HOW SALINITY INFLUENCES SOIL ORGANISMS: EARTHWORMS, ARCHAEA, BACTERIA AND FUNGI

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Cecelia Castleberry

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

MASTER OF SCIENCE

SUPERVISORY COMMITTEE:

Dr. Caley Gasch
Chair

Dr. Samiran Banerjee
Co-Chair

Dr. Brian Darby

Dr. Jason Harmon

Approved:

07/06/2023
Date

Dr. Thomas DeSutter
Department Chair

ABSTRACT

Soil salinity is a naturally-occurring issue in the Great Plains. Current standards for saline soil designation are based on plant tolerance levels. This thesis expands salinity knowledge into salinity responses of other soil organisms. I used laboratory mesocosms to examine survival and fecundity or cocoon production of earthworms in the *Apporectodea* complex to increasing levels of salinity, with or without supplemental organic matter (OM). I then used a split-bin design to examine earthworm choice between combinations of saline and non-saline soils, with and without supplemental OM. I found that earthworms avoided saline soils, but survival and production was steady across salinity levels and in some cases decreased with added OM. I then quantified abundance and diversity of microbial groups in field-collected saline and non-saline soils in increments to 120 cm depth. The response of microbes to depth were stronger than their responses to salinity. Both important groups of soil organisms appear tolerant to salinity levels.

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DEDICATION

To my friend Robert Gianduzzo, thank you for listening to me prattle about earthworms.

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LIST OF ABBREVIATIONS

A.....Aneta

E .....Eldridge

EC .....Electrical Conductivity

Log .....Logarithmic

ND.....North Dakota

NS .....Non-saline soil

NS+ .....Non-saline soil with added organic matter

NS- .....Non-saline soil without added organic matter

M .....Midway

N.....Northwood

OM .....Organic Matter

S+ .....Saline soil with added organic matter

S- .....Saline soil without added organic matter

SS .....Saline soil

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1. GENERAL INTRODUCTION

1.1. Soil as an ecosystem

The soil ecosystem, like many other ecosystems, is shaped by several different abiotic and biotic factors. Abiotic features like hydrology, soil chemistry, texture, and aggregation work in tandem with microorganisms like archaea, bacteria, and fungi (Bisen, 2012; Bruslind, 2020; Yates, 2016); and macro-organisms like earthworms (Blouin, et al., 2013 Bottinelli et al., 2010; Edwards et al., 1996; Shutenko et al., 2022; Zexuan et al., 2019) to create varying soil ecosystems all over the world (Paul, 2015). Together these factors can promote or inhibit the soil's ability to promote growth and sustain life, which is why so many scientists work to expand upon this complex ecosystem.

The soil beneath our feet does more for us than many may think about on a daily basis. Not only does it provide a steady foundation for architecture, but it also plays a role in the nutrients we consume (Butcher, 2016; Hadrich, 2012; Zorb et al., 2019). Everything we eat can be tied to soil, from vegetables to meat. Soil even effects the air we breathe through carbon sequestration and other nutrient and gas fluxes (Paul, 2015). Everything relies heavily on soil ecosystems every day, which is why it is vital to combat rising soil threats like salinity.

1.2. Salinity and its management

Salinity is a term often used when discussing soil health, but what exactly is salinity, and how does it apply to soil ecosystems? When discussing soil salinity, we must first discuss the difference between soil salinity and sodicity, as both terms are applied when discussing salty soils (Seelig, 2000; Shahid, 2018). Saline and sodic soils are often treated like the same thing when it comes to management solutions, both can contain salts, but the salts differ in chemical composition. Saline soils differ in that there can be sodium salts like sodium sulfates (Na_2SO_4)

and sodium chloride (NaCl), or other salts like calcium carbonate ($CaCO_3$) and calcium sulfate ($CaSO_4$) (Gasch et al., 2021; Seelig, 2000). Sodic soils are characterized as being high in sodium (Seelig, 2000).

The U.S. Salinity Laboratory created a system in 1954 to classify saline and sodic soils based on electrical conductivity (EC) and sodium absorption ratio (SAR) (U.S. Salinity Laboratory Staff, 1954). Saline soil can have an EC greater than 4 dS/m and a SAR less than 13, whereas sodic soils are the opposite, having an EC less than 4 dS/m but a SAR greater than 13 (Seelig 2000; U.S. Salinity Laboratory Staff, 1954). Understanding the difference between salinity and sodicity is essential because both present different issues for soil health. In our study we are characterizing the soil based on salinity, this is because our salt composition is not primarily of sodium salts but rather a mix of other salts like sulfate salts.

There are many sources of salinity, both natural and man-made (Gasch et al., 2021; Keller et al.,1986; El-Ashry, 1985; Munns, 2002; Seelig, 2000; Shahid, 2018; Zexuan et al., 2019). Naturally, saline soil can occur due to coastal and brackish water intrusion (Shahid, 2018), weathering of parent material (Gasch et al., 2021; Keller et al., 1986; Seelig 2000; Regasamy, 2010), rising water tables and drainage (Seelig, 2000; Shahid, 2018), and surface and plant transpiration (Shahid, 2018). Human-made salinity is mainly caused by poor agricultural irrigation; an issue felt from Australia to the United States (El-Ashry, 1985; Munns, 2002; Shahid, 2018; Regasamy, 2010). Commercial agriculture has exacerbated this issue, but farmers have faced it for thousands of years, even in ancient Mesopotamia (El-Ashry, 1985). Several other agricultural causes, like the overuse of fertilizers and certain soil amendments, can lead to salty soils (Shahid, 2018). Other human-made salinity causes can include general pollution like kitchen waste, as seen in countries with exponential human population growth like China

(Zexuan et al., 2019). In the Midwest, particularly, farming practices have exacerbated salinity issues. Increased evaporation from tilling practices has increased salinity along with the use of monocrops (Hadrich, 2012; Ries et al., 2020; Seelig, 2000).

With so many different causes behind salinity, it is no surprise that many countries worldwide experience economic issues due to saline soil. Globally it is estimated that over 800 million hectares of land are affected by salts (Regasamy, 2010). For example, Europe contains only 3.3% of the world's saline soils, but it still contains areas that are limited agriculturally by salinity (Daliakopoulos et al., 2016). Australia is considerably more afflicted by saline soil, as a third of the continent's land-area is affected by salinity (Munns, 2002).

Salinity is restricting the availability of land for agriculture and has become a global food security threat by causing osmotic stress to crops (Butcher, 2016). Salinity is especially problematic because world agricultural demand will likely continue to increase (USDA, 2016). While there are more salt-tolerant crops, 50% of all arable land is predicted to eventually be saline by 2050 (Wang et al., 2003). Roughly 27.3 billion dollars worldwide is being lost due to decreased crop production concerning saline soil land degradation; one can only imagine how this cost will increase as the global salinization of soil grows (Qadir, 2014; Wang et al., 2003).

The importance of agriculture has heavily influenced how 3haraoniy is defined. Many studies focus on plant responses to increasing salinity (Zorb et al., 2019). While it is essential to examine this relationship, it has created soil standards that may not apply to many non-plant organisms. Saline soil is characterized as having an EC greater than 4 dS/m (Seelig 2000; U.S. Salinity Laboratory Staff, 1954). Corn (*Zea mays*), for example, can experience decreasing yields once EC reaches 1.7 dS/m (Butcher, 2016). Many organisms call soil ecosystems home, and they all work to create an environment where life can thrive (Paul, 2015).

We must understand how salinity affects various organisms, from plants to microscopic organisms. Beyond that, the methods for analyzing how some organisms are affected could be more specific and standardized. A salinity level that would negatively impact plants may have no effect on other soil-dwelling groups. Standardizing salinity can be detrimental in protecting other organisms that may be imperative in remediating and managing the growing threats posed by salinity.

While 4 dS/m is relatively high for most crops, many soil organisms react differently to this salinity level, with some even being able to withstand higher salinity levels. For example, in one study, earthworms survived in soils containing an EC of 6 dS/cm (Gasch et al., 2021). Nematodes (phylum *Nematoda*) are a very numerous and diverse group of multicellular organisms that regulate soil decomposition. A few sources have demonstrated that nematodes have a weaker tolerance up to 1.2 dS/m depending on the salt type, so an EC of 4dS/m would be far too saline (Nkem, 2006; Paul, 2015).

Even single-celled organisms have different responses to varying salinity levels. Some microbes can thrive in very saline environments like Halotolerant *Alcanivorax* sp. Strain (Dastgheib, 2011) or archaea like *Halorhabdus utahensis*, *Natronomonas 4haraonic*, *Haloferax sulfurifontis* and *Halobaculum gomorrense* (Dawson 2012). One halotolerant bacterium was found in NaCl solution of 5% weight by volume (w/M), which is so high that they have become a promising microbe for salinity remediation (Dastgheib, 2011). Meanwhile, the archaea mentioned could grow in soil solutions of 10-30% NaCl (w/M) (Dawson, 2012). The 4 dS/m standard also does not account for organisms that live in coastal regions that are saltier, and therefore are home to a plethora of microbes that can thrive in higher salinity concentrations (Cecchi, 2021; Chen, 2021; Chung, 2019). It is especially important to study the relationship

between single celled organisms and salinity because research has suggested that microbial diversity is more influenced by salinity than pH or temperature (Lozupone and Knight, 2007; Ruhl et al., 2018; Yang et al., 2016).

There are different management approaches for addressing excess salinity, but many depend on the cause and type of salinity experienced in the area. For example, regarding human-made pollution like kitchen waste, vermicomposting by earthworms can help decrease salinity (Zexuan et al., 2019). Microbes can also be utilized to metabolize salts that can be used to regulate or decrease salts in soils (Arora, 2019; Dastgheib, 2011; Flowers, 2015; Makzum, 2016; Shrivastava, 2015). One example is *Thioalkalivibrio versutus*, which has helped reduce sodium thiosulfate (Makzum, 2016).

Modified agricultural practices can reduce the effects of salinity on crops for farmers. One study found that using plastic mulching and convex plant beds could reduce salinity in soil caused by seawater intrusion (Haque, 2020). Using crops or plant species that are salt resistant or that metabolize salts can also help. For example, licorice, a shrub plant, helped remediate the soil and increase wheat yields (Kushiev et al., 2005). Adding chemical amendments like calcium chloride ($CaCl_2$) or gypsum ($CaSO_4$) can also help depending on the chemical structure of the salts found in the soil, although it is normally recommended that other amendment techniques be used first as some chemical amendments can make salinity worse or affect other soil characteristics (Seelig, 2000). Planting cover crops is also a commonly used tactic that can directly benefit microbes in the soil (Dasgupta, 2023). In cases of irrigation caused salinity, applying more water can help dissolve salts and using irrigation that is high quality (low salinity) can help leach the salts (Seelig, 2000).

While there are many ways to remediate saline soils, it is best to not have saline soils to begin with, so prevention should be prioritized. Regular soil monitoring, like sending in soil samples for analysis can show farmers how saline their soils are and help them understand how it may be occurring (Shahid, 2018; U.S. Salinity Laboratory Staff). When it comes to salinity caused by saline seep, reducing local groundwater recharge, designing ditches to move water can help manage and prevent saline soils (Seelig, 2000). Adding tile drainage and rotating crops has also shown to help manage and reduce salinity in fields (Hadrich, 2012).

1.3. Agriculture in North Dakota

North Dakota is a state that values agriculture, which makes sense as it contributes to a big part of the state's economy. In 2017, crops in North Dakota created over 6 billion dollars in market value of products sold. (Census of Agriculture, 2017). Agriculture is tied to thousands of jobs, and contributes food supplies to millions of Americans (Census of Agriculture, 2017). North Dakota is capable of growing potatoes, sugar beets, corn, wheat and many other crops, the relatively low population in North Dakota also means that there is a lot of land that can be used for agriculture as well, with the state population being less than 800,000, ranking the 48th most populated state out of 50 (U.S Census, 2020). Agriculture is such an important economic driver for the state it is no surprise that millions of dollars in funding have gone towards increasing yields or solving state-wide agricultural hurdles, like salinity (U.S. Salinity Lab, 2022; Ulmer, 2010).

As with other countries and states, salinity is a costly issue in North Dakota and the Great Plains region in general. In North Dakota, there are over 1.2 million acres classified as slightly saline and 275,000 acres are considered moderately saline (Ulmer, 2010). The extent of the saline soil has led to over 150 million dollars in lost crop revenue (Hadrich, 2012). Much of

North Dakota's soil has inherited salinity from the bedrock below which consists of sandstone and marine shales (Seelig, 2000) The discharge/recharge system weathers the salt parent material to bring salts to the surface and spread them throughout the soil (Gasch et al., 2021; Keller et al. 1986). As a result, saline soil here is unique compared to the salinity found in other soils around the world. Other soils have chloride-based salts but North Dakota's saline soils consist of sulfates (SO_4^{-2}), carbonates (CO_3^{-2}), and chloride (Cl^-) salts, sulfate salts being the most abundant (Gasch et al. 2021; Seelig, 2000).

While there has been a lot of research done on salinity in North Dakota, most research has been on the direct effect it has on plants, especially agricultural crops (Gasch et al., 2021; Seelig, 2000). This information is valuable, but many factors influence how plants thrive, including other organismal interactions in the soil. Understanding how salinity in North Dakota affects other organisms can help discover more management practices specific to our soils.

1.4. Earthworms

Earthworms are a staple of healthy soil. Many gardeners' guides praise the immense benefits that earthworms provide in promoting plant growth (Edwards et al., 1996; Hale, 2007). Earthworms are responsible for facilitating several different soil processes that alter and rejuvenate soil ecosystems. Earthworms can influence soil porosity, water management (Bottineli et al., 2010), nutrient cycling (Shutenko et al., 2022), climate regulation, pollution remediation (Zexuan et al., 2019), and primary production (Blouin, et al., 2013; Edwards et al., 1996; Paul, 2015).

In the Great Plains region, a plethora of earthworms have made soils their homes (Edwards et al., 1996; Hale 2007). These vital decomposers play a big role in agriculture. Earthworms can process nutrients into useable forms for crops via their waste, or vermicasts

(Faiza et al., 2016). Even their burrowing behavior can loosen up compacted soil to release trapped gases, weather minerals, or make room for roots (Bottineli et al., 2010; Carpenter et al., 2007; Paul, 2015). Promoting earthworms in agriculture can help farmers in maintaining fertile soil and profitable yield.

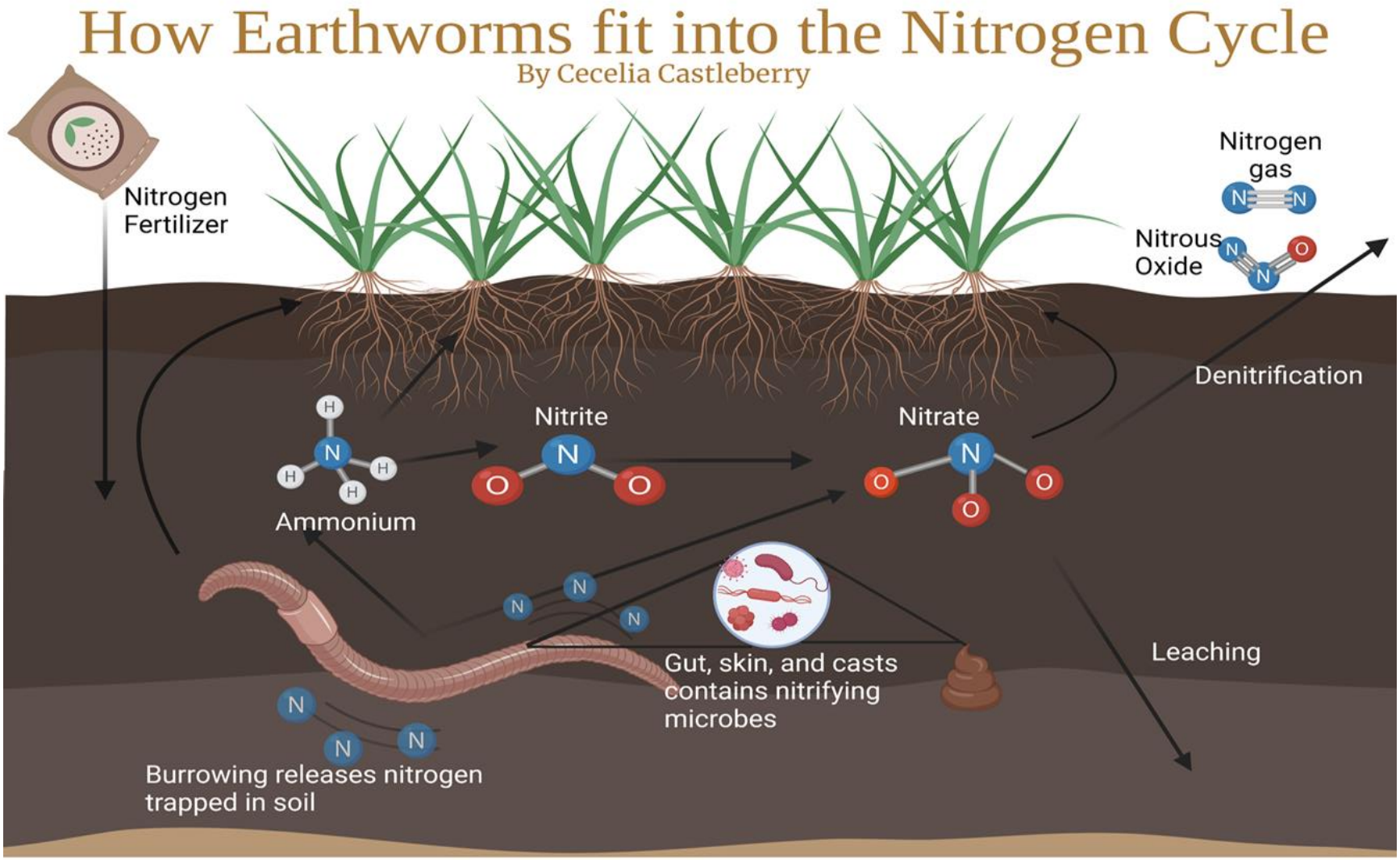

Figure 1. Earthworms and the nitrogen cycle.
Illustration of how earthworms fit in the nitrogen soil cycle, and their interactions with other organisms in converting nitrogen into a usable form. This demonstrates some of the important functions that earthworms play in the soil ecosystem.

Similarly, to our crops, earthworms are also influenced by salinity. High salinity levels can have harmful effects on earthworm health. It can limit their ability to perform vital soil processes, cause osmotic stress, and reduce the survivability of their offspring (Faiza et al., 2016; Gasch et al., 2021; Karimi et al., 2020; Wu et al., 2019). Earthworms are key players in keeping soil healthy, and they are being impacted by the rising issue of saline soil. Understanding how these decomposers react to saline soil can better help farmers better understand how their crops are being influenced.

1.5. Microbes

Microbes are the backbone of many environments and soils are no exception. Soils are home to microbes from all three domains, archaea, bacteria, and eukaryote (Bruslind, 2020; Woese et al., 1990). Archaea and bacteria are very similar single celled organisms. Eukaryotes can be both unicellular and multicellular and consist of macroscopic organisms like plants and animals, but all organisms from this domain share the common characteristic that they contain a membrane bound nucleus (Woese et al., 1990).

