with NET and public once they are available.

### Analysis of pharmaceuticals in soil matrices

Reagents used in soil samples include analytical grade chemicals purchased at Fisher Scientific (Waltham, MA, USA) such as methanol, acetonitrile and disodium hydrogen phosphate; sand white quartz and citric acid monohydrate were purchased at Acros Organics (Geel, Belgium) and ethylenediaminetetraacetic acid (EDTA) disodium salt was purchased at Sigma-Aldrich (St. Louis, MO, USA). Targeted analytes and buffer solution are the same that were used for water matrices.

EPA 1694 method (EPA, 2007) was used to determine the occurrence of antibiotics in soil samples. As described below, a method detection limit (MDL) for the EPA 1694 G1 procedure for solid matrices was conducted at the Water Sciences Laboratory, University of Nebraska-Lincoln, Lincoln, Nebraska, USA. For this method, labeled 50 mL centrifuge tubes were used to weight out 2 gram of sand white quartz. Similarly, the Laboratory Reagent Blank (LRB) was prepared using 2 grams of sand white quartz. All MDL samples were spiked with 100 µL of 0.02 ng/ µL Analyte Spike for a total of 2 ng. LRB and MDL samples were then spiked with 100 µL of 0.1 ng/µL Surrogate Spike. Then, 20 mL of

acetonitrile and 15 mL of McIlvain-EDTA buffer solution were added to all centrifuge tubes and mixed using a wrist action shaker (Burrell Scientific LLC, Zelienople, PA, USA) for 30 minutes. These samples were then centrifuged using an Allegra 6R Centrifuge (Beckman Coulter, Inc., Brea, CA, USA) for 10 minutes at 2000 rpm.

The resultant supernatant was decanted into labelled RapidVap tubes (Labconco Corporation, Kansas City, MO, USA). To avoid any loss of samples, 20 mL centrifuge tubes and 15 mL of McIlvain-EDTA were added to the previously used centrifuge tubes, shaken using a wrist action shaker for 30 minutes and combined with the supernatant previously decanted into labelled RapidVap tubes. These samples were evaporated to 20 mL using a RapidVap $N_2$ evaporation system (Labconco Corporation, Kansas City, MO, USA). DDI water was added to each RapidVap tube up to the 50 mL mark and were extracted onto OASIS HLB 6cc (200 mg) solid phase extraction cartridges (Waters Corporation, Milford, MA, USA), preconditioned with 6 mL of acetonitrile (ACN) and 6 mL of distilled de-ionized water (DDI), by aspirating the samples at no more than 1 mL/min.

SPE cartridges were slowly eluted in labeled glass test tubes with 10 mL of acetonitrile at 1 mL/min using a Visiprep™ SPE Vacuum Manifold (Sigma-Aldrich Co. LLC, Darmstadt, Germany). Although acetonitrile was used in this method, methanol is also used to elute OASIS HLB 200mg/6cc solid phase extraction cartridges. A Visidry™ Drying Attachement with Visiprep 12-port model (Sigma-Aldrich Co. LLC, Darmstadt, Germany) was used to evaporate these samples under nitrogen at 35°C to 500 µL. Then, all samples were spiked with 100 µL of 0.1 ng/µL Internal Standard. Samples were evaporated for a second time under nitrogen at 35°C to 40 µL.

As final step, 160 µL of DDI water was added to each glass test tube and each sample was transferred to labeled glass insert using disposable pipettes. These samples were analyzed by the WSL personnel using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) by Xevo LC/MS with UniSpray source (Waters Corporation, Milford, MA, USA).

