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PLANT RESPONSES TO TOXIC ELEMENTS AND SALT STRESS OF CONTAMINATION FROM THE PETROLEUM INDUSTRY

By

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DECLARATION

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed:

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PREFACE

The main content of this thesis is presented in chapters consisting of an introduction, methods used, results, discussion, and conclusions. Each chapter was prepared for publication in journals using peer-review in a similar format as it appears in this thesis:

Chapters 2

Phatchani Srikhumsuk, Tanya Peshkur, Joanna C. Renshaw, Charles W. Knapp (2019-2020). Characterization, and bioaccumulation of *Festuca rubra* L. (red fescue) and *Trifolium pratense* L. (red clover) for distribution of excess strontium in artificially contaminated soil from experimental microcosms in terms of produced water from the oil industry. To be submitted to *Archives of Environmental Contamination and Toxicology*.

Chapter 3

Phatchani Srikhumsuk, Tanya Peshkur, Joanna C. Renshaw, Charles W. Knapp (2019-2020). Comparing toxicities of produced waters and solutions of similar salinity; the growth of red fescue and red clover plants. To be submitted to *Environmental Geochemistry and Health.*

Chapter 4

Phatchani Srikhumsuk, Tanya Peshkur, Joanna C. Renshaw, Charles W. Knapp (2019-2020). The alternative floating culture technique of *Cyperus longus* (sweet galingale) and *Phragmites australis;* (common reed) for remediation of soluble salts from produced water from the oil industry. To be submitted to *Environmental Chemistry Letters*.

Some of the results of this work have also been used at conference presentations:

- Phatchani Srikhumsuk, Charles W. Knapp, and Joanna C. Renshaw (2016). *Efficacy of Plants to Phytoremediate Metals from Hydraulic Fracturing Wastewaters*. Research

Presentation Day, Faculty of Engineering University of Strathclyde 22nd June 2016. (Appendix E1)

- Phatchani Srikhumsuk, Charles W. Knapp, and Joanna C. Renshaw (2018). *Comparing the growth of fescue and clover plants in petroleum industrial effluents and solutions of similar salinity.* SETAC Europe 28th Annual Meeting in Rome, Italy on 13-17 May 2018. (Appendix E2)

ABSTRACT

***Background & Aims*:** Several studies have sought to explore the toxicity of heavy metals and high salinity from wastewaters, especially produced water (PW) from the petroleum industry, both in laboratory and field trials; however, there is little information available regarding *F. rubra, C. longus, P. australis, and T. pratense* species. This thesis investigated these plant species' ability to be used as an alternative method of removing trace elements and salt ions from contaminated soil and water emanating from produced water.

***Methodology*:** Several methods were used to investigate trace elements, in particular, strontium and salt ions. Analysis included the physicochemical characteristics of both soil and water samples. Soil analyses focused on sequential extraction and batch adsorption methods while water samples were used to analyze the value of trace elements and nutrient concentrations. In addition, biomass harvesting and plant material digestion were used to evaluate the phytoremediation technique, which played an essential role in this thesis to explain exposure-response with metal toxicity and high salinity. This work performed investigations mainly using Inductively Coupled Plasma- Optical Emission Spectrometry (ICP-OES) to determine the types and amounts of trace elements.

Results: Freundlich and Langmuir models describe strontium sorption in South Lanarkshire farm in Glasgow, UK, soils. Apparent equilibrium was reached within 24 hours. The interesting thing about the efficiency of *F. rubra* and *T. pratense* is their ability to grow and respond quickly. It seems possible that these plants will accumulate strontium composition in their tissues. On the other hand, stressful environments with high salinity of contaminated produced water and similar salinity solutions generated the most concentrated element. However, the results of this study suggested that *C. longus* and *P.*

australis can absorb and eliminate excess salt ions from their leaves both in produced water and solutions of similar salinity.

Conclusions: The main focus was two-fold: soil and water contamination. Soil was identified as acidic, sodic, slightly silty to coarse sand with low organic matter content. In sorption, Sr ions prefer to associate within residual and carbonate fractions, rather than other fractions in this soil. The best efficiency of retention time for adsorption was more than 24h, which was employed to fit both of the Langmuir and Freundlich equilibria models. Germination tests showed that *F. rubra* emerged quickly at low concentrations of both calcium and strontium ions; *T. pratense* responded similarly. The upper parts of *T. pratense* showed a greater translocation factor of Sr ions than *F. rubra*. However, in *F. rubra,* made chlorophyll content adjustments in response to elevated concentrations of strontium in the soils. Therefore, both plants can be considered as accumulator species. On the other hand, species-specific responses to different levels of salinity concentrations were also identified. *T. pratense* was a more sensitive species than *F. rubra* in high salinity. In contrast, both *C. longus* and *P. australis* were suitable to grow in the high salinity of produced water effluents, illustrating their potential to remove salts in produced waters via salt exclusion.

The results of this study show responses to salt stress and changes in productivity (including death). These findings enhance our understanding of *F. rubra, C. longus, P. australis, and T. pratense* and their potential uses in mitigation to high salt ions from produced water. Future studies of other potentially useful plant species in this field may also identify important technical difficulties at the genetic or molecular level in salt tolerance of halophyte species.

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LIST OF ABBREVIATIONS & ACRONYMS

°C	degree celcius
µg	microgramme (1µg = $1x10^{-6}$g)
µm	micrometre (1µm = $1x10^{-6}$m)
CEC	cation exchange capacity
cm	centrimetre
Conc.	Concentration
D	day
df	dilution factor
DM	dry matter
$dS.m^{-1}$	deciSiemens per metre
DW	dry weight
EC	electrical conductivity
FGP	final germination percentage
FW	fresh weight
$g\ L^{-1}$	gram per litre
g	gramme
h	hour
$kg.m^{-2}$	kilogram per square metre
L	litre

LOI	loss on ignition
LSD	least significant difference
lux	one lumen per square metre
m^2	square metre
m^3	cubic metre
MDG	mean daily germination
mg L^{-1}	milligram per litre
mg	milligram
mg.g^{-1}	milligram per gram
MGT	mean germination time
Min	minute
ml	millilitre
mm	millimetre
mM	Millimolar
MQL	Method Quantification Limits
n	number
N/A	not available
PW	produced water
RGR	relative growth rate
S.E.	Standard Error
TC	total carbon

TF	translocation factor
UK	United Kingdom
V	volume
v/v	volume per volume
VI	vigor index

CHAPTER 1

INTRODUCTION

1.1 Background

Recently, numerous studies have increasingly focused on addressing the problem of effluents discarded by the petroleum industry, either by improving treatment technologies or management strategies for wastewater its treatment systems. Additionally, efforts are being made to remove toxic elements and salinity from wastewaters known as 'produced waters' (PWs), which emanate from conventional and unconventional oil- and gas-production industries, to meet regulation requirements relating to discharged water and drinking water safety (Mair et al., 2012, Gordalla et al., 2013, Jiang et al., 2014, Kuwayama et al., 2015, Vidic, 2015).

Potentially toxic elements (PTE) are a class of chemical substances with possible adverse impacts on the environment. PWs, which are the fluids from drilling sites that have accumulated in the borehole and eventually returned to surface, are reported to cause contamination of wastewaters with its complex chemical constituents in the flowback (Røe Utvik, 1999, Camus et al., 2015, Arthur et al., 2009, Hayes, 2009, Jackson et al., 2011), of which PTE represent a major problem. The toxic substances in PWs can be released to aquifers, surface waters, and soils in the vicinity of petroleum industry sites (Elias-Samlalsingh and Agard, 2004).

PWs are also excessively saline (Jiménez et al., 2018), which can adversely impact the environment and human health over time (Greenberg et al., 2007). This issue presents a challenge and requires further investigation of treatment techniques to allow safe discharge into the environment. In recent years, attempts have been made to develop wastewater treatment systems to reduce pollutants in PWs. In some cases, advanced technologies, such as electro-flocculation and reverse osmosis, have been effectively used to eliminate trace

elements and brines in effluents, while less advanced techniques such as bioreactors and wetlands have also been tried (Hinchman et al., 1995, Çakmakce et al., 2008).

Salinity reduction by higher technology does not eliminate the salt (Greenberg et al., 2007), rather it concentrates it into smaller volumes. There is a debate about which remediation technology is most appropriate for PW effluents in terms of greater efficiency and lower costs, as well as being environmentally-friendly. The ultimate aims are to be aware of the effluents' impacts to the environment, and to reduce the concentrations of toxic chemical substances and salinity over the long term.

One possibility is phytoremediation, where plants remove pollutants from industrial discharges (Greenberg et al., 2007). This could represent a way of reducing the cost of treatment technology for the removal of chemical toxicity such as PTE and excessive salinity of PWs. Several models have been suggested in the last few decades, including constructing a wetland (Ji et al., 2007). It is from this approach, that I pursue further investigations into the role of plants as indicators of pollution impact, and also their possible role in the remediation of contaminated soils and waters.

1.2 Purpose and scope of research

Vegetation can be used as a environmentally-friendly and cost-effective method to remediate contaminated soil and water. An additional benefit of this methodology is that it would also serve as a toxicological indicator of environmental exposure to salinity from PWs. Phytoremediation is in general, have involved numerous species, and studies have modeled the response of plant roots and shoots to toxicants. I aimed to investigate how plant species respond and uptake specific pollutants, as well as understand the chemical, physical, and biological fate of these contaminants.

This research has been done at microcosm scale with a view to develop full-scale operations in the future. The microcosms represent pseudo-realistic environmental conditions, but with the ability to replicate treatment conditions for statistical analysis.

The investigations occurred in three parts and are presented in the subsequent chapters.

Firstly, soil properties were investigated (Chapter 2) to determine their role in PTE fate and possible pathways to exposure—in this case, I investigated strontium (Sr^{2+}), a major contaminant in PWs (Olsson, et al. 2013, Capo, et al., 2014). Solubilities and availabilities of Sr^{2+} change depending on the character of soils and time (soil aging). Soil character was determined by British Standards; contaminant fate was investigated using soil-batch tests; and sequential extraction methods determined their possible bioavailability to plants.

Secondly, a preliminary test (Chapter 2) was conducted to compare seed germination of plants versus the timing of their exposure to a toxicant (again, using Sr^{2+} as a model compound). This examined how biological indicators of plant species were influenced by timing and conditions associated during their germination, whether in pre-contaminated soil or exposed post-germination during their growth. This experiment was intended to inform the methodologies of subsequent soil-batch experiments.

Finally, I carried out experiments (Chapters 3 and 4) to investigate the toxicity and salt-stress levels from PW effluents. Here, I took a different approach to experimental studies. Rather than using soil-batches (as in previous chapters), I determined the efficiency of plant species in cleaning up wastewater using a floating-plant hydroponic system. This was to avoid any physical/chemical interferences with soil structure (as informed by previous investigations). From these experiments, the responses of various plant species were investigated to varying doses of contaminated water.

1.3 Aims and objectives

1.3.1 Aims

The aims of this research were to investigate properties of contaminated soil and water of synthetic waste solution and PWs from the petroleum industry, and to assess salt-stress, which has become a common issue resulting from oil-industry effluent discharge. Various plant species were examined to assess dose-response behaviour in phytotoxicity tests. The objectives are set out in the next section.

1.3.2 Objectives

- To investigate the properties of artificially contaminated soil and water, representing various diluted dosages of salts (e.g., Sr^{2+}) commonly discharged from the petroleum industry.
- Use eco-toxicological methods to determine the toxicity of effluents and their effects on plant growth and responses.
- To evaluate the potential of UK-native plant species to accumulate toxic elements and salinity from effluent-contaminated waters.

1.3.3 Research gap and idea development

Research to date has focused on 'higher' rather than low-technologies for elimination of trace elements and salinity from wastewater. This thesis will examine the three key themes as possible explanations for the effect of produced water from the oil industry, as represented by a 3Ps model with preliminary, progression, and proof phases (Figure 1.1). This research serves as a foundation for future studies of this challenging task, and helps to gain a deeper understanding of the strategic application of phytoremediation for salt stress. The

results of this study include the identification and evaluation of some plant species that could be used in actual contaminated sites.

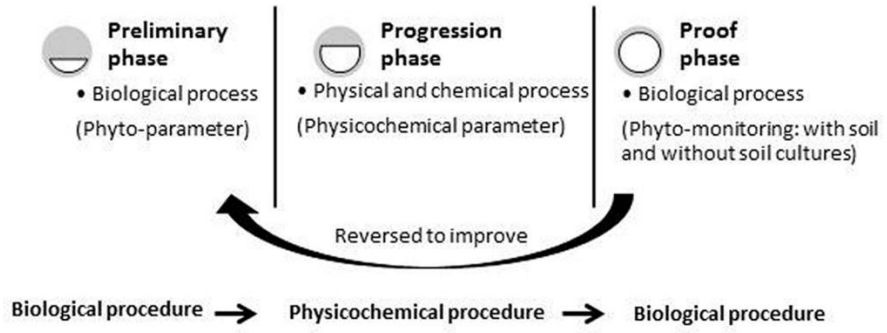

Figure 1.1 Schematic of the 3Ps strategy model (preliminary, progression, and proof phases) to improve phytoremediation techniques.

1.4 Produced waters (PWs) from the petroleum industry

At the present time, PWs are among the huge volumes of wastewaters discharged by the oil and gas industries, and these effluents are increasing significantly worldwide (Somerville et al., 1987, Abhilash et al., 2009, Camus et al., 2015, Jiménez et al., 2018). Wastewater production, both during and after the extraction of oil and gas processes, is also known as 'flowback' (Çakmakce et al., 2008, Camus et al., 2015). PWs are mainly generated from oil and gas activities, which inject water into the underground geological formations (Somerville et al., 1987, Røe Utvik, 1999, Gurska et al., 2009, Alley et al., 2011, Camus et al., 2015, Jiménez et al., 2018), and about 70 per cent of PWs that potentially contribute to underground water contamination are brought to the surface area during oil and gas operations (Çakmakce et al., 2008), with 30% either remaining in boreholes or slowly discharging much later. Underground wells are often used to store wastewater that would otherwise have to be kept on land (Bakke et al., 2013).

According to Veil et al. (2004) and Alley et al. (2011), the estimated volume of PWs usually discharged, in the USA for example, is around 3.3 million m^3. Camus et al. (2015) reported that, in the Norwegian continental shelf, 131 million m^3 of PWs were generated in 2012 and discharged into the marine environment. In detailed analysis of PWs, Bakke et al. (2013) and Jiménez et al. (2018) concluded that PWs contain complex mixtures of toxic inorganic and organic compounds. Neto and Costa (2009) highlight that the main environmental problem from oil- and gas-production effluents is the increased salinity. However, inorganic chemicals such as boron, chlorides, iron, manganese, and sodium have serious adverse effects on the environment (Veil et al., 2004). The salts and trace elements cause co-contamination of soil and water, and Çakmakce et al. (2008) assert that it is very difficult to treat these PW characteristics. They further note it remains essential to remove these pollutants during pre-treatment to successfully treat PWs.

Many areas globally face salt-impacted soil and water. Extreme salinity causes detrimental environmental impacts, especially in the agricultural sector which re-uses treated PWs for irrigation (Ravindran et al., 2007). Sites in Western Canada experience problems resulting from oil and gas exploration PWs, with elevated cations of Ca^{2+}, Mg^{2+}, and Na^{+} and anions such as bicarbonates, chlorides, and sulphates (Greenberg et al., 2007). To address this issue, regulations have been imposed by organisations, such as The United States Environmental Protection Agency (USEPA), stipulating that the maximum amount of pollution (various organic and inorganic components) allowed from PWs discharged by the oil and gas industries are 42 $mg.L^{-1}$ daily and 29 $mg.L^{-1}$ monthly, in order to achieve environmentally acceptable effluent discharges (Fakhru'l-Razi et al., 2009).

1.5 Salinity stress on plant species

The definition of salinity is the presence of salt in rock or soil, or its soluble salts in groundwater and surface water (Lanyon, 2011). As mentioned earlier, salinity is one of the most serious environmental problems, especially in terms of its effects on plants. The hazards and effects of salinity vary depending on the climate and the ability of plants to grow in saline environments (Acosta-Motos et al., 2017), and the risk of rising salinity levels directly affects the plant yields (Lanyon, 2011). The data published by Rasool et al. (2013) clearly demonstrate that it is important to indicate soil sodicity classes. Table 1.1 illustrates the electrical conductivity values of the extracted solutions (Table 1.1). These results suggest that it is possible to measure the severity level of salinity in the environment, and salinization can be introduced by both natural and human activities. Rasool et al. (2013) categorized salinity levels as presented in Table 1.1.

Table 1.1 Range of value electrical conductivities and associated salinity levels, adapted from Rasool et al. (2013).

EC_e range ($dS.m^{-1}$)	**Salinity levels**
0-2	Non-saline
2-4	Low salinity
4-8	Moderate salinity
8-16	High salinity
16-32	Severe salinity
>32	Extreme salinity

Plants can be classified by their response to salt stress into two groups: glycophytes and halophytes. Halophytes are native species that are able to tolerate salinity in their life cycle,

while glycophytes are non-halophytes that are not resistant to saline solutions (Mohammadi and Kardan, 2016). Stofberg et al. (2014) note that plant responses vary depending on salinity and their tolerance. Table 1.2 shows the mechanisms involved, the time required to produce an effect, and the responses of plants once exposed to salinity. Reduced growth was seen in the early osmotic phase, followed by limited nutrient uptake or nutrient imbalance, and finally ion toxicity (Munns, 2002, Munns and Tester, 2008, Parvaiz and Satyawati, 2008, Grieve et al., 2012, Ashkan and Jalal, 2013, Stofberg et al., 2014, Julkowska and Testerink, 2015, Kotagiri and Kolluru, 2017, Terletskaya et al., 2017).

Table 1.2 Effects of salinity on plant response, adapted from Munns and Tester (2008), as cited in Stofberg et al. (2014).

Parameters	**Salt-impacts on plants**		
	Osmotic potential	**Nutrient interaction**	**Toxicity**
Duration	A day, quickly	A day or weeks	Day or weeks, slowly
Mechanisms	Obstructed water absorption	Uptake Ca^{2+}, K^{+}, and NO_3^{-}	Na^{+} (Cl^{-}) toxicity
Response	Reduced growth, new shoot development , and photosynthesis	Deficiency of nutrient	Leaf burn and mortality

There are several possible explanations for plant-growth suppression under salt-stress conditions. It can be caused by reduced enzyme activities, energy metabolism, growth, and photosynthesis (Parida et al., 2004). These are referred to as the physiological responses of salt. Munns (2005) published a paper which explained that these physiological mechanisms are the main influences on growth of plants species. Figure 1.2 presents two phases of the mechanisms of responses of plants grown under salt stress. Phase one illustrates how plant

mechanisms become affected, and how growth will become impeded due to osmotic stress in plant cells. The second phase reflects specific toxicity responses inside the plant structure; some plants do not develop salt resistance, and these sensitive species experience reduced leaf growth and ultimately mortality.

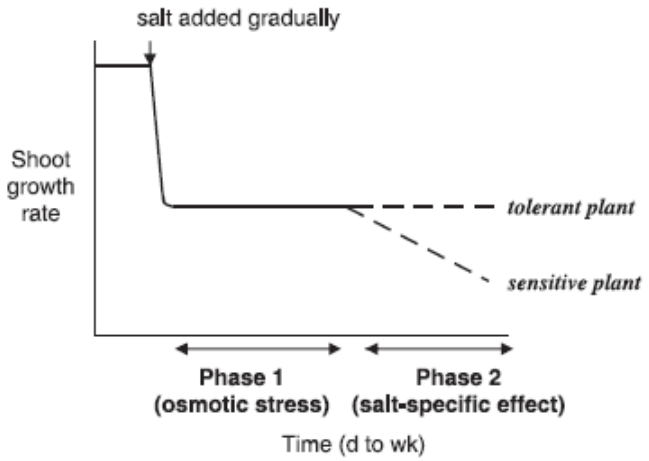

Figure 1.2 Phases of plant response to salt-stress versus time scale, adapted from Munns (2005).

1.6 Phytoremediation technology

Phytoremediation has been defined as a green technology, which has been used to clean-up contaminated water, groundwater, and soil in various habitats using vegetation such as herbs, shrubs, and trees (annual, biennial or perennial plants) (Dzantor and Beauchamp, 2002, Ouyang, 2002, Ghavzan and Trivedy, 2005). Microbes are also induced from root activities, assisting in the removal of pollutants in contaminated sites (Jones et al., 2004, Sharma et al., 2015).

According to Ghavzan and Trivedy (2005), phytoremediation has been mostly used to deal with the accumulation of inorganic compounds. However, this technique has also been shown to help reduce nutrients, organic matter, polycyclic aromatic hydrocarbons,

radionuclides, petroleum hydrocarbons, diesel and salinity (Alkorta and Garbisu, 2001, McCutcheon and Schnoor, 2003, Jones et al., 2004, Sun et al., 2004, Eapen et al., 2006, Euliss et al., 2008, Lin and Mendelssohn, 2009, Asaolu, 2010, Kanter et al., 2010). It involves plant mechanisms, known as an 'excluders' or 'hyperaccumulators' (Sharma et al., 2015), which are defined by the extent of metals are uptaken into the aerial shoots compared to the roots.

Frick et al. (1999) found that the tolerance levels of plants vary with different levels of concentrations. The characteristics of plants for phytoremediation include (i) fast growth rate; (ii) greater yields with more biomass, especially in the shoots; (iii) tolerance of heavy metals at high concentrations; (iv) resistance to pathogens, animals and pests, and (v) the avoidance of becoming part of their food chain (Couselo et al., 2012, Ali et al., 2013, Sharma et al., 2015).

There are various mechanisms, by which plants uptake trace elements, including phytoextraction, phytofiltration, phytovolatilization, and phytostabilization (Kidd et al., 2009, Sharma et al., 2015). The most important fact is that the root system plays a leading role in absorbing pollutants from the contaminated site, and then transferring and accumulating them firmly in the aerial part of the plant (Ali et al., 2013, Sharma et al., 2015).

Rai (2012) found that phytoremediation technologies provide physiological mechanisms including phytodegradation, phytoextraction, phytostabilization, and rhizofiltration. One of the most significant current discussions regarding the mechanisms of phytoremediation involves phytovolatilization (Pantola and Alam, 2014). Mani and Kumar (2014) describe the characteristics of rhizodegradation, rhizoremediation, blastofiltration, and genoremediation, and these are shown in Table 1.3.

Sarwar et al. (2017) state that a plant's resilience to PTEs depends on various mechanisms, in particular, the movement of metal ions into the vacuoles, which requires transport through the cell wall. Plant uptake is affected by its characteristics, environmental

conditions, and chemical and physical properties of the compounds (Rai, 2012). A key point from an investigation of the phytoremediation method (McCutcheon and Schnoor, 2003) was that this represents a relatively new experimental area of environmental engineering.

Table 1.3 Mechanisms of phytoremediation.

Mechanisms	Activities	References
Phytodegradation	Plant tissue metabolic processes	Jadia and Fulekar (2009)
Phytoextraction	Metal accumulation in root, shoot, and leaf	Rai (2012), Mani and
Phytostabilization	Root-zone immobilization of pollutants	Kumar (2014), Pantola
Phytovolatilization	Extraction of volatile metal from soil and release	and Alam (2014)
	to air (e.g., mercury and selenium)	
Rhizofiltration	Root absorption of metal waste solution	
Rhizodegradation	Breakdown of metal-containing compounds in	Mani and Kumar (2014)
	the rhizosphere	
Rhizoremediation	Plant, microbe, and metal interactions	
Blastofiltration	Seedling absorption of pollutants	
Genoremediation	Genetic manipulation towards metal-tolerance	
	in plants	

To elaborate further on the mechanisms involved in phytoremediation:

i) Phytodegradation is a process that breaks down contaminants by plant metabolic activity which is referred as phytotransformation. These terms can also apply to breakdown of contaminants outside the plant through the release of enzymes produced by the plant. Both Sandermann Jr. (1994) and Abhilash, et al. (2009) have discussed this mechanism, which is known as the ‘green liver model.’

ii) Phytoextraction or phytoaccumulation (Kidd et al., 2009) is one of the most studied plant mechanisms that involves toxin accumulation in plant tissues. The response mechanism consists of five main steps. Firstly, PTEs are mobilized in the soil or water, after which (secondly) they are absorbed into the tissues via the roots. The third stage is the transfer of PTE, which then accumulate in different aerial plant tissues and leads to (fourth stage) various plant-tissue response mechanisms. Finally, plant tissues phenotypically display the ability to tolerate PTEs in various tissues of the plant (Pivetz, 2001, Sabir et al., 2014).

iii) Phytostabilization involves root and microbial interactions that immobilize organic and (some) inorganic contaminants by binding them to soil particles. As a result, there is reduced migration of the contaminant to groundwater (Susarla et al., 2002, Kidd et al., 2009).

iv) Phytovolatilization has been defined as volatile toxic compounds (e.g. As, Hg, and Se) that are released into the atmosphere. It can also be used plant structures (e.g. leaves) to absorb vaporous compounds into their tissues (Raskin et al., 1997).

v) Rhizofiltration is the process whereby PTE contaminants in water are absorbed into or precipitated onto plants roots. Plants may, or may not, uptake and translocate the contaminant. More often, this application is associated with contamination occurring in water than in soil particles, and it is dependent on solution pH levels. Plants used in this process will often be harvested to reduce the reintroduction of the contaminants.

vi) Rhizodegradation represents the breaking down of organic pollutants by microorganisms within the rhizosphere. It is also known as ‘assisted degradation’ (Mani and Kumar, 2014).

vii) Rhizoremediation has been mentioned by numerous researchers, including Mani and Kumar's (2014) review of mycorrhizal fungi function in the soil. Mechanisms by microorganisms encourage plant growth and tolerance to contaminants (Mani and Kumar, 2014).

viii) Blastofiltration is where PTE are accumulated as the seedling grow and later distributed throughout plant tissues (Lin et al., 2002; Mani and Kumar, 2014).

ix) Genoremediation is a new form of phytoremediation technology, which through genetic manipulation, plant accumulation and PTE tolerance become enhanced. Gene expression and PTE tolerance in some species such as cottonwood, tobacco, and yellow poplar have been investigated. However, much of the research up to now have described transgenic bacteria remediating wastes with clones that might also be effective in phytoremediation (Pilon-Smits and LeDuc, 2009, Mani and Kumar, 2014).

Phytoremediation technology has some limitations: i) it requires time to rehabilitate soil or contaminated water; ii) phytoextraction is not effective in some plants due to their low biomass production; iii) minor toxicities of substances become useful for organic matter content in the soil; and iv) improper post-harvest use could contaminate the food chain (Ghavzan and Trivedy, 2005).

Various articles have been presented to support the use of plant species that accumulate metals. Toxicologists have been interested in establishing the efficiency of some species of plants to treat chemical toxicities. Unfortunately, removing contaminants from nature is a slow process, as observed by Dzantor and Beauchamp (2002), who found that this remediation system requires long-term use to achieve success. A reasonable approach to tackle this issue could be the appropriate choice of plant species. Jordahl, et al. (2003) claimed that trees were more useful than herbaceous plant, as they have more roots capable of surviving in depth soil horizons (Jordahl et al., 2003); however, they take longer to grow.

Researchers have demonstrated that plant uptake and absorption of contaminants are cost-effective, environmentally-friendly and effective in waste sites (e.g., agriculture, crude oil and areas of hydraulic fracturing) (Jadia and Fulekar, 2009, Rai, 2012, Pantola and Alam, 2014, Mani and Kumar, 2014), generally. In addition, the plants perform photosynthesis,

taking carbon from the air, and utilizing inorganic compounds and macronutrients to promote plant growth (Kumar et al., 1995). Additionally, Rai (2012) highlight some plants produce peptide, exo-molecules that actively sequester PTE to enhance plant growth.

Jadia and Fulekar (2009) report that plant species can sorb at metal concentrations (e.g., Zn>Cu>Cd>Ni>Pb) of 40-50 ppm, while lower concentrations (5-20 ppm) activate plant growth. Moreover, the biomass of some plants, even with high concentrations, has been used in the wicker industry (such as hats, mats, and basket), and the handmade paper business (Ramana et al., 2007).

While essential metals homogenized into the soil (e.g., Ca, Co, Cu, Fe, K, Mg, Mo, Mn, Na, Se, V, and Zn) accumulate in the plant structure, non-essential metals (i.e., Al, As, Au, Cd, Cr, Pb, Pd, Pt, Sb, Te, Ti, and U) also incorporate throughout plant tissues (Jadia and Fulekar, 2009). The presence of non-essential metals can non-specifically inhibit biochemical processes that are (normally) mediated by essential metals. Excessive concentrations of metal could act as direct or indirect poisons destroying plant structures and/or inhibit enzymes. For example, chlorophyll stress indicates that a plant is responding to non-essential elements (Pivetz, 2001). Further, their toxicities cannot be broken down, which (if not appropriately monitored) can have lasting effects on the growth and development of the plant

It is clear that phytoremediation technology offers an effective method for the development of the natural environment as a treatment system. It could remove inorganic and organic compounds. In fact, it can reduce the number of toxic pollutants and salinity in landfills without expensive equipment, and is cheaper than most other conventional remediation processes. The restoration of ecosystems contaminated by PTE can be accomplished by physical and chemical processes such as deposition of ionization, sedimentation, reverse osmosis, and chemical evaporation (Tang et al., 2007); however, the

use of such methods entails high cost and contamination of equipment. The most important features of phytoremediation are its key attributes: low cost, environmentally-friendly, and renewable (Rai, 2012).

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CHAPTER 2

CHARACTERIZATION OF *FESTUCA RUBRA* L. (RED FESCUE) AND *TRIFOLIUM PRATENSE* L. (RED CLOVER) AND THE FATE OF STRONTIUM IN ARTIFICIALLY CONTAMINATED SOIL MICROCOSMS

2.1 Preface

This chapter will be adapted into a manuscript for submission to *Archives of Environmental Contamination and Toxicology*. It investigates the fate of different concentrations of strontium (Sr^{2+}) in soils, intended to better elucidate the interactions between soil and chemical substances. Further, the chapter demonstrates how test plants respond to the timing of the addition of Sr^{2+} and calcium (Ca^{2+}) salts; three different exposure treatments were investigated: pre-germination, post-germination, and a 'combined' (simultaneous) irrigation and germination. Srikhumsuk, as the primary author, was responsible for the experimental design, laboratory work, data analysis, and writing the paper. Dr. Peshkur provided training and initial data analysis of the ICP-OES (metal analysis). Drs. Knapp and Renshaw, as supervisors, provided advice on experimental ideas and design, helped with data analysis, and editorial comments to this chapter.

2.2 Summary

The purpose of this experiment is to investigate Sr^{2+} speciation of strontium contamination in soils. Sr^{2+} ions were chosen as an element of interest from produced wastewaters of petroleum industries. Determinations of soil properties, sequential extraction, and batch exposure methods were used in the study. Soil-batch tests were conducted using various aqueous solutions (0, 10, 20, and 40 mM $SrCl_2$) and exposure-contact times (0, 1, 24, 168, and 504h). From this information, adsorption isotherms with Langmuir and Freundlich models were determined to estimate adsorption capacity.

Additionally, this research experiment aimed to determine the toxicological effects of Ca^{2+} and Sr^{2+} exposures on *Festuca rubra* L. (red fescue) and *Trifolium pratense* L. (red clover), which were native plant species selected for this initial exposure study. Plant biometrics

included mean germination time (MGT), mean daily germination (MDG), relative growth rate (RGR), vigor index (VI), final germination percentage (FGP), as well as biomass chlorophyll extraction and Sr^{2+} concentration in plant tissues.

Sr^{2+} sorption directly related with solution dosage. Efficient adsorption of soil was achieved in 24h of contact time. Importantly, the kinetic model of Sr^{2+} sorption can be appropriately fitted by either Langmuir or Freundlich models. When Sr^{2+} concentrations increased, the FGP and growth rates of both species significantly declined (p<0.05). In contrast, Ca^{2+} treatment responded differently to different concentrations and contact times; with *F. rubra* seedlings, there was no significant (p>0.05) differences in terms of the viability and vigor of the seedlings; while *T. pratense* seedlings only experienced statistically significant (p<0.05) adverse effects at the highest concentration of $CaCl_2$.

Furthermore, the experiment also investigated the impact that exposure timing had on the plants: pre-germination exposure (Pre-GEx), post-germination exposure (Post-GEx), and combined germination exposure (Com-GEx). Experiments were carried out for ten weeks. Pre-GEx conditions affected root-nodule development of the *T. pratense*; whereas the Com-GEx condition had greatest impacts on both plants in terms of germination and root/shoot development.

More significantly, the translocation factor (TF), which measures the ratio of transfer of trace elements (e.g., Sr^{2+}) concentrations between root to shoot, showed different results between the plants. The TF indicated that *T. pratense* was an efficient accumulator of excess Sr^{2+} ions; meanwhile, *F. rubra* was an excluder species—restricting the Sr^{2+} from their aerial structures. While the TF suggest that *T. pratense* should be promoted as a bio-accumulator plant, these results should be interpreted with caution as the experiment only examined a single metal at small scale.

2.3 Introduction

2.3.1 Treatment of wastewaters from petroleum industries

Various human activities cause soil contamination via potentially toxic elements (PTE). Recently notable are the conventional and unconventional onshore development of natural oil and gas industry processes, known as fracking (Gregory et al., 2011, Jackson et al., 2011, Vengosh et al., 2014), which are being increasingly used worldwide and are creating environmental concerns. PTE from these operations impact soils, water, and groundwater (Arthur et al., 2009, Vengosh et al., 2013). They become available in the form of fluid flowback and produced waters, which are high in concentrations of dissolved ions (e.g. barium (Ba^{2+}), bromide (Br^{-}), calcium (Ca^{2+}), chloride (Cl^{-}), sodium (Na^{+}), and strontium (Sr^{2+})) (Olsson et al., 2013, Capo et al., 2014).

These fluids contain a range of chemicals, which have pervaded the deep subsurface geology, and returned to the surface with greater (among other dissolved substances) concentrations of salts and PTE. The fluids are then stored in frac-tanks or temporary borewells (Howarth et al., 2011, King, 2012) until they are transported to a treatment system prior to final disposal (Mair et al., 2012). A concerning risk factor is that some contamination does occur, such as runoffs and spills (Chapman et al., 2012). As a result, an international debate has been triggered on the severity of contamination and issues that could threaten local areas, and also constitute a risk to local people living around the operation site (Kargbo et al., 2010, Olsson et al., 2013).

There have been several reports on the activity of natural gas developments, which have been identified of excess concentrations in some elements, such as calcium (Ca^{2+}) and strontium (Sr^{2+}) (Sangani, 2012, Haluszczak et al., 2013, Ferrar et al., 2013, Lester et al., 2015, Thacker et al., 2015, Vidic, 2015). Sr^{2+} is one of the primary elements widely distributed in the environment (Bowen and Dymond, 1955, Smith, 1971, Koss and Kim, 1990, Lefèvre et al.,

1993), and it is one of the chemical substances commonly found in hydro-fracture wastewaters (Chapman et al., 2012).

An important concern of Sr^{2+} to human health is its radioactive isotopes (Chen, 1997) (Kabata-Pendias and Mukherjee, 2007). According to Sasmaz and Sasmaz (2009), Sr concentrations remain stable in the soil, in exchangeable form. As such, they can become easily mobilized with changing pH (Kabata-Pendias and Mukherjee, 2007). Further, Sr^{2+} competing with Ca^{2+} in soils can be damaging to the soils with low-nutrient quality (Savinkov et al., 2007).

Flowback and produced waters pose an interesting challenge, which needs to be addressed by researchers, to find ways of remedying their environmental toxicity. Primary studies are needed to gain a better understanding how the metals exist in soil, of their complex dynamic behavior, and their associations with soil distribution (Melin et al., 1994), because each wellbore is different and so are the chemical properties of flowback fluids. One approach to tackle wastewater issues with salinity and PTE (Gregory et al., 2011) is crystallization or evaporation; moreover, further actions should be performed to eliminate hydrocarbons and radioactive nuclides from flowback fluids (Mair et al., 2012). The wastes represent complex mixtures, and there has not been any single, address-all approach to its treatment. Even fewer treatments have focused on using “green” treatment methods (Sangani, 2012).

Numerous studies have been carried out on a variety of vegetation types to determine the ability of plants to uptake or absorb substances. For example, various inorganic, organic, and radionuclide substances have been studied (Jadia and Fulekar, 2009, Rai, 2012, Mani and Kumar, 2014, Pantola and Alam, 2014). Potentially, these wastewaters create severe problems for the environment and have detrimental effects on plants. Higher levels of contamination can be toxic to plants and microorganisms, via direct or indirect exposures

(Bamberger and Oswald, 2012). However, the vegetation could serve a purpose to treat smaller spills and/or diluted solutions (e.g., spills in surface waters); it is the scenario I envision that this type of treatment aims to address.

2.3.2 Strontium: a case of PTE and interactions with soils

Firstly, the interactions between PTE and soil are complicated. Numerous studies have attempted to explain the complicated roles that physical and chemical characteristics of soils have on the fate of PTE. The fate of trace elements is affected by characteristics such as soil texture; mineral composition; organic matter; pH; temperature; moisture content, competitive interaction between elements; and microbiological activity (Harmsen, 1977). For example, pH, organic-matter content, and cation exchange capacity have been repeatedly reported being influential in the interaction between soils and metals (Van Bergeijk et al., 1992, Chen, 1997). The equilibrium adsorption or continuous adsorption with sediment surfaces is also significant to understand, along with the chemical equilibria of contaminated soils; pH, mineral composition and surface area of soils affect chemical equilibria of contaminated soils and can strongly affect strontium distribution (Pace et al., 2001, Kamel, 2010).

One principal research objective was to investigate the interaction between strontium and the soil in a laboratory (microcosm) scale. A vital aspect of this work involved the investigation of soil properties, extraction fractionation, and batch adsorption equilibria. Basically, batch tests were conducted using different concentrations of synthesized aqueous Sr^{2+} solutions over time to understand its absorbency and fate in these soils. The approach will elucidate the mobility and bio-availability of Sr, rather than focusing on “total” concentrations. As such, the present study contributes to understanding the possible

interactions of Sr in the soils. This will inform what is possible to phytoremediate Sr^{2+} in dilute (environmental) concentrations of wastewaters and its possible toxicity effect.

Several articles promote plant species that accumulate Sr (Rediske and Selders, 1953, Bowen and Dymond, 1955, Fox and Lipps, 1964, Smith, 1971, Franceschi and Schueren, 1986, Herren and Feller, 1997, Mazen and El Maghraby, 1997, Seregin and Kozhevnikova, 2004, Twining et al., 2004, Seregin and Kozhevnikova, 2005, Kozhevnikova et al., 2007, Savinkov et al., 2007, Beauregard and Côté, 2008, Seregin and Kozhevnikova, 2008, Kozhevnikova et al., 2009, Sasmaz and Sasmaz, 2009, Moyen and Roblin, 2010, He et al., 2012). Rediske and Selders (1953) reported that Sr uptake is the lowest in Gramineae or Poaceae (e.g., fescue), while the highest Sr transfer was found in Leguminosae or Fabaceae plants (e.g., red clover). However, Sr content and distribution are dependent on soil type, and experiments remain needed to demonstrate Sr content for the soil type.

2.3.3 Development of phytoremediation technique

As mentioned previously, several researchers have tried to develop and improve phytoremediation techniques to provide economic efficiency, ecological safety, and environmental aesthetics (Dzantor and Beauchamp, 2002, Ghavzan and Trivedy, 2005). According to Ghavzan and Trivedy (2005). Phytoremediation techniques have been used to accumulate compounds, and McCutcheon and Schnoor (2003) have highlighted this method of cleaning up pollutants such as nutrients, organic matter, petroleum hydrocarbons, and polycyclic aromatic hydrocarbons. It is possible, therefore, that the use of plant species may become vital to assess the environmental risks caused by natural gas development.

This present study investigated some of the member representatives of those families, namely the Poaceae and Fabaceae families. *Festuca rubra* L. (red fescue) and *Trifolium pratense* L., (red clover) were chosen for this experiment to represent the Poaceae and

Fabaceae families respectively. I examined the efficiency of those plants when treated with different concentrations of strontium in studied soil, and assess whether these plant species would be among the choices for use in further phytoremediation technology, in terms of germination ability, viability, and growth. Hanslin and Eggen (2005) list *F. rubra,* as a "marginally salt-tolerant representative of monocotyledonous species" (Hanslin and Eggen, 2005). Among the dicotyledons, *Trifolium* spp. perform better under conditional imbalances such as salinity (Ab-Shukor et al., 1988, Zhang et al., 2008), and *T. pratense* L. (red clover) grows rapidly and broadly in various habitats and is also a very beneficial cultivar for animal feed (Bowley et al., 1984).