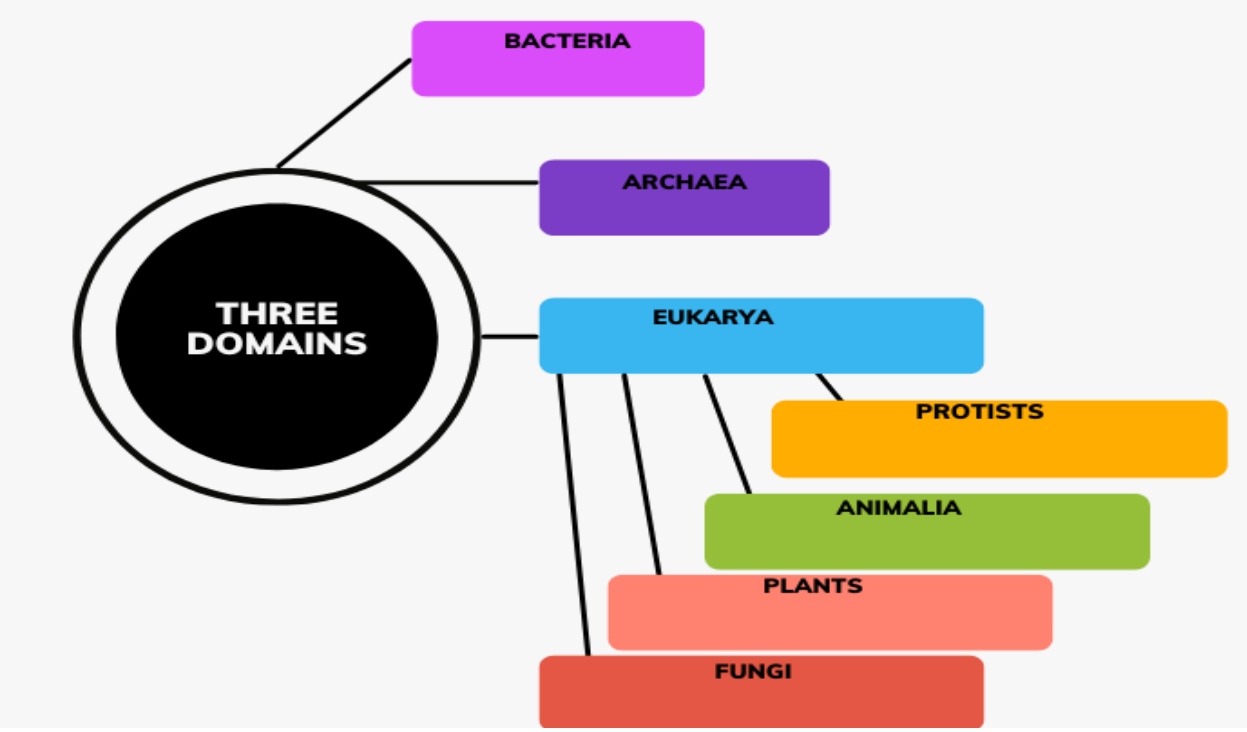

Figure 2. Illustration of the distinct three domains of life: Bacteria, Archaea, and Eukarya. Diagram showing the three domains along with groups that make up the Eukarya domain.

While all macroscopic life falls within the Eukarya, soil communities are dominated by microscopic members in all domains. The soil microbiome contains many microbial members. Many farmers are familiar with fungi from the eucarya domain, as many farmers every year must find new ways to combat yeast and other fungi that are harmful for crops (Somboon, 2017). There are also beneficial microbes living in the soil, many bacteria, archaea, and fungi contribute to maintaining a fertile and healthy soil (Paul, 2015).

Microbes are responsible for many different soils process either on their own or through forming beneficial relationships. Microbes living within larger organisms aid in digesting and processing nutrients (Boyrahmadi et al., 2018; Carpenter et al., 2007; Dasgupta, 2023; Drake et al., 2007; Guofan et al., 2022; Hartman et al., 2018; Horn et al., 2003; Ihssen et al., 2003; Paul, 2015; Yuejian et al., 2014). Fungi in the roots can aid plants in utilizing nutrients like carbon

(Mao, 2014), and bacteria in the guts of earthworms help them digest nutrients (Carpenter et al., 2007; Drake et al., 2007; Guofan et al., 2022; Horn et al., 2003; Ihssen et al., 2003). Microbes are also important for remediating soils that are saline (Dasgupta, 2023).

Microbes are everywhere in soil making them irreplaceable. Soils cannot sustain life without them, for this reason, it is crucial to understand how issues like salinity can affect microbes. It's also important to understand how different groups are influenced by salinity. Extremophiles like archaea may be more capable of adapting to high salinity, but they aren't responsible or capable of mediating all soil processes (Woese et al., 1990).

1.6. Research objectives

This thesis aims to examine the interactions and effects of salinity in the Northern Great Plains on microbes and earthworms using a combination of lab and field techniques. Two separate studies were performed to better understand how these organisms behave in North Dakota's specific soil conditions.

The first study examines the survivability and production and choice in saline soils of the *Apporectodea* earthworms in the presence or absence of additional OM. Experiments were designed in a laboratory setting with the following objectives in mind:

Objective 1: To assess earthworms' survivability and production at varying salinity levels in the absence and presence of organic matter.

Objective 2: To assess earthworm choice for saline versus non-saline soils when given free choice.

Objective 3: To assess earthworm choice for saline versus non-saline soil when supplemented with organic matter.

The second study examined the diversity and abundance of microbial communities at various salinity and depth levels, by analyzing field samples taken from four different fields in Eastern North Dakota. Microbial and soil analysis were performed with the following objectives in mind:

Objective 1: To compare the abundance of microbes found across saline and non-saline soil environments and across depths.

Objective 2: To identify how microbial communities (taxa and diversity) shift across saline and non-saline soil environments in increasing soil depths.

These studies can help paint a more detailed picture of how salinity influences organisms throughout the whole soil profile and will encourage more discussion and research concerning how other soil characteristics could be influencing other organisms besides crops as they play a crucial role in soil health.

Below is a conceptual model which visualizes what is known about salinity, questions we aim to answer, our approach, and a hypothesis in relation to plants, earthworms, and microbes. This model serves as a visualization of key themes and messages that will be discussed throughout this thesis.

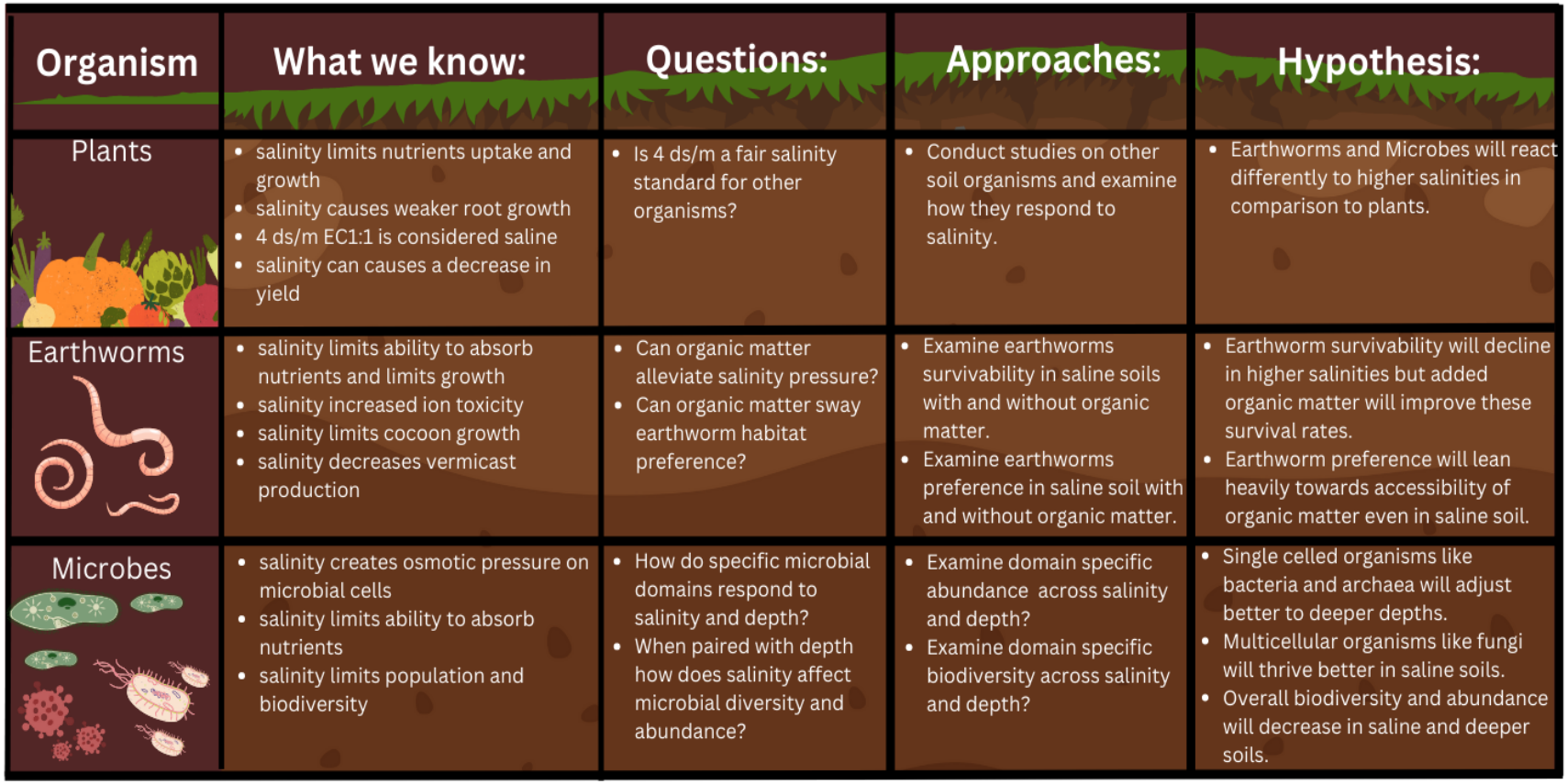

Figure 3. Thesis conceptual model.
Demonstrates what is known about salinity, questions we hope to answer, approaches, and general hypothesis in relation to plants, earthworms, and microbes. This model demonstrates many of the key messages and themes that will be discussed through this thesis.

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2. OBSERVING THE RESPONSE OF APPORECTODEA EARTHWORMS' GENUS TO SALINE SOILS AND ORGANIC MATTER ENHANCEMENTS

2.1. Introduction

Salinity is a major environmental issue in soils across the world from Europe to China to here in the Great Plains (Daliakopoulos, et al., 2016; Zexuan et al., 2019). Saline soils impact members of soil communities, including plants and important soil decomposers like earthworms (Boyrahmadi et al., 2018; Owojori et al., 2009; Zorb et al., 2019). Salts in soil typically cause low crop yields by reducing plant growth (Zorb et al., 2019). For other belowground organisms that perform vital soil processes, like the earthworm, salinity can reduce survivability (Owojori et al., 2009; Owojori et al., 2014). Due to negative effects of high salinity on living organisms, this issue has led to extensive economic loss, sometimes totaling millions of dollars in losses for farmers in the Red River Valley alone (Hadrich, 2012).

The saline soil in North Dakota is unique compared to the salinity found in other soils around the world. Typically, salts in soil are chloride-based, but North Dakota soils can have sulfate (SO_4^{-2}), carbonate (CO_3^{-2}), and chloride (Cl^-) ions, with sulfate salts as the most common (Gasch et al. 2021; Keller et al., 1986; Seelig, 2000). In some ecosystems, salinity is a human-made problem but in the Great Plains soil, salinity occurs naturally due to the parent material and hydrology. The discharge and recharge system in the soil brings salts to the surface of the soil (Keller et al. 1986).

Understanding the chemical composition of soil salts is important because it may influence the other characteristics of the soil like organism growth and activity, toxicity, and nutrient availability. Many studies have been done to examine how chloride salts in soil impact soil processes but there is limited knowledge about sulfate and carbonate salinity. Chloride salts

are toxic to organisms in high concentrations, but very little is known about the toxicity of other salts (Flowers et al., 2015). For example, there are several experiments analyzing how sodium chloride (NaCl) can increase the toxicity of heavy metals such as lead in soil ecosystems (Owojori et al., 2014; Raiesi et al., 2020; Stevens et al., 2003; Sujetoviene et al., 2019). Heavy metal pollution alone is very toxic for *Eisenia fetida* earthworms, but when combined with salts it can be more lethal (Raiesi et al., 2020; Sujetoviene et al., 2019). Salts can increase the solubility of heavy metals like lead and cadmium (Raiesi et al., 2020). Microorganisms and plants metabolize sulfate and carbonates, so their responses may be different to sulfate and carbonate heavy soils (Breazeale, 1923; Muyzer, 2008; Om et al., 2022). More research should be done by soil scientists, across different saline soil conditions to better understand the impacts of salts, especially sulfate and carbonate-based salts, on living organisms.

Agriculture is a vital part of the economy in the Great Plains area and the salinity has resulted in significant crop yield losses (Hadrich, 2012). As a result, the majority of previous research focus in North Dakota has been on studying the influence salinity has on crops (Munns 2002; Seelig 2000). What is less known is the impact the Great Plains' unique salinity has on other belowground organisms. In understanding the impact of salinity on the whole soil ecosystem, we may gain insight into how salinity changes soil function, belowground habitat suitability, and how we might adjust management practices in saline soils.

Many people understand that earthworms are essential to soil systems, and especially agricultural systems, but they might not know how these decomposers contribute to their environment. The presence of these crucial decomposers in soil can be seen as an indicator of soil health (Edwards et al., 1996; Hirano et al., 2011; Paul, 2015). Earthworms play a vital role in many different aspects from soil structure, water management (Bottineli et al., 2010), nutrient

cycling (Shutenko et al., 2022), climate regulation, pollution remediation (Zexuan et al., 2019), primary production, and cultural services (Blouin, et al., 2013; Edwards et al., 1996; Paul, 2015).

Most earthworms accomplish these tasks by contributing vermicasts (their fecal waste) to the environment. Vermicasts are composed of OM or environmental waste and mineral soil that is consumed and deposited by earthworms. These vermicasts are rich in nutrients that can be used by microorganisms and plants (Faiza et al., 2016). The earthworms' ability to form vermicasts has become an essential tool in restoring soil ecosystems; many reclamation projects have used earthworm vermicasts to restore soil nutrients by recycling industrial waste. One such project in China studied how *Eisenia fetida* earthworms could be used to recycle kitchen waste in effort to remediate the soil (Zexuan et al., 2019). This study demonstrates the invaluable services that earthworms can provide.

As part of the remedial benefits, earthworm activity also plays a role in structuring and increasing porosity in soil. Their burrows and castings create nutrient rich areas that support roots and microbial growth, this in turn allows mineralization to occur at a faster rate (Carpenter et al, 2008; Paul, 2015). The ability of the earthworms to aggregate and change the porosity of the soil also influences the flow of water through the soil (Bottineli, 2010; Hendrix, 1995). These actions from the earthworms have lasting effects not just on soil structure but on every aspect of the soil ecosystem.

Quite a few studies have focused on how salinity affects earthworms, and typically the response is negative (Faiza et al., 2016; Gasch et al., 2021; Karimi et al., 2020; Wu et al., 2019). Salinity has been shown to have both direct and indirect influences on earthworms. Like plants, salinity can influence osmotic processes in earthworms, but it can also damage the neurosecretory system (Faiza et al., 2016). One experiment noted that salinity reduced the

survivability of the *Eisenia fetida* earthworm species in the presence of other environmental toxins such as zinc and copper (Karimi et al., 2020). Other studies have found that salinity leads to decreased juvenile earthworms (Gasch et al., 2021). High salinity levels in soil can even halt reclamation projects that utilize earthworms (Wu et al., 2019).

In North Dakota, there are many different species of earthworms (Hale 2007; Schwert, 1991). While many studies on salinity have used *Eisenia fetida* species as a representative study species, this species is not found naturally in the Great Plains area. However, the *Apporectodea* genus is commonly found in the state, although it is not native to North Dakota (Schwert, 1991). It is essential to distinguish between the two genera as different species of earthworms have physiological differences and feeding choices, and they occupy different habitats in the soil profile (Curry et al., 2007). For example, *Eisenia fetida*, prefer manure whereas *Apporectodea* prefer plant based OM like peat. (Reynolds et al., 1977). At the same time, *Eisenia* and *Apporectodea* live in different parts of the soil, the *Eisenia* lives in the litter layer whereas the *Apporectodea* lives in the top six inches of mineral soil (Edwards et al., 1996). These niche differences can lead to the two genera interacting differently with different soil components and properties like OM and porosity.

The response of *Apporectodea* species to salinity has been researched in a field study conducted by North Dakota State University. The study aimed to examine earthworm abundance and growth stages in naturally occurring salinity gradients in a sulfate salt affected field soil. While adults and cocoon numbers were even across salinity gradients, juveniles steeply declined in plots that had an EC 1:1 of over 4 dS/m (Gasch et al., 2021). In the field study, soil OM decreased with salt concentration from about 7% in the non-saline soil plots to about 3% in the

saline plots. So, the earthworm decline may be a result of increasing salt concentration, reduced OM, or both.

Organic matter is the source of many soil nutrients such as organic carbon and nitrogen (Angst et al., 2017; Paul, 2016; van Vliet, 2007). Many organisms rely on OM for these nutrients. Earthworms rely on OM for nutrients but they also fragment the OM and recycle it into vermicasts, which are used by other organisms (Faiza et al., 2016). Adding OM to a saline environment can facilitate higher nitrification and carbon sequestration, which can provide much needed nutrients to facilitate growth for earthworms in saline soils (Angst et al., 2017; van Vlient et al., 2007). This alteration in chemical properties also alters the physical characteristics of soil such as porosity, aggregation, air flow, and water making it easier for soil organisms to thrive (Gasch et al., 2015; Paul, 2016; van Vlient et al., 2007)

While data is abundant on the relationship between *Eisenia fetida* and higher salinity levels caused by chloride salts, our study aims to evaluate salinity impacts specific to North Dakota's saline soil conditions using a species found in the region. Hopefully, the data presented in this study will build upon what was found in the NDSU field study and generate more interest in North Dakotan soils and reveal how the state's unique salts affect earthworm survival and production and choice. In looking at this problem we also hope to assess if OM can alleviate negative effects of salts on earthworms. In order to answer this overarching goal, we aim to pursue three objectives under controlled laboratory conditions:

Objective 1: To assess earthworms' survivability and production at varying salinity levels in the absence and presence of OM.

Objective 2: To assess earthworm choice for saline versus non-saline soils when given free choice.

Objective 3: To assess earthworm choice for saline versus non-saline soil when supplemented with OM.

2.2. Methods and materials

A series of earthworm experiments were conducted with the overall objective of determining salinity threshold values and what salinity levels earthworms prefer across low and high OM concentrations. In order to accomplish this goal, experiments were designed using soil, salinity characteristics, and earthworms from North Dakota.

2.2.1. Soil and salt materials

In order to evaluate earthworm salinity tolerance and choices under controlled settings, we used mesocosms (specific details of mesocosm designs are included below for each objective). Our test soil for all experiments was the Glyndon series, collected from western Minnesota. It is a coarse-silty, mixed, superactive, frigid Aeric Calciaquoll (Soil Survey Staff NRCS, 2022). The soil used for the experiments was collected from the top 15 cm of soil, the 'Ap' horizon, where the *Apporectodea* species typically reside (Edwards et al., 1996). According to the official soil description, this soil is primarily used for small grain crops, sugar beets, and potatoes but is historically home to native tall grass prairies (OSDS NRCS, 2022).