**EPA 1694 G1 procedure for soil matrices**

At the Irrigation and Digital Ag Lab, soil samples were processed following the EPA 1694 G1 procedure for solid matrices (EPA, 2007). Labeled clean 50 mL centrifuge tubes were used to weight out 1 gram of soil sample. A Laboratory Fortified Matrix (LFM), Laboratory Fortified Blank (LFB), and Laboratory Reagent Blank (LRB) samples were prepared. To prepare the LFM, 2 grams of any soil sample collected in field were weighted out in a labeled 50 mL centrifuge tube. To prepare the LFB and LRB samples, 2 grams of sand white quartz were weighted out in labeled 50 mL centrifuge tubes. All samples were then spiked with 100 µL of 0.1 ng/µL Surrogate Spike. LFM and LFB samples were additionally spiked with 100 µL of 0.1 ng/µL Analyte Spike. Then, 20 mL of acetonitrile and 15 mL of McIlvain-EDTA buffer solution were added to all centrifuge tubes and mixed using a wrist action shaker (Burrell Scientific LLC, Zelienople, PA, USA) for 30 minutes. These samples were then centrifuged using an Allegra 6R Centrifuge (Beckman Coulter, Inc., Brea, CA, USA) for 10 minutes at 2000 rpm. The resultant supernatant was decanted into labelled RapidVap tubes (Labconco Corporation, Kansas City, MO, USA). Again, 20 mL centrifuge tubes and 15 mL of McIlvain-EDTA were added to the previously used centrifuge tubes, shaken using a wrist action shaker for 30 minutes and combined with the supernatant previously decanted into labelled RapidVap tubes (Labconco Corporation,

Kansas City, MO, USA). These samples were evaporated to 20 mL using a RapidVap $N_2$ evaporation system (Labconco Corporation, Kansas City, MO, USA), then DDI water was added to each RapidVap tube up to the 50 mL mark. These samples were extracted onto OASIS HLB 6cc (200 mg) solid phase extraction cartridges (Waters Corporation, Milford, MA, USA), preconditioned with 6 mL of acetonitrile (ACN) and 6 mL of distilled de-ionized water (DDI), by aspirating the samples at no more than 1 mL/min.

At the Arid Land Agricultural Research Center, SPE cartridges were slowly eluted in labeled glass test tubes with 3 mL of methanol at 1 mL/min, and 3 mL of acetonitrile in case some compounds elute more complete with a less polar solvent. A CentriVap Centrifugal Concentrator (Labconco Corporation, Kansas City, MO, USA) was used to evaporate these samples at 35°C to 40 µL. Then, all samples were reconstituted using 160 µL of 15% acetonitrile solution containing 100 µL of 0.1 ng/µL Internal Standard. As the final step, each sample was transferred to a labeled glass insert using disposable pipettes and vortexed. These samples were analyzed by the USDA-ALARC personnel using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS).

**Analysis of pharmaceuticals in plant matrices**

Reagents used in plant samples include analytical grade chemicals purchased at Fisher Scientific (Waltham, MA, USA) such as methanol, acetonitrile, methyl tertiary-butyl ether (MTBE) and sodium citrate dihydrate; citric acid monohydrate and cellulose microcrystalline were purchased at Acros Organics (Geel, Belgium); SupelQuE Citrate Extraction Tubes (4 g $MgSO_4$, 1 g NaCl, 0.5 g NaCitrate dibasic sesquihydrate, 1 g

NaCitrate tribasic dihydrate) and Supel QuE PSA Tube (900 mg $MgSO_4$ + 150 mg $C_{18}$) were purchased at Sigma-Aldrich (St. Louis, MO, USA).

Like water and soil matrices, targeted analytes for this experiment are included in the Group 1 of the EPA 1694 method, so their extraction is under acidic conditions. A citrate buffer containing sodium citrate dihydrate and citric acid was used to keep the pH of the plant matrices around 3.0. To prepare the citrate buffer, 25.703 grams of sodium citrate dihydrate and 2.421 grams of citric acid were added to 800 mL of distilled de-ionized (DDI) water. Then, the pH was adjusted to 3.0 using hydrochloric acid or sodium hydroxide solution. This solution was transferred to a 1 L flask and filled up with DDI water up to the mark. For this experiment, the three spikes used for water samples were also used for plant matrices.