An essential, initial step to achieve this is seed performance (Roos and Wiesner, 1991, Hill, 1999); at germination under stressful environmental conditions. Various factors critically affect seed germination, such as light, water, salinity, and temperature (Khan et al., 2000). Seed vigor and viability represent two biomarker determinants of quality and ability to germination performance (Mayer and Poljakoff-Mayber, 1963, Marcos Filho, 2015). Generally, seedlings are more sensitive to excess PTE than mature plants. As such, the first symptoms of environmental damage could be the plants' abilility and timeliness to germinate. Seed vigor, such as germination timing, seedling growth rate and ability to germinate during less optimal conditions, determines seed performance to emerge (Roos and Wiesner, 1991). Broadley et al., (2003) have pointed out that calcium ions act as nutrients to cell plants (Broadley et al., 2003), so the seed radicles and plumules emerge and develop as normal seedlings under these stressful conditions.

In terms of the plants' responses to environmental contamination, one possible biometric is their viability – the ability to persist over time and grow during periods of environmental stress. However, another is their ability to accumulate PTE in different tissues of the plant—the translocation factor. While not a toxicological metric, it is however deemed important

for remediation efforts. For example, the root systems are vital in absorbing contaminants; after which, the plant may translocate the contaminants via vascular tissues to the stem and leaves. It is, at this point, that the plant can help remove salts from the soils.

2.4 Materials and methods

2.4.1 Materials

2.4.1.1 Soil sampling

Soil samples were collected from layers at a depth of <100 cm from a South Lanarkshire farm in Glasgow, UK, in 2014. They were air-dried at room temperature at approximately 22 °C. Then, the dried-soils were sieved through a 2-mm stainless-steel mesh screen and stored in a dried polyethylene bottle.

2.4.1.2 Reagents

Strontium solutions used in this experiment were prepared at different concentrations: 0 (control), 5 (in seed-germination study), 10, 20, and 40 mM of strontium chloride hexahydrate ($SrCl_2 \cdot 6H_2O$) (Sigma-Aldrich) dissolved in nanopure water (Barnstead™ Nanopure D3-Hollow Fibre Filter; Triple Red Limited, UK). Calcium chloride hexahydrate ($CaCl_2.6H_2O$) solutions were similarly prepared to be used for washing in the batch-leaching assays. All labwares used in experiments were pre-soaked with 10% nitric acid overnight and rinsed three-times with nanopure water.

2.4.1.3 Seeds

The seeds for *F. rubra* L. and *T. pratense* L. were purchased from OMC seeds® (Cartagena, Spain) and Sow Seeds® (South cave, United Kingdom) seeds, respectively.

2.4.2 Instrumentation

Element concentrations were determined with an Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES) by (Thermo Scientific iCAP 6000 series), equipped with a mini-torch nebulizer and argon plasma. Samples for PTE (or "metals") were prepared in different dilutions using 1% HNO_3 solution (as further described in experiments) to avoid exceeding detection limits.

ICP was performed with at least two wavelengths (Appendix C). The instrument was calibrated with internal standards (Fisher Chemicals), and a standard curve. The standard solutions for ICP-OES for quantitation included 0, 10, and 50 ppm concentrations of each element; the curve remained within the instrument's quantification limits and had good linearity (R^2 >0.9980). Blanks consisted of 5% nitric acid dissolved in nanopure water treated in a similar manner; the blank concentrations were all below 10% of the lowest sample concentrations, and were subtracted from instrument's determined concentration-value for each sample.

The MQL (minimum quantification limit) is the lowest concentration where a concentration can be reported with adequate confidence as according to Thermo Fisher Scientific (2000). The Quality Control Check Standards (QCCS) were prepared and used at the start, middle and end of each analytical sequence (Appendix C). During the measurement of trace elements, Analytical Quality Control (AQC) standards were used. Analytical precision (RSD, relative standard deviations) for each analysed elements ranged between 0.1 and 5 % (n=3), and were deemed acceptable.

2.4.3 Methods

2.4.3.1 Soil properties and Sr^{2+} fate

("Experiment 1" in the Results, but also contributes to "Experiment #3")

The experiment was carried out in three steps to determine soil properties, and their interactions with Sr^{2+} ions at different concentrations and contact times. First, the physical and chemical characteristics of the soil samples were examined; after which, sequential soil extraction was investigated; and finally, the adsorption of soil was determined using batch-tests.

- *Soil properties*

The physicochemical characteristics of soils were measured for pH, EC (electrical conductivity), TC (total carbon), LOI (loss on ignition), CEC (cation exchange capacity), soil moisture, and soil texture. The texture of soil was determined according to the methodology of British Standard (1990). pH and EC were examined simultaneously; pH was measured in deionized water adapted from USEPA (1986) with 10-g soils added to 20 ml water; it was then mixed with a magnetic bar for 10 min and left to settle for one hour; after which, pH and EC were measured using a METTLER TOLEDO SevenMulti^{TM} probe. Moisture was investigated by weighing soils before and after drying at 55 °C for 48 h. TC and LOI were investigated with a muffle furnace at 550 °C (BSI, 2012) to constant weight. Cation exchange capacity (CEC) was also determined in accordance with BSI (2011).

- *Soil sequential extractions*

Tessier et al. (1979) procedure was used in this study to extract different fractions of PTEs on the soils. As such, this method involved five steps, one for each fraction: (i) exchangeable, (ii) bound to carbonates, (iii) bound to Fe-Mn oxides, (iv) bound to organic matter, and (v) residual fraction. The sequential extractions were determined in triplicate as below; after

each step, samples were centrifuged, and their supernatants were processed and analysed via ICP-OES (Section 2.4.2).

- For fraction (i): 1 g of dried soil was weighed and extracted in 1*M* $MgCl_2$ (8 ml) solution at pH 7 in a 50-ml centrifuge tube; the mixture was shaken end-over-end for 1h at room temperature (20 ˚C).
- Fraction (ii): the residuals from fraction (i) were extracted with 1*M* NaOAc (sodium acetate) solution (8 ml) adjusted to pH 5 with HOAc (acetic acid). Shaking was performed end-over-end at room temperature for 5h.
- Fraction (iii): residuals from fraction (ii) were extracted with 0.4*M* NH_2OH-HCl (hydroxylammonium chloride) in 25% (v/v) HOAc (20 ml), using a horizontal incubator shaker (Thermo Scientific MaxQ5000™) for 24h at 60 ±1°C.
- Fraction (iv): the residual fraction (iii) was extracted with 0.02*M* HNO_3 (nitric acid, 3 ml) and 30% H_2O_2 (hydrogen peroxide, 5 ml) using HNO_3 adjusted to pH 2 for 4h at 60 ±1°C. After another 30% H_2O_2 (3 ml) was added and adjusted to pH 2 with HNO_3 continuously to shake for 6h at 60 ±1°C using an incubator shaker. After cooling, down these samples had 3.2*M* NH_4OAc in 20% (v/v) HNO_3 added and were shaken end-over-end at room temperature for 24h.
- Fraction (v): fraction (iv) was extracted with *aqua regia* (1:3, HNO_3 to HCl) by microwave digestion technique; then the extracts were diluted with 1% nitric acid to 12 ml.

- *Soil batch tests*

A batch experiment was carried out to determine time required for Sr^{2+} (PTE of choice) equilibrium to be reached (as analysed at 0h, 1h, 24h, 168h, and 504h). From the reagent, $SrCl_2$ stock solutions (adsorbate) involved 25ml each concentration (10, 20, and 40 mM $SrCl_2$)

were used as triplicate spikes to 10g of soil. Treatments included controls (Nanopure water to soil) and water blanks (no soil). The experiment was carried out in a closed system and on a horizontal shaker for three weeks at room temperature.

After the defined incubation times, a washing procedure was carried out. 1 g of the soil sample was placed in the centrifuge tube (50 ml). Then, $CaCl_2$ solution (0, 10, 20, 40 mM) was added in each at 25 ml and then using a horizontal shaker for 15 min at room temperature. After centrifugation for 5 min at 1200 x g to settle soil particles; the washing was repeated twice. Next, the soils were put into filter paper (90 mm), settled and dried at 50-55 °C for 48h.

One gram of dried soil was digested and leached with 1% nitric acid (HNO_3), filtered (Whatman No. 1 paper filter), made up to 50-ml volume with 1% HNO_3, and then filtered (45-µm syringe filter) into a 50 ml centrifuge tube. Finally, to prepare the sample for ICP-OES analysis (Section 2.4.2), dilutions ranged from non-diluted factor (0 mM $SrCl_2$), and diluted factor (df) at 1:10 (for 5mM $SrCl_2$), 1:100 (for 10 and 20mM $SrCl_2$), and 1:1000 (for 40mM $SrCl_2$). The experiments included 'blanks' with original solutions, treated under same conditions, but without soil.

- *Adsorption model*

An adsorption model was determined, and Sr equilibrium was calculated over the concentration range (triplicate values) using equations by Ahmadpour et al. (2010) and Kaçan and Kütahyalı (2012) as follows (eq. 2.1):

$$q_e = \frac{V \text{ x } (C_0 - C_e)}{W} \quad (2.1)$$

Where: q_e (mg.g^{-1}) at equilibrium phase presented the amount of metal sorbed in the adsorbate; V is volume of solution (litres); C_0 is concentrations of metal ion (mg.L^{-1}) at the

initial phase; C_e is the concentrations of metal ion (mg.L^{-1}) at equilibrium phase; and W is the mass of adsorbent (g).

The efficiency of Sr adsorption was determined using an equation utilised by Dada et al. (2012) and Kaçan and Kütahyalı (2012), as follows (eq. 2.2)

$$\% \text{ Adsorption} = \frac{(C_0 - C_e)}{C_0} \text{ x } 100 \quad (2.2)$$

Where C_e is the concentration of Sr ion after adsorption and C_0 is the concentration of Sr ion before adsorption.

Adsorption isotherms for this study were considered using the Langmuir and Freundlich equations that are widely used to determine the ability of soils to sorb Sr^{2+}, thus estimating the mobility and retention of Sr ions. The adsorption isotherms were estimated as follows:

(a) Langmuir isotherm

Langmuir isotherms, both the non-linear and linear forms, are given by equations (eq. 2.3) and (eq. 2.4), respectively (Ahmadpour et al., 2010, Dada et al., 2012).

$$R_L = \frac{1}{(1+K_L \text{x } C_o)} \quad (2.3)$$

$$\frac{1}{q_e} = \frac{1}{Q_0} + \frac{1}{K_L \text{x } Q_0 \text{ x} C_e} \quad (2.4)$$

where C_e is the equilibrium concentration (mg.L^{-1}), C_o is the initial metal ion concentration (mg.L^{-1}), K_L is the Langmuir constant related to energy adsorption (L.mg^{-1}), q_e is the amount of adsorbed material at equilibrium concentration (mg.L^{-1}), and Q_o is the maximum monolayer coverage adsorption capacity (Langmuir constant) (mg.g^{-1}). The values of C_e and q_e were computed to find an intercept and slope of the Langmuir plot using $1/C_e$ versus $1/q_e$.

(b) Freundlich isotherm

The Freundlich model was used to express the characteristic adsorption for a heterogeneous surface. Freundlich model is used with the logarithmic form as given by following equation non-linear (eq. 2.5) and linear forms (eq. 2.6):

$$Q_e = K_f \text{ x } (C_e)^{\frac{1}{n}} \quad (2.5)$$

$$\log Q_e = \log K_f + \frac{1}{n} \text{ x } (\log C_e) \quad (2.6)$$

Where C_e is the equilibrium concentration of the adsorbate ($mg.L^{-1}$), Q_e is the amount of adsorbed material at equilibrium ($mg.g^{-1}$), and Freundlich constants were related to K_f is adsorption capacity, and n is adsorption intensity. The value of constant n is approximately the strength of adsorption in the process, where $1/n$ if value < 1 indicates normal adsorption, in contrast, $1/n$ value > 1 indicates cooperative adsorption. When $1/n$ presents values between one and ten, it indicates favorable adsorption.

2.4.3.2 Seed germination assays ("Experiment #2")

Seed germination tests were used to investigate the effect of different concentrations of saline solutions on the efficiency of seed germination as follows:

Experimental design and procedure 1 – "strontium germination": For each plant species, four seeds of two replicates were sown in different concentration of $SrCl_2$ to determine their germination rates. The germination test was carried out in a plastic petri dish (150 x 15 mm) covered with filter papers (Whatman No.1), which supported plant growth. The concentrations were 0 (control), 5, 10, 20, and 40 mM and were provided for four weeks from 23rd April to 20th May 2016.

Experimental design and procedure 2 – "*the calcium exposure assay*" ("Experiment #2" continued, in the Results): Three replicates of two seeds from each plant species were sown in plastic round pots (of 10-cm diameter) with 150g soil per pot. Calcium chloride ($CaCl_2$) solution was added as a treatment factor to examine responses at different concentrations: 0 (control), 20, and 40 mM. The effect of each concentration on plant growth was also investigated for different timings as to when seeds/plants become exposed to the salts (10th November and 20th December 2016). There were three conditions:

i) "Pre-GEx" (pre-germination expsoure): $CaCl_2$ solution was initially mixed at 25 ml into the soil, after which the seeds were sown;

ii) "Post-GEx" (post-germination exposure): $CaCl_2$ solution at 25 ml was added into the soil the day after the seeds were germinated at week three; and

iii) "Com-GEx": the combination was consistently provided water and $CaCl_2$ solution each 25 ml In each of these conditions, irrigation was provided every two days throughout the experiment.

All experiments were conducted at 21±1 °C. Light and dark photoperiod cycles were provided at 16 h to 8 h; illumination intensity was 4,930 lux (as Sylvania GRO-LUX white fluorescent tubes, Germany).

Experimental design and procedure 3 ("Experiment #3" in the Results)– "*the strontium exposure assay*": Again, 150 g soils per pot (10 cm/4'' approx. diameter) were contaminated with 50 ml of Sr^{2+} solutions at different concentrations (0, 5, 10, 20, and 40 mM $SrCl_2$). As in the previous experiment (#2, mentioned previously), the timing of Sr-exposure were altered, except in "Post-GEx" Sr^{2+} was added after 6 weeks. Similar to the $CaCl_2$ experiment (#2), this experiment aimed to determine the responses of seeds and plants to the timing and extent

(concentration) of Sr^{2+} exposure. The soil-fractions of strontium were also determined (as in experiment #1; Section 2.4.3.1).

Environmental conditions had the same temperature (at 21 ±3 °C) and light photoperiod. Soils were irrigated with 50 ml/pot, twice per week. Fortnightly, biomass productivity of plant (e.g., shoot height) was measured (see Section 2.4.4.3, below). These experiments lasted ten weeks (5th April to 15th June 2016).

2.4.3.3 Plant biometrics

- *Biomass production*

At the end of any plant-growth experiment, the plants' responses to Sr^{2+} dosages were assessed by various morphological parameters: e.g., root and shoot length, fresh and dry weight, leaf number, and amount of root nodules. While shoot lengths were estimated once the first plumule emerged, the length of the most extended shoot and root were measured using a vernier caliper (Mitutoyo Absolute®). Fresh material was weighed and then oven-dried at 105 °C for 24 h, after which dry weight was recorded.

- *Chlorophyll extraction*

Chlorophyll *a* and *b* (Chlo *a* and *b*) are possible measures of plant viability. Numerous studies have compared chlorophyll content in a few different forms of Chlo *a* and *b*. They found that they are essentially identical; however Chlo *a* is the primary pigment of photosynthesis. It absorbs light energy from wavelengths 430 and 660 nm, whereas, Chlo *b* is another pigment that absorbs energy in wavelengths 450 and 640 nm.

Leaves of *F. rubra* and *T. pratense* were dried overnight at 80 °C for 48h, and then weighed (g). These samples were ground with mortar and pestle with 5 ml of 80% acetone solution. Homogenised extracts were then transferred into a centrifuge tube and filled to 10 ml with

80% acetone. The samples were centrifuged twice at 5 °C for 5 minutes at 4500 rpm, and 3000 rpm. Filtration was performed using Whatman No.1 filters, and then each sample was measured with a spectrophotometer at different absorbances (*A*): 663, 646, and 470 nm wavelengths (Wellburn, 1994).

Chlorophyll *a* (eq. 2.7) and *b* (eq. 2.8) concentrations were calculated according to equations adapted from Porra et al. (1989), as cited in Wellburn (1994):

$$C_a = 12.21A_{663} - 2.81A_{646} \quad (2.7)$$

$$C_b = 20.13A_{646} - 5.03A_{663} \quad (2.8)$$

Where: C_a = Chlorophyll *a*, and C_b = Chlorophyll *b*.

- *Plant digestion (for Sr^{2+}) and translocation factor*

Plants were digested for Sr^{2+} analysis. First, dry plant samples were ground with a mortar and pestle, then digested using a microwave oven (MARSXpress 240/50 CEM, Mathews, NC, USA) in accordance with the USEPA (1996) guidelines. The samples were weighed 0.01-0.1 g as powdered dry roots or shoot sampling into closed digestion vessels, with 12-ml (1:3 v/v, HNO_3 : HCl) aqua regia. After that, the plants' extracts were filtered with a syringe filter at 0.45 µm (Millex® syringe driven filter unit). Finally, analysis was conducted to determine the total Sr concentration in the solution extracts (diluted at ten times) with ICP-OES (Thermo Scientific iCAP 6000 series ICP Spectrometer).

Translocation factors (TF) of metals into plant tissues were calculated using the ratio of metal in the above-ground tissues to metal in the underground tissues. From these ratios, one can determine whether plants were accumulator species, which is indicated by TF greater than 1; whereas, TF lower than one is referred as an excluder species. The ratio was based on Sasmaz and Sasmaz (2009) with an equation adapted as follows (eq. 2.9):

$$\text{Translocation Factor (TF)} = \frac{\text{Metal in the shoot}}{\text{Metal in the root}} \quad (2.9)$$

- *Calculations*

Germination was observed by radicle and plumule formations, which were monitored and measured daily in terms of the germination count, root, and shoot growth. The mean germination time (MGT) was calculated according to the formula by Ellis and Roberts (1981), modified by Farooq et al. (2005) as follows (eq. 2.10):

$$\mathrm{MGT} = \frac{\sum \mathrm{Dn}}{\sum \mathrm{n}} \tag{2.10}$$

Where *D* is the number of the days counted from the first day of germination, and *n* is the number of seeds that were germinated on day *D*.

This experiment recorded the total amount of germination, which is used to calculate: the 'final-germination percentage' (FGP, eq. 2.11), adapted from Bae et al. (2016), the vigor index (VI, eq. 2.12), and mean daily germination (MDG, eq. 2.13; Kheloufi et al., 2017). The VI indicates the quality of germination of the plant under stress conditions, according to the Association of Official Seed Analysis (1983); the equation was adapted from Geetha et al. (2014).

$$\mathrm{FGP} = \left(\frac{n \text{ germinated seeds}}{\text{total } n \text{ seeds}}\right) \mathrm{x}\ 100 \tag{2.11}$$

$$\mathrm{VI} = \mathrm{FGP}\ (\%)\ \mathrm{x}\ \text{total seedling length (cm)} \tag{2.12}$$

$$\mathrm{MDG} = \frac{\mathrm{FGP}}{\mathrm{D}} \tag{2.13}$$

Where *n* is total number, *D* is a day of maximum germination in the period of this experiment.

After each experiment, both of the root and shoot length, fresh weight (FW), and nodule count in the root part were measured as additional plant responses. The vigor index (VI) and

relative growth rate (RGR) were reported according to equations (eq. 2.12) and (eq. 2.14), respectively (Bernstein, 1975, Association of Official Seed Analysis, 1983, Azevedo Neto et al., 2004, Geetha et al., 2014):

$$\mathrm{RGR} = \frac{(\ln X_2 - \ln X_1)}{(t_2 - t_1)} \quad (2.14)$$

Where X_1 is the initial total shoot height on the first day of germination, X_2 is the final total shoot height in the experiment period, and $(t_2\text{-}t_1)$ is the difference in time interval plant growth of this experiment.

2.4.4 Statistical analysis

Minitab®17 software was used to determine variances and statistical tests at the level $p \leq 0.05$ for this experiment, which included basic descriptive statistics and one-way *ANOVA* test. Using the least significant difference (LSD) method, means were compared to the group of the results and were presented as mean value, standard errors, and *F*-value of the results. Fisher's LSD post-hoc test was used to compare the mean values of treatment, denoted in the form of different letters, which were considered significant at the level of $p \leq 0.05$. The graphs of this research were illustrated using Origin® 2017 Graphing & Analysis computing package.

2.5 Results and discussion

2.5.1 Experiment 1: Soil properties and strontium fate

2.5.1.1 Soil properties

The texture of this soil was slightly silty to coarse sand. The chemical and physical characteristics of this soil were reported as the mean of three replicates analyses as results shown in Table 2.1. The pH of this soil was considered neutral. Interestingly, the EC result of

this soil was considered strongly saline. The high CEC values, as seen in Table 2.1, represent values typical of those from agriculture soil—the origin of these samples. The exchangeable elements range as $Ca^{2+} > Mg^{2+} > K^{+} > Na^{+}$. Moreover, Table 2.1 provides the summary statistics results for estimates of TC, LOI, and MC. TC and LOI represent the total organic content of the soils.

These results were consistent with those of previous research and suggested, in agreement with Najm et al. (2000), that TC could be a determinant for binding metals on the soil particles. On the other hand, LOI and TC are complex, these amounts cannot be accurately used to estimate soil content and its behavior. However, Yasuda et al. (1995) claim that ^{85}Sr concentration and its distribution coefficients (K_d) between the soil properties correlated with CEC and water content. Therefore, CEC and exchangeable Ca^{2+} ions would be particularly considered in the soil mechanics. The change of soil pH was directly influenced from CEC (Aprile and Lorandi, 2012).

Moreover, retained moisture may possibly reflect the amount of the clay in the soil structure. Loch et al. (1981) suggest that the clay represents the most critical information required to predict and know the behavior of metal in the soil, and it should also be considered along with the equilibrium capability of the soil. This work will be presented in a next section.

Table 2.1 Physicochemical properties of soil used in this study (*n=3*)

Parameters		Mean	S.E.
pH	(H_2O)	6.98	±0.15
EC	($mS.cm^{-1}$)	38.1	±1.13
CEC	($cMol^{+}.kg^{-1}$)	11000	±2600
Exchangeable Ca	($cMol.kg^{-1}$)	137	±6.2
Exchangeable K	($cMol^{+}.kg^{-1}$)	4.23	±0.16
Exchangeable Mg	($cMol^{+}.kg^{-1}$)	36.6	±1.39
Exchangeable Na	($cMol^{+}.kg^{-1}$)	0.92	±0.05
TC	(%)	5.02	±0.23
MC	(%)	3.93	±1.01
LOI	(%)	9.04	±0.42

2.5.1.2 Strontium: sequential fractionation

The speciation of Sr was determined following the methods by Tessier et al. (1979), and the results are shown in Table 2.2. The average relative abundance of different soil fractionation forms of Sr were as follows: residual (76.8%) > exchangeable (9.13%) > Fe-Mn oxides (5.83%) > organic matter (5.27%) > carbonates (2.94%) of the average total Sr determined in the soil.

Figure 2.1 displays the relative distribution of Sr bound in this soil before further supplementation, which concluded with a significant portion in the residual fraction. Pearson's product correlation coefficient showed a correlation of soil fractionation between the residual fraction and the carbonates complex phase (r^2 = 0.807). On the other hand, there

was a weak, negative correlation between residual phase and Fe-Mn oxides (r^2 = -0.788). Similarly, residual and organic matter phase closely related with values of Sr bound with the Fe-Mn oxides phase, as seen in Table 2.2, trending negatively with r^2 = 0.120. Therefore, among the Fe-Mn oxides and organic fraction, Sr was restricted or had minimal migration through the soil. Previous studies found that the majority of Sr was found bound with the carbonate fraction (Puhakainen et al., 2001, Kamel, 2010). Even though Sr would not migrate from this soil; nevertheless, identifying soil fractions was useful to understand and predict Sr availabilities in the environment.

Table 2.2 Concentration of initial Sr (mg.kg^{-1}) in each soil fraction (n=4).

Soil Fraction	Mean	S.E.	95% CI	***p*-value in *t*-test***	**Average total Sr** (%)
(i) Exchangeable	2.34	±0.08	(2.08, 2.60)	0.31	9.13
(ii) Carbonates	0.75	±0.03	(0.67, 0.84)	0.15	2.94
(iii) Fe-Mn oxides	1.50	±0.49	(-0.06, 3.05)	0.07	5.83
(iv) Organic matter	1.35	±0.14	(0.90, 1.80)	0.34	5.27
(v) Residual	19.7	±0.78	(17.2, 22.1)	0.55	76.8

*[*Each distribution type was determined by the Anderson-Darling normality test with the level of significance at p≤0.05]*

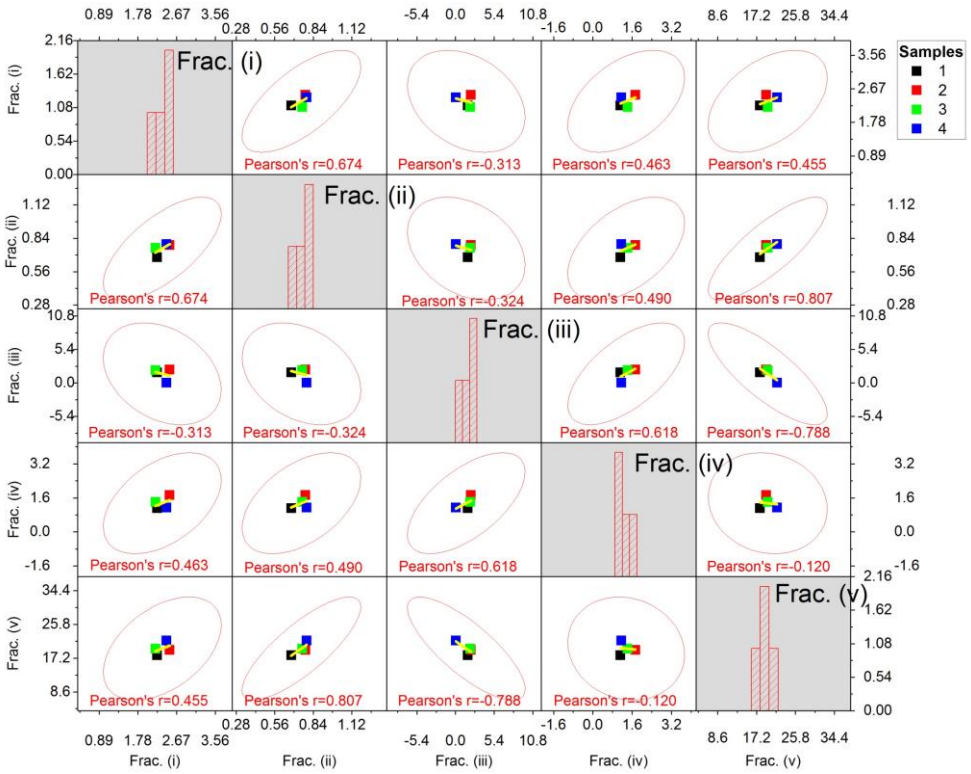

Figure 2.1 Correlation between extracted Sr concentration in the soil sample and in each soil fractionation; samples (1-4) represents soil studies with Sr initially added.

2.5.1.3 Strontium sorption: effect of concentration and contact time

Figure 2.2 presents two dimensions. Figure 2.2(a) is an illustration of the adsorbed optimum concentration, and Figure 2.2(b) exhibits of incubation time for adsorption. Different concentration of $SrCl_2$ in the range of 0, 10, 20, and 40 mM were used in this study. Adsorption efficiency increased as the concentration increased (Figure 2.2(a)), agreeing with previous works (Ahmadpour et al. (2010), Li et al. (2010), Guan et al. (2011)), and time (Figure 2.2(b)). Results for 10mM were rather inconsistent; however concentrations 20 and 40 mM remained very stable after 168h, and remained higher than 10mM.

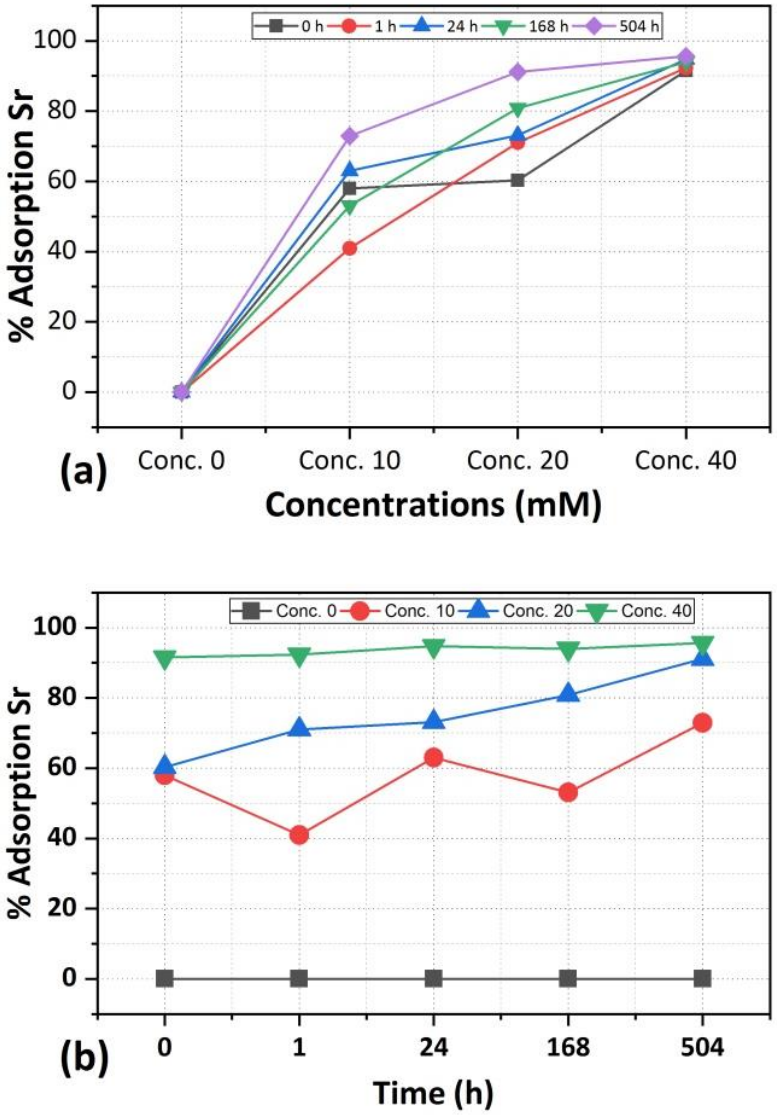

Figure 2.2 The percentage of Sr adsorption under different conditions: **a)** absorption trends by aqueous concentration, **b)** absorption trends by contact time.

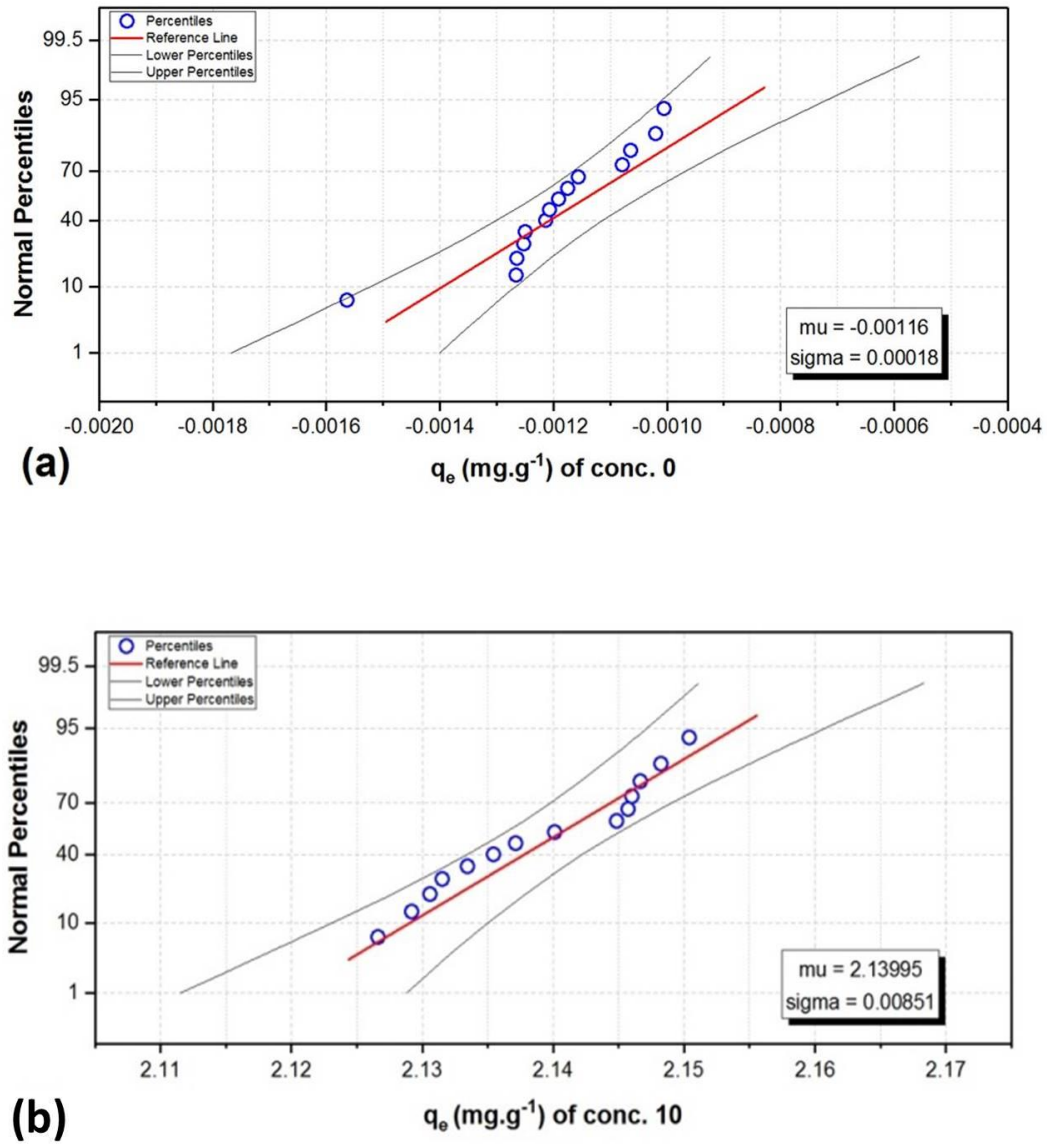

Figure 2.3 Probability distribution of equilibrium adsorption (q_e) of Sr in the soil with different adsorbate; **a)** water (control, 0), and **b)** conc. 10 mM of $SrCl_2$, as measured at different contact times (0, 1, 24, 168, and 504h).

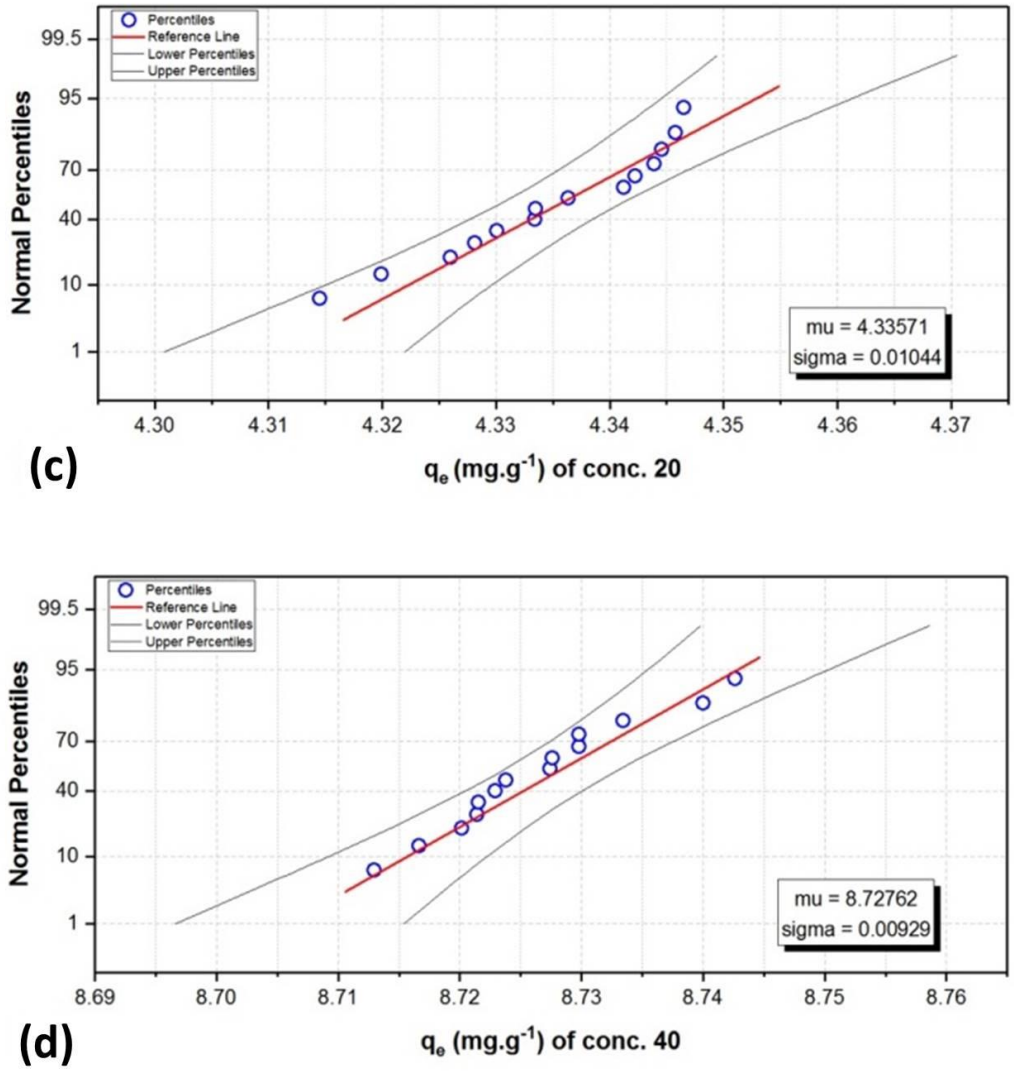

Figure 2.3(cont.) Probability distribution of equilibrium adsorption (q_e) of Sr in the soil with different adsorbate; **c)** conc. 20 mM of $SrCl_2$, and **d)** conc. 40 mM of $SrCl_2$ as measured at different contact times (0, 1, 24, 168, and 504h).

The probability distribution of Sr amounts (q_e) per unit adsorbent in different aqueous concentrations are shown in Figure 2.3. These graphs were produced using percentiles with Kaplan-Meier score method at a confidence level of 95%. The residuals, which had a normal distribution in all experiments, are represented as the vertical distance between each of the points and fitted line, with the slope of its distribution curve near the 50 percentile value. Table 2.3 shows the *t*-test values of Sr distribution versus a Normal probability distribution (Anderson-Darling normality test); there were no significant differences ($p \leq 0.05$), meaning the means were normally distributed.

Table 2.3 Statistical descriptors of equilibrium capacity (q_e) of Sr in different concentration of adsorbate at a different contact time.

$SrCl_2$ Conc. (mM)	Distri-bution	Min	Median	Max	Mean	S.E.	*p*-value in *t*-test
0 (control)	Normal	-0.0016	-0.0012	-0.0007	-0.0012	0	0.071
10	Normal	2.127	2.140	2.153	2.140	0.002	0.330
20	Normal	4.315	4.336	4.350	4.336	0.003	0.447
40	Normal	8.713	8.727	8.744	8.723	0.002	0.491

[Each distribution type determined by the Anderson-Darling normality test with the level of significance at $p \leq 0.05$]

2.5.1.4 Adsorption isotherm

Among the Langmuir model constants calculated, the values of Q_o are presented in Figure 2.4(a) and K_L, which are given in Appendix D. R_L parameter was essentially expressed in terms of equilibrium (Figure 2.5(a)), which is the Langmuir dimensionless constant factor as shown in Appendix D. R_L value is indicative of the characteristics of adsorption isotherm as follows:

R_L > 1 represents unfavorable adsorption; R_L = 1 corresponds to a linear relationship; R_L = 0 is irreversible; and 0 < R_L < 1 is favorable adsorption.

The values of log C_e against log Q_e were used to estimate the slope and interception in linear form; the regression results and its fit (r^2) are shown in Appendix D, along with the adsorption constants of the Freundlich model.

The Freundlich parameters are also given in Figure 2.4(b) for K_f and Figure 2.5(b) for Q_e values. The sorption of Sr ions of this model shows the amount of adsorbed very close to the amount of equilibrium adsorption. Therefore, the correlation coefficients (Appendix D) show whether Langmuir or Freundlich's models are favorable and suitable for predicting the adsorption equilibrium state of strontium ions for this studied soil.