First, we examined the chemical properties of the soil (Raiesi et al., 2020). We tested the electrical conductivity (EC) and pH using the slurry method of measurement which is a 1:1 ratio of soil:water (Rhoades, 1996; Thomas, 1996), and OM (Combs 2011) of the soil. The test soil had an initial EC1:1 of 0.397 dS/m, a pH of 8.15 and an OM of 3.4%. After measuring these characteristics, we dried the soil and sifted out any large rock pieces.

To create a saline soil condition, a salt mixture was added to increase soil salt concentrations; the salt composition was created to be similar to that from a field study (Gasch et

al., 2021): 5% KCl, 15% $CaCO_3$, 25% Na_2SO_4, 20% $CaSO_4$, and 35% $MgSO_4$. This salt recipe was used for all experiments (exact soil and salt proportions for each experiment are listed in the appendix).

The *Apporectodea* genus prefers plant OM, so corn husks were used as a material to increase OM content in some treatments. These corn husks were collected from a field near Fargo, ND, air dried, ground into powder, and mixed into the soil (exact OM masses used in each experiment are listed in the appendix).

2.2.2. Earthworms

The *Apporectodea* earthworms for our experiment were collected from the Breker farm (Rutland, ND) and acclimated in a lab setting for a few months. Earthworms were housed in tubs containing a mixture of Glyndon soil and composted manure, also collected from the Breker farm in southern North Dakota. Earthworm tubs were stored in the dark at approximately 14 deg. C, and earthworms were fed and watered twice a week or as needed. Food consisted of dried, finely-ground grass clippings.

The individual earthworms used in the experiments were identified as being members of the *Apporectodea* species complex, which is a grouping of closely related species, including those common in North Dakota. The complex includes *Apporectodea calignosa, trapezoid,* and *tuberculate,* species recovered from saline field soils (Edwards et al., 1996-2004; Gasch et al., 2021). Adult earthworms were identified and selected for the experiment based on a prominent tubercula pubertatis (Figure 4). These markings are used as indicators of earthworm sexual maturity (Edwards et al., 1996). Adults were chosen for the experiment because they have a higher salt tolerance and chance of survivability (Edwards et al., 1996).

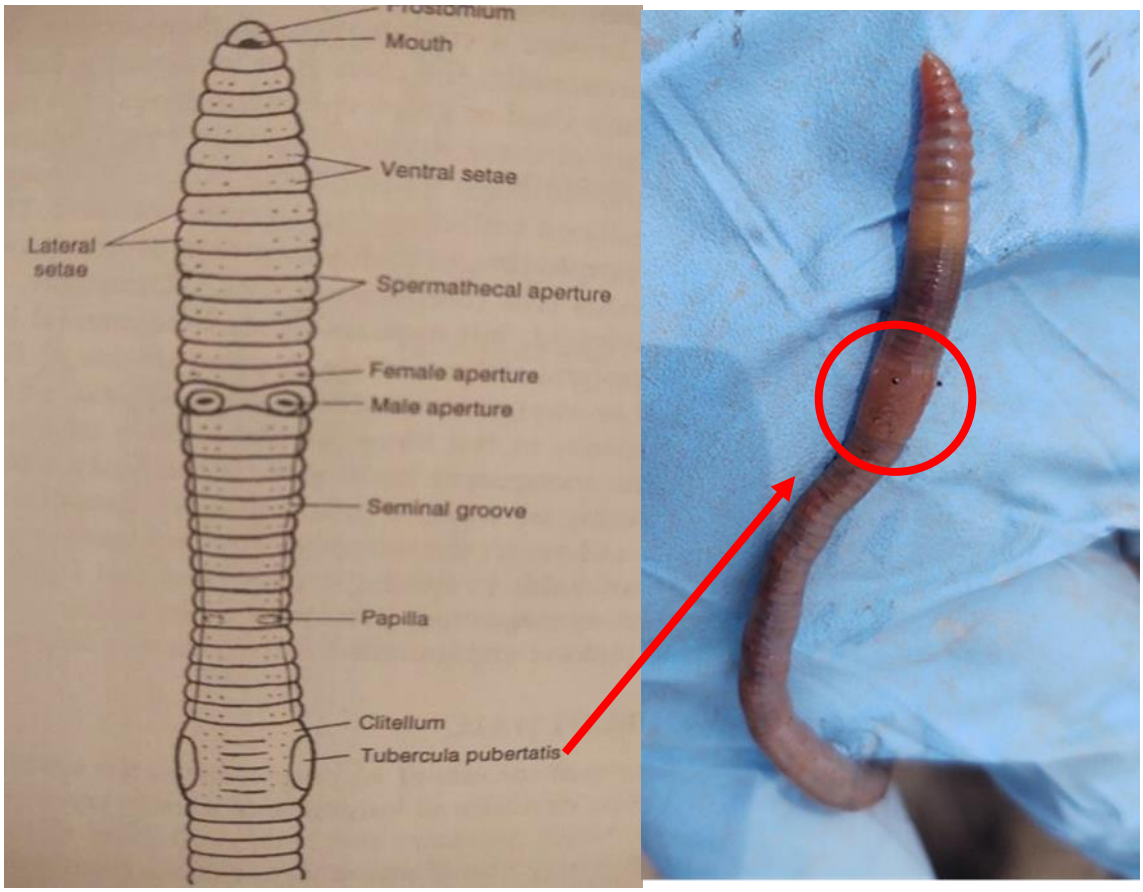

Figure 4. Adult earthworms.
Ventral view of the anterior region of *L. terrestris* illustrating the tubercula pubertatis, which was similar in form for the *Apporectodea* individuals used in the study (image from Edwards et al., 1996). Photo showing the pubertatis on an *Apporectodea* adult earthworm from the field.

For our experiments we counted adults, juveniles, and cocoons. Adults were defined on the presence of a Tubercula pubertatis, which demonstrates their ability to lay cocoons, or eggs sacks (Edwards et al., 1996; Hale, 2007). Juveniles do not contain these markings. Below is a figure demonstrating the general life cycle of earthworms. Information was taken from published studies and personal observations (Bart, 2019). There is limited information on earthworm development for the *Apporectodea* genus, and it is also dependent on the laboratory conditions and what earthworms are being fed. Based on what we noticed in tending to the earthworms we

wanted to give enough time in our experiments for earthworms to lay cocoons, and hopefully have the cocoons hatch into juveniles.

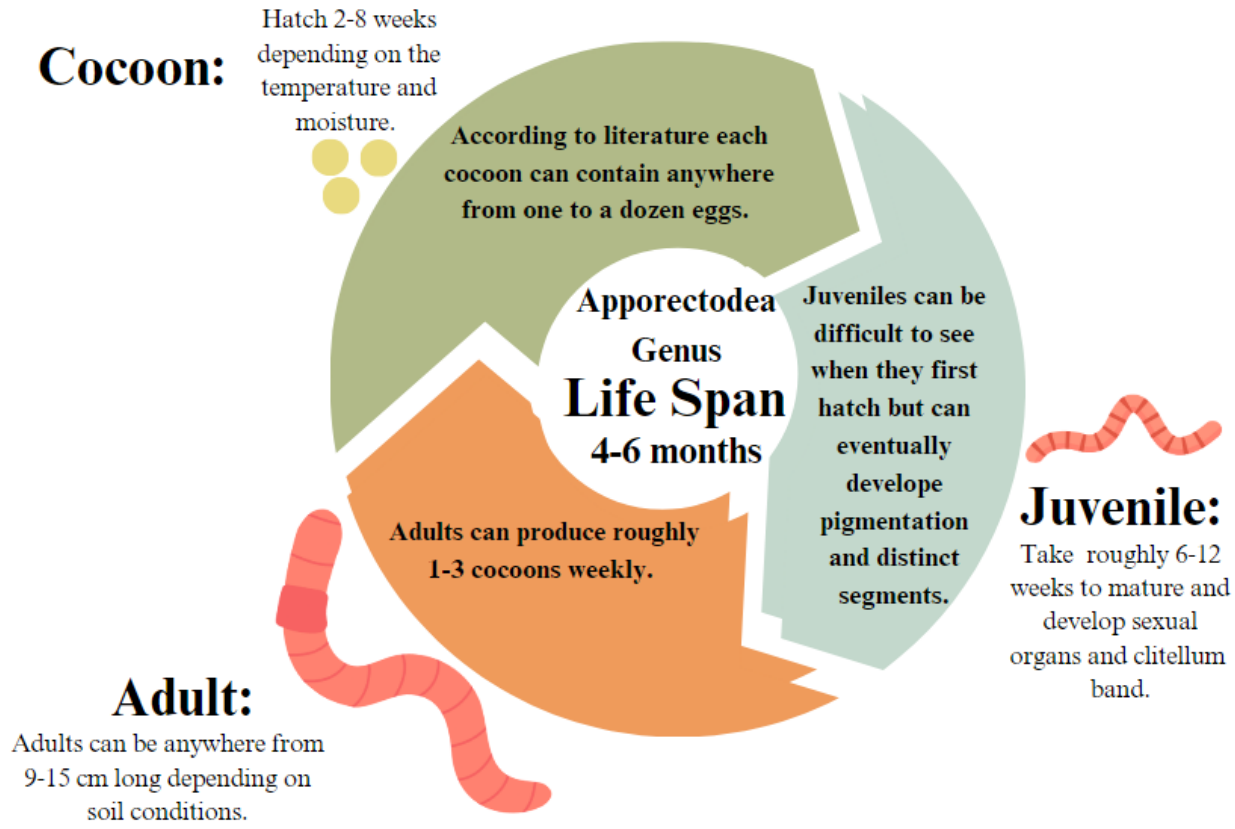

Figure 5. Earthworm life cycle.
Illustrates the three major stages of the earthworm life cycle; adult, juveniles, and cocoons specific to the *Apporectodea* earthworm genus.

Before and after each experiment, the EC1:1 and pH were taken from each container. This data was gathered to see if the salinity or pH changed over the duration of the experiment. We took this measurement because we were concerned that there would be salt migration within split treatment tubs, or salt leeching from the treatment during watering. In all experiments, the post-experiment EC1:1 and pH were consistent with the pre-experiment values. These measurements were taken to ensure there were no sudden shifts in the soil mesocosms. When

watering as needed EC can decrease or in the case of our choice experiments leech into non-saline soil.

2.2.3. Objective 1: survivability and production experiment

Earthworm performance in saline and non-saline soils was assessed by creating 30 small soil treatment mixtures, each plastic container held 200 grams of the material. To evaluate earthworm salt tolerance levels, mesocosms were created for a range of salt concentrations. Salts were added to the test soil to achieve the following EC1:1 values: 1, 2.6, 3.3, 3.5, and 4.5 dS/m. These values were chosen because they are likely to be encountered in field soils and they fall within the range of salinity levels where plants experience stress. Each salt level was replicated six times. Half of the containers then had corn husk OM added to them so that the OM concentrations reached 10% by weight. So, in this experiment, each treatment had three replicates and incorporated two factors: salinity value and presence or absence of elevated OM (Figure 6).

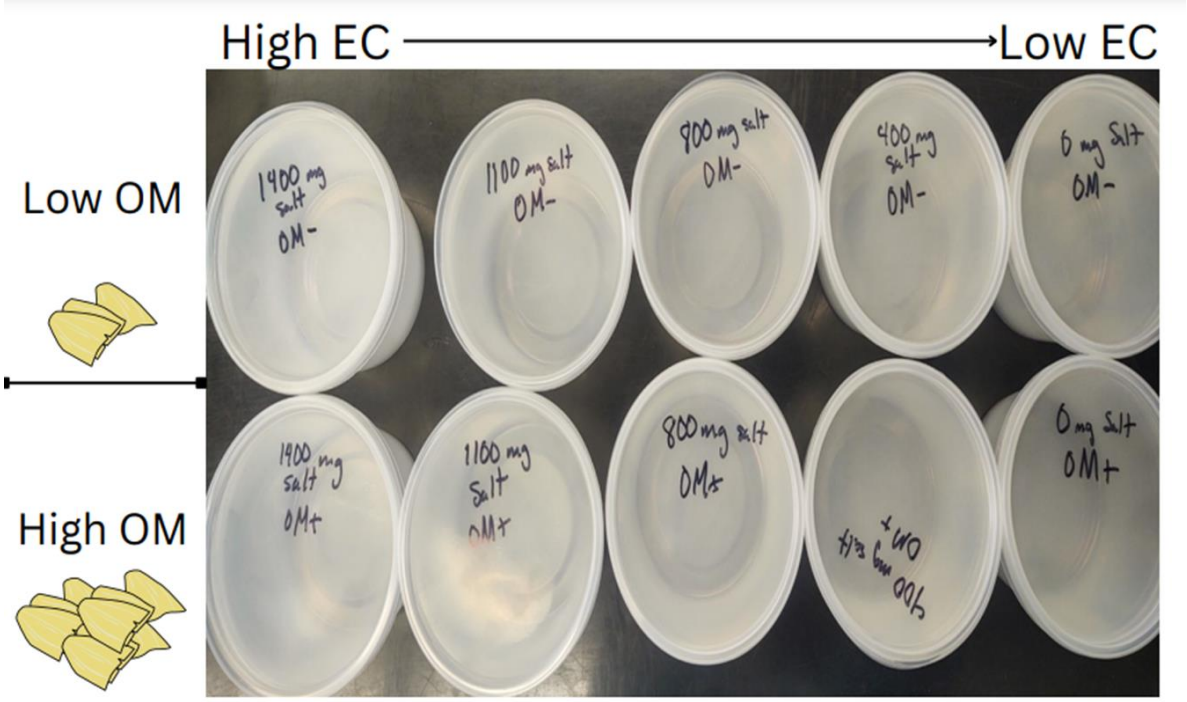

Figure 6. Survival and production experiment treatments.
Depicts the treatments used in the first and second round of the survival and production experiment for objective one. Demonstrates the mg of salt per 100 grams of soil. The top bins are low OM (6%) while the bottom is high OM(10%).

At least 100 ml of water was added to the containers before the containers were placed in the dark incubator at 14 deg C and excess water was allowed to drain. Once the soil treatments had been chilled, three *Apporectodea* adult earthworms were added to each container. Containers were covered with perforated lids and placed back in the incubator. During the second and 4th week, 10 ml of water was added as needed.

After six weeks, containers were deconstructed and living adults, dead earthworms, and cocoons from each bin were tallied and recorded. We chose this amount of time because based on what was seen in our earthworm incubators, six weeks would be sufficient time for adults to lay cocoons, have cocoons hatch, and die by the salinity (Figure 5.) We assume that all recovered cocoons were deposited during the duration of this study. The experiment was conducted twice. The second round was identical to the first round except with two additions. In the second round,

the total earthworm mass for each container was measured before and after the experiment. This measurement was taken to provide a more in depth look at the potential impact OM and salinity may have on the growth of the earthworms. Additionally, six bins with an EC of 4.5 dS/m (three without OM additions, and three with OM additions) were also created without earthworms so any potential interactions between OM and high salt concentrations on the physical and chemical characteristics of the soil could be observed.

2.2.4. Objective 2: choice experiment

In order to evaluate earthworm response when given the option to migrate between saline and non-saline soils, larger bins were created that had saline and non-saline conditions (Figure 7). We created two soil mixtures (saline and non-saline). Each tub contained 30 kgs of material, with each side totaling roughly 15 kg. All large tubs were filled at least 15 cm deep with soil mixture to allow sufficient earthworm movement. A divider was used during bin creation so that the treatments would not mix.

The first treatment was the control treatment with non-saline soil on both the right and left side of the tubs. The second experimental treatment had a saline side and a non-saline side. Each treatment configuration was replicated three times. The Glyndon soil was used for the non-saline soil. To create the saline soil, 15 kg of the Glyndon was mixed with the salt mixture described above, to create a soil with EC1:1 of 6 dS/m (see appendix for recipes). This salinity was chosen because it was expected to be intolerable for the earthworms, based on literature and field observations.

After the soil mixes were added, enough water to moisten the soil was added to the bins. Once the water was settled, the barrier separating the treatments was removed. Thirty adult

earthworms were then placed in the centerline of each tub. These worms were monitored twice a week for a month, and given supplemental water as needed.

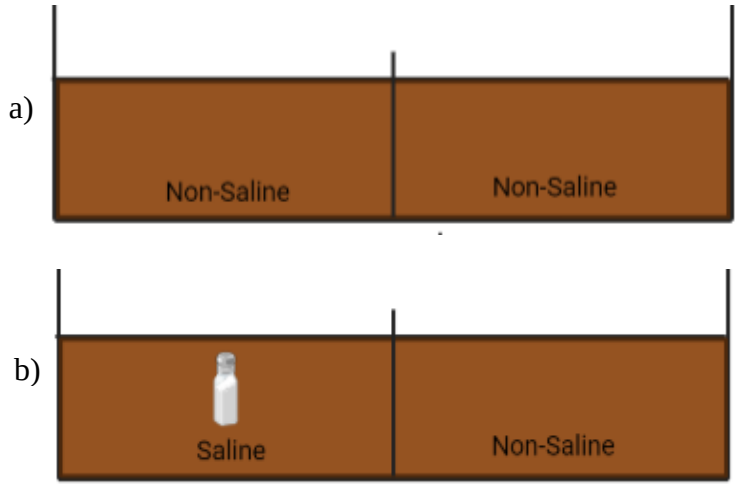

Figure 7. Design for the choice experiment.
Treatment a) is the control treatment with only non-saline soil (EC1:1 = 0.397 dS/m). Treatment b) is the experimental treatment with non-saline and saline (EC1:1 = 6 dS/m) sides. Each treatment bin was replicated three times. In each bin, thirty earthworms were allowed to freely navigate to both sides for one month and then evaluated for survival and location.

After a month, the divider was placed in the tubs to separate the treatments and block the earthworms from changing sides. A month was chosen because we believed based on out life cycle data that this would give the adults enough time to chose between sides, and lay cocoons that would hatch into juveniles. Containers were deconstructed and counts of living adults, juveniles, and cocoons from each side in each bin were tallied and recorded. For this experiment, and the following (for Objective 3), only adult earthworms were added to the bins, so all cocoons and juveniles were assumed to be deposited and hatched during the duration of the studies

2.2.5. Objective 3: organic matter dependence experiment

To evaluate the effect additional OM had on earthworm choice in saline soil, and to see if OM alleviated the avoidance of saline soil, we created four split-bin treatments (Figure 8), each replicated three times. As in the previous experiment, each tub contained 30 kgs of material, with

each side totaling roughly 15 kg, and providing about 15 cm of soil depth. A divider was placed during the creation process so that the treatments would not mix. In all mixtures, the OM and salt additions to the Glyndon soil were similar as in the experiment for the first objective (10% ground cornhusk by mass and target EC1:1 of 6 dS/m, see appendix for all soil mix recipes).

The first experimental treatment had saline and non-saline sides, each with added OM. The goal of this treatment comparison was to evaluate if elevated OM would facilitate earthworms moving into saline soils. The second experimental treatment consisted of non-saline soil with no OM addition and a non-saline soil with added OM. The purpose of this comparison was to evaluate if OM level alone would influence earthworm presence.

The third treatment was modeled off of the conditions typically found in nature, where low OM accompanies high salinity. This treatment consisted of a side with non-saline soil and elevated OM content, and a side with saline soil with no added OM. The purpose of this comparison was to evaluate if earthworm choice in the bins reflected field observations (avoidance of saline soils with lower OM). The last experimental treatment consisted of a non-saline soil with no added OM and a saline side with elevated OM. This treatment is the opposite of what is found in nature and was included to evaluate if the addition of OM can attract earthworms despite higher salt concentrations.

