Phytoremediation of contaminated soils: The effects of soil salinity on the phytoremediation rate of polyaromatic hydrocarbons by alfalfa (*Medicago sativa L.*).

by

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Abstract

Phytoremediation poses an ecologically friendly and cost-effective alternative to other remediation methods such as chemical or thermal treatment. However, in contaminated sites such as retired oil wells and brine spills, it is common to have a co-contamination of salt and polyaromatic hydrocarbons (PAHs). The co-contamination of salt and PAHs may decrease the rate and effectiveness of bioremediation. Here we investigated the effect soil salinity has on the rate of phytoremediation, plant survivability and biomass. A 90-day greenhouse study was performed, growing alfalfa (*Medicago sativa L.*) in soils treated with varying salt (NaCl) concentrations in the presence of pyrene and benzo[a]pyrene. No significant differences were observed in the presence or absence of PAHs. Salt treatments has significant affects on plant biomass, nodulation, and successful germination.

Keywords: Phytoremediation; bioremediation; polyaromatic hydrocarbons; alfalfa; salt

Dedication

To Ingrid Glossop (1937-2019). An inspiration to never stop learning.

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List of Acronyms

SFU	Simon Fraser University
LAC	Library and Archives Canada
PAHs	Polyaromatic hydrocarbons
BaP	Benzo[a]pyrene
AHR	Aryl hydrocarbon receptors
Na^+	Sodium ion
SAR	Sodium Absorption Ration
DI	Deionized water
SNNFF	Saskatchewan Pulse Growers Nodulation and Nitrogen Fixation Field Assessment Guide
SOM	Soil organic matter
CYP1A1	Cytochrome p450 1A1 gene
CYP1B1	Cytochrome p450 1B1 gene

Glossary

Bioremediation	The use of organisms including microbes, fungi, plants, and animals to remediate or remove contaminants from a site
Phytoremediation	The process of using plants and their associated rhizosphere to degrade or accumulate contaminants
Mycoremeditaion	The process of using fungi to degrade or accumulate contaminants
Polyaromatic hydrocarbon	A class of organic compounds which is characterised by possess >2 aromatic ring structures
Rhizosphere	Community of microbes and fungi that live within the root network of plants.
Nodule	A swelling in plant root tissue that provides areas for endophytic fungi and microbes to inhabit
Endophyte	A fungal or microbial organism that lives within the tissues of its host plant species and does not cause apparent harm or disease to the host
Bioaccumulation	An increase in concentration of a compound within an organism that is faster than the removal or use of said compound
Biomagnification	The process in which a compound increases in concentration at a greater rate in organisms that belong to a higher trophic level
Bioavailability	The degree to which a compound is available for uptake to an organism
Anabolic pathway	A biochemical pathway which synthesises molecules

Chapter 1. Soil Contamination and Phytoremediation

1.1. Introduction to soil contamination and phytoremediation

Pollutants such as polyaromatic hydrocarbons (PAHs), pesticides, chlorophenols, heavy metals and other salts are a major issue in contaminated soils (Samanta et al. 2002, Chen et al. 2015). With increasing populations and urbanization, it is crucial to protect the remaining ecosystems we have and restore areas contaminated by anthropogenic activities (Carré et al. 2017). Contaminated soils can pose a serious risk to both human and ecosystem health (Samanta et al. 2002, Buha and Williams 2003, Gandolfi et al. 2010). PAHs, such as phenanthrene, pyrene, and benzo[a]pyrene (BaP) are considered environmental contaminants and possess carcinogenic, teratogenic, and mutagenic properties (Samanta et al. 2002, Buha and Williams 2003, National Pollutant Release Inventory 2018). The contamination of soils can also change physical and biogeochemical processes that occur within an area (Rath and Rousk 2015).

Phytoremediation is one possible technique to remediate and restore contaminated soils (Frick et al. 1999, Nyer et al. 2000, Margesin and Schinner 2001a, Haritash and Kaushik 2009, *North Dakota Remediation Resource Manual* 2016, Petrová et al. 2017). The process of phytoremediation involves using plants and their associated microbial and fungal communities to break down, detoxify, and / or remove the contaminant from the soil. The process of phytoremediation is contaminant dependent, but can be applied to organic contaminants, heavy metals, and other salts (Huang et al. 2004, Haritash and Kaushik 2009, Greenberg et al. 2012, Jesus et al. 2015). There are several key issues facing phytoremediation including the location of contaminated soils, the degree of contamination, the presence of co-contaminants, and the rate at which remediation and restoration occurs (Margesin and Schinner 2001b, Gerhardt et al. 2009, Greenberg et al. 2012, Jesus et al. 2015). Sites containing both organic and salt contamination can be challenging to restore using phytoremediation as both contaminants negatively affect plant growth and inhibit germination (Greenberg et al. 2012, Sirguey and Ouvrard 2013). Despite this, research continues to better understand the conditions required for successful remediation and techniques that can be used to

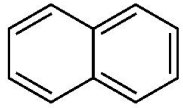
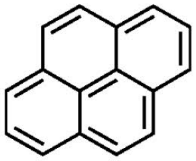
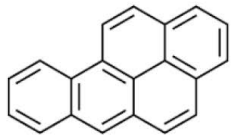
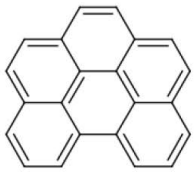
improve remediation rate and success (Huang et al. 2004, 2005, Gerhardt et al. 2009, 2017b, Cristaldi et al. 2017).

1.2. Polyaromatic Hydrocarbons

Polyaromatic hydrocarbons (PAHs) are a class of persistent organic compounds that can be found in the environment from both natural and anthropogenic sources (Samanta et al. 2002, Buha and Williams 2003). Many different PAHs have been classified as toxic, carcinogenic, and teratogenic; making it necessary to remove them from the environment. PAH contamination originates from three different source types: pyrogenic, petrogenic, and biological (Abdel-Shafy and Mansour 2016). Pyrogenic PAH creation results from the incomplete combustion of organic compounds, such as vehicle exhaust, burning of coal, and other industrial processes such as oil refinement. Petrogenic sources occur from the introduction of oil into the environment, typically spills or leaks from oil tanks. Lastly, the biological creation of PAHs occurs through the incomplete combustion of biological tissues, during events such as forest fires or volcanoes, or the synthesis of biological compounds by different biota such as plants, animals, fungi and bacteria (El-Shahawi et al. 2010, Abdel-Shafy and Mansour 2016). The source type will generally dictate which PAHs will be present in the contaminated area and can impact how the contaminated site is treated during restoration.

PAHs consist of a minimum of two aromatic rings (naphthalene) and can possess a wide variety of functional groups (Chen et al. 2015). Due to the diversity of structures within the PAH family it is important to consider what contaminants are present. As PAHs grow, additional ring structures are added to their base form (Table 1-1). The ring structures (typically benzene rings) make them very stable compounds with low water solubility and vapor pressure, especially as they increase in size (Abdel-Shafy and Mansour 2016, Varjani et al. 2017). Increasing in size allows for additional bay and K-regions to exist (Samanta et al. 2002). The Bay and K-regions are important as they can form Bay and K region epoxides which are thought to account for the carcinogenic properties of PAHs in animals via these epoxide moeties interacting with macromolecules (i.e. DNA, proteins, RNA, etc.; Lehr et al. 1985, Samanta et al. 2002). As the number of rings increase, their solubility and vapor pressure will also decrease further, making them more difficult to degrade by limiting their bioavailability (Haritash and Kaushik 2009).

Table 1-1. Example polycyclic aromatic hydrocarbons of varying size. As the number of benzene rings increase the water solubility, vapor pressure decreases (Juhasz and Naidu 2000, Physical Constants of Organic Molecules 2018).

Compound	Chemical Formula	Number of Rings	Water Solubility (mg L-1)	Vapor Pressure (torr at 20°C)	Compound Structure
Naphthalene	C_8H_8	2	Insoluble	0.082	
Pyrene	$C_{16}H_{10}$	4	0.14	6.8×10^{-7}	
Benzo[a]pyrene	$C_{20}H_{12}$	5	0.0038	5.0×10^{-7}	
Benzo[g,h,i]perylene	$C_{22}H_{12}$	6	0.0003	1.0×10^{-10}	

Many PAHs, such as benzo[a]pyrene are not innately toxic, but only become toxic after undergoing bioactivation within an organism (Sims et al. 1974, Gelboin 1980, Uppstad et al. 2010). The lipophilic nature of PAHs allow for diffusion through cell membranes (Meudec et al. 2006, Czub et al. 2008). After diffusing into the cell, aryl hydrocarbon receptors (AHR) can bind to the PAHs, initiating a cellular response upregulating phase I and phase II detoxification enzymes (Safe 2001, Syed et al. 2010, Tsuji et al. 2011). CYP1A1 and CYP1B1 are two genes, from the cytochrome p450 family, that becomes upregulated and function in the initial epoxidation of PAHs such as benzo[a]pyrene. The first step to both the bioactivation and detoxification of PAHs is the formation of epoxides (Sims et al. 1974, Gelboin 1980, Uppstad et al. 2010). In the example of benzo[a]pyrene, an epoxide will form at the 7,8 position (Figure 1-1) and become hydrolysed by epoxide hydrolase (Jiang et al. 2007, Uppstad et al. 2010). If the benzo[a]pyrene-7,8-dihydrol is not conjugated with a polar group such as glutathione, a second round of epoxidation can occur, resulting in a benzo[a]pyrene-7,8-dihydrol-9,10-epoxide (Sims et al. 1974, Gelboin 1980, Harvey 1985, Lehr et al. 1985, Safe 2001, Karle et al. 2004, Jiang et al. 2007, Uppstad et al. 2010, Abdel-Shafy and Mansour 2016). The formation of second a second epoxide (9,10-epoxide) is what creates a bay region (Figure 1-1) allowing for the formation of DNA adducts.

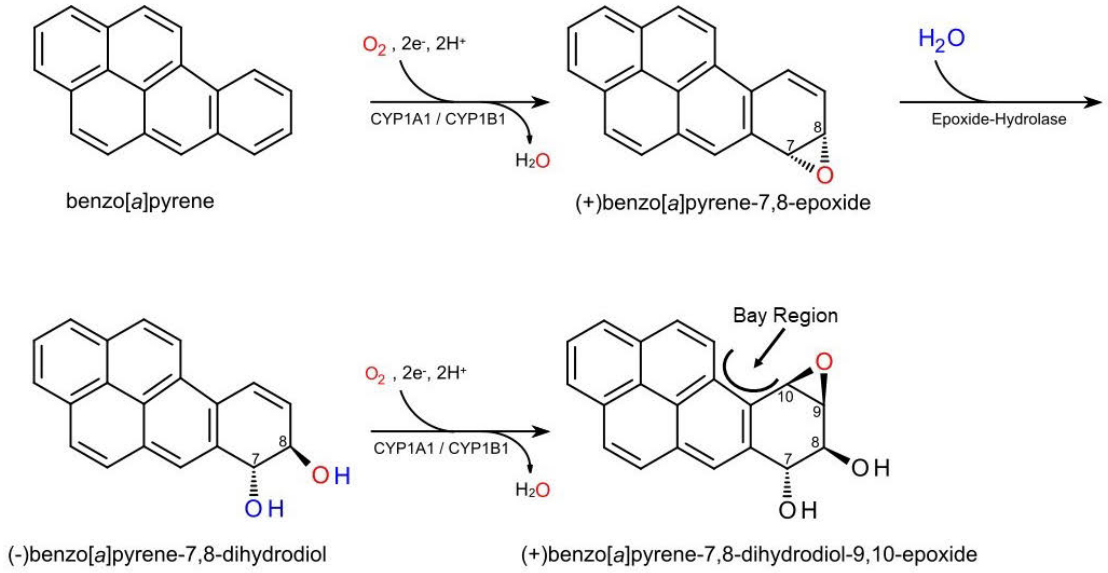


Figure 1-1. Benzo[a]pyrene bioactivation pathway through CYP1A1 and CYP1B1 epoxidation. CYP1A1/CYC1B1 create an initial epoxide at the 7,8 position which is then hydrolyzed by epoxide-hydrolase. A second epoxidation occurs by CYP1A1/CYP1B1 which results in forming benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BaP-7,8-dihydrodiol-9,10-epoxide). In its bioactivated from BaP-7,8-dihydrodiol-9,10-epoxide is carcinogenic and will for DNA adducts. Adapted from *Uppstad et al. 2010 and Tsuji et al. 2011*.

It has been well established that PAHs can bioaccumulate within organisms exposed (D'Adamo et al. 1997, Sacco and James 2004, Meudec et al. 2006, Morin et al. 2007, Czub et al. 2008, Takeuchi et al. 2009, Net et al. 2015). Bioaccumulation is the process in which toxins are accumulated within an organism's body through all possible routes (contact, ingestions, inhalation; Alexander 1999). It has the potential to decrease organism fitness due to the carcinogenic and teratogenic capabilities of PAHs (Juhasz and Naidu 2000, Pereira et al. 2009, Abdel-Shafy and Mansour 2016, Alegbeleye et al. 2017, Varjani et al. 2017). The degree PAHs bioaccumulate within an ecosystem is still under debate, and research regarding the biomagnification of PAHs is split. Both biomagnification and bio-dilution have been reported when examining the accumulation of BaP within a food web (D'Adamo et al. 1997, Frick et al. 1999, Morin et al. 2007, Takeuchi et al. 2009, Net et al. 2015). Biomagnification occurs when toxins accumulate to a higher degree in species with high trophic levels, and bio-dilution is the opposite, where low trophic level species tend to accumulate a higher concentration of toxin (Alexander 1999, Morin et al. 2007). The majority of the research regarding the

bioaccumulation and biomagnification of PAHs has occurred in aquatic and marine ecosystems (D'Adamo et al. 1997, Takeuchi et al. 2009, Net et al. 2015). There is limited research regarding the bioaccumulation and biomagnification of PAHs in the terrestrial environment (Morin et al. 2007, Czub et al. 2008, Abdel-Shafy and Mansour 2016). Additional research needs to examine the potential and occurrence of both bioaccumulation and biomagnification within terrestrial ecosystems to ensure processes such as phytoremediation do not cause toxins to increasingly accumulate in higher trophic levels species (Reid et al. 2000, Meudec et al. 2006, Morin et al. 2007, Czub et al. 2008, Pereira et al. 2009, Gerhardt et al. 2017a, Sushkova et al. 2018).

PAHs mobility within soil is dependent on the soil and PAH type (Abdel-Shafy and Mansour 2016). Soil particles may bind to the PAHs acting as a sorbent (Weissenfels et al. 1992, Reid et al. 2000, Ren et al. 2018a). Bound PAH molecules will not move through the soil systems and tend to be less bioavailable (Reid et al. 2000, Wang et al. 2007, Abdel-Shafy and Mansour 2016). Any PAH molecules that have not bound to the soil can move through the pore space and be transported into different regions of the soil or into an aquifer or other low lying water bodies (Neyer et al. 2000, Abdel-Shafy and Mansour 2016). Clay dominant soils have very small pore spaces that prevent the movement of PAHs, whereas sand based soils have large pore spaces resulting in a higher mobility (Ren et al. 2018b). Thus, restoration needs will vary considerably in soils contaminated by PAHs depending on the type of soil present and must be considered during remediation planning.

Remediation of PAH contaminated soils can be done using several *in situ* and *ex situ* techniques (Nyer et al. 2000). All *ex situ* techniques involve the removal of the contaminated soil from the site, and is then followed by thermal, chemical, or biological treatments or enters permanent soil storage (Kuppusamy et al. 2016, Carré et al. 2017). These techniques can be extremely efficient at nearly or completely removing all contaminants within the soil, but they are also very destructive to other essential soil components such as soil organic content, mycelium networks, soil microbial communities, seed banks and soil structure (Kuppusamy et al. 2016). In situ techniques such as bioremediation pose an alternative to destructive *ex situ* techniques, and in some situations may be more cost effective (Nyer et al. 2000).

1.3. Salt contamination events

Salt contamination results from high dissolved salt concentrations and / or high concentrations of absorbed sodium ions (Na^+, Jesus et al. 2015). This results in impacts to local plant populations, and soil biological, physical, and biogeochemical properties (Del Amor and Cuadra-Crespo 2012, Greenberg et al. 2012, Jesus et al. 2015, Arora et al. 2017). Contamination can be most readily observed in plant populations present on the site. High salt concentrations result in reduced plant germination, changes in osmotic potential, and Na^+ shock, or even plant death (Fougere et al. 1991, Jouyban 2004, Li et al. 2010). Salt contamination also has effects on the physical soil properties; causing poor aggregate formation and aggregate breakdown, resulting in soil compaction and reduced water and air penetration (Jesus et al. 2015). As soil particles become saturated in salts it alters the soil pH and electrical conductivity resulting in changes to biogeochemical processes within the soil (Rath and Rousk 2015, Arora et al. 2017). As the amount of dissolved Na^+ increases they bind to negatively charged soil particles making interactions between microbes and soil particles challenging as well as decreasing the availability of nutrients and other organic molecules (Chowdhury et al. 2011, Asghar et al. 2012, Ebadi et al. 2018). This can significantly alter the microbial community within the soil and impact organic carbon cycling (Rath and Rousk 2015).