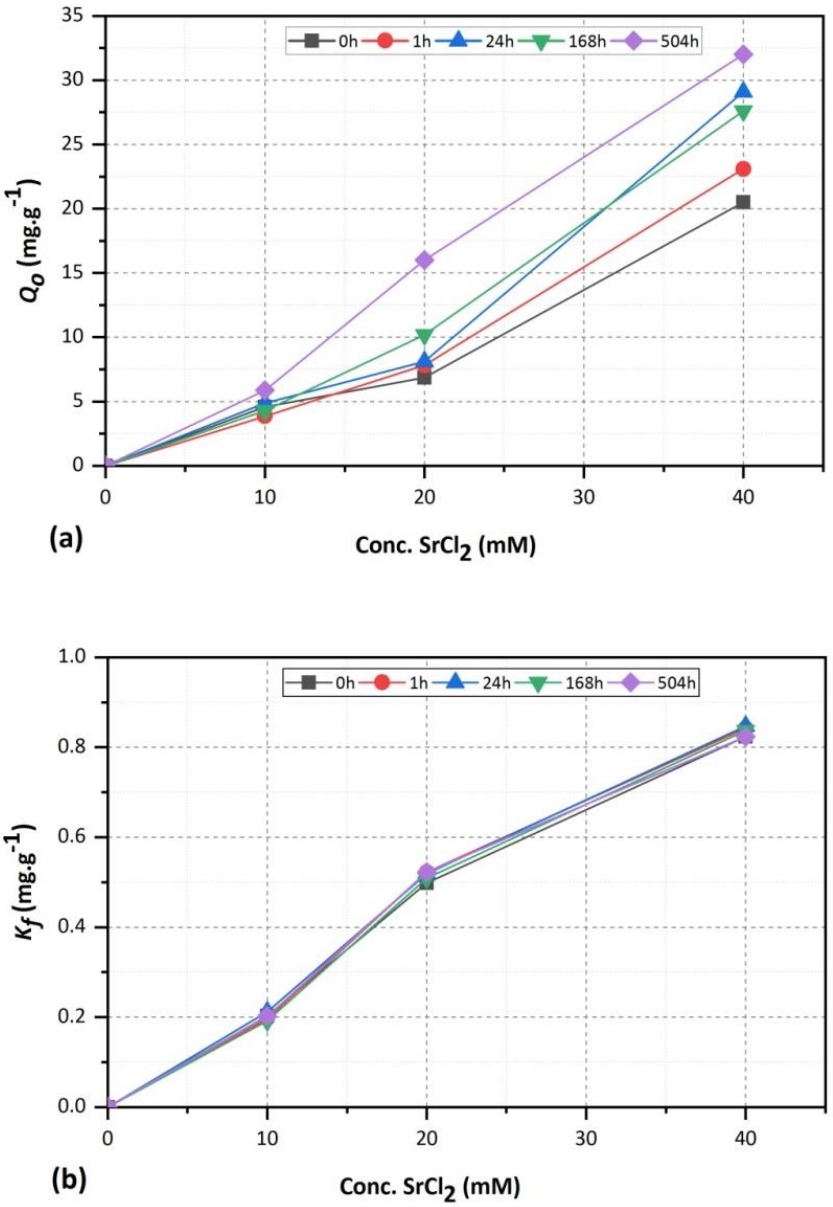

Figure 2.4 (a) Langmuir and **(b)** Freundlich adsorption constants related to the adsorption concentrations of Sr^{2+} in the studied soil.

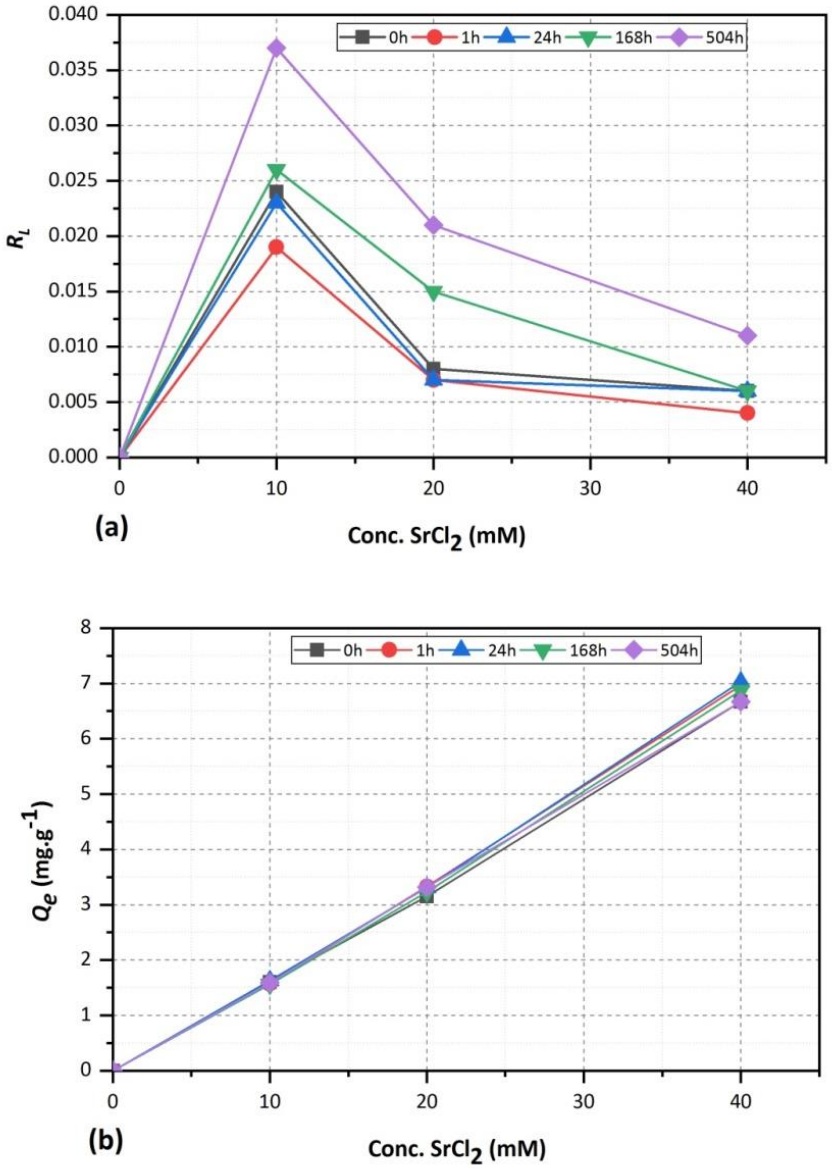

Figure 2.5 (a) R_L values for Langmuir and **(b)** Q_e values for Freundlich adsorption isotherm for Sr^{2+} in the studied soil.

2.5.2 Experiment 2: Seed germination assays

2.5.2.1 Seed germination in $SrCl_2$ solutions

In this initial experiment, plants were germinated in petri-dishes with water to different strontium chloride ($SrCl_2$) concentration; this was done to determine the germination dynamics at different salinities.

Figures 2.6 and 2.7 show the results obtained from the preliminary germination test for *F. rubra* and *T. pratense*, respectively, whose radicle and plumule were measured and recorded on a daily basis. As shown in Figures 2.6a and 2.6b, for *F. rubra* seeds, there was a relationship between concentration and extent of both shoot and root production. Similarly, *T. pratense* showed comparable relationships among all concentrations of treatments as seen in Figures 2.7a and 2.7b. However, for this species at 40 mM $SrCl_2$, there was no germination.

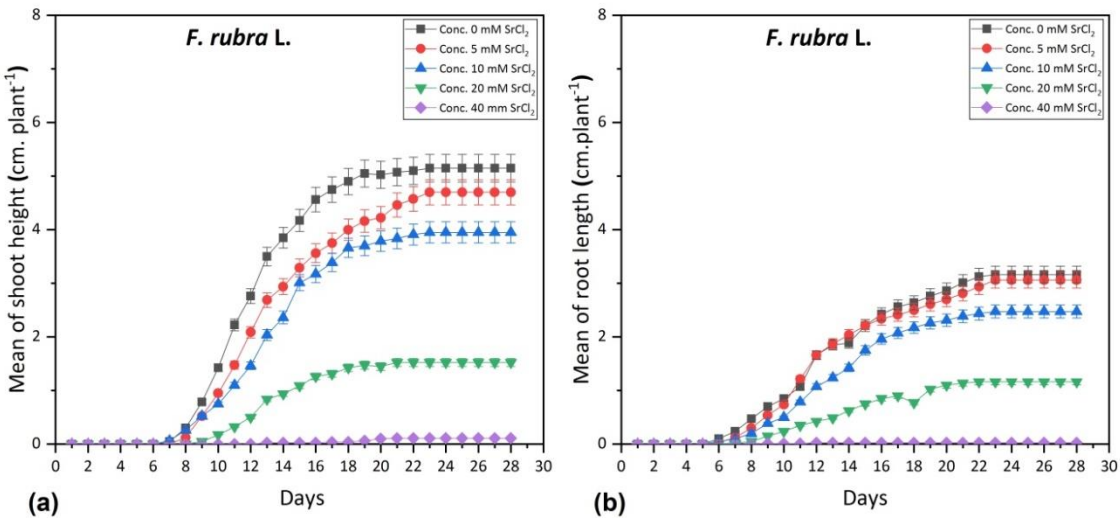

Figure 2.6 Mean values of seedling length over time for *F. rubra* grown in different concentrations of $SrCl_2$ (0, 5, 10, 20, and 40 mM) for four weeks: **(a)** shoot height, and **(b)** root length.

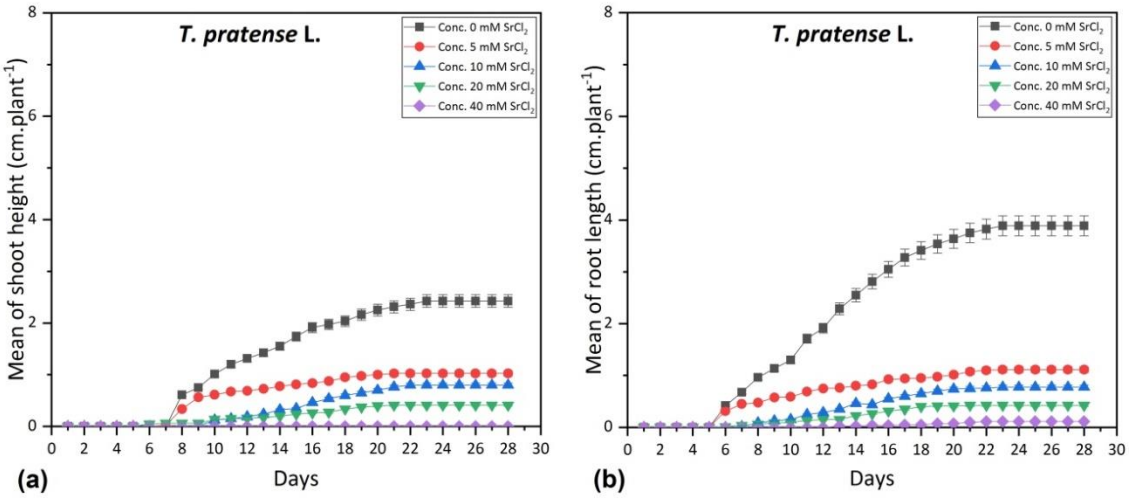

Figure 2.7 Mean values of seedling length over time for *T. pratense* grown in different concentrations of $SrCl_2$ (0, 5, 10, 20, and 40 mM) for four weeks: **(a)** shoot height, and **(b)** root length.

Table 2.4 shows the seeds' ability to germinate in different concentrations of $SrCl_2$. The table reveals that *F. rubra's* final germination percentage (FGP) (*ANOVA*, $F_{(4,135)}$ = 22.14, p<0.001) dropped significantly as $SrCl_2$ concentrations increased, and was related to the percentage of mean daily germination (MDG) (*ANOVA*, $F_{(4,135)}$ = 22.14, p<0.001). Similarly, the vigour index (VI) (*ANOVA,* $F_{(4,135)}$ = 19, p<0.001) was significantly different under the varied $SrCl_2$ concentrations. Seeds subjected to 40 mM $SrCl_2$ had a longer mean germination time (eight days) than seeds subjected to 0, 5, 10, and 20 mM $SrCl_2$ (6-7 days).

Table 2.4 Effect of $SrCl_2$ concentrations on germination of *F. rubra* and *T. pratense.*

Conditions	*F. rubra*				*T. pratense*			
	FGP (%)	MDG (%)	MGT (d)	VI	FGP (%)	MDG (%)	MGT (d)	VI
0 mM $SrCl_2$ (Control)	74.6[a]	2.66[a]	6	448[a]	71.9[a]	2.57[a]	6	321[a]
5 mM $SrCl_2$	72.8[a]	2.60[a]	6	408[a,b]	53.1[b]	1.90[b]	6	92[b]
10 mM $SrCl_2$	64.7[a]	2.31[a]	6	306[b]	34.4[c]	1.23[c]	7	39[b,c]
20 mM $SrCl_2$	47.3[b]	1.69[b]	6	90[c]	39.7[c]	1.42[c]	6	26[c]
40 mM $SrCl_2$	9.38[c]	0.33[c]	8	0.76[c]	11.6[d]	0.41[d]	13	1.03[c]

[FGP = final germination percentage, MDG = means daily germination, MGT = mean germination time, and VI = vigor index; different letters in each column denote significantly different group using LSD post-hoc test with significance set at p<0.05 (one-way ANOVA)]

Similarly, *T. pratense* had a significantly lower final germination percentage (FGP) (*ANOVA,* $F_{(4,135)}$ = 22.4, p<0.001) as concentrations of $SrCl_2$ were increased, and related to the percentage of mean daily germination (MDG) (*ANOVA,* $F_{(4,135)}$ = 22.4, p<0.001). The vigour index (VI) (*ANOVA,* $F_{(4,135)}$ = 44.4, p<0.001) exhibited significant differences in varied $SrCl_2$ concentrations of conditional treatments. Seeds subjected to 40 mM $SrCl_2$ had mean germination time of 13 days compared to seven days at 10 mM $SrCl_2$ and six days when subjected to 0, 5, and 20 mM $SrCl_2$.

Overall, the germination rates decreased as the $SrCl_2$ solution increased. This study confirmed the findings of Jadia and Fulekar (2009), who found that lower concentrations of metal did not directly affect plant growth, while the higher (>40 mM) concentrations adversely influenced growth. However, a clear difference was observed in different types of seeds: *F. rubra* seeds seemed to have better germination responses in all conditional treatments, while the *T. pratense* plumule did not appear at 40 mM of $SrCl_2$. Therefore, the

final rate of germination showed that the seeds of *F. rubra* had significantly greater chemical tolerance.

2.5.2.2 Seed germination in $CaCl_2$ solutions

In this experiment, seeds were subjected to different salinities, but in this case, calcium chloride (less toxicity) was used to create the saline conditions (0, 20 and 40 mM). Further, the timing of the addition of the saline solutions differed as experimental treatments:

i) "Pre-GEx" (pre-germination exposure): $CaCl_2$ solution was initially mixed into the soil before the seeds were sown;

ii) "Post-GEx" (post-germination exposure): $CaCl_2$ solution was irrigated into the soil after germination; and

iii) "Com-GEx": the combination was consistently irrigated with $CaCl_2$ solutions.

Figure 2.8 compares the biometric measurements of the seedlings after their harvest at week six from each $CaCl_2$ and exposure-timing treatment, presented as mean values of shoot height and root length. As shown in Figures 2.8(a), and 2.8(c) for *F. rubra*, concentrations of $SrCl_2$ solutions significantly affected the viability of germination in shoot only for the Com-GEx treatment (*ANOVA*; $F_{(2,29)} = 3.36$, $p=0.05$).

On the other hand, for *T. pratense*, the shoot height ($F_{(2,29)} = 29.32$, $p<0.001$) and root length ($F_{(2,29)} = 14.49$, $p<0.001$) decreased significantly in the Com-GEx treatment. In addition, the germination rates also declined when $CaCl_2$ solution was increased to 40 mM in the Pre-GEx treatment, which resulted in significant difference in the shoot (*ANOVA*, $F_{(2,29)} = 3.90$, $p = 0.032$) as seen in Figure 2.8(b). In the introduction to their study, Broadley et al. (2003) reported that high concentrations of calcium acted as a nutrient for seedling development; however, the findings of this study do not support this previous research, as detrimental results can be seen in *T. pratense*.

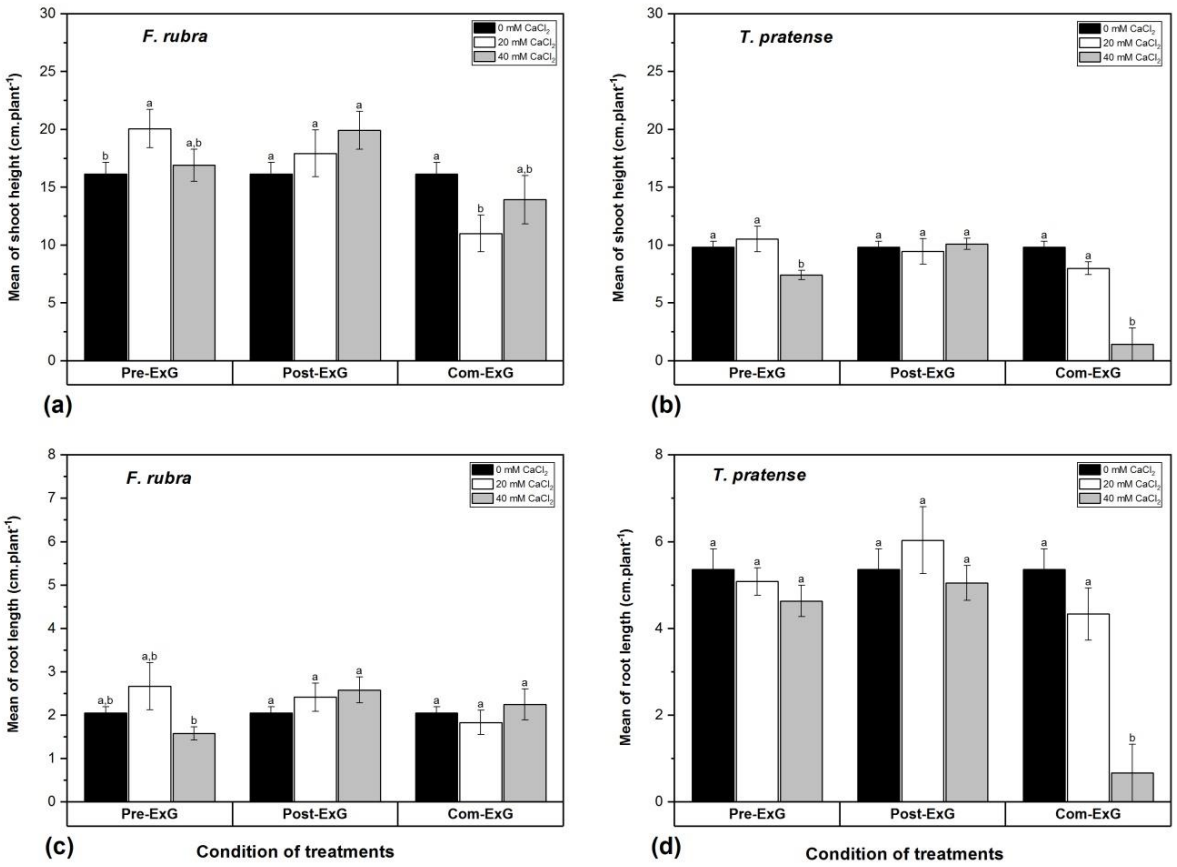

Figure 2.8 The effect of different $CaCl_2$ solutions (0, 20, and 40 mM) on the growth rates of shoot and roots of *F. rubra* (**a**, and **c**) and *T. pratense* (**b**, and **d**) after six weeks. Results are mean values (±S.E.) of shoot height and root length (Pre-GEx = $CaCl_2$ mixed into the soils before germination, Post-GEx = added $CaCl_2$ after germination for three weeks, and Com-GEx = consistently mixed water and $CaCl_2$ throughout the experiment; different letters in each vertical bar denote differences in groups at the significance level $p \leq 0.05$ using Fisher's LSD post-hoc test).

Table 2.5 indicates the relative growth rate (RGR), fresh weight (FW), nodule, and vigor index (VI) in each $CaCl_2$ concentration for both *F. rubra and T. pratense*, summarized as follows:

- *Relative growth rate (RGR)*

F. rubra showed no significant difference in the shoot height at any treatment. For *T. pratense* there was only a difference at the highest (40 mM) concentration of $CaCl_2$ in the Com-GEx treatment (*ANOVA*; $F_{(2,29)} = 15.46$, $p<0.01$).

- *Fresh weight (FW)*

FW had a significant difference in both *F. rubra* (*ANOVA*; $F_{(2,14)} = 33.36$, $p<0.01$) and *T. pratense* (*ANOVA*; $F_{(2,14)} = 5.87$, $p = 0.02$) for the Com-GEx treatment. However, *F. rubra* also showed a significantly different response in FW in the Pre-GEx treatment (*ANOVA;* $F_{(2,14)} = 6.86$, $p=0.01$) and Post-GEx (*ANOVA*; $F_{(2,14)} = 9.60$, $p=0.003$) (Table 2.5).

-*Nodule*

These results can only be found in *T. pratense*, which is an unique member of the Fabaceae family and could develop nodules. As shown in Table 2.5, *T. pratense* had significant differences of nodule content in the Com-GEx treatment (*ANOVA*; $F_{(2,29)} = 12.05$, $p<0.01$). These cultivars had relatively reduced nodule counts when the concentration of $CaCl_2$ was increased, especially in the conditional treatment of Com-GEx, without any nodules generated at 40 mM of $CaCl_2$.

-*Vigour index (VI)*

The seeds responded differently to different levels of $CaCl_2$ stress, as reflected in the vigor index of six-week old seedlings. The application of the Pre-GEx (20 mM) and Post-GEx treatment (40 mM) enhanced the seedling vigor, especially in *F. rubra*, when compared each treatment of the Com-GEx for either *F. rubra* or *T. pratense*.

Table 2.5 Mean values (±S.E.) of relative growth rate (RGR) of shoot height, fresh weight (FW), nodules in the root parts, and vigour index (VI) of *F. rubra* and *T. pratense*.

Parameters	Conc. (mM)	*F. rubra*						*T. pratense*					
		Pre-GEx		Post-GEx		Com-GEx		Pre-GEx		Post-GEx		Com-GEx	
RGR	0	0.15^{a}	±0.03	0.15^{a}	±0.03	0.15^{a}	±0.03	0.17^{a}	±0.02	0.17^{a}	±0.02	0.17^{a*}	±0.02
	20	0.17^{a}	±0.02	0.16^{a}	±0.04	0.12^{a}	±0.04	0.22^{a}	±0.02	0.19^{a}	±0.02	0.14^{a*}	±0.03
	40	0.18^{a}	±0.02	0.20^{a}	±0.02	0.18^{a}	±0.03	0.17^{a}	±0.02	0.17^{a}	±0.01	0.02^{b*}	±0.02
FW	0	0.10^{b*}	±0	0.10^{a*}	±0	0.10^{a*}	±0	0.62^{a}	±0.07	0.62^{a}	±0.07	0.62^{a*}	±0.07
	20	0.14^{a*}	±0.01	0.10^{a*}	±0	0.08^{b*}	±0	0.61^{a}	±0.09	0.63^{a}	±0.08	$0.35^{a,b*}$	±0.03
	40	0.07^{b*}	±0.02	0.09^{b*}	±0	0.06^{c*}	±0.01	0.36^{a}	±0.08	0.69^{a}	±0.07	0.08^{b*}	±0.06
Nodules	0	N/A		N/A		N/A		6.44^{a}	±0.86	6.44^{a}	±0.86	6.44^{a*}	±0.86
	20	N/A		N/A		N/A		6.00^{a}	±1.40	7.50^{a}	±2.10	2.00^{b*}	±0.49
	40	N/A		N/A		N/A		4.67^{a}	±1.28	7.00^{a}	±2.02	0.00^{b*}	±0
VI	0	1822^{b}	±100	1822^{a}	±100	1822^{a}	±100	1519^{a}	±84	1519^{a}	±84	1519^{a*}	±84
	20	2273^{a}	±186	2033^{a}	±233	1283^{b}	±166	$1562^{a,b}$	±134	1548^{a}	±127	1233^{a*}	±100
	40	$1848^{a,b}$	±131	2250^{a}	±175	$1618^{a,b}$	±237	1205^{b}	±65	1515^{a}	±67	35^{b*}	±35

*[pre-germination exposure (Pre-GEx), post-germination exposure (Post-GEx), and combined-germination exposure (Com-GEx)] in different concentration of $CaCl_2$ (0, 20, 40 mM) solutions for 6 weeks, sets of controls (0 mM) are the same repeated in 3 different treatments; N/A = not available, * = statistically different at p≤0.05, and different letter in each column denoted significant difference with using LSD post-hoc test at p<0.05 (one-way ANOVA)]*

2.5.3 Experiment 3: Strontium exposure

Growth experiments were conducted, similarly as the previous $CaCl_2$ trials, except $SrCl_2$ were used. Concentrations of $SrCl_2$ included (0, 5, 10, 20 and 40 mM), and similar exposure-timing treatment were applied (Pre-GEx, Post-GEx and Com-GEx). However, in these assays, as Sr was the element of interest, more supporting information was provide to support the experiment, e.g.: soil properties, Sr-fate, a greater emphasis on plant biometrics, and determination of translocation factor (i.e., plant uptake and mobilization of Sr). The experiment was also run for 10 weeks.

2.5.3.1 Physicochemical properties and Sr fractionation in the soil

After treatment with different strontium chloride concentrations for ten weeks, there were significant pH declines in soils with *T. pratense* plants in treatments: Pre-GEx (ANOVA; $F_{(4,20)}$=7.47, p=0.001), Post-GEx (ANOVA; $F_{(4,20)}$=133, p<0.000), and Com-GEx (ANOVA; $F_{(4,20)}$=29.9, p<0.001). In contrast, in treated soils with *F. rubra,* there were no significant differences in pH for any conditions (Table 2.6).

The EC values in all exposure conditions after treatment became higher than previously. Interestingly, EC results suggest that the soil values were saline sodic (Vanatta, 2000), or equivalent to “brines” when compared with the typical water (Walton, 1989). In regards to the CEC content, the soils were not significantly affected by excess Sr^{2+}. As Table 2.7 shows, only the CEC results for *F. rubra* growth in the Com-GEx treatment was significantly different. The CEC represents Ca^{2+}, Mg^{2+}, K^{+}, Na^{+} exchangeable concentrations, which were greater in soils with *F. rubra* than those with *T. pratense*.

It is apparent from Table 2.7 that soils had concentrations of exchangeable ions in the following order: $Ca^{2+} > Mg^{2+} > K^{+} > Na^{+}$. In particular, *F. rubra* had significantly different concentrations of exchangeable K^{+} in the Pre-GEx condition (ANOVA; $F_{(4,20)}$=6.48, p=0.003),

Post-GEx condition (ANOVA; $F_{(4,20)}$=6.68, p=0.002), and Com-GEx condition (ANOVA; $F_{(4,20)}$=7.37, p=0.001). In contrast, the results for pH, and moisture content were not significantly different among the varying exposure conditions. These findings agree with those of Rediske and Selders (1953), which showed strontium was more adsorbed in soils with increased acidity, and this was illustrated in soils with *T. pratense.* There were significantly different concentrations of exchangeable Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} (Table 2.7), especially within the Com-GEx exposure treatment, exchangeable Ca^{2+} (ANOVA; $F_{(4,20)}$=14.5, p<0.001), Mg^{2+} (ANOVA; $F_{(4,20)}$=3.26, p=0.039), and K^{+} (ANOVA; $F_{(4,20)}$=21.2, p<0.001). In contrast, exchangeable Na^{+} was significantly different in the Pre-GEx (ANOVA; $F_{(4,20)}$=3.15, p=0.043), and Post-GEx (ANOVA; $F_{(4,20)}$=3.61, p=0.028) treatment. Interestingly, in the Post-GEx treatment at 40 mM, pH value was lower at 5.78. This was in accordance with Harmsen (1977); the interaction of trace elements can be complicated by the physical properties of the soils. As mentioned by IAEA (1994) and Savinkov et al. (2007), the types of soil, especially sandy soil, affect the mobilty of Sr^{2+}. Their results are consistent with mine, which found soils with acidic pH allowing Sr content to easily bind with soil and possibly transfer into the plant roots (IAEA, 1994, Savinkov et al., 2007).

However, some authors have explained that soils with greater CEC indicate a soil structure with high levels of clay and organic matter (Busenberg and Clemency, 1973, Gillespie et al., 2001, Aprile and Lorandi, 2012). These results are consistent with those of other studies and suggest that soil samples after treatment had slightly higher CEC values (Table 2.7).

Table 2.6 Chemical and physical characteristics of the soil following the growth of *F. rubra* and *T. pratense* under different exposure-timings and concentrations of strontium chloride.

Treated Soil	Conc.[Sr^{2+}] (mM)	pH (H_2O)	EC ($mS.cm^{-1}$)	TC (%)	LOI (%)	Moisture content (%)
F. rubra						
Pre-GEx	0	6.51^{a}	81^{d*}	5.37^{a}	9.66^{a}	19.6^{a}
	5	6.66^{a}	114^{c*}	5.31^{a}	9.56^{a}	26.1^{a}
	10	6.62^{a}	117^{c*}	5.37^{a}	9.66^{a}	26.9^{a}
	20	6.62^{a}	167^{b*}	5.30^{a}	9.54^{a}	28.5^{a}
	40	6.65^{a}	284^{a*}	5.28^{a}	9.50^{a}	25.7^{a}
Post-GEx	0	6.51^{a}	$81^{b,c*}$	5.37^{a}	9.66^{a}	19.6^{a}
	5	6.52^{a}	72^{c*}	5.13^{b}	9.24^{b}	22.6^{a}
	10	6.48^{a}	$76^{b,c*}$	$5.26^{a,b}$	$9.47^{a,b}$	28.4^{a}
	20	6.43^{a}	100^{b*}	$5.23^{a,b}$	$9.41^{a,b}$	27.8^{a}
	40	6.40^{a}	164^{a*}	5.10^{b}	9.18^{b}	21.2^{a}
Com-GEx	0	6.51^{a}	81^{d*}	5.37^{b*}	9.66^{b*}	19.6^{b}
	5	6.52^{a}	210^{c*}	5.63^{a*}	10.1^{a*}	$27.2^{a,b}$
	10	6.53^{a}	255^{c*}	5.64^{a*}	10.1^{a*}	31.7^{a}
	20	6.46^{a}	508^{b*}	$5.51^{a,b*}$	$9.91^{a,b*}$	$28.5^{a,b}$
	40	6.37^{a}	853^{a*}	$5.40^{a,b*}$	$9.71^{a,b*}$	31.8^{a}
T. pratense						
Pre-GEx	0	6.45^{a*}	41^{d*}	4.98^{a}	8.97^{a}	15.4^{a}
	5	6.48^{a*}	40^{d*}	4.99^{a}	8.99^{a}	15.7^{a}
	10	6.52^{a*}	60^{c*}	4.97^{a}	8.95^{a}	16.8^{a}
	20	6.50^{a*}	99^{b*}	4.98^{a}	8.96^{a}	14.0^{a}
	40	6.12^{b*}	211^{a*}	4.95^{a}	8.92^{a}	13.7^{a}
Post-GEx	0	6.45^{a*}	41^{b*}	4.98^{a}	8.97^{a}	15.4^{a}
	5	6.30^{b*}	37^{b*}	5.05^{a}	9.10^{a}	18.0^{a}
	10	6.24^{b*}	40^{b*}	5.14^{a}	9.25^{a}	13.1^{a}
	20	6.25^{b*}	94^{a*}	5.01^{a}	9.01^{a}	9.47^{a}
	40	5.78^{c*}	116^{a*}	4.93^{a}	8.87^{a}	13.8^{a}
Com-GEx	0	6.45^{a*}	41^{e*}	4.98^{b*}	8.97^{b*}	15.4^{a}
	5	6.39^{a*}	153^{d*}	$5.12^{a,b*}$	$9.22^{a,b*}$	11.2^{a}
	10	6.24^{b*}	288^{c*}	$5.15^{a,b*}$	$9.28^{a,b*}$	8.37^{a}
	20	5.93^{c*}	466^{b*}	5.25^{a*}	9.45^{a*}	20^{a}
	40	6.04^{c*}	643^{a*}	$5.06^{a,b*}$	$9.11^{a,b*}$	17.3^{a}

*Mean values (samples n=3, control n=9), denoted by a different letter based on LSD post-hoc test similarities, * = statistically different at p≤0.05 (one-way ANOVA), Pre-GEx=pre-germination exposure, Post-GEx=post-germination exposure, and Com-GEx=combined-germination exposure*

Table 2.7 CEC and exchangeable of Ca^{2+}, K^{+}, Mg^{2+}, and Na^{+} values in the soil samples following the growth of *F. rubra* and *T. pratense* under different exposure-timings and concentrations of strontium chloride.

Treated Soil	Conc.[Sr^{2+}] (mM)	CEC	Ca^{2+}	K^{+} ($cMol^{+}.kg^{-1}$)	Mg^{2+}	Na^{+}
MQL (mg L^{-1})			0.05	0.08	0.001	0.03
F. rubra						
Pre-GEx	0	296^{a}	125^{a}	1.69^{b*}	37.1^{a}	-0.37^{b*}
	5	217^{a}	156^{a}	2.90^{a*}	45.7^{a}	0.98^{a*}
	10	232^{a}	151^{a}	3.06^{a*}	45.3^{a}	1.40^{a*}
	20	262^{a}	148^{a}	2.50^{a*}	41.5^{a}	$0.54^{a,b*}$
	40	263^{a}	145^{a}	2.90^{a*}	42.0^{a}	1.04^{a*}
Post-GEx	0	296^{a}	125^{b}	1.69^{c*}	37.1^{b}	-0.37^{b*}
	5	308^{a}	$153^{a,b}$	$2.86^{a,b*}$	$46.6^{a,b}$	1.03^{a*}
	10	330^{a}	160^{a}	3.20^{a*}	47.9^{a}	1.66^{a*}
	20	346^{a}	$147^{a,b}$	$2.30^{b,c*}$	$43.5^{a,b}$	$0.67^{a,b*}$
	40	380^{a}	$149^{a,b}$	$2.61^{a,b*}$	$43.7^{a,b}$	1.25^{a*}
Com-GEx	0	296^{a}	125^{a}	1.69^{c*}	37.1^{b}	-0.37^{b*}
	5	214^{a}	152^{a}	$2.83^{a,b*}$	47.1^{a}	1.31^{a*}
	10	219^{a}	129^{a}	1.09^{c*}	$36.8^{a,b}$	$0.85^{a,b*}$
	20	275^{a}	134^{a}	$2.02^{b,c*}$	$37.4^{a,b}$	$0.89^{a,b*}$
	40	244^{a}	118^{a}	3.10^{a*}	32.3^{b}	0.97^{a*}
T. pratense						
Pre-GEx	0	300^{a}	138^{a}	1.53^{a}	41.3^{a}	0.79^{a*}
	5	249^{a}	$134^{a,b}$	1.11^{a}	$40.8^{a,b}$	1.04^{a*}
	10	267^{a}	$131^{a,b}$	1.0^{a}	$39.6^{a,b}$	0.26^{b*}
	20	265^{a}	$131^{a,b}$	0.89^{a}	$39.0^{a,b}$	$0.58^{a,b*}$
	40	210^{a}	130^{b}	1.30^{a}	37.8^{b}	1.18^{a*}
Post-GEx	0	300^{a}	$138^{a,b}$	1.53^{a}	41.3^{a*}	$0.79^{a,b*}$
	5	340^{a}	143^{a}	1.46^{a}	43.3^{a*}	1.10^{a*}
	10	355^{a}	130^{b}	0.86^{a}	37.1^{b*}	0.19^{c*}
	20	328^{a}	$132^{a,b}$	1.07^{a}	37.4^{b*}	$0.35^{b,c*}$
	40	286^{a}	129^{b}	1.90^{a}	36.8^{b*}	$0.80^{a,b*}$
Com-GEx	0	300^{a}	138^{a*}	1.53^{b*}	41.3^{a*}	0.79^{a}
	5	274^{a}	$134^{a,b*}$	1.40^{b*}	39.3^{a*}	1.03^{a}
	10	250^{a}	$135^{a,b*}$	2.42^{a*}	$38.6^{a,b*}$	0.91^{a}
	20	245^{a}	125^{b*}	$1.84^{a,b*}$	35.6^{b*}	1.12^{a}
	40	238^{a}	110^{c*}	2.29^{a*}	28.9^{c*}	0.92^{a}

*Mean values (samples n=3, control n=9), denoted by a different letter based on LSD post-hoc test similarities, MQL=method quantification limit * = statistically different at p≤0.05 (one-way ANOVA), Pre-GEx=pre-germination exposure, Post-GEx=post-germination exposure, and Com-GEx=combined-germination exposure*

Figure 2.9 presents the percentage of Sr in each fraction per total-Sr among the various treatments. Most Sr in all treated soils were in the exchangeable fraction, which was found to be significantly greater in soils with *F. rubra* (Figure 2.9(a), (c), and (e)) than with *T. pratense* (Figure 2.9(b), (d), and (f)). The largest proportion the exchangeable phase was found at the treated condition of Com-GEx, which reflects the mass loadings of $SrCl_2$.

Puhakainen et al. (2001) suggested that Sr becomes distributed among the first three extractable fractions, which includes the "exchangeable" one. However, this study did not exhibit any carbonate fractions, which was the highest fraction in a similar experiment by Kamel (2010). As such, this experiments yielded exchangeable, soluble strontium under slightly acidic conditions, which can become easily mobilized as an environmental contaminant (Guogang et al., 1998, Lerouge et al., 2010).

2.5.3.2 Biomass production

Table 2.8 reports the total biomass of root and shoot, number of leaves and nodules after ten weeks of exposure. The results following harvest indicate a statistically significant difference in germination rate during different strontium exposures. The significant difference among *F. rubra* treatments were Pre-GEx (root weight (ANOVA; $F_{(4,41)}$=2.67, p=0.047); shoot height (ANOVA; $F_{(4,41)}$=3.12, p=0.026); shoot weight (ANOVA; $F_{(4,41)}$=6.12, p=0.001); number of leaves (ANOVA; $F_{(4,41)}$=3.94, p=0.009)) and Com-GEx (root weight (ANOVA; $F_{(4,41)}$=3.22, p=0.023), and shoot height (ANOVA; $F_{(4,41)}$=6.64, p<0.001)). In contrast, *T. pratense* had statistically difference in the Com-GEx treatment (in terms of: root length (ANOVA; $F_{(4,41)}$=2.65, p=0.049), shoot height (ANOVA; $F_{(4,41)}$=4.48, p=0.005), number of leaves (ANOVA; $F_{(4,41)}$=3.72, p=0.012), nodules (ANOVA; $F_{(4,41)}$=3.73, p=0.012)). Besides, at the Pre-GEx was illustrated the significant difference in shoot weight (ANOVA; $F_{(4,41)}$=2.67, p=0.047) and number of leaves (ANOVA; $F_{(4,41)}$=3.05, p=0.029). However, the root weight within only was statistically different (ANOVA; $F_{(4,41)}$=3.10, p=0.027) in the Com-GEx treatment.

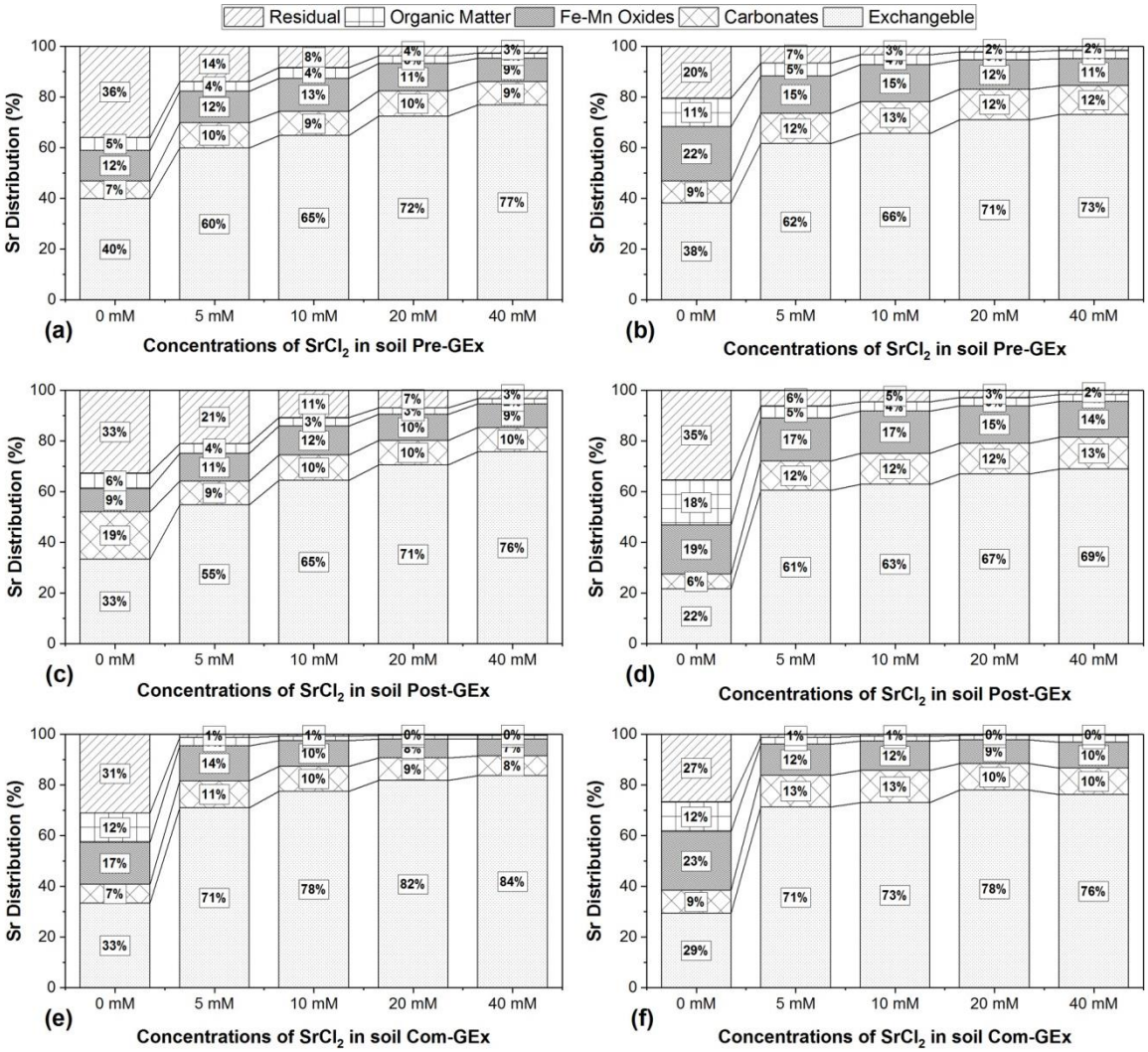

Figure 2.9 Relative distributions of Sr fractions; **(a)** *F. rubra* and **(b)** *T. pratense* with pre-germination exposures (Pre-GEx), **(c)** *F. rubra* and **(d)** *T. pratense* with post-germination exposures (Post-GEx), and **(e)** *F. rubra* and **(f)** *T. pratense* at combined-germination exposures (Com-GEx). The vertical bars represent average percentages calculated from three replicated measurements.