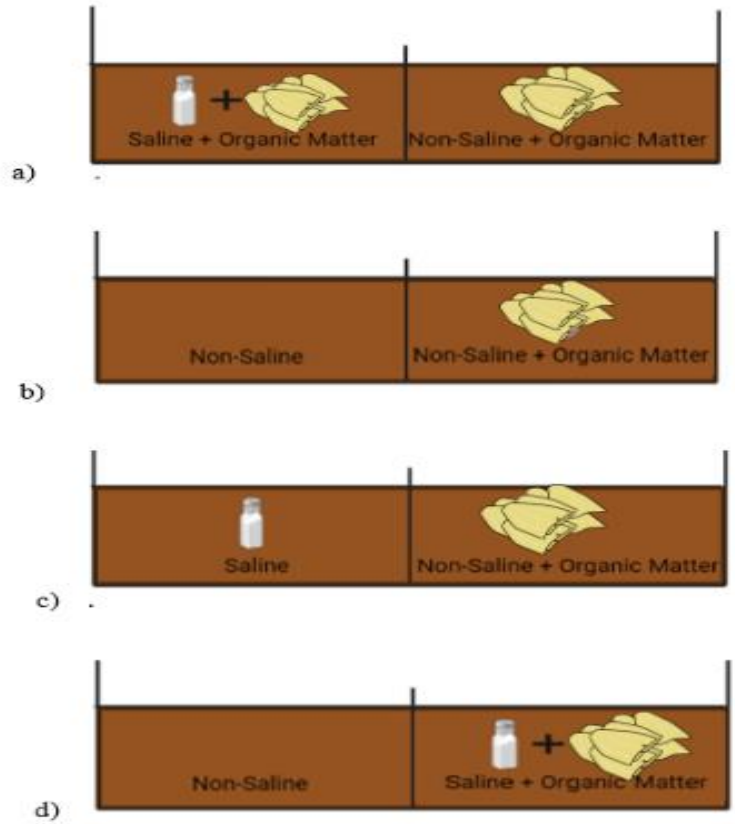

Figure 8. Design for the second choice experiment.
Treatment a) with saline soil (6 dS/m) + OM (10%) and non-saline soil (0.397 dS/m) + OM (10%). Treatment b) with non-saline soil (0.397 dS/m) and baseline OM (3.4%) and non-saline soil (0.397 dS/m) + OM (10%). Treatment c) with saline soil (6 dS/m) and baseline OM (3.4%) and non-saline soil (0.397 dS/m) + OM (10%). Treatment d) non-saline soil (0.397 dS/m) and baseline OM (3.4%) and saline soil (6 dS/m) + OM (10%). Each treatment bin was replicated three times. In each bin, thirty earthworms were allowed to freely navigate to both sides for one month and then evaluated for survival and location.

After the soil mixes were added, enough water to moisten the soil was added. Once the water was settled, the barrier separating the treatments was removed. Thirty earthworms were then placed in the center of each tub. These worms were monitored twice a week for a month, and given water as needed.

After a month, the divider was placed in the tubs again to separate the treatments and block the earthworms from changing sides. Containers were deconstructed and living adults, juveniles, and cocoons from each tub were tallied and recorded.

2.2.6. Statistical analysis

In order to analyze the data from the three earthworm experiments, statistical analyses were performed in RStudio (RStudio Team, 2021). The packages used for the analysis were 'ggplot2' (Wickham, 2016), 'tidyr' (Wickham and Girlich, 2022), 'dplyr' (Wickham et al., 2022), 'readxl' (Wickham and Bryan, 2022), 'grid Extra' (Auguie, 2017).

For the survival and production experiment a Two Sample T-test was used to evaluate if pooling the two experimental rounds was acceptable. A two factor ANOVA analysis was conducted on the adult, cocoon and dead earthworm counts and mass data to identify if earthworm counts and average mass gain differed across salt amount, presence of OM, and the two factors together.

To analyze the data collected in the experiments for Objective 2, and Objective 3, a paired t-test was used to compare two treatments within a bin and a standard t-test was used to compare treatments from different bins. This test determined if there was a significant difference in adult, cocoon, and juvenile counts between treatments. Our goal was to compare the earthworm and cocoon counts between the non-saline and saline sides of the bins and between the two treatments and the control. Since the non-saline versus saline treatments occurred within the same bin, we used a paired t-test for that comparison. For the comparisons to the control, we used a standard t-test.

2.3. Results

2.3.1. Objective 1: survival and production experiment results

The first experiment evaluated survival and production, growth, and cocoon deposition of earthworms across different salinity with or without added OM. Overall, earthworm survivability and production (based on earthworm counts) was not different across increasing salt concentrations, or with additional OM, based on the data collected. There were two rounds of this experiment. The counts for adults, cocoons, and dead earthworms were combined after Two Sample T-tests indicated that the counts within treatments were not different between the two rounds, so each treatment had six replicates. A two factor ANOVA analysis was conducted on the adult, cocoon and dead earthworm counts. This analysis indicates if the response variables (counts) differ across salt amount, presence of OM, and the two factors together. There were generally no significant differences between the number of adult earthworms, cocoons, or dead earthworms across treatments as can be seen in Figure 9 and Table 1. The only significant difference was between adult counts across OM levels (p-value = 0.0318), where the mean adult count in all treatments with added OM was lower than the mean adult count in treatments without OM. This finding was opposite of expectations.

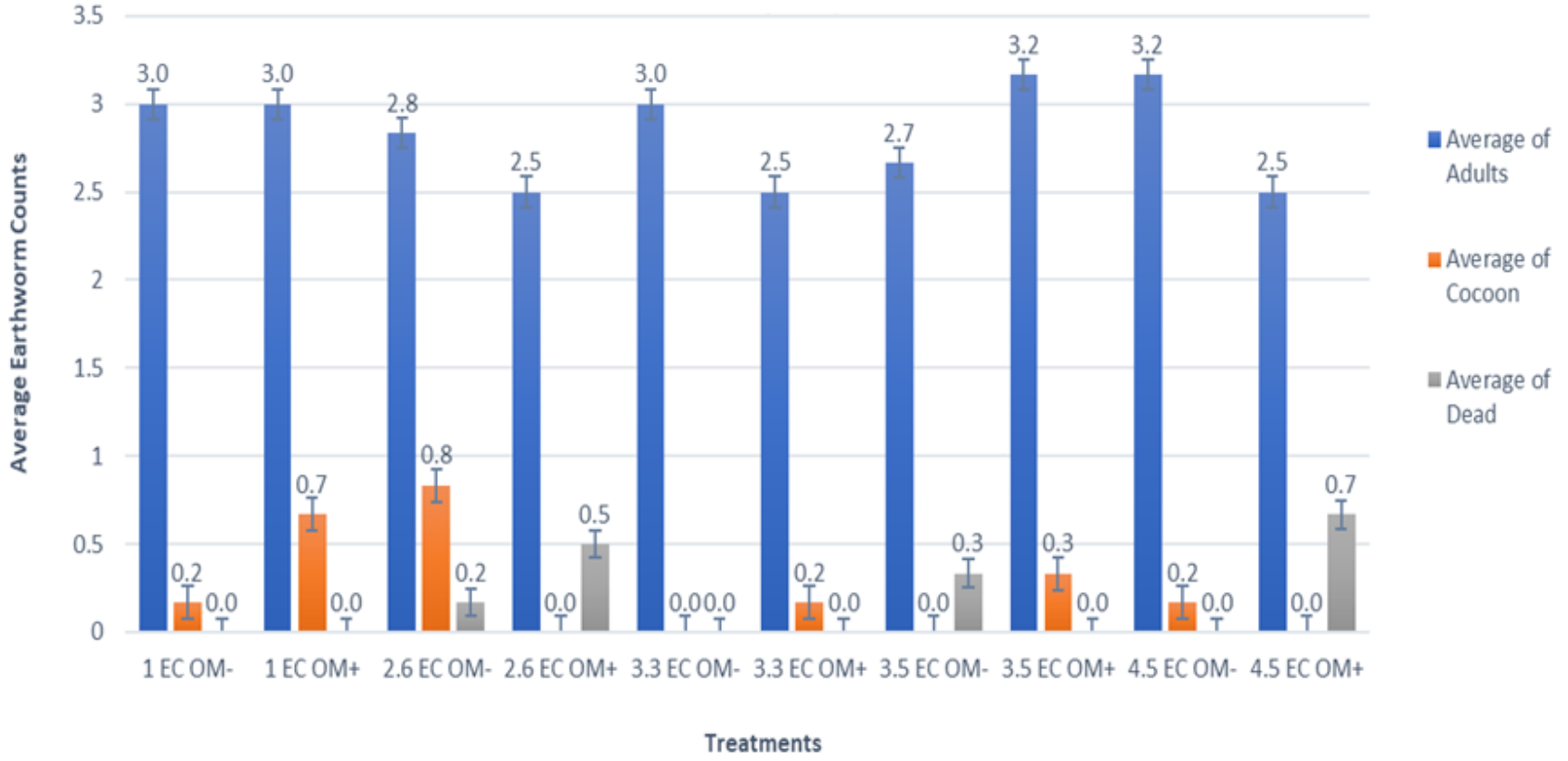

Figure 9. Average earthworms counts survival and production experiment.
Bar graph depicting average earthworm counts for Adult, Cocoon, and Dead earthworms with standard deviation as error bars from two rounds of survivability and production experiments (n=6).

Table 1. Survival experiment count results.
Two Factor ANOVA results table for adult earthworms, cocoons, and dead earthworms counted across salinity and OM levels (n=6). An asterisk (*) is used to highlight p-values < 0.05.

		Df	Sum Sq	Mean Sq	F value	Pr (>F)
Adult earthworms	***Salt***	4	1.433	0.3583	1.295	0.285
	OM	1	1.350	1.3500	4.880	0.032*
	Salt: OM	4	1.567	0.3917	1.416	0.242
	Residuals	50	13.833	0.2767		
Cocoons	***Salt***	4	3.57	0.89	1.305	0.281
	OM	1	0.82	0.81	1.195	0.280
	Salt: OM	4	2.43	0.61	0.890	0.477
	Residuals	50	34.17	0.68		
Dead earthworms	***Salt***	4	1.833	0.4583	1.858	0.132
	OM	1	0.600	0.600	2.432	0.125
	Salt: OM	4	1.567	0.3917	1.588	0.192
	Residuals	50	12.333	0.2467		

Earthworm mass data was only taken in the second round. Mass data was averaged within each treatment and analyzed with a two factor ANOVA to identify differences across salt amount, OM presence, and their interaction. Average masses are included in Figure 6 and ANOVA results are listed in Table 2. The ANOVA indicated that the interaction between salinity level and OM was significant (p-value = 0.0234, Table 2), and a post-hoc test indicated that earthworm mass was significantly higher in the elevated OM treatments at the lowest salinity level (see Figure 10). The low weight was seen in the 1 dS/m bin with no organic matter.

This may be because the earthworms were still able to burrow unlike in higher salinity levels but were not being supplemented with additional OM.

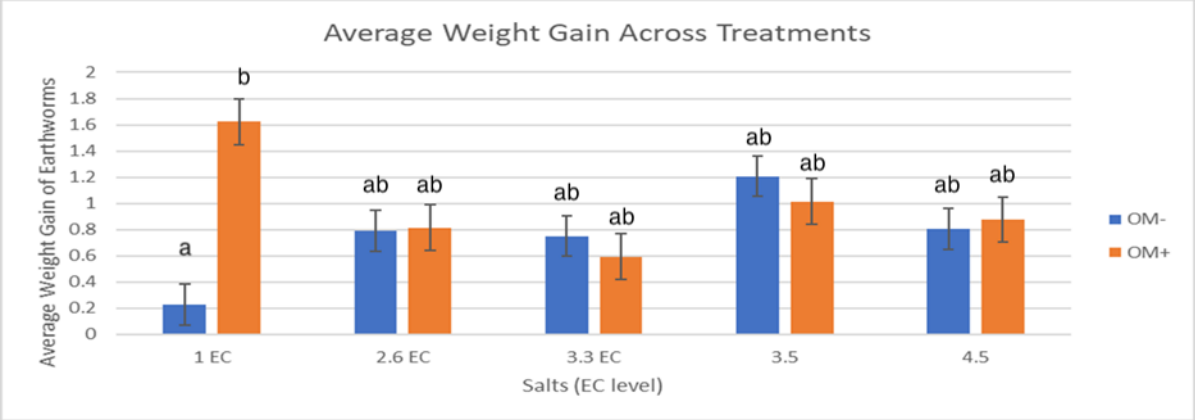

Figure 10. Average weight gain across treatment.
Bar graph showing mean earthworm mass across treatments after 1 month of growth (n=3, standard deviation as error bars). The same letters indicate that the averages were not significantly different to each other.

Table 2. Survival experiment mass results.
Two Factor ANOVA results table for earthworm mass across salinity and OM levels (n=6). An asterisk (*) is used to highlight p-values < 0.05.

	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Salt	4	0.633	.1582	0.858	0.506
OM	1	0.390	0.3899	2.116	0.161
Salt: OM	4	2.637	0.6593	3.577	0.023*
Residuals	20	3.686	0.1843		

2.3.2. Objective 2: choice experiment 1

The second experiment evaluated earthworm behavior when given a choice between saline and non-saline soil without added OM. Overall, adults, cocoons, and juveniles preferred non-saline soil, as indicated by higher counts in non-saline soils.

There was no difference between the control bin and the non-saline sides for mean counts of adults, juveniles, and cocoons (Table 3). This is also true for the juveniles found in the saline sides of the bins. In general, juvenile numbers were low across all treatments. A paired t-test was done to compare the sides in the experimental bin to the control bin. There was no significant difference between the non-saline side counts and the control bin. The saline side of the experimental bin was different from the control bin. There were significantly fewer adults (p-value = 0.006) and cocoons (p-value = 0.039) in the saline side compared to the control and non-saline side. These results support our hypothesis that salt does influence adult movement and cocoon production in non-saline soils.

Table 3. Choice experiment 1 count results.
The mean counts and standard deviation (n=3) are listed according to treatment for the adults, cocoons, and juveniles. The letters a and b indicate the significance within the row based on a) paired t-test between the two treatment sides saline and non-saline and b) a standard t-test between the treatment sides and the control.

	Control	**Non-saline**	**Saline**
Adults	24.3 (5.13) a	26.3 (3.06) a	0.67 (0.58) b
Cocoons	23 (2) a	29 (8.19) a	2.67 (3.79) b
Juveniles	4 (2) a	3.67 (1.53) a	0.33 (0.58) a

2.3.3. Objective 3: organic matter dependence experiment

The third experiment evaluated earthworm choice between varying treatments of saline soil with or without OM and non-saline soil with or without added OM. Overall, adults, cocoons, and juveniles preferred non-saline soil with added OM (Table 4), based on counts. Earthworms preferred the non-saline soil with high OM even when the saline side also had high OM. More cocoons, adults, and juveniles were found in non-saline sides for every treatment. Along with preferring non-saline soil, counts were higher in soils containing elevated OM. In the treatment

that included non-saline soil without OM versus saline soil with OM, the mean differences for juveniles, cocoons, and adults were lower than in any other treatment. While OM may not fully convince the earthworms to prefer saline soil, it did close the gap in that specific comparison.

Table 4. Choice experiment 2 count results.
Tables depicting the mean, standard deviation, and difference in means between treatments for adults, cocoon, and juveniles. (NS+=Non-saline+OM) ,(NS-=Non-saline), (SS+=Saline+OM), (SS-=Saline). An asterisk (*) is used to highlight p-values < 0.05.

	Treatment		**Difference Between Treatment Means**	**P-value**
	NS-	**NS+**		
Adult	5.22(4.03)	20.7 (5.77)	15.33	0.10
Cocoon	4.33(1.15)	18 (1.73)	13.67	0.01*
Juvenile	1.67 (1.53)	5.67 (2.08)	4	0.18
	NS+	**S-**		
Adult	4.33(1.53)	0.33 (0.57)	4	<0.001*
Cocoon	25 (8.54)	2.33(0.57)	22.66	0.04*
Juvenile	26 (2)	0 (0)	26	0.07
	NS+	**S+**		
Adult	26.3 (1.53)	0.67 (1.15)	25.66	<0.001*
Cocoon	20.7 (5.69)	3 (1.73)	17.66	0.02*
Juvenile	4.67 (2.52)	0.33 (0.57)	4.33	0.07
	NS-	**S+**		
Adult	13 (5.57)	10 (4.58)	3	0.61
Cocoon	2(1.73)	1.67(1.53)	0.33	0.67
Juvenile	2.67(2.52)	0 (0)	2.67	0.21

2.4. Discussion

While we expected earthworms to have an aversion to saline soil, we speculated that extra OM may alter saline effects on earthworm behavior and ability to function. The goals of this chapter were to first assess earthworms' survivability and production at varying salinity levels in the absence and presence of OM; second, assess earthworm behavior in saline versus non-saline soils when given free choice; and third assess earthworm choice for saline versus non-saline soil when supplemented with additional OM. These objectives were accomplished but our results for both objectives 1 and 3 were unexpected.

In objective 1, we expected added OM to aid earthworms in surviving increasing salinities; however, earthworm survival and production remained similar to non-saline soils despite OM level. In a field study, saline soils lacked OM, and this was a possible reason behind earthworm absence in these areas (Gasch et al., 2021). We were curious if the absence was due to avoidance or mortality. Our results indicate that earthworms survive in soils with increasing salinity regardless of OM levels. While salinity alters soil structure by reducing porosity and increasing soil plasticity, OM influences structure by increasing porosity, aggregation, air flow, and hydrology (Gasch et al., 2015; Paul, 2016; van Vlient et al., 2007). Many studies have shown that adding OM can improve earthworm habitat and provide earthworms with much needed carbon and nitrogen in harsh soil ecosystems (Angst et al., 2017; van Vlient et al., 2007). The treatment bins with an EC of 1 dS/m did show a difference in average mass gain between OM levels. Earthworms in the bin with elevated OM gained more weight than earthworms in tubs with no OM. Adult counts also decreased with the addition of OM. This experiment indicated that earthworms can survive in soils with salt concentrations up to 4.5 dS/m, and suggests that OM can be beneficial for earthworm growth in some conditions, and perhaps their absence in

field saline soils is due to avoidance. The experiments for the second and third objectives aimed to examine this choice when mobility is allowed.

The results for the survival and production experiments told an interesting story about the relationship between salts, OM, and earthworms. In the survival and production experiments neither increasing salt level nor reduced OM had an overall significant reduction in adults and cocoons or a significant increase in dead earthworms. This didn't meet the original hypothesis that increased salinity would decrease earthworm's survivability and production or that OM would aid the earthworms in surviving. It could be that our earthworm species is more tolerable to salt or that the OM concentration was too high for such small containers.

Our first choice experiment did follow expectations: earthworms avoided saline soil. As many studies suggest the cons of saline soil don't appear worth traversing especially if there is less food (OM) (Gasch et al., 2015; Seelig 2000). In the lab it was noted that the saline soil was difficult to dig through, which is to be expected as salinity can alter soil through influencing how aggregates bind (Hanson et al., 1999; Paul 2015; Seelig 2000). These physical changes likely influenced the earthworms in both choice experiments.

In the second choice experiment, both young and old earthworms preferred non-saline soil with elevated OM, followed by non-saline soil with normal OM, followed very closely by saline soil with elevated OM. The normal OM/saline soil treatment was the earthworm's least favorite environment. This choice was also represented in the number of cocoons. The main point from the choice experiments is that earthworms avoid saline soils, and that elevated OM does not necessarily allow them to overcome this avoidance.