There are three general types of salt contaminated soils: (1) saline, (2) saline-sodic, and (3) sodic soils which are defined by their electrical conductivity and sodium absorption potential (Table 1-2; Jesus et al. 2015). Salt contamination occurs through irrigation, altering of hydrological regimes and industrial activities such as drilling and usage of brine solutions (Greenberg et al. 2012, Jesus et al. 2015). Different activities will result in different degrees of contamination. Altering hydrological conditions can cause soils to dry, bringing salts up to the soil surface through a capillary affect, resulting in slight to moderate salt contamination levels (Zhang et al. 2008). Events such as brine spills can cause serious salt contamination resulting in complete death of plants and microbes in an area (Tomlinson 2016, Meehan et al. 2017).

Table 1-2. Salt contamination categories in soils, based on electrical conductivity (dS m^{-1}) and sodium absorption ration (SAR). The SAR is the ratio of dissolved sodium ion to square root of one half the sum of dissolved calcium and magnesium ions. (Jesus et al. 2015).

Contamination Type	Electrical Conductivity (dS m^{-1})	Sodium absorption ration ($SAR = \frac{Na^+}{\sqrt{1/2(Ca^{2+}+Mg^{2+})}}$)
Saline	>4	<13
Saline – Sodic	>4	>13
Sodic	<4	>13

A common occurrence is the co-contamination of soils with PAHs and salts (Sei and Fathepure 2009, Greenberg et al. 2012, Konkel 2016). During oil extraction it is common to have soils contaminated with both hydrocarbons from the oil and salt, used in the extraction processes, brines, maintenance of equipment, and alteration of natural ground water levels or flows (Whittemore 1995, Caenn and Chillingar 1996, Franzen 2013, Tomlinson 2016, Meehan et al. 2017). The co-contamination can make phytoremediation difficult due to the accumulative toxic affects limiting plant growth (Greenberg et al. 2012, Jesus et al. 2015, Gerhardt et al. 2017b). Also, both salts and PAHs can alter the soil quality and biogeochemical processes that are occurring (Zhang et al. 2008, Jesus et al. 2015). When soils are contaminated with PAHs they can alter hydrological processes by making soil particles hydrophobic, thereby preventing water infiltration and salt leaching (Zhang et al. 2008).

1.4. Bioremediation

Bioremediation is the process of using biota such as plants, fungi, and bacteria to degrade or sequester contaminants removing them from the environment or reducing their negative affects (Haritash and Kaushik 2009). The process of bioremediation can be implemented *in situ* using individual bacterial, fungal, or plant species; and has been shown to remediate and restore soils with organic, salt, and heavy metal contaminants (Salt et al. 1998, Neyer et al. 2000, Gutiérrez-Ginés et al. 2014, Jesus et al. 2015, Cristaldi et al. 2017). Microbial and fungal remediation alone often can not produce enough biomass to sufficiently remediate sites completely (Greenberg et al. 2012). Using plants in bioremediation, also known as phytoremediation, increases the biomass performing restoration, and helps to stabilize soil conditions (Nannipieri et al. 2007,

Jonathan et al. 2017, Ren et al. 2018a). It has been demonstrated in many studies that the symbiotic relationship between plants and their rhizospheres have a positive impact on the remediation process, allowing for faster and more complete remediation (Liu et al. 2004, Gerhardt et al. 2009, Greenberg et al. 2012, Hamdi et al. 2012, Wang et al. 2012, Muratova et al. 2015, Jonathan et al. 2017). Plants will release exudates such as sugars, amino acids, organic acids, fatty acids, sterols, growth factors, enzymes, flavonones, nucleotides, and other molecules into the soil. The exudates released act to acquire nutrients, breakdown organic molecules such as PAHs, assist in root growth, and provide some nutrients to the rhizosphere (Fan et al. 2007, Nannipieri et al. 2007). The rhizosphere will then assist in the breakdown of PAHs within the soil, and release plant growth promoting hormones and enzymes to assist in the growth of the plant (Huang et al. 2004, Nannipieri et al. 2007, Saleem et al. 2007, Maqbool et al. 2012, Hou et al. 2015, Muratova et al. 2015, Gerhardt et al. 2017b).

The mechanism of PAH remediation will vary between PAHs but typically follows similar processes (Juhasz and Naidu 2000, Newman et al. 2004, Liu et al. 2017). The first step to degrading PAHs is to hydrolyze one of the benzene rings replacing a double bond with two hydroxyl groups (Juhasz and Naidu 2000). The addition of hydroxyl groups creates an area which can be further targeted in enzymatic reactions allowing for mineralization (Figure 1-2). As the size of PAHs increases they become more difficult to remediate using bio-, phyto-, and mycoremediation (Juhasz and Naidu 2000, Gkorezis et al. 2016). Polyaromatic hydrocarbons greater than 3 aromatic rings in size tend to be very difficult to remediate due their high molecular weight and typically poor bioavailability to microbial, fungal, and plant species (Juhasz and Naidu 2000, Varjani et al. 2017, Jonathan et al. 2017). The complete biochemical pathway involved in remediating high molecular weight PAHs (>4 aromatic rings) are still unsolved (Juhasz and Naidu 2000, Samanta et al. 2002, Sushkova et al. 2018). However, several processes involved in the initial remediation of some PAHs have been elucidated. With enough time, and the correct species of microbes, fungi, and or plants it is theorized that all PAHs can be completely mineralized or degraded to the point where it can be used in anabolic biochemical pathways (Margesin and Schinner 2001b, Chen et al. 2015, Liu et al. 2017).

The initial steps to degrading benzo[a]pyrene are species and strain specific, but in general follow similar steps. First a double bond is broken and hydroxyl groups are

added (Juhasz and Naidu 2000, Varjani et al. 2017). The hydroxyl groups then allow for polar interactions to occur and undergo further enzymatic reactions to split the ring; similarly, to benzene degradation (Figure 1-2). A second double bond will then be attacked and oxidized resulting in the ring becoming split. This process will continue until the molecule is broken down into components used in other biochemical processes or it is completely mineralized into CO_2 (Juhasz and Naidu 2000, Gieg et al. 2014, Varjani et al. 2017, Jonathan et al. 2017, Sushkova et al. 2018). The entire process is unlikely to be performed by a single species or strain of plant, fungi, or microbe. Due to the recalcitrant nature of PAHs and the number of possible metabolites produced it is likely that the mineralization of PAHs, especially large ones, occurs in a syntrophic pathway (Nyer et al. 2000, Gieg et al. 2014, Varjani et al. 2017). Fungi have been shown to be more effective at degrading larger PAHs due to their initial ring cleavage mechanism (Gerhardt et al. 2009, Adenipekun and Lawal 2012, Liu et al. 2017). Several species of fungi, such as the white rot fungi, excrete peroxidases and laccases (Juhasz and Naidu 2000, Adenipekun and Lawal 2012). The peroxidases and laccases will oxidize the ring structures of PAHs resulting in cleavage (Juhasz and Naidu 2000, Adenipekun and Lawal 2012). PAHs can also be degraded through pathways involving the cytochrome P450 family. This however is a less advantageous pathway as it can lead to the formation of epoxides and the bioactivation of the PAHs (Gelboin 1980, Juhasz and Naidu 2000, Sushkova et al. 2018).

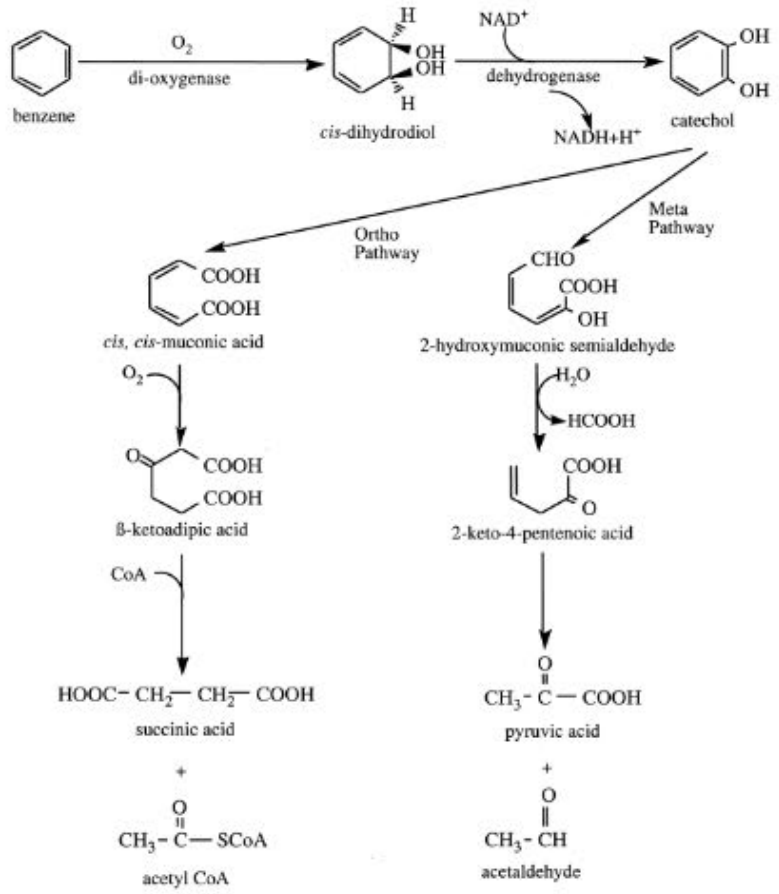


Figure 1-2. Microbial degradation of benzene via ortho and meta cleavage pathways. The initial step involves breaking a double bond and adding to hydroxyl groups, which is capable of being performed by many different species and strains of microbes. Subsequently, the hydroxyl groups provide polar regions for further enzymatic attack breaking the ring structure down further, to the point where it can be shuttled into another metabolic pathway. Figure generated by *Juhasz and Naidu 2000*.

1.5. Implications of phytoremediation on ecological restoration and the environment

The process of phytoremediation has pros and cons like any other remediation technique (Neyer et al. 2000). Using an *in situ* technique allows you to maintain the native seed bank, microbial networks within the soil, natural soil structure, and prevents erosion from the wind and rain. By maintaining the soil structure and biogeochemical processes you reduce the likelihood of alien species invasion and increase ecosystem

regeneration by native species (Yang et al. 2007, Brittingham et al. 2014). Currently, many agronomic species, such as alfalfa, are used as phytoremediators (Salt et al. 1998, Frick et al. 1999, Gerhardt et al. 2009, Wang et al. 2012, Cristaldi et al. 2017). This however, can cause issues after the remediation process is complete and native species are desired on site instead of the phytoremediator. Many of the characteristics that make a good phytoremediator, such as their ability to acquire biomass quickly and grow in sub-par soil conditions, also make them persistent in sites once established (Salt et al. 1998). This can make it difficult to re-establish native communities when the remediators are no longer desired on site. Research into native species is essential in order to make phytoremediation an effective restoration tool (Gerhardt et al. 2009, Kuppusamy et al. 2016).

Currently, it is common practice to add soil amendments, such as organic matter, during reclamation and restoration (Gerhardt et al. 2009, Gandolfi et al. 2010, Rieger et al. 2014, Field et al. 2017, Ren et al. 2018a). Indeed, soil amendments vary in composition, and can contain nutrients such as organic nitrogen, phosphorus, carbonaceous material as well as organic matter. They can be helpful in re-establishing plant growth and provide the essential nutrients biota in the soil require to undergo bio and phytoremediation. However, increasing the soil organic content (SOC) with materials such as biochar or compost can reduce the bioavailability of PAHs to microbes, fungi, and plants due to its high sorption capabilities (Ren et al. 2018a). Once bound to the organic matter the PAHs will become less available to microbes and plants but can increase the bioavailability to organisms that consume the organic matter within the soil such as earth worms (Reid et al. 2000).

The possibility of contaminants accumulating within plant tissues during phytoremediation also needs to be considered in restoration plans (Juhasz and Naidu 2000, Jesus et al. 2015, Kuppusamy et al. 2016). Sites that are contaminated with salts and heavy metals require plant biomass to be removed post remediation, to permanently remove the contaminant from the site. If left on site, heavy metals and salts will be slowly reintroduced as plant tissues breakdown (Salt et al. 1998). Organic contaminants such as PAHs can also accumulate within plant tissues, resulting in a contaminated food source for herbivores and ultimately bioaccumulation in animals and potential contamination throughout an the ecosystem (Juhasz and Naidu 2000, Abdel-Shafy and Mansour 2016, Sushkova et al. 2018). However, the amount of PAHs that accumulate

within plant tissues without undergoing degradation is much smaller than the amount that is actively remediated, posing a far smaller risk than leaving the PAHs untreated within the soil (Zhang et al. 2008, Kuppusamy et al. 2016, Gerhardt et al. 2017a).

Objectives

Although phytoremediation takes longer, the expense, logistics, and destructive nature of *ex situ* techniques warrant considering phytoremediation for restoring contaminated sites (Rojas-Avelizapa et al. 2007, Gerhardt et al. 2009, Chen et al. 2015). The degree of contamination, type, and soil characteristics will all influence the ability of the plants to remediate the contaminant. Further research needs to better understand the biochemical process involved in PAH degradation, the risk of bioaccumulation occurring in ecosystems, the identification of new native species that are capable of acting as phytoremediators, and the effects co-contamination of salts and PAHs have on the phytoremediation process (Greenberg et al. 2012, Hamdi et al. 2012, Jesus et al. 2015, Abdel-Shafy and Mansour 2016, Gkorezis et al. 2016, Gerhardt et al. 2017a). The objective of this study was to examine how soil salinity affects the rate of phytoremediation of two PAHs, pyrene and benzo[a]pyrene, in a 90-day greenhouse study using alfalfa (*Medicago sativa L.*).

Chapter 2. The effect co-contamination of salt and polyaromatic hydrocarbons on the rate of phytoremediation by *Medicago sativa* (alfalfa).

2.1. Introduction

Areas contaminated with both polyaromatic hydrocarbons (PAHs) and salt pose a threat to the environment and human health (Carré et al. 2017, Field et al. 2017, Ebadi et al. 2018). Due to the possible widespread contamination of PAHs and salt it is unrealistic to remediate extensive areas using *ex situ* remediation techniques (Neyer et al. 2000, Czub et al. 2008). Using phytoremediation, sites can be decontaminated while maintaining the integrity of the soil. Both pyrene and benzo[a]pyrene (BaP) are registered environmental contaminants and are believed to be carcinogens, teratogens, and possess acute toxicity (Samanta et al. 2002, National Pollutant Release Inventory 2018). Alfalfa (*Medicago sativa L.*) and its associated rhizobia and microbial communities have demonstrated the ability to degrade PAHs including pyrene and benzo[a]pyrene (Liu et al. 2004, Fan et al. 2007, Gkorezis et al. 2016). In this study, a 90 day greenhouse exposure was conducted to examine the impacts soil salinity has on the rate of phytoremediation of pyrene and benzo[a]pyrene in alfalfa (*Medicago sativa L.*).

2.2. Methods

2.2.1. Soil characterization and creation for phytoremediation trials

The soil used was a 50:50 mixture of Black Gold: Seedling Mix and organic garden topsoil (Davidson Farms, Maple Ridge, BC, Canada). Dolomite lime was added to the soil to increase the pH to a suitable level to grow alfalfa (Evergro, Dolomite Lime Soil Amendment, Abbotsford, BC, Canada). After mixing, the soil was passed through a 2 mm sieve to ensure there was no clumping or large debris within the soil.

Table 2-1. Soil grain size distribution and percent organic content in soil used for salt and polycyclic hydrocarbon phytoremediation x day study. The soil was purchased from Davidson Farms in Maple Ridge BC and was a 50:50 mixture of Black Gold: Seedling Mix and organic garden topsoil.

Particle Size distribution	Percent Composition
> 600 µm (Medium sands and larger)	17.92 %
212 – 600 µm (Medium Sands)	65.61 %
63 – 212 µm (Fine Sands)	10.98 %
< 63 µm (Silts and Clays)	1.83 %
Organic Matter	21.03 %

The electrical conductivity (EC) and pH were measured using a HACH HQ40D Portable multi meter probe (Hach, London, ON, Canada), with Intellical CDC401 Laboratory 4-Poles Graphite Conductivity Cell and Intellical PHC201 Laboratory General Purpose Gel Filled pH Electrode, respectively. Measurements where taken from a 5:1 v:v soil slurry of boiled deionized water, using methods adapted from Daniel et al. (2006). The slurries were agitated for 5 minutes using a magnetic stirring rod and then left to sit for 2 hours prior to measurement.

