Tolerance, uptake, and translocation of platinum (Pt), nickel (Ni), and cobalt (Co) by *Tamarix usneoides* E. Mey. ex Bunge

By

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I. Declaration

I declare that this thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

(Anthony Edward Mader)

23rd day of October 2022

II. Abstract

The intensification of platinum (Pt), cobalt (Co) and nickel (Ni) mining and processing results in the release of salts and metals into the environment. This calls for the identification of halophytes with an ability to tolerate and desalinate metal-contaminated sites while simultaneously allocating metals (Pt, Ni, and Co) into harvestable biomass. *Tamarix usneoides* E. Mey ex Bunge is an indigenous exo-recretohalophyte that has been used for erosion control and for the desalination and allocation of metals from gold and uranium mine tailings and land contaminated by metallurgical effluent. The aim of this study was to investigate the uptake, translocation, and tolerance of Pt, Ni, and Co by *T. usneoides* from liquid medium (*in vitro*) and soil contaminated by base metal refinery effluent spillages and previous overspray from the enhanced evaporation spray system (*in situ*). More specifically, the *in situ* study investigated the utility of mature *T. usneoides* trees in the desalination of soil contaminated by previous metallurgical spillages and overspray emissions through the extraction of sulphur and metals Pt, Ni, and Co into harvestable biomass. Four *T. usneoides* trees were categorised into different size classes based on tree measurements and allometric equations. Seven soil pits (four "planted" and three "unplanted" – control) were excavated and opposite faces of the soil profile were sampled at 20 different intervals (0 – 340 cm). Soil samples were freeze-dried and analysed for total element concentrations. Root systems were harvested by excavating soil pits (maximum depth of 3.5 meters) using a mechanical excavator. Trees were harvested and immediately separated into above (leaves, twigs, wood, and flowers) and belowground (coarse and fine roots) plant organs. Tree biomass was further separated into different above (outer bark, inner bark, and sapwood and heartwood) and belowground (epidermis, cortex, and stele) tissue types. Plant material was rinsed three times in tap water to remove unbound residual metals and residual substrate from root and shoot surfaces. It must be noted that the determined metal concentrations are a combined measure of metals adsorbed on the root surface, assimilated *in planta*, and excreted on the plant surface from the foliar salt glands. Metals were allocated in trees (across plant organs and tissue types) in the order: Ni (59.46 ± 4.67 mg/kg) > Co (2.65 ± 0.34 mg/kg) > Pt (50 ± 6 µg/kg) whereas sulphur (S) was hyperaccumulated in tree leaves [39 900 ± 861 mg/kg (3.9% ± 0.7 %)]. Platinum was bioaccumulated

[bioconcentration factor (BCF) > 1.5] and translocated [translocation factor (TF) > 1] in the leafy shoots of one individual tree, Ni in one (BCF = 1.03), and Co in another replicate (BCF = 1.02). Soil chemical properties (pH, electrical conductivity, and redox potential) differed between planted and unplanted pits whereby pH and EC were lower in planted pits [pH 6.0; EC = 3 499 µS/cm (34.99 mM NaCl)] compared with unplanted [pH 7.6; EC = 9 644 µS/cm (96.44 mM NaCl)] (ANOVA, $p < 0.01$). The lower EC, along with S hyperaccumulation (BCF > 20; TF > 1), supports the potential use of *T. usneoides* for phytoextraction of S and Ni in shoot tissues and Co and Pt in roots. At a spacing of 1333 trees / ha, *T. usneoides* trees could remove an estimated 2.23 ± 0.30 mg Pt/ha, 3.02 ± 0.83 kg Ni/ha, 1.28 ± 0.90 kg Co/ha, and 1.28 ± 0.09 tons S/ha, excluding excreted salts. Excreted salts were visible but could not be quantified without confounding surface dust contamination. The first *in vitro* study determined factors influencing the rhizogenesis of *T. usneoides* in order to develop a mass propagation protocol. Explant establishment *in vitro* was influenced by various donor plant factors, *viz.* growing conditions (contaminated < non-contaminated; Kruskal-Wallis (KW), $p < 0.05$), physiological age (younger > older donor plants; ANOVA, $p < 0.05$), genotype (KW, $p < 0.001$), season of culturing (higher establishment in winter; KW, $p < 0.05$), length of explant (40 mm > 25 mm; KW, $p < 0.05$), and volume of growth vial (50 mL > 15 mL; KW, $p < 0.05$) but not pH, chronological age, strength of plant growth medium, or auxin pulse treatments. This study indicates that propagation protocols can be developed by controlling factors influencing explant establishment. A standardised and rapid *in vitro* protocol was developed for the mass propagation of *T. usneoides* explants. This *in vitro* protocol was used for the metal uptake studies whereby established explants were exposed to 25 % Murashige and Skoog standard plant growth medium supplemented with Pt, Co, or Ni (as sulphate complexes) at 0, 25, 50, or 100 mg/L at pH 5.5 or 7.5 over a 14-day exposure period. On completion of the metal exposure period, plantlets were harvested, separated into roots and shoots, freeze-dried, and analysed for metal concentrations. Higher metal concentrations (Ni > Co >> Pt) were accumulated in roots (combined measure of metals adsorbed on the root surface and assimilated *in planta*) compared with shoots whereby BCF > 1 (excluding Pt) and TF < 1. Metal BCF (Ni > Co >> Pt; KW, $p < 0.05$) and TF (Co > Ni >> Pt; KW, $p < 0.05$) increased in a dose-dependent fashion and were not influenced by pH level. Cobalt and Ni (≤ 50 mg/L) uptake dynamics did not

differ suggesting similar uptake dynamics, when treated separately. Platinum (defined in this study as Pt > 1 – 4 mg/kg), Ni (> 1 000 mg/kg), and Co (> 300 mg/kg) were hyperaccumulated in roots ("rhizo-hyperaccumulation") across treatments with possible Co-hyperaccumulation in shoots by two genotypes. Genotype influenced Co allocation in shoots but not Ni or Pt. Tolerance indices did not differ [Co (97 %) > Pt (82 %) > Ni (77 %)] between pH, metal, treatment concentration, or the interplay between these factors. Metal treatments did not impact measured morphological parameters (excluding Ni treatments which promoted shoot length increment) (KW, $p < 0.05$). Plantlet survival differed between pH and metals [Pt (90 %) > Ni (81 %) > Co (62 %)] (KW, $p < 0.05$). Variability in Co accumulation capacity between genotypes indicated that selective breeding, using the developed *in vitro* mass propagation protocol, for improved rhizofiltration and phytoextraction traits is feasible. Results demonstrate that *T. usneoides* has the potential for recovery of Ni and Co (and Pt to a lesser degree) from effluents, exhibiting a tolerance to Ni, Co, and Pt at 1, 10, and 10,000 times the average soil crustal abundance, respectively, under moderately acidic (pH 5.5) and alkaline (pH 7.5) conditions and across a wide metal concentration range. Results from the *in situ* study indicate that 9-year-old *T. usneoides* trees can be used for the decontamination of sulphate-contaminated soils under study site conditions which are more conducive to the survival of glycophytes. *Tamarix usneoides* is thus able to assimilate, translocate, and tolerate Ni, Co, and Pt (to a lesser degree) when exposed to metals across a wide pH and metal concentration range, under different (*in situ* and *in vitro*) experimental conditions. This opens the possibility for the species to be used in a range of phytotechnologies.

Keywords: halophyte, metallurgical effluent, micropropagation, phytomining, phytoremediation

III. Acknowledgments

Firstly, I would like to thank Professor David Mycock for his endless support, encouragement, and creating an environment to explore and challenge ideas, and for throwing the odd "curve ball" to expand my thinking. I would also like to thank Ms. Isabel Weiersbye for the invaluable insightful discussions and extensive support in the laboratory and the field, as well as for providing the scope of the project and sourcing funding.

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in my research and for all the love and support. Lastly, thank you to all my family and friends who have encouraged me and always been there to share laughs and make this journey memorable.

"*We are going to die, and that makes us the lucky ones. Most people are never going to die because they are never going to be born. The potential people who could have been here in my place but who will in fact never see the light of day outnumber the sand grains of Arabia. Certainly those unborn ghosts include greater poets than Keats, scientists greater than Newton. We know this because the set of possible people allowed by our DNA so massively exceeds the set of actual people. In the teeth of these stupefying odds it is you and I, in our ordinariness, that are here*" – Richard Dawkins (Unweaving the Rainbow)

IV. List of abbreviations

A

AAS	Atomic absorption spectroscopy
AFLP	Amplified polymorphism technique
AG	Aboveground
Ag	Silver
AlO	Aluminium oxide
AMD	Acid mine drainage
AMF	Arbuscular mycorrhiza fungi
ANOVA	Analysis of variance
Ar	Argon
ARD	Acid rock drainage
ARF	Adventitious root formation
As	Arsenic
ATP	Adenosine triphosphate
Au	Gold

B

B	Bark
Ba	Barium
BCF	Bioconcentration factor
BG	Belowground
BIC	Bushveld Igneous Complex
BMR	Base metal refinery

C

C	Cortex
Ca	Calcium
Cd	Cadmium
CDF	Cation diffusion facilitator
CEC	Cation exchange capacity
CIP	Coefficient of Industrial Pollution
Cl	Chloride
CO	Carbon monoxide
Co	Cobalt
CO_2	Carbon dioxide
$CoSO_4$	Cobalt sulphate
$CoSO_4 \cdot 7H_2O$	Cobalt sulphate heptahydrate
Cr	Chromium
CRM	Certified reference material
CS	Contaminated site
Cu	Copper
Cys	Cysteine

D

D_2	Canopy diameter
DM	Dry mass
DNA	Deoxyribose nucleic acid
DSB	Degree of shoot branching

E

E	Epidermis
E°	Standard potential
EC	Electrical conductivity
EDTA	Ethylenediaminetetraacetic acid
ED-XRF	Energy dispersive X-ray fluorescence
EES	Enhanced evaporation system
EESS	Enhanced evaporation spray system
E_h	Redox potential
ET	Evapotranspiration

F

Fe	Iron
FeO	Iron oxide

G

g	Gram
g/cm^3	Gram per cubic centimeter
g/mol	Grams per mole
GIS	Geographic information system
GSH	Glutathione

H

H^+	Proton
H_2O	Water
ha	Hectare
HCl	Hydrochloric acid
HF	Hydrofluoric acid
Hg	Mercury
HNO_3	Nitric acid
HSAB	Hard and soft acids and bases
HSD	Honest significant difference

I

IAA	Indole-3-acetic acid
IB	Inner bark
IBA	Indole-3-butyric acid
ICP-MS	Inductively coupled plasma-mass spectrometry
Ir	Iridium

K

kg	Kilogram
kg/m^3	Kilogram per cubic metre
kJ/mol	Kilojoule per mole
KPa	Kilopascal
KW	Kruskal-Wallis

L

L	Litre
La	Lanthanum
LD_{50}	Lethal dose
LDA	Linear discriminant analysis
LoA	Level of agreement

LoD	Limit of detection

M

m^2	Square metre
m^3	Cubic metre
MANOVA	Multivariate analysis of variance
MAPK	Mitogen-activated protein kinase
MDA	Malondialdehyde
Mg	Magnesium
mg	Milligram
mg/kg	Milligram per kilogram
mg/L	Milligram per litre
MgO	Magnesium oxide
mL	Millilitre
Mn	Manganese
Mo	Molybdenum
MS	Murashige and Skoog
MTI	Mass tolerance index
MTP	Metal transporter proteins
mV	Millivolts

N

Na	Sodium
NAA	Naphthaleneacetic acid
NaClO	Sodium hypochlorite
NaOH	Sodium hydroxide
$NaSO_3$	Sodium sulphite
NCS	Non-contaminated site
Ne	Neon
NEMA	National Environmental Management Act (Act No.107 of 1998)
NEM:WA	National Environmental Management: Waste Act (Act No. 59 of 2008)
NH_3	Ammonia
Ni	Nickel
$NiSO_4$	Nickel sulphate
$NiSO_4 \cdot 6H_2O$	Nickel sulphate hexahydrate
NORM	Naturally occurring radioactive material
NR	Number of roots

O

O_2	Oxygen
OB	Outer bark
Os	Osmium

P

P	Phosphorous
Pb	Lead
PC	Phytochelatins
Pd	Palladium
PGE	Platinum group element
pH	Potential of hydrogen
PMR	Precious metal refinery

ppm	Parts per million
Pt	Platinum
PTC	Plant tissue culture
PTFE	Polytetrafluoroethylene
$PtSO_4$	Platinum sulphate

Q

QC	Quality control
QGIS	Quantum geographic information systems

R

RB	Degree of root branching
Rh	Rhodium
RL	Root length
ROS	Reactive oxygen species
RS	Rocky soil
RSA	Root system architecture
RSL	Rustenburg Layer Suite
Ru	Ruthenium

S

S	Sulphur
SA	South Africa
SB	Shoot branching
SBD	Stem basal diameter
Sc	Scandium
SD	Stem diameter
Se	Selenium
SE	Standard error
SH	Sapwood and heartwood
Si	Silica
SL	Shoot length
SO_2	Sulphur dioxide
$SO4^{2-}$	Sulphate
SOM	Soil organic matter
Sr	Strontium

T

Te	Tellurium
TF	Translocation factor
Th	Thallium
TI	Tolerance index
Ti	Titanium
TLB	Tractor loader backhoe
TPSA	Topological polar surface area
TR	Taproot
TSF	Tailing storage facilities

U

U	Uranium
UG2	Upper ground 2

V

V	Vanadium

VB	Vascular bundle

X

Xe	Xenon

Y

Y	Yttrium

Z

Zn	Zinc
Zr	Zirconium

Symbols

%	Percentage
°C	Degree Celsius
µg	Microgram
µg/kg	Microgram per kilogram
µM	Micromolar
µm	Micrometre
µS/cm	MicroSiemens per centimetre
χ^2	Chi-squared

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V. Structure of the Thesis

This thesis is comprised of five chapters. Three of these chapters are data chapters and Chapter 1 and Chapter 5 are the introductory and concluding chapter, respectively.

Chapter 1 contextualises the thesis relative to the platinum (Pt) mining industry, the introduction of contaminants into the environment, negative impacts associated with the presence of these metals in the environment, physicochemical and biological (i.e., phytoremediation) technologies for the removal of these metals, introducing and providing a rational for the use of the study species, namely *Tamarix usneoides*. This chapter also provides the rationale, aims, and objectives of this thesis.