2.4.1. Pitfalls

While the *Apporectodea* genus of earthworms were selected from the field and used in the experiment, we used several different members of the genus, including *A. calignosa* and *A. tuberculatae*. These different species could have different characteristics with regard to salt tolerances. Research has shown that different species of earthworms experience different levels of toxicity to different chemicals (Edwards et al., 1996). Perhaps some of the earthworms found in the more saline sides of the tubs had a higher salinity tolerance then other *Apporectodea* species. It is difficult to distinguish the difference between *Apporectodea* species; *A calignosa* and *A. tuberculata*, as there is a very subtle anatomical difference in their genital tumenscences (Hale, 2007; Edwards et al., 2022).

Another pitfall of our experiment could have been the experimental design of our mesocosms. Most *Apporectodea* prefers moist soil, so our mesocosms had to be watered at least once a week. While we did check the salinity before and after to ensure that the EC1:1didn't change, there could have been some leaching or shifting of the OM concentrations in our mesocosm. Other experiments addressed this by keeping the *Eisenia fetida* earthworms in an aquatic mesocosm so that the salinity was consistent during the entirety of the experiment (Owojori et al., 2009). This experiment only addressed growth, survival, and cocoon deposition and not choice.

Throughout this experiment it is assumed that all juveniles were born during the experiment, which is why numbers are likely so low, with more time, perhaps they could have been more divergence between the saline and non-saline treatments. A lengthier experiment would allow populations to further develop which could uncover key difference between soil conditions. This would better approximate the earthworm's activities as they achieve maturity.

2.4.2. Significance

Despite the pitfalls these projects created more information on earthworms and on saline soils in North Dakota. Before there was limited information in the Great Plains area about how the unique salinity influenced earthworms, which are vital to soil ecosystems.

Our earthworm experiment provides more information about salinity tolerance and choice in *Apporectodea* species. For local farmers like Joe Breker, this information could aid in understanding how salinity influences important decomposers; and combatting local field salinity to improve crop yields (Zorb et al., 2019). Laying down large amounts of OM is not enough to inspire earthworm migration to saline soils with a high EC. Despite this revelation we also discovered that the *Apporectodea* are very resilient when it comes to saline soil after living here for a very long time.

Hopefully the findings in this paper create renewed interest in researching inhibitory conditions like salinity especially here in North Dakota not just in terms or crops and yields, but also for the little guys who make it all possible.

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3. ANALYZING MICROBIAL ABUNDANCE AND BIODIVERSITY IN SALINE SOIL ACROSS A VERTICAL PROFILE

3.1. Introduction

Elevated salinity in soil is a major environmental issue that many countries worldwide struggle to solve (Daliakopoulos et al., 2016; Zexuan et al., 2019). Salinity has many causes, whether naturally occurring or man-made (Keller et al., 1986; Rengasamy, 2006; Rhoades, 1996; Qadir, 2008; Stavi, 2021; Zexuan et al., 2019). It can be caused via the weathering of bedrock, seawater intrusion, or poor irrigation (Keller et al., 1986; Rhoades, 1996). No matter the cause, scientists, and the U.S. Salinity Laboratory, categorize saline soil based on an EC of 4 dS/m (Seelig 2000; U.S. Salinity Laboratory Staff, 1954).

Saline soils impact everything from crops to microscopic organisms (Ries et al., 2020; Zorb et al., 2019). Salinity can change many aspects of a soil ecosystem, leading to a shift in the organisms that can thrive there (Asghar et al., 2012; Canfora et al., 2016). The detrimental impact salinity has on crop growth and yield is very well studied, from the direct influence to the indirect influences of salinity (Maas et al., 1999; Muller et al., 2006; Munns et al., 2008; Parid, 2005; Ries et al., 2019; Zorb et al., 2019). Salinity negatively impacts many plants' ability to absorb water (and nutrients) and can kill invaluable decomposers and organisms that aid the plant's growth (Zexuan et al., 2019; Zheng et al., 2017). Other organisms, such as earthworms, are similarly harmed by salinity (Faiza et al., 2016; Gasch et al., 2021; Karimi et al., 2020; Sharif et al., 2016; Wu et al., 2019). If macro-organisms consistently respond negatively to saline soils, it begs the question of how their smaller counterparts (micro-organisms) fare in salty soil.

In North Dakota, soil salinity is distinctly different when compared to the salinity found in many soils worldwide. Salts in soil ecosystems are normally chloride-based and caused by

poor irrigation or coastal water intrusion, but North Dakota soils can have sulfate (SO_4^{-2}), carbonate (CO_3^{-2}), and chloride (Cl^-) ions, with sulfate salts being the most abundant throughout saline soils (Gasch et al., 2021; Seelig, 2000). This salinity process occurs naturally through the discharge and recharge system in the soil that brings up salts to the surface (Gasch et al., 2021; Keller et al., 1986). Due to how salinity in soil occurs in North Dakota, it can change across the soil profile; the deeper into the soil profile, the nature of the parent material and hydrology cause higher salt concentrations (Bardgett et al., 2014; Blume et al., 2002, Chu et al., 2016; Eilers et al., 2012; Keller et al., 1986; Xu et al., 2021).

As with many areas across the world, agriculture is a vital part of the economy in the Great Plains area, and the salinity has resulted in significant crop yield and economic losses (Hadrich et al., 2012; Zorb et al., 2019). As a result, most previous research focus in North Dakota has been on studying salinity's influence on crops (Munns 2002; Seelig 2000). Less known is the impact of the Great Plain's unique salinity on the organisms below ground, especially organisms that can't be seen without a microscope (Ries et al., 2020).

Microbes play crucial roles in soil environments, and they are especially important in agricultural soils. Microbes span three domains; archaea, fungi, and bacteria, and exist in all environments from within other living organisms to volcanic springs in the oceans (Bruslind, 2020; Caroll et al., 1981.; Woese et al., 1990; Yates, 2016). Despite being microscopic, all three microbial domains perform essential roles, especially in soils (Agata, 2020; Noah et al., 2007; Paul, 2015). They can form various relationships that can influence other organisms like plants and earthworms, who are also important members in soil ecosystems. Several microbes assist plants (Boyrahmadi et al., 2018; Hartman et al., 2018; Yuejian et al., 2014) and earthworms in

performing processes like nitrification, fermentation and carbon cycling (Drake et al., 2007; Guofan et al., 2022; Horn et al., 2003; Ihssen et al., 2003).

A wide range of different groups take part in these microbial interactions to help facilitate growth in soil ecosystems (Gryta et al., 2020; Noah et al., 2007; Paul, 2015; Wagg et al., 2019). These microbes are the backbone of soil ecosystems, but like plants and earthworms, they can be susceptible to changing soil conditions like pH (Zhao et al, 2019), temperature (Xiaoya et al., 2017), and nutrient availability (Aliasgharzadeh et al., 2001; Banerjee et al., 2019; Specht et al., 1989; Stiles et al., 1996). Salinity is an especially strong factor in agriculture ecosystems, so it is vital to understand how it effects microbes across all three domains.

In other saline soils, studies have found that high salinity levels harm several different microbial groups. Several studies showed a change in both microbial biodiversity and biomass in saline soils (Asghar et al., 2012; Boyrahmadi et al., 2018; Canfora et al., 2017; Chowdhury et al., 2011; Dasgupta, 2023; Egamberdieva et al., 2010; Georgieva et al., 2012). One field experiment found that coastal salt intrusion significantly decreased the richness and diversity of all three microbial domains (Canfora et al., 2017). Another study performed in cotton fields looked at how salinity at varying levels from carbonate and chloride salts and found that microbial biomass decreased as salinity levels reached "strong" levels (7.1 ± 0.6 dS m^{-1}) (Egamberdieva et al., 2020). Salinity negatively impacts microbes both directly and indirectly, whether it be by limiting nutrient availability or affecting the microbe's ability to take in nutrients by destroying lipid layers (Asghar et al., 2012; Batra et al., 1997; Chowdhury et al., 2011; Elmajdoub et al., 2012; Ruhl et al., 2018). This phenomenon was seen in a study using field soil with an EC (electrical conductivity) ranging from 0.3-6.0 dS m^{-1}, which found that increasing salinity decreased the ability of microbes to decompose particulate organic carbon (Asghar et al., 2012).

While many studies have been done on how salinity influences microbes, few studies have been done globally on how depth alters microbial groups when paired with salinity, but they often call for more research to be done across deeper soil profiles (Becerra et al., 2014). Even less has been done in North Dakota or the Great Plains concerning how microbes in the region react to the unique salinity (Jakubowski, 2021; Ries et., 2020). Due to the importance of agriculture in this region, many studies have analyzed how plant life reacts (Combs et al., 2011; Hadrich, 2012; Langseth et al., 2015; Munns, 2002; Seelig, 2000), and a few studies have been done on earthworms as well (Gasch et al., 2021). The groundwork was started by a project in 2020 that analyzed how major microbial groups and nematodes reacted to saline soils in the region. While this study found a decrease in nematode abundance in the more saline soil, there was an increase in microbial group abundance, as measured with phospholipid fatty acid analysis (Ries et al., 2020). These unexpected results may be because of the unique salinity composition found in the area (Kellers et al., 1986).

Perhaps different microbial groups are more able to adapt to sulfate and carbonate salts. Because the 2020 study examined broad taxonomic microbial groups, it is unknown what genera can survive and thrive more effectively. Therefore, this study examines abundance and diversity of microbial groups (bacteria, fungi, and archaea) found throughout the soil profile of saline and non-saline field soils. Answering these questions may provide more insight into what microbial groups can handle salt stress at different depths.

In this study, 160 soil samples were analyzed in a lab from non-saline soil and saline soil at the following depths: 0-15 cm, 15-30 cm, 30-60 cm, 60-91 cm, and 91-121 cm. Analyses were performed with the following objectives in mind:

Objective 1: To compare the abundance of microbes found across saline and non-saline soil environments and across depths.

Objective 2: To identify how microbial communities (taxa and diversity) shift across saline and non-saline soil environments in increasing soil depths.

We hypothesize that saline soil will have less biodiversity than non-saline soil because the osmotic stress presented by the salinity will create selective pressures where only specific microbial species adapted to saline niches will thrive. Our second hypothesis is that smaller unicellular microbial species like bacteria and archaea will occur in higher abundance in deeper soil due to reduced nutrients like oxygen. Thirdly, we predict that fungi will be hardier and therefore more abundant in gene abundance and biodiversity in saline environments owing to them being multicellular.

3.2. Methods

Field sites were established in 2017 to study salinity and soil biology in agricultural fields. Sites were named after nearby towns: Aneta, Eldridge, Midway, and Northwood, North Dakota. These farms were under no tillage management and followed a corn and soybean (*Glycine max (L.))* crop rotation from 2017 – 2020 (Ries et al., 2020). Only Aneta had an actively growing soybean crop during sampling in 2020. The other fields (Eldridge, Midway, and Northwood) were fallow due to poor planting conditions in the spring of 2020.

Within each field, we identified areas within two salinity levels: Non-saline soils were classified according to an electrical conductivity of a 1:1 soil:water slurry (EC1:1) < 1 dS/m and saline soils were classified according to an EC1:1 2-4 dS/m. These areas were identified based on an apparent electrical conductivity survey using a Veris cart, conducted at the inception of the

project in 2017. Four sample locations were identified within each field, and within each salinity class for a total of 32 sample locations (16 at each salinity level).

In early August of 2020, soil samples were collected from each sample location to investigate the microbial community structure in field soils, to 121 cm depth. Soil cores were separated into the following depth increments: 0-15 cm, 15-30 cm, 30-60 cm, 60-91 cm, and 91-121 cm. Samples were collected using a hydraulic soil probe (Giddings, 2021) before being sliced into the depth increments listed above. Then these samples were frozen and stored at -80 deg, C until analysis. Soil samples were also analyzed for pH (Thomas et al., 1996), total, inorganic, and organic carbon (Nelson and Sommers, 1996), and EC1:1 (Rhoades et al., 1996). Due to samples being from field sites measuring the before mentioned chemical properties can help us interpret the microbial data. We can also examine the trend of chemical properties shifting across saline and non-saline soils or depth.

3.2.1. Microbial Analysis

3.2.1.1. DNA extraction protocol

In order to assess biodiversity and genetic composition these samples had DNA extracted before being analyzed using metanalysis. DNA extraction allows for just DNA to be isolated from our samples removing minerals and soil (Quiagen, 2013). An acceptable extracted DNA concentration has 280/260 ratio of 1.6-1.9 and 230/260 ratio of 1.0-1.6. DNA extractions were performed using the DNAeasy Power Soil Pro Kit protocol, with a few alterations to better suit the available lab equipment.

In the PowerBead Pro Tube 300 mg of soil and 800 µl of Solution CD1 were added before being spun in an MP tissue lyser for 20 seconds. Then the PowerBead Pro tube was centrifuged at 15,000 x g for 1 min. Then 500-600 µl of supernatant was then transferred to a

clean 2 ml Microcentrifuge Tube, then 200 µl of Solution CD2 was added and centrifuged at 15,000 x g for 1 min. Then, 700 µl of supernatant was transferred to a clean 2 ml Microcentrifuge Tube along with 600 µl of Solution CD3 before being vortexed for 5 seconds.

The contents were then transferred to an MB spin Column in increments of 650 µl before being centrifuged at 15,000 x g for 1 min and discarding the flow through. This step was repeated until all the lysate had passed through the MB Spin Column. The MB Spin Column was placed into a clean 2 ml Collection Tube along with 500 µl of Solution EA to the MB Spin Column. This was centrifuged at 15,000 x g for 1 min and flow through discarded. Then 500 µl of Solution C5 to the MB Spin Column and centrifuge at 15,000 x g for 1 min before this step was repeated again. The MB Spin Column was moved into a new 2 ml Collection Tube before being centrifuged at up to 16,000 x g for 2 min. The MB Spin Column was then placed into a new 1.5 ml Elution Tube. Then 30 µl of water was added to the center of the filter membrane, before being centrifuged again at 15,000 x g for 1 min. The MB Spin column was discarded and the DNA was nanodropped to ensure DNA quality (QIAGEN, 2019). These extractions were then standardized so that each contained 2 ng per 50 ul of DNA.

3.2.1.2. QPCR protocol

Quantitative Polymerase Chain Reaction (QPCR) analysis was performed on all the samples to find the ITS and 16s content. This allows for specific gene abundance to be determined. ITS is a rRNA present in fungi whereas 16s is a rRNA found in bacteria and archaea. A QPCR analysis can tell you the log gene abundance and gene copy number per ng of DNA. Only four depths were analyzed for QPCR; 0-15 cm, 15-30 cm, 60-91 cm, and 91-121 cm.

The QPCR analysis protocol was developed by Kim Zitnick in Dr. Banerjee's lab. First a tube of master mix was created using the following amounts per plate: 96 µl of 338 forward

primer and 518 reverse primer or 96 µl of ITS1 forward and ITS2 reverse primer depending on the analysis (16s or ITS) and , 960 µl of Mastermix, and 576 µl of water. Each well of the QPCR plate contained 2 µl of DNA Extractions or ITS/ QPCR ladder and 18 µl of the Mastermix solution mentioned previously. Three replicates were made per sample. The completed plates were placed in the QuantStudio Machine. Depending on the analysis the machine then ran the pre-made 16s or ITS temperatures and times.

3.2.1.3. Amplicon sequencing

Samples from all five depths were analyzed for sequencing and bioinformatics to examine bacterial, archaeal and fungal communities. In order to acquire amplicon sequencing data, samples were sent to the University of Minnesota Genomic Center (https://genomics.umn.edu). Various primers were used to target specific amplicons targeting the bacterial 16S rRNA gene, archaeal 16S rRNA gene and fungal ITS1 gene region. The bacterial and archaeal communities were studied by amplifying the primers 515F modified (5′-GTGYCAGCMGCCGCGGTAA -3′) and 806R (5′- GGACTACNVGGGTWTCTAAT -3′) (Walters et al., 2015). To study the fungal communities the primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA -3′) and ITS2R (5′- GCTGCGTTCTTCATCGATGC -3′) were used (Gardes & Bruns, 1993; White et al., 1990).

An Illumina 300 bp paired-end sequencing was performed at the University of Minnesota for all amplicons. In bacterial and archaeal 16S rRNA genes, the quality of R1 and R2 reads were determined using FastQC (Andrews, 2010). Once sample quality declined, the reads were trimmed by removing base pairs at the ends. These reads were then merged using FLASH (Magoˇc & Salzberg, 2011), with the range overlap being 15 and 70. FASTA format sequences were extracted from FASTQ files, and sequences < 400 bp, ambiguous bases or homopolymers

higher than 8 bp, were removed using Mothur (V1.38.0) (Schloss et al., 2009). In order to define operational taxonomic units (OTUs), sequences with a 97% similarity were clustered with the "cluster_otus" function in UPARSE, before being assessed for chimeras (Edgar, 2013).

De novo OTUs were combined from UPARSE output and chimeric OTUs to form OTU FASTA mapping files. After identifying chimeric sequences using the UPARSE outputs, they were clustered to create a sequence file for each OTU cluster. Then sequences were mapped on the OTUs to produce an OTU abundance table with the USEARCH function "usearch_global" (Edgar, 2010). After, it was classified according to SILVA v123 using the Naïve Bayesian classifier with a 60% sequence similarity requirement (Wang et al., 2007).

In the fungal ITS1 region sequences, FASTA files were extracted from FASTQ files. Using ITSx, complete ITS1 regions were extracted from R1 reads (Bengtsson-Palme et al., 2013). Any partial ITS1 sequences or sequences lacking ITS1 were removed. ITS1 sequences leftover were used for OTU picking and to create a table as described above. With the UNITE database (V6), ITS1 OTUs were classified classified in the same manner as mentioned above. (Koljalg et al., 2005).

Below is a table with some of the information gathered from the analysis. The table below features information on 16s and ITS amplicon sequences; number of reads, number of quality reads, input sequences, reads for the OTU tables, and the number of OTUs found in the analysis.

Table 5. Amplicon table.
Table demonstrating Amplicon sequencing information for both ITS and 16s rRNA. Shows number of reads, quality filtered reads, input sequences, Reads OTU table, Number of OTU.

Amplicon Sequencing	ITS	16s
Number of Reads	8558541	
Quality Filtered Reads	7508544	6495695
Input Sequences	7508544	8238596
Reads OTU table	7210629	
Number of OTUs	5950	

3.2.2. Statistical analysis

For the response variables, a two factor ANOVA analysis was conducted on the fungal: bacterial ratio, ITS, 16s, EC, inorganic carbon, organic carbon, and total nitrogen to determine differences in mean response variables across depth, salinity, or both. This was conducted to better understand the chemistry around the microbes and the microbial response. Tukey's post hoc was performed when a significant result arose in the analysis of variance.

To analyze the amplicon results, the amplicon data was combined with the QPCR and general data to create three metadata set, one for archaea, bacteria and fungi groups. These new datasets had blanks removed before the Shannon diversity index, Chao1, and Richness were calculated to measure alpha diversity. Alpha diversity is the measurement of the number of taxa and the relative abundance of the taxa (Walters, 2020). The Shannon diversity index accounts for both richness and evenness of a population (Kim, 2017) . Chao is a non-parametric method of determining the number of species in a community with the idea that more can be gained from accounting for rare species (Kim, 2017). Richness is the number of species in the community (Pyron, 2010). All three were run to develop a bigger picture of the alpha diversity of the specific

domains. A two-factor ANOVA was then run on the Shannon diversity index, Chao1, and Richness to determine what factors; salinity, depth, or both had different means of the alpha diversity measurements. The Chao1 and Richness figures mirror closely the trends seen in our Shannon Diversity Index figure, so they were placed in the Appendix section.