Ten samples (one of which was spilled and eliminated from the data set) were taken from stock uncontaminated soil were used to calculate organic content and particle size distributions. Organic content was measured by drying soil samples (10.83 g ± 0.67 g of wet soil, N=9) for 48 hours at 60^{o}C to remove any water content from the soil. The dry weight was then measured, and the soil moved to a blast furnace to bake for 1 hour at 400^{o}C. Organic content was calculated using Equation 2-1, as per Daniel et. al. (2006).

Equation 2-1. Percent Organic Matter Equation (Daniel et al., 2006)

$$\%\ Organics = \frac{(Dry\ Weight - baked\ weight)}{Wet\ weight} * 100\%$$

Grain size was measured according methods from Daniel et al. (2006), and classified using the ISO 14688-1:2017(E) Identification and classification of soil (2017). Post baking and removal of high heat organics, soil samples were subjected to a

sequential sieving process using 3 sieve sizes (600 µm, 212 µm and 63 µm) to provide an estimate of grain size distribution in the soil used for this study (Table 2-1).

2.2.2. Spiking of soil with pyrene, benzo[a]pyrene and NaCl for alfalfa phytoremediation trials

The overall experimental design of this study included quadruplicate 1 L pots per soil treatment, and each was seeded with 50 alfalfa seeds. The soil treatments were as follows: a single control (with no PAH and no NaCl added); 20 mM NaCl; 80 mM NaCl; 160 mM NaCl; PAH only; 20 mM NaCl+PAH; 80 mM NaCl+PAH; and 160 mM NaCl+PAH. Sodium chloride concentrations were chosen according to previous research regarding the tolerance of alfalfa. Previous agricultural studies have indicated that a decrease in biomass can be observed at 20 mM NaCl, with a decrease in harvestable yields occurring around the 80 mM range (Li et al. 2010, Putnam et al. 2017).

To create these 8 different treatments, the soil (50:50 mixture of Black Gold: Seedling Mix and Davidson's Farm organic garden topsoil) was first split into two 8 kg portions, of air-dried soil, that were untreated or underwent addition or spiking with PAHs. Spiking of the soil with the PAHs followed methods previously used by Hamdi et al. (2006) and Wang et al. (2012), with some minor changes and aimed to achieve concentrations 73 mg/kg of pyrene and 3.5 mg/kg of benzo[a]pyrene (a nominal concentration of 76 mg/kg of PAHs). Specifically, PAH treated soils were created by mixing 9.280 L of deionized water (DI), creating a soil slurry. A PAH solution was created by dissolving 587 mg pyrene and 48 mg benzo[a]pyrene in 160 mL of acetone and 50 ml, respectively. The PAH solutions were then added to the soil slurry and stirred over the next hour to create a homogenous mixture. Equal portions of the spiked soil slurry were then distributed among the 1 L pots (600 g of soil slurry per pot). The salt-only treated soil received 9.280 L of DI water and 210 mL of acetone to create a soil slurry. The soil slurry was mixed by hand until all portions of the soil was wet and incorporated into the slurry. Equal portions of the untreated soil slurry were then distributed among the 1 L pots (600 g of soil slurry per pot). Both the untreated and PAH treated soils were then spiked with NaCl to create the four NaCl conditions (i.e.0 mM, 20 mM, 80 mM and 160 mM NaCl). Additions of varying volumes of a 1 M NaCl solution were added to each treatment and mixed by hand to make the varying salt concentrations. All individual pots

where then mixed again to ensure the soil slurries were homogenous and the spiked NaCl and / or PAH solutions were fully incorporated and distributed throughout the soil.

All the pots used where lined with plastic bags to prevent salts and PAHs from draining from the pots during watering, as recommended by Wang et. al. (2012). After addition of the soil slurries to each 1 L pot for all treatments, the pots were then dried in a fume hood for one week and hand stirred daily with a stainless-steel spoon to assist in the drying process and loosen any compacted soil. The air-dried pots were then moved to a greenhouse research facility at SFU, and randomly distributed on work benches. Fifty alfalfa (*Medicago sativa L.*) seeds were planted in the top 1.5 cm of the soil and watered with 100 mL of DI water. Throughout the entire study, the green house had a mean temperature of 19.7°C ± 2.53°C (9.8°C – 30.2°C) and humidity of 46.7% ± 11.36% (20.9% – 84.4%). A 12:12 hour light to dark period was used throughout the 90-day growth period. Watering initially occurred every two days (August 13th to September 7th) but switched to every third day (September 10th to November 11th) as temperatures and evaporation decreased in the cooler months. After the 90-day growth period the plants were harvested for measurement.

2.2.3. Soil hydrocarbon analysis

Samples were submitted to ALS Environmental in Burnaby, British Columbia for polyaromatic hydrocarbon analysis. Samples (minimum 50 mL of soil) were collected by removing a vertical profile of soil from the surface of the soil to the bottom of the pot. The soil was transported to ALS the same day the samples were collected, in glass jars with a Teflon lined lid, kept on ice in a cooler. Day 0 samples were collected just prior to seeding with alfalfa. Day 0 samples were collected from each replicate and pooled to form two samples, one from soils spiked with PAHs and the other from soils spiked only with salt. Day 90 samples where submitted the day the plants were harvested. Two samples were submitted, on Day 90, from separate replicates of the 160 mM NaCl+PAH treatment.

2.2.4. Plant growth and survival measurements

Throughout the growth period the number of plants per pot was recorded every two to three days. This study was terminated on day 90 in the greenhouse and plant

growth and survival were recorded. Specifically, the number of surviving plants per pot, plant wet and dry biomass, and plant nodulation were recorded. Plant tissue was separated into above and below ground tissue by cutting the plants at the root - shoot junction (

Figure 2-1). The root - shoot junction is the point at which the root tissue transitions from a brow/white colour to green (S.E.R.A.S 1994). The number of surviving plants were counted prior to removing them from the soil to account for plant survival. The percent of successful germination was calculated by dividing the maximum number of plants observed per pot throughout the greenhouse experiment by the number of seeds planted (Equation 2-2). The maximum number of plants per pot differs from the abundance of plants at day 90 as some plants died throughout the experiment.

Equation 2-2. Percent successful germination.

$$Percent\ successful\ germination = \left(\left(\frac{Xmax_{ij}}{T}\right) * 100\right)$$

$Where\ X\ is\ the\ maximum\ number\ of\ living\ plants\ counted\ within\ a\ pot\ thoughout\ the\ experiment$
$T\ is\ the\ total\ number\ of\ seeds\ planted\ (50)$

Equation 2-3. Seed viability equation using control (0 mM NaCl, No PAH) as references. Percent successful germination was calculated using the highest number of plants per pot observed throughout the experiment (Equation 2-2).

$$Seed\ Viability = Median\ (Percent\ Successful\ Germination\ of\ Controls)$$

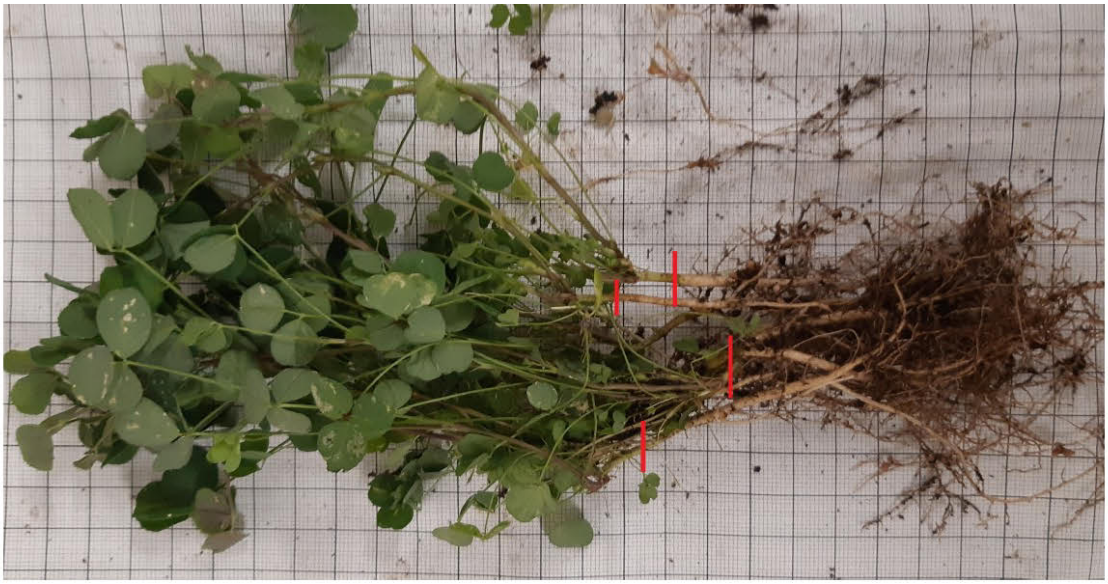


Figure 2-1. Root - shoot junction is located at the transition of root to shoot tissue. The cut locations are shown in red and occur where the root tissue begins to express chlorophyll, changing into shoot tissue.

Biomass of the wet shoot and root tissue was weighed immediately after removing the plants from the pot. After measurement the above and below ground tissue were placed into individual pre-weighed and labelled paper bags for drying. After all samples were prepared, they were dried in an oven for 48 hours (44 hr + 4hr) at 70 °C. After 44 hours the samples were removed from the oven and cooled in desiccator jars prior to taking the dry weight (g) and replacing in the oven for an additional 4 hours to ensure the weight does not change, as per S.E.R.A.S (1994). The percent moisture content was calculated by using Equation 2-4.

Equation 2-4. Percent moisture content of the alfalfa plant biomass per pot (S.E.R.A.S, 1994).

$$Percent\ Moisture\ Content = \frac{(wet\ shoot\ biomass + wet\ root\ biomass) - (dry\ shoot\ biomass + dry\ root\ biomass)}{(wet\ shoot\ biomass + wet\ root\ biomass)} * 100\%$$

Prior to drying, the plant colour and vigour, number of nodules (≥2mm in size), number of nodule clusters, nodule colour, and the nodule position were recorded. Following the Saskatchewan Pulse Growers Nodulation and Nitrogen Fixation Field Assessment Guide (SNNFF) the nodulation and general nitrogen fixation potential was estimated. The SNNFF breaks down estimated nitrogen fixation into three categories: (1)

Plant Vigour and Health, (2) Nodule Colour and Abundance, and (3) Nodule Position. Scores are given to each category and summed allowing for a score between 0 and 13. A score of 0 indicates no plant growth, 1-6 indicates poor nodulation and nitrogen fixation potential, 7-10 less effective nodulation, and 11-13 effective nodulation. The nodule colour was broken into two categories: (1) nodules with pink pigmentation, and (2) nodules that are green, brown, or white. Nodule location was split into 4 categories: (1) both crown and lateral positions, (2) crown only, (3) lateral only, (4) nodules absent. Figure 2-2 depicts examples of crown and lateral nodule positions. The total number of nodules (greater than 2 mm in size) per pot were also compared against treatment types.

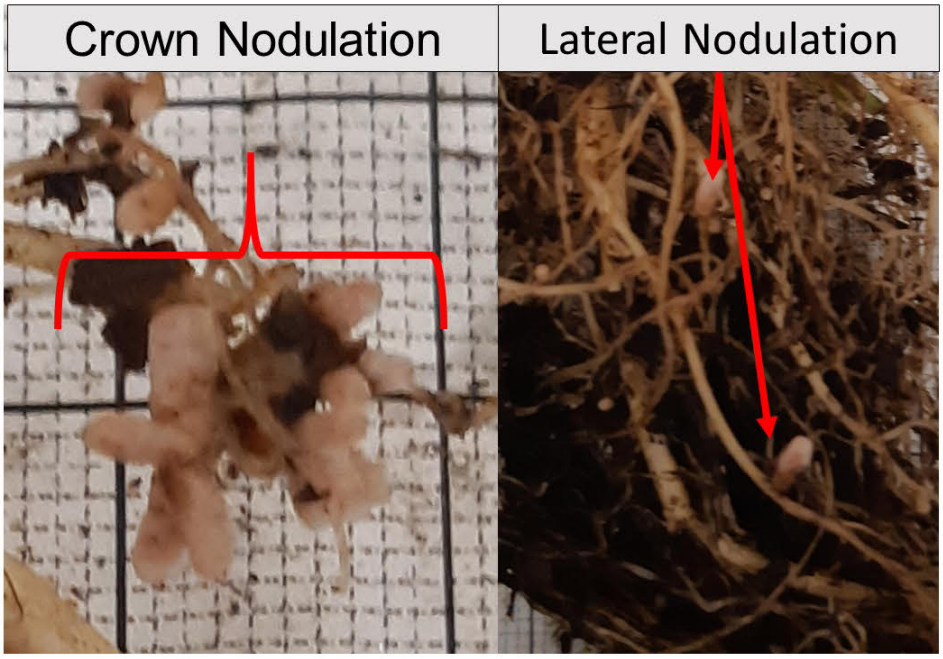


Figure 2-2. Crown (left) and lateral (right) nodule position along roots. Crown positions form groupings of nodules and typically occur at joints in the roots. Lateral nodules are typically singular and are found along the length of the root.

2.2.5. Statistical Analysis

All statistical procedures were performed in JMP Version 13.1.0. Wilcoxon/Kruskal – Wallis Rank Sum tests were performed on alfalfa percent germination success, abundance at day 90 and maximum abundance observed throughout the experiment, biomass, number of nodules and nitrogen fixation potential scores. A Steel-Dwass analysis was used to determine specific relationships between

salt treatments. Due to a lack of normality and unequal variances parametric tests were unsuitable. Due to the very low/absent PAH concentrations measured at day 0 and day 90, as well as the consistent lack of significance, PAH and non-PAH treated soils of the same NaCl treatments are pooled for analysis of plant biomass, and nodule abundance.

A two-way ANOVA was performed on percent plant moisture content (data were normal and exhibited equal variance), followed by a pot-hoc Tukey HSD. All treatments where no growth was observed were excluded during percent moisture analysis.

2.3. Results

2.3.1. Polyaromatic hydrocarbon measured soil concentrations

Initial day 0 concentrations prior to the addition of alfalfa to the pots reported from ALS were 0.011 mg/kg of pyrene and <0.010 mg/kg of benzo[a]pyrene in the PAH spiked soil samples, and <0.2000 mg/kg of pyrene and <0.010 mg/kg of benzo[a]pyrene in the control samples (Table 2-2). The reported PAH levels in the PAH spiked samples are considerably lower than the expected nominal concentration of 76.875 mg/kg (73.375 mg/kg of pyrene and 3.5 mg/kg of benzo[a]pyrene). At the end of the exposure experiment, the day 90 concentrations followed a similar trend with the PAH spiked soil treatments reporting no measurable concentrations of pyrene and benzo[a]pyrene (<0.050 mg/kg of pyrene and <0.050 mg/kg of benzo[a]pyrene). Due to these dramatic differences between measured and the nominal PAHs concentrations in any of the soils deliberately spiked with PAHs on day 0 and 90, respectively, it is not possible to make any conclusive observations regarding the effects of PAHs in this study.

2.3.2. Percent successful Germination

As expected, based on the low or non-detectable PAHs measured in this study, there were no significant differences observed between PAH treatments and germination success. However, there was significant differences between salt treatments and germination success (p-value: <0.05). As NaCl concentrations increased the germination success decreased. Percent successful germination per pot ranged from 0 to 74%. The highest concetration NaCl treatment, 160 mM of NaCl, exhibited a mean 2% successful germination (Figure 2-3). The seed viability (median percent successful germination for

the control treatment; no PAHs and no salt) was 58% (Figure 2-3 indicated by red line). Two values exceeded the estimated seed viability, one in the control treatments (no PAHs, no salt; 74%) and one in the PAH only treatment (60%).

2.3.3. Plant Survival

The number of surviving plants per pot, at day 90, varied between treatments, ranging from 0 to 27 plants per pot. Salt treatments showed a significant effect on the number of surviving plants per pot with significant differences between each NaCl treatment (p-value: <0.05, Figure 2-4). No significant effects on survival were observed between the PAH treatments. The dramatically low/absent measured PAHs throughout the experiment combined with the same survival profiles evident between NaCl treatments with and without PAHs clearly demonstrate no significant effect. These data strongly suggest that it was the increasing NaCl concentrations that decreased the number of surviving plants and germination success. Differences between the percent successful germination and plant abundance at day 90 are caused by plant death during the growth period. When comparing the difference between maximum numbers of plants per pot observed throughout the experiment and the abundance at day 90 you see a significant effect between the 0 mM NaCl and 160 mM NaCl salt treatments as well as the 20 mM and 160 mM salt treatments. No significant effects were observed between PAH treatments (Figure 2-5).