Chapter 2 investigates the allocation of sulphur (S), platinum (Pt), nickel (Ni), and cobalt (Co) in 9-year-old *Tamarix usneoides* (salt cedar) grown on soil contaminated by base metal refinery effluent. Two manuscripts will be prepared from this chapter, namely (1) *Fate of sulphur, platinum, nickel, and cobalt in a 9-year-old phytoremediation trial with Tamarix usneoides (salt cedar)*: in process of being prepared as a manuscript for publication in the *International Journal of Phytoremediation*, and (2) *Estimating Tamarix usneoides tree biomass, grown in platinum- and gold-mining sites, using allometric equations – applications in phytoremediation*: in process of being prepared as a manuscript for publication in the *Journal of Plant Ecology*.

Chapter 3 explores numerous variables which may have accounted for the low establishment percentage of *T. usneoides* explants cultured *in vitro*. This data chapter investigates aspects of the different stages of plant tissue culture which may have limited the number of established explants required for the metal recovery experiment (Chapter 4). This chapter will be prepared, entitled *Development of a protocol for in vitro mass propagation of Tamarix usneoides – an exo-recretohalophyte used for phytoremediation*, for publication in *Plant Cell, Tissue and Organ Culture (PCTOC).*

Chapter 4 investigates the recovery of Pt, Ni, and Co (as metal-sulphate complexes) by *T. usneoides* from solution across a wide pH (pH 5.5 or 7.5) and concentration range (25, 50, or 100

mg/L). A manuscript, entitled *Recovery of platinum (Pt), cobalt (Co), and nickel (Ni) from solution by Tamarix usneoides*, has been prepared and submitted to the journal *Plant Physiology and Biochemistry*.

Chapter 5 summarises the findings of preceding chapters, providing the biological implications of this research.

The primary author of this thesis has published a review paper, entitled *Treatment wetlands and phyto-technologies for remediation of winery effluent: Challenges and opportunities*, in the journal, *Science of the Total Environment* (https://doi.org/10.1016/j.scitotenv.2021.150544). This paper reviews the remediation of winery wastewater (WWW) by treatment wetlands (TWs). A major challenge associated with wine production includes the ineffective removal of persistent inorganics, namely salts and their constituents, by TWs. A review of available literature indicates that glycophytes have been extensively used in TWs due to their ability to hyperaccumulate a range of elements from contaminated land and water. However, glycophytes are ineffective at assimilating these elements in the presence of salts due to the lack of efficient salt tolerance mechanisms. The predicted increase in primary (amid global climate change) and secondary (introduction of salts into the environment via anthropogenic processes) salinisation limits the ability to use glycophytes for the phytoremediation of elements from saline land or water. This presents a direct link to the scope of this thesis whereby *Tamarix usneoides* (study species) was planted on a salt and metal cross-contaminated site (*refer to Chapter* 2) to advance the survivable conditions for *Searsia lancea* and *S. pendulina* (to provide hydraulic control) and *Berkheya coddii* (to extract Ni and Co). A similar challenge, relative to the wine production industry, is detailed in the Author's publication. The Author presents the case for the use of halophytes in TWs to reduce the salt load (i.e. phytodesalination) of WWW – providing a novel, potential solution to a major challenge associated with the wine production. Chapters 3 and 4 present a protocol for the propagation of *T. usneoides*. Based on the design of TWs (e.g. substrateless system), *T. usneoides* may be mass propagated (and screened) for use in TWs. The scope of this thesis and the published paper investigate the use of halophytes to reduce the salt load of saline land or water (i.e. phytodesalination) cross-contaminated with various elements.

Each data chapter comprises an Abstract, Introduction, Materials and Methods, Results, Discussion, Reference, and Supplementary Data. Please note that due to the scope of each chapter, some repetition of introductory information exists between chapters. The author has attempted to minimise such repetition throughout the chapters; however, it is acknowledged that some repetition may still occur throughout this thesis.

VI. Contribution of authors (primary author, supervisor, advisor, and other co-authors)

The contribution of authors mentioned in the following data chapters are summarised as follows:

Anthony E Mader: Primary author who conducted the field and laboratory research and prepared the thesis (75 %)

Prof. David J. Mycock: Supervisor, reviewed thesis, held numerous discussions, and provided tissue culture facilities and advice (15 %)

Ms Isabel M. Weiersbye: Provided project scope, laboratory facilities, PhD advisor and reviewed the thesis and prepared manuscripts (10 %)

The following co-authors are acknowledged and will be included in manuscripts to be published.

- Mr Chris Davies (Chapter 2) provided field assistance and logistical support.
- Dr Hayden T. Wilson (Chapter 3) mentored the primary author with regards to plant tissue culture protocol during the primary author's honours research.

Chapter 1

Introduction

1.1. Platinum, nickel, and cobalt mining and production

South Africa (SA) has abundant natural resources. Platinum (Pt), as well as nickel (Ni) and cobalt (Co) (which co-occur with Pt) are major contributors to the South African economy (Glaister and Mudd, 2010). Approximately 75 % of the earth's platinum group elements (PGEs), which includes Pt, palladium (Pd), rhodium (Rh), iridium (Ir), osmium (Os), and ruthenium (Ru), are located in the Bushveld Igneous Complex (BIC), within SA (Cawthorn, 2010, Matthey, 2016). Within these layered mafic and ultramafic igneous horizons, magmatic base metal sulphide (S^{2-}) minerals are commonly Pt-dominated and contain significant amounts of Ni and copper (Cu), and to a lesser extent Co (Figure 1.1) (Cawthorn, 2010).

Platinum, as well as Ni and Co, have a high affinity for sulphur (S) and may naturally occur as S complexes. These metals may be adsorbed to the surface of S species (such as the surface of pyrrhotite [Fe_8S_9], chalcopyrite [$CuFeS_2$], and/or pentlandite [$(Ni, Fe)_9S_8$] particles), dissolved in base metal S^{2-} solutions (such as pentlandite), and/or present as mineral particles (such as braggite [(Pt, Pd, Ni)S]) (Langa *et al.*, 2021).

Our world is becoming increasingly dependent on PGEs, Ni, and Co for their use in various industries (Table S1.1), where the scarcity, physicochemical properties, and low substitutability of these critical elements increases their demand in such industries (Faucon *et al.*, 2018, Won *et al.*, 2014). For example, the transition to the hydrogen economy (i.e., renewable energy industry) will elevate future demand for Pt and Co.

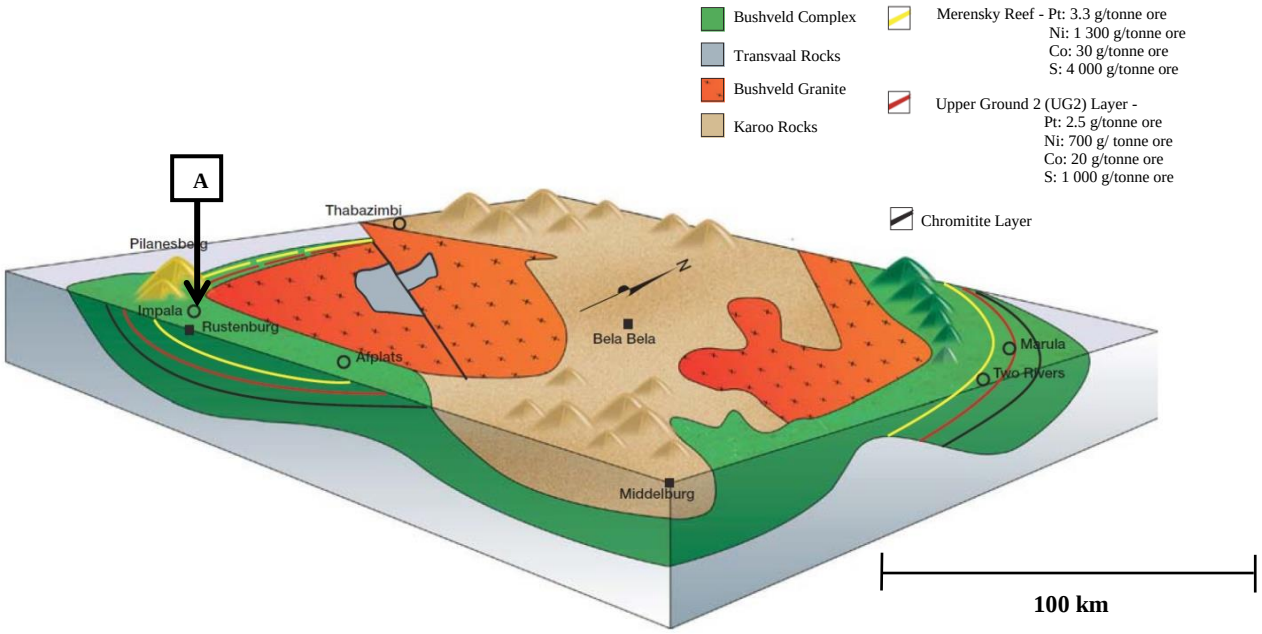

Figure 1.1. Bushveld Igneous Complex (BIC) with associated geology and horizons of economic importance. A: Rustenburg Layer Suite (RLS), the most economically important layer consisting of Merensky Reef and Upper Ground 2 (UG2). Adapted from Langa *et al.* (2021), Cawthorn (2010), and Implats (2021). Note, Pt = platinum, Ni: nickel, Co: cobalt, S: sulphur.

The mining and processing (concentrating, smelting, and refining) of Pt, Ni, and Co differ based on the elemental composition of the mined ore (Figure S.1.1) (Crundwell *et al.*, 2011). Tailings, present in solid, liquid, and / or gaseous forms, produced by the metal mining and processing industries are transported to TSF, such as storage ponds or mine dumps (Tutu *et al.*, 2008). The increased demand for critical elements (Mudd *et al.*, 2018) has consequently elevated the production and storage of tailings.

1.2. Introduction of metals into the environment

Platinum, Ni, and Co may be present or released into the environment via numerous natural and anthropogenic sources. Natural pathways include atmospheric (e.g., geothermal events such as volcanic eruptions) and geological (parent rock material) sources whilst anthropogenic sources include metal mining and processing (Rauch and Fatoki, 2013), vehicle emissions (thermal and mechanical attrition of vehicle catalytic converters) (Juan *et al.*, 2007), as well as the release of

domestic effluents (such as sewage and hospital effluent) (Kümmerer *et al.*, 1999) into the environment. Heavy metals and salts are released into the environment via TSF wall failure, metallurgical effluent spillages, historical mining activities (e.g. TSF footprints), TSF reclamation, aeolian erosion (dust degeneration/ emission), evaporation spray systems, storage pond leakages, tailing weathering (precipitation and subsequent oxidation of tailings), and metal end-use activities, use in renewable technologies (e.g. hydrogen fuel cells, medical waste, and electronics), and e-waste (Figure 1.2).

Tailing storage facilities constitute a major source of release of metal/loids and salts into the environment. Tailing storage facilities, TSF footprints, as well as areas of contaminant spillages may contain multiple contaminants such as inorganics [salts, metals, naturally occurring radioactive materials (NORMs, such as thorium and uranium (U)) and other radionuclides] and organics (cyanide complexes and explosive residues) produced by the mining and precious metal production processes (Naicker *et al.*, 2003, Tutu *et al.*, 2008).

Rauch and Fatoki (2013) demonstrated that elevated levels of Pt (698 ± 178 µg/kg) were present within the topsoil (0 - 2 cm) of areas surrounding smelters compared with surrounding towns (where vehicle emissions were a major contributor to Pt release). These results are supported by other studies indicating low mobility of Pt down the soil profile. Soils located near Ni smelters were reported to contain elevated Ni concentrations ranging from 200 – 22 000 mg/kg, compared with uncontaminated soils (10 – 1 000 mg/kg) (Everhart *et al.*, 2006). Various studies have reported that metal production and refining activities are major contributors to the introduction of PGEs and other metals into the environment, influencing the global biogeochemical cycle of these elements (Almécija *et al.*, 2017, Li *et al.*, 2011, Tutu *et al.*, 2008) (Figure 1.2). This indicates that metal refineries and smelters are important anthropogenic sources of metal pollution, contributing to the introduction of elevated levels of contaminants into the environment.

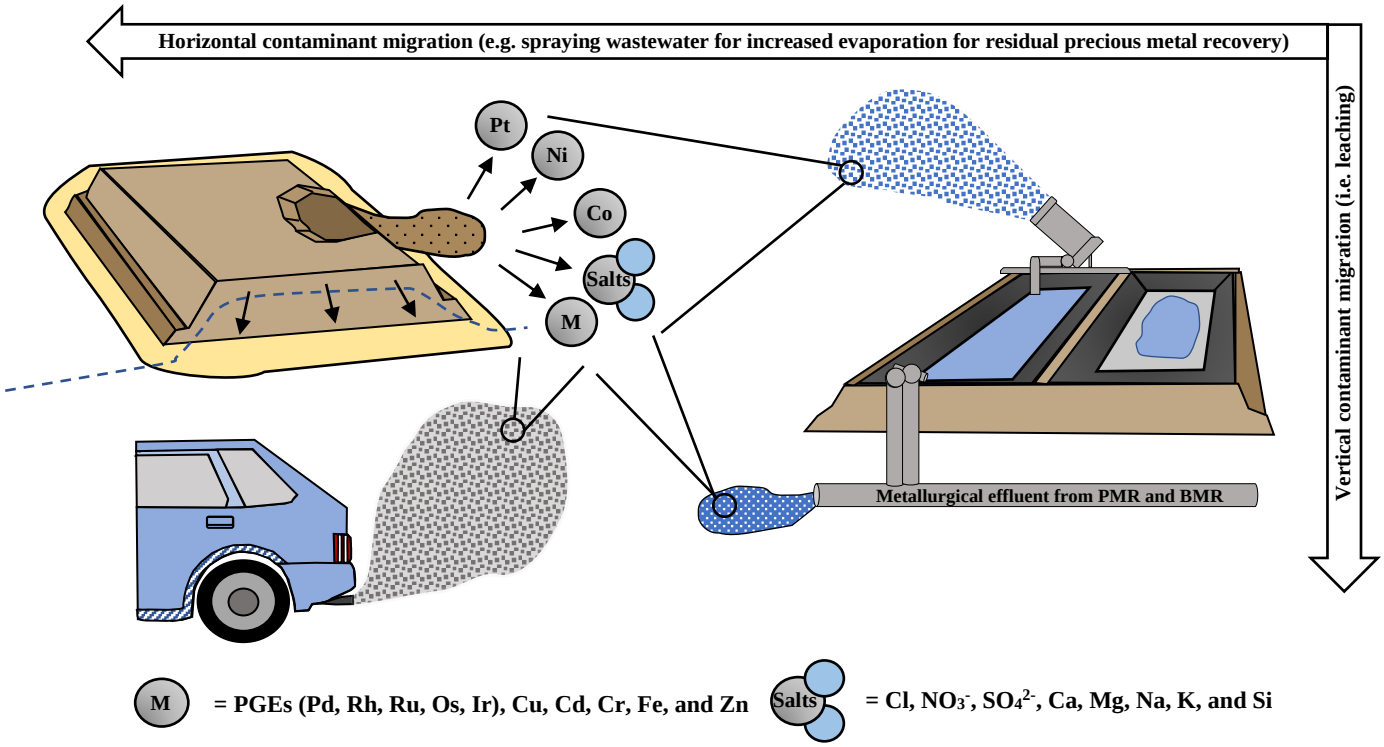

Figure 1.2. Introduction of platinum (Pt), nickel (Ni), cobalt (Co), and salts into the environment by activities associated with the storage of tailings (e.g., tailing storage facilities (TSFs)) and metal end-use activities. Salts present in Pt TSFs include sulphates (SO_4^{2-}), chlorides (Cl^-), nitrates (NO_3^-), sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), and silicon (Si). Metals include platinum (Pt), nickel (Ni), cobalt (Co), PGEs, copper (Cu), cadmium (Cd), chromium (Cr), iron (Fe), and zinc (Zn). PMR: Precious metal refinery; BMR: Base metal refinery. References: Tutu *et al.* (2008), Kümmerer *et al.* (1999), Juan *et al.* (2007), and Almécija *et al.* (2017).