A Principal Coordinate Analysis (PCOA) was used to determine the clustering of archaea, bacteria, and fungal groups as a measure of beta diversity. Beta diversity accounts for communal composition per sample in the habitat (Walters, 2020). PCOA are useful in that they can help create a gradient analysis for ordination methods within the communal composition (Manly & Navarro, 2017) This was then used to create a PCOA plot to visualize the clustering across salinity and depth. Lastly, a PERMANOVA or permutational multivariate analysis of variance, was performed on all three microbial group datasets to determine the spread of the samples as a result of salinity, depth, or both. PERMANOVA is a distance-based method that tests the association of microbial covariates, it uses a distance matrix to separate the diversity based on sources of variation, in our case salinity, depth, and their interaction as dependent variables (Tang, 2016).

Rstudio was used or statistical analysis on both the QPCR, response variables, and Amplicon data were (RStudio Team, 2021). The packages used for the QPCR analysis were ‘ggplot2’ (Wickham, 2016), ‘tidyr’ (Wickham and Girlich, 2022), ‘dplyr’ (Wickham et al., 2022), ‘readxl’ (Wickham and Bryan, 2022), ‘scales’ (Wickham et al., 2022). The packages used for Amplicon analysis were ‘vegan’ (Oksanen et al., 2022), phyloseq (McMurdie and Holmes, 2013), tidyverse (Wickham et al., 2019), and microbiome (Leo Lahti et al., 2012-2019).

3.3. Results

3.3.1. Soil chemical properties

We expected that salinity would increase as soil depth increased in our field soils. Summaries of field-measured EC are listed in Table 6 for the purpose of describing the field soils. In general, salinity slightly increases with depth in non-saline soils, and remains high or slightly decreases with depth in saline soils. The EC for all depths is also well under what is considered saline by the U.S. Salinity Lab at 4.0 dS/m (Seelig 2000; U.S. Salinity Laboratory Staff, 1954). For certain crops like corn, which was a rotational crop for our test fields, this EC would result in decreasing crop yield (Butcher, 2016; Maas, 1997). After analyzing this trend and for the sake of simplicity, microbial data from all fields were pooled for analyses.

Table 6. EC measurements across depths by field.
Mean and standard deviation for EC1:1 across depths for all fields combined (n =16) and within each field site (n = 4).

Electrical Conductivity (EC1:1) dS/m		
Depth (cm)	**Non-saline**	**Saline**
All Fields		
0-15	0.27 (0.08)	2.26 (0.77)
15-30	0.29 (0.10)	2.13 (0.72)
60-91	0.63 (0.40)	1.87 (0.80)
91-121	0.76 (0.49)	1.85 (0.82)
Aneta		
0-15	0.25 (0.15)	2.15 (0.24)
15-30	0.20 (0.06)	2.12 (0.33)
60-91	0.36 (0.14)	1.61 (0.59)
91-121	0.48 (0.17)	1.56 (1.36)
Eldridge		
0-15	0.22 (0.02)	2.84 (0.20)
15-30	0.29 (0.08)	2.48 (0.37)
60-91	1.14 (0.29)	1.96 (0.55)
91-121	1.33 (0.55)	2.10 (0.42)

Table 6. EC measurements across depths by field (Continued).

Midway		
0-15	0.27 (0.09)	2.16 (0.82)
15-30	0.3 (0.12)	2.19 (1.00)
60-91	0.6 (0.38)	1.63 (0.62)
91-121	0.77 (0.44)	1.73 (0.60)
Northwood		
0-15	0.35 (0.08)	1.88 (1.22)
15-30	0.37 (0.04)	1.75 (1.01)
60-91	0.42 (0.14)	2.29 (1.31)
91-121	0.47 (0.23)	1.99 (0.86)

Additional soil chemical characteristics, provided for descriptive purposes, are listed in Table 7. In general, soil properties were similar across saline and non-saline sample locations. pH and inorganic carbon increased with depth, while organic carbon decreased with depth. Total nitrogen slightly decreased in the shallow soil depths measured.

Table 7. Soil chemical properties across depth.
Table depicting mean and standard deviation across depths and saline/ non-saline soil for pH, total nitrogen, inorganic carbon, organic carbon.

	pH		**Total Nitrogen**		**Inorganic Carbon**		**Organic Carbon**	
Depth (cm)	**Non-saline**	**Saline**	**Non-saline**	**Saline**	**Non-saline**	**Saline**	**Non-saline**	**Saline**
0-15	7.09 (1.14)	7.77 (0.47)	0.22 (0.04)	0.27 (0.04)	0.3 (0.49)	0.18 (0.17)	1.87 (0.54)	2.42 (0.37)
15-30	7.61 (0.89)	7.93 (0.26)	0.16 (0.05)	0.18 (0.04)	0.67 (0.91)	0.49 (0.44)	1.15 (0.59)	1.42 (0.40)
60-91	8.43 (0.36)	8.38 (0.24)	Not measured	Not measured	1.96 (0.57)	2.15 (0.59)	0.15 (0.16)	0.17 (0.12)
91-121	8.4 (0.35)	8.33 (0.16)	Not measured	Not measured	1.8 (0.46)	1.79 (0.55)	0.11 (0.09)	0.08 (0.07)

3.3.2. QPCR results

The following QPCR results present the log gene abundance for the 16s rRNA gene and the ITS rRNA gene. The 16s rRNA gene can be found in both archaea and bacteria so the results measuring the 16s gene abundance account for both of these groups. The ITS rRNA gene is found in fungi.

Figure 11 shows that the gene abundance for bacteria and archaea decreased with depths but there is a slight increase where 91-121 cm has an equal or higher gene abundance than the results from 60-91 cm depths. Gene abundance for bacteria and archaea don't appear to differ across salinity and this is also supported by our ANOVA results listed in Table 8, below.

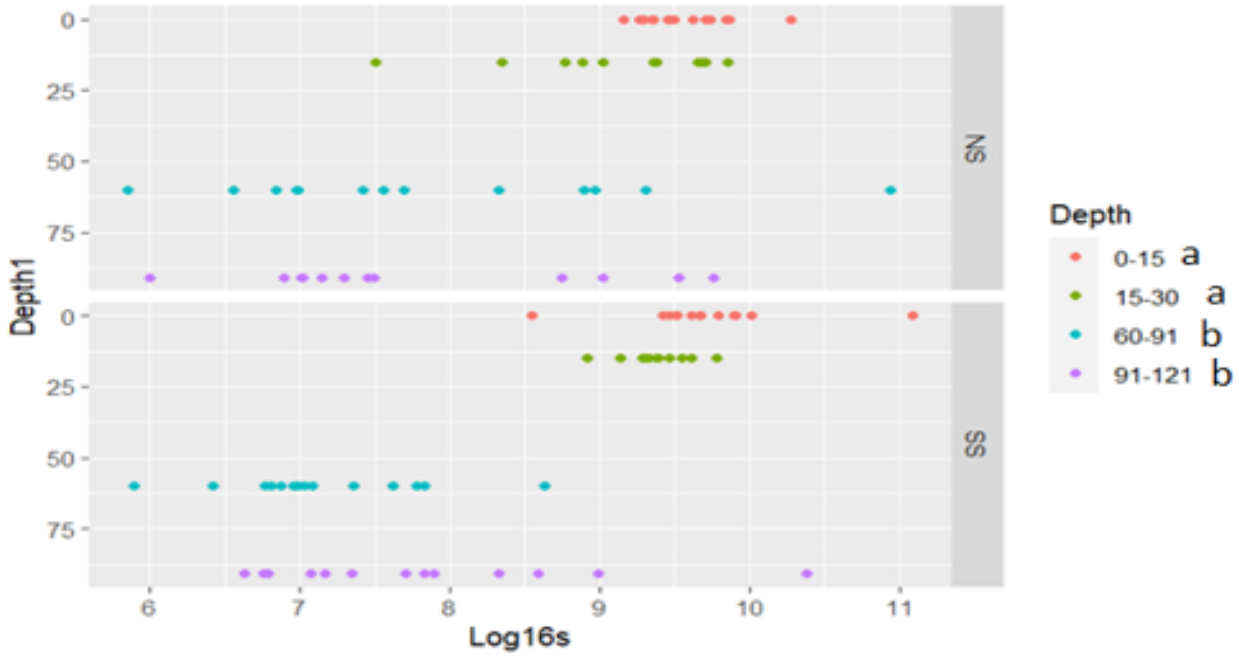

Figure 11. 16s gene abundance.
Scatterplot showing log 16s gene abundance across depths and salinity. A Two-Factor ANOVA was conducted to determine if mean 16s gene abundance was significantly different across depth and/or salinity. The depth factor was significant (p-values < 0.001). Same letters in the legend indicate depths that were statistically similar to each other, regardless of salinity level.

Figure 12 below shows that the gene abundance for fungi also decreased with depths. Similar to Figure 11, there is a slight increase where 91-121 cm has an equal or higher gene abundance than the results from 60-91 cm depths. Similarly, to bacterial gene abundance, fungal gene abundance was not effected by salinity, which can be seen in Table 8., in our ANOVA results and in Figure 12.

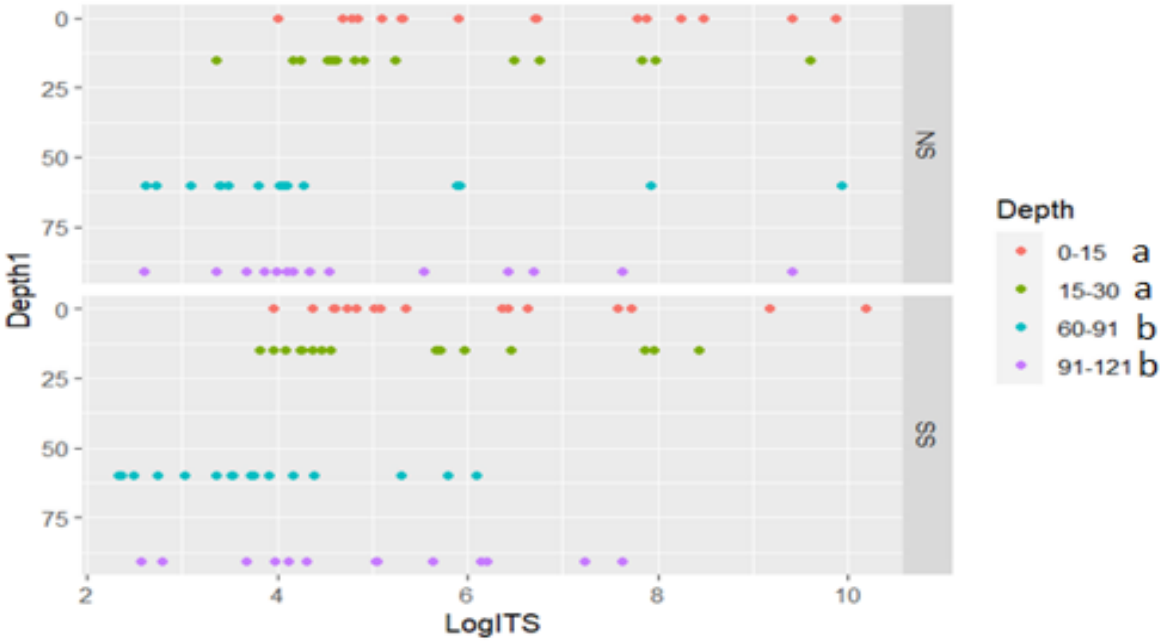

Figure 12. ITS gene abundance.
Scatterplot showing log ITS gene abundance across depths and salinity. A Two-Factor Anova was conducted to determine if depth and/or salinity was significant in influence ITS gene abundance. Depth had a significant p-value p-values < 0.001. The a and b in the legend represent depths that were statistically similar to each other.

The fungal bacterial ratio, which is represented here as the ITS gene abundance divided by 16s gene abundance, did not significantly change across salinity levels, but the depth factor was significant (p-value was < 0.001) as seen in Table 8.

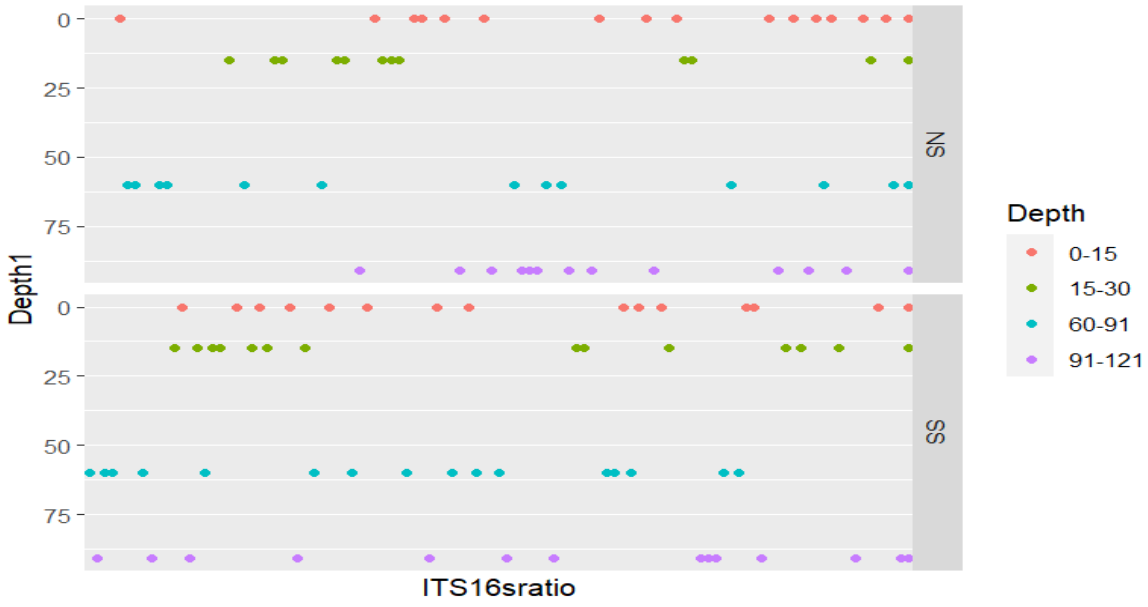

Figure 13. Fungal: bacterial ratio.
Scatterplot showing Log ITS to Log16s ratio across depths and salinity. (X axis is 0 to 1).

A two factor ANOVA was run on the 16s (bacterial and archaeal) data and ITS (fungal) data collected via QPCR specifically on the log gene abundance. The goal of this was to evaluate the abundance of different microbial groups in each salinity level and within the soil profiles. It was determined that salinity did not have a significant effect on LogITS or Log16s numbers but depth on the other hand did play a significant role on both. (p-value <0.001, Table 8). As depth increased the bacterial and archaeal 16s gene abundance decreased. The p-value for ITS also demonstrated that increasing depth decreased the fungal gene abundance more than salinity alone (Table 8).

Table 8. Log gene abundance results.
Two Factor ANOVA results table for log gene abundance for 16s and ITS across salinity and depth. An asterisk (***) is used to highlight p-values < 0.001.

		Df	Sum Sq	Mean Sq	F value	Pr (>F)
Log16s	***Salinity***	1	1.18	1.18	0.72	0.42
	Depth	3	95.74	31.91	46.31	< 0.001***
	Salinity:Depth	3	4.19	1.40	2.03	0.12
	Residuals	101	69.60	0.69		
LogITS	***Salinity***	1	5.3	5.29	1.79	0.18
	Depth	3	77.7	25.90	8.77	< 0.001***
	Salinity:Depth	3	2.3	0.77	0.26	0.84
	Residuals	113	333.7	2.95		
Fungal/Bacterial	***Salinity***	1	0.05	0.05	1.40	0.24
ratio	***Depth***	3	0.19	0.06	1.90	< 0.001***
	Salinity:Depth	3	0.04	0.14	0.44	0.73
	Residuals	98	3.13	0.03		

3.3.3. Amplicon results

3.3.3.1. Alpha diversity

Shannon diversity is a measurement of alpha biodiversity that accounts for both richness and evenness. ANOVA and post-hoc tests were run to determine what depths and salinity were most similar in diversity, which was then recorded on the Shannon Diversity figures. A post-hoc was also run to compare what depths had significant differences in biodiversity on the Shannon Diversity Index, Chao1, and Richness results. These results can be found in Figures 10-14 (Appendix B).

3.3.3.1.1. Archaea

Unfortunately, archaeal alpha diversity could not be reported. Due to the procedure used for Amplicon sequencing, out archaeal reads were incredibly low after being separated from bacterial reads. Archaea were not sequenced separately a subset of 515-806 was used to find archaeal ASVs from SILVA Archaea, causing archaeal reads to be low.

3.3.3.1.2. Bacteria

The non-saline samples had a higher mean Shannon diversity of 5.6, compared to saline samples that had a mean of 5.3 (p-value <0.05). With bacterial communities, Shannon diversity decreased with increasing depth (p-value <0.05) and slightly increased in the deepest depth (91-121 cm) compared to the second deepest depth (60-91 cm), although they are still statistically similar (Figure 14).

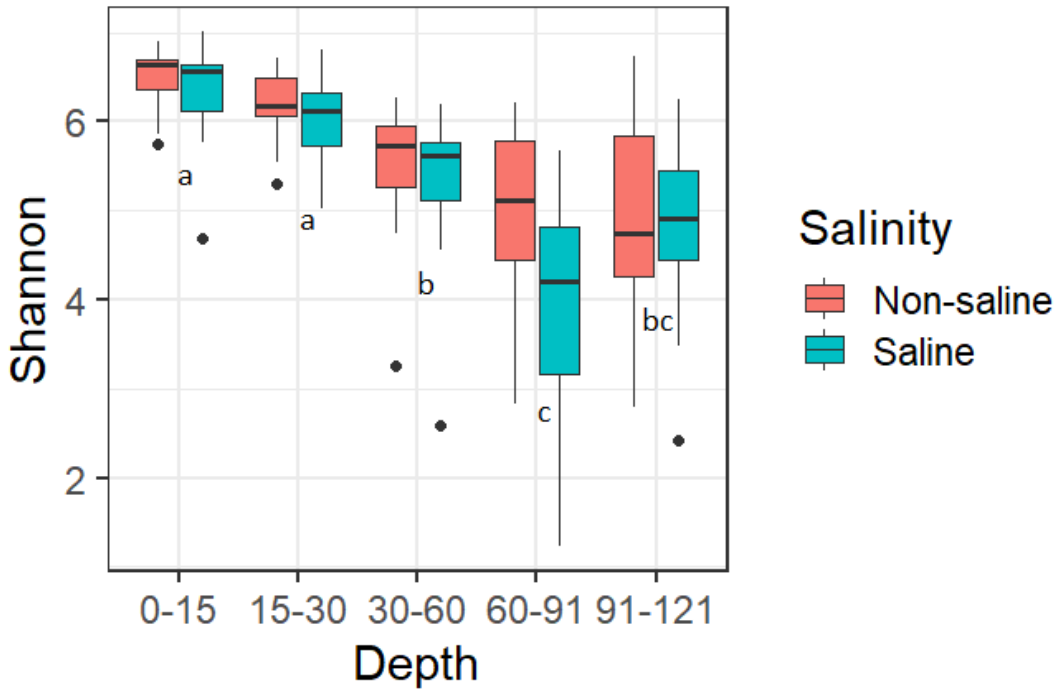

Figure 14. Bacterial Shannon diversity.
Boxplot demonstrating bacterial Shannon diversity index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).