2.3.4. Above and Below Ground Biomass

Wet shoot biomass showed significant differences between salt treatments (p-value: <0.001) but not PAH treatments (p-value: 0.9533; Figure 2-6). Biomass per pot values ranged from 0 g (no growth) to 10.7 g. No growth at day 90 was observed in pots with 160 mM NaCl treatments for both PAH and non-PAH treated soils. The 20 mM NaCl treatment possessed the highest mean shoot wet biomass (7.25 g ± 2.99 g, Figure 2-7). Differences between all salt treatments can be observed except when comparing 20 mM NaCl and 0 mM NaCl, as well as 80 mM NaCl and 160 mM NaCl treatments, where there is no significant difference (Figure 2-7).

Dry shoot biomass showed similar trends to the wet shoot biomass. There was no significant difference between PAH treated soils and a significant difference between

salt treatments (p-value: <0.001,Figure 2-6). The 0 mM NaCl treatment had the highest mean biomass (1.75 g ± 0.47 g) followed by the 20 mM NaCl treatment (1.72 g ± 0.81 g), but the 20 mM NaCl treatments possessed a higher maximum and median biomass (Figure 2-8). There was a significant difference between the 0 mM NaCl treatment and the 80 mM and 160 mM treatments. Also, there was a significant difference between the 20 mM NaCl treatment and the 80 mM and 160 mM treatments (p-value: <0.05). There was no significant difference when comparing the 0 mM NaCl and 20 mM NaCl; and when comparing the 80 mM and 160 mM NaCl treatments (Figure 2-8). The highest salt treatment (160 mM NaCl) resulted in no growth or biomass at day 90.

The wet root biomass ranged from 0 to 13.195 grams, with no growth occurring in the highest salt treatments (160 mM NaCl) at day 90. Treatments containing 0 mM NaCl had the highest maximum and mean wet root biomass observed (Figure 2-9). A significant difference can be observed between salt treatments (p-value: <0.001), but not between PAH treatments (Figure 2-6). When comparing different salt treatments, there is a significant difference between all treatments (p-value: <0.05) except for 0 mM and 20 mM groups, as well as the 80 mM NaCl and 160 mM NaCl treatments (Figure 2-9).

Dry root biomass ranged from 0 g, in the 160 mM treatments, to 4.92 g, in the 0 mM NaCl treatment. The 0 mM treatments showed the highest mean and maximum value but possess a lower median value than the 20 mM treatments (Figure 2-10). When comparing individual salt treatments significant differences can be seen between all treatments except for 0 mM and 20 mM NaCl treatments, and the 80 mM NaCl and 160 mM treatments. No significant differences can be observed between PAH treatments (Figure 2-6).

2.3.5. Percent moisture content of plants

Percent moisture content ranged from 49.7% - 92.2%, with the minimum and maximum values belonging to the 80 mM with PAH and 80 mM without PAH treatments, respectively. No significant differences were observed between any treatments (Figure 2-11). Any pots resulting in no growth were not considered during analysis.

2.3.6. Nodule Abundance, and Nitrogen Fixation Potential

Nodule Abundance

The number of nodules ranged from 0 – 124 per pot, with the lowest value in the 160 mM NaCl treatment and the highest in the 0 mM treatment. The 0 mM salt treatment possess the highest mean and median number of nodules (Figure 2-12). No nodule growth occurred in the 160 mM treatments. A significant difference can be observed between salt treatments (p-value: <0.001). Individual salt treatments, except for the 0 mM and 20 mM, as well as the 80 mM and 160 mM possessed a significant difference. No significant observations can be made between PAH treatments and the interactions between PAH and salt treatments.

Estimated Nitrogen Fixation Potential

The estimated nitrogen fixation potential total scores ranged from 0 (minimum score possible) to 13 (maximum score possible). The 0 mM NaCl, no PAH treatment received the highest score (13). Treatments containing 160 mM NaCl resulted in no growth and therefore a score of 0. There was no observable difference between the 0 mM and 20 mM NaCl treatments, as well as the 20 mM and 80 mM treatments (Figure 2-13). However, there was a significant difference between the remaining salt treatments (p-value: <0.05).

Table 2-2. Polyaromatic hydrocarbon concentration results from Day 0, Day 90, and expected values. (*) Indicates results where the detection limit has been raised due to chromatographic interference as a result of co-elution.

Treatment Type	Pyrene (mg/kg)	Benzo[a]pyrene (mg/kg)
Day 0: Non-PAH treated soil	<0.200*	<0.010
Day 0: PAH treated soil	0.011	<0.010
Day 90: PAH treated soil	<0.050*	<0.050*
Day 90: PAH treated soil	<0.040	<0.040*
Expected Values Day 0	73.375	3.5

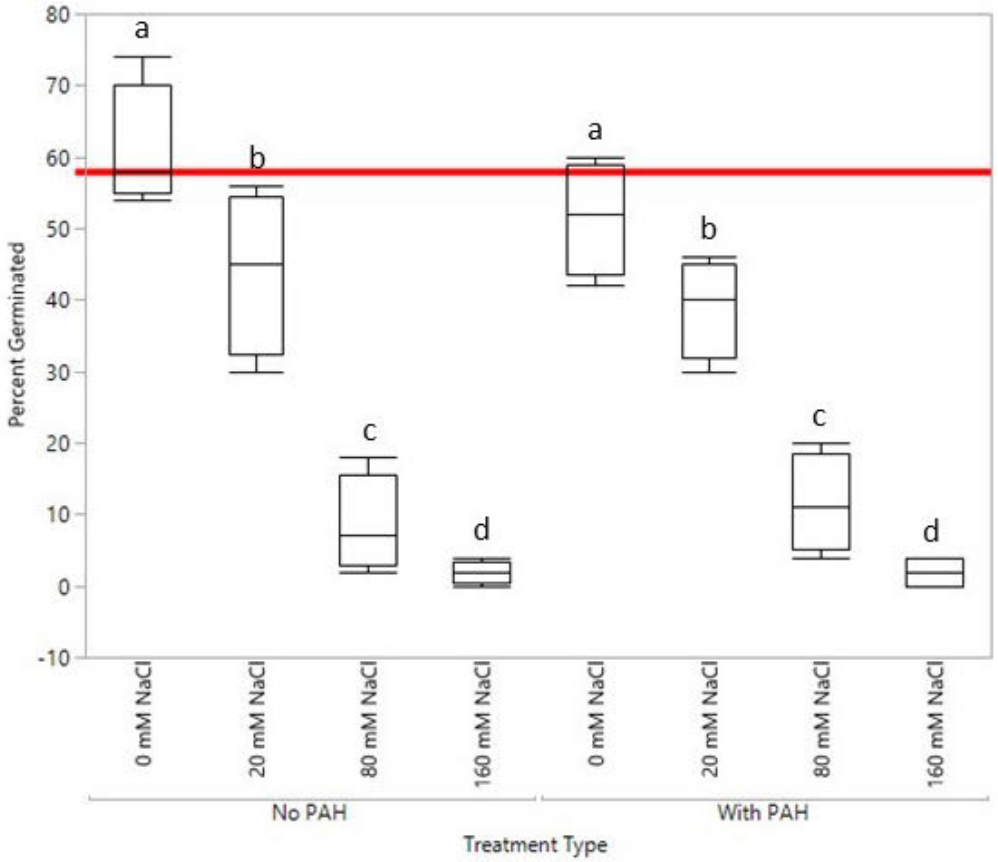


Figure 2-3. The percent successful germination of *Medicago sativa* L. seeds using the highest number of living plants counted throughout the phytoremediation trials. Box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles, represented by the box boundaries, are depicted. Data extending to the 1st quartile - 1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. N = 4 for all treatments. The red line indicates the seed viability (58%) based on the median maximum number of plants per pot that germinated in the control treatments throughout the experiment. Day 90 measurements indicate <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects. Significance between treatments is represented by superscripts.

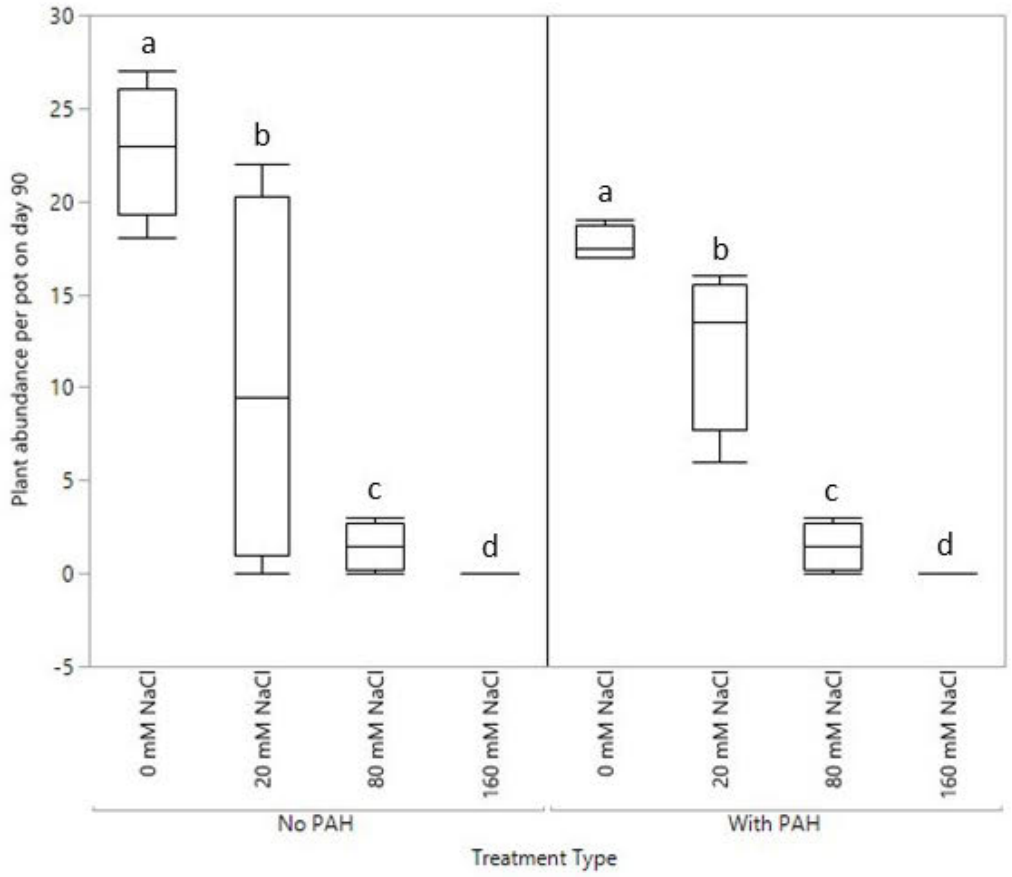


Figure 2-4. Number of surviving *Medicago sativa L.* plants per pot treated with varying salt concentrations and in the presence or absence of PAHs over 90 days. Number of plants is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. N = 4 for all treatments. Measured values of PAHs indicate extremely low or absent PAHs, day 90 measurements were <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects. Significance between treatments is represented by superscripts.

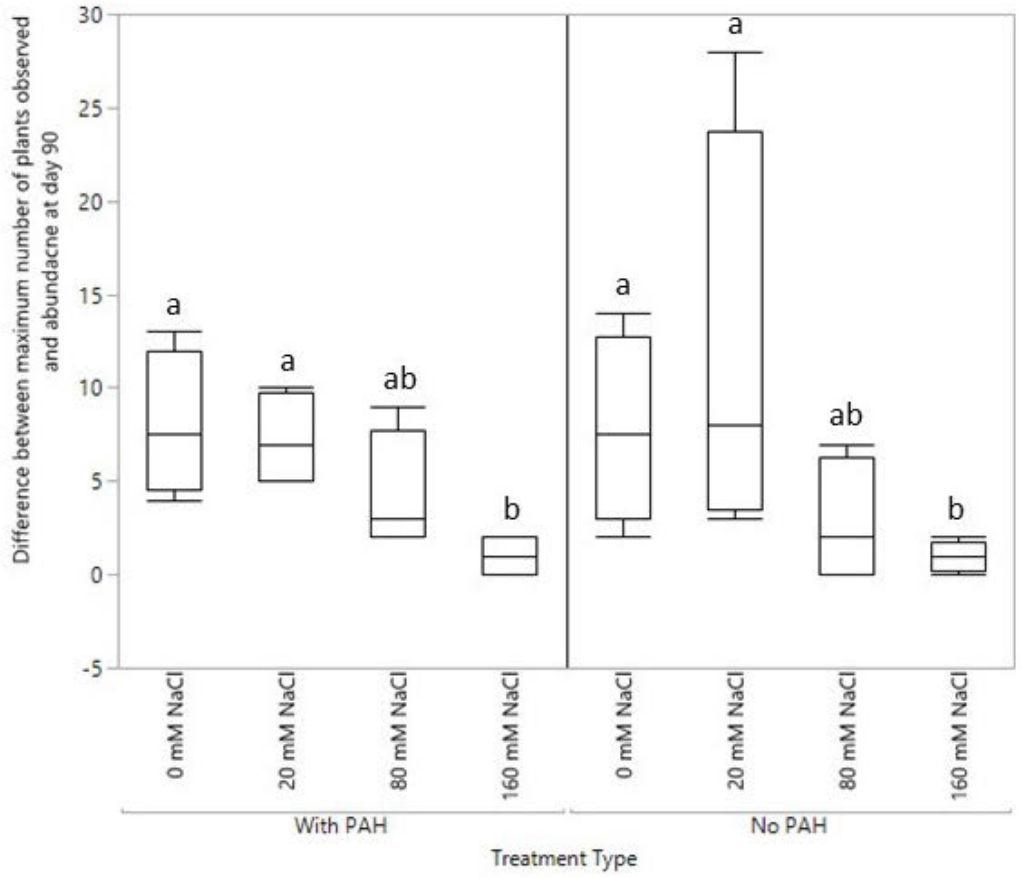


Figure 2-5. The difference between the maximum plants per pot observed during the growth period and the plant abundance at day 90. Number of plants is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. N = 4 pots with 50 seeds per pot for each treatment. Measured values of PAHs indicate extremely low or absent PAHs, day 90 measurements were <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects.

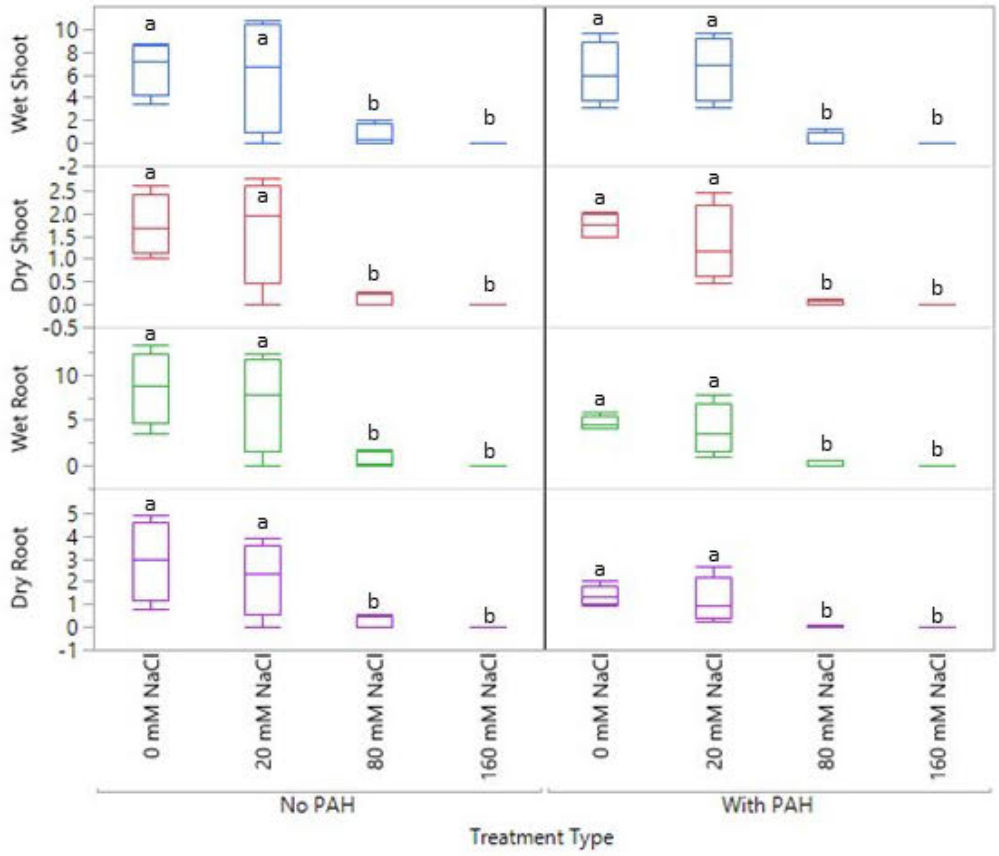


Figure 2-6. The (A) wet shoot biomass, (B) dry shoot biomass, (C) wet root biomass, (D) and dry root biomass are shown. The wet shoot and root biomass (A, C) were weighed immediately after removal from the soil. Dry biomass (B, D) was measured after drying at 70°C for 48 hours. The biomass of *Medicago sativa L.* per pot is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are shown by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. N = 4 pots with 50 seeds planted per pot for all treatments. Day 90 measurements indicate <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects. Significance between treatments is represented by superscripts.