An optimized antibiotic extraction method developed by Chen et al. (2020), which determines the occurrence of 28 antibiotics in plant tissues, was used as reference for a method proposed for this experiment. In this method, Chen et al. (2020) freeze-dried, homogenized, and kept the plant tissue samples at -20°C in the dark until extraction. For the extraction, 0.2 grams of plant tissue samples were weighted out and placed into labeled 50 mL centrifuge tubes. Then, 50 µL of Internal Standard (2 mg/L each) and 100 µL of the Antibiotic Standard (1 mg/L each) were added to each centrifuge tube and vortexed with the plant tissue sample for 30 seconds using a vortex mixer. It was pointed out the importance of the Internal Standard and Antibiotic Standard contact with the plant tissue samples, so these centrifuge tubes were placed in the dark within a refrigerator at 4°C overnight. Then, 5 mL of citric acid buffer (pH 3) were added to each centrifuge tube and vortexed for 1 minute using a vortex mixer. Additionally, 10 mL of acetonitrile with 1.0%

of acetic acid and the content of a QuEChERS extraction salt packet (4 g of anhydrous MgSO4, 1 g of sodium chloride, 1 g of citrate acid and 1 g of sodium hydrogen citrate) were added to each centrifuge tube and vortexed for 1 minute. These matrices were centrifuged at 3500 rpm for 5 minutes, and 7 mL of the resultant supernatant was transferred from each centrifuge tube into labeled 10 mL centrifuge tube containing dispersive solid-phase extraction sorbents. In the method, it was observed the highest recovery rate using the dispersive solid-phase extraction sorbent containing 900 mg MgSO4 + 150 mg $C_{18}$. These tubes with sorbents went through a vortex shaker for 30 seconds and centrifuged for 5 minutes at 3500 rpm. Then, 5 mL of the resultant supernatant was transferred into labeled glass tubes and evaporated to near dryness under a gentle stream of nitrogen. As final step, and before the instrument analysis, these samples were redissolved in 1 mL of methanol and filtered through a 0.22 µm nylon filter.

**Proposed method 1: QuEChERS pretreatment with LC/MS/MS detection**

Method detection limit

For our experiment, the method developed by Chen et al. (2020) was modified to reflect the current practices of the Water Sciences Laboratory, University of Nebraska-Lincoln. Therefore, a method detection limit (MDL) was conducted. For the MDL procedure, labeled 50 mL centrifuge tubes were used to weight out 0.2 gram of cellulose. Similarly, the Laboratory Reagent Blank (LRB) was prepared using 0.2 grams of cellulose. All MDL samples were spiked with 100 µL of 0.02 ng/ µL Analyte Spike for a total of 2 ng. LRB and MDL samples were then spiked with 100 µL of 0.1 ng/µL Surrogate Spike. Then, 5 mL of citric acid buffer (pH 3) were added to each centrifuge tube and vortexed for 1 minute using a vortex mixer. Additionally, 10 mL of acetonitrile with 1.0% of acetic

acid and the content of a SupelQuE Citrate Extraction Tube (4 g $MgSO_4$, 1 g NaCl, 0.5 g NaCitrate dibasic sesquihydrate, 1 g NaCitrate tribasic dihydrate) were added to each centrifuge tube and vortexed for 1 minute. These matrices were centrifuged at 3500 rpm for 5 minutes, and the resultant supernatant was transferred from each centrifuge tube into labeled 10 mL centrifuge tube containing Supel QuE PSA Tube (900 mg $MgSO_4$ + 150 mg $C_{18}$). These tubes with sorbents went through a vortex shaker for 30 seconds and centrifuged for 5 minutes at 3500 rpm. Then, the resultant supernatant was transferred into labeled glass tubes and evaporated at 35°C to 1 mL under a gentle stream of nitrogen using a Visidry™ Drying Attachement with Visiprep 12-port model (Sigma-Aldrich Co. LLC, Darmstadt, Germany). These samples were filtered using GMF filters into clean, labeled glass tubes; the old test tubes were rinsed with 1 mL of acetonitrile and filtered for a second time. Additionally, filters were rinsed with a small amount of acetonitrile. Samples were evaporated for a second time under nitrogen at 35°C to 500 µL. Then, all samples were spiked with 100 µL of 0.1 ng/µL Internal Standard and evaporated for a third time under nitrogen at 35°C to 40 µL. As final step, 160 µL of DDI water was added to each glass test tube and each sample was transferred to labeled glass insert using disposable pipettes. These samples were analyzed by the WSL personnel using liquid chromatography combined with tandem mass spectrometry (LC/MS/MS) by Xevo LC/MS with UniSpray source (Waters Corporation, Milford, MA, USA).