1.3. Impact of Pt, Ni, and Co in the environment

In SA, mining has major short- and long-term environmental impacts (e.g., land and water (surface and groundwater contamination) (Erasmus *et al.*, 2020), and socio-economic impacts (*viz.* human health, village relocations, compensation challenges, and drinking water pollution) (Farrell *et al.*, 2012, Glaister and Mudd, 2010). Moreover, approximately 96 – 98 % of mined ore is discarded as tailings or waste rock resulting in various environmental impacts, such as TSF spillages, leakages, and failures, the generation of acid rock drainage (ARD), and dust production, negatively impacting fauna (including humans) and flora health and survival (Tutu *et al.*, 2008).

1.3.1. Impact on fauna

Fauna require certain levels of macro- and micronutrients (Ni) to function and survive however, exposure to excess concentrations of such elements (as well as non-essential elements – Co and Pt) may cause varying degrees of skin irritations, sensitisation of airways, mutagenic and carcinogenic effects including increased frequency of tumours, as well as hepatic and renal damage and neurological disorders (Genchi *et al.*, 2020, Lison *et al.*, 2018). Platinum, Ni, and Co can enter the food chain by the bioaccumulation of these metals in edible plant species [see Khan *et al.* (2015) for review]. This highlights the need to determine the composition and concentration of metal/loids in the soil as well as plant species' ability to assimilate these elements. As outlined in Khan *et al.* (2015), excess metals may negatively impact the nutrient status, growth and survival of plants.

1.3.2. Impact on flora

Essential micronutrients [Ni, boron (B), Cl^-, Cu, Fe, manganese (Mn), molybdenum (Mo), and Zn] are defined as (i) elements that the plant requires to complete its life cycle, (ii) being directly involved in metabolic functioning, and (iii) where the function of the nutrient cannot be replaced by any other nutrient (Arnon and Stout, 1939). These essential elements are involved in important biochemical and physiological processes, such as redox reactions, and are vital constituents of numerous molecules including enzymes. For example, Ni is incorporated in urease, an enzyme responsible for hydrolysing urea into carbon dioxide (CO_2) and NH_3 (Eskew *et al.*, 1984), whereas the role of Co in non-leguminous plants is yet to be determined (Akeel and Jahan, 2020). However, Co is an essential micronutrient (incorporated into vitamin B12) required by animals. Although micronutrients are required by plants, excess concentrations of these elements are phytotoxic. Phytotoxicity levels are metal-, site-, and plant species- dependent ranging from 10 mg Ni/kg (sensitive plant species) to > 50 mg Ni/kg (indicator species), to > 1 000 mg Ni/kg (tolerant plant species such as *Alyssum spp.* and *Thalspi spp.*) (Assunção *et al.*, 2001), whereas Co phytotoxicity levels for Co-sensitive plant species is approximately 0.4 mg/kg.

Platinum, Ni, and Co are classified as trace elements[1] which is attributed to their relatively low natural concentrations in the environment compared with other elements. However, excess concentrations of these elements have been documented to negatively impact numerous plant species relative to genetic, molecular, cellular, and physiological factors (Table S1.3). Heavy metal toxicity induces osmotic and ionic stress in plants (Munns and Tester, 2008), impacting the plant's ion homeostasis network[2] (Egorova *et al.*, 2019). Platinum exposure has been reported to reduce plant growth and water content of *Arabidopsis thaliana* (Gawrońska *et al.*, 2018b) and *Lemna minor* (Bednarova *et al.*, 2014), resulting in chlorosis and necrosis. Exposure to elevated Ni concentrations has been demonstrated to disrupt membrane lipid composition (Ros *et al.*, 1992), nutrient imbalances (Sabir *et al.*, 2011), reduction in water content (Llamas *et al.*, 2008), as well as leaf chlorosis and necrosis (Shukla and Gopal, 2009). Li *et al*. (2009) reported that the growth of *Lycopersicon esculentum* (tomato), *Hordeum vulgare* L. (barley), and *Brassica napus* L. (oilseed rape) was reduced by 50 % when plants were exposed to 57 mg/kg, 89 mg/kg, and 169 mg/kg Co, respectively. Cobalt phytotoxicity symptoms also include nutrient imbalances and decrease water content (Chatterjee and Chatterjee, 2000), generation of reactive oxygen species (Tewari *et al.*, 2002) as well as reduce the rate of transpiration and photosynthesis (Lwalaba *et al.*, 2017). Thus, the ecotoxicity of these elements highlights the need to alleviate the negative impacts associated with toxic metal(s) assimilation, and subsequent exposure, in the environment

1.4. Phytoremediation

Numerous physical, chemical, thermal and biological remediation mechanisms have been studied and implemented for the remediation of a range of elements (ITRC, 2009). The cost of physical, chemical, and thermal remediation technologies ranges from 1 384 580 – 41 537 400 ZAR/ha whereas biological remediation (such as phytoremediation) offers a relatively inexpensive (2 769 – 1 384 580 ZAR/ha) remediation technology which, unlike physical, chemical and thermal

[1]In the context of this study, a *trace element* is defined as an element found in low concentrations/ mass fractions (e.g. mg/kg) in a particular matrix such as soil or plant tissue.

[2]The plant's ability to control the distribution of the exact amount of metals needed by metal-requiring molecules at a specific time whilst avoiding exposure to metal-sensitive areas within the plant cell.

remediation, confers simultaneous *in situ* decontamination and rehabilitation of contaminated land or water (Garbisu and Alkorta, 2001, Weiersbye, 2007).

Phytoremediation involves the use of plants to sequester contaminants in the rhizosphere, degrade contaminants in the rhizosphere and / or *in planta*, and accumulate, extract, volatilise contaminants, stabilise substrate and/or control water content and movement (surface and sub-surface water) of a particular contaminated site (Table 1.1) (Salt *et al.*, 1998). Phytoremediation has been used to remediate the soil, surface- and groundwater contaminated by acid rock drainage (ARD) and metallurgical effluent spillages (Tutu *et al.*, 2008, Winde *et al.*, 2004). For example, the Mine Woodlands Project (MWP) is a large-scale phytoremediation program whereby various tree species (including *Tamarix usneoides*, *Searsia lancea*, and *S. pendulina*) have been planted in seepage pathways to reduce the horizontal and vertical migration of contaminants (Dye and Weiersbye, 2010). These species were selected to provide hydraulic control, as well as stabilise and extract contaminants from polluted sites (Table 1.1). Various phytotechnologies, *viz.* rhizofiltration, phytoextraction, phytodesalination, phytostabilisation, and phytohydraulics, are used as tools for phytoremediation (Table 1.1). Phytoremediation (and potentially phytomining) is a cost-effective process that may extract a desirable contaminant of economic value whilst simultaneously decontaminating and rehabilitating contaminated sites (Weiersbye and Witkowski, 2007).

Table 1.1. Phytotechnologies used as tools for phytoremediation. Phytotechnologies described below are applicable to the study species and site conditions. References: (ITRC, 2009, Pulford and Watson, 2003).

Phytotechnology	Description of potential mechanism(s)	References
Rhizofiltration	Sorption (adsorption / absorption) of metals by plant roots from waste streams (e.g. metallurgical effluent). Metals may be (hyper)accumulated within roots ("*rhizoaccumulators*").	(Dushenkov *et al.*, 1995)
Phytoextraction	Allocation of elevated metals (BCF > 1, TF > 1) from soil into harvestable plant organs (e.g. leaves, stem, etc.).	(Kumar *et al.*, 1995)
Phytodesalination	Removal of elevated salt loads from contaminated land and / or aqueous solution. Halophytes are utilised due to various molecular, morphological, and/or physiological adaptations (e.g. excretion of salts via salt glands) which maintains ion homeostasis network. Phytodesalination may be used to advance favourable conditions for glycophytes (salinity-intolerant plant species).	(Flowers and Colmer, 2015, Rabhi *et al.*, 2010)
Phytostabilisation (and phytosequestration)	Reduction in the mobility and bioavailability of metals by the immobilisation of metals via the (i) release of exudates which bind with metals, and / or adsorption by plant roots. Plants establish vegetative covers – promoting erosion control.	(Shackira and Puthur, 2019, Smith and Bradshaw, 1992)
Phytohydraulics	Plant roots intercept and prevent the horizontal migration of contaminants within groundwater. This involves the reversal of the hydraulic gradient into a cone of depression (by extracting groundwater down the hydraulic gradient), creating a contaminant 'capture zone'. This reduces the hydraulic gradient within the plume when groundwater is extracted up-gradient of the plume.	(Ahlfeld and Heidari, 1994, ITRC, 2009)

1.4.1. The case for rhizofiltration

Aqueous waste streams, generated by mining and metallurgical (i.e., PMR and BMR) industries (Figure S.1.1), contain economically significant concentrations of critical elements (Chojnacka *et al.*, 2004). Along with other metals, waste streams produced by metal refineries are characterised by high salt content (namely Cl^- and SO_4^{2-}), low pH levels, and elevated ammonia levels. The recovery of these precious metals from secondary resources (e.g. metallurgical effluent and e-waste) has gained increased attention from academic and industrial sectors due to the (i)

economic significance of these critical elements [elucidated by their current trading prices [Co: USD \$33.50 kg^{-1}; Ni: USD \$14.99 kg^{-1}; and Pt: USD \$24 958.94 g^{-1} (LME, 2022)], (ii) ecotoxicity of these elements, (iii) broad range of recovery technologies (Falagán *et al.*, 2017), and (iii) residual concentrations of these critical elements in natural ore and mine dumps (i.e. cost-benefit ratios) (Sapsford *et al.*, 2017). As the production of metallurgical waste streams is consequentially linked to the increased demand for precious metals, the effective recovery of these metals from secondary sources (e.g. metallurgical effluent, contaminated sites, e-waste) presents an economically viable case.

Ion exchange, precipitation, bioleaching, and solvent extraction are conventional methods utilised for the recovery of precious metals from metallurgical waste streams (Kurniawan *et al.*, 2006). This includes the reclamation of residual precious metals, present in metallurgical wastewater, via the evaporation of wastewater and subsequent re-working of the sludge through metallurgical processes (Gunarathne *et al.*, 2022). However, these methods incur high capital and operational costs, are ineffective when metal concentrations in wastewater streams are low, utilise hazardous materials, and produce hazardous, concentrated waste products which require specialised disposal (Bashir *et al.*, 2019), reducing the use of conventional metal recovery techniques. These disadvantages favour the development of cost-effective and efficient methods of metal recovery.

Biosorption involves the use of biological materials (e.g. plants, algae, and bacteria) to remove metals from solution via adsorption processes (Escudero *et al.*, 2019). The use of plant roots (i.e. rhizofiltration) has gained interest for its advantages over conventional methods due to its relatively low cost, production of less waste, and elevated (targeted) metal recovery potential (Bashir *et al.*, 2019). This is especially the case when residual metal concentrations are present in the growth media where traditional techniques may not be economically viable (Calderón *et al.*, 2020).

Numerous plant species have been utilised for rhizofiltration of heavy metals, e.g., *Eichhornia crassipes* (Ni, Cu, Zn – Hammad *et al.*, (2011)) and *Typha domingensis* (Zn, Al, Fe – Hegazy *et al.* (2011)). A review of the available literature shows that plant species commonly selected for heavy metal rhizofiltration are aquatic species. The rhizofiltration potential of many aquatic plant

species is low due to the small size of the root system (i.e. root system architecture) compared to terrestrial species which produce longer, fibrous roots which increases the surface area for metal adsorption. Moreover, the effective recovery of metals during processing (e.g. drying and incineration) from aquatic plants is also limited due to the high water content of aquatic plant material (Dushenkov *et al.*, 1995). Various studies have demonstrated the ability of terrestrial plant species, such as *Salicornia europaea* and *Salsola crassa* (Farzi *et al.*, 2017), *Atriplex halimus* (Fountoulakis *et al.*, 2017, Shelef *et al.*, 2013), and *Helianthus annuus* (Lee and Yang, 2010), to remove salts and / or metals, and successfully propagate these plant species under hydroponic conditions. Please see Mader *et al.* (2021) for a review on this aspect. It must be noted that rhizofiltration is pH-dependent where the optimal pH range is pH 3 – 7 (Mack *et al.*, 2007). The efficacy of phytoremediation trials depends on a multiplicity of soil, aqueous solution, metal, and plant factors, as well as the interaction between these factors which vary through space and time.

1.4.2. Factors influencing the phytoremediation of Pt, Ni, and Co

1.4.2.1 Metal factors

Platinum, Ni, and Co are classified as heavy metals[3] (typically defined by their atomic density > 5 g/cm^3) sharing similar physicochemical properties (Table 1.2) (Savignan *et al.*, 2021). These elements are used in various industries (Table S1.1) and are considered economically strategic metals due to their physicochemical properties, namely their high resistance to oxidation and corrosion, high conductivity, thermoelectrical properties, and stability at a range of temperatures (Wang *et al.*, 2017). In the absence of complexing agents, the standard potentials (E°) of Pt (Pt^{2+}: 1.2 V) and Co (Co^{2+}: 1.8 V), compared with water (H_2O: 1.2 V) and Ni (Ni^{2+}: - 0.25 V), indicate that Pt and Co are relatively stable and insoluble in aqueous solutions across pH levels (Figure 1.3). Platinum is a noble element

[3]The term "*heavy metal*" is inconsistently used in scientific literature to classify a group of elements based on their physical properties, e.g. density, as such properties do not inform the reader about the biological, physicochemical, or toxicological importance of the particular element. Therefore, in the context of this study, the term 'metal' and 'heavy metal' will be used to described a group of elements based on their biological, physicochemical and toxicological properties and is concerned with the pure *metal* and its compounds. It must be noted that in this study, elements and their compounds, categorized as *metals* and *heavy metals* differ from non-metals relative to their biological, physicochemical, and toxicological properties – see: Duffus, J. H. 2002. " Heavy metals" a meaningless term? (IUPAC Technical Report). *Pure and Applied Chemistry*, 74, 793-807.

stable over a wide range of pH levels, whereby Pt solubility increases as pH decreases (Mitsushima *et al.*, 2007).