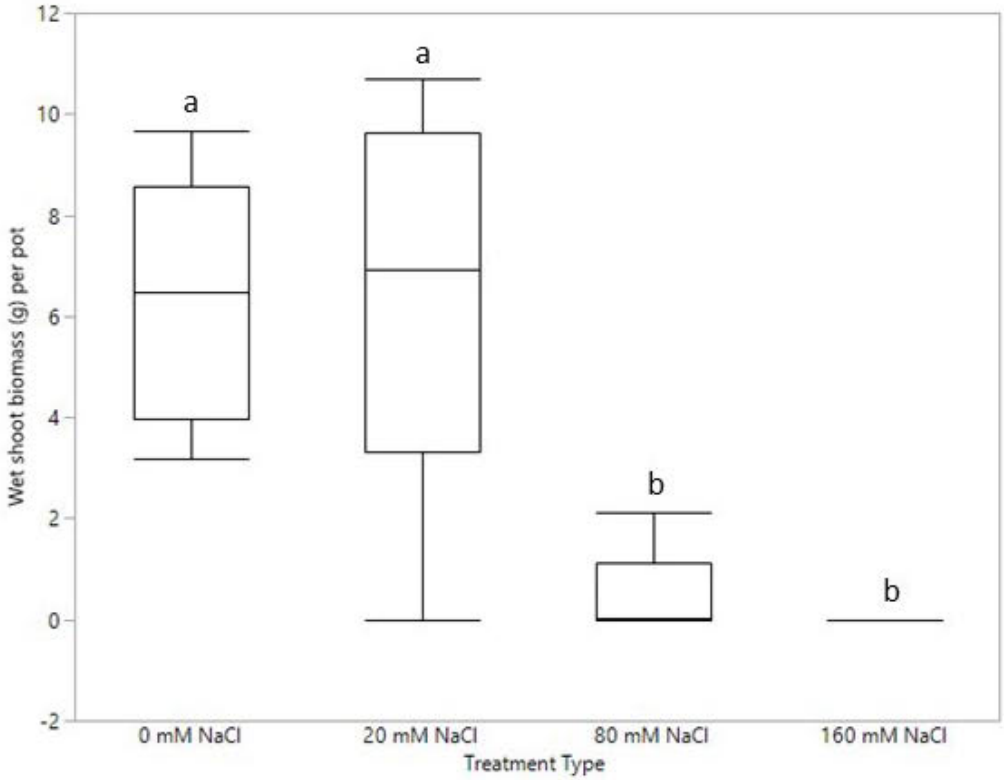


Figure 2-7. Wet shoot biomass (g) of *Medicago sativa L.* per pot comparing salt treatments. Due to insignificant PAH effects, PAH and non-PAH treated soil are pooled (N=8 pots with 50 seeds per pot for each treatment). The wet shoot biomass is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5*(interquartile range) or 3rd quartile +1.5*(interquartile range) are shown by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. Significance between treatments is represented by superscripts.

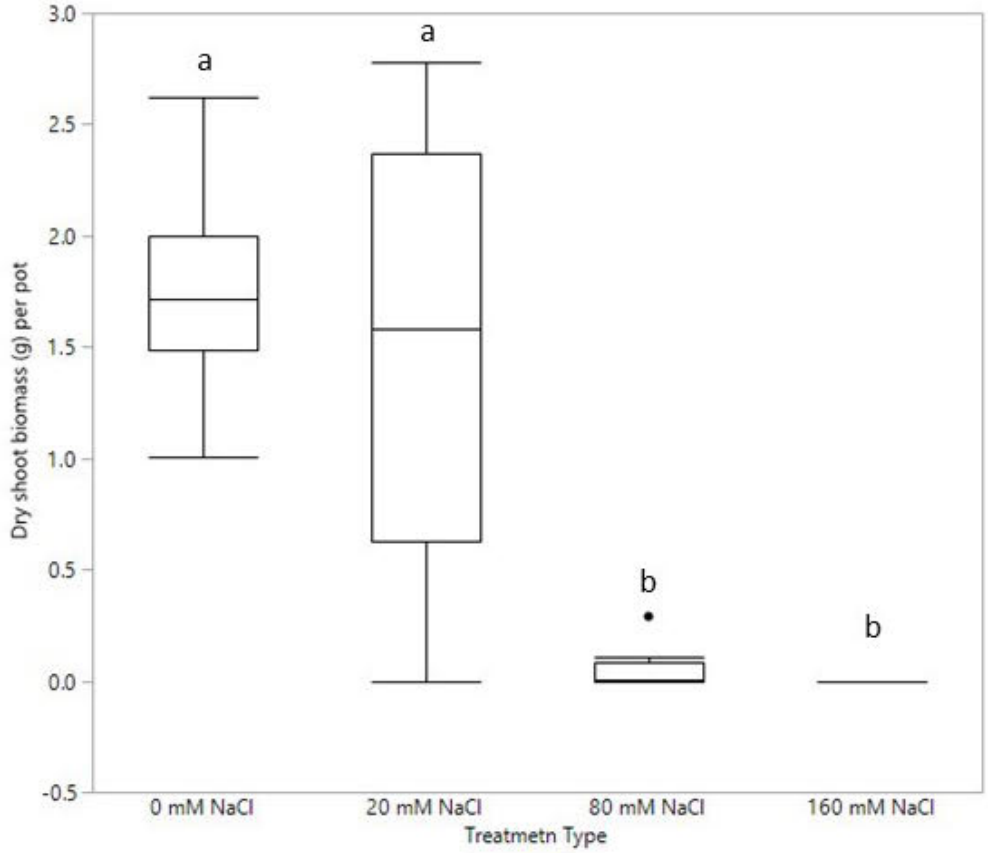


Figure 2-8. Dry shoot biomass (g) of *Medicago sativa L.* per pot comparing salt treatments. Due to insignificant PAH effects, PAH and non-PAH treated soil are pooled (N=8 pots with 50 seeds per pot for each treatment). The wet shoot biomass is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. Significance between treatments is represented by superscripts.

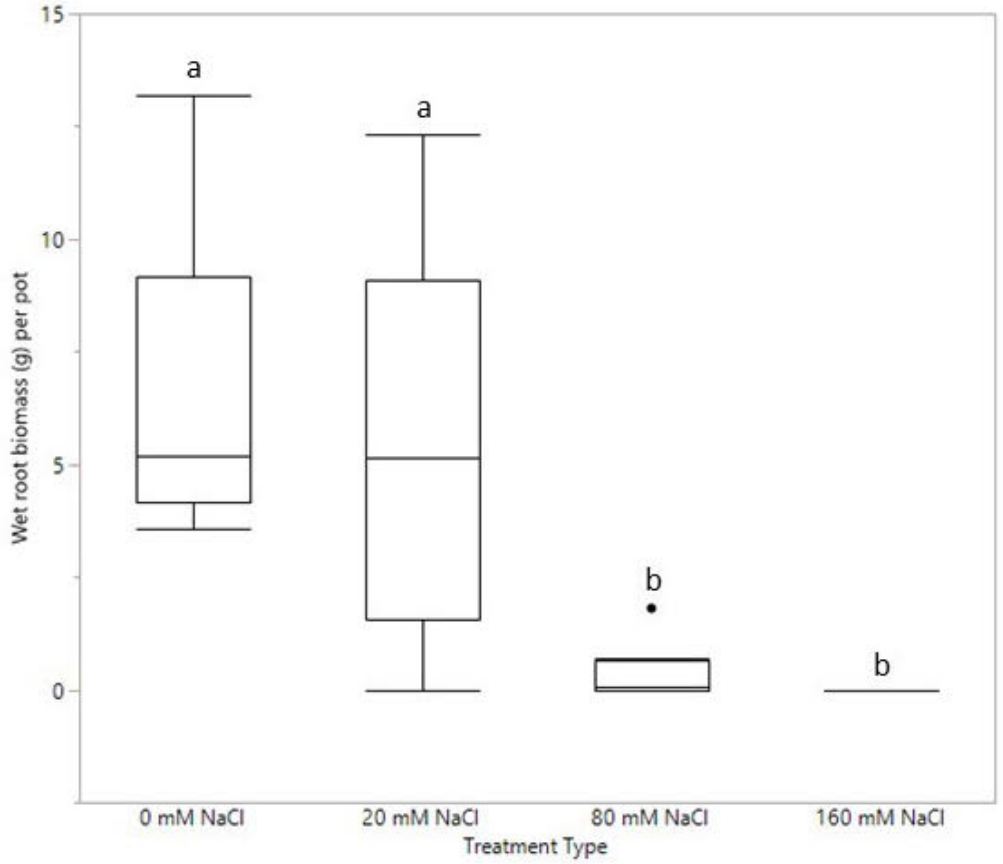


Figure 2-9. Wet root biomass (g) per pot comparing salt treatments. Due to insignificant PAH effects, PAH and non-PAH treated soil are pooled (N=8 pots with 50 seeds per pot for each treatment). The wet root biomass is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile - 1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are shown by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. Significance between treatments is represented by superscripts.

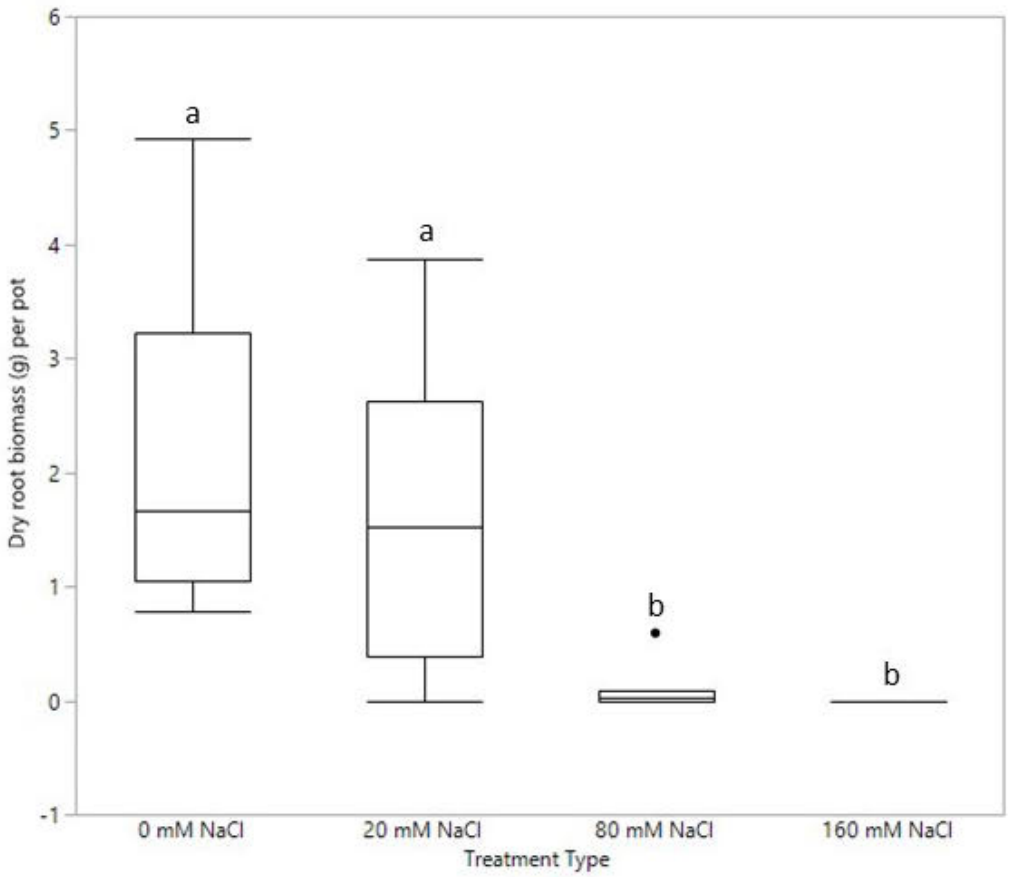


Figure 2-10. Dry root biomass (g) per pot of *Medicago sativa L.,* comparing salt treatments. Due to insignificant PAH effects, PAH and non-PAH treated soil are pooled (N=8 pots with 50 seeds per pot for each treatment). The dry root biomass is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are shown by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. Significance between treatments is represented by superscripts.

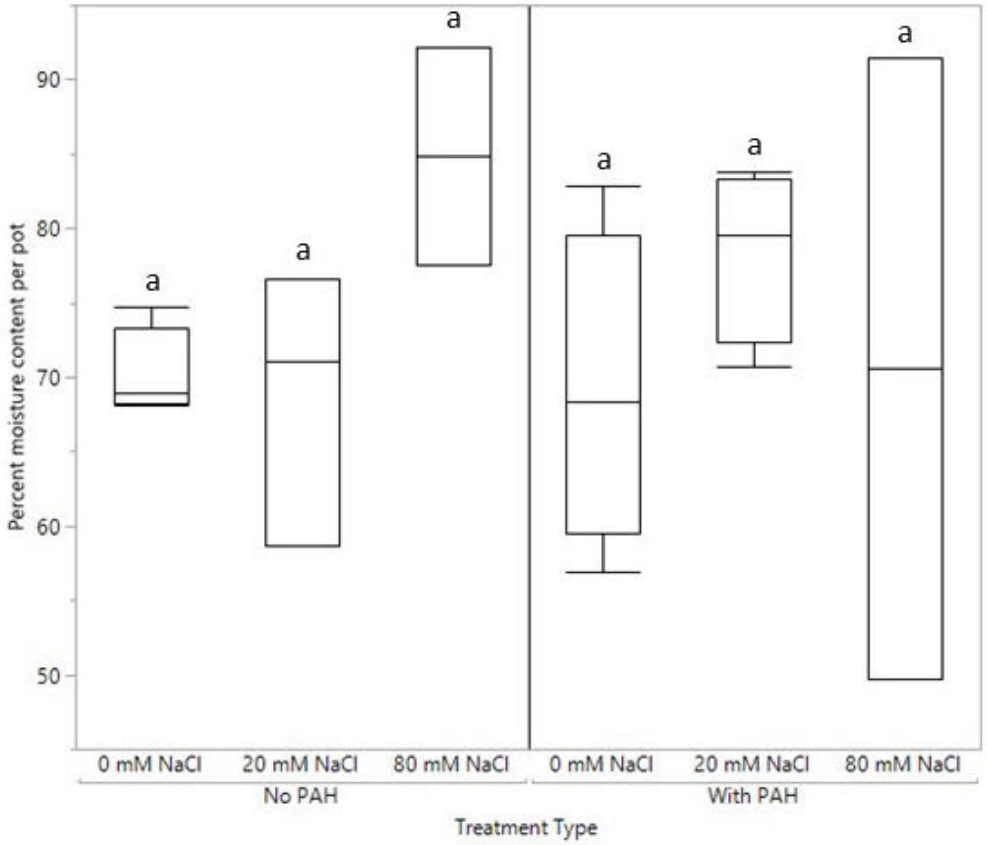


Figure 2-11. The percent moisture content of *Medicago sativa L.* is depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. N = 4 pots with 50 seeds per pot for each treatment. Day 90 measurements indicate <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects. Significance between treatments is represented by superscripts.