Table 2.8 Biometric measurements of freshly harvested *F. rubra* and *T. pratense* grown in different concentrations of $SrCl_2$ and exposure timings at week 10.

Treated Soil	Conc. $SrCl_2$ (mM)	Root Length (cm.plant^{-1})	Root Weight (mg.plant^{-1})	Shoot height (cm.plant^{-1})	Shoot weight (mg.plant^{-1})	No. of leaves (plant^{-1})	No. nodules
F. rubra							
Pre-GEx	0	10.7^{a}±0.77	0.21^{a*}±0.03	36.4^{a*}±1.63	0.54^{a*}±0.06	20.7^{a*}±1.59	NA
	5	10.7^{a}±1.91	0.11^{b*}±0.04	$31.3^{a,b*}$±2.62	0.23^{b*}±0.05	11.0^{b*}±1.44	NA
	10	12.4^{a}±1.03	$0.17^{a,b*}$±0.03	29.4^{b*}±1.62	0.30^{b*}±0.05	$15.3^{a,b*}$±3.16	NA
	20	10.6^{a}±1.09	0.12^{b*}±0.02	$31.3^{a,b*}$±1.40	0.26^{b*}±0.03	12.8^{b*}±1.05	NA
	40	8.60^{a}±1.47	0.09^{b*}±0.02	26.2^{b*}±4.40	0.21^{b*}±0.04	14.3^{b*}±2.97	NA
Post-	0	$10.7^{a,b}$±0.77	0.21^{b}±0.03	36.4^{a}±1.63	0.54^{a}±0.06	$20.7^{a,b}$±1.59	NA
GEx	5	8.55^{b}±0.79	0.31^{a}±0.05	31.8^{a}±0.83	0.48^{a}±0.07	$21.7^{a,b}$±1.96	NA
	10	8.25^{b}±0.44	$0.22^{a,b}$±0.03	31.3^{a}±1.45	0.42^{a}±0.07	$19.3^{a,b}$±2.29	NA
	20	12.2^{a}±1.33	$0.23^{a,b}$±0.02	31.7^{a}±2.64	0.51^{a}±0.03	24.3^{a}±3.83	NA
	40	$9.03^{a,b}$±0.82	0.16^{b}±0.02	37.0^{a}±1.32	0.50^{a}±0.05	15.7^{b}±1.98	NA
Com-	0	10.7^{a}±0.77	0.21^{a*}±0.03	36.4^{a*}±1.63	0.54^{a}±0.06	$20.7^{a,b}$±1.59	NA
GEx	5	$9.75^{a,b}$±0.92	0.30^{a*}±0.10	35.3^{a*}±1.80	0.57^{a}±0.11	$23.7^{a,b}$±5.51	NA
	10	7.17^{b}±0.76	$0.19^{a,b*}$±0.05	31.2^{a*}±1.46	0.46^{a}±0.13	$23.7^{a,b}$±5.67	NA
	20	$8.67^{a,b}$±1.44	0.38^{a*}±0.13	32.2^{a*}±0.59	0.72^{a}±0.27	28.2^{a}±9.74	NA
	40	$7.70^{a,b}$±2.49	0.03^{b*}±0.01	20.4^{b*}±4.82	0.13^{b}±0.04	9.83^{b}±2.09	NA
T. pratense							
Pre-GEx	0	12.0^{a}±1.23	0.40^{a}±0.06	24.1^{a}±1.48	1.99^{b*}±0.17	9.67^{b*}±0.36	50.0^{a}±7.6
	5	11.0^{a}±0.80	0.53^{a}±0.07	21.5^{a}±0.81	2.90^{a*}±0.21	12.0^{a*}±0.73	49.3^{a}±4.94
	10	11.8^{a}±1.25	0.57^{a}±0.09	22.2^{a}±1.31	$2.48^{a,b*}$±0.38	$11.7^{a,b*}$±1.40	49.3^{a}±4.36
	20	10.0^{a}±0.62	0.45^{a}±0.09	22.3^{a}±1.68	2.78^{a*}±0.27	12.0^{a*}±1.03	38.2^{a}±4.38
	40	10.3^{a}±1.24	0.36^{a}±0.10	22.7^{a}±1.36	$2.11^{a,b*}$±0.34	9.33^{b*}±0.84	37.8^{a}±7.81
Post-	0	12.0^{a}±1.23	0.40^{c*}±0.06	24.1^{a}±1.48	1.99^{b}±0.17	$9.67^{a,b}$±0.36	50.0^{a}±7.6
GEx	5	8.25^{a}±1.76	$0.42^{b,c*}$±0.09	24.2^{a}±5.07	$2.34^{a,b}$±0.57	7.67^{b}±1.58	$47.8^{a,b}$±11.6
	10	12.9^{a}±1.16	0.79^{a*}±0.15	25.1^{a}±3.21	3.11^{a}±0.37	12.0^{a}±1.71	$41.2^{a,b}$±5.56
	20	12.9^{a}±1.71	$0.70^{a,b*}$±0.07	$23.2^{a,b}$±1.49	3.02^{a}±0.16	$10.7^{a,b}$±0.99	$46.2^{a,b}$±5.08
	40	9.17^{a}±3.40	$0.42^{b,c*}$±0.18	14.4^{b}±4.69	$1.94^{a,b}$±0.77	7.33^{b}±2.86	23.5^{b}±8.83
Com-	0	12.0^{a*}±1.23	0.40^{a}±0.06	24.1^{a*}±1.48	$1.99^{a,b}$±0.17	$9.67^{a,b*}$±0.36	50.0^{a*}±7.6
GEx	5	$9.92^{a,b*}$±1.07	0.42^{a}±0.17	$19.9^{a,b*}$±1.29	$2.14^{a,b}$±0.57	11.3^{a*}±1.91	$39.8^{a,b*}$±10.5
	10	$9.50^{a,b*}$±0.56	0.55^{a}±0.17	$20.3^{a,b*}$±3.65	2.57^{a}±0.33	12.7^{a*}±1.84	$34.8^{a,b*}$±8.15
	20	6.58^{b*}±2.28	0.30^{a}±0.12	13.3^{b*}±4.58	$1.49^{a,b}$±0.63	6.33^{c*}±2.27	13.5^{b*}±6.96
	40	7.00^{b*}±0.43	0.46^{a}±0.11	12.7^{b*}±1.78	1.06^{b}±0.24	$7.0^{b,c*}$±1.13	12.2^{b*}±4.12

*Mean values (±S.E.) (Samples n=6, Control n=18), denoted by a different letter based on LSD post-hoc test similarities, * = statistically different at p≤0.05 (one-way ANOVA), NA=not available, Pre-GEx=pre-germination exposure, Post-GEx=post-germination exposure, and Com-GEx=combined-germination exposure*

Results recording shoot height in all treatment exposures are shown in Figures 2.10 – 2.12. From the graphs, it is apparent that there is a relationship between shoot elongation and the concentration level of strontium at every two weeks. However, as it can be seen from Figures 2.10(a) and 2.11(a), *F. rubra* does not clearly indicate any differences among the Sr concentrations. Further, there were no significantly differences between Pre-GEx and Post-GEx treatments; however, the Com-GEx treatment was significantly different for both *F. rubra* and *T. pratense* (Figures 2.12 (a) and (b)). In summary, these results demonstrate that Sr^{2+} has an effect on the growth of the plants as contaminants in soil, both in terms of concentrations and timing of their exposure.

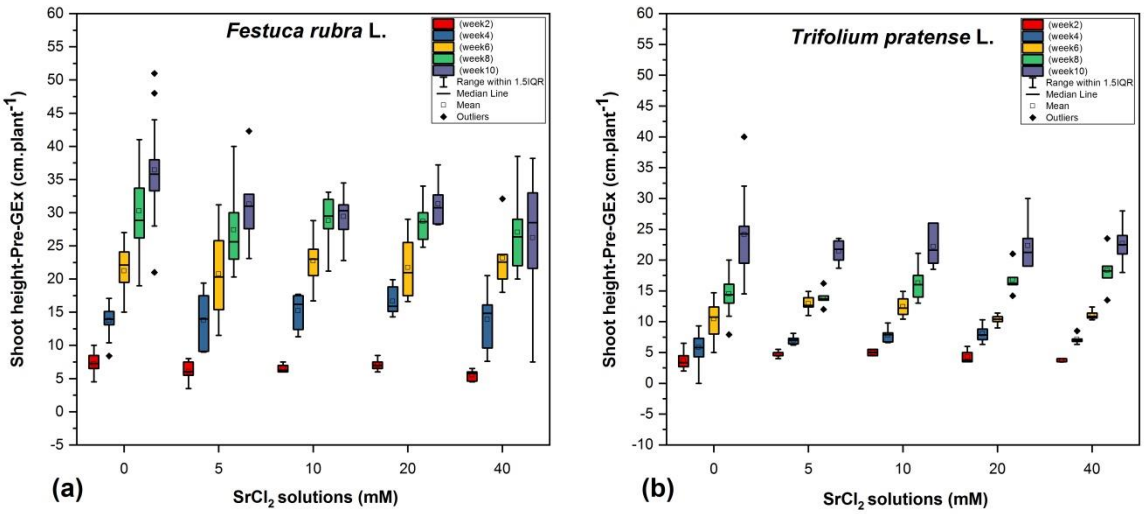

Figure 2.10 Boxplot of shoot height of plants (n=6, control n=18) grown in different concentrations of $SrCl_2$ over a period of time (sampled at weeks 2, 4, 6, 8, and 10) at pre-germination exposure (Pre-GEx): **(a)** *F. rubra*, and **(b)** *T. pratense.*

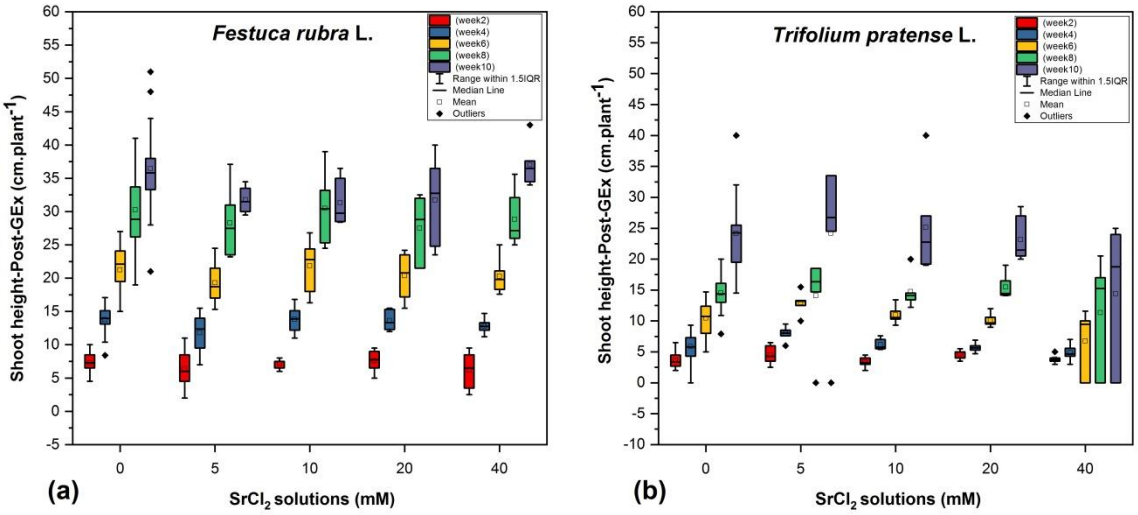

Figure 2.11 Boxplot of shoot height of plants (n=6, control n=18) grown in different concentrations of $SrCl_2$ over a period of time (sampled at weeks 2, 4, 6, 8, and 10) at post-germination exposure (Post-GEx); **(a)** *F. rubra*, and **(b)** *T. pratense*.

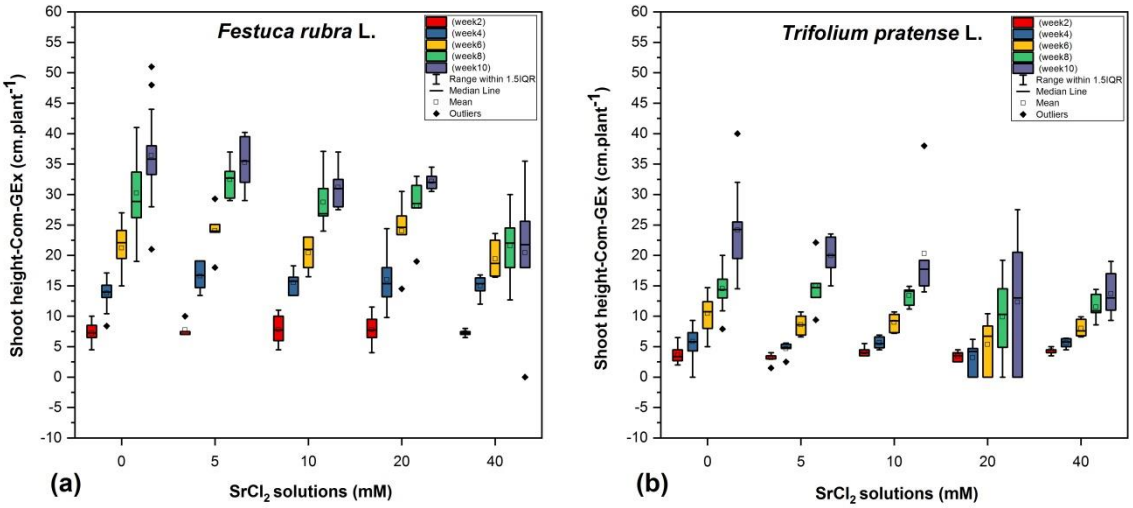

Figure 2.12 Boxplot of shoot height of plants (n=6, control n=18), grown in different concentrations of $SrCl_2$ over a period of time (sampled at weeks 2, 4, 6, 8, and 10) at combined-germination exposure (Com-GEx); **(a)** *F. rubra*, and **(b)** *T. pratense.*

2.5.3.3 Chlorophyll a, and b

Figure 2.13 presents the chlorophyll content, indicating how Sr^{+2} ions affected plant growth at different exposures. Figure 2.13(a) show that *F. rubra* had significantly greater chlorophyll concentrations (both Chlo *a* and *b*) than *T. pratense* in Figure 2.13(b). In Figure 2.13(a) for *F. rubra,* Chlo *a* responded differently depending on the timing of strontium exposure. Chlorophyll *a* was highest when the strontium was pre-added to the soils; whereas chlorophyll *b* trended inversely with strontium concentrations – except for the highest (40mM) concentration, in which it increased. In contrast, *T. pratense* showed no significant differences between Chlo *a* and *b* in all of the conditional exposures (Figure 2.13(b)). Interestingly, chlorophyll *b* trended inversely with strontium conditions without any increase. However, the chlorophyll *a*, after declining at initial strontium amendments, began to increase at elevated Sr^{2+} additions.

These results were confirmed with a two-way ANOVA as displayed in Table 2.9. There were greater explanations of variances represented (by two-way ANOVA model) in *F. rubra* than in *T. pratense*, as shown by r^2 value for both Chlo *a* and Chlo *b.* However, the most surprising aspect of these results is that they are in disagreement with those of Moyen and Roblin (2010), who asserted that Sr ions were often associated with a decrease of Chlo *a* and Chlo *b* content when Sr^{2+} is greater than 10 mM on maize leaves, which is the same family as *F. rubra.* These findings also show the association between carbon fixation pathways like C_3 (e.g., most trees, grasses) and C_4 (e.g., maize, sugar cane) plant species that are mainly employed in a process known as photorespiration (Sivaram et.al, 2018). These factors may explain the relationship between chlorophyll concentration and the photorespiration process. *F. rubra* likely follows a C_3 pathway with a higher photorespiration rate than those with a C_4 pathway. These results are different from those of Moyen and Roblin (2010); however, their study utilized a C_4 species (maize). On the other hand, *T. pratense* had minimal

significant response in chlorophyll content from increasing concentrations of $SrCl_2$. Overall, these results indicate that plants (even within the same family) may be affected differently by strontium stress.

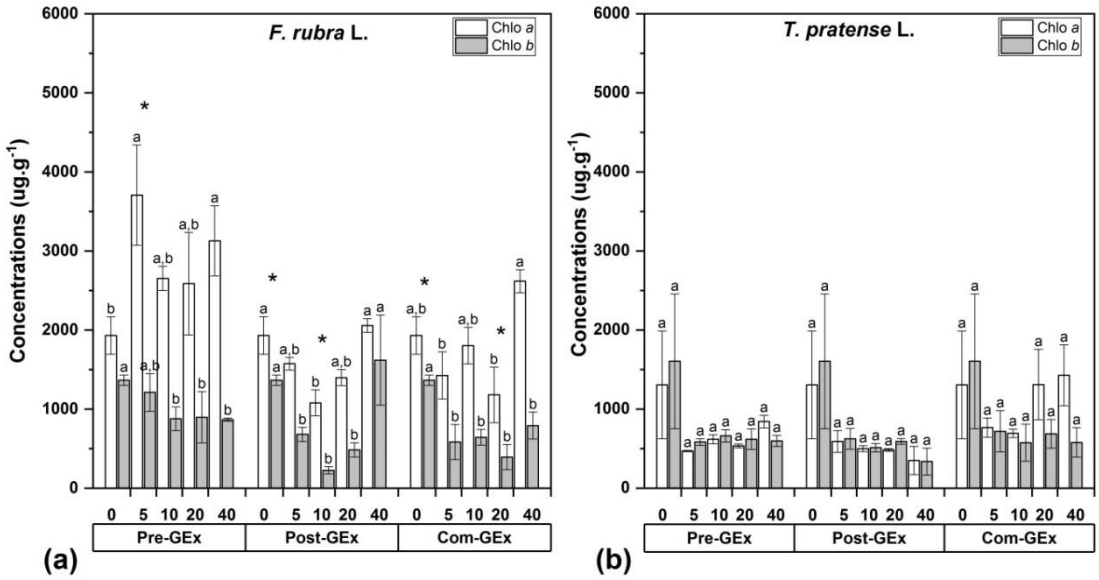

Figure 2.13 Concentrations of chlorophyll *a* and *b* of **(a)** *F. rubra* and **(b)** *T. pratense* grown under different concentrations of $SrCl_2$ solutions in different exposure conditions [Pre-GEx = pre-germination exposure, Post-GEx = post-germination exposure, and Com-GEx = combined-germination exposure]. The vertical bars are mean values (±S.E.) of triplicate measurements, denoted by a different letter based on LSD post-hoc test similarities, * = statistically different at $p \leq 0.05$ (one-way ANOVA).

Table 2.9 Two-way ANOVA representing effects of exposure-timings and concentrations of strontium solution on chlorophyll contents of *F. rubra* and *T. pratense.*

Source of variation	df	Concentration per plant			
		Chlo *a*		Chlo *b*	
		***F*-value**	***p*-value**	***F*-value**	***p*-value**
F. rubra					
Treatment	2	26.4	<0.001	1.51	0.237
Concentration	4	3.47	0.019	7.61	<0.001
Treatment × Concentration	8	1.59	0.170	2.56	0.030
Error	30				
r^2 (%)		72.6		64.2	
T. pratense					
Treatment	2	4.18	0.025	1.43	0.255
Concentration	4	0.86	0.497	1.46	0.238
Treatment × Concentration	8	0.87	0.552	1.15	0.363
Error	30				
r^2 (%)		38.5		37.3	

2.5.3.4 Sr content in plant tissues

In order to determine whether plants have any value for reducing salinity and/or strontium, tissues were assayed for Sr content; from these assay, one can compare translocation factor. Table 2.10 gives the results of plant-tissue digestions following their growth in differently-treated soils (Pre-GEx, Post-GEx, and Com-GEx). Shoot and root tissues uptook Sr (as $g.kg^{-1}$) and trended according to exposure concentrations, including the translocation factor (TF), representing the movement of Sr^{2+} from the root to the aerial structures of the plant. In most cases, there was greater accumulation of Sr in tissue among *T. pratense* than in *F. rubra*. Additionally, *T. pratense* had significantly greater values at each concentration within all exposure timings: Pre-GEx condition (ANOVA; $F_{(4,20)}$=17.99, p<0.001), Post-GEx (ANOVA; $F_{(4,20)}$=4.12, p=0.018), and Com-GEx condition (ANOVA; $F_{(4,20)}$=8.35,

p=0.001). On the other hand, while *F. rubra* shoots and roots responded to treatment conditions, there were no significant differences in their accumulation of Sr in their tissues. However, *F. rubra* was found to exclude Sr in the roots for the treated soil in Post-GEx, which had no significant difference at $F_{(4,20)}$=2.68, p=0.069.

In this experiment, the findings were in agreement with those of Rediske and Selders (1953), which showed strontium was best accumulated in the roots in slightly acidic soils. Some results have asserted that strontium concentrations correlated, with leaves and root increasing when the pH decreases in five plant species (e.g., Red Kidney bush bean, Rutgers tomatoes, White Russian wheat, Belsford Beardless Barley, and Russian thistle) (Rediske and Selders, 1953).

It is clear that *T. pratense* was better than *F. rubra* to absorb Sr in these soils. The adsorption capacity improved significantly in Post-GEx, and Com-GEx treatments (as Sr^{2+} was added when plants existed) as shown in Table 2.10, reinforcing the idea of Sr accumulation by *T. pratense.*

Table 2.10 Mean values (±S.E.) of Sr concentration in the aboveground and underground tissues of *F. rubra* and *T. pratense* and their translocation factors (TF) [MQL=0.0001 mg.L^{-1}].

Treated Soil	Conc. $SrCl_2$ (mM)	Sr concentrations (g.kg^{-1}) aboveground (shoot part)		underground (root part)		TF
F. rubra						
Pre-GEx	0	0.024^{c*}	±0.005	0.028^{e*}	±0.003	1.28ac
	5	0.123^{c*}	±0.027	0.066^{d*}	±0.009	1.80ac
	10	0.187^{c*}	±0.020	0.112^{c*}	±0.006	1.66ac
	20	0.449^{b*}	±0.070	0.182^{b*}	±0.015	2.55ac
	40	1.08^{a*}	±0.132	0.376^{a*}	±0.009	2.87ac
Post-	0	0.024^{c*}	±0.005	0.028^{c*}	±0.003	1.28ac
GEx	5	0.049^{c*}	±0.008	0.055^{b,c*}	±0.002	0.89ex
	10	0.057^{c*}	±0.003	0.089^{b*}	±0.005	0.64ex
	20	0.126^{b*}	±0.012	0.121^{b*}	±0.008	1.08ac
	40	0.944^{a*}	±0.031	0.275^{a*}	±0.043	3.68ac
Com-	0	0.024^{c*}	±0.005	0.028^{c*}	±0.003	1.28ac
GEx	5	0.432^{c*}	±0.048	0.278^{b,c*}	±0.004	1.55ac
	10	1.05^{b,c*}	±0.059	0.630^{b,c*}	±0.082	1.78ac
	20	2.61^{b*}	±0.521	0.963^{b*}	±0.113	2.79ac
	40	12.0^{a*}	±1.77	4.00^{a*}	±0.568	2.99ac
T. pratense						
Pre-GEx	0	0.036^{d*}	±0.003	0.012^{c*}	±0.002	2.65ac*
	5	0.506^{c,d*}	±0.038	0.065^{b,c*}	±0.001	7.81ac*
	10	0.930^{c*}	±0.148	0.087^{b,c*}	±0.009	11.2ac*
	20	1.94^{b*}	±0.086	0.148^{b*}	±0.021	14.0ac*
	40	6.05^{a*}	±0.513	0.281^{a*}	±0.059	24.6ac*
Post-	0	0.036^{b*}	±0.003	0.012^{c*}	±0.002	2.65ac*
GEx	5	0.265^{b*}	±0.022	0.037^{b,c*}	±0.002	7.04ac*
	10	0.530^{b*}	±0.032	0.063^{a,b,c*}	±0.003	8.58ac*
	20	1.00^{b*}	±0.044	0.101^{a,b*}	±0.003	9.99ac*
	40	2.99^{a*}	±1.06	0.120^{a*}	±0.044	17.0ac*
Com-	0	0.036^{d*}	±0.003	0.012^{d*}	±0.002	2.65ac*
GEx	5	2.08^{c,d*}	±0.067	0.213^{c*}	±0.016	10.0ac*
	10	8.72^{b,c*}	±1.38	0.356^{b*}	±0.019	24.1ac*
	20	11.3^{a,b*}	±0.84	0.660^{a*}	±0.058	17.5ac*
	40	17.4^{a*}	±5.31	0.726^{a*}	±0.036	25.3ac*

*Mean values (Samples n=3, Control n=9), denoted by a different letter based on LSD post-hoc test similarities, Pre-GEx=pre-germination exposure, Post-GEx=post-germination exposure, and Com-GEx=combined-germination exposure, * = statistically different at p≤0.05 (one-way ANOVA), ac= accumulator, ex = excluder*

2.6 Conclusions

An investigation of the laboratory seed test showed that the germination percentage of seedlings was significantly affected by differences in concentrations of chemical solutions. *F. rubra* showed the ability to germinate quickly at low concentrations of both calcium and strontium ions; similarly *T. pratense* was more sensitive at high concentrations. Both species, particularly, *T. pratense,* were unfavorably affected at the highest concentration (40 mM) of both $CaCl_2$ and $SrCl_2$, and there was a significant negative correlation between plant growth and concentrations of solutions; suggesting that the plants are affected by the increased salinity.

However, during the pre-test of soil, it was determined to be acidic and sodic. Also, this study found that Sr ions prefer to associate within residual and carbonate fractions, rather than other (more tightly bound) fractions in this soil. Moreover, from results of the batch sorption experiment, the distribution depended on the strontium aqueous concentration in this studied soil. The best efficiency of retention time for adsorption was >24h. Kinetic data of adsorption were employed to fit with both of the Langmuir and Freundlich equilibria models.

Hence, after soil testing with sequential extractions, additional Sr bounded mostly within the exchangeable fraction. It is possible, therefore, that the strontium bound easily (but loosely) with the soils. Interestingly, this study revealed that $SrCl_2$ solution, especially, at 40 mM, contributed to a high level of Sr in the soils and ultimately with greater accumulations in the plants. In particular, elevated concentrations in the upper parts of *T. pratense* suggest a greater translocation factor (TF) of Sr ions than *F. rubra*. Therefore, these plants can be considered as accumulator species. However, *F. rubra* was also suggest that the plants may help with their chlorophyll content adjustments (being impacted differently) to added

concentrations of strontium in the soils. It facilitates plant growth even in environmentally stressful conditions.

Further research may include whether to investigate plant germination using water-culture systems (e.g., hydroponics) in order to avoid interference with characteristics of soil structure, as there are many additional factors to consider about soils to understand bioavailability and Sr dosages. This procedure will focus on the specific reactions of plants to metal ions (e.g., strontium) in wastewaters contamination. It may enhance the efficiency to determine plant-related effects, and provide an accurate capacity of plants to directly accumulate elemental contaminants using the phytoremediation technique.

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CHAPTER 3

COMPARING TOXICITIES OF PRODUCED WATERS WITH SOLUTIONS OF SIMILAR SALINITY: THE GROWTH OF RED FESCUE AND RED CLOVER PLANTS

3.1 Preface

This chapter is part of a paper that will be submitted for publication in *Environmental Geochemistry and Health*. From previous chapters, significant interference in the soil structure was found, and this could affect the results. Basically, the interaction between salinity and soil are complex and could impact interpretations; it was decided to try an alternate method. Using hydroponic culture systems, I investigated the activity of plant mechanisms without the soil-related effects. With this, I examined produced waters (PWs) from an oil industry in an effort to gain insights into using plants for mitigating its salinity. The same plant species used in previous chapters were employed.

The main author, P. Srikhumsuk, was responsible for experimental design and practice, data collection and analysis, and writing the paper. Dr Peshkur trained and assisted me in the analysis and preparation of both standard solutions and ICP-OES equipment. Dr Knapp, via a consultancy contract, provided industrial samples for the experiment, while Dr Renshaw provided expertise of trace elements. The aforementioned mentors also were project supervisors; they provided advice on experimental ideas and design, data analysis, and editorial comments regarding the preparation of the manuscript.

3.2 Summary

Produced waters (PWs) from the natural oil industry were investigated for their potential toxicity to the environment, particularly in regards to chemical composition and salinity. The purpose of this study was to investigate whether their acute toxicity to plants is any greater than its exposure to solutions of similar salinity, i.e., determine whether the waters contain any substances other than dissolved salts that may be toxic downstream. For eight weeks, I compared the growth and tolerance of *Festuca rubra* L. (red fescue) and *Trifolium pratense*

L. (red clover) in hydroponic dilutions of wastewaters and synthetic solutions of similar salinities.

Higher salinities were innately toxic, although red fescue exhibited better growth responses than red clover, which was generally more sensitive. Interestingly, when comparing solutions (at 1:100 dilution), the biomass of red fescue was greater in industrial wastewater in than the comparable brine solution. Although salinity limited plant growth, the industrial wastewaters contained substances that may have aided plant survival and salt-tolerance. Therefore, red fescue grew under salts stress, and even exhibited salt crystals on its leaves. Hence, plant uptake, under certain conditions in this case, may be promoted as a treatment for higher salt concentrations.

3.3 Introduction

A current challenge in the petroleum industry is the environmentally-acceptable handling of flow-back wastewaters, also known as produced waters, which contain (among water additives). Basically, flow-back is fluid that returns to the surface after gas production (Ziemkiewicz and Thomas He, 2015). These waters contain substances sequestered from the subsurface (depending on geologic conditions and residence times) and involve the dissolution of naturally occurring mineral salts such as sodium, calcium, and magnesium during the tertiary oil and gas recovery efforts (Greenberg et al., 2007, Bakke et al., 2013); these constituents often have the greatest impact on water quality (Ayers and Westcot, 1985). Flow-back or produced waters contain additional complex toxic inorganic and organic compounds (Bakke et al., 2013, Jiménez et al., 2018), including chemicals added to maintain oil production: formaldehyde, ammonium bisulfates, biocides, methanol and hydrochloric acid (Veil et al., 2004, Kargbo et al., 2010, Venkatesan and Wankat, 2017). Further, they acquire compounds from the petroleum (Fakhru'l-Razi et al., 2009, Camus et al., 2015):

hydrocarbon compounds ranging from alkanes to aromatics (including those with nitrogen, oxygen and sulfur side-groups),

The compositions of chemical products used, operated by steam injection (Venkatesan and Wankat, 2017) for oil and gas development, often remain confidential (Colborn et al., 2011). The chemical components of produced waters have been reported to have detrimental impacts both on people's health and the environment; for example, radioactive radium, often found in wastewaters, is carcinogenic and can have chronic adverse effects on human health (e.g., eyes, intestines, lungs, liver, skin, etc. (Kargbo et al. (2010)). Colborn et al. (2011) and Saunders et al. (2018) have asserted that the toxic chemicals used in oil and gas operations account for 25% of cancers and mutations, 37% of defects in the endocrine system, 40-50% of diseases of the brain, kidney, and immune and cardiovascular system, and over 75% of harmful effects on the eyes, skin, and respiratory systems. Saunders et al. (2018) have revealed, in a toxicological assessment that increased chloride (Cl^-) concentrations in flow-back wastewater contribute to total suspended solids (TSS) downstream.

The question whether this is accountable for these environmental and human health problems is under debate; however, these issues are increasingly being addressed. Several natural oil and gas companies are attempting to manage and dispose of wastewater employing high-cost wastewater treatment systems. The use of plants for clean-up of wastewater is well-accepted as a green- or gentle-remediation modality because of its cost-effectiveness and environmentally friendly nature (Stoltz and Greger, 2002, Schröder et al., 2007). Further, a number of studies have investigated the use of hydroponic systems for remediation (Kurth et al., 1986, Ab-Shukor et al., 1988, Zavoda et al., 2001, Stoltz and Greger, 2002, Trajkova et al., 2006, Wu et al., 2008, López-Chuken, 2012). Phytoremediation has the potential to remove of heavy metals, trace elements, inorganic and organic compounds, and

salinity from wastewater (Bañuelos, 2006, Ji et al., 2007, Schröder et al., 2007, Gurska et al., 2009, Khan et al., 2009, Neto and Costa, 2009, Yeh et al., 2009, Sood et al., 2012).

Several studies have been carried out, mainly to investigate the toxicity of produced water from oil fields, such as its physicochemical characteristics. It has been found that produced water from oil and gas fields can present varying characteristics from well to well, and these can also change over the lifetime of a well (Çakmakce et al., 2008, Bakke et al., 2013). For this reason, I sought to toxicologically compare the impact of wastewaters at different dilutions, simulating inadvertent discharge levels to the environment, that is, when they become diluted. Further, I wanted to determine whether it would be possible to assess effluent toxicity without detailed, expensive chemical analysis. As such, a comparison trial was developed to experimentally investigate this with salinity as a covariate.

Two common plant species were selected as potential phytoremediation indicators: *Festuca* spp. (Zavoda et al., 2001, Zhang et al., 2008, Zhang et al., 2013) and *Trifolium* spp. (Ab-Shukor et al., 1988, Zhang et al., 2008), which are two species that are capable of growing rapidly in soluble salts. Red fescue is the second most abundant grass species, and as Marcum (1999) reported, creeping fescues are broadly tolerant of salinity stress, although creeping-red fescues have lower tolerance. The other species is known as red clover, or meadow clover, which is widely cultivated globally for agricultural animal feed (Bowley et al., 1984). Both have been previously used to assess total-petroleum-hydrocarbon (TPH) phytoremediation (Kaimi et al., 2007).

3.4 Materials and methods

3.4.1 Plant materials

Two plant species *F. rubra* L. (red fescue) and *T. pratense* L. (red clover) were used for this study. All seeds were purchased from OMC seeds® (Cartagena, Spain) and Sow Seeds® (South Cave, United Kingdom), as they are capable of rapid growth in all seasons.

3.4.2 Hydroponic culture and data collection

A hydroponic system was selected for the experiment. Plant growth without soil has been used as an alternative research methodology to reduce the influence of soil (Marchiol et al., 1999, Alshammary et al., 2004), which was explored in the previous chapter.

3.4.2.1 Plant germination and screening

Eighty seeds of each species were germinated in a tray [36 cm (length) x 23 cm (wide) x 4 cm (deep)] with water on a waterlogged sponge [2.5 cm (length) x 2.5 cm (wide) x 2 cm (deep)]. Once germinated, they were grown for two weeks. *T. pratense* germinated in 1-2 days, while *F. rubra* emerged after one week. In total, six representative seedlings of each species were randomly selected and used for each set of experimental treatments.

3.4.2.2 Preparation of experimental solutions

Wastewater samples were provided by a consultancy as collected samples from storage lagoons in Ontario. The waters (produced and flow-back) have been used to tertiarily extract natural gas from the underground, and have, overtime, become very saline (brine). The identity of the client, and the sample train have been purposely omitted per company request.

Experiments compared the plants' growth in serial dilutions of different solutions: wastewater sample ('WS') from a produced water storage pond belonging to the petroleum industry, and a synthetic solution of similar salinity ('SS'), which intended to contain dissolved NaCl and $CaCl_2$ to match sodium and calcium concentrations (principal cations) of WS, but

ended up with ten times higher calcium concentration of artificially salinized media. Chemical characteristics of the undiluted stock solutions were analysed (Table 3.1), and five levels of serially dilution were experimentally examined: 1% (WS-1 and SS-1), 0.1% (WS-2 and SS-2), 0.01% (WS-3 and SS-3), 0.001% (WS-4 and SS-4), and 0.0001% (WS-5 and SS-5). At the same time, a control (deionized water) test was carried out in a similar manner to that of the experimental treatment. Each treatment was one litre in volume, and all treatments received 5 ml of macro- and micro-nutrients (Table 3.2), adapted from Hoagland and Arnon (1950) nutrient solution.

Table 3.1 Chemical characteristics of original industrial wastewater samples (WS) and synthetic saline solutions (SS) in this experiment (before nutrient supplementation), compared with permissible limits for Drinking Water Directive [98/83/EC] (Environmental Protection Agency, 2001).

Parameters	Values		Standard of water quality acceptability
	WS	SS	Drinking Water Directive [98/83/EC]
pH	7.3	5.6	6.5≤ pH ≤9.5
EC (Scm^{-1})	0.3	0.2	0.0025
Cl^{-} ($g\ L^{-1}$)	110	410	0.25
SO^{2-}_{4} ($g\ L^{-1}$)	2		0.25
Ca^{2+} ($g\ L^{-1}$)	1.4	14	None available
Mg^{2+} ($g\ L^{-1}$)	0.07		None available
Na^{+} ($g\ L^{-1}$)	79	79	0.2
K^{+} ($g\ L^{-1}$)	0.05		None available
Sodium Adsorption Ratio	550		
Hardness ($g\ CaCO_3\ L^{-1}$)	3.8		>0.35
TDS, Calculated ($g\ L^{-1}$)	270		None available

Table 3.2 Nutrient stock solutions to be used for this experiment (adapted from Hoagland and Arnon (1950)).

Ingredients of nutrients	Conc. of solution used (g L^{-1})
Macro-nutrients	
KH_2PO_4	136
KNO_3	101
$Ca(NO_3)_2.4H_2O$	236
$MgSO_4.7H_2O$	246
Micro-nutrients	
Boron (B)	0.5
Copper (Cu)	1.25
Iron (Fe)	16.5
Magnesium (MgO)	21
Manganese (Mn)	15
Molybdenum (Mo)	0.025
Zine (Zn)	5

3.4.2.3 Experimental growth conditions

Experiments were conducted in round plastic garden pots approximately 22 cm in diameter and 22.5 cm tall, holding one litre of solution. Foam discs (with 2-cm diameter holes) were placed on top of each flowerpot to support sponges with plant seedlings (Figure 3.1(a)). Three representative seedlings of each species were transplanted to each hydroponic culture. Each seedling (with sponge cube) was inserted into a foam disc soaked in the appropriate wastewater or saline solution (Figure 3.1(b)). For each set of conditions, there were two hydroponic cultures. Experiments involved factorial design: wastewater vs. synthetic brine solution, each serially diluted, with completely random sampling of plants. An aeration pump was used to provide oxygen in the root zone. Temperature was controlled at 19-21°C and lights provided luminous intensity of 9,900 lux using white fluorescent tubes

(Sylvania GRO-LUX F58W/GRO, Germany) at 16/8 h cycles in the indoor ecotoxicology facility at the University of Strathclyde, Glasgow, UK (16 June to 11 August, 2017) (Figure 3.1(c)). The pots were topped up with deionized water daily to maintain volume and replace evaporative losses, and the flowerpots were rotated weekly under the lights. The plants were incubated for 8 weeks.