The solubility of these metals is mediated by metal binding processes. Platinum, Ni, and Co are classified as siderophiles (affinity to bind with salt-derived anions, such as SO_4^{2-}) which naturally occur as sulphide (S^{2-}) compounds. The oxidation of these complexes contributes to the introduction of SO_4^{2-} into wastewater streams (Hamilton, 1994b). The complexation of these metals with SO_4^{2-} therefore increases the solubility (Barceloux and Barceloux, 1999a, b) and bioavailability of these metals in solution (Amari *et al.*, 2014).

Metal bioavailability may be defined as the fraction of the total metal content present in the soil which is available to the plant for uptake. This portion of available metal may be categorised into different speciation classes including portions of available and/or exchangeable metals (Class I), metals adsorbed to reducible phases such as Al-, Fe-, and/or Mn-oxides and sulphides (Class II), or as a major constituent of the primary mineral (Class III) (Salomons, 1995). The presence of other metals (ion competition) may reduce the bioavailability of metals by inhibiting the binding and subsequent uptake of the other metal(s) (Irving and Williams, 1953).

Ion competition occurs between metals with similar physicochemical properties, such as similar valences and diameters (Table 1.2), where competition between cations is influenced by rhizosphere pH and metal form. The theory of Hard and Soft Acids and Bases (HSAB) classifies Pt as a soft acid and Co and Ni as soft borderline acids (Pearson, 1968). Nickel and Co are categorised within the same class – the biological implication of this classification is that elements with similar properties have similar binding site preferences (Mack *et al.*, 2007). This highlights the influence of how the physicochemical properties dictate their bioavailability, as well as subsequent sorption of such elements. According to the HSAB theory, Pt, Ni, and Co bind with sulphates (SO_4^{2-}) and chlorides (Cl^-). Other physicochemical properties such as metal form also influence the solubility of the metal complex in water as solubilised metal complexes, such as Pt salts (especially salts consisting of sulphur, nitrogen-, or halogen-donor ligands) whereby salts may be more bioaccessible for plant

uptake compared with free metal ions (Feichtmeier and Leopold, 2015). This has been reported by Omrani *et al.* (2020) whereby Pt, emitted by vehicle exhausts (i.e., abrasion of catalytic converters), was bound with S - increasing the solubility and bioavailability of Pt (as a S-complex) in the environment.

According to the Irving-Williams series, a transition metal[4] may displace another ion downstream from its specific binding site: $Zn^{2+} < Cu^{+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Fe^{2+} > Mn^{2+} > Mg^{2+} > Ca^{2+}$. For example, Ni^{2+} displaces Co^{2+} at a particular binding site (e.g. transport proteins) which may account for the inhibitory effect (antagonistic interaction) of Ni on Co uptake, which has been demonstrated in numerous studies (Boros-Lajszner *et al.*, 2021, Paul *et al.*, 2020, Tappero *et al.*, 2007, van der Ent *et al.*, 2018, Yamaguchi *et al.*, 2015). Nickel may outcompete Co, Ca, Cu, Fe, Mg, and Zn for sorption at binding sites for plant uptake, translocation, and storage, thereby resulting in essential metal deficiencies (Ameen *et al.*, 2019, Tang *et al.*, 2019). The Ni hyperaccumulator, *Alyssum murale*, has been demonstrated to hyperaccumulate Ni and Co whereas *Berkheya coddii* (also a Ni hyperaccumulator) accumulates Ni and Co. However, Co concentrations > 111 mg/kg were shown to decrease Ni uptake, indicating a possible antagonistic interaction between these metals due to their similar physicochemical properties (Keeling *et al.*, 2003, Tappero *et al.*, 2007). Metal bioavailability may be enhanced by modulating soil and plant physiological factors (Sheoran *et al.*, 2013).

[4] Metals within Group 4 - 12 of the Periodic Table. Transition metals have variable valences with a strong tendency to form coordination compounds. IUPAC defines a transition metal as "*an element whose atom has a partially filled d sub-shell, or which can give rise to cations with an incomplete d sub-shell*".

Table 1.2. Physicochemical properties of platinum (Pt), nickel (Ni), cobalt (Co), and sulphur (S). Asterisks (*), bold and highlighted values represent metals with similar physicochemical properties. Similarities are based on physicochemical properties implicated in element-binding / complexation. HSAB = Pearson's hard and soft acids and bases theory (Hamilton, 1994a, Kabata-Pendias, 2011, Pearson, 1968, Zereini and Wiseman, 2015).

Property	**Platinum**	**Nickel**	**Cobalt**	**Sulphur**
Atomic number	78	28	27	16
Group	**10***	**10***	9	16
Period	6	**4***	**4***	3
Relative atomic mass	195.08	**58.69***	**58.93***	32.065
Atomic radius (pm)	138.5	**124.6***	**125.3***	103.5
Ionisation energy (1^{st};2^{nd};3^{rd}) (kJ/mol)	870; 1791	737.1; 1753.0; 3395	760.4; 1648; 3232	999.6; 2252; 3357
Crystal structure	**Face-centred cubic***	**Face-centred cubic***	Hexagonal close packed	Orthorhombic
Electron configuration	[Xe] $4f^1$ $4sd^9$ $6s^1$	**[Ar] $3d^8$ $4s^2$***	**[Ar] $3d^7$ $4s^2$***	**[Ne] $3s^2$ $3p^4$**
Oxidative states	+2; +4; +5	+2; +3; +4; +5	+2; +3; +4	-2; 2; 4; 6
Electronegativity	1.42	**1.75***	**1.7***	2.44
Density (Kg.m^{-3})	21.45	**8.91***	**8.89***	2.06
Resistivity (microhm.cm at 20 °C)	9.85	7.63	6.24	2 x 10 23
Melting point (°C)	1772	1455	1495	115.21
Colour	Silvery-white	Silvery-white	Greyish-white	Yellow
HSAB classification	Soft/ borderline acid*	Soft/ borderline acid*	Soft/ borderline acid*	Borderline base

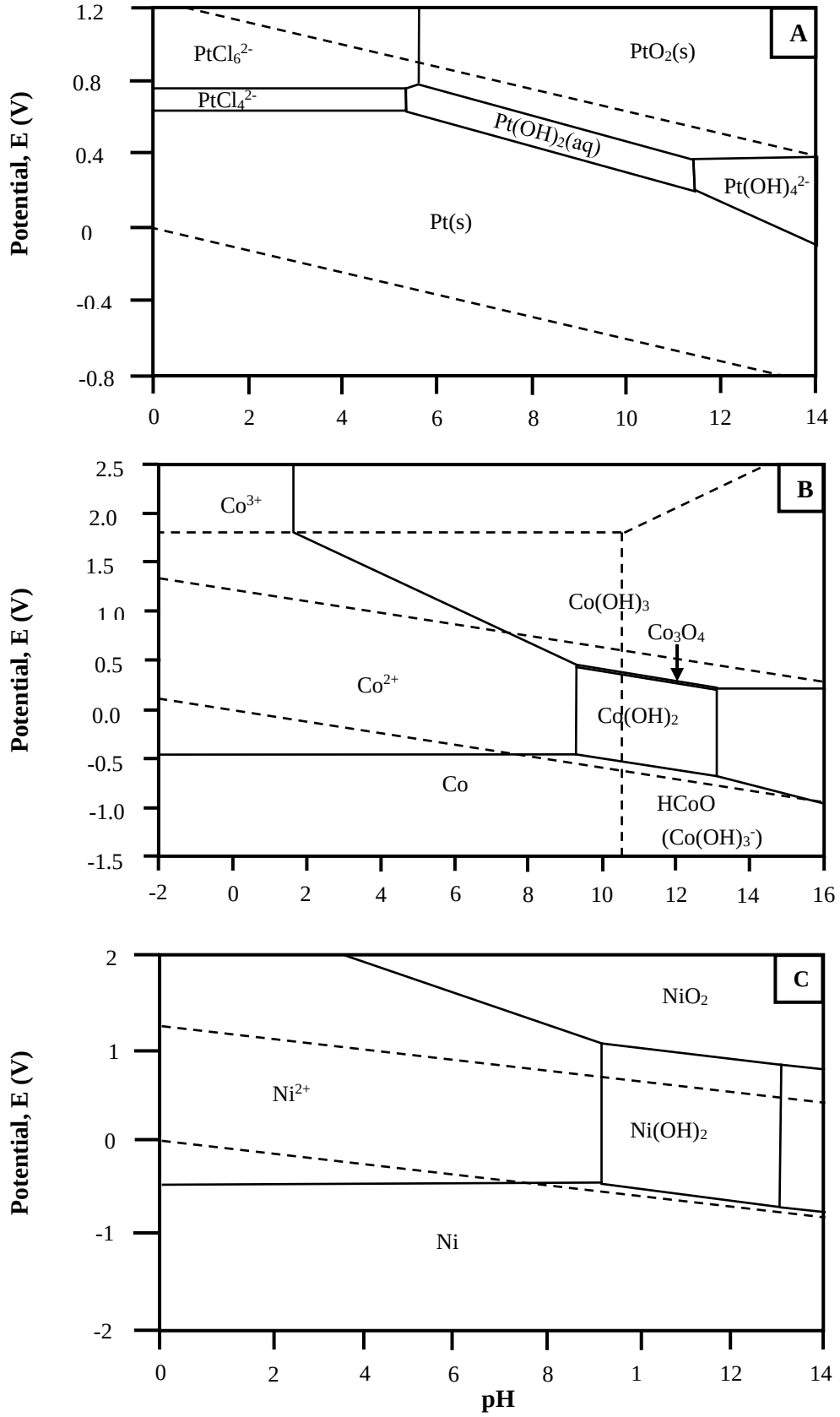

Figure 1.3. Potential-pH diagrams (Pourbaix diagrams) for (A) platinum, Pt (in 0.5 M HCl), (B) cobalt, Co, and (C) nickel, Ni, in aqueous solution. Solid lines represent zones of stability for the relative metal whereas dashed lines represent the stability zones of water. For example, as per the Ni Pourbaix diagram, Ni oxidises under acidic conditions. Adapted from Topalov *et al.* (2014), Pourbaix *et al.* (1959), and Garcia *et al.* (2008).

1.4.2.2. Soil and aqueous solution factors

Physical (e.g. soil compaction) and chemical (e.g. pH, electrical conductivity (EC), and oxidation-reduction potential (ORP or E_h)) soil and aqueous solution properties have been documented to influence the phytoremediation of contaminated sites. The compaction of soil has been reported to impact root growth and development (Correa *et al.*, 2019), limiting access to contaminants by plant roots. Chemical factors such as pH, EC, and E_h, influence the bioavailability of metals (Esposito *et al.*, 2002). As pH level increases, the bioavailability and sorption of metals decrease as metals are precipitated out of solution (Adriano *et al.*, 2004, Schneider *et al.*, 2001). These results are supported by Panda *et al.* (2007) whereby lower pH levels (pH 5) increased Ni^{2+} uptake by *Lathyrus sativus*, compared with higher pH levels (pH 8). In alkaline environments, plant biomass becomes negatively charged thereby attracting cations whereas in acidic environments the opposite occurs, i.e. plant biomass becomes positively charged thereby attracting anions (Schneider *et al.*, 2001).

The E_h is defined as the tendency of solution to oxidise or reduce available substances (loss or gain in electrons), whereby metals may be present in multiple redox forms (Kabata-Pendias, 2011). Metals bound to S species in aqueous solutions are maintained at low E_h. However, as E_h increases the potential for the dissociation of salts within the solution increases (Chuan *et al.*, 1996). Another factor that needs to be considered is salinity and the presence of salts and their constituents. Electrical conductivity is commonly used as a measure of soil salinity or soluble salt concentration in solution (Visconti and de Paz, 2016).

Soil salinity, where EC exceeds 4 dSm^{-1} or 2 338 mg/kg NaCl (Carter and Nippert, 2011, Committee and America, 2008), is an increasing problem in arid and semi-arid environments. Primary (natural) and secondary (via anthropogenic processes such as mining) salinity is the primary factor contributing to reduced agricultural productivity (Tomaz *et al.*, 2020). Salinity influences the physicochemical properties of soil and may destabilise soil aggregations, lowering water availability (i.e. reduced hydraulic conductivity), and root penetration whilst increasing anoxic conditions (due to salt crust formation) (Acosta *et al.*, 2011, Sun *et al.*, 2017). The main drivers of salt accumulation are evaporation and leaching processes, where salt complexes accumulate according to their solubility.

For example, soluble salts such as calcium chloride (CaCl) and nitrate-salts, accumulate lower down in the soil profile whereas less soluble salts (such as calcium sulphate/ gypsum ($CaSO_4^{2-}$)) accumulate closer to the surface (Tobergte and Curtis, 2013). Salinity has been demonstrated to increase metal mobility via the complexation of metal ions with anions derived from salts, as well as ion competition between salt and metal cations for solid-phase adsorption binding sites (Acosta *et al.*, 2011, Paalman *et al.*, 1994). These physicochemical parameters have been demonstrated to influence metal bioavailability and thus, the efficacy of phytoremediation trials. Therefore, plants selected for phytoremediation must be able to (i) tolerate site-specific conditions (salinity, element composition and concentrations), (ii) ad/absorb target contaminants, (iii) produce large biomass, and (iv) access zone of contamination (i.e. depth of root system) (Pulford and Watson, 2003).

1.4.2.3. Plant factors

Element tolerance, uptake, and translocation

As plants are sessile organisms, they must be able to avoid or tolerate metals and salts at phytotoxic levels present in the growing medium. Plants can tolerate and allocate a range of elements, to varying degrees, by adopting avoidance and / or tolerance defence strategies to maintain ion homeostasis network (Hall, 2002). These defence strategies occur on the genetic, molecular, cellular, and whole-plant scales, as well as via the interplay between these levels (Talebi *et al.*, 2019, Thakur *et al.*, 2016).

The upregulation of numerous metal transporters, including Zn-regulated, Fe-regulated transporter proteins (ZIP), cation diffusion facilitator (CDF), and heavy metal-associated proteins (HMAs), have been implicated in elevated rates of metal uptake by plant roots (Jogawat *et al.*, 2021, Krämer *et al.*, 2007). Metals may be taken up, passively or actively by specific and / or general metal ion transporters, such as SO_4^{2-} transporters (SULTR1) (Takahashi, 2019). Thus, the interplay between the up- and down-regulation of members of metal transporter families mediates the degree of metal(s) uptake by plants.