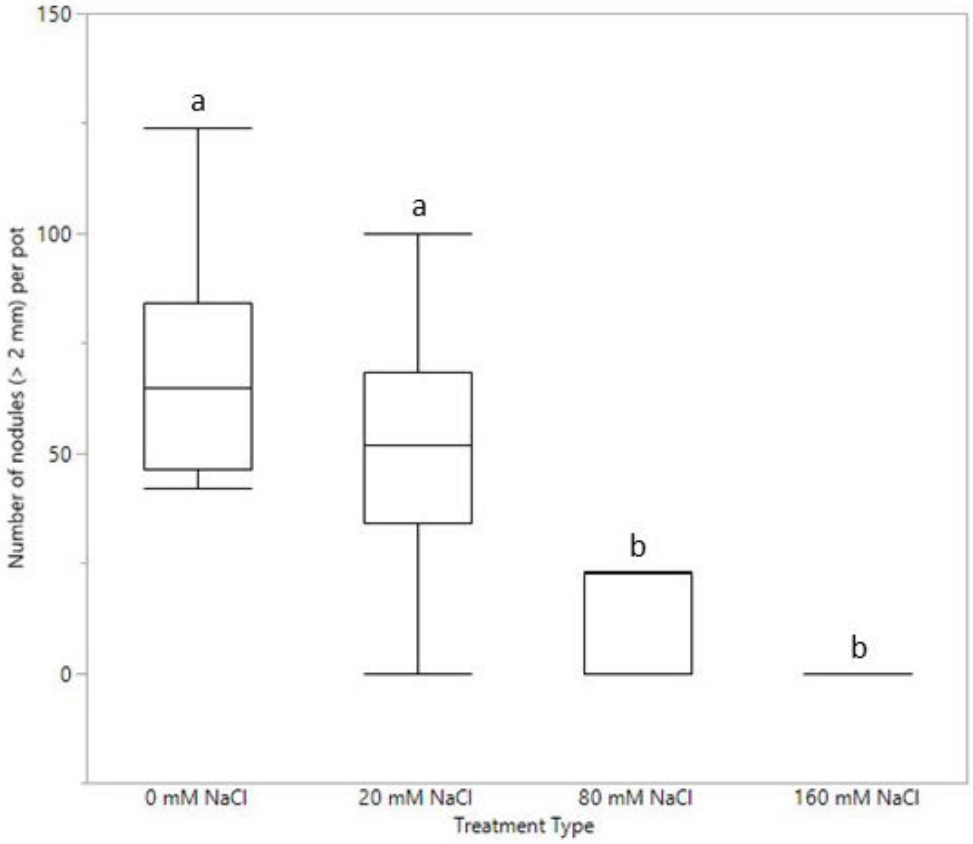


Figure 2-12.The number of nodules (> 2 mm) per pot are depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile -1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are shown by the whiskers. Any points outside the whisker range are considered outliers and are represented by a black dot. Due to insignificant PAH effects, PAH and non-PAH treated soil are pooled (N=8 pots with 50 seeds per pot for each treatment). Significance between treatments is represented by superscripts.

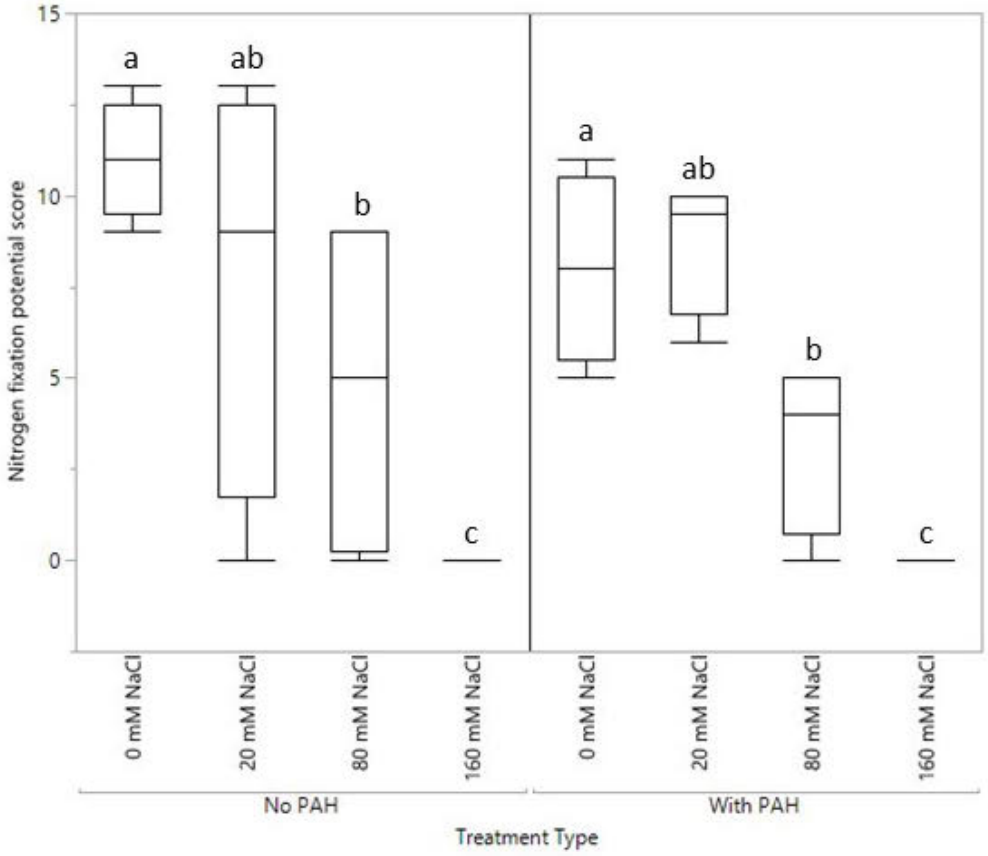


Figure 2-13. The nitrogen fixation potential is estimated using the Nodulation and Nitrogen Fixation Field Assessment Guide. Scores from per treatment are depicted by box plots including medians (horizontal lines), upper 75th and lower 25th percent quartiles are represented by the box boundaries. Data extending to the 1st quartile - 1.5x(interquartile range) or 3rd quartile +1.5x(interquartile range) are show by the whiskers. N = 4 pots with 50 seeds per pot for each treatment. Day 90 measurements indicate <0.050 mg/kg pyrene and <0.040 mg/kg of benzo[a]pyrene in PAH treated soils and had no significant effects. Significance between treatments is represented by superscripts.

2.4. Discussion

Alfalfa (*Medicago sativa* L.) was grown over 90 days to determine the effects of soil salinity on the phytoremediation of pyrene and benzo[a]pyrene. Do to inconclusive PAH measurements no insights may be gained regarding the phytoremediation potential of alfalfa when soils contain salts and PAHs as co-contaminants. However, the salt

treatments clearly demonstrated an effect on alfalfas ability to grow. Therefore, future studies are required to examine the direct impact of soil salinity on the phytoremediation of PAHs by alfalfa. This study clearly demonstrates that salinity would influence the phytoremediation potential of alfalfa, by limiting growth and seed germination, and an optimal soil salinity should be incorporated into ecological restoration plans for this plant species.

The lack of measurable pyrene and benzo[a]pyrene may have been caused by mishandling of soil during the spiking process or by the high sorption capabilities of the soil organic matter (SOM). Since the soil was created using a 50:50 mixture of Black Gold: Seedling Mix and organic garden topsoil (Davidson Farms, Maple Ridge, BC, Canada) it contained a very high organic content (21%) that would be atypical for areas without a thick organic soil horizon (Dumanski et al. 1970, *Soil and Terrain Environmental Setting Report for the Suncor Voyageur South Project* 2007). Organic molecules, such as PAHs can bind to SOM, in a process called sorption (Weissenfels et al. 1992, Reid et al. 2000, Zhang et al. 2008, Ren et al. 2018a, 2018b). When bound to SOM, PAHs become less bioavailable (to some species) and difficult to degrade or extract (Reid et al. 2000). However, having a high SOM is ideal in greenhouse experiments as they create large pore spaces between soil molecules preventing soil compression after repeated watering. This may have caused the PAHs spiked in the soil to become bound to the OM particles and show the lower than expected measured values. However, since there were no significant differences with respect to effects on growth and abundance between PAH spiked soil and non-PAH spiked soils in this study, it is likely that experimental error was the likely culprit for the non-detectable or low levels of PAHs measured compared to the target nominal concentrations. Indeed, many other studies have demonstrated decreased plant growth during similar phytoremediation trials with PAHs (Smreczak and Maliszewska-Kordybach 2003, Gerhardt et al. 2009, Saharan and Nehra 2011). For example, Fan et al. (2007) grew alfalfa (*Medicago sativa L.*) over 60 days in various concentrations of pyrene (approximately 10 mg/kg, 50 mg/kg, 100 mg/kg, 200 mg/kg, and 500 mg/kg). Fan et al. (2007) observed a decrease in both root and shoot growth, with significant decreases in shoot growth occurring at 100 mg/kg of pyrene and significant decreases in root growth at 49 mg/kg of pyrene. Thus, replication of this study and comparing soils containing less SOM are warranted.

This study did demonstrate a significant effect on alfalfa endpoints between salt treatments, except for percent moisture content, where no significant effects were observed. Specifically, the soil salinity had a significant affect on successful germination, growth, and nodulation of alfalfa over the 90 day growth period, with variables decreasing as salt concentrations increased. Germination and plant abundance significantly decreased at each NaCl concentration, resulting in the lowest day 90 abundances being observed in the highest salt treatments (160 mM). The effects of salt on germination and plant growth have been well established for alfalfa (Bernstein 1975, Allen et al. 1986, Lai and Mckersie 1995, Li et al. 2010, Undersander et al. 2011, Sirguey and Ouvrard 2013, Arora et al. 2017). Established plants that have been pre-germinated in clean soil can be used to bi-pass the effects salts have on seed germination (Hamdi et al. 2012, Jesus et al. 2015, Arora et al. 2017, Gerhardt et al. 2017b). However, using established plants can be unrealistic over large areas, due to their cost and time to plant. Little to no germination within the 160 mM treatments in the present study was observed and this was expected. Previous research by Li et al. (2010) indicates a 50% reduction in successful germination of alfalfa seeds (*Medicago sativa L.*) when exposed to 150 mM NaCl, where as research by Ayers et al. (1994) indicate no successful germination at the 160 mM salt concentration. Thus, the present study concurs with the existing literature that salt concentrations of equal to or greater than 160 mM would not be suitable for alfalfa phytoremediation if germinated on site and transplanting of established alfalfa would be necessary.

The root and shoot biomass showed a similar pattern, to germination and plant abundance, decreasing in mass as the NaCl concentrations increased. However, there was no significant differences observed between the 0 mM and 20 mM salt treatments and the 80 mM and 160 mM salt treatments. Alfalfa is a low to moderate salt tolerant species that generally prefers electrical conductivities below 2 dS m^{-1}, but can thrive in soils greater than that (Allen et al. 1986, Putnam et al. 2017). Growth yields are expected to decrease between 5-7 dS m^{-1}, but some reports have shown yields to be stable up to 8 dS m^{-1} (Putnam et al. 2017).The range of electrical conductivities and therefore salt concentrations in which alfalfa can grow is most likely what caused no significant difference to be observed between the 0 mM and 20 mM salt treatments. When comparing NaCl concentrations to electrical conductivity every 10 mM NaCl will increase in the electrical conductivity by approximately 1 dS m^{-1} (Electrical Conductivity

of Electrolytes in Aqueous Solution 2018). As the NaCl concentrations and electrical conductivity moved past this acceptable range for alfalfa, decreased biomass was observed. In treatments where no biomass was recorded the ion toxicity and osmotic stress caused by salt contamination was too high and resulted in plant death or complete inhibition of germination (Li et al. 2010, Putnam et al. 2017). This was also demonstrated by Li et al. (2010) who observed a significant decrease in relative growth rate of alfalfa exposed to increasing salt concentrations. The use of halophytes, plants that specialize in saline environments, has been suggested as a possible alternative when the salt concentrations are two high for native species, and / or other phytoremediators (Margesin and Schinner 2001b, Arora et al. 2017, Ebadi et al. 2018). Ebadi et al. (2018) investigated the potential of using halophyte *Salicornia persica* as a phytoremediator as well as the effects of bacterial assisted phytoremediation on PAH degradation. Indeed, this study demonstrates that utilizing alfalfa at concentrations greater than 20 mM NaCl is not an efficient process and utilizing halophytes should be considered to achieve higher phytoremediation rates.

The number of nodules also showed an inverse relationship with salt concentration, showing a decrease in the number of nodules as salt concentrations increased. There was no significant difference observed between the 0 and 20 mM NaCl concentrations and no significant difference observed between the 80 and 160 mM NaCl concentrations. This may be an artifact due to the decrease of biomass with increasing salt concentrations. Research by Djilianov et al. (2003) observed no significant changes in the number of nodules per alfalfa plant when exposed to four different salt treatments (0 mM, 37.5 mM, 75 mM, and 150 mM), but did observe a significant difference in shoot biomass when comparing their control (0 mM) and 150 mM treatments. Within the 80 mM and 160 mM treatments, of this study, only four of possible 16 pots had observable biomass, and only three of those four possessed any nodulation (nodules >2mm in size). The low number of nodules may be caused by an inability of the plants to produce the biomass necessary for nodule formation or salt concentrations may have inhibited the growth of nodule inhabiting species of rhizobacteria (Djilianov et al. 2003, Hamdi et al. 2012, Rajtor and Piotrowska-Seget 2016, Arora et al. 2017). Future studies verifying that above 20 mM NaCl would inhibit nodule growth in alfalfa could be obtained by testing a narrower range of concentrations, particularly between 20 mM and 80 mM NaCl.

Preparation of soils for research involved in phytoremediation is a challenging task. Using isolated contaminants such as specific PAHs, and specific salts allow you to evaluate more specific interactions but are less realistic and applicable to restoration practices. Use of soils from the field are a great way to test field applications but can be difficult to obtain and transport. Ideally, soils from a contaminated site can be evaluated in both a field and laboratory setting to gain a full perspective on the issues and challenges present. Future studies should incorporate a smaller range of NaCl concentrations below 160 mM to gain a better understanding of the interactions between salt contamination and its affect on phytoremediators. By using too high a concentration of NaCl treatments and creating numerous zero data points it can be difficult to statistically observe patterns involving biomass and abundance (McCune and Grace 2002, Rajtor and Piotrowska-Seget 2016, Arora et al. 2017).

Phytoremediation poses as an excellent tool to be used in the remediation of contaminated sites (Frick et al. 1999, Huang et al. 2004, 2005, Gerhardt et al. 2009, 2017a, Wang et al. 2012). It can be a low-cost option to remediate almost any contaminant. Applicable strategies for phytoremediation are still young and being developed. This study was not able to conclusively examine the effects soil salinity on the phytoremediation of pyrene and benzo[a]pyrene. The lack of measurable PAHs makes conclusions regarding the phytoremediation of pyrene and benzo[a]pyrene impossible. However, the effects of varying salt concentrations (0 mM, 20 mM, 80 mM, and 160 mM) were evident in this study. High NaCl concentrations limited both plant growth and germination significantly with several pots resulting in a complete lack of germination or plant death. Repeating this study along with lower concentrations of NaCl, a comparison of PAH soil spiking methods, and varying soil types is recommended to improve this experiment and achieve the objective of assessing the effects of salinity on PAH phytoremediation in alfalfa. Research into the complete biochemical degradation pathways of larger PAHs would also help to better understand any limitations of phytoremediation and may offer insights into the speed and efficiency of the remediation of different PAHs (Fan et al. 2007, Ying et al. 2011, Varjani et al. 2017, Sushkova et al. 2018). Future research into the remediation of co-contaminated sites will also be necessary due to the ever increasing amount of organic, salt, and heavy metal contamination (Field et al. 2017). The increased frequency of organic, inorganic and salt contamination from non-point source pollution means areas that are

not actively involved in industry are more likely to be contaminated with multiple pollutants (Sacco and James 2004, Carré et al. 2017, Field et al. 2017), and optimizing phytoremediation in this context, as attempted in the present study, is of paramount importance.

References

Abdel-Shafy, H. I. and M. S. M. Mansour. 2016. A review on polycyclic aromatic hydrocarbons: Source, environmental impact, effect on human health and remediation. *Egyptian Journal of Petroleum* 25:107–123.

Adenipekun, C. O. and R. Lawal. 2012. Uses of mushrooms in bioremediation: A review. *Biotechnology and Molecular Biology Reviews* 7:62–68.

Alegbeleye, O. O., B. O. Opeolu, and V. A. Jackson. 2017. Polycyclic Aromatic Hydrocarbons: A Critical Review of Environmental Occurrence and Bioremediation. *Environmental Management* 60:758–783.

Alexander, D. E. 1999. Bioaccumulation, bioconcentration, biomagnification. Pages 43–44 *Environmental Geology*. Dordrecht: Springer Netherlands.

Allen, S. G., A. K. Dobrenz, and P. G. Bartels. 1986. Physiological Response of Salt-Tolerant and Nontolerant Alfalfa to Salinity during Germination. *Crop Science* 26:1004.

Del Amor, F. M. and P. Cuadra-Crespo. 2012. Plant growth-promoting bacteria as a tool to improve salinity tolerance in sweet pepper. *Functional Plant Biology* 39:82–90.