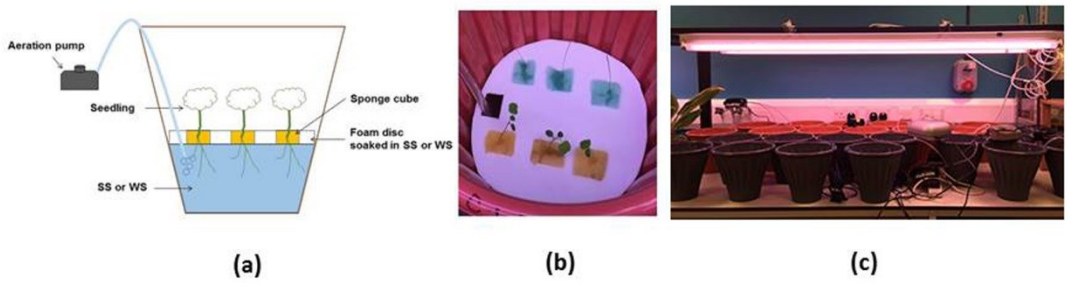

Figure 3.1 Hydroponic culture setup **(a)** diagram, **(b)** plant soaked in SS or WS solutions, and **(c)** incubation of plant growth.

3.4.2.4 Data collection

Determination of physicochemical analysis

EC and pH of the wastewater and saline solutions in each pot were measured before the seedlings were transferred (pre-treatment) and after they have been grown for 8 weeks and harvested (post-treatment), using METTLER TOLEDO SevenMulti^{TM} and VWR pH100 probes, respectively. Measurements were carried out in accordance with the international standard for water analysis (ISO 5667) (Madrid and Zayas, 2007).

Determination of chemical content

Samples of wastewater and saline solutions, following the experiment, were collected and preserved with concentrated HNO_3 (1-2 drops) (BS, 1993). The samples were then filtered

with Millipore membrane 0.45 µm (Millex® syringe driven filter unit). Trace element contents including Ca, Cu, Fe, K, Mg, Mn, Mo, Na, P, Sr, and Zn were measured by ICP-OES (Thermo Scientific iCAP 6000 series ICP spectrometer). Minimum quantification levels (MQL) represents the lowest concentration (Appendix C) where a concentration can be reported with adequate confidence (2000), and this is based on the lowest concentration used on the standard curve for the instrument.

Plant sampling

Root and shoot lengths of each plant were measured, along with plant biomass. Shoot extensions were monitored weekly, whilst root length and biomass were determined after plants (n=6) were harvested at the end of the experiment (8 weeks).

After harvesting at the conclusion of the experiment, plants were washed using deionized water and gently dried. The longest root of each plantlet was measured, and root and shoot weights were determined – both in terms of fresh and dry weights. For dry weight, biomass was dried at 80 °C for 48h (Alshammary et al., 2004). The estimated yields were used equation by Bell and Fischer (1994) as follows (eq. 3.1). The relative growth rate (RGR) was calculated from the shoot height measurements using the following equation (eq. 3.2):

$$\text{Yield}\left(\frac{kg}{ha}\right) = \text{Yield}\left(\frac{g}{m^2}\right) * 10 \quad (3.1)$$

$$\text{RGR} = \frac{[ln(X_2) - ln(X_1)]}{(t_2 - t_1)} \quad (3.2)$$

Where X_1 and t_1 are total shoot height at initial time, and X_2 and t_2 are the same parameters at final plant growth (Bernstein, 1975, Hoffmann and Poorter, 2002).

3.4.3 Statistical analysis

Minitab® 17 software was used for statistical analysis. ANOVA (analysis of variance) was used to determine differences among treatment groups, with LSD post-hoc test to determine significantly different treatment groups. Paired *t*-test was also used to compare solutions at each concentration. Mean values with standard errors (S.E.) were presented, and probabilities (*p*-values) of less than 5% were considered significant. The graphs were illustrated using Origin®2017 Graphing & Analysis.

3.5 Results and discussion

3.5.1 Physicochemical characteristics

Electrical conductivity (EC) of water samples in almost all serial dilutions increased, with post-treatment mean values being significantly greater than the pre-treatment ones ($p<0.05$; Figure 3.2). In contrast, at 0.1% concentration, both SS and WS solutions showed EC values, which were statistically significantly lower following treatment with the plants. Interestingly, at the highest concentration of 1%WS, there was no significant difference between the mean values over the course of the experiment (comparing pre-treatment and post-treatment). Two *t*-tests were performed to compare SS and WS concentrations, and SS had significantly greater EC values than WS at 0.0001% ($t = 22.83$, $p = 0.028$), 0.001% ($t = 46.5$, $p = 0.014$), and 0.01% ($t = 20.61$, $p = 0.031$) when comparing dilutions at pre-treatment ($p<0.05$). However, at post-treatment, EC values for SS became slightly (but significantly) lower than WS at 0.0001% dilutions ($t = -26.79$, $p = 0.024$). These results suggest that the EC values were significantly different between SS and WS concentrations. It seems that the growth of these plants were impacted differently depending on the percentage of salinity concentration, despite the plants having resistance to salt stress, as reported previously.

Generally, pH values were slightly higher post-treatment when compared to pre-treatment for all solution concentrations (Figure 3.2). A significantly greater increase in pH post-treatment were observed (p<0.05) for WS at 0.0001% and 1%. The pH in SS solutions increased for 0.1% and 1% dilutions. By comparison, with two *t*–test, SS and WS solutions did not have significantly different pH values.

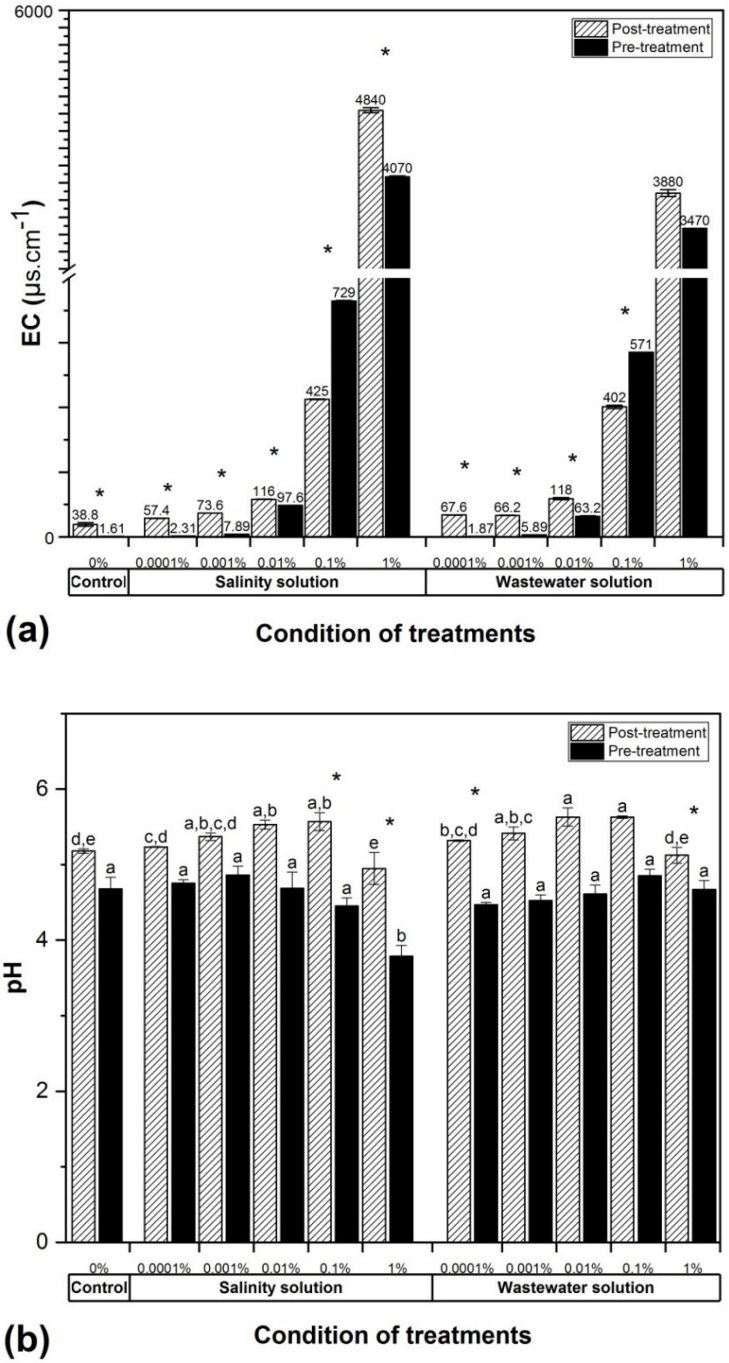

Figure 3.2 Mean value (and S.E.) of **(a)** EC, and **(b)** pH among different concentrations of salinity (SS) and wastewater solutions (WS), which were measured before- and after treatment of eight weeks.

3.5.2 Composition of wastewater and similar salinity solutions

Table 3.3 compares experimental data regarding the elemental composition of water samples pre- and post-treatment and also their nutrient composition. The results obtained from the analysis of the 1% concentration SS and WS solutions suggest that the concentration of individual elements in the samples, both pre- and post-treatment, increased in concentrations. The highest increments were observed for Ca^{2+} and Na^{+} ions in both WS and SS solutions. This may be explained by the fact that calcium concentrations measured in the chemical composition of similar salinity solutions were approximately ten times higher than in WS solutions.

Table 3.4 highlights the fact that calcium and sodium concentrations in water samples differed considerably between the analyzed experimental periods. Interestingly, at the 1% concentration threshold in each solution, the ratio of Na^{+} to Ca^{2+} increased significantly ($p<0.05$) after treatment for 8 weeks, as shown in the table as values of difference. Calcium and sodium levels were significantly greater in the SS solution than in the WS. The fact that the solution of 'similar salinity' was higher in calcium than the wastewater could have influenced water conditions.

These findings seem to be consistent with those of Potasznik and Szymczyk (2015), who reported that high occurrence of calcium and mineral levels can significantly affect the carbonate balance, leading to calcium precipitation and deposition in the water. One unanticipated finding was that calcium and sodium also appeared in the control conditions. It is quite possible that some contamination may have occurred in this experiment from dusty concrete experiments in a neighbouring laboratory.

The higher values of calcium may also suggest variations in evaporation and precipitation. But, atmospheric deposition was also found and affected both quantity and quality of trace elements in this hydroponic experiment (Ehret et al., 1990, Potasznik and Szymczyk, 2015).

3.5.3 Plant growth and survival

The percentage of plant survival in each treatment is shown in Table 3.5. Red fescue showed a higher percentage of age-related survival in most treatments; it was only with 1% of SS that percentage of age-related survival decreased significantly. Similarly, percentage of age-related survival of red clover was high for both SS (0.0001-0.1% dilutions) and WS (0.0001-0.01% dilutions). However, reduced survival of red clover was observed in 0.1% concentration of WS, and with 1% concentration of WS and SS; red clover survival was greatly reduced. However, different concentrations of both WS and SS significantly inhibited seedling survival of both red fescue ($F_{(10,61)}$ = 6.73, p<0.001) and red clover ($F_{(10,61)}$ = 21.4, p<0.001). These findings enhance our understanding of previous studies, which noted that high salinity may be a major growth factor for the creeping fescue species and red clover (Marcum, 1999). Moreover, Shannon and Grieve (1998) suggested that salinity concentrations reduce crop yields, and this appears to be a mechanism of plant response — ion toxicity (Kurth et al., 1986, Hu and Schmidhalter, 2005).

Table 3.3 Summary of mean values (±S.E.) performance of trace elements in different sample types of experimental treatment over 8 weeks.

Ele.	MQL (mg L^{-1})	Mac-N in 5 ml (mg L^{-1})	Mic-N in 5 ml (mg L^{-1})	Total Ori.SS 1%	Total Ori.WS 1%	Mean conc. of pre-treatment (mg L^{-1})						Mean conc. of post-treatment (mg L^{-1})					
						Control		SS		WS		Control		SS		WS	
Ca	0.06	1.358	0.0158	114	10	2.9	±0.03	123	±0.993	10.3	±0.182	5.03	±0.073	161	±1.45	16.7	±0.461
Cu	0.001	*<MQL*	0.4738	0.0173	0.0227	0.729	±0.007	0.021	±0.0009	0.034	±0.006	0.418	±0.008	0.515	±0.045	0.449	±0.014
Fe	0.008	*<MQL*	1.105	0.08	*<MQL*	2.22	±0.024	*<MQL*		*<MQL*		0.916	±0.032	0.807	±0.123	0.997	±0.087
K	0.2	1.715	0.0873	3.46	3.56	3.43	±0.026	6.27	±0.155	7.08	±0.376	0.318	±0.039	7.0	±0.117	7.29	±0.134
Mg	0.002	0.4319	1.03	0.02	0.633	5.63	±0.053	*<MQL*		0.716	±0.03	3.02	±0.045	2.72	±0.059	3.65	±0.034
Mn	0.0001	*<MQL*	*<MQL*	0.001	*<MQL*	2.16	±0.02	*<MQL*		0.028	±0.018	1.17	±0.022	1.243	±0.041	1.3	±0.017
Mo	0.0006	*<MQL*	0.0012	0.006	*<MQL*	0.008	±0.0004	*<MQL*		*<MQL*		0.006	±0.0006	*<MQL*		0.007	±0.0004
Na	0.05	0.0455	0.885	750	758	1.34	±0.012	801	±6.36	772	±9.33	2.437	±0.158	1028	±9.29	953	±26.7
P	0.007	0.4309	*<MQL*	0.07	*<MQL*	0.863	±0.01	*<MQL*		*<MQL*		*<MQL*		*<MQL*		*<MQL*	
Sr	0.0001	*<MQL*	0.0002	0.0457	0.188	0.0004	±0.00002	0.047	±0.0005	0.188	±0.002	0.003	±0.00004	0.066	±0.0007	0.229	±0.007
Zn	0.0002	*<MQL*	0.227	0.002	*<MQL*	0.558	±0.005	*<MQL*		*<MQL*		0.334	±0.007	0.731	±0.189	0.336	±0.003

[Ele.= elements, Mac-N= macro-nutrient, Mic-N= micro-nutrient, Ori.= origin, SS= salinity solution, WS= wastewater solution, and <MQL= lower than Method Quantification Limits]

Table 3.4 Values of difference and subtracting out of trace elements (growth media) with a comparison between pre-treatment (before) and post-treatment (after).

Elements	MQL (mg L^{-1})	Values of difference (mg L^{-1})						Subtracting out (mg L^{-1})				*t*-test		*p*-value	
		Control		SS		WS		SS(Pre)	SS(Post)	WS(Pre)	WS(Post)	SS	WS	SS	WS
		(Before-After)		*(Before-After)*		*(Before-After)*		*(Before-control)*	*(After-control)*	*(Before-control)*	*(After-control)*				
Ca	0.06	-2.12	±0.055	-37.7	±2.18	-6.44	± 0.488	120	156	7.40	11.7	-16.6	-8.78	0	0
Cu	0.001	0.312	± 0.007	-0.4942	±0.045	-0.415	±0.010	-0.708	0.097	-0.695	0.031	-15.5	-43.9	0	0
Fe	0.008	1.31	±0.035	*<MQL*		*<MQL*		0	-0.109	0	0.081	0	0	0	0
K	0.2	3.12	±0.030	-0.732	± 0.199	-0.21	± 0.429	2.84	6.68	3.65	6.97	-17.5	-7.73	0	0.001
Mg	0.002	2.61	± 0.026	*<MQL*		-2.94	± 0.041	0	-0.300	-4.91	0.630	0	-177	0	0
Mn	0.0001	0.992	±0.016	*<MQL*		-1.27	± 0.014	0	0.073	-2.13	0.130	-39.7	-105	0	0
Mo	0.0006	0.002	±0.001	*<MQL*		*<MQL*		0	0	0	0	0	0	0	0
Na	0.05	-1.09	± 0.152	-227	±13.7	-181	±27.3	800	1026	771	951	-16.5	-6.55	0	0.001
P	0.007	0.857	± 0.010	*<MQL*		*<MQL*		0	0	0	0	0	0	0	0
Sr	0.0001	-0.003	± 0.0001	-0.0187	±0.001	-0.041	±0.007	0.047	0.063	0.188	0.226	-14.8	-5.42	0	0.003
Zn	0.0002	0.223	±0.008	*<MQL*		*<MQL*		0	0.397	0	0.002	0	-71.1	0	0

[Mean (±S.E.), <MQL= lower than Method Quantification Limits, paired-t test, significant differences (p≤0.05), Pre=pre-treatment, Post=post-treatment, SS=saline solution, WS=wastewater solution]

Table 3.5 Average percentage of plant survivability during 8 weeks in different dilutions of wastewater and salinity solutions.

Conditions	Percentage of survivorship			
	Red fescue		Red clover	
	Mean	S.E.	Mean	S.E.
0%Control	100[a]	±0	100[a]	±0
0.0001%SS-5	100[a]	±0	88[a]	±13
0.0001%WS-5	100[a]	±0	100[a]	±0
0.001%SS-4	100[a]	±0	100[a]	±0
0.001%WS-4	100[a]	±0	100[a]	±0
0.01%SS-3	100[a]	±0	100[a]	±0
0.01%WS-3	100[a]	±0	100[a]	±0
0.1%SS-2	100[a]	±0	88[a]	±13
0.1%WS-2	100[a]	±0	63[b]	±12
1%SS-1	58[b]	±15	29[c]	±2.63
1%WS-1	88[a]	±8.53	29[c]	±2.63

(One-way ANOVA, letters denote significant differences ($p \leq 0.05$) based on LSD post-hoc test, n = 6)

3.5.4 Plant growth responses in hydroponic systems

3.5.4.1 Root and shoot responses

RGR of shoot height

In weeks 4 and 8, I calculated the relative growth rates (RGR) of shoot height, as shown in Figure 3.3. At week 4, the RGR value of red fescue showed a positive result at all treatment levels. The highest RGR was measured with WS at 1% concentration; however, there was no significant difference in the means of RGR in the first four weeks in all serial dilutions of WS and SS solutions ($F_{(10,61)} = 1.2$, $p = 0.31$) when compared with controls. Similarly, by week 8,

RGR of red fescue was still positive in all dilutions of WS and SS solutions. There were no significant differences (all $p>0.05$; two population *t*–test) in each concentration of diluted solutions; although the ANOVA results showed significant differences in some solution types between WS and SS ($F_{(10,61)} = 5.04$, $p<0.001$) compared with deionized water (control) at $p<0.05$. However, as shown by the *t*–test results in Figure 3.3, red fescue had significantly different ($p<0.05$) shoot growth rate in the following solutions: 0%, 0.0001% of SS, 0.001% of SS, 0.01% of SS, and 1% of WS.

In contrast to red fescue, the RGR for red clover was zero or negative with all treatments at four weeks. However, comparable solutions of WS and SS to deionized water (control) were significantly different ($F_{(10,61)} = 2.75$, $p = 0.007$). This suggests that red clover was the more sensitive species, with very little salt-tolerance. Some wilt was observed by week three and mortality occurred at 1% of WS and SS solutions over the duration of experiment. In addition, by week 8, the mean of RGR for red clover was significantly different at the level ($F_{(10,61)} = 3.88$, $p<0.001$). Red clover showed a recovery of growth rate that led to the *t*–test results showing negative values, mostly in WS solution ($p<0.05$). Positive RGR in WS and SS solutions were found in the order 0.001% > 0.1% > 0.0001% of WS and at 0.1% of SS. Although *t*–test indicates that in red clover, there was a significant difference in the mean value of RGR only at 0.001% of SS solution. However, during weeks 4-8, red clover succumbed when salinity exceeded 1% concentration in both effluent and salinity solution (Appendix D).

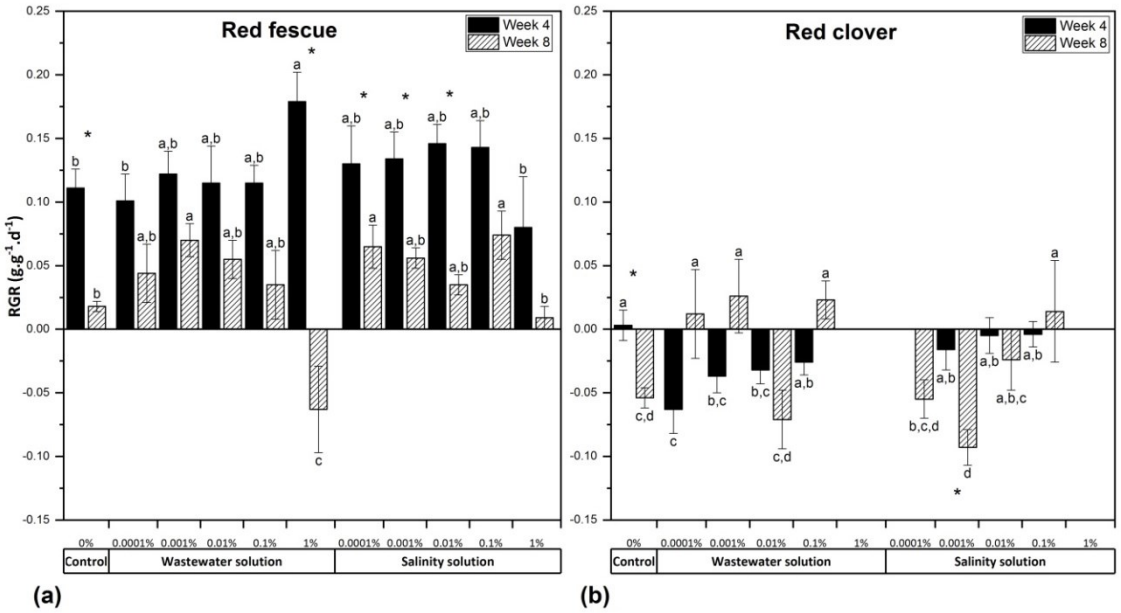

Figure 3.3 Relative growth rate (RGR) of shoot height at 4 weeks and 8 weeks in different dilutions of wastewater and salinity solution **(a)** *F. rubra* and **(b)** *T. pratense*.

Root length and shoot height

Figure 3.4 shows root and shoot responses under different concentrations of WS and SS solutions after 8 weeks. Data from this figure illustrates an increasing trend of root growth of red fescue ($F_{(10,61)}$ = 2.53, p = 0.013). As it can be seen, in SS solution, growth was significantly greatest at 0.1%. On the other hand, at 1% of SS, there was significantly lower root growth compared with effluent solution (WS) (Figure 3.4(a)). Red clover had a similar trend of root growth to that of red fescue. At 0.1% of SS solution, it was significantly greater at the level p<0.05. However, in WS, it was greatest at 0.001% solution.

Red fescue had significant differences in shoot height when treated with <1% concentrations of WS compared with SS ($F_{(10,61)}$ = 4.25, p<0.001) while red clover had no growth responses at 1% concentration of both solutions (Figure 3.4(b)); however, in all

conditional treatments, shoot height of red clover was significantly higher ($F_{(10,61)}$ = 9.07, p<0.001).

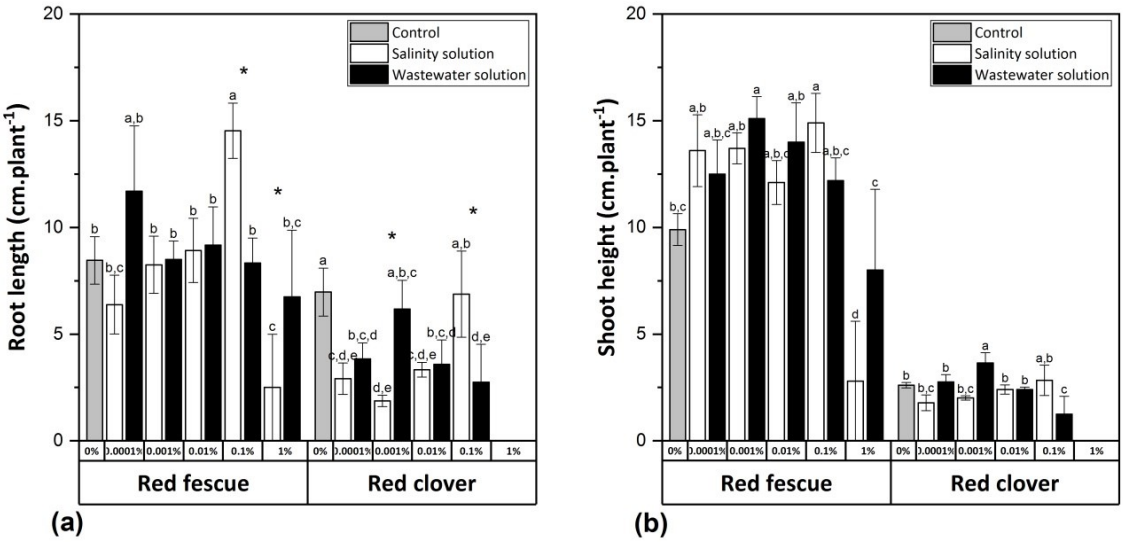

Figure 3.4 Mean values of root and shoot responses **(a)** root length, and **(b)** shoot height after treatment for 8 weeks of red fescue and red clover, in different dilutions of solutions [wastewater (WS), and salinity synthetic solution (SS)]. Errors represent 95% confidence intervals, and different letters indicate significant differences between the mean values of each condition of treatment using one-way ANOVA with LSD post-hoc test.

The results of this investigation showed that with the red clover species, there was significantly less growth in all treatments when compared to 0% control, especially in the roots. This work observed significant plant growth at a high salinity of both solutions with red fescue growing significantly (p<0.05) in both root and shoot. It is somewhat surprising that neither Kurth et al. (1986) nor Hu and Schmidhalter (2005) noted the complexity of the relationship between constituents of trace elements and salinity that affected root growth. It was clear that, in many conditions, there may have been more components in wastewater effluents, even with high salinity, but there were other elements that could have affected

plant responses. As a result, this work's findings seem to be consistent with those of other research which found nutrient imbalance as deleterious effects of ion imbalance at high sodium ions (Ayers and Westcot, 1985, Kurth et al., 1986, Hu and Schmidhalter, 2005).

3.5.4.2 Yields

Weight of the biological yield after being dried is the total dry matter product. This research investigated the percentage of root and shoot dry matter (%DW). Estimated dry matter yield (DM) was then calculated to estimate amount of dry matter per hectare. The results in this experiment indicate that there was a significant difference between the two conditions of WS and SS solutions both in %DW and DM yield. As shown in Figure 3.5 for red fescue, the %DW both in the root ($F_{(10,61)}$ = 5.06, p<0.001), and shoot ($F_{(10,61)}$ = 3.95, p<0.001) were significant at the p≤0.05 level. The results of DM yield of this study (Table 3.6) show that in red fescue, there was no significant difference between any of the populations' means at p<0.05 level in different solutions of WS and SS ($F_{(10,61)}$ = 1.38, p = 0.212). However, in LSD post-hoc test (Table 3.6), with some of the main groups, the greatest actual DM yield was seen in the high sodium ions of WS, e.g. 0.01%, 0.001%, and 1% WS, which produced 98, 97, and 92.4 kg.ha^{-1} respectively. Moreover, the observed increase in red fescue productive DM yields in an excess salt soluble was greatest at 0.1% SS at 94.8 kg.ha^{-1}.

It is concluded that red clover varieties probably do not all produce the same mean of %DW at p≤0.05 level of root ($F_{(10,61)}$ = 13.17, p<0.001) and shoot ($F_{(10,61)}$ = 12.69, p<0.001) (Figure 3.5). Actual DM yields in all conditions (Table 3.6) showed significantly different means ($F_{(10,61)}$ = 4.85, p<0.001). Interestingly, WS solutions produced the highest DM yield in red clover at 0.001%, and 0.01%WS, at 100 kg.ha^{-1}, and 93.4 kg.ha^{-1} respectively. Like red fescue, the salinity solution of 0.1% produced the greatest DM yield (95.6 kg.ha^{-1}); however,

no data was available for red clover at 1% concentration ($p \leq 0.05$), as this sensitive species does not grow at this concentration, as mentioned in the report of Zavoda et al. (2001).

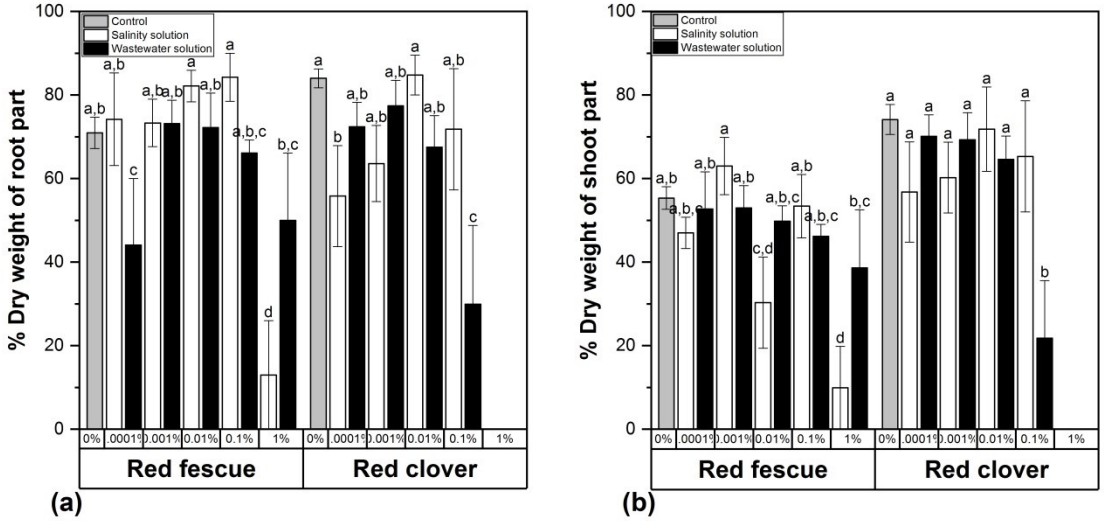

Figure 3.5 Mean values of the percentage of dry weight of **(a)** root and **(b)** shoot after 8 weeks of treatment, showing a comparison between red fescue and red clover in different solution sources and concentrations [wastewater (WS), and saline solution (SS)]. Errors represent 95% confidence intervals, and different letters indicate significant differences between the mean values of each condition of treatment using LSD post-hoc test.

The empirical finding in this study was a clearly perceptible increased actual DM yield of red clover similar to the trend of red fescue, although it had lower productive growth responses. A possible explanation for this is that red fescue may adapt to absorb water with high concentrations of salinity more readily than red clover. As a result, there were totally different fresh weights between the two plant species. On the other hand, when considering the value of DM yield, it was found that red fescue's percentage of dry weight at the highest concentration of EC (1%) of wastewater solution was no higher than that of saline solutions,

and one may suppose that a 1% concentration of salinity index would be too great (Trajkova et al., 2006); however, these results do demonstrate that red fescue has the ability to grow in high salinity concentrations.

Table 3.6 Dry matter (DM) of whole plants yield of load within the field.

Conditions	DM yields (kg.ha^{-1})			
	Red fescue		Red clover	
0%Control	49.3^{a,b}	±9.9	95.5^{a}	±14
0.0001%SS	61.4^{a,b}	±13	44.3^{b,c}	±11
0.0001%WS	81.9^{a,b}	±19	75.5^{a,b}	±19
0.001%SS	76.5^{a,b}	±16	55.6^{a,b}	±18
0.001%WS	97.0^{a}	±20	100^{a}	±14
0.01%SS	69.2^{a,b}	±11	79.7^{a,b}	±15
0.01%WS	98.0^{a}	±18	93.4^{a}	±19
0.1%SS	94.8^{a}	±16	95.6^{a}	±20
0.1%WS	67.0^{a,b}	±8.3	43.3^{b,c}	±28
1%SS	26.2^{b}	±26	-	
1%WS	92.4^{a}	±43.4	-	

(Different letters in each column denote significant differences (p≤0.05) based on LSD post-hoc test; - = plant dead, control (n=12), samples (n=6))

3.6 Conclusions

One of the more significant findings of this experiment was that the number of constituents of effluent (WS) from the oil industry (mostly unknown), which were greater than those in the synthesized salinity (SS) solutions, acted as nutrients for plant growth. Moreover, species-specific different levels of salinity concentrations to induce plant response

were identified. The results of this study indicate that determination of the effect was changes in productivity (including death). The most obvious finding to emerge from this study is that plant species have flexibility and success in adapting to increasing salinity. In particular, this study showed that the enhancement of root and shoot elongation in high salinity is greater in WS than SS solutions.

These hydroponic experiments have shown the efficiency of red fescue and red clover in growing in different solutions (synthetic salinity vs. wastewater) at different dilutions, and reported their responses to salt from petroleum wastewater and synthetic salinity. Our results suggest that red clover is a very sensitive species based on the ranges of plant response (Bernstein, 1975), and the inability to implement a cultivar of red clover at higher salinity solutions. However, the DM yield of this species was greater than *red fescue*, which may be used to enhance biomass productivity to adsorb trace substances. Certainly, not all plants can be used for hydroponic culture because it has been shown that they have different physiological uptake and responses in terms of salt stress. It is clear that red fescue may be a good representative for salt response with other conditions in the future; however, it would be beneficial to test seedling germination over a longer period of time prior to transplanting into waste site or exposing to salt stress.

3.7 Acknowledgements

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CHAPTER 4

SALINITY TOLERANCE TO PRODUCED WATERS BY HALOPHYTE SPECIES: *CYPERUS LONGUS* L. AND *PHRAGMITES AUSTRALIS* (CAV.) TRIN. EX STEUD.

4.1 Preface

This chapter is part of a study that will be submitted for publication in the *Environmental Chemistry Letters*. Previous chapters revealed that plant species *F. rubra* and *T. pratense* did not reduce the salinity of solutions of produced waters from the petroleum industry. This chapter continues the investigation to find some way of removing salinity, focusing on two new plants. The primary author, P. Srikhumsuk, was responsible for experimental design and work, data collection and analyses, and writing the paper. T. Peshkur trained and assisted me in the preparation and analysis of samples and standards for the ICP-OES. Dr. Knapp provided industrial samples for this experiment, and Dr. Renshaw provided expertise in trace elements. They were project supervisors, also providing advice on experimental ideas and design, data analysis, and editorial comments during preparation of this manuscript.

4.2 Summary

Produced waters from oil and gas operations generate highly saline wastewaters, and salt-tolerant mechanisms are often needed for remediation, and halophyte species may represent an environmentally-friendly method for doing this. *Cyperus longus* L. and *Phragmites australis* (Cav.) Trin. Ex Steud. were investigated to determine their ability to grow in tertiary wastewater samples (WS) from the petroleum industry and in synthesised solutions with similar salinities (SS).

Plants were grown in different dilutions of the wastewater: 0% (deionized water, “control”), 0.1%, and 1% concentrations, for both WS and SS solutions. Growth experiments were carried out in pots under laboratory conditions over eight weeks. Plant growth biometrics included root and shoot lengths of the new sprouts, relative growth rates (RGR),

fresh- (FW) and dry-weights (DW), plant length, number of leaves, and chlorophyll-*a* and pheophytin concentrations.

The results showed that *C. longus* had greater survival ability than *P. australis* in both wastewater and synthetic salinity solutions. However, among the plants that survived, there were no significant differences in the total biomass production, RGR, or pigment contents in different concentrations of treatment solutions. Interestingly, the *C. longus* sprouts did not have any toxicity response at the highest level of salinity. Further results suggest that both plant species eliminated excess salinity by excreting salt through their leaves.

4.3 Introduction

The harmful nature of produced waters (PWs) from tertiary wastewater treatment systems of the petroleum industries has been debated, particularly in regards to their salinity. The major characteristics of PWs include soluble salts and potentially hazardous chemicals (Çakmakce et al., 2008, Bakke et al., 2013). Dissolved minerals originate from both introduced chemicals and natural salinity (Veil et al., 2004, Kargbo et al., 2010).

Several researchers have claimed that salinity is the biggest environmental risk during effluent handling and disposal from oil and gas operations (Greenberg et al., 2007, Bakke et al., 2013). Fakhru'l-Razi et al. (2009) hold the view that salt concentration of produced waters from the oil field mainly consists of Na^+ and Cl^- from dissolved mineral compounds, and this is in agreement with the findings of Jacoby (1999), who reported that the same ions were the main contributors of the salinity into the environment. Excessive soluble salts accumulated in the water contribute to its toxicity (Ayers and Westcot, 1985) and also to the degradation of the biosphere, including plant growth (Greenberg et al., 2007).

Research is increasingly attempting to find methods to remediate sites contaminated with PW that maintain effectiveness in high salinity conditions. Interestingly, most have focused

on using high-end technology for the removal of trace elements and organic compounds; however, soluble salts cannot be eliminated in this way (Usman et al., 2013); instead, they tend to become concentrated. The ultimate goals are to achieve environmental responsibility and meet environmental health regulations with social acceptability. One possible solution could be developing natural resources that can help to remediate these pollutants. One alternative method involves using plants for phytoremediation (Stoltz and Greger, 2002, Schröder et al., 2007). A number of studies have explored the properties of various plants to determine their usefulness.

Often, wetlands are used to treat contaminated water (Ayaz and Akça, 2001, Liu et al., 2010), and most commonly submerged or floating aquatics species are preferred, as they are easier to grow and have the ability to survive in different conditions such as in areas extremely contaminated with toxic metals, organic compounds, and salinity. Mostly, these species are monocotyledonous plants belonging to families Poaceae and Cyperaceae. Among such types of useful wetland plants are the halophytes, which are plants that can survive in highly saline environments (Mohammadi and Kardan, 2016).

Being specially adapted to grow in salinity is a requirement of any vegetation that is to treat produced wastewaters. Some researchers have claimed that wetland plant species that re-vegetate from root-to-shoot under low nutrients would be beneficial as a low-cost method without fertilizers (Deng et al., 2004).

Recent interest has included *Phragmites australis* (Cav.) Trin. Ex Steud., also known as common reed, a member of the family Poaceae. It is widely used and has tolerance to heavy metals in wetlands (Ayaz and Akça, 2001, Stoltz and Greger, 2002, Liu et al., 2010), and it has been found to absorb toxic substances (Ennabili et al., 1998, Shardendu et al., 2003, Vymazal and Krőpfelová, 2005, Engloner, 2009, Khan et al., 2009, Kumari and Tripathi, 2015, Rezania et al., 2016, Vymazal, 2016, Vymazal and Březinová, 2016, Gill et al., 2017).

Cyperus longus L., also known as sweet galingale, belongs to the Cyperaceae genus (Larridon et al., 2013). It is grass-like, originating from cool temperate areas, and is particularly productive with high-growth rates and photosynthetic efficiency by virtue of its triangular stem arrangement (Collins and Jones, 1986). Studies of *C. longus* have demonstrated their importance in heavy-metal assessments (Núñez et al., 2011, Alikaj and Brahushi, 2017, Bonanno et al., 2018) and uptake (Núñez et al., 2011, Cordeiro et al., 2016, Alikaj and Brahushi, 2017, Bonanno et al., 2018). However, this species is less commonly used in constructed wetlands than *P. australis*.

While there have been more published studies describing the role of *P. australis* for environmental pollution remediation than *C. longus*, they have tended to focus mostly on the accumulation of heavy metals (Vymazal and Krőpfelová, 2005, Núñez et al., 2011, Březinová and Vymazal, 2014, Kumari and Tripathi, 2015, Alikaj and Brahushi, 2017, Bonanno et al., 2018). Little attention has been paid to the relationship between these plant species and highly saline wastewaters; even more so, fewer researchers have been able to draw on the ability of *C. longus* to grow in saline soils. Some research has been carried out on similar plants within the same family – for example, Maas and Grattan (1999) reported that the growth of corn and wheat were reduced under lower concentrations of salinity. It remains, therefore, a challenge to study contaminated waters with high salinity, and to test the usefulness of these plant species.

The purpose of this study was to evaluate these different species and their ability to grow under the brine conditions of petroleum industry PWs, and to establish whether their suitability for phytoremedition. The primary objective of this study was to investigate these aspects using a water-culture system to determine the efficiency of these plant species to uptake saline ions from PWs and compare their performance with synthetic solutions of similar salinity. Another aim was to develop a simple and rapid floating-culture technique,

without soils, to determine their dose-responses. Therefore, the purpose of this research was to biologically assess the toxicity and treatment feasibility of PWs using an environmentally-friendly and cost-effective method.

4.4 Materials and methods

4.4.1 Hydroponic experiment

4.4.1.1 Plant samples

C. longus and *P. australis* were selected for this experiment, and were purchased from the Honeysome Aquatic Nursery® and Trees by Post®, respectively. The propagations were grown in a wetland microcosm for almost one year in the greenhouse laboratory at the Department of Civil and Environmental Engineering, University of Strathclyde, Glasgow, UK. New juveniles were germinated with deionized water, and cuttings of each new sprout included whole structures, both root and shoot (only green shoots), and were 5-10 cm in length. They were then transferred to study pots.

4.4.1.2 Preparation of wastewater sample and nutrient solutions

Wastewater samples were collected from PW of the petroleum industry (WS), in a similar manner to the wastewater for previous studies (Chapter 3). The synthetic salinity solution (SS) was created based on the composition of the wastewater (Chapter 3) with dissolved NaCl to $CaCl_2$ at ratio 1:10. Both solutions were diluted to two treatment grades: 0.1% and 1% concentration of original stock solutions. In addition, a 0% control (deionized water) was also used as an experimental treatment. The standard nutrients were adapted from those used by Hoagland and Arnon (1950). Each 1*M* of macro-nutrient stock solution, within which was dissolved 13.6 g of KH_2PO_4, 10 g of KNO_3, 23.6 g of $Ca(NO_3)_2.4H_2O$, and 24.6 g of $MgSO_4.7H_2O$ in 100 ml deionized water. The working solution was then made involving volumes of each stock solution: 1, 5, 5, and 2 ml respectively, mixed and filled to 1-litre (nutrient solution *a*).