Reduction of data obtained from the Liquid Chromatography-Tandem Mass analysis was necessary prior their validation and delivery of final reports from the WSL personnel. Data reduction was conducted at the WSL using the MassLynx v4.2 software (Waters Corporation, Milford, MA, USA).

QuEChERS pretreatment with LC/MS/MS detection (modified)

This method was sketched following the EPA 1694 G1 procedure for solid matrices (EPA, 2007). Plant tissue samples should be freeze-dried, homogenized, and kept at -20°C in the dark until extraction. For the extraction process, weigh out 0.2 grams of plant tissue sample into a labeled 50 mL centrifuge tube, spike all samples (including LFM, LFB and LRB samples) with 100 µL of 0.1 ng/µL Surrogate Spike for a total of 10 ng, and spike LFM and LFB with 100 µL of 0.1 ng/µL Analyte Spike for a total of 10 ng. Then, add 5 mL of citric acid buffer (pH 3) into the centrifuge tube and vortex the samples in the vortex mixer for 1 minute. Additionally, add 10 mL of acetonitrile with 1.0% acetic acid and the contents of a Supel QuE Citrate Extraction Tube (4 g $MgSO_4$, 1 g NaCl, 0.5 g NaCitrate dibasic sesquihydrate, 1 g NaCitrate tribasic dihydrate) and vortex for 1 minute on the vortex mixer. Centrifuge these samples at 3500 rpm for 5 minutes, transfer the resultant supernatant from each tube into a labeled 50 mL centrifuge tube containing the contents of a Supel QuE PSA Tube (900 mg $MgSO_4$ + 150 mg $C_{18}$) and vortex-shake tubes with sorbents for 30 seconds. Centrifuge these samples for 5 minutes at 3500 rpm and transfer the entirety of the supernatant from each centrifuge tube to a labeled glass tube, then evaporate to 1 mL under a gentle stream of nitrogen. Filter each sample into a new labeled glass tube using a GMF filter, rinse the old test tube with a small amount of acetonitrile and filter as well. In addition, rinse the filter with a small amount of acetonitrile. Evaporate these samples for a second time under nitrogen to 500 µL, then spike each concentrated extract with 100 µL of 0.1 ng/µL Internal Standard Spike and continue evaporating to 40 µL. Finally, add 160 µL of DDI water to each glass tube, vortex for 30 seconds and transfer to a glass insert.

**Proposed method 2: Optimized protocol coupling ultrasonic extraction, SPE cleanup and LC/MS/MS detection**

Method detection limit

An optimized antibiotic extraction method developed by Wu et al. (2012), which determines the occurrence of 19 pharmaceutical and personal care products in vegetables, was used as reference for an alternative method proposed for this experiment. As described by Wu et al. (2012), vegetables were cut into small pieces and freeze-dried for 36 hours. Then, these samples were ground to powder and stored at -20°C until extraction. For extraction, 0.2 grams of the grounded sample was weighted out into a centrifuge tube, spiked with deuterated standards, and extracted in a sonication bath for 20 minutes with 20 mL of methyl tert-butyl ether (MTBE) and 20 mL of acetonitrile. These samples were centrifuged at 1000 *x g* for 20 minutes. These matrices were evaporated to near dryness under nitrogen at 40°C, recovered with 1 mL of methanol, and diluted with 20 mL ultrapure water. Then, these matrices were extracted onto OASIS HLB 6cc (150 mg) solid phase extraction cartridges (Waters Corporation, Milford, MA, USA), preconditioned with 6 mL of methanol and 6 mL of ultrapure water, and dried under nitrogen. SPE cartridges were slowly eluted with 7 mL of methanol, and the extract was evaporated to near dryness under nitrogen at 40°C. As final step, and before the instrument analysis, these samples were recovered in 0.5 mL of methanol and filtered through a syringe PTFE filter.