Arora, S., A. K. Singh, and D. Sahni. 2017. Bioremediation of salt-affected soils: Challenges and opportunities. Page in S. Arora, A. K. Singh, and D. Sahni (eds), *Bioremediation of Salt Affected Soils: An Indian Perspective*.

Asghar, H. N., R. Setia, and P. Marschner. 2012. Community composition and activity of microbes from saline soils and non-saline soils respond similarly to changes in salinity. *Soil Biology and Biochemistry* 47:175–178.

Ayers, R. S. and D. W. Westcot. 1994. *Water quality for agriculture*. Rev.1. Rome: Food and Agriculture Organization of the United Nations.

Bernstein, L. 1975. *Effects of salinity and sodicity on plant growth*. Riverside, California.

Brittingham, M. C., K. O. Maloney, A. M. Farag, D. D. Harper, and Z. H. Bowen. 2014. Ecological risks of shale oil and gas development to wildlife, aquatic resources and their habitats. *Environmental Science and Technology* 48:11034–11047.

Buha, A. and M. M. Williams. 2003. Polycyclic Aromatic Hydrocarbons. Pages 4616–4625 in B. Caballero, L. Turgo, and P. M. Finglas (eds), *Encyclopedia of Food Sciences and Nutrition*. 2nd edition.

Caenn, R. and G. V. Chillingar. 1996. Drilling fluids: State of the art. *Journal of Petroleum Science and Engineering* 14:221–230.

Carré, F., J. Caudeville, R. Bonnard, V. Bert, P. Boucard, and M. Ramel. 2017. Soil Contamination and Human Health: A Major Challenge for Global Soil Security. Pages 275–295 in D. J. Field, C. L. S. Morgan, and A. B. McBratney (eds), *Global Soil Security*. : Springer, Cham.

Chen, M., P. Xu, G. Zeng, C. Yang, D. Huang, and J. Zhang. 2015. Bioremediation of soils contaminated with polycyclic aromatic hydrocarbons, petroleum, pesticides, chlorophenols and heavy metals by composting: Applications, microbes and future research needs. *Biotechnology Advances* 33:745–755.

Chowdhury, N., P. Marschner, and R. G. Burns. 2011. Soil microbial activity and community composition: Impact of changes in matric and osmotic potential. *Soil Biology and Biochemistry* 43:1229–1236.

Cristaldi, A., G. O. Conti, E. H. Jho, P. Zuccarello, A. Grasso, C. Copat, and M. Ferrante. 2017. Phytoremediation of contaminated soils by heavy metals and PAHs. A brief review. *Environmental Technology & Innovation* 8:309–326.

Czub, G., F. Wania, and M. S. Mclachlan. 2008. Combining Long-Range Transport and Bioaccumulation Considerations to Identify Potential Arctic Contaminants. *Environ. Sci. Technol.* 42:3704–3709.

D'Adamo, R., S. Pelosi, P. Trotta, and G. Sansone. 1997. Bioaccumulation and biomagnification of polycyclic aromatic hydrocarbons in aquatic organisms. *Marine Chemistry* 56:45–49.

Daniel, C., J. Louis, and F. F. C. 2006. *Handbook of Soil Analysis*. Heidelberg: Springer-Verlag Berlin Heidelberg.

Djilianov, D., E. Prinsen, S. Oden, H. Van Onckelen, and J. Mü. 2003. Nodulation under salt stress of alfalfa lines obtained after in vitro selection for osmotic tolerance. *Plant Science* 165:887–894.

Dumanski, J., R. Newton, and J. D. Lindsay. 1970. Geographic zonation in selected characteristics of surface mineral horizons in Alberta soils. *Canadian Journal of Soil Science* 50:131–139.

Ebadi, A., N. Azam, K. Sima, M. Olamaee, M. Hashemi, and R. G. Nasrabadi. 2018. Remediation of saline soils contaminated with crude oil using the halophyte Salicornia persica in conjunction with hydrocarbon-degrading bacteria. *Journal of Environmental Management* 219:260–268.

El-Shahawi, M. S., A. Hamza, A. S. Bashammakh, and W. T. Al-Saggaf. 2010. An overview on the accumulation, distribution, transformations, toxicity and analytical methods for the monitoring of persistent organic pollutants. *Talanta* 80:1587–1597.

Electrical Conductivity of Electrolytes in Aqueous Solution. 2018. CRC Handbook of Chemistry and Physics. http://hbcponline.com/faces/contents/InteractiveTable.xhtml?search=true&tableId=2

9.

Fan, S., P. Li, Z. Gong, W. Ren, and N. He. 2007. Promotion of pyrene degradation in rhizosphere of alfalfa (Medicago sativa L.) DOI 10.1016/j.chemosphere.2007.10.068.

Field, D. J., C. L. S. Morgan, and A. B. McBratney, editors. 2017. *Global Soil Security*. Cham: Springer International Publishing.

Fougere, F., D. Le Rudulier, J. G. Streeter, F. Fougère, D. Le Rudulier, J. G. Streeter, F. Fougere, D. Le Rudulier, J. G. Streeter, F. Fougère, D. Le Rudulier, and J. G. Streeter. 1991. Effects of Salt Stress on Amino Acid, Organic Acid, and Carbohydrate Composition of Roots, Bacteroids, and Cytosol of Alfalfa (Medicago sativa L.). *Plant Physiology* 96:1228–1236.

Franzen, D. 2013. *Managing Saline Soils In North Dakota (SF-1087)*.

Frick, C. M., J. J. Germida, R. E. Farrell, J. J. Germida, R. E. Farrell, and J. J. Germida. 1999. *Assessment of phytoremediation as an in-situ technique for cleaning oil-contaminated sites*. Page *Technical Seminar on Chemical Spills*. Saskatoon.

Gandolfi, I., M. Sicolo, A. Franzetti, E. Fontanarosa, A. Santagostino, and G. Bestetti. 2010. Influence of compost amendment on microbial community and ecotoxicity of hydrocarbon-contaminated soils. *Bioresource Technology* 101:568–575.

Gelboin, H. V. 1980. *Benzo[a]pyrene Metabolism, Activation, and Carcinogenesis: Role and Regulation of Mixed-Function Oxidases and Related Enzymes*. Page *PHYSIOLOGICAL REVIEWS*.

Gerhardt, K. E., P. D. Gerwing, and B. M. Greenberg. 2017a. Opinion: Taking phytoremediation from proven technology to accepted practice. *Plant Science* 256:170–185.

Gerhardt, K. E., X. D. Huang, B. R. Glick, and B. M. Greenberg. 2009. Phytoremediation and rhizoremediation of organic soil contaminants: Potential and challenges. *Plant Science* 176:20–30.

Gerhardt, K. E., G. J. MacNeill, P. D. Gerwing, and B. M. Greenberg. 2017b. Phytoremediation of Salt-Impacted Soils and Use of Plant Growth-Promoting Rhizobacteria (PGPR) to Enhance Phytoremediation. Pages 19–51 in A. A. Ansari, S. S. Gill, R. Gill, G. R. Lanza, and L. Newman (eds), *Phytoremediation: Management of Environmental Contaminants, Volume 5*. Cham: Springer International Publishing.

Gieg, L. M., S. J. Fowler, and C. Berdugo-Clavijo. 2014. Syntrophic biodegradation of hydrocarbon contaminants. *Current Opinion in Biotechnology* 27:21–29.

Gkorezis, P., M. Daghio, A. Franzetti, J. D. Van Hamme, W. Sillen, and J. Vangronsveld.

2016. The Interaction between Plants and Bacteria in the Remediation of Petroleum Hydrocarbons: An Environmental Perspective. *Frontiers in Microbiology* 7:1836.

Greenberg, B., X. Huang, K. Gerhardt, P. Mosley, X. Yu, S. Liddycoat, X. Lu, B. McCallum, G. MacNeill, N. Knezevich, M. Hannaberg, P. Gerwing, T. Obal, and B. Chubb. 2012. Phytoremediation of Petroleum and Salt Impacted Soils: A Scientifically-based Innovative Remediation Process. Pages 420–433 *35th AMOP Technical Seminar on Environmental Contamination and Response*.

Gutiérrez-Ginés, M. J., A. J. Hernández, M. I. Pérez-Leblic, J. Pastor, and J. Vangronsveld. 2014. Phytoremediation of soils co-contaminated by organic compounds and heavy metals: Bioassays with Lupinus luteus L. and associated endophytic bacteria. *Journal of Environmental Management* 143:197–207.

Hamdi, H., S. Benzarti, I. Aoyama, and N. Jedidi. 2012. Rehabilitation of degraded soils containing aged PAHs based on phytoremediation with alfalfa (Medicago sativa L.). *International Biodeterioration and Biodegradation* 67:40–47.

Hamdi, H., L. Manusadžianas, I. Aoyama, and N. Jedidi. 2006. Effects of anthracene, pyrene and benzo[a]pyrene spiking and sewage sludge compost amendment on soil ecotoxicity during a bioremediation process. *Chemosphere* 65:1153–1162.

Haritash, A. K. and C. P. Kaushik. 2009. Biodegradation aspects of Polycyclic Aromatic Hydrocarbons (PAHs): A review. *Journal of Hazardous Materials* 169:1–15.

Harvey, R. G. 1985. Polycyclic Hydrocarbons and Carcinogenesis. *ACS Symposium Series* 283:420.

Hou, J., W. Liu, B. Wang, Q. Wang, Y. Luo, and A. E. Franks. 2015. PGPR enhanced phytoremediation of petroleum contaminated soil and rhizosphere microbial community response DOI 10.1016/j.chemosphere.2015.07.025.

Huang, X. D., Y. El-Alawi, J. Gurska, B. R. Glick, and B. M. Greenberg. 2005. A multi-process phytoremediation system for decontamination of persistent total petroleum hydrocarbons (TPHs) from soils. *Microchemical Journal* 81:139–147.

Huang, X. D., Y. El-Alawi, D. M. Penrose, B. R. Glick, and B. M. Greenberg. 2004. A multi-process phytoremediation system for removal of polycyclic aromatic hydrocarbons from contaminated soils. *Environmental Pollution* 130:465–476.

ISO 14688-1:2017(E) Identification and classification of soil - Part 1: Identification and description. 2017. . Pages 2–7 *Geotechnical inverstigation and testing*.

Jesus, J. M., A. S. Danko, A. Fiúza, and M. T. Borges. 2015. Phytoremediation of salt-affected soils: a review of processes, applicability, and the impact of climate change. *Environmental Science and Pollution Research* 22:6511–6525.

Jiang, H., S. L. Gelhaus, D. Mangal, R. G. Harvey, I. A. Blair, and T. M. Penning. 2007.

Metabolism of Benzo[a]pyrene in Human Bronchoalveolar H358 Cells Using Liquid Chromatography-Mass Spectrometry. *Chem. Res. Toxicol.* 20:1331–1341.

JMP. 2019. . Cary, NC: SAS Institute Inc.

Jonathan, S. G., M. D. Asemoloye, and R. Ahmad. 2017. Synergistic Plant-Microbial Rhizosphere Mechanism: A Potential Headway for Remediation of Hydrocarbon Polluted Soils. *SSRN Electronic Journal* DOI 10.2139/ssrn.3086229.

Jouyban, Z. 2004. *Salt stress in plants: Signalling, Omics and Adaptations*. Page (P. Ahmad, M. M. Azooz, and M. N. V. Prasad, editors). : Springer.

Juhasz, A. L. and R. Naidu. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo[a]pyrene. *International Biodeterioration & Biodegradation* 45:57–88.

Karle, I. L., H. Yagi, J. M. Sayer, and D. M. Jerina. 2004. *Crystal and molecular structure of a benzo[a]pyrene 7,8-diol 9,10-epoxide N 2-deoxyguanosine adduct: Absolute configuration and conformation*. Page *PNAS February*.

Konkel, L. 2016. Salting the Earth: The Environmental Impact of Oil and Gas Wastewater Spills. *Environmental Health Perspectives* DOI 10.1289/ehp.124-A230.

Kuppusamy, S., T. Palanisami, M. Megharaj, K. Venkateswarlu, and R. Naidu. 2016. Ex-Situ Remediation Technologies for Environmental Pollutants: A Critical Perspective. Pages 117–192 in P. de Voogt (ed), *Reviews of Environmental Contamination and Toxicology Volume 236*. Cham: Springer International Publishing.

Lai, F.-M. M. and B. D. Mckersie. 1995. Germination of Alfalfa (Medicago sativa L.) Seeds and Desiccated Somatic Embryos. II. Effect of Nutrient Supplements. *Journal of Plant Physiology* 146:731–735.

Lehr, R. E., S. Kumar, W. Levin, A. W. Wood, R. L. Chang, A. H. Conney, H. Yagi, J. M. Sayer, and D. M. Jerina. 1985. The Bay Region Theory of Poly Aromatic Hydrocarbon Carcinogenesis. Pages 63–84 in R. G. Harvey (ed), *Polycycilic Hydrocarbons and Carcinogenesis*. Philadelphia, Pa.

Li, R., F. Shi, K. Fukuda, and Y. Yang. 2010. Effects of salt and alkali stresses on germination, growth, photosynthesis and ion accumulation in alfalfa (Medicago sativa L.). *Soil Science and Plant Nutrition* 56:725–733.

Liu, S.-H., G.-M. Zeng, Q.-Y. Niu, Y. Liu, L. Zhou, L.-H. Jiang, X.-F. Tan, P. Xu, C. Zhang, and M. Cheng. 2017. Bioremediation mechanisms of combined pollution of PAHs and heavy metals by bacteria and fungi: A mini review. *Bioresource Technology* 224:25–33.

Liu, S. L., Y. M. Luo, Z. H. Cao, L. H. Wu, K. Q. Ding, and & P. Christie. 2004. Degradation of benzo[a]pyrene in soil with arbuscular mycorrhizal alfalfa.

Environmental Geochemistry and Health 26:285–293.

Maqbool, F., Z. Wang, Y. Xu, J. Zhao, D. Gao, Y.-G. Zhao, Z. A. Bhatti, and B. Xing. 2012. Rhizodegradation of petroleum hydrocarbons by Sesbania cannabina in bioaugmented soil with free and immobilized consortium. *Journal of Hazardous Materials* 237–238:262–269.

Margesin, R. and F. Schinner. 2001a. Biodegradation and bioremeditaion of hydrocarbons in extreme environments. *Appl Microbiol Biotechnol* 56:650–663.

Margesin, R. and F. Schinner. 2001b. Biodegradation and bioremediation of hydrocarbons in extreme environments. *Applied Microbiology and Biotechnology* 56:650–663.

McCune, B. P. and J. Grace. 2002. Chapter 9: Data Transforamtions. Pages 67–79 *Analysis of Ecological Communities*. Gleneden Beach, Oregon, USA: MjM Software.

Meehan, M., K. Sedivec, T. Desutter, C. Augustin, A. Daigh, E. Livestock, E. Stewardship, K. Sedivec, E. Rangeland, M. Specialist, T. Desutter, S. Scientist, C. Augustin, E. Area, S. Health, A. Daigh, S. Scientist, and A. Daigh. 2017. *Environmental Impacts of Brine (Produced Water)*. Grand Forks .

Meudec, A., J. Dussauze, E. Deslandes, and N. Poupart. 2006. Evidence for bioaccumulation of PAHs within internal shoot tissues by a halophytic plant artificially exposed to petroleum-polluted sediments. *Chemosphere* 65:474–481.

Morin, A. E., J. D. Blair, M. G. Ikonomou, F. A. P. C. Gobas, and B. C. Kelly. 2007. Food Web-Specific Biomagnification of Persistent Organic Pollutants. *Science* 317:236–239.

Muratova, A., E. Dubrovskaya, S. Golubev, V. Grinev, M. Chernyshova, and O. Turkovskaya. 2015. The coupling of the plant and microbial catabolisms of phenanthrene in the rhizosphere of Medicago sativa. *Journal of Plant Physiology* 188:1–8.

Nannipieri, P., R. Pinton, and Z. Varanini. 2007. *The rhizosphere : biochemistry and organic substances at the soil-plant interface / edited by Roberto Pinton, Zeno Varanini, Paolo Nannipieri.* 2nd ed. : Boca Raton, FL : CRC Press.

National Pollutant Release Inventory. 2018. Government of Canada. https://www.canada.ca/en/environment-climate-change/services/national-pollutant-release-inventory/substances-list/threshold.html.

Net, S., F. Henry, S. Rabodonirina, M. Diop, D. Merhaby, C. Mahfouz, Amara, and Ouddane. 2015. Accumulation of PAHs, Me-PAHs, PCBs and total Mercury in sediments and Marine Species in Coastal Areas of Dakar, Senegal: Contamination level and impact. *Int. J. Environ. Res* 9:419–432.