Another micro-nutrient solution was prepared (nutrient solution *b)* using mixed commercial solid compost (Table 4.1) 10 g dissolved in 1-litre deionized water. Each pot contained 1,000 ml (fixed water level treatment), and 5 ml of additional macro and micro-nutrient solutions were added per pot.

Table 4.1 Constituents of the micronutrient solution and nutrient stock solutions (Hoagland and Arnon, 1950).

Micro-nutrients		**Macro-nutrients**	
Trace nutrients	**Concentration ($g\ L^{-1}$)**	**Ingredients of nutrients**	**Conc. of solution used ($g\ L^{-1}$)**
Boron (B)	0.5	KH_2PO_4	136
Copper (Cu)	1.25	KNO_3	101
Iron (Fe)	16.5	$Ca(NO_3)_2.4H_2O$	236
Magnesium (MgO)	21	$MgSO_4.7H_2O$	246
Manganese (Mn)	15		
Molybdenum (Mo)	0.025		
Zine (Zn)	5		

4.4.1.3 Plant growth conditions

Plastic flowerpots (approx. 22 cm in diameter and 22.5 cm tall) were used for this experiment, and plastic cups (with 2-cm diameter holes) were placed on top of each flowerpot supported by cotton wool with juvenile vegetation (Figure 4.1). There were two holes of 2-cm width to accommodate plant supported with cotton wool. Oxygen was provided by an aeration pump (Figure 4.1(a)). Three juvenile shoots of *C. longus* and *P. australis* (each) were randomly transplanted to soak in one of five different treatments (2

each of WS and SS, and a control). A black plastic bag was used to cover the top of the container to minimise water evaporation. This experiment lasted for 8 weeks from 13 February to 17 April 2018. Fluorescent lights (Sylvania GRO-LUX F58W/GRO, Germany) provided luminous intensity at 9,900 lux for 16/8 hours cycle (day/night), and the temperature was controlled at 21°C ±1.

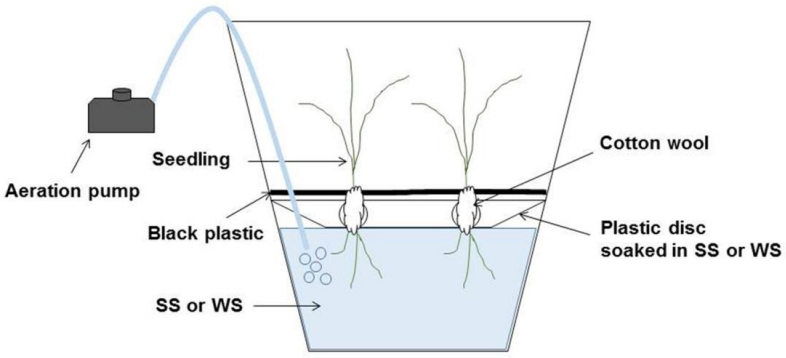

Figure 4.1 Diagram of the experimental setup of hydroponic culture.

4.4.2 Data collection and analysis

4.4.2.1 Plant analysis

(i) Determination of plant growth

After 8 weeks, plants were harvested, and measurement of plant growth was performed based on the values of shoot height, fresh weight, and a number of leaves. From these values, relative growth rates (RGR) were calculated, according to equation (eq. 4.1) for each set of parameters:

$$\mathrm{RGR} = \frac{[\ln(X_2) - \ln(X_1)]}{(t_2 - t_1)} \quad (4.1)$$

where X_1 is either initial shoot height, number of leaves, or fresh weight; X_2 is either the final shoot height, fresh weight, or number of leaves; t_1 and t_2 are the initial and final times of plant growth, respectively (Bernstein, 1975, Lissner and Schierup, 1997).

Additionally, after biomass harvesting was performed, drying was performed at 80 °C for 48 h. The determination of the total sample dry weight, the percentage of dry weight (%DW) and dry matter yield (DM) were measured. Subsampling was used to measure the total sample dry weight (eq. 4.2) and related to the percentage of DW ratio (eq. 4.3). The design of DM measurement in this experiment was also calculated by estimating the circle area (m^2) in the flowerpot (approx. 22 cm in diameter) with formula of πr^2 (eq. 4.4). Criteria for all calculations were made in accordance with those of Bell and Fischer (1994), as shown below:

$$\text{Total sample dry weight } (\text{g. plant}^{-1}) = \text{Total sample fresh weight x } \left(\frac{\text{subsample dry weight}}{\text{subsample fresh weight}}\right) \quad (4.2)$$

$$\text{Percentage of dry weight} = \left(\frac{\text{Dry weight}}{\text{Fresh weight}}\right) \text{x } 100 \quad (4.3)$$

$$\text{DM yield } (\text{kg. m}^{-2}) = \text{amount of biomass weight per area } (\text{kg. m}^{-2}) \text{ x \%DW sample} \quad (4.4)$$

(ii) Determination of chlorophyll and pheophytin concentration

Chlorophyll extractions were carried out with 80% acetone. The leaves were dried at 80 °C for 48 h after which the dried specimens were weighed, ground with a mortar and pestle with 5 ml of 80% acetone solution, transferred into a centrifuge tube, and filled to 10 ml of 80% acetone. The sample solutions were centrifuged twice at 5 °C for 5 minutes at 2500 x g, and 1160 x g respectively (Eppendorf Centrifuge 5804R). Centrates were filtered using Whatman No.1 filters. Chlorophyll and pheophytin concentrations were measured by spectrophotometeric absorbances (Abs) at wavelengths: 664, 665, and 750 nm. Analysis of pheophytin concentration involved chlorophyll acidification adding 1 N HCl (2-3 drops) into the cuvette (Axler and Owen, 1994). The spectrophotometric data were recorded before and

after acidification. The calculations for these pigments were adapted from Lorenzen (1967), as cited in Axler and Owen (1994). Firstly, the required E_{664b} and E_{665a} (corrected absorbance values, for blanks and turbidity) were calculated as equations (eq. 4.5) and (eq. 4.6):

$$E_{664b} = [(Abs_{664b}(\text{sample}) - Abs_{664b}(\text{blank})) - (Abs_{750b}(\text{sample}) - Abs_{750b}(\text{blank}))] \quad (4.5)$$

$$E_{665a} = [(Abs_{665a}(\text{sample}) - Abs_{665a}(\text{blank})) - (Abs_{750a}(\text{sample}) - Abs_{750a}(\text{blank}))] \quad (4.6)$$

Where '*a*' is after acidification, '*b*' is before acidification. Then, the chlorophyll *a* (Chlo *a*) and pheophytin were defined via equations (eq. 4.7) and (eq. 4.8), with: V_{ext} is milliliters of the volume of 80% acetone used in the extraction; M_{sample} is a gram mass of dry weight sample, adapted from Lorenzen (1967) cited in Axler and Owen (1994) as follows:

$$\text{Chlo } a \ (\text{ug. g}^{-1}) = \frac{[26.7 \text{ x } (E_{664b} - E_{665a}) \text{ x } V_{ext}]}{M_{sample}} \quad (4.7)$$

$$\text{Pheophytin } (\text{ug. g}^{-1}) = \frac{[26.7 \text{ x } (1.7 \text{ x } E_{665a} - E_{664b}) \text{ x } V_{ext}]}{M_{sample}} \quad (4.8)$$

4.4.2.2 Water analysis

Measurements of triplicate water-quality samples were carried out for pH, EC, TDS, trace metal and nutrient content according to international standards for water analysis; ISO 5667 (Madrid and Zayas, 2007). Water samples were collected (*n* =3) at the start of the experiment (beginning test; B) and at the end at week 8 (after test; A), after which they were preserved with concentrated HNO_3 (1-2 drops). The Mettler Toledo MPC227™ was used to determine pH, EC, and TDS. Trace element and nutrient content were investigated at the beginning and end of the experiment. These samples were examined using ICP-OES (Thermo Scientific iCAP 6000 series ICP spectrometer). The method quantification limits (MQL) was reported with adequate confidence with the values at correlation coefficients (R^2) >0.9980 (Scientific, 2000) based on internal standard (IS) solutions (Fisher Chemicals). The ICP value content of each trace element was calculated and classified as either "without plant" or "with plant". Also,

dust interference was determined by subtraction of “after treatment” and “before treatment” values.

4.4.3 Statistical analysis

Minitab®17 for Windows was used for statistical analysis. One-way *ANOVA* was used to determine significant differences ($p \leq 0.05$) for different concentrations of solutions for each plant species, after which Fisher’s LSD post-hoc test was performed. Graphs and pictures were produced by Origin® 2017 Graphing and Analysis and Adobe® PhotoshopCS6P respectively.

4.5 Results and discussion

4.5.1 Plant growth responses

Table 4.2 summarises survivorship observed during the eight weeks of plant growth. *C. longus* survived (100%) in all concentration treatments. On the other hand, *P. australis* had 33.3% - 66.6 % survival rates in two salinity treatments (0.1% and 1%) and wastewater (1%) solutions, whilst a 100% mortality rate was found in the number of shoots at 0.1% wastewater treatment levels. In addition, *C. longus* plant, grown in high concentration of 1% of wastewater solution, generated new sprouts. *P. australis* only generated sprouts in controls (0%, deionized water). Terletskaya et al. (2017) pointed out that salts exhibit greater stress on juvenile plants’ roots first.

Table 4.2 Frequency (%) of plant survivorship, new sprout generation, and amount of shoot mortality in each different concentration of different solutions *(n = 3)*.

Conditions	% survivorship		% new sprout		% No. shoot mortality	
	C. longus	*P. australis*	*C. longus*	*P. australis*	*C. longus*	*P. australis*
0% Control	100	66.6	16.6	33.3	0	33.3
0.1% SS	100	33.3	0	0	0	66.6
0.1% WS	100	0	0	0	33.3	100
1% SS	100	33.3	0	0	0	66.6
1% WS	100	66.6	66.6	0	0	33.3

4.5.2 Relative growth rate (RGR)

Table 4.3 demonstrates the results of RGR analyses of *C. longus* and *P. australis*. Between WS and SS solutions at each dilution; there were no significant differences ($p \leq 0.05$). The mean values RGR of fresh weight for *C. longus* illustrated a greater range of variance, and there were significant differences related to solution concentrations ($p \leq 0.05$), but not between WS and SS.

Some results suggested a negative RGR, especially at 1% concentration of either WS or SS solutions for *P. australis*. It can, therefore, be inferred that the plants reacted to toxic levels of salts and reduced their growth during the experiment. In these situations, as a result, growth was stunted and progressively decreased.

Moreover, it was somewhat surprising that no *P. australis* grew at 0.1%WS. However, this was possibly related to the fact that the concentration ratio of Na^+/Ca^{2+} was greater in the WS treatments than in SS (Table 4.6). There is an important relationship between increased concentrations of Ca^{2+} and plant survival; Maas and Grattan (1999) explained that Ca^{2+} can inhibit the toxicity of accumulated levels of Na^+. Therefore, the results at 0.1%WS suggest

that nutritional imbalance and osmotic potential caused by these ions led to the mortality of *P. australis*.

Furthermore, there was an element of experimental error, with saturated cotton wool possibly causing a lack of oxygen. It is important to bear this in mind when interpreting the results, as anoxia can affect conditions (Rengasamy et al., 2003). Rengasamy et al. (2003) point out, anaerobic microbial metabolisms produce toxic substances (e.g. hydrogen sulphide) and could increase the toxicity of soluble salts.

Table 4.3 Mean values of Relative Growth Rates (RGR) of *C. longus* and *P. australis* in terms of shoot height, fresh weight, and number of leaves for each solution treatment.

Conditions	Shoot height		Fresh weight		No. of leaves	
			C. longus			
0% Control	0.032^{a}	±0.037	**-0.01^{c}**	±0.007	0.043^{a}	±0.030
0.1% SS	0.095^{a}	±0.074	0.015^{b,c}	±0.010	0^{a}	±0
0.1% WS	0.104^{a}	±0.054	0.015^{b,c}	±0.013	-0.029^{a}	±0.029
1% SS	0.076^{a}	±0.115	0.049^{a}	±0.007	0^{a}	±0
1% WS	**-0.012^{a}**	±0.082	0.033^{a,b}	±0.008	0.046^{a}	±0.025
			P. australis			
0% Control	**-0.049^{a}**	±0.042	**-0.01^{a,b}**	±0.014	0.03^{a}	±0.018
0.1% SS	0^{a}	±0	**-0.012^{a,b}**	±0.009	0^{a}	±0
0.1% WS	0^{a}	±0	**-0.039^{b}**	±0.013	0^{a}	±0
1% SS	**-0.017^{a}**	±0.017	0.01^{a,b}	±0.023	0^{a}	±0
1% WS	**-0.009^{a}**	±0.033	0.022^{a}	±0.022	0^{a}	±0

[WS: wastewater solution and SS: salinity solution, letters in each column denote groups of similarity, using LSD post-hoc test at p≤0.05 (following One-Way ANOVA), "Bold" represents the values of decreasing response after treatment for eight weeks].

Table 4.4 shows the plant biometrics, including root, rhizome or stem, and leaf biomasses. Data from this table can be compared with the data in Table 4.3, which shows results of the total dry weight of *C. longus* and *P. australis* to make further assessments of plant growth. As can be seen from Figure 4.2, they only showed a significant difference in the percentage of DW leaves ($F_{(4,13)} = 3.36$, $p = 0.043$) in *C. longus*. However, when comparing %DW value in each treatment, both species experienced greater growth in WS solutions than SS solutions.

Ashkan and Jalal (2013) hold the view that both the root and shoot structures play important roles in assessing salinity stress. The root is directly in contact with water and absorbs nutrients for the rest of the plant, leading to the production of shoot lengths affected by wastewater solutions (Table 4.3). However, the most important thing for the plants' growth in high salinities is how the salt exudes from the plants' leaves as the water is transpired. They release salt crystals from the petiole and leaf blade as seen in Figures 4.3(a), and 4.3(b). Bernstein (1975) found this in plants grown slowly with salinity. Moreover, this may possibly lead to stunting and cause leaf burn by the accumulated sodium and chloride (Bernstein, 1975).

Table 4.4 Mean value (±S.E.) of total dry weight (g.plant^{-1}) in each part (root, rhizome or stem, and leaves) of *C. longus* and *P. australis* in different solutions.

Conditions	Total Dry Weight					
	Root		Rhizome/Stem		Leaves	
	Mean	S.E.	Mean	S.E.	Mean	S.E.
			C. longus			
0% Control	0.295[a]	±0.119	0.29[b]	±0.044	0.259[a]	±0.047
0.1% SS	0.456[a]	±0.350	0.384[a,b]	±0.030	0.284[a]	±0.042
0.1% WS	0.287[a]	±0.271	0.297[a,b]	±0.052	0.293[a]	±0.019
1% SS	0.89[a]	±0.334	0.487[a]	±0.066	0.252[a]	±0.089
1% WS	0.439[a]	±0.311	0.436[a,b]	±0.089	0.284[a]	±0.092
			P. australis			
0% Control	0.031[a]	±0.019	0.042[a]	±0.017	0.028[a]	±0.011
0.1% SS	0.039[a]	±0.039	0.016[a]	±0.016	0.001[a]	±0.001
0.1% WS	0		0		0	
1% SS	0.03[a]	±0.030	0.032[a]	±0.032	0.022[a]	±0.022
1% WS	0.022[a]	±0.019	0.035[a]	±0.018	0.007[a]	±0.003

[WS: wastewater solution and SS: salinity solution, letters in each column denote groups of similarity, using LSD post-hoc test at p≤0.05 (following One-Way ANOVA)]

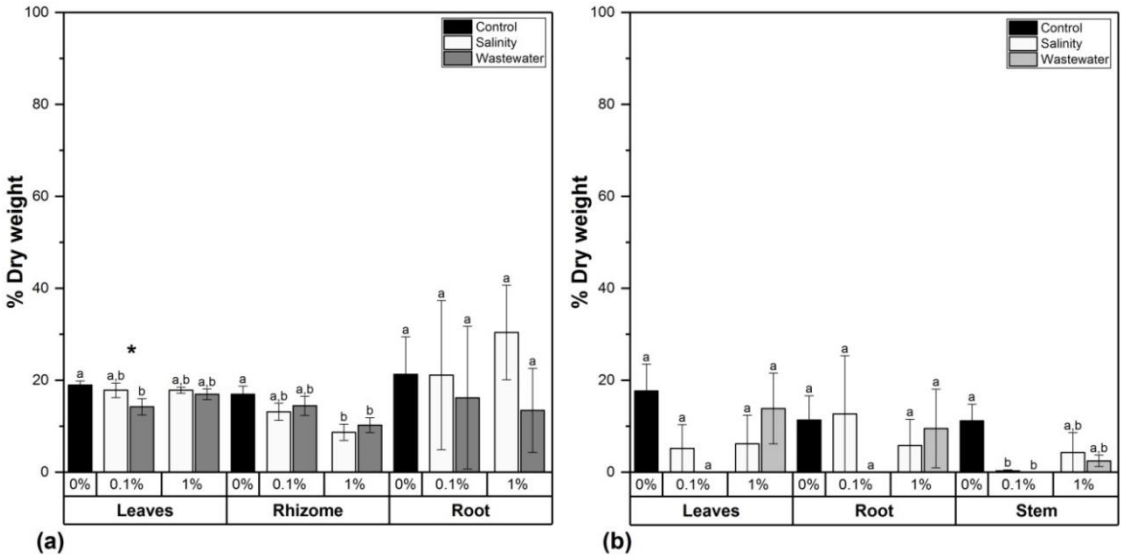

Figure 4.2 The percentage of dry weight in each part of root, rhizome or stem, and leaves; **(a)** *C. longus,* and **(b)** *P. australis* [Mean (±S.E.); the letters in each vertical bar graph denote significant differences among groups using LSD post-hoc].

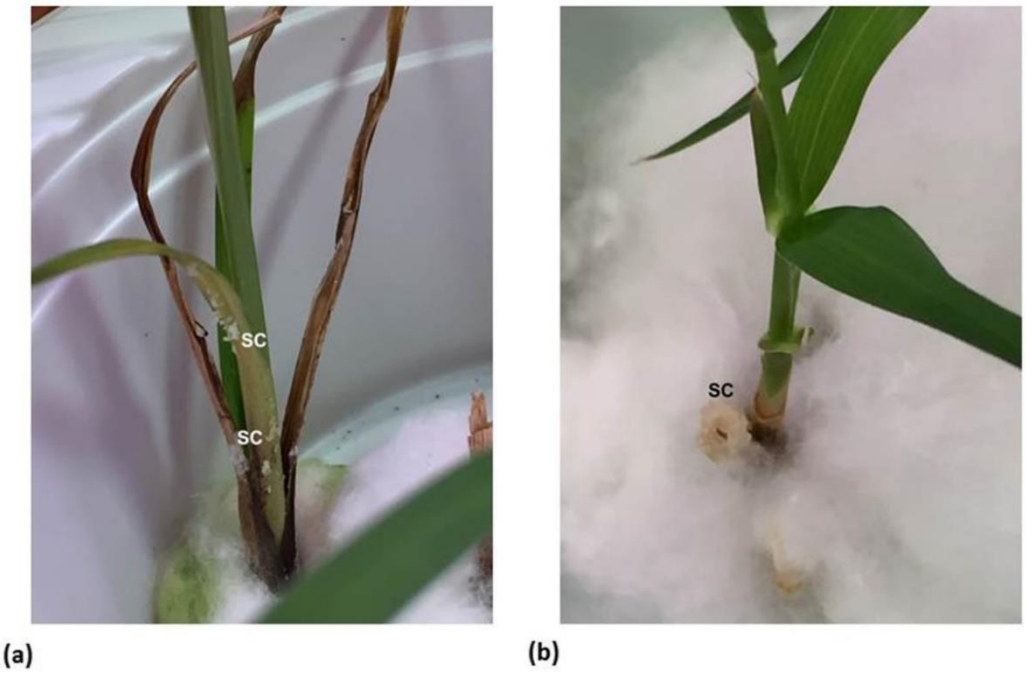

Figure 4.3 Exudes of excess salt crystals (SC) on leaves of; **(a)** *C. longus* at 1% of WS, and **(b)** *P. australis* at 1% of SS.

4.5.3 Dry matter yield (DM)

Figure 4.4 illustrates the total dry matter yield (DM) of the entire *C. longus* ($F_{(4,13)}$ = 0.79, p = 0.55) and *P. australis* ($F_{(4,13)}$ = 0.85, p = 0.52) plant. The results indicate that there were no significant differences (p<0.05) among different concentrations of treatment for each species. Moreover, a comparison of mean values between SS and WS solutions were obtained using the two-sample *t*-test. *C. longus* species was not significantly different at either 0.1% ($t_{(2)}$ = -1.28, p = 0.329) or 1% ($t_{(2)}$ = -0.036, p = 0.975) concentrations. Similarly, *P. australis* species in all concentrations of the *t*-tests were $t_{(2)}$ = -1.000, p = 0.423), and 1% ($t_{(2)}$ = -0.120, p = 0.91), which were 0.1% and 1% respectively.

From the data in Figure 4.4, it is apparent that there were increased yields when concentrations were raised in either WS or SS solution in both species. These findings seem to be consistent with those of Grattan and Grieve (1992), which showed that nutrient deficiency restricted the growth of halophyte species more than high salinity. The results of this study indicate that the different concentrations at 1% salinity level did not affect the salt tolerance threshold of these species. These results differ from those of some previously published studies (Acosta-Motos et al., 2017), where there was consistently reduced DW when under high salt concentrations. Nevertheless, the current study found that the different diluted concentrations did not significantly affect productivity from these growth responses, either in WS or SS.

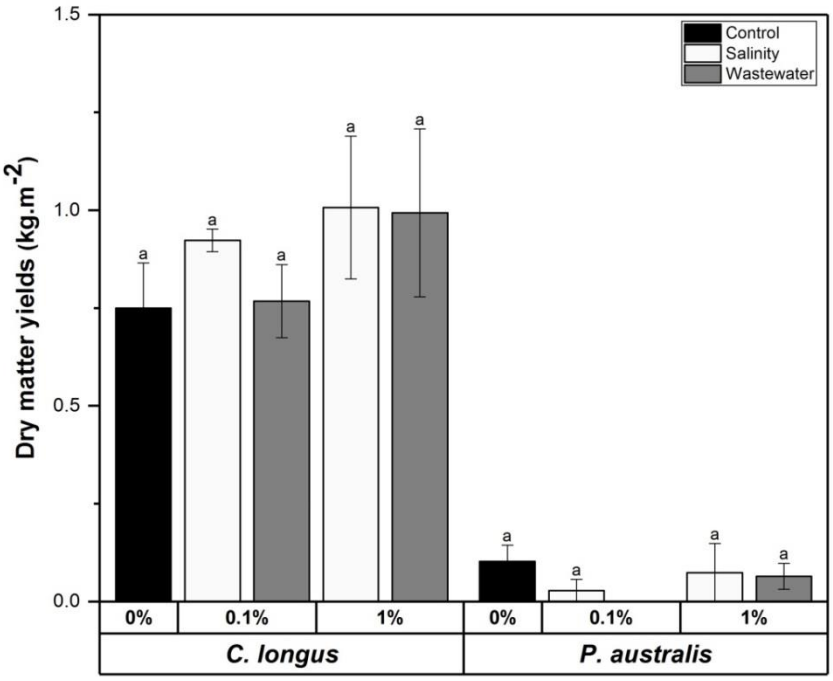

Figure 4.4 Mean values of dry matter yield (kg.m^{-2}) after growth for 8 weeks among the different diluted concentrations of solutions, [the letters in each vertical bar graph denote significant differences among groups using LSD post-hoc].

4.5.4 Chlorophyll and pheophytin concentrations

As shown in Figure 4.5, pigments of *C. longus* were not significantly different for either chlorophyll *a* and pheophytin concentrations, $p>0.05$. Heidari (2012) notes that salinity stress may decrease the chlorophyll content. Salinity was possibly a major cause of reduced chlorophyll content in plants weakly tolerant to increased salt. However, from the results of this study, both *C. longus* and *P. australis* produced more pigment despite their growth in highly saline conditions.

In fact, for this experiment the effect of high salinity on leaves was not significantly different at the $p\leq0.05$ level based on chlorophyll and pheophytin, although slightly increasing trends were observed. Terletskaya et al. (2017) point out that salt-stress

conditions would first have a greater negative effect on the root system and then lead to difficulties in the foliage. Contrary to expectations, this finding is in agreement with Maas and Grattan (1999), demonstrating that increased Na^+ did not have a direct effect on plant growth.

This study has shown that these plants were not immediately affected by Na^+. As a result, there were no significant differences in pigment values in both species, especially, at the highest salinity. However, another important finding was that during increased salinity, plants may accumulate greater concentrations of salt. Estimating pigment can help in evaluating the physical health tolerance of plant tissues (Bernstein, 1975). Therefore, it seems possible that these plants have the ability to protect themselves while growing in high salinity.

Another possible explanation for this is the mechanisms of salt tolerance with the type of "excluder" salts. Figure 4.3(a) and 4.3(b) provide the experimental results on hydroponic culture. This finding supports previous research into the idea of Munns (2002), who suggested that the mechanism known as salt exclusion was greatest in the halophytes species. It is, therefore, likely that these could be effective plant species as salt excluders, which is clearly related with their tissues or cell (e.g. epidermis, xylem), and transpiration stream.

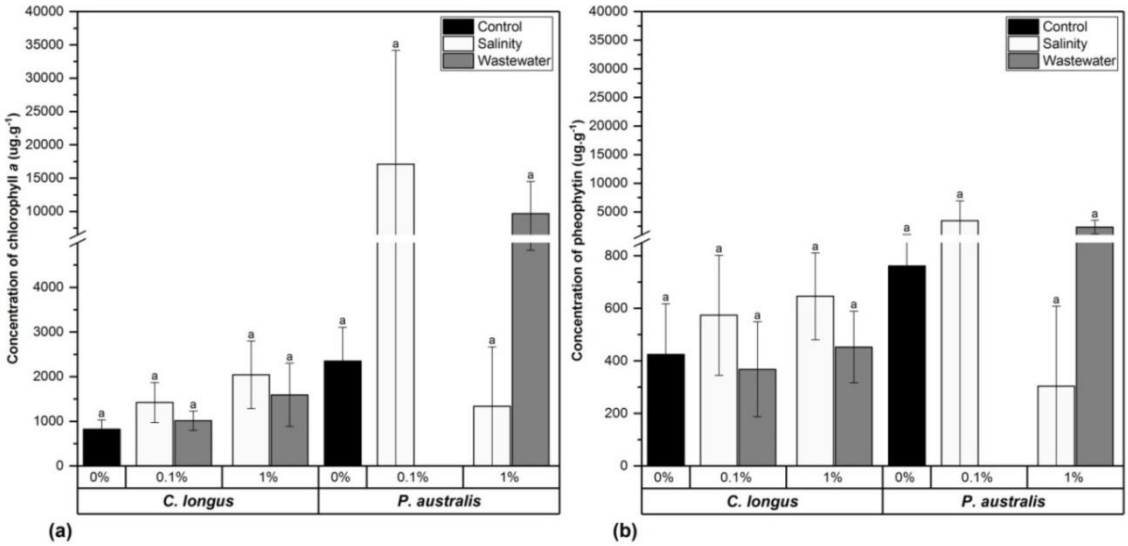

Figure 4.5 Mean values (±S.E.) of pigment concentrations; **(a)** chlorophyll *a*, and **(b)** pheophytin of *C. longus* and *P. australis* after 8 weeks growth among the different diluted concentrations of solutions [the letters in each vertical bar graph denote significant differences among groups using LSD post-hoc].

4.5.5 Physicochemical characteristics

Table 4.5 shows the physico-chemical data of water samples, in which the two sample *t*-test were used to analyse pH, EC, and TDS values between treatments with and without growing plants. pH values were slightly acidic throughout the experiment. There was a dramatic reduction in all conditional treatments ($p \leq 0.05$) at weeks 4 and 8. In contrast, the EC measurement showed greater significant differences ($p \leq 0.05$) among the concentrations in both treatments with and without growing plants. However, at 1% concentration both SS and WS solutions had significantly different ($p \leq 0.05$) EC values in weeks 4 and 8, although after using plant treatment it was slightly increased. TDS showed the greatest values at 1% SS and WS solutions in both treatments.

Values in Table 4.6, are estimates with dust from a nearby concrete lab, which affected experimental and control samples; ion concentrations were subtracted by the amount found in controls. WS solutions were higher than SS solution; this may be from accumulated and excess salt ions from other elements. Data from the table, suggest that treatments without plants had higher values of each trace element. One possible explanation is anoxia (Rengasamy et al., 2003), due to the fact that this experiment had no aeration pump. Oxygen flow significantly improves conditions of the water and plants. Rosenbauer et al. (2005) found that saturated carbon dioxide in water enhanced saline ions, and activities in the roots could produce carbon. Moreover, the composition of macro-or micro-nutrients also lead to increase Na^+ (Grattan and Grieve, 1992).

The data in Table 4.7 indicates that these plant species had a decrease in Ca^+ uptake into plants at 0.1% WS than in SS solution. As mentioned by Grattan and Grieve (1992), increased Na^+ ion into plant tissues may prevent absorption of Ca^{2+} uptake with the vascular system of the root, because of cation exchange or increasing ionic strength. However, these results show that there was no increase in the value of Na^+ and Ca^{2+}, which decreased in 1%WS and SS solutions. In contrast, the data in Table 4.7 illustrates that, in most solutions, there was an increase of Na^+ and Ca^{2+} ratio.

However, the differences between plant treatments (i.e., those with vs. without) are highlighted in Table 4.8. There were greater decreases when using plants for both S solutions in all concentrations. These results suggest that sodium does not directly and immediately affect any plant species when salt concentration remains lower than thresholds (Maas and Grattan, 1999). These studies confirmed the relationship between nutrient and salt concentrations, and that there was no competetition for nutrient balance in either SS or WS solutions. Levels of some elements decreased after testing with plant growth. It is clear from these results that there may be considerable salt tolerance at 1% of WS and SS solutions.

Therefore, these halophyte species may tolerate the extreme ratio of Na^+ and Ca^{2+} without any direct effect.

Table 4.5 Mean value (±S.E.) of pH, EC, and TDS in different concentrations and plant treatment (with plant and without the plant, *n*=3).

Conc.	Plant						Non-plant						*t*-value			*p*-value		
	Pre		Week4		Week8		Pre		Week4		Week8		Pre	Week4	Week8	Pre	Week 4	Week8
pH																		
0% Control	5.36^{a}	±0.03	4.05^{a}	±0.03	4.35^{a}	±0.05	6.07^{a}	±0.4	5.31^{b}	±0.03	5.55^{b}	±0.02	1.77	30.4	22.3	0.219	0*	0.002*
0.1% SS	5.12^{b,c}	±0.03	4.02^{a}	±0.05	4.06^{a,b}	±0.06	5.11^{b}	±0.01	5.05^{c}	±0.02	5.51^{b,c}	±0.03	-0.29	19.5	20.7	0.796	0.003*	0*
0.1% WS	5.22^{a,b}	±0.03	4.03^{a}	±0.09	4.48^{a}	±0.3	5.27^{b}	±0.1	5.13^{c}	±0.01	5.39^{c}	±0.01	0.43	12.6	3.1	0.71	0.006*	0.09
1% SS	5.02^{c}	±0.01	3.69^{b}	±0.04	3.84^{b}	±0.05	5.04^{b}	±0.05	4.69^{d}	±0.07	5.14^{d}	±0.07	0.41	12.4	15.3	0.719	0.006*	0.001*
1% WS	5.26^{a,b}	±0.10	3.95^{a}	±0.14	4.25^{a,b}	±0.12	5.29^{b}	±0.1	5.61^{a}	±0.02	5.89^{a}	±0.03	0.26	11.4	13.7	0.814	0.008*	0.005*
EC (µS cm^{-1})																		
0% Control	75.1^{c}	±2.3	94.7^{c}	±3.1	86.7^{e}	±15	75.1^{c}	±2.3	94.7^{e}	±3.1	86.7^{e}	±15	0	0	0	1	1	1
0.1% SS	469^{b}	±7. 6	488^{b}	±0.6	533^{c}	±0.7	565^{b}	±4.1	903^{c}	±4.6	1092^{c}	±1.9	10.6	89.9	283	0.002*	0*	0*
0.1% WS	475^{b}	±2.9	494^{b}	±7.5	442^{d}	±3.5	568^{b}	±5.8	569^{d}	±24	549^{d}	±32	14.4	2.94	3.3	0.005*	0.099	0.081
1% SS	3170^{a}	±32	3533^{a}	±3.3	3696^{a}	±27	3853^{a}	±64	4220^{b}	±25	4440^{a}	±5.8	9.59	27.1	26.8	0.011*	0.001*	0.001*
1% WS	3187^{a}	±3.3	3647^{a}	±113	3630^{b}	±20	3880^{a}	±36	4607^{a}	±58	3973^{b}	±67	19.2	7.57	4.92	0.003*	0.017*	0.039*
TDS (mg L^{-1})																		
0% Control	37.5^{e}	±0.7	48.8^{d}	±0.2	43.0^{d}	±7.3	37.1^{d}	±1.4	40.3^{e}	±0.1	44.9^{e}	±0.1	-0.28	-38.4	0.26	0.795	0*	0.82
0.1% SS	285^{c}	±3.2	285^{c}	±12	274^{c}	±17	283^{c}	±2.7	453^{c}	±2	548^{c}	±1.3	-0.48	14.0	16.2	0.666	0.005*	0.004*
0.1% WS	238^{d}	±0.9	247^{c}	±3.5	220^{c}	±2.1	234^{c}	±3.3	245^{d}	±1.3	266^{d}	±0.3	-1.16	-0.45	22.0	0.366	0.697	0.002*
1% SS	1927^{a}	±8.8	2307^{a}	±35	1987^{a}	±32	1910^{a}	±29	2090^{a}	±0	2227^{a}	±3.3	-0.55	-	7.51	0.636	-	0.017*
1% WS	1590^{b}	±5.8	1820^{b}	±53	1807^{b}	±13	1580^{b}	±21	1763^{b}	±6.7	1867^{b}	±8.8	-0.46	-1.06	3.75	0.689	0.399	0.033*

*[WS; wastewater solution and SS; salinity solution, different letter in each column denoted significant difference group at the level p≤0.05 with using Fisher's LSD test (one-way ANOVA), * = significant difference p≤0.05 (two samples t-test)]*

Table 4.6 Subtracting out the influence of values by dust between experimental treatments and control.

Parameters	EC	TDS	Ca^{2+}	Cu	Fe	K^{+}	Mg^{2+}	Mn	Mo	Na^{+}	P	Sr	Zn
MQL($mg.L^{-1}$)			0.02	0.001	0.001	0.1	0.0007	0.0002	0.0009	0.05	0.008	0.00003	0.0002
With plant													
0.1% SS	52	-16	-0.889	-0.052	-0.229	-2.604	-0.211	-0.093	-0.002	-0.878	-0.099	0.001	-0.031
0.1% WS	-45	-24	-0.265	-0.086	-0.315	-1.237	-0.403	-0.198	-0.001	-3.145	-0.106	-0.001	-0.058
1% SS	512	55	4.62	-0.061	-0.343	-3.099	-0.320	-0.145	-0.001	34.9	-0.146	0.002	-0.026
1% WS	432	211	2.31	-0.055	-0.156	0.236	0.028	-0.116	-0.005	175	-0.081	0.015	-0.032
Without plant													
0.1%SS-nP	515	257	33.7	0.4	0.92	3.05	2.97	1.12	0.01	165	0.73	0.01	0.35
0.1%WS-nP	-31	24	2.73	0.31	0.8	2.33	2.51	0.92	0	82.9	0.7	0.01	0.29
1%SS-nP	575	309	41	-0.01	-0.191	1.05	0.06	-0.03	*<MQL*	204	0.35	0.01	-0.02
1%WS-nP	81	279	0.97	-0.11	-0.688	0.45	0.1	-0.17	*<MQL*	-13.3	0.24	0.01	-0.08

[Mean values (n=3), <MQL= lower than Method Quantification Limits, nP = no plant growth]

Table 4.7 Values of difference in physicochemical characteristics and trace elements of SS and WS solutions with a comparison between after and before treatments undertaken by plant culture.

Parameters	pH	EC	TDS	Ca^{2+}	Cu	Fe	K^{+}	Mg^{2+}	Mn	Mo	Na^{+}	P	Sr	Zn
0% Control	-1.01^{a}	11.7^{d*}	5.47^{c*}	0.949^{b*}	-0.014^{a}	0.202^{a}	1.34^{a*}	0.345^{a}	0.07^{a}	0.002^{a*}	2.51^{c*}	-0.242^{a}	0.002^{c*}	0.004^{a}
0.1% SS	-1.07^{a}	64^{c*}	-11^{c*}	0.06^{b*}	-0.066^{a}	-0.027^{a}	$-1.26^{b,c*}$	0.135^{a}	-0.023^{a}	$0^{a,b*}$	1.63^{c*}	-0.341^{a}	0.002^{c*}	-0.027^{a}
0.1% WS	-0.75^{a}	-33.3^{d*}	-18.3^{c*}	0.684^{b*}	-0.100^{a}	-0.113^{a}	$0.105^{a,b*}$	-0.058^{a}	-0.128^{a}	0.001^{a*}	-0.63^{c*}	-0.348^{a}	0.001^{d*}	-0.054^{a}
1% SS	-1.18^{a}	523^{a*}	60^{c*}	5.57^{a*}	-0.074^{a}	-0.141^{a}	-1.76^{c*}	0.025^{a}	-0.075^{a}	0.001^{a*}	37.4^{b*}	-0.388^{a}	0.004^{b*}	-0.022^{a}
1% WS	-1.01^{a}	443^{b*}	217^{a*}	3.26^{a*}	-0.068^{a}	0.046^{a}	1.58^{a*}	0.373^{a}	-0.046^{a}	-0.002^{b*}	177^{a*}	-0.323^{a}	0.016^{a*}	-0.028^{a}

[Mean values (n=3), = significant difference by using one-way ANOVA at p<0.05, and different letter in each column denoted significant difference group with using Fisher's LSD test]*

Table 4.8 Values of difference in trace elements between non-plant versus with growing plant.

Conditions	Ca	Cu	Fe	K	Mg	Mn	Mo	Na	P	Sr	Zn
MQL(mg.L^{-1})	0.02	0.001	0.001	0.1	0.0007	0.0002	0.0009	0.05	0.008	0.00003	0.0002
0%Control-B	0.34	0.04	0.12	0.73	0.33	0.14	0	0.81	0.37	0	0.04
0%Control-M	-0.42	0.10	-0.03	-0.61	0.26	0.17	0	-0.59	0.15	0	0.07
0%Control-A	-0.40	0.19	0.16	-0.81	0.82	0.44	0	-2.34	0.29	0	0.16
0.1%SS-B	-15.9	-0.37	-1.08	-1.97	-2.77	-1.08	0	-75.1	-0.39	0	-0.35
0.1%SS-M	-15.2	-0.32	-1.15	-0.77	-2.88	-1.08	0	-71.1	-0.24	0	-0.33
0.1%SS-A	18	0.22	0.11	2.14	0.90	0.44	0	87.8	0.36	0	0.15
0.1%WS-B	-2.46	-0.39	-1.11	-1.84	-2.90	-1.11	0	-69.9	-0.37	-0.01	-0.35
0.1%WS-M	-3.36	-0.34	-1.22	-1.22	-3.11	-1.13	-0.01	-74. 8	-0.09	-0.01	-0.35
0.1%WS-A	-0.21	0.16	0.05	0.19	0.50	0.30	0	12.9	0.35	0	0.11
1%SS-B	-18.6	-0.04	-0.22	-0.95	0.21	0.03	*<MQL*	-189	-0.08	0	0
1%SS-M	-17.8	0.04	-0.32	0.21	0.41	0.17	*<MQL*	-207	*<MQL*	-0.01	0.04
1%SS-A	17	0.15	-0.03	1.67	1.08	0.44	*<MQL*	-23	*<MQL*	0	0.12
1%WS-B	0.54	0.02	0.10	0.93	0.14	0.06	*<MQL*	26.2	0.06	0	0.03
1%WS-M	-0.76	0.08	-0.28	1.49	0.90	0.30	*<MQL*	-119	*<MQL*	0	0.09
1%WS-A	-1.55	0.11	-0.39	-0.40	0.71	0.31	*<MQL*	-165	*<MQL*	0	0.10

[WS; wastewater, SS; salinity, A= after treatment (week 8), B = before treatment, M= median treatment (week 4), <MQL= lower than Method Quantification Limits]

4.6 Conclusions

The aim of this study was to evaluate the feasibility to grow these species under high salinity concentrations of PWs caused by the petroleum industry. The results indicated the plants' ability to accumulate and remove ions from solutions. Interestingly, the two tested plants displayed varied performance throughout this study.