A method detection limit (MDL) using eight samples (MDL samples) and one method blank (Laboratory Reagent Blank) is detailed as follows. For the MDL samples and the Laboratory Reagent Blank (LRB), weight out 0.2 grams of cellulose into labeled 50 mL centrifuge tubes. Spike the MDL samples with 100 µL of 0.02 ng/ µL Analyte Spike

for a total of 2 ng and spike all samples (including the LRB) with 100 µL of 0.1 ng/µL Surrogate Spike. As solvents, add 20 mL of MTBE and 20 mL of acetonitrile to each centrifuge tube, vortex for 1 minute in a vortex mixer, and extract by sonication in an ultrasonic bath for 20 minutes. Centrifuge these matrices at 3000 rpm for 20 minutes. Then, transfer the resultant supernatant from each centrifuge tube into labeled glass tubes. To avoid any loss of materials, add 5 mL of MTBE and 5 mL of acetonitrile to each centrifuge tube, vortex for 1 minute and transfer the resultant supernatant into the labeled glass tubes previously used. Evaporate these matrices to near dryness under nitrogen at 40°C, recover with 1 mL of methanol, and dilute with 20 mL ultrapure water. Then, extract these matrices onto OASIS HLB 6cc (150 mg) solid phase extraction cartridges, preconditioned with 6 mL of methanol and 6 mL of distilled de-ionized water (DDI) by aspirating the cartridge at no more than 5 mL/min. For the elution of SPE cartridges, slowly elute with 7 mL of methanol, and evaporate the extract to near dryness under nitrogen at 40°C. As final step, add 100 µL of 0.1 ng/µL Internal Standard Spike to all glass tubes, add 0.5 mL of methanol to recover the sample, and transfer the solution to labeled glass inserts.

Optimized protocol coupling ultrasonic extraction, SPE cleanup and LC/MS/MS detection (modified).

For the analysis of pharmaceutical and personal care products in vegetables and plant tissues, it is proposed the following alternative method. Cut the vegetables or plant tissue samples in small pieces and place them in a freeze-drier for 16 hours using individual labeled trays. To normalize the concentration of antibiotics to dry weights, record weights of samples before and after the freeze-drying process. Finely ground each sample in a stainless steel coffee grinder, and clean the grinders with soap, water, and acetone after each use. These samples can be stored at -20°C until extraction

For the extraction process, weigh out 0.2 grams of plant tissue sample into a labeled 50 mL centrifuge tube, spike all samples (including LFM, LFB and LRB samples) with 100 µL of 0.1 ng/µL Surrogate Spike for a total of 10 ng, and spike LFM and LFB with 100 µL of 0.1 ng/µL Analyte Spike for a total of 10 ng. Add 20 mL of MTBE and 20 mL of acetonitrile to each centrifuge tube, vortex for 1 minute in a vortex mixer, and extract by sonication in an ultrasonic bath for 20 minutes. Centrifuge these matrices at 3000 rpm for 20 minutes. Then, transfer the resultant supernatant from each centrifuge tube into labeled glass tubes. To avoid any loss of materials, add 5 mL of MTBE and 5 mL of acetonitrile to each centrifuge tube, vortex for 1 minute and transfer the resultant supernatant into the labeled glass tubes previously used. Evaporate these matrices to near dryness under nitrogen at 40°C, recover with 1 mL of methanol, and dilute with 20 mL ultrapure water. Then, extract these matrices onto OASIS HLB 6cc (150 mg) solid phase extraction cartridges, preconditioned with 6 mL of methanol and 6 mL of distilled de-ionized water (DDI) by aspirating the cartridge at no more than 5 mL/min. For the elution of SPE cartridges, slowly elute with 7 mL of methanol, and evaporate the extract to near dryness under nitrogen at 40°C. As final step, add 100 µL of 0.1 ng/µL Internal Standard Spike to all glass tubes, add 0.5 mL of methanol to recover the sample, and transfer the solution to labeled glass inserts.