3.3.3.1.3. Fungi

Fungal diversity decreased with increasing depth (p-value <0.05), and were also impacted by salinity (p-value < 0.05). Saline samples had a lower mean diversity index of 3.1 in comparison to non-saline samples that had a mean of 3.2.

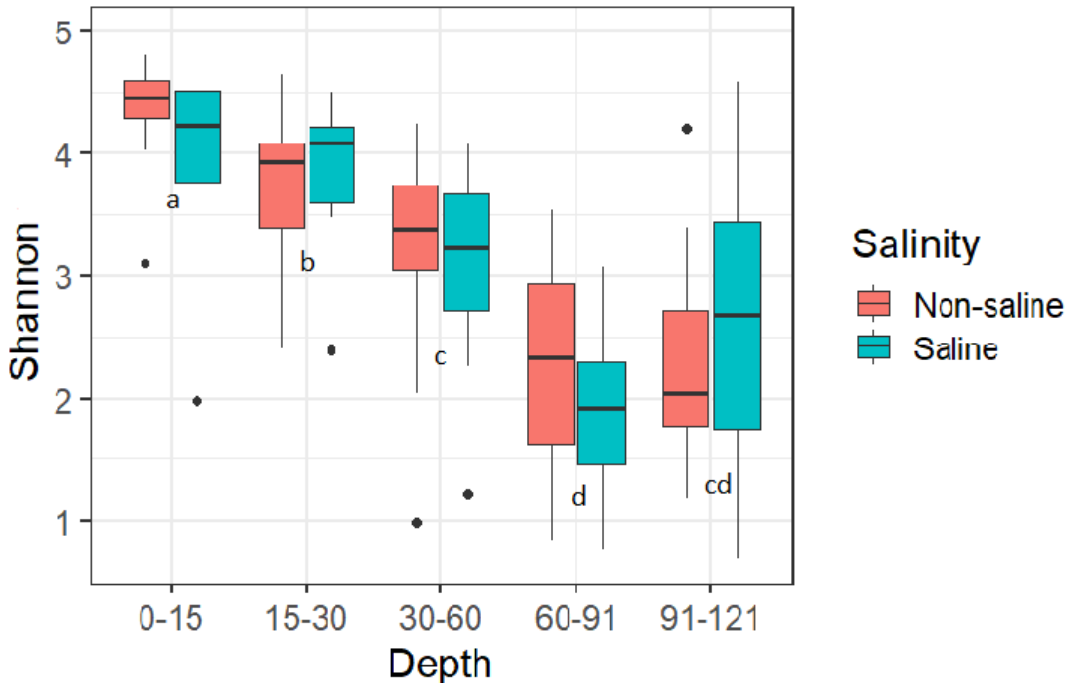

Figure 15. Fungal Shannon diversity.
Boxplot demonstrating fungal Shannon diversity index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).

3.3.3.2. Communal diversity

A Principal Coordinate Analysis (PCOA) Plot was created to visualize the maximum amount of variation present in the dataset by plotting ordinations for each individual community. Individually, each of the communities: bacterial and fungal, did not demonstrate distinct community clusters across depth and salinity.

3.3.3.2.1. Bacteria

When looking at the second cluster plot below, Figure 16., we can see some distinct clustering of the bacteria at the 0-15 cm and 15-30 cm depth keeping relatively to one end of the figure, but the other depths like 60-91 cm and 91-121 cm have less distinct clustering. The communities at 30-60 cm in depth are mostly clustered towards the bottom of the graph the community is made up of members interspersed with the shallower depth communities then with the communities in the deeper depths. This may show that this mid-depth bacteria are more similar to shallower bacteria but have some similarities with deep bacteria.

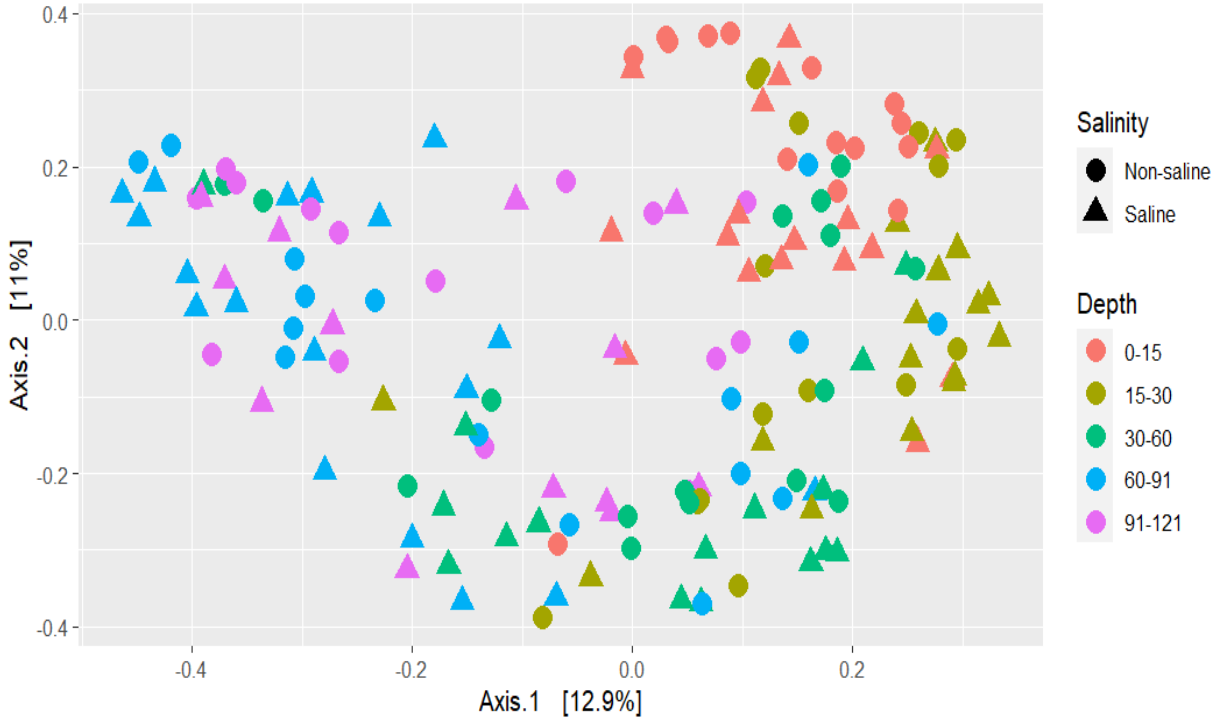

Figure 16. Bacteria PCOA.
Clusterplot demonstrating the variability and dispersion of bacterial microbes across samples.

3.3.3.2.2. Fungi

In Figure 17, there is also distinct clustering for the shallow 0-15 cm and 15-30 cm depth organisms but the deeper fungal communities are less distinct. Salinity appears to have less impact on the clustering as also seen above. The other depths: 15-30 cm, 30-60 cm, 60-91 cm, 91-121 cm, are less distinct being interspersed across the graph.

Figure 17. Fungi PCOA.
Clusterplot demonstrating the variability and dispersion of fungal microbes across samples.

3.3.3.3. Permanova

A permanova or permutational multivariate analysis of variance. It is a non-parametric multivariate statistical test, that also measures beta diversity. The ultimate goal is to compare cohorts of samples and determine if the null hypothesis that the centroids and dispersion of the cohorts as identified in the PCOA, are equivalent for all cohorts. The permanova (Table 9), shows variance between salinity, depth, and the factors combined and how they influence the beta diversity for the bacterial and fungal microbial domains.

In both fungal and bacterial communities both depth and salinity had p-values < 0.001, demonstrating that individually increasing depth and increasing salinity decreased variance.

Table 9. Permanova results.
Permanova table for bacteria and fungi across salinity and depth. An asterisk (***) is used to highlight p-values < 0.001, and (**) is used to highlight p-values <0.01.

		Df	Sum Sq	R2	F value	Pr (>F)
Bacteria	***Salinity***	1	1.35	0.02	4.06	<0.001***
	Depth	4	7.74	0.12	5.81	<0.001***
	Salinity:Depth	4	2.00	0.03	1.50	0.0028**
	Residuals	148	49.28	0.82		
Fungi	***Salinity***	1	1.02	0.02	2.83	<0.001***
	Depth	4	5.83	0.09	4.03	<0.001***
	Salinity:Depth	4	1.54	0.024	1.06	0.26
	Residuals	148	53.5	0.86		

3.4. Discussion

This experiment showed how salinity and depth work in tandem to affect organisms. The salinity across our samples was relatively low, falling under 3.0 dS/m; this EC level is less than what is considered saline by the U.S. Salinity Laboratory (U.S. Salinity Laboratory Staff, 1954).

The saline sampled fields did not demonstrate a strong trend of becoming more saline deeper into the vertical profile (Seelig 2000). Our initial hypothesis assumed that rising salinity in the deeper soils would influence or decrease microbial diversity, but our ability to evaluate it was limited by the narrow range of salinity observed in the soil profile. The EC for the saline samples decreased in the deeper depths instead. In our non-saline samples, the EC did increase as depth increased. The variability of the EC values and trends may explain why for all our tests,

depth played a more significant role in decreasing the microbial biodiversity and abundance present in the soil.

With such a low EC, the shifting of other soil factors across depth likely led to changes in the log gene abundance of bacterial/ archaeal 16srRNA and fungal ITSrRNA. Gene abundance decreased as depth increased, the shallower depths 0-15 cm, 15-30 cm, 30-60 cm, having a higher gene abundance than the deeper depths 60-91 cm and 91-121 cm. These trends can be seen for bacterial/ archaeal gene abundance (Figure 11.), and fungal gene abundance (Figure 12.). In Table 5. and Table 6., soil characteristics like EC, pH, total nitrogen, inorganic carbon, and organic carbon averages shifted with depth. Deeper in the profile, the soil became more basic and lost nitrogen and carbon. These are all factors that contribute to an organism's survival, especially with microbes.

When looking at individual microbial groups, bacteria maintained higher Shannon Diversity Index across depth (Figure 14.) when compared to fungal (Figure 15.). According to alpha diversity, bacteria were more diverse and distinct than fungal communities. It is unfortunate that our tests on archaea did not yield results, but this has been a teaching moment that can be applied to future archaeal research about the ineffectiveness of 515-806 subset in accurately separating archaea from bacteria.

Salinity may not have impacted gene abundance but it did influence alpha and communal diversity. Both fungal and bacterial communities showed that both depth and salinity were important factors for influencing biodiversity.

Our studies like many other studies have found that salinity affects microbial diversity but not biomass or abundance. This may be because their salt concentrations were higher or had a more significant range than what was seen in our experiment (Canfora et al., 2014; Elmajdoub

et al., 2013; Jakubowski, 2021; Zhao et al., 2018.) This could explain why the gene abundance analysis showed that salinity was not a significant factor. When comparing our results to other studies, we see that the changing chemical properties may have impacted the microbes more than salinity; for example, one study found that salinity and pH dictated the dominant bacterial phyla (Zhao et al., 2018).

Other depth studies heavily mirror our findings that depth declined fungi: bacteria ratio and bacterial/fungal diversity (Xu et al., 2021). A few studies even explain trends we saw with fungi fairing the worst out of the three microbial groups, as fungi may be more challenging to distribute spores in deeper depths (Becerra et al., 2014). When comparing our study to these, it is also important to understand that our study analyzed samples from far deeper depths, going as far as 121 cm compared to 40 cm (Becerra et al., 2014) or 70 cm (Xu et al., 2021). Only a few studies have gone further and found that with depth, chemical factors like pH shifted significantly and were likely tied to the exponential decrease in microbial biomass and soil carbon (Eilers et al., 2012).

3.4.1. Pitfalls

Methods for determining saline soil in fields were limited to surface-level tests like surface level EC measurements and crop history. By farmer standards, our test fields for our saline samples were saline. Crops planted there in the past, for example corn, were experiencing yield decreases due to salinity (Butcher, 2016). The salinity history of the fields is part of why they were chosen for sampling. At 3.0 dS/m, corn crops would experience a significant decrease yield despite the EC level being well under the salinity standard of 4.0 dS/m (U.S. Salinity Laboratory Staff, 1954; Butcher, 2016). This issue may have resulted in choosing fields that, at a

glance, appear to struggle with salinity, but actually have low salinity levels and ranges across depths.

Recreating this experiment in a more controlled environment, like a laboratory setting with mesocosms, may allow for a more controlled salinity trend across the soil profile. EC could be increased to 4 dS/m, to see if there is a more significant impact on the three microbial domains from salinity. A controlled environment would also reduce the effects of other soil characteristics like pH, nitrogen, and carbon and can solely focus on the influence of EC and depth.

Archaea are also still difficult to separate from bacteria due to their similarities (Woese et al., 1990). As technology advances, perhaps distinguishing between the two in an amplicon sequencing analysis will become more accurate.

3.4.2. Significance

Limited research examines microbes at such a depth, let alone when paired with salinity (Alejandra et al., 2014; Ries et al.,2020). There are even fewer experiments that focus on more than one microbial domain. The number of studies becomes even smaller when you try to find these microbial studies in the Great Plains region (Ries et al., 2020). Very few studies consider all these factors, which is why this research is crucial in better understanding how microbes adjust to shifting soil conditions, especially salinity, which is a massive issue for this area (Hadrich, 2012; Seelig, 2000).

Although the results was not expected, neither were the field conditions. This variability of the EC demonstrated that soil conditions do not always follow expected trends, mainly because the causes behind salinity are variable. Improvements in measuring deep soil chemical properties are crucial in exploring soil depth for future field studies. This field experiment

analyzed microbial communities in a real-world environment, where several factors, including depth and salinity, work in tandem to host and hinder life.

3.5. References

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4. GENERAL CONCLUSION

The overall goal of this thesis was to study how soil organisms, microbes and earthworms responded to the unique salinity of the Northern Great Plains. This was achieved through two separate studies utilizing both field and laboratory techniques.

For earthworms, our goals were to monitor the survivability and production and choice of *Apporectodea* earthworms in saline soils in the presence or absence of OM. We addressed these objectives by designing three different mesocosm experiments.

The first experiment focused on the survivability and production of earthworm adults, cocoons, and juveniles across increasing salinities with or without added OM. This was assessed by creating 30 small soil treatment mixtures with five different salinity levels: 1, 2.6, 3.3, 3.5, and 4.5 dS/m. Half of these containers had elevated OM. At the beginning of the experiment three adults were placed in each container and after six weeks, cocoons, dead, and adults were counted. The survivability and production did not significantly change across salinity but in adults, survivability and production did decrease in treatments with elevated OM.

The second experiment aimed to measure earthworm behavior between saline and non-saline soils when given a choice. Tubs contained two different treatments, a control with only non-saline soil, and an experimental treatment with a non-saline soil on one side and saline soil on the other. Thirty earthworms were added and allowed to roam freely throughout the mesocosms for a month. After this time cocoons, juveniles, and adults were counted for each side (non-saline and saline.) Earthworms preferred non-saline soil, which affirmed previous research (Gasch et al., 2021).

The third experiment mirrored the second experiment to measure earthworm behavior between saline and non-saline soils, in the presence and absence of added OM. In order to

evaluate the effect additional OM had on choice, four split-bin treatments were created as follows: saline and non-saline side, each with added OM, non-saline soil with no OM addition and a non-saline soil with added OM, non-saline soil and added OM content, and a side with saline soil with no added OM, and the last experimental treatment consisted of a non-saline soil with no added OM and a saline side with added OM. Once again 30 earthworms were placed in each bin, and at the end of a month; cocoons, juveniles, and adults were counted. Earthworms preferred non-saline soil with added OM primarily, followed by non-saline soil with normal OM, then thirdly saline soil with added OM. The normal OM/saline soil treatment was the earthworm's least favorite environment.

With microbes, we wanted to see how depth and salinity influenced the abundance and diversity of the three main microbial domains. From fields near Aneta, Eldridge, Midway, and Northwood, North Dakota, 160 soil samples were analyzed in a lab from non-saline soil and saline soil at the following depths: 0-15 cm, 15-30 cm, 30-60 cm, 60-91 cm, and 91-121 cm.

To address microbial abundance shifts across depth and salinity, Quantitative Polymerase Chain Reaction analysis was performed on the samples to find gene abundance. An ITS QPCR found fungal genes, while a 16s QPCR found bacterial and archaeal genes. While salinity didn't have a significant impact on gene abundance, depth did. Shallower depth: 0-15 cm and 15-30 cm had more microbial gene abundance than deeper depths: 60-91 cm and 91-121 cm.

With biodiversity, both alpha and beta diversity were calculated on amplicon data results. For both alpha and beta diversity of bacteria, and fungi, both salinity and depth were significant factors. Interestingly, bacteria maintained a higher Shannon biodiversity index than fungi, and showed that the combination of salinity and depth were influencing the communal diversity for bacteria.

Understanding how salinity influences crops is crucial to keeping North Dakota's economy afloat, but neglecting the responses of other organisms in saline soil is equally detrimental. Agriculture is a major component of the Great Plains region, not just economically but culturally, many people rely on our crops, but salinity research cannot ignore organisms that play a major component in agriculture. This exclusion is not just an issue for the Great Plains but is consistent with how we measure salinity across the United States (US Salinity Laboratory, 2022). The current measurements for salinity do not account for many of the organisms that call soil home. In order to better understand salinity, salinity standards should be specified according to organism. This sentiment was supported by both experiments.

In our choice experiments, we found adults venturing to saline soil that reached 6 dS/m. While earthworms despise saline soil, they could easily thrive in salinity levels of 4.5 dS/m without OM. Cocoons, adults, and juveniles showed no significant mortality changes across levels of salinity. Many plants would be unable to survive these higher salinity levels, but we noted no significant changes in the *Apporectodea's* survival rate. Other experiments have echoed this, with one field experiment finding cocoons and juveniles in saline soils (Gasch et al., 2021). The *Apporectodea* earthworm genus seems well adjusted not only to North Dakota's unique salinity but to higher levels of salinity as well.

In the microbial experiments, fields did not follow the expected trends of salinity increasing deeper into the vertical soil profile (Seelig, 2000). While increasing depth did influence our results, salinity did not always increase with increasing depth, as seen in our saline samples. Other chemical properties like pH, carbon, and nitrogen displayed stronger changes through depth than EC. As a result, salinity did not alter microbial abundance or biodiversity as significantly as depth. How we determine salinity in field experiments also makes it challenging

to understand salinity across depths. By the standards of the farmers managing the field, the field are saline. The EC levels are high enough, 3.0 dS/m, to harm crop yields and influence the biodiversity of microbes but not their gene abundance.

In this research, we have discovered limitations in how salinity research is conducted and understood, but we have also learned more about soils in North Dakota. Our salinity is unique and interesting; even the smallest organisms have a unique reaction across the whole soil profile. Even though *Apporectodea* earthworm species are non-native to the Great Plains area, they have adjusted and can thrive in our salty soils (Edwards et al, 1996; Edwards et al, 2004; Hale, 2007; Reynolds et al, 1977). This research has painted a fuller picture of the saline soil profile, but more organisms still need to be addressed concerning their reaction to varying salinity levels or types.

In this thesis we have concluded that:

1. Soils that are considered saline based on plant tolerance may not be detrimental to other organisms.
2. High salinities of 4.5 ds/m and added organic matter did not affect earthworm survival and production, growth, and cocoon deposition.
3. Earthworms favored non-saline soil even with added organic matter in saline soil.
4. Salinity levels greater than 3 ds/m did not impact gene abundance in microbes but did influence biodiversity.
5. Depth decreased biodiversity and abundance in all three microbial domains.