At a physiological scale, metal uptake is facilitated by root interception, mass flow, and diffusion. Roots that proliferate through the soil gain access to bioavailable metals, whereby metals bind to the root cell wall, which has a low affinity or selectively. Heavy metal binding and uptake, via sorption (absorption) mechanisms, is facilitated by physicochemical interactions between functional groups and metals, *viz.* electrostatic interactions, ion exchange, and metal complexation processes (Özer *et al.*, 2004). Intracellular binding sites with high binding affinities for certain metals facilitate the transport of ions across the plasma membrane. Negative membrane potential on the inside of the plasma membrane also provides a driving force for cation uptake through secondary transporters, such as H^+-coupled carrier proteins and / or channel proteins (Clemens *et al.*, 2002, Krämer, 2010, Krämer *et al.*, 2007).

On a whole-plant level, mass flow involves the uptake of heavy metals from a solution based on evapotranspirational pull whereby the water potential of the plant, soil, and atmosphere differ. This difference in water potential mediates the passive uptake of water, and dissolved metals present in solution (Colombi *et al.*, 2018). Diffusion also facilitates metal uptake based on differences in metal concentration gradients. Generally, metal selectivity is lower in the transporters facilitating the influx of metals into the cytoplasm. Metal uptake and translocation differ between plant species, plant genotype, plant organs (e.g. leaves, wood, twigs, and roots), as well as different tissue types (e.g. outer bark, inner bark, and sapwood and heartwood) (Tőzsér *et al.*, 2017, Violante *et al.*, 2010). This difference is attributable to the number and type (e.g. selectivity) of binding sites, as well as the tolerance strategy elicited by the plant to maintain the plant's metal homeostasis network (Clemens *et al.*, 2021, Hanikenne *et al.*, 2021).

Heavy metal uptake and translocation is species-dependent and controlled by mechanisms at different scales. Once metal ions have been taken up by the roots, the ions (present as hydrated metals and/or complexes) may be apoplastically (to prevent excess metal concentrations accumulating in sensitive cells) or symplastically transported to the stele. These metals are then loaded into the xylem vessels (i.e., xylem loading) (Ghori *et al.*, 2019, Küpper *et al.*, 2001, Prasad, 2013), where metal ion translocation is facilitated by a pH-dependent equilibrium between free-hydrated metal cations, low

molecular weight chelators, metal-binding sites, and the plant's transpirational stream (Figure 1.4) (Gupta *et al.*, 2019, Hao *et al.*, 2012). Once metals have been translocated to aboveground tissue, metal unloading, trafficking and storage take place. Metallochaperones, that interact with P-type ATPase, maintain metal concentrations within the physiological limits of each cell, mitigating metal phytotoxicity and maintaining the plant's ion homeostasis network (Clemens, 2001).

Although heavy metals may be stored in the roots (i.e., retained) to prevent metal toxicity-related damage, reduced sequestration within root vacuoles may enhance translocation to shoots due to differences in concentration gradients (DalCorso *et al.*, 2013, Lasat *et al.*, 1998). The intracellular uptake of metal ions may also be mediated by secondary transporters, plating a vital role in loading heavy metals into the xylem for translocation to aboveground tissue.

Plant factors influence the physicochemical properties of metals present in soil and aqueous solutions by direct (changes in redox reactions, rhizosphere acidification/ alkalisation, metal uptake, and complexation via chelation) and / or indirect (physicochemical properties of the rhizosphere[5], root system architecture, and microorganism activity) plant-related processes (Chen *et al.*, 2017, Vives-Peris *et al.*, 2020). *Tamarix ramosissima*, an alien invasive *Tamarix spp* distributed throughout southern Africa and the United States (Craine *et al.*, 2016), has been reported to release solubilising agents (e.g., lixiviants) into the rhizosphere, decreasing rhizosphere pH which consequently increasing the bioavailability of metals for plant uptake (Sookbirsingh *et al.*, 2010). The release of solubilising agents increases metal solubility by creating bioavailable complexes whereas certain compounds, such as borderline acidic heavy metals (Table 1.2), may be precipitated out of solution, complexed, or extracted from the solution. The extraction of acidic / alkaline metals alters solution pH levels (i.e., alkalinises / acidifies, respectively) (Bali *et al.*, 2020, Chen *et al.*, 2017, Vives-Peris *et al.*, 2020). Root exudates contribute to mineral weathering, changes in metal mobility, as well as changes in the structure of binding sites.

[5] Layer of soil directly influenced by plant roots (e.g. root exudations) as well as soil microorganisms within the soil.

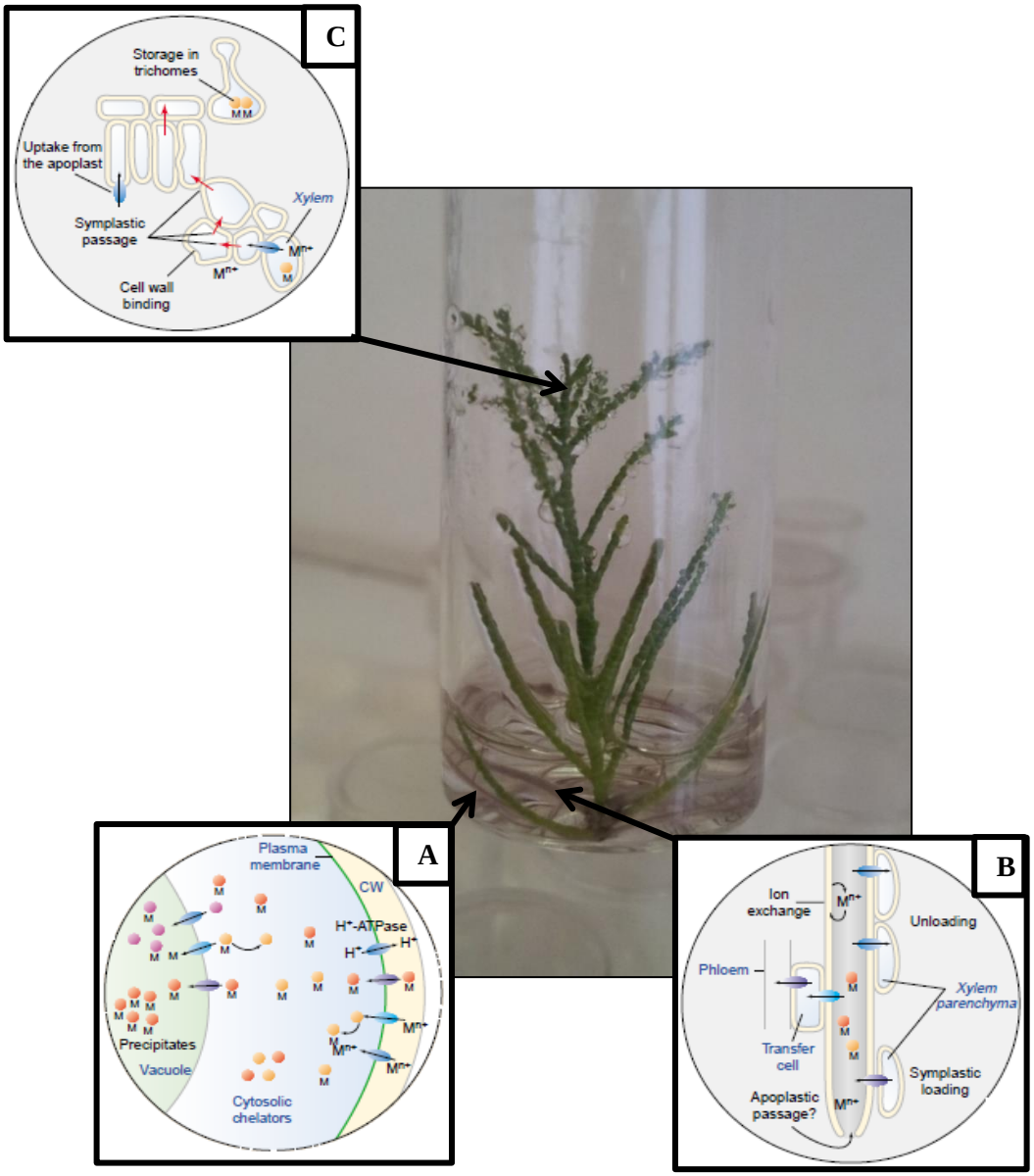

Figure 1.4. Processes involved in metal (M) uptake, translocation, and tolerance. **A:** Lixiviants (solubilizing species) released from plant roots bind to metals, increasing metal bioavailability for plant uptake. Metal complexes are transported into the root via various metal transporters, such as CP_x-type ATPases, H^+-ATPases and channel proteins, present in the plasma membrane of the roots; **B:** In the xylem, metal compounds are translocated to shoots, via apoplastic transport, to reduce metal phytotoxicity in cytoplasm; **C:** Metal compounds are subsequently translocated into shoot cells where metal complexes are compartmentalized in cell vacuoles, trichomes, and / or excreted onto the leaf surface through vesiculated trichomes (such as the salt excretion mechanism by *T. usneoides*) (Wilson *et al.*, 2017). CW: Cell wall. Adapted from Clemens *et al*., (2002).

The selective uptake of metals by roots creates concentration gradients that differ between plant organs and tissue types. These concentration gradients induce variation in metal accumulation between plant organs and tissue types (Marschner, 2011, Violante *et al.*, 2010). Differences in metal accumulation may also be attributed to variation in elemental resistance between plant organs and tissue types (Figure 1.4) (Marschner, 2011). Average concentrations of metals within plants range from 0.0009 to 1.46 mg Pt/kg dry weight (DW), 0.10 to 5 mg Ni/kg DW, and 0.03 to 2 mg Co/kg DW (Herselman *et al.*, 2005, Kabata-Pendias, 2011, Rauch and Fatoki, 2013, Zereini and Wiseman, 2015)

Plant resistance

Complex and intrinsic genetic and molecular factors (quantity, distribution, and degree of upregulation of metal transport proteins (MTP) involved in specific metal storage sites), as well as crosstalk between genes, confer metal tolerance (Ricachenevsky *et al.*, 2013). These MTPs, contributing to Ni^{2+} and Co^{2+} tolerance increase the compartmentalisation of divalent cations into vacuoles – reducing metal phytotoxicity. At a physiological level, plants overcome heavy metal toxicity by excluding or including (avoidance and tolerance) toxic metal ions / concentrations to maintain ion homeostasis. These resistance mechanisms have been described in Figure 1.5 below.

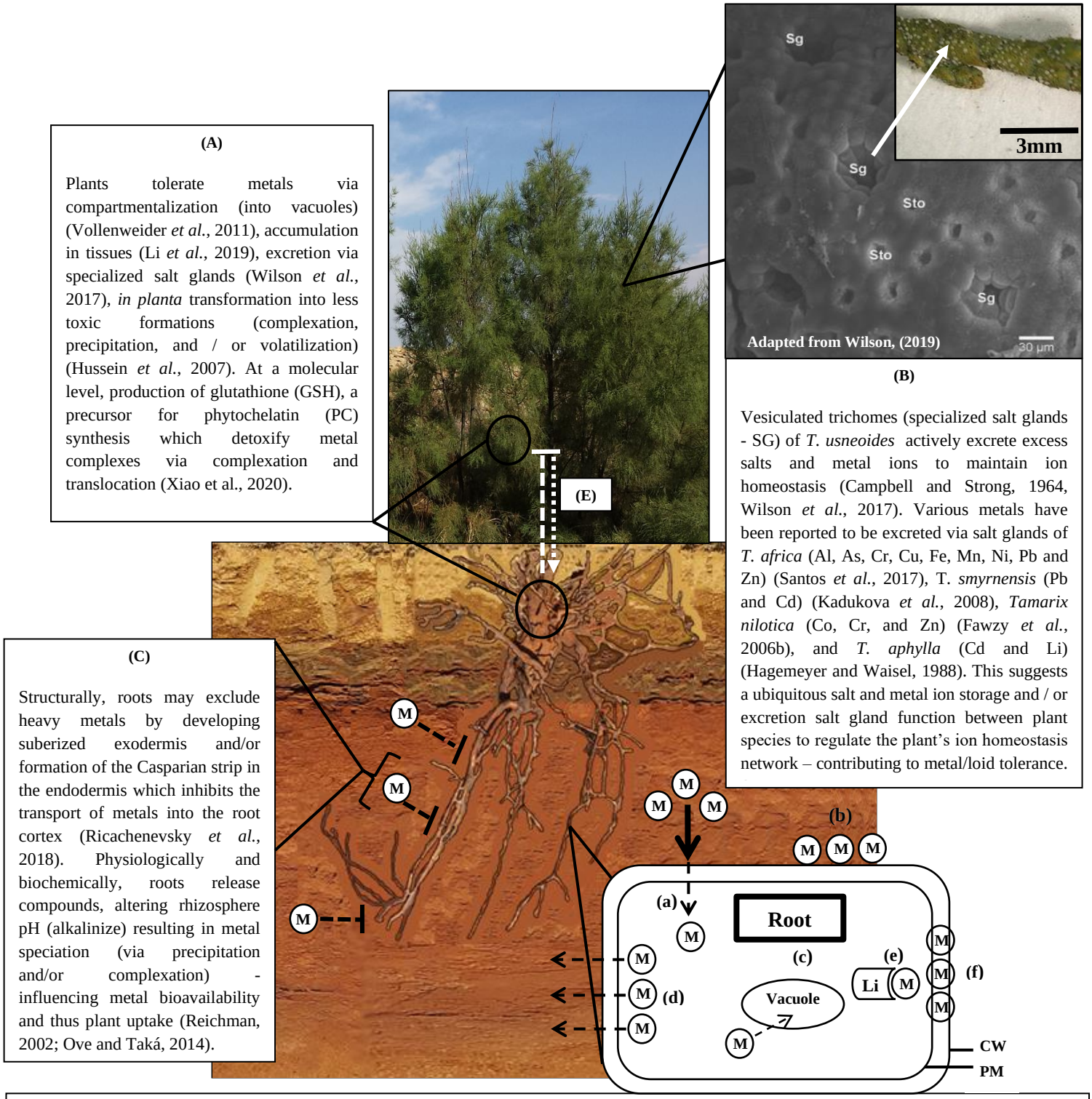

Figure 1.5. Element resistance mechanisms, which may be incorporated by plants including *Tamarix usneoides* (study species), at structural, biochemical, and physiological levels. Metal resistance involves adopting metal exclusion [Figure 1.4 A] and inclusion [tolerance (Figure 1.4 B, C, and D) or avoidance (Figure 1.4 C and D) strategies to maintain the plant's ion homeostasis network.