Firstly, they were capable of surviving at the highest concentrations in both WS and SS solutions (1%). Further, these plant species had mechanisms to exude excess salt ('salt exclusion') along their leaves. Secondly, plants responded with higher pigment concentrations and dry matter yields when salinities were raised; however, high activities of photosynthesis were affected by the salinity. Finally, water content extended our knowledge of *C. longus* and *P. australis* suitabilities to grow in the high salinity of PWs effluents. The results for *P. australis* in this study do not support previous research that found it useful in tackling this issue. In this study, it was less efficient than *C. longus*. However, there was no significant difference between results among different concentrations of either WS or SS solutions.

Overall, this study indicated the potential of the two plants to remove salts in PWs, via salt exclusion. Therefore, the hydroponics were well demonstrated and might prove to be useful as a reference in future work.

Further research in this field may also highlight important technical difficulties at the genetic, or molecular, the level in salt tolerance and to hone in on the mechanisms of plants to accumulate salinity ions. Research should focus on reducing time spans.

4.7 Acknowledgments

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CHAPTER 5

CONCLUSIONS

This final chapter concludes the aims and objectives, discusses the key findings, critically reflects on this research, and makes suggestions for future work. This study investigated the dose-response of different plant species and their efficacy to possibly remediate salinity from soil and water due to potentially toxic elements emanating from the oil industry.

5.1 Summary of the aims and objectives

The major aim of this research was to assess salt-stress, which has become a common issue resulting from oil-industry effluent discharges. To do this, I had to determine the physical-chemical properties of contaminated soil and water. From that, I eco-toxicologically examined the dose-responses of two native plants (i.e., *F. rubra* and *T. pratense*) in a phyto-toxicity test, and then expanded to two additional halophytes: *C. longus, and P. australis*. Finally, the plants' ability to sequester elemental ions were investigated. Basically, the objectives were set out for different aspects of study:

(i) Artificially contaminated soil and water were characterised, and their toxicities were investigated at different concentrations. This research considered salinity and potentially toxic elements as important factors.

(ii) Determined how germination responses changed, depending on the frequency and timing of contaminant exposure.

(iii) Much of the experimental design assessed effluent toxicity on plants, examining their biometrics, including: weight and height of plant components, germination exposure, and chlorophyll content.

(iv) The plants were then evaluated for their potential to accumulate toxic elements and salinity from industry effluent.

5.2 Key findings

This study represents three main investigations, each described in a chapter of this thesis. My experiments investigated the viability of experimental plant species (*F. rubra* and *T. pratense*). Mainly, the exposure to excess calcium and strontium ions were the focus. Chapter 2 aimed to examine the characteristics of soil and their impacts on the growth of plants. Also, contaminated water, as hydroponic experiments, was also investigated as seen in Chapter 3 and 4. This study provides an insight into the concept of high salinity impacts (wastewater vs synthesised salinity). The findings revealed three key criteria: physicochemical characteristics, toxicity, and plant performance as described below.

5.2.1 Physicochemical characteristics

Excessive Sr ions affect plant growth; however, many other external factors also have an impact. The evidence from the sequential extractions showed that strontium was mostly distributed in the residual forms for the soils in the study; that is, the characteristics of soil required aqua regia within 24h of adsorbent contact time to obtain the higher levels of strontium. The isotherms adsorption of both Langmuir and Freundlich models were considered suitable, showing the adsorption capacity of the soils to strontium, and were employed for the studied soil. This experiment achieved the objective of this study, which was to determine contaminant fate of strontium in soil.

5.2.2 Toxicity

The toxicological properties of strontium versus calcium elements were studied, and the results showed that growth and germination decreased significantly in both species when strontium concentrations were raised. In excess calcium, there was no significant growth of either plant species.

The experiments further determined the differential impact of timing of the exposure to excess ions—both in terms of plant growth and germination. Obviously, *F. rubra* had greater

tolerances than *T. pratense*. Exposure to contaminants before and immediately after did not affect germination rates and seedlings. However, irrigation at a high concentration (40 mM) had the greatest impact.

Chapter 2 also examined the effect of vegetation growth in contaminated soil, and my experiment revealed that *T. pratense* showed more efficient accumulation of Sr. It is likely that a connection exists between the internal mechanisms and the unique structure in the *T. pratense* roots; i.e., the nodules can assist with the interaction with elements in the soil and may easily induce metal accumulation in their tissues. On the other hand, *F. rubra* displayed the potential to be an excluder species, which restricted Sr content by transferring it through the aerial part of their structure. It is interesting to note that grasses, such as *F. rubra*, show high saline tolerance, and this is in agreement with several previous studies.

5.2.3 Plant species performance

The plants species were exposed to strong salinities to investigate their performance in uptaking metals and salinity. The laboratory results revealed that industrial wastewater might be nutritionally more suitable for enabling growth of *F. rubra* L. (fescue) and *T. pratense* L. (clover) than solutions of similar salinity (with Na^+ and Ca^{2+}). Notably, finding excess salt being excreted from the shoot of *F. rubra* confirmed that it is a tolerant species at the highest contamination concentration. Another significant finding from this study is that most dry matter (DM) yields from both species were higher in effluent waters than in the salinity solutions. The evidence from this study, therefore, suggests that effluent waters contain more nutrients than the synthetic solutions of similar salinity—a promising factor for phytoremediation.

In the final experiment, I investigated wetland species *C. longus* (sweet galingale) and *P. australis* (common reed). Both showed the ability to growing increasing in high salinity solutions of both wastewater and synthetic saline. In spite of this, they showed low efficiency

in uptaking trace elements from the water. These plant species have the ability to exude excess salt crystals in their leaves. This study found that both *C. longus* and *P. australis* were able to grow and produce more chlorophyll and pheophytin under high-salinity conditions.

It may be concluded, therefore, that these plant species (*F. rubra, C. longus, P. australis,* and *T. pratense*) could play a role in removing excess metal and sodium ions. However, high-salt stress directly affects plant tissues, because sodium and chloride ions can obstruct the distribution of nutrients within plant structures. It can also lead to nutrient imbalances (e.g., Ca^{2+}, K^{+}, and Mg^{2+}), which have limited assimilation when compared to Na^{+}. Also, osmotic stress remains one factor in which excess salts damage the internal structures of plant cells.

5.3 Critique of this research

Several aspects of the undertaken work can be criticized. One main factor is the time limitation imposed by vegetation growth. Certainly, the plants were exposed to different concentrations of salinity, and their responses were assessed, providing very important information. The major criticisms might relate to (i) aspects of representative sampling; (ii) the traits of measured selection; (iii) the period of time to study:

(i) Aspects of representative sampling

A concern might be the species representing the phytoremediation method. There are compared to other common technological approaches. Several plants are well known to be ecologically advantageous and have phyto-accumulation abilities. Their beneficial characteristics include fast rate of growth, ability to grow with poor nutrients, and metal tolerance. It is clear that the four representative species demonstrated an efficiency to meet these requirements, especially, *F. rubra*. However, there may be other 'better' plants.

(ii) The traits of measured selection

The morphological measurements were measured, e.g., length, weight, number of leaves, etc. They were related to biomass and size of the part of the plants studied. Obviously, the consideration of such not only illustrated the morphological traits, but also their tolerance mechanisms.

Certainly, further work would require intensive investigations to define any specific function, for instance pigment contents, or their bioavailability within their tissues. There are also genetic and specific physiological activities that could be investigator further understand tolerances and sequestration. The functional classifications as shown in Chapters 2, 3, and 4 are useable for predicting vegetations to adsorb available metal in contaminated soil and water; more can be done to understand their underlying physiological mechanisms.

(iii) The period of time to study.

This issue was raised in Chapter 3, and 4, due to complex structure of vegetation. Such rhizome structure of *C. longus* is the main composition that impacts of distribution. Longer exposures to contaminated water culture were required. However, the hydrological culture method has a good potential to be successfully applied for these species (*C. longus*, and *P. australis*) for over three years as a research period. Similarly, for *F. rubra* and *T. pretense*, which had reduced levels of seedling germination at higher salinity concentrations, may lead to loss of viability. Therefore, the germination phase of these seeds should be delayed before transplanting to grow at wastewater or salinities solutions. A more thorough understanding of plant life cycles could be explored in relation to salinity exposures.

5.4 Suggestions and future work

5.4.1 Phyto-accumulation of contaminated soil

In future studies, the efficiency of these plant species to remediate contaminated soils should be examined under various soil types. More research is also needed to determine the efficacy of their uptake performance. A natural progression of this work is to determine and support their capacity to accumulate metals and high salinity in contaminated sites. Future studies could be undertaken to assess the long-term effects of cultivars on waste contamination, and the potential of plant structures to act as cost-effective bio-accumulators should be more evaluated. More broadly, research is also needed to determine the leaching and mobility of contaminated soil that pose a risk to the environment.

5.4.2 Phyto-accumulation of contaminated water

Application of hydroponic culture could potentially be useful with wetland species for treatment to investigate the quantity of metals, such as strontium, can be absorbed and accumulated into plant tissues. Another possible area of future research would be to investigate not only in laboratory conditions but also in the field. Further research on a commercial scale might be carried out full-scale with actual effluent productivity. At present, the growth behavior and metabolic mechanism of plants are traditionally considered to be useful in phytoremediation technology. The issue of sensitive species with hydroponic screening for rapid response to environmental stress is an intriguing one, which could be usefully explored in further research.

5.4.3 Species-specific contamination

Pollution by heavy metal and salinity is widespread and often involves vast volumes of wastewater produced by human activities. Phytoremediation strategies for oil industry wastewater must be created to promote the use of these plant species in order to contain

the costs of wastewater treatment. This research underlines the importance of continuing fundamental research in order to the gain the best possible understanding of the mechanisms of phytoaccumulation. Considerably more work needs to be done in a pilot-scale study that would pave the way for its use on an industrial level. Plant species such as *F. rubra* and *T. pratense,* with their translocation factors (TF), could be effective in acting as bio-accumulator plants for other contaminating metals in the soils. Wetland species should also be promoted for this purpose, and *C. longus* and *P. australis* should be investigated in possible translocation factor of heavy metals in their tissues, both in the aerial and root part of the plants. This harvesting may be useful to assess the ratios necessary to maximize removal of heavy metal in the root and shoot.

APPENDICES

Appendix A: The University's Occupational Health and Safety Policy (OHS)

A1: Laboratory access

Appendix B: Standard Procedure

B1: Biological analysis

B2: Chemical analysis

B3: Physical analysis

B4: Physicochemical analysis

Appendix C: ICP-OES Standard

C1: Standard analytical method

C2: Standard for working

C2.1 ICP-OES Standard for Soil Sequential Extraction

C2.2 ICP-OES Standard for CEC and Absorption

C2.3 ICP-OES Standard for Plant Material Digestion

C2.4 ICP-OES Standard for Wastewater Sampling

Appendix D: Photographs of Plant Growth and some analysis of statistics

D1: Chapter 2

D2: Chapter 3

D3: Chapter 4

Appendix E: Publication

E1: Research Presentation Day, Faculty of Engineering University of Strathclyde 22nd June 2016

E2: SETAC Europe 28th Annual Meeting in Rome, Italy on 13-17 May 2018

Appendix A: The University's Occupational Health and Safety Policy (OHS)

A1: Laboratory access

Department of Civil & Environmental Engineering

Laboratory Access

My Name is:	*Phatchani Srikhumsuk*
My contact details:	Phone: 07951502944 Email: phatchani.srikhumsuk@strath.ac.uk
My Academic Supervisor(s) 1)	Dr. Charles Knapp
2)	Dr. Joanna Renshaw

My status in the department is:	☐ UG ☐ Masters ☒ PhD ☐ Post Doc ☐ Visitor	
My supervisor would like for me to work in the following labs:	☒ Cat2 Biology and Chemistry ☐ Analytical ☐ Radiochemistry ☒ Geo Mechanics	
My supervisor is happy for me to have lab access during the following times:	☐ M-F: 9AM-5PM ☐ M-F: 9AM-5PM, W/H: 9AM-5PM (red card required) ☒ M-F: 7AM-10PM, W/H: 8AM-6PM (red card required) ☐ Other: _____	
My supervisor is happy for me to be a lab key holder (list 1):	☐ Yes ☒ Not yet, but soon! ☐ Not eligible	
If badge does not have access to the laboratories, see Mara Knapp		
The following safety has been put in place for my project:	☒ Risk Assessment	Reference(s): CoSHH: *Using plant as toxicological indicators to effects of heavy metal*
	☐ eCoSHH	Reference(s):
These are also required and I have registered:	☒ Biological Protection Registration	☐ Radiological Registration

Signed : Phatchani Srikhumsuk _____
(Researcher)

By signing, I accept the lab status granted to me by my supervisor and understand any unauthorized deviations can result in my lab privileges being removed. I acknowledge CEE has a no lone working policy.

Signed : Charles W Knapp

(Person Responsible for Research—PI, supervisor)

By signing, I accept responsibility for the named researcher and will ensure they have received the proper training for the work they will be undertaking.
Once signed by Person Responsible, take completed form to Mara Knapp and/or Derek McNee to be posted in the write up area of the floor(s) where you will be working

Appendix B: Standard Procedure

B1: Biological analysis

Standard procedure	**Subject:** *Biological analysis*	
	Title: *Chlorophyll and pheophytin extraction*	
	COSHH: *S20-2016, S20-2017*	**Designation for Chapter:** *2, 4*

A. Introduction

Chlorophyll in the chloroplast pigment of green plant is played an important role to converse the sunlight to become a chemical energy. This is the oxidation-reaction characteristic of photosynthesis that can be produce oxygen into the atmosphere. In addition pheophytin is widely known where specific component of the reaction centre of PS II that has also found in the green leaves. An estimated concentration of chlorophyll and pheophytin are the common methods for assessing the primary productivity and indicated plant health in the part of physiological mechanism (Axler and Owen, 1994, Wellburn, 1994).

B. Equipment

1. Mortar and Pestle
2. Volumetic flask 25 ml
3. Beaker 25 ml
4. Filter Whatman No.1
5. Centrifuge tube 15 ml
6. Centrifuge (eppendorf centrifuge 5804R)
7. Spectrophotometer (Thermo scientific Helios Zeta UV-VIS)

C. Reagents

1. 80% Acetone
2. 1*N* HCl
3. Nano pure water produced by Barnstead™ Nanopure D3-Hollow Fibre Filter
4. 10% HNO_3 (soak glassware)

D. Analytical procedure and determination

1. Dried-leaves are ground with mortar and pestle and added 5 ml 80% acetone
2. Harmonized and transferred into the centrifuge tube with added 10 ml of 80% acetone
3. Centrifugation at 4,500, and 3,000 rmp, each for 5 minutes at 5 °C
4. Filtration by using Whatman No.1, made up to 25 ml
5. 5 ml transferred into the cuvette to measurement
6. For Chapter 6 Acidification of chlorophyll with added 1*N* HCl (2-3 drops)
7. Determine the chlorophyll for Chapter 4 is 470, 646, and 663 nm, another measured both of chlorophyll and pheophytin at 664, 665, and 750 nm for Chapter 6 with using spectrophotometer
8. Read and record

E. Calculation / Identification

Chapter 4

$C_a = 12.21A_{663} - 2.81A_{646}$

$C_b = 20.13A_{646} - 5.03A_{663}$

Where:

C_a is chlorophyll *a*, and C_b is chlorophyll *b*

Chapter 6

E_{664b} = [{Abs_{664b}(sample)-Abs_{664b}(blank)}-{Abs_{750b}(sample)-Abs_{750b}(blank)}]

E_{665a} = [{Abs_{665a}(sample)-Abs_{665a}(blank)}-{Abs_{750a}(sample)-Abs_{750a}(blank)}]

Chlorophyll *a* (ug.L^{-1}) = $\frac{[26.7\ x\ (E664b - E665a)\ x\ Vext]}{Vsample\ x\ L}$

Pheophytin (ug.L^{-1}) = $\frac{[26.7\ x\ (1.7 * E665a - E664b)\ x\ Vext]}{Vsample\ x\ L}$

Where:

a is after acidification

Abs is absorbance in different wavelength

b is before acidification

V_{ext} is milliliter of the volume of 80% acetone used in the extraction

V_{sample} is a liter of filtered water volume

F. References

AXLER, R. P. & OWEN, C. J. 1994. Measuring chlorophyll and phaeophytin: whom should you believe? *Lake and Reservoir Management,* 8**,** 143-151.

WELLBURN, A. R. 1994. The spectral determination of chlorophylls a and b, as well as total carotenoids, using various solvents with spectrophotometers of different resolution. *Journal of plant physiology,* 144**,** 307-313.

Standard procedure	**Subject:** *Biological analysis*	
	Title: *Plant material digestion*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

The microwave digestion is one of method to determine amount of metal in plant tissue, which is using aqua regia that is concentration of ration HNO_3 and HCl (3:1, v/v). Digestion method is procedure with heating by microwave radiation (Wang and Jia, 2009, Bonanno, 2011).

B. Equipment

1. Balance
2. Syringe 10 ml
3. Syringe filter 0.45 um
4. Glass pipette 25 ml
5. Polyethylene centrifuge tube 50 ml
6. Glass beaker 25 ml
7. Teflon double wall digestion vessels
8. Microwave digestion (MARSXpress 240/50 CEM, Mathews, NC, USA)
9. ICP-OES model Thermo Scientific iCAP 6000 series ICP Spectrometer

C. Reagents

1. Nanopure water produced by Barnstead™ Nanopure D3-Hollow Fibre Filter
2. Concentration of Nitric acid (HNO_3)
3. Concentration of Hydrochloric acid (HCl)
4. Standard solutions (Ca, Mg, Na, K, Ba, Fe, Sr, Cd, Hg, Mn, Al, Zn, Cu, As, Co, Mo, Pb, Ni, Sb, Se, V)
5. Prepared stock calibration working standards from 1000 mgL^{-1} standard solutions
    a. Sr, and Ba concentration 1 ppm

b. Al, As, Cd, Co, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Sb, Se, Si, V, and Zn concentration of each 10 ppm
c. Ca, K, Mg, and Na concentration of each 50 ppm
d. Dilute solutions with 5% HNO_3

D. Analytical procedure and determination

1. Root and shoot of plant samples grounded with mortar and pestle
2. Weigh powder of dry plant tissue sampling 0.01-0.1 g place into the closed vessel
3. Added aqua regia 12 ml
4. Put into the microwave digestion, with program two stages of digestion (CEM, 2006)
   a. Stage 1 in 15 min, hold 5 min at 170 °C
   b. Stage 2 in 5-10 min, hold 15 min at 180 °C
5. After digestion filtered with syringe filter at 0.45 um
6. Diluted at 10 times
7. Investigated with ICP-OES

E. Calculation / Identification (SOP, 2006)

mg metal/kg sample = $\frac{A \, x \, V}{F \, x \, W} \; x \; DF$

where:

A is metal in processed sample from read-out, ($mg.L^{-1}$ or $ug.L^{-1}$)

F is concentration unit factor

V is final volume of the processed sample, (mL)

W is weight of sample, (g)

DF is dilution factor for diluted samples

F. References

BONANNO, G. 2011. Trace element accumulation and distribution in the organs of Phragmites australis (common reed) and biomonitoring applications. *Ecotoxicology and Environmental Safety,* 74**,** 1057-1064.

CEM. 2006. *Mars Operation Manual*. United States of America patent application Matthews, North Carolina 28106.

SOP 2006. Determination of metals by Inductively Coupled Plasma (ICP) method. *Standard Operating Procedures.*

WANG, H. & JIA, Y. 2009. Bioaccumulation of heavy metals by Phragmites australis cultivated in synthesized substrates. *Journal of Environmental Sciences,* 21**,** 1409-1414.

B2: Chemical analysis

Standard procedure	**Subject:** *Chemical analysis*	
	Title: *Cation Exchange Capacity (CEC)*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

Cation exchange capacity (CEC) is defined as a quality of negative charging on soil surface, which can retain positive charging (cations). For instance, Ca^{+}, K^{+}, and Mg^{2+} with electrostatic forces are maintained. The highest CEC has greater showed the capacity to maintain the exchangeable of Ca^{+}, K^{+}, Mg^{2+}, and Na^{+} (Ross and Ketterings, 1995, BSI, 2011).

B. Equipment

1. End-over-end shaker
2. Volumetic flask 50, 100, 1000 ml
3. 50 ml centrifuge tube
4. Syringe 10 ml
5. 45 um syringe filter
6. Pipettes
7. Centrifuge model eppendorf centrifuge 5804R
8. Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) model Thermo Scientific iCAP 6000 series ICP Spectrometer

C. Reagents

1. $BaCl_2$ solution
    a. 0.1 $mol.L^{-1}$ dissolved 24.43 g of $BaCl_2.2H_2O$ in nano pure water, then make up volume to 1000 ml
    b. 0.0025 $mol.L^{-1}$ diluted 25 ml of 0.1 $mol.L^{-1}$ $BaCl_2$ solutions with nano pure water, then make up volume to 1000 ml
2. $MgSO_4$ solution

a. 0.020 mol.L^{-1} dissolved 4.93 g ±0.01 g of $MgSO_4.7H_2O$ in nano pure water and make up volume to 1000 ml

D. Analytical procedure and determination

Day 1:

1. Transfer 2.5 g of air-dried soil (particle size ≤2 mm) [Balance centrifuge tube + soil] ***(m_1)***
2. Add 30 ml of $BaCl_2$ 0.1 mol.L^{-1} (Shake for 1 h and centrifuge at 3000 g for 10 min)
3. Transfer supernatant liquid to 100 ml volumetric flask
4. Repeat add 30 ml $BaCl_2$ 0.1 mol.L^{-1} (twice more) (Shake for 1 h and centrifuge at 3000 g for 10 min)
5. Transfer supernatant to V. flask 100 ml and make up volume with $BaCl_2$ 0.1 mol.L^{-1} (Mix, filter and store for determination of Na, K, Ca, Mg)

Day 2:

1. Soil cake from day 1 add 30 ml $BaCl_2$ 0.0025 mol.L^{-1} (Shake for 1 h and centrifuge at 3000 g for 10 min)
2. Pour the supernatant
3. Weigh the tube with cover and contents ***(m_2)***
4. Add 30 ml $MgSO_4$ 0.020 mol.L^{-1} (Shake overnight)

Day 3:

1. Centrifuge at 3000 g for 10 min.
2. Pour supernatant and filter with coarse filter paper (7 cm diameter)
3. Store in conical flask 100 ml (to determine Mg excess conc.)

**Prepare blank with procedure completely without soil

E. Calculation / Identification

a. CEC value (cMol$^+$kg^{-1})

$$c_2 = \frac{c_1(30+m_2-m_1)}{30}$$

$$\text{CEC} = \frac{(c_{b1}-c_2)x\ 3000}{m}$$

Where:

c_1 is the magnesium concentration in the sample, (mM.L^{-1})

c_{b1} is the magnesium concentration in the blank, (mM.L^{-1})

c_2 is the corrected magnesium concentration in the sample, (mM.L^{-1})

m is the mass of the air-dried soil sample, (g)

m_1 is the mass of the centrifuge tube with air-dried soil, (g)

m_2 is the mass of the centrifuge tube with wet soil, (g)

b. Exchangeable Ca^+ (cMol$^+$kg^{-1})

Ca, exch = $\frac{4.9903 \text{ x } (p_4 - p_3)}{m}$

Where:

p_4 is the concentration of calcium in the diluted extract, (mg.L^{-1})

p_3 is the concentration calcium in the diluted blank, (mg.L^{-1})

m is the mass of air-dried soil, (g)

c. Exchangeable K^+ (cMol$^+$kg^{-1})

K, exch = $\frac{1.2788 \text{ x } (p_4 - p_3)}{m}$

Where:

p_4 is the concentration of potassium in the diluted extract, (mg.L^{-1})

p_3 is the concentration potassium in the diluted blank, (mg.L^{-1})

m is the mass of air-dried soil, (g)

d. Exchangeable Mg^{2+} (cMol$^+$kg^{-1})

Mg, exch = $\frac{8.2288 \text{ x } (p_4 - p_3)}{m}$

Where:

p_4 is the concentration of magnesium in the diluted extract, (mg.L^{-1})

p_3 is the concentration magnesium in the diluted blank, (mg.L^{-1})

m is the mass of air-dried soil, (g)

e. Exchangeable Na^+ (cMol$^+$kg^{-1})

Na, exch = $\frac{2.1749 \text{ x } (p_4 - p_3)}{m}$

Where:

p_4 is the concentration of sodium in the diluted extract, (mg.L^{-1})

p_3 is the concentration sodium in the diluted blank, (mg.L^{-1})

m is the mass of air-dried soil, (g)

F. References

BSI 2011. *Soil Quality: Determination of Effective Cation Exchange Capacity and Base Saturation Level Using Barium Chloride Solution,* British Standard Institution, BS EN 11260:2011.

ROSS, D. S. & KETTERINGS, Q. 1995. Recommended methods for determining soil cation exchange capacity. *Recommended soil testing procedures for the northeastern United States,* 2**,** 62-70.

Standard procedure	**Subject:** *Chemical analysis*	
	Title: *pH of soil*	
	COSHH: *S20-2015*	**Designation for Chapter:** *2, 3, 4*

A. Introduction

The pH is the value of acidify and alkali of ion in soil. It is a rage from 1 to 14, which is the moderate at 7.0. It can be indicated that neutrality of soil, if it has indicate lower than 7.0, soil was presented with acidity. On the other hand, the value of pH shown more than 7.0 it would be index for a akali of soil. Therefore, the pH of soil can be indicated that the efficient of soil that were accumulated a nutrient in there. It is also become a benefit to plant growing (USEPA, 1986, Thomas, 1996).

B. Equipment

1. pH metre model METTLER TOLEDO SevenMulti^{TM} probe
2. Horizontal shaking machine
3. 3 of 15 ml centrifuge tubes
4. Beakers 50 or 100 ml
5. Glass stick / bar
6. Stirring machine

C. Reagents

1. Deionized water
2. Buffer to calibrate pH
3. 10 % HNO_3 solution (soak glassware)

D. Analytical procedure and determination

1. Weighed 10 g of soils place into 3 beakers
2. Added deionized water 10 ml
3. Mixed by magnetic bar and deposit 10 min (repeat 5 times)
4. Measured pH
5. Read and record

6. Before to use pH probe to read other samples, rinse electrodes with deionize water is required

E. Calculation / Identification

n/a

F. References

THOMAS, G. W. 1996. *Methods of Soil Analysis: Part 3 Chemical Methods. ,* Madison, Wisconsin, USA, Soil Science Society of America.

USEPA, S. W. 1986. Test methods for evaluating solid waste: Physical/chemical methods. *http://www. epa. gov/epaoswer/hazwaste/test/7_series. htm*.

Standard procedure	**Subject:** *Chemical analysis*	
	Title: *Total Dissolved solids (TDS)*	
	COSHH: *S20-2017*	**Designation for Chapter:** *4*

A. Introduction

In water samples are normally determined the amount of total dissolved solids (TDS). Total dissolved solid has defined colloids composition that dissolved in the water (Rhoades, 1996)

B. Equipment

1. TDS metre model METTLER TOLEDO SevenMulti^{TM} probe
2. 3 of 15 ml centrifuge tubes
3. Syringe 10 ml

C. Reagents

D. Analytical procedure and determination

1. Take-up water sample 10 ml
2. Place in 15 ml centrifuge (replicate 3)
3. Measured TDS
4. Read and record

E. Calculation / Identification

n/a

F. References

RHOADES, J. D. 1996. *Methods of Soil Analysis: Part 3 Chemical Methods.,* Madison, Wisconsin, USA, Soil Science Society of America.

Standard procedure	**Subject:** *Chemical analysis*	
	Title: *Total Organic Carbon (TOC) by Loss of weight on Ignition (LOI)*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

The important of soil organic matter contributed to supply nutrient and cation exchange capacity in order to improve soil structure. Analysis TOC is to determine the total organic content in the soil in order to evaluate the quality of soil. This measurement is involved to oxidize organic carbon from the sample by ignition loss procedure (LOI) (BSI, 2012).

B. Equipment

1. Oven
2. Porcelain crucibles (50ml) (adjusted to constant weight)
3. High temperature marker pen
4. Spatula for weighing soil
5. Silica gel
6. Desiccator filled with silica gel
7. Balance with precision to at least 0.0001 g
8. Muffle furnace Carbolite (England ELF 11/14)

C. Reagents

1. 10 % HNO_3 solution (soak crucibles)

D. Analytical procedure and determination

1. Switch on analytical balance and tare.
2. Place porcelain crucible on balance pan and note weight ***(W_1)***
3. Tare balance and carefully transfer 5 - 10 g ± 0.1 g of air-dried soil into crucible using clean spatula. Note weight of the samples ***(W_2)***
4. Cover crucibles with lid
5. Place crucible into muffle furnace at 550 ^oC for 4 h
6. Cool crucibles for 1 h

7. Using a pair of tongs, carefully transfer crucible into desiccator and leave for 1 hour to allow sample to cool down till room temperature at moisture free condition.
8. Then weigh crucible with ashed sample, register the weight ***(W_3)***

E. Calculation / Identification

1. Calculate loss on ignition using the following equation (Allen, 1989):

$$\text{LOI, \%} = \frac{W_2-(W_3-W_1)}{W_2} \times 100$$

2. Total organic carbon (TOC) is determined by applying the formula as following below:

$$\text{TOC, \%} = \frac{\text{LOI}}{1.8}$$

F. References

ALLEN, S. E. 1989. Analysis of vegetation and other organic materials. In 'Chemical analysis of ecological materials'.(Ed. SE Allen) pp. 46–61. Blackwell Scientific Publications: Oxford.

BSI 2012. *Sludge, treated biowaste, soil and waste-Determination of total organic carbon (TOC) by dry combustion,* British Standard Institution, BS EN 15936:2012.

B3: Physical analysis

Standard procedure	**Subject:** *Physical analysis*	
	Title: *Electrical Conductivity (EC)*	
	COSHH: *S20-2015*	**Designation for Chapter:** *2, 3, 4*

A. Introduction

Salinity has been defined as a major total concentration of salt soluble that mainly included inorganic ions e.g. Ca^{2+}, Cl^{-}, CO_3^{2-}, HCO_3^{-}, K^{+}, Mg^{2+}, Na^{+}, and SO_4^{2-}. The investigation of saline soil is used aqueous extract of soil sample. In contrast, for brine water is directly measured. The total salt soluble concentration can be investigated with measurement of electrical conductivity (EC). This experiment is extracted with soil/water ratios of 1:1. (USEPA, 1986, Rhoades, 1996)

B. Equipment

1. EC metre model METTLER TOLEDO SevenMulti^{TM} probe
2. Horizontal shaking machine
3. 3 of 15 ml centrifuge tubes
4. Beakers 50 or 100 ml
5. Glass stick / bar
6. Stirring machine

C. Reagents

1. Deionized water
2. Buffer to calibrate EC
3. 10 % HNO_3 solution (soak glassware)

D. Analytical procedure and determination

1. Weighed soil 10 g into 3 beakers
2. Added deionized water 10 ml
3. Mixed by magnetic bar and deposit 10 min (repeat 5 times)
4. Measured EC

5. Read and record
6. Rinse electrodes with deionize water is required before to use measuring other samples

E. Calculation / Identification

n/a

F. References

RHOADES, J. D. 1996. *Methods of Soil Analysis: Part 3 Chemical Methods.,* Madison, Wisconsin, USA, Soil Science Society of America.

USEPA, S. W. 1986. Test methods for evaluating solid waste: Physical/chemical methods. *http://www. epa. gov/epaoswer/hazwaste/test/7_series. htm*.

Standard procedure	**Subject:** *Physical analysis*	
	Title: *Soil water content or moisture content (MC)*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

Water content in the soil is indicated that how much water available in the soil. The amount of water content in the soil is directly affected to growth of plant. The most commonly soil analysis is moisture content or water content parameter. The measurement of soil moisture content in this experiment is according to Carter and Gregorich (2008).

B. Equipment

1. Oven
2. High temperature marker pen
3. Beaker 50 ml
4. Spatula for weighing soil
5. Silica gel
6. Desiccator filled with silica gel
7. Balance with precision to at least 0.0001 g

C. Reagents

10 % HNO_3 solution (used soak glassware)

D. Analytical procedure and determination

1. Tare balance and carefully transfer 10 g of soil sampling into the beaker using clean spatula. Note weight of the samples
2. Place beaker into oven for 48 h at 105 ^{o}C
3. Cool down beaker into desiccator leave for 1 h
4. Then weigh soil sampling after drying

E. Calculation / Identification

$$\%\text{Water content (mass basis)} = \frac{(\text{mass of moist soil+tin})-(\text{mass of dry soil+tin})}{\text{mass of dry soil}} \text{ x } 100$$

F. References

CARTER, M. R. & GREGORICH, E. G. 2008. Soil sampling and methods of analysis.

B4: Physicochemical analysis

Standard procedure	**Subject:** *Physicochemical analysis*	
	Title: *Batch method*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

Soil interacted with trace element is very complicated to investigate that how elements are remained in the soil. The leaching test is widely tool to investigate and assess long-term of contaminated soil pathway. Soil batch test is one of expression is widely used to determine the potential of waste soluble contaminated soil. The standard procedure according ISO/TS21268-1:2007-07 (2007) was cited in Grathwohl and Susset (2009), and Krüger et al. (2012).

B. Equipment

1. Polyethylene bottles size 125 ml
2. Centrifuge tube size 15 ml, and 50 ml
3. Paper filter Whatman No.1
4. Volumetric flash 50 ml
5. Syringe 10 ml
6. Syringe filter 0.45 um
7. Beaker 50 ml
8. Horizontal shaker
9. Centrifuge (eppendorf centrifuge 5804R)
10. ICP-OES

C. Reagents

1. Strontium hexahydrate ($Sr.6H_2O$) solution at 5, 10, 20, and 40 mM
2. Calcium chloride hepatahydrate ($CaCl.7H_2O$) solution at 5, 10, 20, and 40 mM
3. Nanopure water produced by Barnstead™ Nanopure D3-Hollow Fibre Filter

D. Analytical procedure and determination

1. Dry soil at oven 50-55 ºC for 2 days

2. Weigh 10 g of soils put in polyethylene bottles
3. Added $SrCl_2$ 25 ml in each concentration i.e. 0, 5, 10, 20, 40 mM $SrCl_2$ (3 replicates in each)+ blank solution (0, 5, 10, 20, 40 mM $SrCl_2$)
4. Take in time at 0h, 1h, 24h, 168h, and 504h in closed system & shaking all time
5. Run on time then, take sample out and put in the centrifuge tube (50 ml) and added $CaCl_2$ solution (0, 5, 10, 20, 40 mM) in each at 25ml and shaking for 15 min at room temperature then, bring to centrifuge for 5 min at 3000 g, kept supernatant to analyse the solution out (kept in the polyethylene 75 ml) repeat washing 2 times
6. Sink residual into filter paper 90 mm and dried at 50-55 ºC for 2 days
7. Weigh 1 g powder of soil to digest and leaching with 1% HNO_3 fluid through filter with 90 mm paper filter
8. Make up volume to 50 ml with 1% HNO_3 with volumetric flash 50 ml
9. Filter with 45 um and kept in 50 ml centrifuge tube
10. Preparation the sample for ICP analysis with non-diluted for 0 mM, and diluted df = 10 (5mM), 100 (10, 20 mM), 1000 (40mM)
11. Blank of original solution would be operated in the same condition without soil sample

E. Calculation / Identification (SOP, 2006)

mg metal/kg sample **=** $\frac{A\ x\ V}{F\ x\ W}\ x\ DF$

where:

A is metal in processed sample from read-out, ($mg.L^{-1}$ or $ug.L^{-1}$)

F is concentration unit factor

V is final volume of the processed sample, (mL)

W is weight of sample, (g)

DF is dilution factor for diluted samples

F. References

GRATHWOHL, P. & SUSSET, B. 2009. Comparison of percolation to batch and sequential leaching tests: Theory and data. *Waste Management,* 29**,** 2681-2688.

ISO/TS21268-1:2007-07 2007. Soil quality-Leaching procedures for subsequent chemical and ecotoxicological testing of soil and soil materials. *Part 1: Batch test using a liquid to solid ratio of 2 l/kg dry matter.*

KRÜGER, O., KALBE, U., BERGER, W., SIMON, F. G. & MEZA, S. L. 2012. Leaching experiments on the release of heavy metals and PAH from soil and waste materials. *Journal of Hazardous Materials,* 207-208**,** 51-55.

SOP 2006. Determination of metals by Inductively Coupled Plasma (ICP) method. *Standard Operating Procedures.*

Standard procedure	**Subject:** *Physicochemical analysis*	
	Title: *Sequential Extraction*	
	COSHH: *S20-2016, S21-2016*	**Designation for Chapter:** *2*

A. Introduction

The physiochemical characteristics of soils are important to determine the effects of heavy metals distribution. In order to understand the reaction of many trace elements to bind form with the soil. Sequential extraction method is widely used to investigate the association with specific fraction in the soil. Contaminated soil has greater determined with using an available and mobile values of the trace elements on the particle surface of soil. The standard of this experiment followed the procedure of sequential fractionation according with Tessier et al. (1979).

B. Equipment

1. Centrifuge tube 50 ml
2. Beaker
3. Whatman No.40
4. Volumetric flask
5. Pipette
6. Polypropylene bottle
7. pH meter
8. Hot plate
9. Stirrer
10. Balance
11. Oven
12. Incubator
13. Horizontal shaker
14. End-over-end shaker
15. Horizontal incubator shaker
16. Centrifuge (eppendorf centrifuge 5804R)

17.ICP-OES (Thermo Scientific iCAP 6000 series ICP Spectrometer)

C. Reagents

1. 1*M* of $MgCl_2 \bullet 6H_2O$
2. 1*M* NaOA
3. 0.04*M* of $NH_2OH \bullet HCl$
4. 30% (w/v) of H_2O_2
5. Conc. HCl
6. Conc. HNO_3
7. Nano pure water produced by Barnstead™ Nanopure D3-Hollow Fibre Filter
8. 10% HNO_3 (used soak glassware)

D. Analytical procedure and determination

Fraction	Extract solution	Preparation of solution	V (ml)	Extraction condition
Exchangeable (i)	1*M* $MgCl_2$, pH 7	$MgCl_2$ (203.31 g/mol) 1*M*, pH7 → (1x203.31x100)/1000 = 20.331 g → Adjust pH 7 by 1*M* ammonium hydroxide (35.04 g/mol) → make up volume to 100 ml with nanopure water	8	1 h (room temp.)
Bound to Carbonates (ii)	1*M* NaOAc, pH 5 (HOAc)	NaOAc (Sodium acetate) (82.03 g/mol) 1*M*, pH 5 → (1x82.03x100)/1000 = 8.203 g→Adjust pH by acetic acid→make up volume to 100 ml with nanopure water	8	5 h (room temp.)
Bound to Fe-Mn Oxides (iii)	0.04*M* $NH_2OH \bullet HCl$ in 25% v/v HOAc	$NH_2OH \bullet HCl$ (Hydroxylamine hydrochloride) (69.49 g/mol) 0.04*M* in 25% v/v acetic acid → (0.04x69.49x100)/1000 = 0.278 g Make up volume to 100 ml with 25% v/v acetic acid (→ 250 ml (acetic acid) + 750 ml nanopure water)	20	6 h (96±3 ˚C)
Bound to Organic Matter (iv)	0.02*M* HNO_3	HNO_3 (Nitric acid) (63.0128 g/mol) 0.02*M*→ (0.02x63.0128x100)/1000 = 0.126 g	6	2 h (85±2 ˚C)
	30% w/w H_2O_2, pH 2	H_2O_2 (Hydrogen peroxide) (34.0147 g/mol) →(30 g/100 g)*100 = 30 →Adjust 2 pH with nitric acid	20	3 h (85±2 ˚C)
	3.2*M* NH_4OAc in 20% v/v HNO_3, pH 2	NH_4OAc (Ammonium acetate) (77.08 g/mol) 3.2*M* in 20% v/v nitric acid (246.62 g/mol) → (3.2x77.8x100)/1000 = 24.66 g→ Make up volume to 100 with 20% v/v nitric acid → (200 ml (nitric acid) + 800 ml nanopure water)	5	30 min (room temp.)
Residual (v)	HCl/HNO_3 (9/3 ml)			

E. Calculation / Identification (SOP, 2006)

$$\text{mg metal/kg sample} = \frac{A \times V}{F \times W} \times DF$$

Where:

A is metal in processed sample from read-out, ($mg.L^{-1}$ or $ug.L^{-1}$)

F is concentration unit factor

V is final volume of the processed sample, (mL)

W is weight of sample, (g)

DF is dilution factor for diluted samples

F. References

SOP 2006. Determination of metals by Inductively Coupled Plasma (ICP) method. *Standard Operating Procedures.*

TESSIER, A., CAMPBELL, P. G. C. & BISSON, M. 1979. Sequential extraction procedure for the speciation of particulate trace metals. *Analytical chemistry,* 51**,** 844-851.