Hopefully, this study encourages farmers and other researchers to look at the Great Plain's soil on a larger scale than crops and yields. Soils are vast ecosystems, and everything,

whether biotic or abiotic, plays a role in sustaining life. In order to keep our North Dakotan soil healthy, we need to ensure all the members of this complex community can survive and thrive.

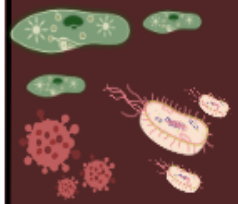
Organism	What we know:	Questions:	Approaches:	Hypothesis:	Conclusion:
Plants 	• salinity limits nutrients uptake and growth • salinity causes weaker root growth • 4 ds/m EC1:1 is considered saline • salinity can causes a decrease in yield	• Is 4 ds/m a fair salinity standard for other organisms?	• Conduct studies on other soil organisms and examine how they respond to salinity.	• Earthworms and Microbes will react differently to higher salinities in comparison to plants.	• Soils that are considered saline for plants may not significantly deter the growth of earthworms and microbes.
Earthworms 	• salinity limits ability to absorb nutrients and limits growth • salinity increased ion toxicity • salinity limits cocoon growth • salinity decreases vermicast production	• Can organic matter alleviate salinity pressure? • Can organic matter sway earthworm habitat preference?	• Examine earthworms survivability in saline soils with and without organic matter. • Examine earthworms preference in saline soil with and without organic matter.	• Earthworm survivability will decline in higher salinities but added organic matter will improve these survival rates. • Earthworm preference will lean heavily towards accessibility of organic matter even in saline soil.	• High salinities of 4.5 ds/m and added organic matter did not affect earthworm survivability. • Earthworms favored non-saline soil even with added organic matter in saline soil.
Microbes 	• salinity creates osmotic pressure on microbial cells • salinity limits ability to absorb nutrients • salinity limits population and biodiversity	• How do specific microbial domains respond to salinity and depth? • When paired with depth how does salinity affect microbial diversity and abundance?	• Examine domain specific abundance across salinity and depth? • Examine domain specific biodiversity across salinity and depth?	• Single celled organisms like bacteria and archaea will adjust better to deeper depths. • Multicellular organisms like fungi will thrive better in saline soils. • Overall biodiversity and abundance will decrease in saline and deeper soils.	• Salinity levels > 3 ds/m did not impact gene abundance in microbes but did influence biodiversity. • Depth did decrease biodiveristy and abundance in all three microbial domains.

Figure 18. Final thesis conceptual model.
Demonstrates what is known about salinity, questions we hope to answer, approaches, general hypotheses, and general conclusions in relation to plants, earthworms, and microbes. This conceptual model aims to reiterate the key messages, themes, and findings found in this thesis.

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APPENDIX A. SOIL SALINITY AND OM RECIPES FOR EACH EXPERIMENT

The first objective's tubs contained the following salt masses to create the EC1:1 values of 1, 2.6, 3.3, 3.5, and 4.5 dS/m. For every 200 grams of soil, roughly 10 grams of OM was added to the OM+ treatments.

Table A1. First objectives soil mixtures.
Depicts salt, OM, and soil mixture for tubs used in the first objective experiment. The experiment included 30 tubs (3 replicates for each treatment) and was repeated twice, with fresh materials in each round.

Tubs	EC	Treatment	Soil Mass	Salt Mass	Organic Matter
1,2,3	0 EC	0gOM-	200 g	0g	0g
4,5,6	0 EC	0gOM+	200 g	0g	9.349g
7,8,9	2.6 EC	0.04gOM-	200 g	0.04g	0g
10,11,12	2.6 EC	0.04gOM+	200 g	0.04g	9.349g
13,14,15	3.3 EC	0.08gOM-	200 g	0.08g	0g
16,17,18	3.3 EC	0.08gOM+	200 g	0.08g	9.349g
19,20,21	3.5 EC	0.12gOM-	200 g	0.12g	0g
22,23,34	3.5 EC	0.12gOM+	200 g	0.12g	9.349g
25,26,27	4.5 EC	0.14gOM-	200 g	0.14g	0g
28,29,30	4.5 EC	0.14gOM+	200 g	0.14g	9.349g

For the second objective experiment, two grams of salt for every 100 grams of soil was used to raise the salinity of the test soil to 4.5 dS/m. Our mesocosms consisted of 68,026.7 grams of soil, which required 1361 grams of the salt mixture.

Table A2. Second objective soil mixtures.
Depicts salt and soil mixture for tubs used in the second objective experiment. The experiment included 6 tubs (3 control, and 3 split).

Tub #	Treatment	Side	Soil mass	Salt Mass
1	Control	Control	29.56kg	0
2	Control	Control	29.52kg	0
3	Control	Control	29.54kg	0
4	Non-saline/Saline	Non-saline	15.04kg	0
4	Non-saline/Saline	Saline	15.04kg	278.61g
5	Non-saline/Saline	Non-saline	15.04kg	0
5	Non-saline/Saline	Saline	15.04kg	278.61g
6	Non-saline/Saline	Non-Saline	15.04kg	0
6	Non-saline/Saline	Saline	15.04kg	278.61g

For the third objective experiment, two grams of salt for every 100 grams of soil was used to raise the salinity of the test soil to 6 dS/m. To achieve the 6-10% OM concentration, five grams was added per 100 grams of soil. Our mesocosms consisted of 68,026.7 grams of soil, which required 1361 grams of the salt mixture.

Table A3. Third objective soil mixtures.
Depicts salt and soil mixture for tubs used in the third objective experiment. The experiment included 9 tubs total.

Tub #	Treatment	Side	Soil mass	OM	Salt Mass
1	Non-saline /Non-saline +OM	Non-saline	14.77kg	0	0
1	Non-saline /Non-saline +OM	Non-saline+OM	14.06kg	703.052g	0
2	Non-saline /Non-saline +OM	Non-saline	14.77kg	0	0
2	Non-saline /Non-saline +OM	Non-saline +OM	14.06kg	703.052g	0
3	Non-saline /Non-saline +OM	Non-saline	14.77kg	0	0
3	Non-saline /Non-saline +OM	Non-saline +OM	14.06kg	703.052g	0
4	Non-saline+OM/Saline+OM	Non-saline+OM	14.77kg	703.052g	0
4	Non-saline+OM/Saline+OM	Saline+OM	14.06kg	703.052g	265.21g
5	Non-saline+OM/Saline+OM	Non-saline+OM	14.06kg	703.052g	0
5	Non-saline+OM/Saline+OM	Saline+OM	14.06kg	703.052g	265.21g
6	Non-saline+OM/Saline+OM	Non-saline+OM	14.06kg	703.052g	0
6	Non-saline+OM/Saline+OM	Saline+OM	14.06kg	703.052g	265.21g
7	Non-saline + OM/ Saline Side	Non-saline+OM	14.06kg	703.052g	0
7	Non-saline + OM/ Saline Side	Saline	14.77kg	0	278.61g
8	Non-saline + OM/ Saline Side	Non-saline+OM	14.06kg	703.052g	0
8	Non-saline + OM/ Saline Side	Saline	14.77kg	0	278.61g
9	Non-saline + OM/ Saline Side	Non-saline+OM	14.06kg	703.052g	0
9	Non-saline + OM/ Saline Side	Saline	14.77kg	0	278.61g

APPENDIX B. ADDITIONAL ALPHA DIVERSITY FIGURES

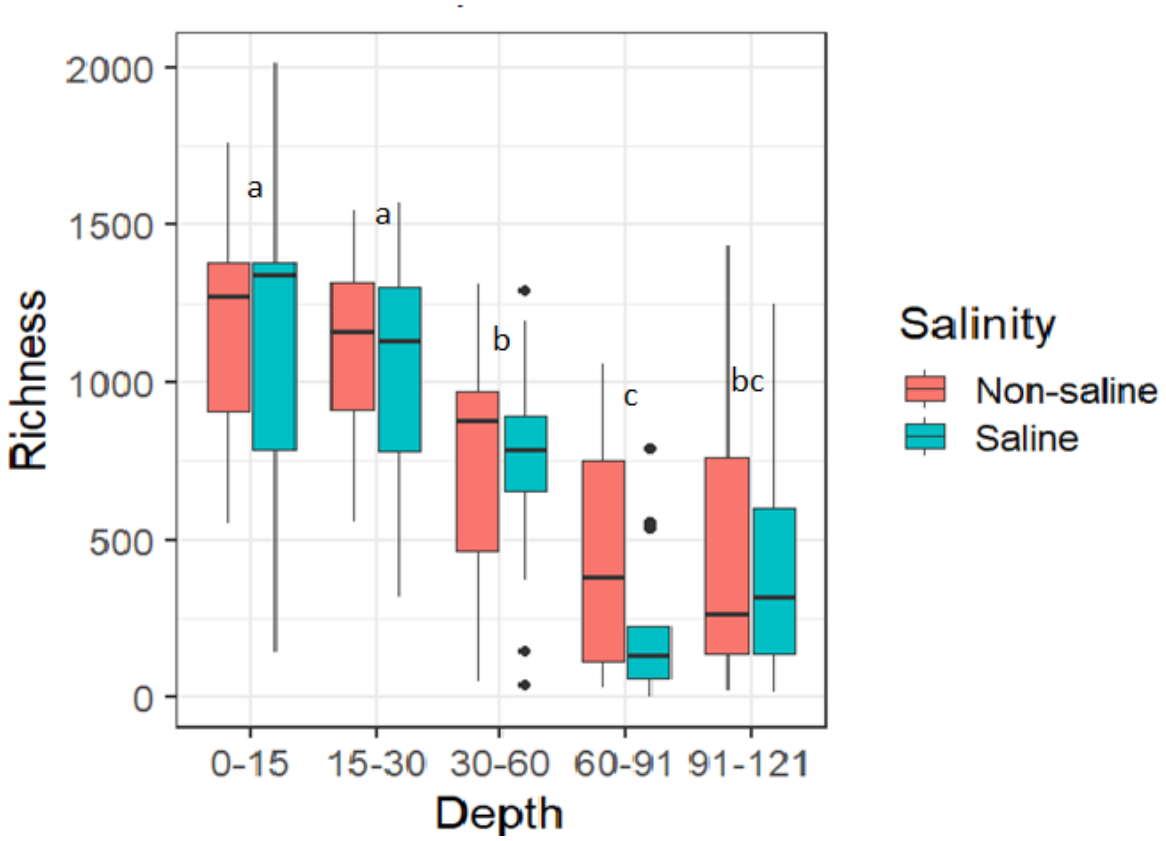

Figure B1. Bacteria richness index.
Boxplot demonstrating bacterial Richness index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).

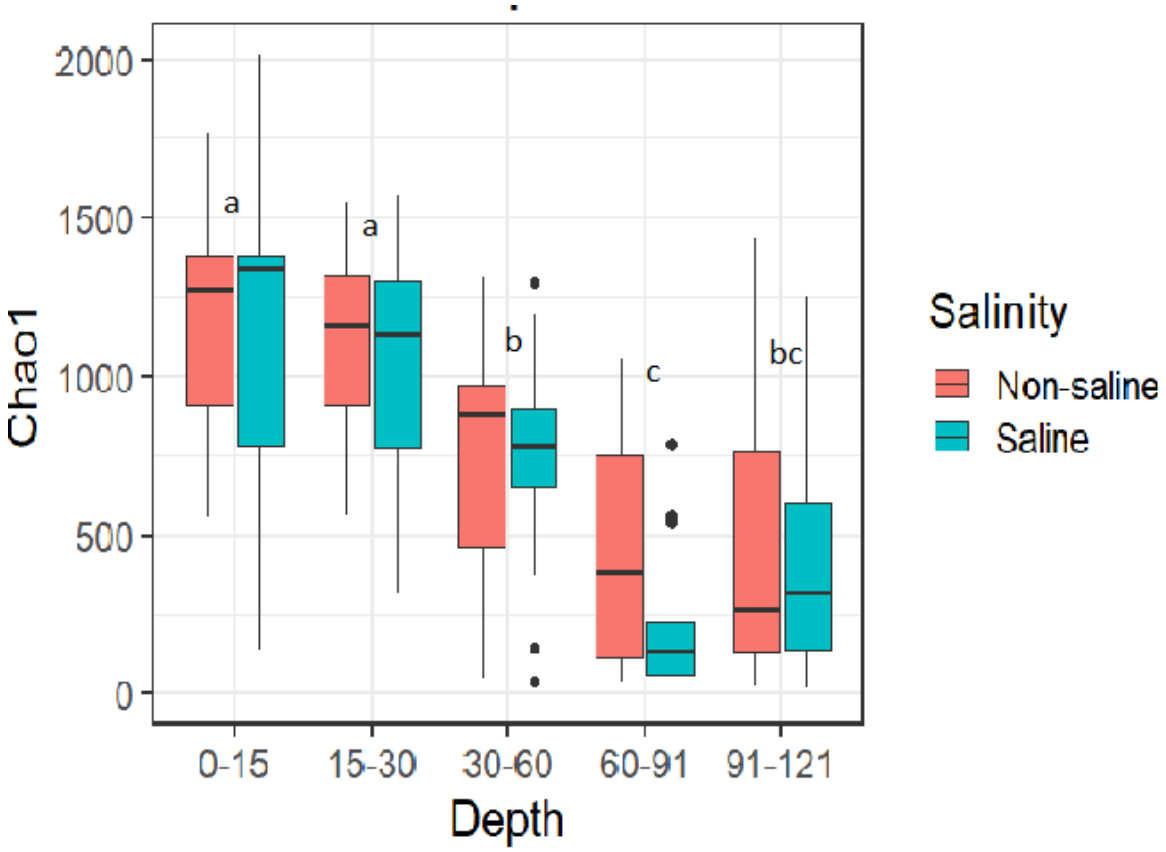

Figure B2. Bacteria chao1 index.
Boxplot demonstrating bacterial Chao1 index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).

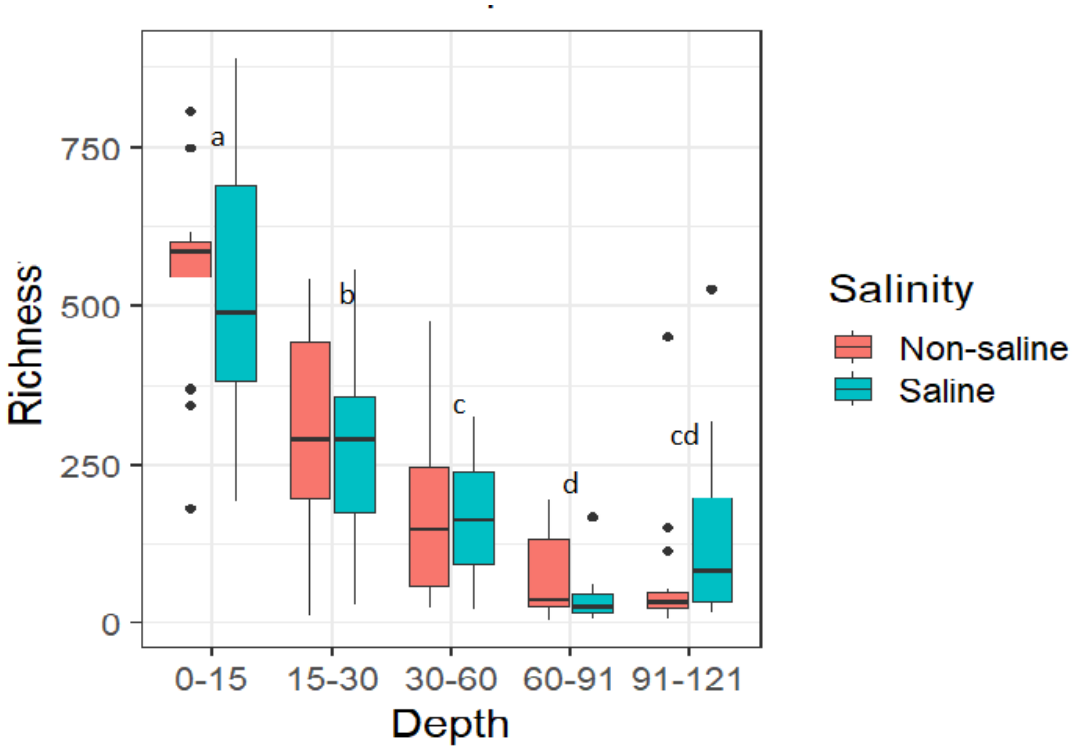

Figure B3. Fungi richness index.
Boxplot demonstrating fungal Richness index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).

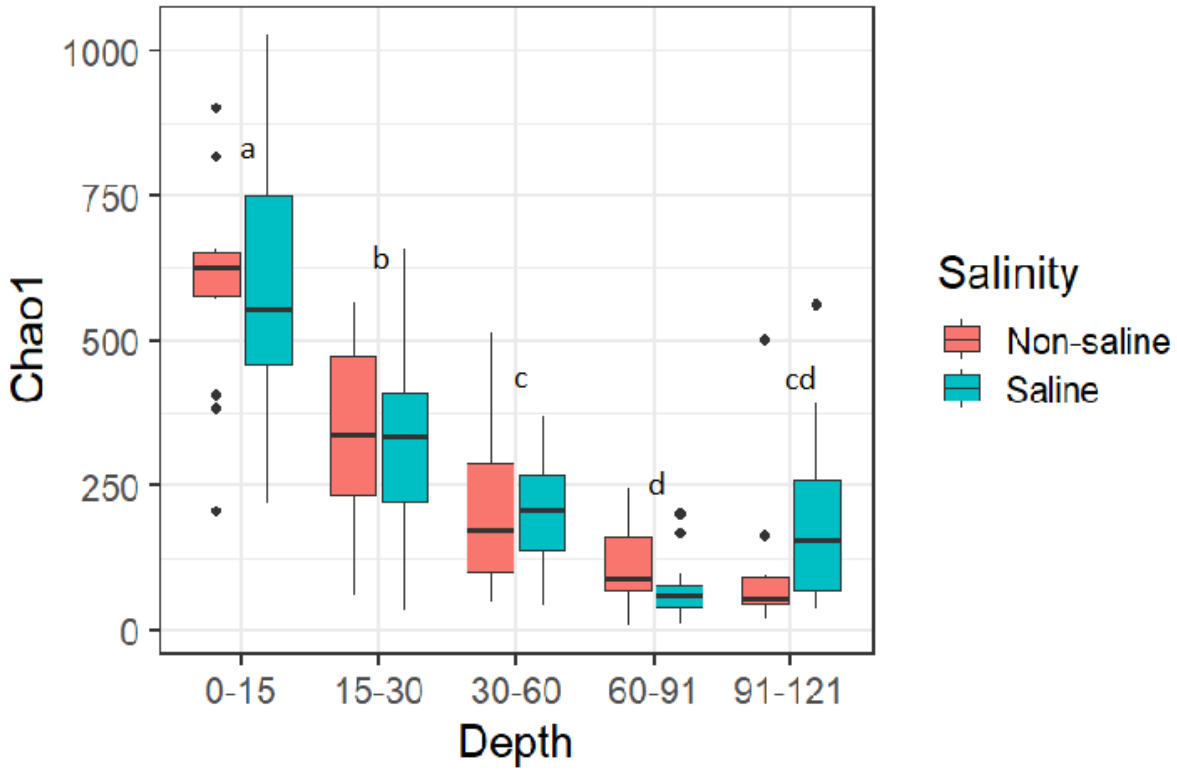

Figure B4. Fungi chao1 index.
Boxplot demonstrating fungal Chao1 index across salinity and depth, along with letters indicating significant differences across depths (not by salinity level).