Degree of metal uptake, translocation, and tolerance

Numerous studies have demonstrated the ability of various plant species to take up Pt, Ni, and Co to varying degrees (Table S1.3). Based on the degree of accumulation in different plant organs, plants may be categorised as excluders (survive on metal contaminated soils), indicators (internal metal concentration is similar to soil concentrations), accumulators (bioconcentrate metals to aboveground plant organs, such as leaves), and hyperaccumulators (Baker, 1981, Brooks *et al.*, 1977).

Metal hyperaccumulation may be defined as the plant's ability to accumulate elevated concentrations [generally > 1 000 mg/kg (dry mass)] of a particular metal into aboveground plant organs, namely leaves, which has been recorded in at least one specimen growing in its natural habitat (Baker, 1981, Brooks *et al.*, 1977). However, the hyperaccumulation threshold has since be revised to be metal-dependent [see (Van der Ent *et al.*, 2013) for review]. For example, the hyperaccumulation threshold for Ni and Co are > 1 000 mg/kg and > 300 mg/kg, respectively. Other criteria used to define hyperaccumulation includes (i) a high bioconcentration[6] factor (BCF > 1) (shoot: soil metal ratio), (ii) translocation factor (TF) > 1 (root-to-shoot metal translocation), and (iii) edaphic conditions (such as the presence of other elements in the soil – i.e. ion competition) (Macnair, 2003, Van der Ent *et al.*, 2013). The plant's metal homeostasis network mediates the plant's ability to (hyper)accumulate and/or (hyper)tolerate metals at elevated concentrations (Hanikenne and Nouet, 2011).

Limited studies have reported platinum accumulation in trees. Platinum has been reported to accumulate in bark and twigs of *Picea* spp. (6 µg/kg), *Cedrus* spp. (7 µg/kg), and *Pseudotsuga menziesii* (9 µg/kg) (Fletcher *et al.*, 1995), *Picea mariana* (77 µg/kg) (Dunn *et al.*, 1989) and pine needles of *Pinus pinea* (1 – 102 µg/kg) (Dongarra *et al.*, 2003) as well as various herbaceous species including *Medicago sativa* (94 mg/g) and *Brassica juncea* (39 mg/g) (Bali *et al.*, 2010), *Lolium perenne* (0.0033 – 1.63 µg/kg) (Verstraete *et al.*, 1998) and *Arabidopsis thaliana* (100 – 425 µg/kg) (Gawrońska *et al.*, 2018a). However, as the Pt hyperaccumulation threshold is yet to be defined – no Pt hyperaccumulators have been reported in the literature.

[6] In the context of this thesis, the terms "*bioconcentration*" and "*bioaccumulation*" are used synonymously.

Nickel hyperaccumulation has been reported for approximately 390 plants whereas 26 plant species have been reported to hyperaccumulate Co (Reeves *et al.*, 2018). Lange *et al.*, (2017) demonstrated that Co accumulation by *Anisopappus chinensis* increased in a dose-dependent fashion. However, Co hyperaccumulation is a rare phenomenon whereby Co concentrations in plants (growing on metalliferous soils) range from 0.03 – 20 mg/kg. The relatively lower cases of Co hyperaccumulation by plants is attributed to the low bioavailability of Co and elevated Ni concentrations in the environment (Malik *et al.*, 2000). Nickel has been demonstrated to outcompete Co for binding sites (competitive ion adsorption), restricting the allocation of Co by plants (Keeling *et al.*, 2003). Nickel and Co hyperaccumulation by plants has been reported by numerous studies (Table S1.3).

From an anthropocentric standpoint, the plant's ability to tolerate and hyperaccumulate elements allows for phytomining (Chaney *et al.*, 1998). Phytomining is the process whereby plants are used to extract and recover metals of economic value, such as Pt, from shallow surface ore bodies and contaminated areas, in which the conventional method of secondary mining (reclamation of TSF) is not feasible (Chaney and Baklanov, 2017). The extracted desired metal, in some cases, present within the plant as nanoparticles, may be recovered from plant tissues and used in various industries (Mudd *et al.*, 2018) (Table S1.1). Plants used for phytomining must produce large biomass, be fast-growing, possess a root system capable of reaching the depth of metal contamination, and allocate elevated metal ions to plant organs which can be harvested and precious metals recovered (Dinh *et al.*, 2022).

1.4.3. Salt and metal cross-tolerance

Primary (increase in the rate of evaporation and frequency of droughts) and secondary (e.g. release of salts by mining processes) salinisation compromises crop growth, survival, and ultimately food security (Munns and Tester, 2008, Tomaz *et al.*, 2020). Due to the similarities in salinity and metal stresses (i.e. osmotic, oxidative, and ionic stress), salt tolerance mechanisms may confer metal tolerance (Flowers and Colmer, 2015, Joshi *et al.*, 2020). Halophytes have gained attention over the past few decades due to their ability to tolerate elevated salinity whilst simultaneously allocating

metals to varying degrees (Van Oosten and Maggio, 2015). As plants differ in degree of salt tolerance (Flowers and Colmer, 2008), there is a growing need to investigate halophytes for the remediation of saline metal contaminated sites whereby 'traditional' plant species utilised for phytoremediation are herbaceous plant species intolerant to saline environments (i.e. glycophytes).

Cross-tolerance refers to specific and general tolerance mechanisms elicited by plants to tolerate multiple abiotic and/or biotic stresses (Nikalje and Suprasanna, 2018, Wiszniewska *et al.*, 2019). These cross-tolerance mechanisms occur at the genetic (e.g. upregulation of genes, such as *OsCBSX4*, under salinity and heavy metal stress) (Singh *et al.*, 2012), molecular (e.g. cross-talk between mitogen-activated protein kinase (MAPK) cascades and hormone signalling) (Cardinale *et al.*, 2002), cellular, and whole-plant levels (salt excretion via salt glands) (Manousaki and Kalogerakis, 2011, Nikalje and Suprasanna, 2018), as well as the interplay between these levels (Figure 1.5). The presence of salt glands of members of the genus *Tamarix*, as well as other plant species (e.g. *Spartina alterniflora* Weis and Weis (2004)), have been demonstrated to contribute to the tolerance of saline sites via the accumulation and / or excretion of elevated salt loads (Flowers and Colmer, 2015, Wei *et al.*, 2020, Wilson *et al.*, 2017). Therefore, it may be hypothesised that the presence of salt glands may enable halophytes to effectively assimilate, translocate, and subsequently excrete salts, metal ions, and metal-salt compounds in order to maintain ion homeostasis (Table 1.3). These studies highlight the potential use of halophytes, and more specifically *T. usneoides*, on saline metal- contaminated sites (Table 1.3).

Halophytes differ in their mode of salt transport, storage, and potential excretion mechanisms. Based on these differences in salt allocation, halophytes may be categorised as recretohalophytes [excretion (exo-recretohalophytes) or storage (endo-recretohalophyte) of salts from / in specialised salt glands or bladders], euhalophytes (compartmentalisation of salts in leaf / stem vacuoles), and pseudohalophytes (accumulation of salts in vacuoles present in the roots) (Breckle, 1995, Wei *et al.*, 2020). Halophytes may also be further categorised based on the environments they inhabit (such as xero-halophyte, hydro-halophyte, etc) and their salt dependency (facultative vs obligate halophytes). The biological implication of these categorisations involves the need to consider the type of halophyte

selected for phytoremediation. For example, Mader *et al.* (2021) presented the case that the effective removal of salts (i.e., persistent contaminants) from agricultural wastewater is mediated by the halophyte's salt allocation mechanisms. For example, exo-recretohalophytes may excrete salts onto the surface of their leaves via salt gland excretion. Under certain environmental conditions (e.g. rainfall), these salts may be re-introduced into the wastewater being treated.

Sulphur is an essential macronutrient incorporated into various biomolecules within the plant. Sulphur has many biochemical functions including lipid synthesis, formation of S-S bridges, reactive oxygen species (ROS) signalling, as well as metal detoxification (Gill and Tuteja, 2011). Sulphur acts as a ligand which binds heavy metals, such as Ni and Co, to metalloproteins and enzymes (Hawkesford and De Kok, 2006, Hossain *et al.*, 2012)

Sulphate is the most oxidative yet stable form of S and is the primary form of S present in the soil as well as *in planta* (xylem and phloem vessels). As halophytes, such as *Tamarix usneoides*, have been demonstrated to (hyper)accumulate SO_4^{2-} (Dennis, 2008, Kendall, 2010, Wilson, 2019), elevated *in planta* S concentrations may amplify the biosynthetic pathways of S-incorporated molecules and compounds such as cysteine (Cys) and glutathione (GSH). These molecular protectants have elevated affinities for toxic metal ion binding - conferring heavy metal tolerance via metal detoxification (Hossain *et al.*, 2012, Sun *et al.*, 2005). This may be a potential tolerance mechanism contributing to metal and salt cross-tolerance by halophytes.

Table 1.3. Studies investigating the phytoremediation of heavy metals by halophytes.

Study number	Halophyte	Element accumulated	Results	Phytotechnology	References
1	*Tamarix usneoides*	Au (as C_2AuKN_2, $HAuCl_4$, and $Na_3Au(S_2O_3)_2 \cdot 2H_2O$)	Elevated gold (Au) accumulated in roots > shoots across Au treatment concentrations (0.5 – 50 mg/L). Au accumulation differed between Au species ($HAuCl_4$ > C_2AuKN_2 > $Na_3Au(S_2O_3)_2 \cdot 2H_2O$), increasing in a metal dose-dependent fashion. However, BCF decreased as metal treatment concentration increased. pH level impacted Au translocation but not Au uptake by plantlets. Plantlets exhibited phytotoxic symptoms for C_2AuKN_2 treatments.	Phytoextraction and Phytostabilisation	(Wilson, 2019)
2	*Tamarix usneoides*	Al, Cr, Fe, Co, Ni, Cu, Zn, Ag, Cd, Cs, Au, U	Elevated metal concentrations were accumulated in leaves of 3-year-old trees compared with 5-year-old *T. usneoides* trees. Metal accumulation in *T. usneoides* leaves ranged from 384 – 467 mg Al/kg, 219 – 461 mg Fe/kg, 74 – 503 mg Zn/kg, 14.5 – 15.8 mg Cu/kg, 10.8 - 13.5 mg Cr/kg, 8.3 – 13.7 mg Cs/kg, 3.7 – 25 mg Au/kg, 3.6 – 11.9 mg Ni/kg, 1.2 – 4.4 mg Co/kg, 0.9 – 1.7 mg U/kg, 0.1 – 1.2 mg Ag/kg, and 0.3 – 0.6 mg Cd/kg.	Phytoextraction and phytostabilisation	(Dennis, 2008)
3	*Tamarix africana*	As, Cd, Cr, Cu, Pb, and Zn	Elevated concentrations of metal/loids (Al, As, Cd, Cr, Fe, Ni, Pb, and Zn) accumulated in roots compared with shoots, whereas elevated levels of Cu and Zn accumulated in shoots > roots. TF > 1 for Cu, Pb, and Zn. BCF > 100 for Al, As, Cd, Cr, Cu, Fe, Ni, Pb, and Zn. All investigated metal/loids were present in salts secreted by salt glands of *T. africana* where Al, Fe, Mn, Na, and Zn were the main metals contributing to salt crystal composition. Salt excretion by *T. africana* was reported to confer metal tolerance.	Phytostabilisation	Santos *et al.* (2017)
4	*Tamarix aphylla*	Cd and Lithium (Li)	Cd and Li were allocated and excreted onto the leaf surface by salt glands. The rate of Cd allocation and excretion increased over time.	Phytoexcretion	Hagemeyer and Waisel (1988)
5	*Tamarix aphylla* and *Phragmites australis*	Cu, Fe, Zn, Mn, Ni, Cd, and Pb	Elevated concentrations of all metals were accumulated in roots > shoots. Root BCF > 1 for Cu (> 6), Zn (> 9), Mn (> 8), Ni (> 1.7), Cd (> 5), and Pb (> 6). Root-to-shoot TF < 1 for all metals indicating retention of metals in roots.	Phytostabilisation	Wafa'a (2009)
6	*Tamarix articulata*		Ni, Fe, Pb, Cd, Mn, and Zn were retained in roots compared with aboveground biomass (stems and leaves) whereas elevated concentrations of Cu and Cr were translocated and subsequently accumulated in		

		Ni, Pb, Zn, Mn, Cu, Cr, Cd, and Fe	aboveground biomass (when plants were growing in polluted soil).	Phytoextraction (Cu and Cd) and phytostabilisation	Shah *et al.* (2013)
7	*Tamarix gallica*	As and Hg	As and Hg accumulation increased in a dose-dependent fashion. As and Hg were concentrated in roots with low TF (< 1). Exposure to As and Hg compromised the nutrient status (namely Mn and P – where accumulation decreased as As and Hg treatment concentrations increased) and photosynthetic activity (reduction in chlorophyll *a*) of the plant. Elevated concentrations of As and Hg concentrations accumulated by *T. gallica*, compared with another halophyte, *Pistacia lentiscus*.	Phytostabilisation	Moreno-Jiménez *et al.* (2009)
8	*Tamarix gallica*	As	As accumulation increased in a dose-dependent fashion. Salinity increased As accumulation in roots but decreased translocation, and accumulation in shoots. Elevated Na concentrations accumulated in shoots > roots. *T. gallica* tolerated As treatment where the plant's nutrient status (relative to K, Ca, and Mg) was not negatively impacted.	Phytostabilisation	Sghaier *et al.* (2015)
9	*Tamarix gallica*	Al and As	Accumulation of Al in roots increased in a (i) salinity and (ii) metal dose-dependent fashion. As accumulation decreased as salinity increased. Al and As TF < 1 indicating element retention in roots. Al concentrations in roots ranged from 575 - 785 mg/kg and 667 – 1 253 mg/kg for non-saline and saline treatments, respectively, whereas Al in shoots ranged from 104 – 106 mg/kg and 83 – 107 mg/kg in non-saline and saline conditions, respectively. Majority of accumulated Al was immobilised in cell walls, potentially restricting Al translocation to aboveground biomass. As was mainly compartmentalised in vacuoles.	Phytostabilisation	Sghaier *et al.* (2016)
10	*Tamarix hispida*	Ni, Co, Cu, Cr, Fe, Mn, Zn, Ti, and V	Ni (5.7 mg/kg), Cr (8.5 mg/kg), strontium (Sr, 264 mg/kg), Fe (2 960 mg/kg), Mn (74 mg/kg), Zn (200 mg/kg), Cu (6.4 mg/kg), vanadium (8 mg/kg), Co (3.8 mg/kg) were accumulated by *T. hispida*, with an elevated Sr translocation factor. *T. hispida* accumulated elevated concentrations of Fe, titanium (Ti), Cu, Sr, Zn, and Co in aboveground biomass. Authors described *T. hispida* as a potential metal hyperaccumulator.	Phytoextraction and phytostabilisation	Toderich *et al.* (2010)
11	*Tamarix hispida*	Pb, Cd	*T. hispida* accumulated low concentrations of Pb and Cd from wastewater ranging from 500 - 660 µg Pb/L and 70 – 120 µg Cd/L, respectively.	Phytoextraction and Rhizofiltration	Sasan *et al.* (2017)
12	*Tamarix nilotica*	Fe, Mn, Cr, Cu, Ni, Zn, Cd and Pb	Root-to-shoot metal translocation mobility followed the series; Fe > Mg > Cr > Zn > Mn > Ni > Pb > Co where Cd was below limit of detection. Elevated concentrations of Fe, Mn, Mg, and Cr (as well as the cations Ca, Na and K) were accumulated in leaves > roots. Metals (excluding Co, Cr, and Zn) were present in salts excreted by salt glands. The uptake of Pb, Ni, and Co had strong positive correlations with soil metal concentrations.	Phytoextraction and phytostabilisation	Fawzy *et al.* (2006a)