Newman, L. A., C. M. Reynolds, M. Y. Galperin, and A. Jm Baker. 2004. Phytodegradation of organic compounds This review comes from a themed issue on Environmental biotechnology Edited. *Current Opinion in Biotechnology* 15:225–230.

Neyer, E., G. Rorech, P. Palmer, J. Bedessem, D. Kidd, J. Boettcher, F. Lenzo, T. Crossman, E. Carman, K. Thalman, P. Fierro, and O. Braids. 2000. *In situ treatment technologies*. Second edition. : Nyer, Evan.

North Dakota Remediation Resource Manual. 2016. . Grand Forks: Energy & Environmental Research Center.

Nyer, E., P. Palmer, E. Carman, G. Boettcher, J. Bedessem, D. Kidd, F. Lenzo, G. Rorech, and T. Crossman, editors. 2000. *In Situ Treatment Technology, Second Edition*. 2nd edition. : CRC Press.

Pereira, M. G., L. A. Walker, J. Wright, J. Best, and R. F. Shore. 2009. Concentrations of Polycyclic Aromatic Hydrocarbons (PAHs) in the Eggs of Predatory Birds in Britain. *Environ. Sci. Technol.* 43:9010–9015.

Petrová, Š., J. Rezek, P. Soudek, and T. Vaněk. 2017. Preliminary study of phytoremediation of brownfield soil contaminated by PAHs. *Science of the Total Environment* 599–600:572–580.

Physical Constants of Organic Molecules. 2018. CRC Handbook of Chemistry and Physics. : CRC Press. http://hbcponline.com/faces/contents/ContentsResults.xhtml.

Putnam, D. H., S. Benes, G. Galdi, B. Hutmacher, and S. Grattan. 2017. Alfalfa (Medicago sativa L.) is tolerant to higher levels of salinity than previous guidelines indicated: Implications of field and greenhouse studies. *Geophysical Research Abstracts* 19:2017–18266.

Rajtor, M. and Z. Piotrowska-Seget. 2016. Prospects for arbuscular mycorrhizal fungi (AMF) to assist in phytoremediation of soil hydrocarbon contaminants. *Chemosphere* 162:105–116.

Rath, K. M. and J. Rousk. 2015. Salt effects on the soil microbial decomposer community and their role in organic carbon cycling: A review. *Soil Biology and Biochemistry* 81:108–123.

Reid, B. J., K. C. Jones, and K. T. Semple. 2000. Bioavailability of persistent organic pollutants in soils and sediments: a perspective on mechanisms, consequences and assessment. *Environmental Pollution* 108:103–112.

Ren, X., G. Zeng, L. Tang, J. Wang, J. Wan, H. Feng, B. Song, C. Huang, and X. Tang. 2018a. Effect of exogenous carbonaceous materials on the bioavailability of organic pollutants and their ecological risks. *Soil Biology and Biochemistry* 116:70–81.

Ren, X., G. Zeng, L. Tang, J. Wang, J. Wan, Y. Liu, J. Yu, H. Yi, S. Ye, and R. Deng. 2018b. Sorption, transport and biodegradation - An insight into bioavailability of persistent organic pollutants in soil. *Science of the Total Environment* 610–611:1154–1163.

Rieger, J., J. Stanley, and R. A. Y. Traynor. 2014. *Project Planning and Management for Ecological Restoration*. Page (J. Aronson, K. D. Holl, D. A. Falk, R. J. Hobbs, and M. A. Palmer, editors). First edition. Washington, DC: IslandPress.

Risula, D. (n.d.). *Nodulation and Nitrogen Fixation Field Assessment Guide*.

Rojas-Avelizapa, N. G., T. Roldán-Carrillo, H. Zegarra-Martínez, A. M. Muñoz-Colunga, L. C. Fernández-Linares, A. M. Muñ Oz-Colunga, L. C. Fernández-Linares, A. M. Muñoz-Colunga, and L. C. Fernández-Linares. 2007. A field trial for an ex-situ bioremediation of a drilling mud-polluted site. *Chemosphere* 66:1595–1600.

S.E.R.A.S. 1994. *Standard Operating Procedures: Plant Biomass Determination*.

Sacco, J. C. and M. O. James. 2004. Glucuronidation in the polar bear (Ursus maritimus). *Marine Environmental Research* 58:475–479.

Safe, S. 2001. Molecular biology of the Ah receptor and its role in carcinogenesis. *Toxicology Letters* 120:1–7.

Saharan, B. S. and V. Nehra. 2011. Plant Growth Promoting Rhizobacteria : A Critical Review. *Life Sciences and Medicine Research* 2011:1–30.

Saleem, M., M. Arshad, S. Hussain, and A. S. Bhatti. 2007. Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. *Journal of Industrial Microbiology and Biotechnology* 34:635–648.

Salt, D. E., R. D. Smith, and I. Raskin. 1998. Phytoremediation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49:643–668.

Samanta, S. K., O. V Singh, and R. K. Jain. 2002. Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends in Biotechnology* 20:243–248.

Sei, A. and B. Z. Fathepure. 2009. Biodegradation of BTEX at high salinity by an enrichment culture from hypersaline sediments of Rozel Point at Great Salt Lake. *Journal of Applied Microbiology* 107:2001–2008.

Sims, P., P. L. Grover, A. Swaisland, K. Pal, and A. Hewer. 1974. Metabolic activation of benzo(a)pyrene proceeds by a diol-epoxide. *Nature* 252:326–328.

Sirguey, C. and S. Ouvrard. 2013. Contaminated soils salinity, a threat for phytoextraction? *Chemosphere* 91:269–274.

Smreczak, B. and B. Maliszewska-Kordybach. 2003. Seed germination and root growth

of selected plants in PAH contaminated soil. *Fresenius Environmental Bulletin* 12:946–949.

Soil and Terrain Environmental Setting Report for the Suncor Voyageur South Project. 2007. .

Sushkova, S., I. Deryabkina, E. Antonenko, R. Kizilkaya, V. Rajput, and G. Vasilyeva. 2018. Benzo[a]pyrene degradation and bioaccumulation in soil-plant system under artificial contamination. *Science of the Total Environment* 633:1386–1391.

Syed, K., H. Doddapaneni, V. Subramanian, Y. W. Lam, and J. S. Yadav. 2010. Genome-to-function characterization of novel fungal P450 monooxygenases oxidizing polycyclic aromatic hydrocarbons (PAHs). *Biochemical and Biophysical Research Communications* 399:492–497.

Takeuchi, I., N. Miyoshi, K. Mizukawa, H. Takada, T. Ikemoto, K. Omori, and K. Tsuchiya. 2009. Biomagnification profiles of polycyclic aromatic hydrocarbons, alkylphenols and polychlorinated biphenyls in Tokyo Bay elucidated by δ13C and δ15N isotope ratios as guides to trophic web structure. *Marine Pollution Bulletin* 58:663–671.

Tomlinson, H. A. K. 2016. Brine impacted soils in semi-arid ragelands: Greenhosue electrical conductivity thresholds and ex situ / in situ remediation comparisons. : North Dakota State University.

Tsuji, G., M. Takahara, H. Uchi, S. Takeuchi, C. Mitoma, Y. Moroi, and M. Furue. 2011. An environmental contaminant, benzo(a)pyrene, induces oxidative stress-mediated interleukin-8 production in human keratinocytes via the aryl hydrocarbon receptor signaling pathway. *Journal of Dermatological Science* 62:42–49.

Undersander, D., M. H. Hall, P. Vassalotti, and D. Cosgrove. 2011. Alfalfa germination & growth. *National Alfalfa and Forage Alliance and University of Wisconsin Cooperative extension*.

Uppstad, H., S. Øvrebø, A. Haugen, and S. Mollerup. 2010. Importance of CYP1A1 and CYP1B1 in bioactivation of benzo[a]pyrene in human lung cell lines. *Toxicology Letters* 192:221–228.

Varjani, S. J., E. Gnansounou, and A. Pandey. 2017. Comprehensive review on toxicity of persistent organic pollutants from petroleum refinery waste and their degradation by microorganisms. *Chemosphere* 188:280–291.

Wang, M. C. C., Y. T. T. Chen, S. H. H. Chen, S. W. W. Chang Chien, and S. V. V. Sunkara. 2012. Phytoremediation of pyrene contaminated soils amended with compost and planted with ryegrass and alfalfa. *Chemosphere* 87:217–225.

Wang, X., K. Yang, S. Tao, and B. Xing. 2007. Sorption of aromatic organic contaminants by biopolymers: Effects of pH, copper (II) complexation, and cellulose coating. *Environmental Science and Technology* 41:185–191.

Weissenfels, W. D., H.-J. Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: influence on biodegradability and biotoxicity. *Appl Microbiol Biotechnol Applied Microbiology Biotechnology* 36:689–696.

Whittemore, D. O. 1995. Geochemical Differentiation of Oil and Gas Brine from Other Saltwater Sources Contaminating Water Resources: Case Studies from Kansas and Oklahoma. *Environmental Geosciences* 2:15–31.

Yang, R.-Y., J.-J. Tang, Y.-S. Yang, and X. Chen. 2007. Invasive and non-invasive plants differ in response to soil heavy metal lead contamination. *Botanical Studies* 48:453–458.

Ying, X., G. Dongmei, L. Judong, and W. Zhenyu. 2011. Plant-microbe Interactions to Improve Crude Oil Degradation. *Energy Procedia* 5:844–848.

Zhang, K., X. F. Hua, H. L. Han, J. Wang, C. C. Miao, Y. Y. Xu, Z. D. Huang, H. Zhang, J. M. Yang, W. B. Jin, Y. M. Liu, and Z. Liu. 2008. Enhanced bioaugmentation of petroleum- and salt-contaminated soil using wheat straw. *Chemosphere* 73:1387–1392.

Appendix A.

Pot Dimension

Dimensions of the pots used during the 90 day growth period are shown (Figure A1).

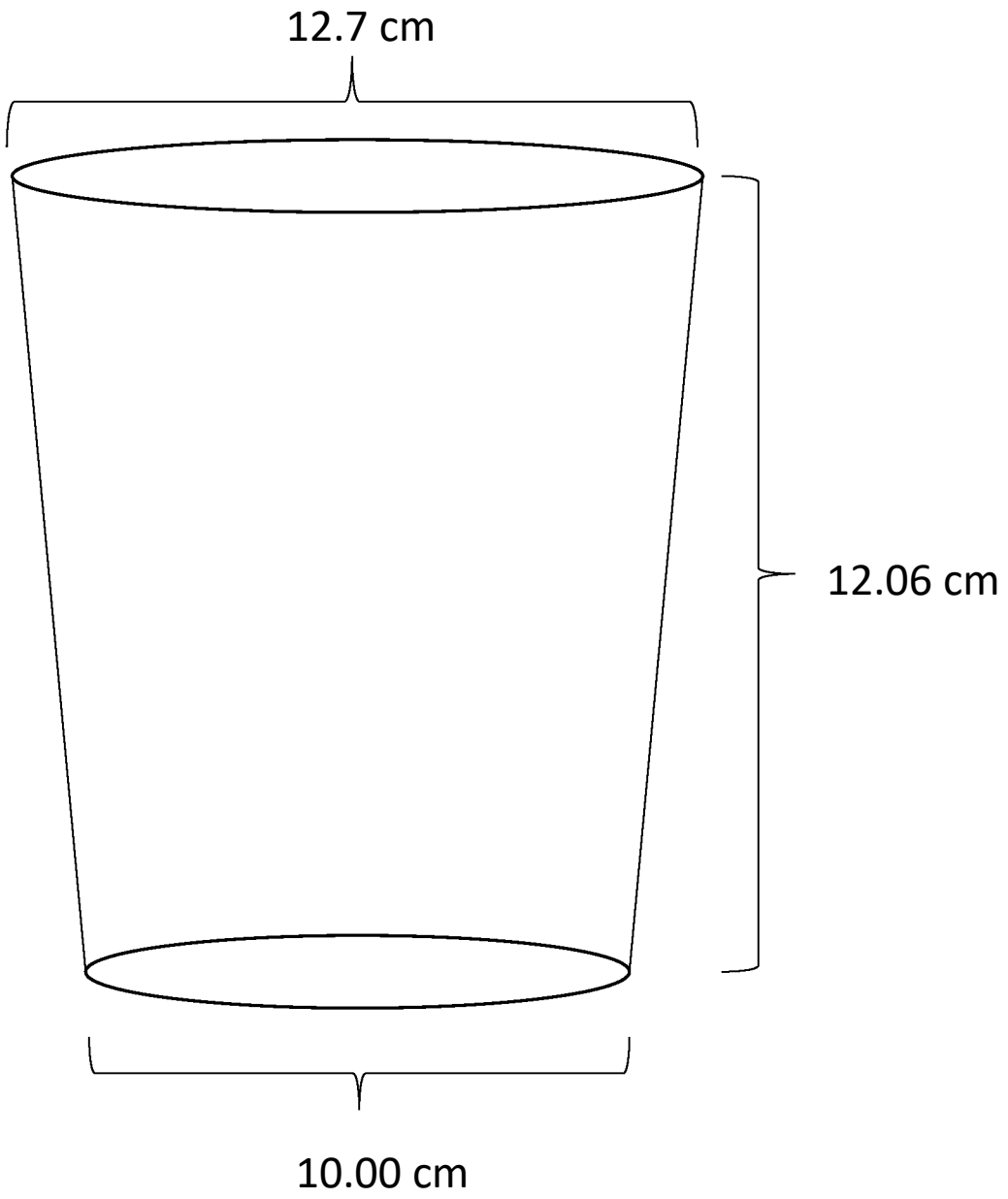


Figure A1. Dimensions of pots used for greenhouse growth experiment.

Appendix B.

Summary data per treatment and variable observed

Table B1. Mean results of dependent variables ± standard deviation per treatment.

Dependent Variable	Control (0 PAH, 0 NaCl)	Treatment 1 (0 PAH, 20 mM NaCl)	Treatment 2 (0 PAH, 80 mM NaCl)	Treatment 3 (0 PAH, 160 mM NaCl)	Treatment 4 (Added PAH, 20 mM NaCl)	Treatment 5 (Added PAH, 80 mM NaCl)	Treatment 6 (Added PAH, 160 mM NaCl)	Treatment 7 (Added PAH, 0 mM NaCl)
Maximum plant abundance observed	30.50±4.43	22.00±5.72	4.25±3.40	1.00±0.82	19.50±3.42	5.75±3.50	1.00±1.15	25.75±4.03
Plant Abundance at termination	22.75±3.69	13.67±9.07	1.50±1.29		12.25	4.35		17.75±0.96
Wet Shoot Biomass (g)	6.65±2.31	8.04±3.65	0.67±1.00	No Growth (Net 0.00±0.00)	6.65±2.82	0.33±0.32	No Growth (Net 0.00±0.00)	6.16±2.69
Dry Shoot Biomass (g)	1.75±0.67	2.23±0.48	0.08±0.14		1.33±0.84	0.03±0.05		1.74±0.26
Wet Root Biomass (g)	8.58±3.97	9.35±3.04	0.58±0.85		3.99±2.81	0.22±0.33		4.69±0.77
Dry Root Biomass (g)	2.90±1.75	2.87±0.90	0.16±0.28		1.17±1.03	0.04±0.05		1.39±0.44
Percent Moisture Content	70.2±3.04	68.7±9.14	84.9±10.4		78.4±5.79	70.61±29.5		69.1±10.7
Abundance of Nodules	64.3±22.4	73.7±24.3	11.3±12.9		49.0±15.3	5.75±11.5		75.0±34.4

Appendix C.

Potential nodulation and nitrogen fixation potential scoring

Potential nodulation and nitrogen fixation scores were estimated using the Saskatchewan Pulse Growers Nodulation and Nitrogen Fixation Field Assessment Manual. Score were provided using the criteria presented in Table C1.

Table C1. Potential nodulation and nitrogen fixation estimate score criteria. Methods adapted from Risula's Nodulation and Nitrogen Fixation Field Assessment Guide

Plant Vigor Assessment Scoring	
Plants Green and vigorous	5
Plants green and relatively small	3
Plants chlorotic (yellow)	2
Plants very chlorotic (Yellow)	1
No Growth	0
Nodule Colour and Abundance Scoring	
>5 clusters of pink nodules	5
3-5 clusters of predominately pink nodules	3
< 3 clusters of pink nodules or nodules are white/green	1
No nodules or only white/green nodules present	0
Nodule Location Scoring	
Both crown and lateral nodule positions	3
Mostly crown nodule positions	2
Mostly lateral nodule positions	1
No Nodules	0
Sum Score Categories	
Sum Score: 11-13	Effective Nodulation
Sum Score: 7-10	Moderate Nodulation
Sum Score: 1-6	Poor Nodulation
Sum Score: 0	No Growth