Appendix C: ICP-OES Standard

C1: Standard analytical method

Table C1.1 List of elements, Method Quantification Limits (MQL) and plasma view

ELEMENT	MQL, mg/L	PLASMA VIEW
1. Arsenic	0.0090	Axial
2. Barium	0.0001	Axial
3. Calcium	0.0710	Radial
4. Cadmium	0.0003	Axial
5. Cobalt	0.0008	Axial
6. Chromium	0.0011	Axial
7. Copper	0.0007	Axial
8. Iron	0.0009	Axial
9. Mercury	0.0025	Axial
10. Potassium	0.1190	Radial
11. Lithium	0.00004	Axial
12. Magnesium	0.0028	Radial
13. Manganese	0.0002	Axial
14. Sodium	0.0370	Radial
15. Nickel	0.0010	Axial
16. Lead	0.0034	Axial
17. Silica	0.0024	Axial
18. Strontium	0.00003	Axial
19. Zinc	0.0005	Axial

Table C1.2 ICP-OES operating conditions

PARAMETERS	SETTINGS
RF power, w	1150
Plasma gas flow rate, l/min	15
Auxiliary gas flow rate, l/min	0.5
Nebulizer argon gas flow rate, l/min	0.5
Purge gas	Nitrogen
Plasma gas	Argon
Flush pump rate, rpm	100
Analyses pump rate, rpm	50
Pump stabilisation time, s	5
Pump sample flush time, s	30
Pump wash time, s	30
Pump tubing type	Tygon orange/white
Drain tubing	Tygon white/white
Wash solution	Nitric acid, 3 % (v/v)
Wash time, s	15
Spray chamber	Glass cyclonic
Nebulizer	Mira mist
Centre tube, mm	2
Replicates reading	3
Wavelength range	Low and High
Analysis Integration time Low Wavelength range, s	Axial - 15; Radial - 15
Analysis Integration time High Wavelength range, s	Axial - 15; Radial - 5
Plasma view mode	For details see Table 1

Table C1.3 Calibration curve, internal and quality control standards preparation details

STANDARD CODE	ELEMENTS	CONCENTRATION, mg/L
CALIBRATION STANDARDS		
Std-1	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	0.01 0.10 0.50
Std-2	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	0.10 1.00 5.00
Std-3	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	1.00 10.0 50.0
ANALYTICAL QUALITY CONTROL		
AQC	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	0.5 2.0 2.5
QUALITY CONTROL CHECK STANDARDS		
QCCS-Low	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	0.01 0.10 0.50
QCCS - Medium	Ba, Sr As, Cd, Co, Cr, Cu, Fe, Hg, Li, Mn, Ni, Pb, Si, Zn, Ca, K, Mg, Na	0.10 1.00 5.00

References

Determination of dissolved elements in ground water samples using inductively coupled plasma optical emission spectrometry. SETN's Standard Operating Procedure (2013). Scottish Environmental Technology Network, The University of Strathclyde, Glasgow.

iTEVA Software Operation Manual (2000) Thermo Fisher Scientific, Cambridge, UK.

iCAP 6000 Series ICP-OES Spectrometer Technical Reference manual (2005). Thermo Fisher Scientific, Cambridge, UK.

C2: Standard for working

Table C2.1 ICP-OES Standard for Soil sequential Extraction

Date	Standard Code	Elements	Concentration, ppm	Stock Concentration, ppm	DF	Flask Volume, ml	Stock Volume, ml	Media	Media
22.11.16		**Calibration Working Standards**						5% HNO_3	Prepared by dilution of a top standard
	Nui_1	Sr, Ba	0.01	1	100	100	1.000		
		19 elements	0.1	10	100	100	1.000		
		K, Na, Ca, Mg,	0.5	50	100	100	1.000		
	Nui_2	Sr, Ba	0.1	1	10	100	10.000		
		19 elements	1	10	10	100	10.000		
		K, Na, Ca, Mg,	5	50	10	100	10.000		
	Nui_3	Sr, Ba	1	1000	1000	100	0.100		From stock
		19 elements	10	1000	100	100	1.000		
		K, Na, Ca, Mg,	50	1000	20	100	5.000		
	Independent Standard - AQC								
	AQC	Sr, Ba	0.5	1000	2000	250	0.125		
		19 elements	2	1000	500	250	0.500		
		K, Na, Ca, Mg,	2.5	1000	400	250	0.625		

Table C2.2 ICP-OES Standard for CEC and Absorption

Date	Standard Code	Elements	Concentration, ppm	Stock Concentration, ppm	DF	Flask Volume, ml	Stock Volume, ml	Media	Media
13.03. 17		**Calibration Working Standards**						5% HNO_3	Prepared by dilution of a top standard
	Nui_Abs1	Sr	0.01	1	100	100	1.000		
		K, Na, Ca, Mg,	0.5	50	100	100	1.000		
	Nui_Abs2	Sr	0.1	1	10	100	10.000		
		K, Na, Ca, Mg,	5	50	10	100	10.000		
	Nui_Abs3	Sr	1	1000	1000	100	0.100		From stock
		K, Na, Ca, Mg,	50	1000	20	100	5.000		
	Independent Standard - AQC								
	AQC	Sr	0.5	1000	2000	250	0.125		
		K, Na, Ca, Mg,	2.5	1000	400	250	0.625		

Table C2.3 ICP-OES Standard for Plant material digestion

Date	Standard Code	Elements	Concentration, ppm	Stock Concentration, ppm	DF	Flask Volume, ml	Stock Volume, ml	Media	Media
30.08.17		**Calibration Working Standards**						3.6% HCL 1.2 HNO_3	Prepared by dilution of a top standard
	Nui_1	Sr	0.001	1	1000	100	0.100		
		Ca	0.5	50	100	100	1.000		
	Nui_2	Sr	0.1	1	10	100	10.000		
		Ca	5	50	10	100	10.000		
	Nui_3	Sr	1	1000	1000	100	0.100		From stock
		Ca	50	1000	20	100	5.000		
	Independent Standard - AQC								
	AQC	Sr	0.5	1000	2000	100	0.050		
		Ca	2.5	1000	400	100	0.250		

Table C2.4 ICP-OES Standard for Wastewater sampling

Date	Standard Code	Elements	Concentration, ppm	Stock Concentration, ppm	DF	Flask Volume, ml	Stock Volume, ml	Media	Media
12.1.18		**Calibration Working Standards**						20% HNO_3	Prepared by dilution of a top standard
	Nui_1	Sr	0.01	1	100	100	1.000		
		Cu, Fe, Mn, Mo, P, Zn	0.1	10	100	100	1.000		
		K, Na, Ca, Mg,	0.5	50	100	100	1.000		
	Nui_2	Sr	0.1	1	10	100	10.000		
		Cu, Fe, Mn, Mo, P, Zn	1	10	10	100	10.000		
		K, Na, Ca, Mg,	5	50	10	100	10.000		
	Nui_3	Sr	1	1000	1000	100	0.100		From stock
		Cu, Fe, Mn, Mo, P, Zn	10	1000	100	100	1.000		
		K, Na, Ca, Mg,	50	1000	20	100	5.000		
	Independent Standard - AQC								
	AQC	Sr	0.5	1000	2000	250	0.125		
		Cu, Fe, Mn, Mo, P, Zn	2	1000	500	250	0.500		
		K, Na, Ca, Mg,	2.5	1000	400	250	0.625		

Appendix D: Photographs of plant growth and some analysis of statistics

D1: Chapter 2

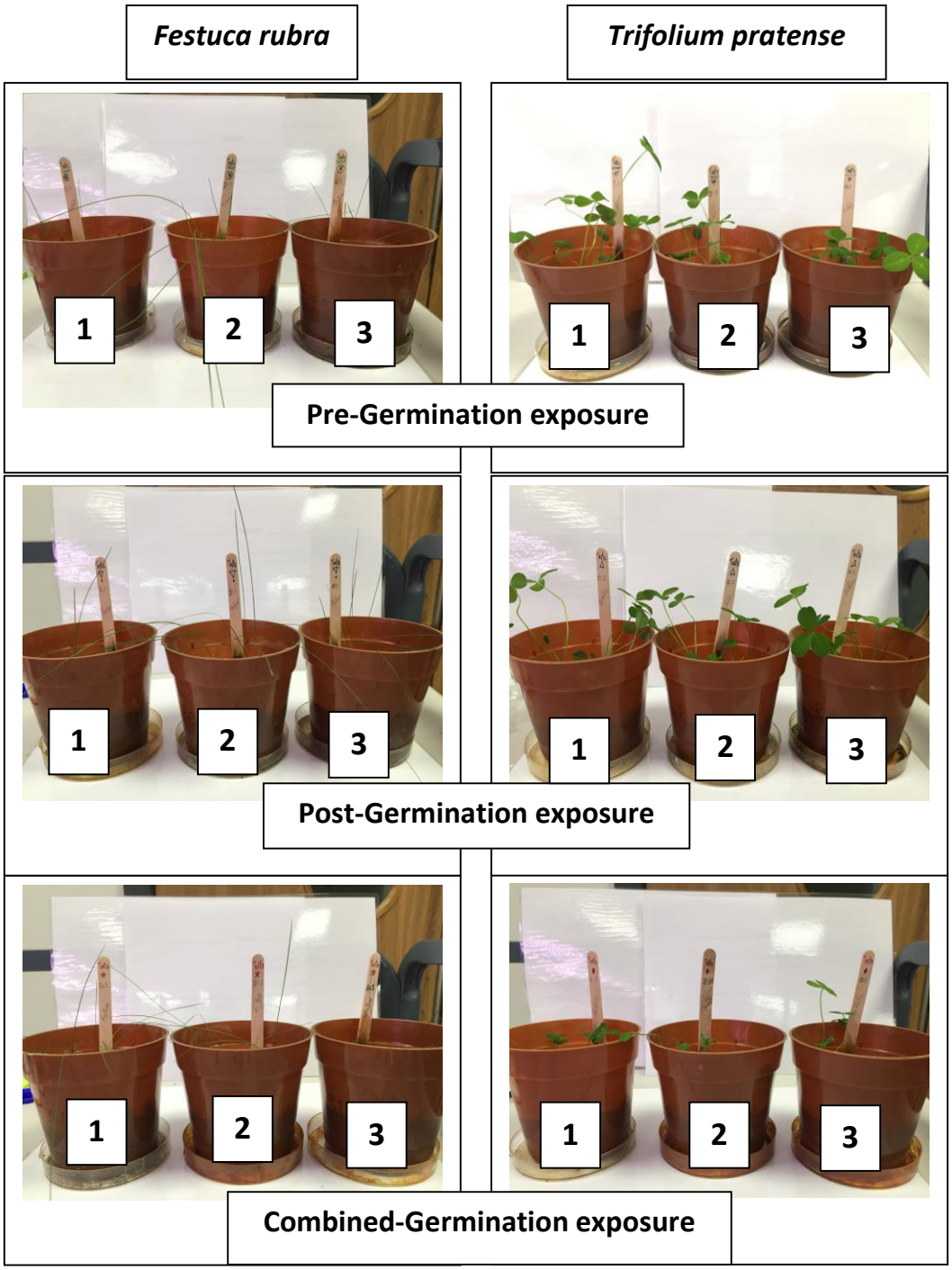


Figure D1.1 Preliminary test of growing *F. rubra* and *T. pratense* in different conditional treatment of $CaCl_2$ solution (1 = control, 2 = 20 mM $CaCl_2$, and 3 = 40 mM $CaCl_2$) at contaminated soil

Root length (*Festuca rubra*)

Experiment 1: Pre-Germination exposure

One-way ANOVA:

```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2   3.583  1.7917     2.43    0.121
Error       15  11.042  0.7361
Total       17  14.625

Model Summary
       S    R-sq  R-sq(adj)  R-sq(pred)
0.857969  24.50%     14.43%       0.00%

Pooled StDev = 0.857969
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)    N   Mean  Grouping
20      6  2.667  A
0       6  2.000  A B
40      6  1.583    B
```

Experiment 2: Post-Germination Exposure

One-way ANOVA:

```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2   1.444  0.7222     1.42    0.272
Error       15   7.625  0.5083
Total       17   9.069

Model Summary
       S    R-sq  R-sq(adj)  R-sq(pred)
0.712975  15.93%      4.72%       0.00%

Pooled StDev = 0.712975
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)    N   Mean  Grouping
40      6  2.583  A
20      6  2.417  A
0       6  1.917  A
```

Experiment 3: Combined-Germination Exposure

One-way ANOVA:

```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2  0.6944  0.3472     0.61    0.558
Error       15  8.5833  0.5722
Total       17  9.2778

Model Summary
       S   R-sq  R-sq(adj)  R-sq(pred)
0.756454  7.49%      0.00%       0.00%

Pooled StDev = 0.756454
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)   N   Mean  Grouping
40     6  2.250  A
0      6  2.250  A
20     6  1.833  A
```

Root length (*Trifolium pratense*)

Experiment 1: Pre-Germination exposure

One-way ANOVA:

```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2   8.541  4.2706     4.39    0.032
Error       15  14.595  0.9730
Total       17  23.136

Model Summary
       S    R-sq  R-sq(adj)  R-sq(pred)
0.986408  36.92%     28.51%       9.16%

Pooled StDev = 0.986408
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)   N   Mean  Grouping
0      6  6.267  A
20     6  5.083  A B
40     6  4.633    B
```

Experiment 2: Post-Germination Exposure

One-way ANOVA:

```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2   6.201   3.101     1.10    0.358
Error       15  42.263   2.818
Total       17  48.464

Model Summary
      S    R-sq  R-sq(adj)  R-sq(pred)
1.67856  12.80%      1.17%       0.00%

Pooled StDev = 1.67856
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)   N   Mean  Grouping
0      6  6.450  A
20     6  6.033  A
40     6  5.050  A
```

Experiment 3: Combined-Germination Exposure

One-way ANOVA:

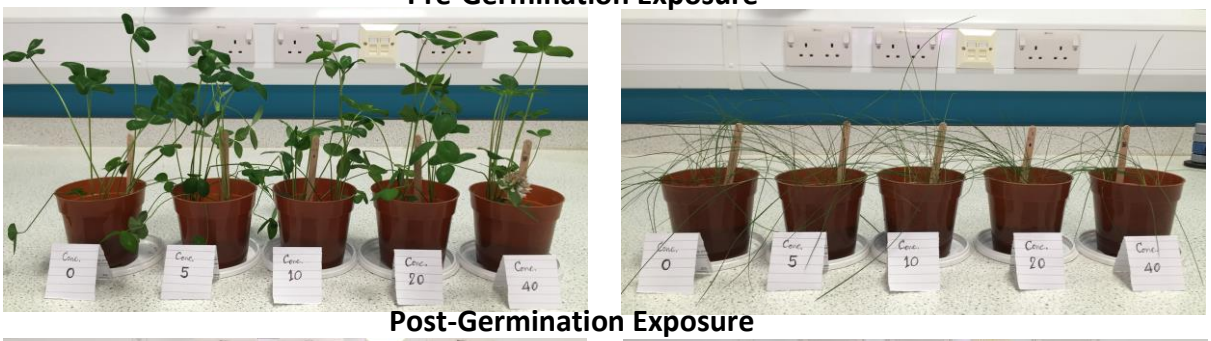
```
Significance level      α = 0.05

Factor      Levels  Values
Conc. (mM)       3  0, 20, 40

Analysis of Variance
Source      DF  Adj SS  Adj MS  F-Value  P-Value
Conc. (mM)   2   43.34  21.669    11.08    0.001
Error       15   29.34   1.956
Total       17   72.68

Model Summary
      S    R-sq  R-sq(adj)  R-sq(pred)
1.39857  59.63%     54.25%      41.87%

Pooled StDev = 1.39857
```

Fisher Pairwise Comparisons

```
Grouping Information Using the Fisher LSD Method and 95% Confidence
Conc.
(mM)   N   Mean  Grouping
20     6  4.333  A
0      6  3.367  A
40     6  0.667    B
```

Trifolium pratense ***Festuca rubra***

Pre-Germination Exposure



Post-Germination Exposure

Combined-Germination Exposure

Figure D1.2 *F. rubra* and *T. pratense* grown in different conditional treatment of contaminated soil at different concentration of $SrCl_2$ (0 = control, 5, 10, 20, and 40 mM $SrCl_2$)

Table D2.1 Langmuir and Freundlich adsorption constant related to the adsorption isotherms of Sr ion in the studied soil

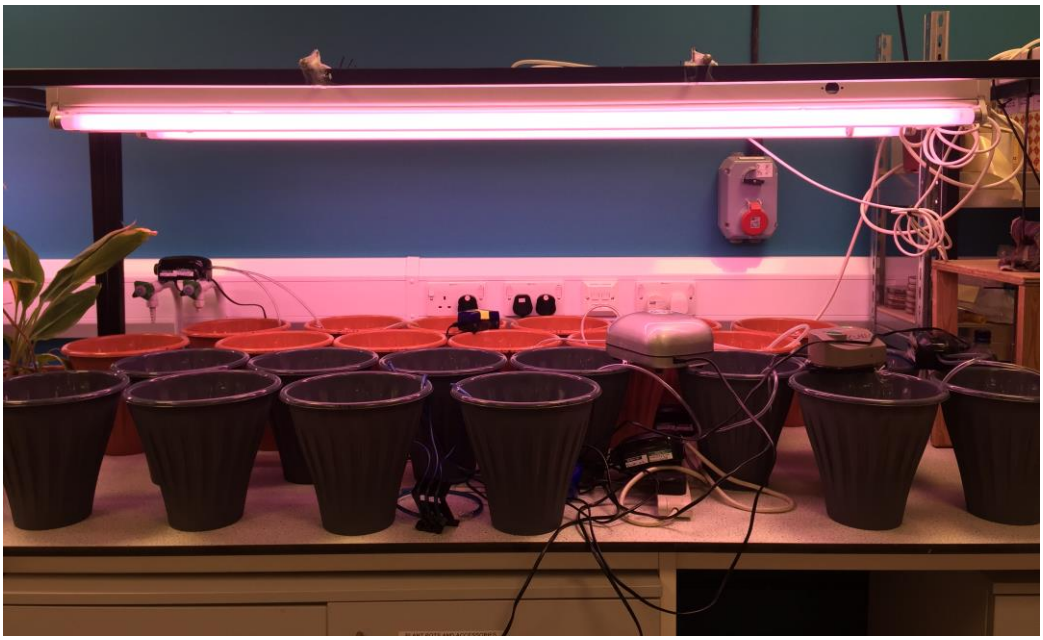
Time	Conc.	Langmuir constants			Freundlich constants		
	$SrCl_2$	Q_o	K_L	r^2	K_f	1/n	r^2
(h)	(mM)	($mg.g^{-1}$)	($L.m^{-1}$)		($mg.g^{-1}$)		
0	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	10	4.58	0.047	0.9953	0.203	0.09996	0.9984
	20	6.86	0.072	0.9759	0.498	0.09996	0.9833
	40	20.5	0.050	0.8532	0.824	0.09995	0.8656
1	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	10	3.86	0.059	0.9944	0.197	0.09996	0.9978
	20	7.81	0.087	0.9965	0.522	0.09996	0.9938
	40	23.1	0.066	0.9999	0.842	0.09995	0.9978
24	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	10	4.87	0.050	0.9888	0.212	0.09996	0.9942
	20	8.12	0.076	0.9796	0.519	0.09996	0.9942
	40	29.1	0.050	0.9991	0.847	0.09995	0.9855
168	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	10	4.28	0.043	0.9996	0.192	0.09996	0.9999
	20	10.2	0.038	0.9967	0.509	0.09996	0.9943
	40	27.6	0.045	0.9526	0.837	0.09995	0.9912
504	0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	10	5.87	0.030	0.9914	0.202	0.09996	0.9961
	20	16.0	0.026	0.9997	0.522	0.09996	0.9991
	40	32.0	0.026	0.9573	0.824	0.09995	0.9619

[n.d.= not detection]

Table D2.2 R_L and Q_e values for Langmuir and Freundlich isotherm adsorption of Sr ions on studied soil

Time (h)	**Conc. $SrCl_2$** (mM)	**Initial conc.** ($mg.L^{-1}$)	R_L	Q_e ($mg.g^{-1}$)
0	0	0	0	0
	10	45.9	0.024	1.60
	20	59.8	0.008	3.15
	40	174	0.006	6.67
1	0	0	0	0
	10	36.2	0.019	1.57
	20	49.8	0.007	3.33
	40	133	0.004	6.96
24	0	0	0	0
	10	43.4	0.023	1.63
	20	57.1	0.007	3.31
	40	173	0.006	7.03
168	0	0	0	0
	10	49.4	0.026	1.56
	20	113	0.015	3.23
	40	195	0.006	6.87
504	0	0	0	0
	10	72.3	0.037	1.59
	20	166	0.021	3.32
	40	337	0.011	6.67

D2: Chapter 3



Festuca rubra* and *Trifolium pratense

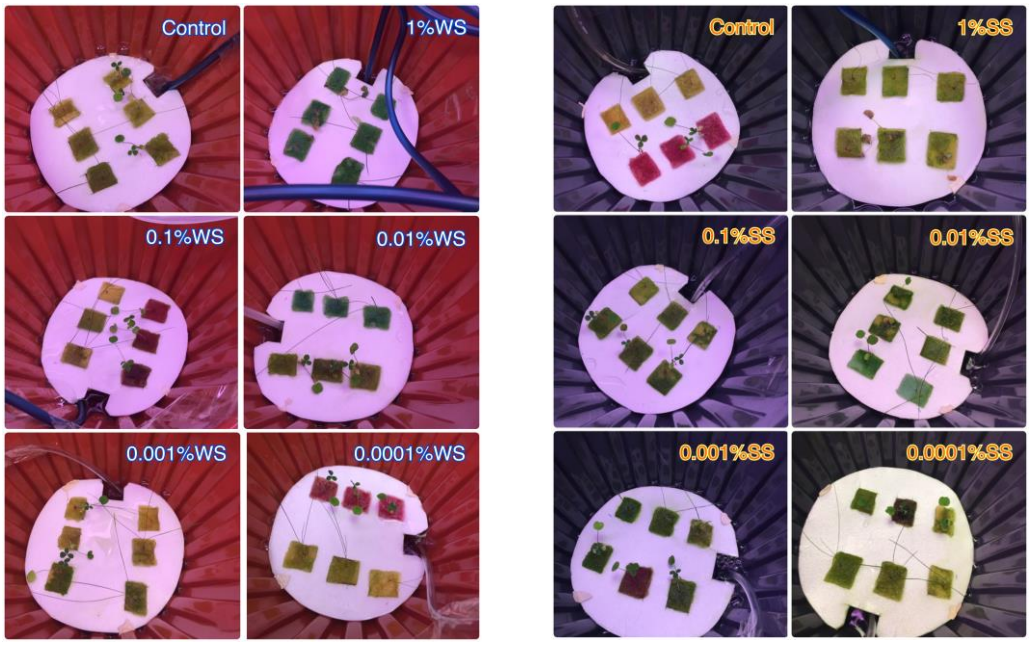


Figure D2.1 *F. rubra* and *T. pratense* growth in water culture system in different sources of solution and concentrations [wastewater (WS), and salinity synthetic solution (SS)]

Table D2.1 Mean value (±S.E.) of EC and pH in the conditions of different concentration between salinity (SS) and wastewater solutions (WS), with measured before and after hydroponic treatment for eight weeks

Conditions	EC (µs cm^{-1})						pH					
	Mean				*t*-value	*p*-value	Mean				*t*-value	*p*-value
	Pre-treatment		Post-treatment				Pre-treatment		Post-treatment			
Control	1.61	±0.102	38.8	±5.45	-6.79*	0.007	4.68	±0.155	5.18	±0.031	-2.7	0.074
0.0001%SS	2.31	±0.010	57.4	±0.350	-153*	0.004	4.76	±0.045	5.24	±0.005	-9.6	0.066
0.0001%WS	1.87	±0.016	67.6	±0.150	-395*	0.002	4.47	±0.030	5.32	±0.010	-21.3*	0.030
0.001%SS	7.89	±0.025	73.6	±0.150	-525*	0.001	4.86	±0.120	5.37	±0.050	-7.29	0.087
0.001%WS	5.89	±0.035	66.2	±0.550	-117*	0.005	4.53	±0.075	5.42	±0.085	-5.56	0.113
0.01%SS	97.6	±0.250	116	±0.450	-91*	0.007	4.69	±0.215	5.53	±0.060	-5.45	0.115
0.01%WS	63.2	±1.65	118	±3.20	-35.2*	0.018	4.61	±0.120	5.63	±0.120	-4.25	0.147
0.1%SS	729	±2.00	425	±0.997	304*	0.002	4.46	±0.105	5.57	±0.120	-74.3*	0.009
0.1%WS	571	±0	402	±4.99	33.8*	0.019	4.86	±0.085	5.63	±0.015	-7.7	0.082
1%SS	4070	±9.97	4840	±29.9	-38.5*	0.017	3.79	±0.140	4.95	±0.210	-16.6*	0.038
1%WS	3470	±0	3880	±40.0	-10.3	0.062	4.67	±0.120	5.13	±0.105	-30.3*	0.021

**Statistically different p≤0.05 with using paired t-test*

Table D2.2 Relative growth rate (RGR) of shoot height at 4 weeks and 8 weeks in different dilutions of wastewater and salinity solutions

Conditions	RGR ($g.g^{-1}.d^{-1}$)											
	Red fescue						Red clover					
	Week 4		Week 8		*t*-value	*p*-value	Week 4		Week 8		*t*-value	*p*-value
0%Control	0.111^{b}	±0.02	0.018^{b}	±0	7.11*	0.000	0.003^{a}	±0.01	$-0.054^{c,d}$	±0.01	4.43*	0.001
0.0001%SS	$0.130^{a,b}$	±0.03	0.065^{a}	±0.02	3.21*	0.024	$0.000^{a,b}$	±0.01	$-0.055^{b,c,d}$	±0.02	2.44	0.059
0.0001%WS	0.101^{b}	±0.02	$0.044^{a,b}$	±0.02	1.97	0.106	-0.063^{c}	±0.02	0.012^{a}	±0.04	-1.48	0.198
0.001%SS	$0.134^{a,b}$	±0.02	$0.056^{a,b}$	±0.01	3.22*	0.024	$-0.016^{a,b}$	±0.02	-0.093^{d}	±0.01	3.99*	0.010
0.001%WS	$0.122^{a,b}$	±0.02	0.070^{a}	±0.01	1.68	0.154	$-0.037^{b,c}$	±0.01	0.026^{a}	±0.03	-2.02	0.099
0.01%SS	$0.146^{a,b}$	±0.02	$0.035^{a,b}$	±0.01	5.64*	0.002	$-0.005^{a,b}$	±0.01	$-0.024^{a,b,c}$	±0.02	0.51	0.631
0.01%WS	$0.115^{a,b}$	±0.03	$0.055^{a,b}$	±0.02	1.81	0.130	$-0.032^{b,c}$	±0.01	$-0.071^{c,d}$	±0.02	1.23	0.272
0.1%SS	$0.143^{a,b}$	±0.02	0.074^{a}	±0.02	2.53	0.052	$-0.004^{a,b}$	±0.01	0.014^{a}	±0.04	-0.38	0.722
0.1%WS	$0.115^{a,b}$	±0.01	$0.035^{a,b}$	±0.03	2.16	0.083	$-0.026^{a,b}$	±0.01	0.023^{a}	±0.02	-2.17	0.082
1%SS	0.080^{b}	±0.04	0.009^{b}	±0.01	1.91	0.115	$0.000^{a,b}$	±0	$0.000^{a,b}$	±0	-	-
1%WS	0.179^{a}	±0.02	-0.063^{c}	±0.03	6.88*	0.001	$0.000^{a,b}$	±0	$0.000^{a,b}$	±0	-	-

*(Different letters in each column denoted significant differences (p≤0.05) based on LSD post-hoc test; *statistically different p≤0.05 with using paired t-test)*

D3: Chapter 4

(a)

(b)

(c)

Figure D3.1 *C. longus* and *P. australis* **(a)** juveniles germinated with deionized water, **(b)** conditional treatment with water culture system, **(c)** harvesting after 8 weeks [wastewater (WS), and salinity synthetic solution (SS)]

Table D3.1 Mean values (±S.E.) of concentration (mg.L^{-1}) 11 elements in different condition solutions with using plant growth for 8 weeks,

Conditions	**Ca**		**Cu**		**Fe**		**K**		**Mg**		**Mn**		**Mo**		**Na**		**P**		**Sr**		**Zn**	
MQL (mg.L^{-1})	0.02		0.001		0.001		0.1		0.0007		0.0002		0.0009		0.05		0.008		0.00003		0.0002	
0%Control-B	1.32	±0.26	0.296	±0.06	1.00	±0.2	1.43	±0.29	2.37	±0.47	0.923	±0.18	0.004	±0.001	0.831	±0.17	0.328	±0.06	0.000	±0	0.292	±0.06
0%Control-M	2.13	±0.08	0.312	±0.01	1.35	±0.04	2.59	±0.17	2.91	±0.05	1.11	±0.02	0.006	±0	1.54	±0.09	0.213	±0.03	0.001	±0	0.337	±0.01
0%Control-A	2.27	±0.11	0.282	±0.01	1.20	±0.05	2.78	±0.29	2.72	±0.06	0.993	±0.04	0.006	±0.001	3.34	±1.04	0.085	±0.04	0.002	±0	0.296	±0.01
0.1%SS-B	17.7	±0.05	0.414	±0.004	1.20	±0.03	2.12	±0.04	3.08	±0.02	1.20	±0.004	0.005	±0	83.6	±0.59	0.430	±0.01	0.003	±0	0.383	±0.002
0.1%SS-M	17.8	±0.58	0.363	±0.004	1.25	±0.02	0.898	±0.25	3.18	±0.03	1.19	±0.01	0.005	±0	83.5	±3.47	0.280	±0.004	0.005	±0	0.367	±0.004
0.1%SS-A	17.8	±0.91	0.349	±0.01	1.17	±0.04	0.859	±0.42	3.21	±0.09	1.18	±0.04	0.005	±0	85.2	±4.84	0.089	±0.02	0.005	±0	0.356	±0.01
0.1%WS-B	2.72	±0.03	0.426	±0.004	1.23	±0.01	2.00	±0.03	3.22	±0.02	1.24	±0.01	0.005	±0	77.9	±0.7	0.407	±0.001	0.011	±0	0.390	±0.001
0.1%WS-M	3.60	±0.09	0.376	±0.01	1.32	±0.01	1.35	±0.53	3.43	±0.09	1.25	±0.02	0.005	±0.001	82.2	±1.91	0.117	±0.07	0.013	±0	0.391	±0.02
0.1%WS-A	3.40	±0.04	0.326	±0.005	1.12	±0.02	2.10	±0.79	3.17	±0.09	1.11	±0.02	0.006	±0.001	77.2	±1.59	0.059	±0.05	0.012	±0	0.336	±0.01
1%SS-B	148	±2.60	0.453	±0.01	1.33	±0.02	4.77	±0.12	2.78	±0.05	1.12	±0.02	*<MQL*		839	±15	0.441	±0.01	0.026	±0	0.358	±0.01
1%SS-M	171	±3.48	0.442	±0.01	1.46	±0.03	3.60	±0.79	3.12	±0.10	1.19	±0.03	*<MQL*		975	±21	*<MQL*		0.032	±0.001	0.381	±0.01
1%SS-A	154	±1.87	0.378	±0.004	1.19	±0.01	3.01	±0.31	2.81	±0.1	1.04	±0.01	*<MQL*		876	±13	*<MQL*		0.030	±0	0.336	±0.01
1%WS-B	14.1	±0.17	0.467	±0.003	1.25	±0.01	3.73	±0.3	3.55	±0.03	1.20	±0.01	0.004	±0	786	±6.42	0.371	±0.01	0.098	±0.001	0.380	±0.004
1%WS-M	16.4	±0.78	0.409	±0.02	1.32	±0.04	3.72	±0.23	3.61	±0.15	1.13	±0.05	*<MQL*		887	±38	*<MQL*		0.109	±0.01	0.357	±0.01
1%WS-A	17.4	±0.12	0.398	±0.01	1.30	±0.03	5.31	±0.51	3.92	±0.06	1.16	±0.03	*<MQL*		964	±1.82	*<MQL*		0.115	±0.001	0.352	±0.01

[A= after treatment (week 8), B = before treatment, M= median procedure (week 4), <MQL = lower than Method Quantification Limits, SS= salinity solution, and WS= wastewater solution]

Appendix E: Publication

E1: Research Presentation Day, Faculty of Engineering University of Strathclyde 22nd June 2016

Efficacy of Plants to Phytoremediate Metals from Hydraulic Fracturing Wastewaters

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ABSTRACT

Many people have concerns regarding hydraulic fracturing technology and its potential environmental impact on soil and water. The main problems include toxic chemicals in flowback waters, and this can potentially pollute the environment as runoff and spills during transport. Phytoremediation technology has been used cleaning up heavy metal contaminants from the environment. It is both economic and effective, and it may be useful in this situation. The first aim of this research project focuses on the toxicological properties of strontium exposure on native species, *Trifolium pratense* L. (red clover) and *Festuca rubra* L. (red fescue), which have been selected for initial exposure study. Other studies will include the exposure effects of strontium to plant species associated with many treatment wetlands (e.g., *Phragmites* spp. or common reeds), and the ability of these plants to remove earth metals from the environment will also be examined.

INTRODUCTION

Currently, unconventional onshore development of natural gas is significantly increasing worldwide, particularly in the United States. This involves hydraulic fracturing (or 'fracking') to release gas from shale gas basins[2 & 3]. Hydraulic fracturing involves drilling vertical and horizontal wells, then injecting at high pressure fluids containing a range of chemicals into the shale gas formation to cause fracturing[1]. This method can cause the release of heavy metal contaminants into the injected fluids and groundwater. These fluids, containing the heavy metals, are extracted to the surface and could potentially pollute the environment as runoff and spills during transport or storage. The phytoremediation technique is one of the method was picked up. In order to use some vegetation to explain how much efficiency of plant structure that can be uptake or absorb substance of accumulation of heavy metal from fracking wastes. In this investigation, the aim was to assess an efficiency of these plants that can be indicated strontium exposure in the short-time.

METHODOLOGY

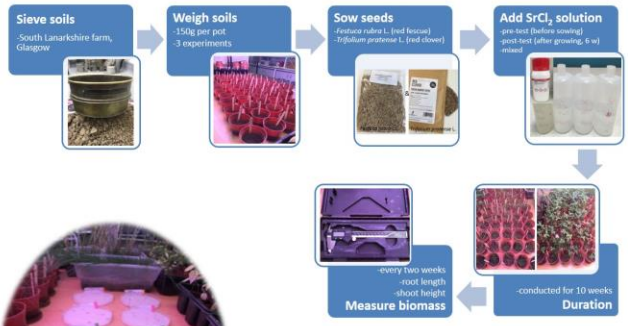

Figure 1: Schematic of research procedure

Figure 2: Showing preliminary test with sowing eight seeds on each paper tray in different $SrCl_2$ concentration in three weeks

RESULTS

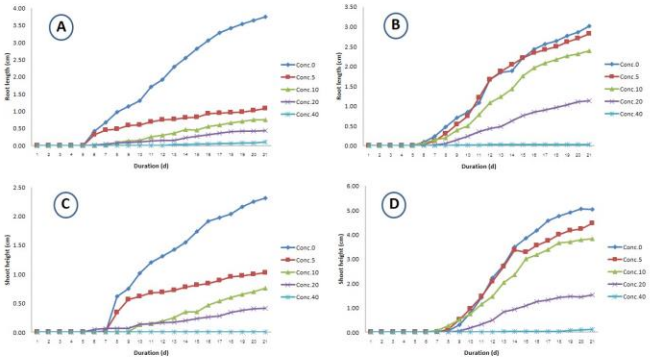

Figure 3: Showing the line charts of plant growth in different $SrCl_2$ solution (mM) in the paper trays; comparison of the root length in each concentration A= *T. pratense* and B= *F. rubra*, comparison of the shoot height C= *T. pratense* and D= *F. rubra*

DISCUSSION & CONCLUSIONS

There was a significant negative correlation between plant growth and $SrCl_2$ concentrations (Figure 3). This study has confirmed the findings of Jadia and Fulekar (2009) which found that at the lower concentration of heavy metal can activate increased plant growth, meanwhile the higher concentration was adversely affected to grow up[4]. However, these findings are limited that is only measure with biomass productively. Future studies will investigate Sr distribution in soil and plant tissues.

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E2: SETAC Europe 28th Annual Meeting in Rome, Italy on 13-17 May 2018

Comparing the growth of fescue and clover plants in petroleum industrial effluents and solutions of similar salinity

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1. Introduction & Objective

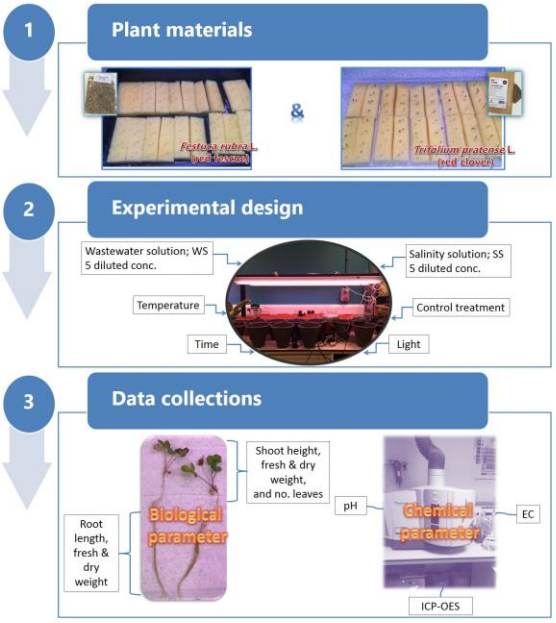
A current challenge of petroleum industry is environmentally acceptable discharge of wastewater, particularly the impact of its produced-water components. Certainly, salinity is one of the problems of produced water disposal, which is a major contributor of toxicity; however, it remains questionable whether other constituents in wastewater may also be toxic. As such, we examine differentially the effects of wastewater exposure to synthetic solutions of similar salinity. Therefore, this aim and objective is intended to as following;

(i) to investigate the efficiency of plant species (*Festuca rubra* and *Trifolium pratense*) to grow in different salinities;

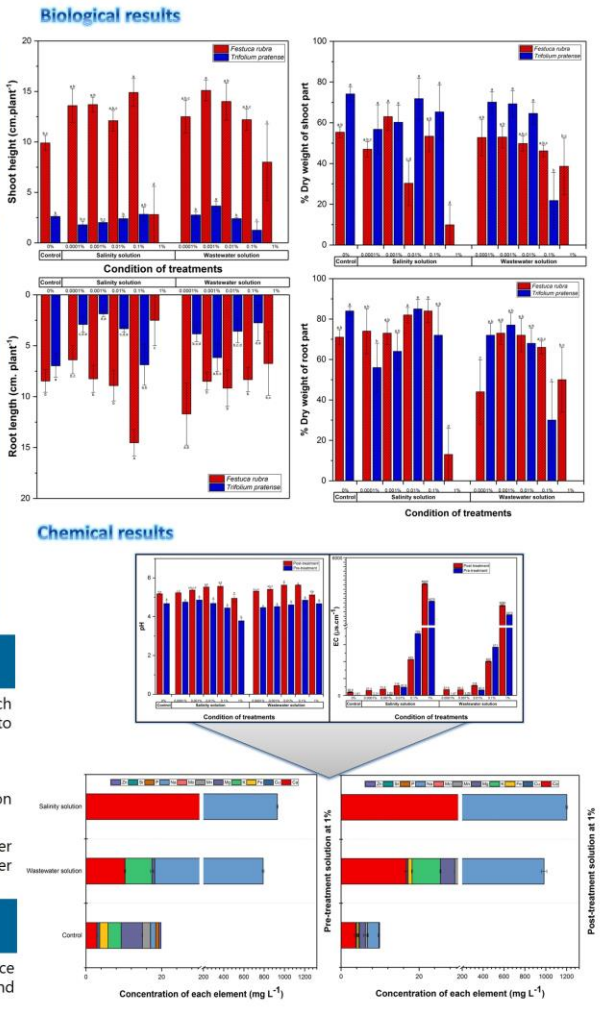
(ii) to determine whether similar growth patterns exist in brine wastewaters from a petroleum industry; and

(iii) to evaluate whether observable effect concentrations are comparable (or differentially toxic).

2. Materials & Methods



3. Results



4. Conclusions

- As expected, plants required dilutions for survival (0.1%-0.001%), which would be representative of downstream dilutions following discharge into surface water
- *F. rubra* was more halo-tolerant, and actually reduced salinity levels

  Further investigations with this other halophilic plants for remediation potential are underway.
- No indications of additional toxicity (other than salinity) in the wastewater from petroleum industry—at least at dilutions tested (being further examined)

5. Acknowledgements

The authors are grateful to acknowledgements the Ministry of Science and Technology under the Royal Thai Government for financial funding and the University of Strathclyde for laboratory facilities.