13	*Tamarix nilotica*	Al, B, Cr, Cu, Fe, Mn, Mo, Pb, V, and Zn	Elevated concentrations of Al, Cr, Cu, V, Mo, Fe, Pb, and Mn were accumulated in roots compared with shoots, where Al Cr, Fe, Mo, and V concentrations differed between plant organs. TF series was Cr > Cu > Mo > Fe > Pb > Zn > Mn > Al > V > B.	Phytoextraction	Osman and Badawy (2013)
14	*Tamarix smyrnensis*	Pb	Elevated Pb accumulated in plant roots (83 – 94 % of total Pb accumulated by plant) compared with shoots, regardless of salinity. Elevated salinity increased Pb accumulation in leaves (which differed between salt treatments), whereas salinity decreased Pb accumulation by roots.	Phytostabilisation	Kadukova and Kalogerakis (2007)
15	*Tamarix smyrnensis*	Cd, Pb	Cd accumulation increased in roots and shoots in a salinity-dose dependent fashion. Soil salinity promoted Cd allocation as well as Cd excretion by salt glands present on leaf surface. Presence of 0.5 % NaCl resulted in 3.5 times greater rate of excretion compared with non-saline growing media. This disagrees with results obtained by Lefèvre *et al.* (2009) where salinity inhibited salt / Cd excretion by *Atriplex* spp. Presence of Cd, in combination with elevated salt loads, did not negatively impact the physiological growth of *T. smyrnensis*.	Phytoexcretion	Kadukova *et al.* (2008)
16	*Tamarix smyrnensis*	Pb and Cd	Metal accumulation in shoots of *T. smyrnensis* ranged from 150 - 270 mg Pb/kg and 7.5 - 42 mg Cd/kg, respectively. Pb accumulation in shoots, under salinity, showed a hormetic response where Cd accumulation increased in a salinity-dose-dependent fashion.	Phytoextraction and phytostabilisation	Manousaki *et al.* (2008)
17	*Tamarix* spp (*Tamarix* plants were not identified to species level)	Cu, Pb, Cd, Cr, Co, Mn, Ni, Mo, Be, La, Cs, Pr, Nd, Zr.	Elevated concentrations of Cu, Pb, Cd (three orders of magnitude greater), and Zn (and lower Cr, Co, Mn, Ni, Mo, and beryllium (Be)) were accumulated by *Tamarix* spp in this study. *Tamarix* individuals also accumulated low concentrations of cerium (Ce), lanthanum (La), caesium (Cs), praseodymium (Pr), neodymium (Nd), and zirconium (Zr), indicating the ability of *Tamarix* species to accumulate a range of metals.	Phytoextraction (Cu, Pb, Cd) and phytostabilisation	Suska-Malawska *et al.* (2019)
18	*Mesembryanthemum crystallinum* (Halophyte) and *Brassica juncea* (glycophyte)	Ni	Ni accumulated in roots (roots > shoots) for both plant species, where Ni accumulation in roots and shoots increased in a dose-dependent fashion. Elevated Ni was translocated to and accumulated in shoots of *M. crystallinum* compared with *B. juncea*. Although authors concluded that *M. crystallinum* was an ideal candidate for Ni phytoextraction, the highest Ni concentration in roots (371 ± 8.00 mg/kg) and shoots (78 ± 1.4 mg/kg), along with a BCF of 0.78 and a low translocation factor (i.e. TF < 1) to harvestable plant material suggests that *M. crystallinum* is an ideal candidate for phytostabilisation (as defined in the context of this thesis) of Ni-contaminated sites.	Phytostabilisation	Amari *et al.* (2017)
19	*Sesuvium*	Pb	Plant physiological growth increased in a salinity dose-dependent fashion. The combination of salinity (8 000 mg/kg) and Pb (ranging from 0 – 300 mg/kg) promoted biomass production. Elevated Pb treatments	Phytoextraction (when soil is supplemented with EDTA)	Khella

	portulacastrum		(e.g., 600 mg/kg), at 8 000 mg/kg salinity, increased Pb accumulation by roots and shoots (elevated BCF), regardless of season. Pb accumulation in roots and shoots was directly proportional to increasing salinity concentration. Pb TF decreased as salinity increased. It must be noted that pots were supplemented, once off, with EDTA which may have promoted Pb accumulation.	and phytodesalination	(2018)
20	*Bolboschoenus maritimus*	Cd	Cd was accumulated in the series; belowground biomass (roots and rhizomes) > stems > leaves. Although elevated Cd was accumulated at lower salinity levels, elevated salinity reduced Cd accumulation and resulted in plant mortality.	Phytostabilisation (at lower salinity levels)	Santos *et al.* (2015)
21	*Kosteletzkya virginica*	Cd, Zn	Exposure to Cd negatively impacted physiological growth of *K. virginica*. Elevated Cd and Zn concentrations accumulated in plant roots > shoots. Salinity reduced Cd allocation in shoots. Zn increased Na accumulation whereas exposure to Cd reduced Na concentrations. Although salinity reduced Cd and Zn accumulation, salinity did not influence Zn accumulation in leaves.	Phytostabilisation and phytohydraulics (rhizofiltration)	Han *et al.* (2012)
22	*Halimione portulacoides*	Zn, Pb, Co, Cd, Ni and Cu	Accumulation of metals differed between plant organs where Zn, Pb, Co, Cd, Ni and Cu were accumulated in root > stems > leaves. Metals accumulated in cell walls (> 50 %), reducing metal translocation to aboveground biomass.	Phytostabilisation	Sousa *et al.* (2008)
23	*Halimione portulacoides* and *Spartina maritima*	Cd, Cu	Elevated concentrations of all metals were present in the rhizosphere compared with sampled sediment. The bioavailable fraction of metals differed between sediments, inhabited by the halophytes, suggesting that these halophytes influence metal fractionation in the rhizosphere.	Phytostabilisation	Reboreda and Cacador (2007)
24	*Sesuvium portulacastrum* and *Mesembryanthemum crystallinum*	Cd	Cd exposure compromised the uptake of K^+ and Ca^{2+} which limited the growth of both halophytes. Elevated Cd accumulation in shoots of *M. crystallinum* compared with *S. portulacastrum*. Cd accumulation was influenced by biomass production and Cd treatment concentration. Uptake of N was not inhibited by Cd exposure.	Phytoextraction	Ghnaya *et al.* (2007)
25	*Halogeton glomeratus*	Hg, Ni, Cd, Cr, Cu, and Zn.	Plants had a total salt yield of ~2 105 kg/ha. Elevated concentrations of metal/loids (Ni, Cu, Cd, Cr, Hg, As, and Zn), were accumulated under saline conditions.	Phytoextraction	Li *et al.* (2019)
26	*Suaeda glauca* and *Limonium aureum*	Cd	Cd exposure did not negatively impact plant nutrient status. Presence of salt (NaCl) enhanced Cd accumulation by both halophytes, where elevated concentrations of Na accumulated in both plants.	Phytostabilisation	Zhang *et al.* (2020)
27	*Salicornia europaea, Suaeda maritima, Salsola soda,* and	Al, Co, Cr, Cu, Fe, Mn, Ni, and Zn	All metals were concentrated in roots > stems whereas elevated concentrations of Zn (TF > 1) accumulated in leaves. Salinity influenced Co and Ni uptake by *H. portulacoides* and *S. maritima*, where Co and Ni uptake was positively correlated to salinity in roots, stems, and leaves of *H. portulacoides* (and in the roots of *S. maritima*). Co and Ni accumulated in roots > stems > leaves. Co was retained in roots	Phytostabilisation and phytoextraction (Zn)	Milić *et al.* (2012)

	Halimione portulacoides		(TF < 1) by all halophytes whereas *S. maritima* had a TF > 1 for Ni. Elevated metal concentrations were accumulated by plants sampled from coastal saline areas compared with inland areas.		
28	*Sesuvium portulacastrum*	Cd and Ni	Exposure to Cd did not negatively impact plant biomass production. Elevated concentrations of Ni and Cd were accumulated when growing medium was supplemented separately, compared with metals in combination (i.e., binary treatments). This indicates a potential antagonistic interaction (competitive ion adsorption) between Cd and Ni. TF < 1 for Ni and Cd when plants were treated with metals separately or in combination.	Phytostabilisation	Mnasri *et al.* (2015)
29	*Salicornia ramosissima*	Cd	Elevated Cd concentrations accumulated in roots > shoots. Cd accumulation decreased in a salinity dose-dependent fashion but increased in a metal dose-dependent. TF < 1 across Cd treatments.	Phytostabilisation	Pedro *et al.* (2013)
30	*Halimione portulacoides*	Ni, Co, As, Cd, Cu, Cr, Mn, Fe, Pb, Zn	Elevated metal concentrations (excluding Pb) accumulated in *H. portulacoides* roots > stems > leaves whereas Zn accumulated in roots > leaves > stems. BCF < 1 and TF < 1. *H. portulacoides* is an ideal candidate for phytostabilisation of metal contaminated sites.	Phytostabilisation	Andrades-Moreno *et al.* (2013)
31	*Arthrocnemum macrostachym*	Cd	Cd BCF > 1 but decreased in a metal dose dependent fashion. Elevated Cd translocation to aboveground biomass at lower Cd treatment concentrations. Relative growth rate decreased as Cd concentration increased. TF < 1 for all Cd treatments.	Phytostabilisation	Redondo-Gómez *et al.* (2010)
32	*Spartina alterniflora*	Cu	Cu accumulated in roots in the following series: fine roots > leaves > rhizomes > stems. Highest Cu treatment (1 000 mg/kg) reduced biomass production and Cu accumulation. Elevated Cu was accumulated at 800 mg/kg treatment which was attributed to stimulation of biomass production (possible hormetic response).	Phytostabilisation	Chai *et al.* (2014)
33	*Atriplex hortensis* var. *purpurea*, *A. hortensis* var. *rubra* and *A. rosea*	Ni, Cu, Pb, and Zn	Metals accumulated in roots > shoots in a dose-dependent fashion. Elevated Pb and Cu concentrations accumulated in the roots of *A. hortensis* var. *rubra* and *A. rosea*. Although some plants had an elevated BCF (> 1), plants had a low TF < 1. *Atriplex hortensis* var. *purpurea* was an ideal candidate for allocation of Pb and Zn in shoots whereas *A. hortensis* var. *rubra* was most suitable for Ni and Cu phytostabilisation.	Phytostabilisation	Kachout *et al.* (2012)
34	*Juncus maritimus* and *Phragmites australis*	Cd	Elevated Cd levels were accumulated in belowground biomass (roots and rhizomes) > aboveground biomass (stems and leaves) by both plant species. Addition of Cd-tolerant rhizobacteria increased Cd uptake and accumulation in roots.	Phytostabilisation	da Silva *et al.* (2014)
35	*Atriplex amnicola*, *A. undulate* and *A.*	Cd, Pb, and Ni	Cd, Pb, and Ni were retained in roots > shoots where elevated metal allocation by *A. lentiformis*. Cd concentrations ranged from 34 - 269 mg/kg and 13 - 141 mg/kg; Pb ranged from 71 - 615 mg/kg and 25 -	Cd phytoextraction by *A. lentiformis*; Phytostabilisation	Eissa and Almaroai

	lentiformis		337 mg/kg; and Ni ranged from 51 - 59 mg/kg and 24 - 31 mg/kg, in roots and shoots, respectively. Therefore, all investigated *Atriplex* spp accumulated similar Ni concentrations compared with Cd and Pb.	(Cd, Pb, and Ni).	(2019)
36	*Sesuvium portulacastrum* and *Cakile maritima*	Ni	Ni was accumulated in a dose-dependent fashion in shoots by both plant species. *S. portulacastrum* accumulated elevated Ni concentrations in shoots (1 050 mg/kg) compared with *C. maritima* (550 mg/kg) under hydroponic conditions at the highest Ni treatment – 100 mg/kg. Ni (65 %) was accumulated as a soluble fraction by *C. maritima* whereas ~ 25 % was complexed with cell walls. *S. portulacastrum* accumulated 44 % of Ni as a soluble fraction and 43 % complexed to cell wall. Authors concluded that elevated Ni-cell wall binding may have enabled *S. portulacastrum* to tolerate elevated Ni treatments compared with *C. maritima*.	Phytoextraction of Ni from Ni-contaminated water (i.e. (phytofiltration/ rhizofiltration)	Fourati *et al.* (2016)
37	*Atriplex halimus*	Cd	Elevated Cd concentrations accumulated in roots > shoots. Salinity, as chlorides (NaCl and KCl) reduced Cd accumulation in roots and shoots whereas salinity (as $NaNO_3$) increased Cd accumulation in the leaves of *A. halimus*. Cd and Zn were excreted via trichomes onto the leaf surface. However, salinity decreased the phytoexcretion efficiency of the halophyte where the presence of $NaNO_3$ salts restricted salt excretion compared with chloride salts. Presence of salinity (excluding nitrate salts) increased the Cd tolerance which was attributed to the reduction in Cd uptake and production of osmoprotectants.	Phytostabilisation	Lefèvre *et al.* (2009)
38	*Triglochin maritima*, *Juncus maritimus*, *Sarcocornia perennis*, and *Halimione portulacoides*	Hg	Hg was concentrated in the roots > shoots) of *T. maritima*, *J. maritimus*, *S. perennis*, and *H. portulacoides*. Roots and rhizomes were the main sites of Hg retention where the two monocots (*T. maritima* and *J. maritimus*) had an approximate Hg removal efficiency of 98 % of total Hg. Investigated monocots retained Hg in belowground biomass, reducing the release of Hg back into the environment. Dicots (*H. portulacoides* and especially, *S. perennis*) had an elevated root-to-shoot Hg translocation, indicating that Hg may be released (e.g., phytovolatilisation) and may therefore be a potential source of Hg in the environment.	Phytostabilisation/ Phytoextraction	Castro *et al.* (2009)

1.5. Types of studies investigating phytoremediation potential

Results obtained from different types of phytoremediation studies (e.g. field, pot, and hydroponic studies) are not directly comparable due to variation in abiotic and biotic factors which fluctuate through space and time (Table S.1.3) (Fitz and Wenzel, 2002, Watson *et al.*, 2003, Yamato *et al.*, 2008). For example, phytoremediation studies are influenced by soil (e.g., elemental concentration gradients, root access to depth of contamination), metal (e.g., species and bioavailability), and plant (e.g., metal allocation) factors (Pulford and Watson, 2003), limiting the comparability and repeatability of studies conducted on the same plant species. Under controlled experimental conditions, the plant's response to a particular treatment can be distinguished from other influencing factors, such as the impact of arbuscular mycorrhizal fungi (AMF) to metal allocation by the investigated plant species (Spruyt *et al.*, 2014). This gap in knowledge calls for systems with controlled and quantifiable variables (such as growth medium pH, nutrient composition and concentration, temperature, and relative humidity) (Di Lonardo *et al.*, 2011, Shahzad *et al.*, 2017).

Plant tissue culture (PTC), the aseptic culture of explants (material excised from a donor or parent plant), has been widely used to expose explants to a set of known, and quantified, physical, and chemical conditions over time (García-Gonzáles *et al.*, 2010). Consequently, PTC is a valuable tool which has been used for screening plant species, and their genotypes, for specific traits such as phytoremediation potential (Table S4.1) (Watson *et al.*, 2003, Yongpisanphop *et al.*, 2017). Various studies have concluded that results obtained from field-based studies are comparative but should be confirmed and verified by tissue culture experiments and vice-versa (Capuana, 2011, Di Lonardo *et al.*, 2011, Gatti, 2008, Kališová-Špirochová *et al.*, 2003). Validating results, by undertaking studies differing in experimental conditions, is crucial to developing a network of cumulative evidence to create consilience. Numerous tissue culture studies have also successfully identified plant species, and their genotypes, as potential candidates for their use in phytoremediation trials, i.e., as tools for phytoextraction, phytostabilisation, and phytoextraction (Table 1.1).

1.6. Study species

1.6.1. The genus Tamarix

The genus *Tamarix* (commonly known as salt cedar), belonging to the family *Tamaricaceae*, are a group of halophytic (recretohalophytes) shrubs and trees indigenous to Africa and Eurasia (Sookbirsingh *et al.*, 2010). Alien *Tamarix* species, namely *T. ramosissima* and *T. chinensis*, were introduced to South Africa and Namibia for gardens, agriculture, stabilisation of sand as well as Au mine dust suppression in the late 1800s / early 1900s however, this has resulted in riparian habitats and saline areas being invaded by these *Tamarix* species (Mayonde *et al.*, 2019, Weiersbye *et al.*, 2006a). *Tamarix* spp. provide habitat for fauna, such as birds, as well as stabilises water body banks (Shafroth and Briggs, 2008, Sookbirsingh *et al.*, 2010).

Halophytes are of interest due to their ability to control erosion in arid areas, tolerate salinity, and accumulate a wide range of elements (Dye and Weiersbye, 2010, Flowers and Colmer, 2015, Manousaki and Kalogerakis, 2011). Members of the Tamaricaceae and Chenopodioideae (Amaranthaceae) can tolerate and accumulate a wide range of elements in tissues, including halogens and metals. For example, *T. usneoides* has been demonstrated to (hyper)accumulate Al, Cr, Fe, Co, Ni, Cu, Zn, Ag, Cd, Cs, Au, U, Na, Ca, Cl, Se, and SO_4^{2-} (Dennis, 2008, Dye *et al.*, 2008, Kendall, 2010 , Wilson, 2019); *Tamarix aphylla* - Li and Cd (Hagemeyer and Waisel, 1988); and Cd and Pb (Manousaki *et al.*, 2009) (Table 1.3). The presence of salt glands and ion efflux appears to contribute to salt and metal hyper- and cross-tolerance by recretohalophyte species (Table 1.3) (Wei *et al.*, 2020).

1.6.2. Tamarix usneoides

Tamarix usneoides E. Mey ex Bunge 1843 (Tamaricaceae: Southern African Salt Cedar) (Figure 1.6) is an indigenous, evergreen halophyte tree of semi-arid regions, growing along rivers, and over shallow saline water in paleochannels and pans (Baum, 1978). The indigenous *T. usneoides*

occurs in the southern and western region of Southern Africa, from the Cape to Angola. *Tamarix usneoides* has also been planted on Au/ uranium (U), Pt and Cu mines in South Africa for dust control and abstraction of S and other contaminants from ARD (Weiersbye *et al.*, 2006b). Like many halophyte species, *T. usneoides* possesses ion uptake, translocation and excretion mechanisms to maintain cellular ion homeostasis, as evidenced by the presence of foliar salt glands (Wilson *et al.*, 2017, Wilson *et al.*, 2022). Although some species of *Tamarix*, such as *T. ramosissima*, have been reported to deplete water tables, *T. usneoides* has been demonstrated to have relatively low water use requirements, and therefore is a water cost-effective species which can be utilised for phytodesalination and acid rock drainage decontamination (Dye *et al.*, 2022). Therefore, *T. usneoides* was selected for this study based on the species' ability to tolerate and accumulate a range of elements found in Pt metallurgical effluent and contaminated sites, namely salts (and their metal constituents) including Ni, Co, SO_4^{2-}, Cl^-, Ca, Mg, and Na.

1.7. Rationale

To the knowledge of the author, no previous studies have determined the tolerance, uptake, and translocation of Pt, Ni, and Co by *T. usneoides* from an aqueous solution or under field conditions. This study forms part of a larger study investigating the phytoremediation potential of *T. usneoides* to reduce and recover elements (Pt, Ni, Co, Au, S, Cl^-, Se, and NH_3) present in Au/ uranium (U), Pt and Cu mines and land contaminated by metallurgical effluent produced by base metal refineries (Table S.1.2). The combination of spillages associated with the effluent storage ponds, residual contamination from historic mining activities, and previous evaporation spraying methods have resulted in the contamination of approximately 3.4 ha of land surrounding the ponds (study site). Moreover, the predicted increase in secondary salinisation augments the extent of land cross-contaminated with salts and metal constituents. This highlights the need to identify halophytes as candidates for the simultaneous decontamination (relative to EC) and allocation of precious metals from land contaminated by base metal refinery effluent.

Thus, *T. usneoides* was investigated for its potential to reduce site salinity (i.e. phytodesalination) to advance survivable conditions for salinity intolerant plant species (glycophytes), namely *Searsia lancea* and *S. pendulina* (to provide hydraulic control - (Dye *et al.*, 2017)) and *Berkheya coddii* (extraction of Ni and Co from the study site - (Nethengwe, 2013)). This study also investigated the potential use of *T. usneoides* for rhizofiltration, relative to the recovery of Pt, Ni, and Co, from an aqueous solution across a wide range of pH levels and metal concentrations. This would determine whether *T. usneoides* has the potential to be used for the rhizofiltration (and phytoextraction) of residual precious metals (Pt, Ni, and Co) from effluent produced by base metal refineries – reducing the horizontal and vertical migration of contaminants in sites contaminated by metal refinery activities. Therefore, the findings of this study will create a better understanding of *T. usneoides*' underlying biology, relative to the plant's degree of element tolerance, uptake, and translocation under different experimental conditions.

Two types of studies, namely *in situ* (field trial - Chapter 2) and *in vitro* (PTC - Chapter 4) experiments were conducted and compared with previously published literature. The rational for these studies were to build a network of cumulative evidence to create consilience (Saad, 2020), relative to the ability of *T. usneoides* to accumulate, translocate, and tolerate Pt, Ni, and Co across a wide pH and concentration range. Results obtained were compared between experimental conditions (i.e., controlled and field studies), plant genotypes (Table S4.1), as well as halophytic (Table 1.3) and metal hyperaccumulating (Table S.1.3) plant species to determine the efficacy of *T. usneoides* as a potential candidate to reduce site salinity (phytodesalination) and decontaminate metallurgical effluent spillages - reducing the impact on fauna and flora.

Figure 1.6. *Tamarix usneoides* E. Mey ex Bunge tree growing on an area contaminated by base metal refinery effluent, surrounding the Enhanced Evaporation Spray System (EESS), Springs, Ekurhuleni, South Africa. A: overlapping scales; B: white monecious flowers; C: genetic, molecular, cellular, and physiological, variation in salt accumulation in which (2) ("snow tree") may have elevated salt accumulation adaptation compared with (1). Note C (1) and (2) are different plants growing approximately 1 m apart.

1.8. Aim and objectives

The aim of this thesis was to determine the tolerance, uptake, and translocation of Pt, Ni, and Co by the indigenous exo-recretohalophyte *T. usneoides*, under field (*in situ*) and controlled (*in vitro*) conditions. In order to achieve this aim, specific objectives were set out. The objectives of this study, relative to each chapter, were to:

Chapter 2

(i) Characterise soil chemical properties which are influenced by metallurgical effluent spillages, and in turn mediate the fate of metals between tree biomass and soil profiles;

(ii) Determine S, Pt, Ni, and Co concentrations within harvested *T. usneoides* biomass (plant organs and tissue types) and soil pit profiles;

(iii) Establish whether there is evidence for hyperaccumulation of elements of interest (i.e. potential for phytomining) through the derivation of the bioconcentration factor (BCF) and translocation factor (TF)

(iv) Investigate the effect of tree size (size categories based on tree measurements and allometric equations) on the amount of S, Pt, Ni, and Co extracted by tree replicates via applying the BCF and TF.

Chapter 3

(i) Develop a standardised and rapid direct organogenesis *in vitro* procedure for establishment of *T. usneoides* explants, based on identifying plant tissue culture factors which can be controlled to increase explant establishment.

Chapter 4

(i) Determine the range in Pt, Ni, and Co tolerance and recovery (i.e. potential for phytomining) by *T. usneoides* through the derivation of metal BCF, TF, and tolerance index (TI);

(ii) Assess the impact of aqueous solution pH, plant genotype, and metal concentrations on the tolerance and recovery of Pt, Ni, and Co by *T. usneoides*;

(iii) Investigate the range in tolerance by *T. usneoides* relative to the impact of metal treatments on measured morphological growth parameters (shoot length, number of shoot branches, root length, number of roots, degree of root branching, position of root growth, callus production, phenolic production, and presence of infections).

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Chapter 2

Fate of sulphur, platinum, nickel, and cobalt in a 9-year-old phytoremediation trial with *Tamarix usneoides* (salt cedar)

Aspects of this chapter will form part of two manuscripts:

1. Fate of sulphur, platinum, nickel, and cobalt in a 9-year-old phytoremediation trial with Tamarix usneoides (salt cedar): in process of being prepared as a manuscript for publication in the *International Journal of Phytoremediation.*
2. *Estimating Tamarix usneoides tree biomass, grown in platinum- and gold-mining sites, using allometric equations – applications in phytoremediation*: in process of being prepared as a manuscript for publication in the *Journal of Plant Ecology*.

The first manuscript will benefit the scientific, and non-scientific communities by determining the utility of mature *T. usneoides* trees for the desalination of an industrially polluted technosol through the extraction of S and precious metals (Pt, Ni, and Co). The aim of the second manuscript is to present an alternative, non-invasive biomass measurement protocol for *in situ* estimation of biomass.

2.1. Abstract

Pipe failures and overspray from an enhanced evaporation spray system (EESS) at the Impala Platinum refinery on the Highveld of South Africa, designed to reduce the volume of metallurgical effluents in ponds, resulted in the contamination of surrounding soil. In 2007, a phytoremediation trial was implemented by the University of the Witwatersrand whereby *Tamarix usneoides*, a southern African exo-recretohalophyte, was planted with the objectives of removing ammonia, chloride and sulphate salts in order to protect groundwater from pollution, and to test the trees capacity for removal of contaminant metals. The soils, tree foliage and excreted salts were analysed for chemical properties at intervals. This chapter describes assessment of non-coppiced trees and soils at 9 years after planting, in terms of the fate of S, Co, Ni and Pt. Four trees, different genotypes, that were representative of the range in canopy heights present were selected for harvesting. Tree dimensions were measured in order to categorise trees into different size classes based on allometric equations. Harvested biomass was immediately separated into leafy shoots, twigs, branches, and trunks (wood), and flowers. The root was then excavated to a maximum depth of 3.5 metres using a mechanical excavator. All biomass was weighed immediately upon harvest (fresh mass). Roots were separated into different soil depth intervals. Seven soil pits, comprising four around the harvested root systems and three unplanted control pits, were excavated, and opposite faces were sampled at 20 different intervals (n = 40 soil samples per pit). In the laboratory, fresh subsamples were first washed, and then separated into tissues, namely epidermis, cortex and stele for roots, and outer bark, whereas twigs, branches and wood (trunk) were separated into outer bark, inner bark, and sapwood and heartwood combined. The sub-samples were then dried, milled, and analysed for elemental concentrations. The soil moisture content, pH_{aq}, electrical conductivity (EC) and oxidative reductive potential (ORP) of the fresh soil samples were measured. Dried soil samples were analysed for total elemental content. Overall, the four trees contained metal in the order of: Ni (59.46 ± 4.67 mg/kg) > Co (2.65 ± 0.34 mg/kg) > Pt (50 ± 6 µg/kg). Elevated Ni was allocated by *T. usneoides* compared with Co or Pt (ANOVA, $P < 0.001$). Sulphur was hyperaccumulated (3.9 % ± 0.7 %) in the leaves, followed by coarse roots > twigs > wood > flowers (ANOVA, $p < 0.05$). The pattern of metal bioaccumulation was not consistent between the four replicate trees. Platinum was bioaccumulated (BCF > 1, TF > 1)

in the leafy shoots of one individual tree (BCF = 1.54), Ni in one (BCF = 1.03), and Co in another (BCF = 1.02). Accumulation was in the order of Ni > Co >> Pt, where metals were allocated in the series: flowers > coarse roots > twigs > wood > leaves. Nickel and Co were co-accumulated (BCF > 1; TF < 1) in the roots of three trees. Only one replicate tree accumulated Pt to a greater extent in the leaves, exhibiting both BCF > 1 and TF > 1. There was a trend for higher element concentrations in tissues with tree size, with most Pt and Co in the root tissues, and most S and Ni in the shoot tissues. This supports the potential use of *T. usneoides* for phytoextraction of Co and Pt in the root, and of S and Ni in the shoot. The medium and large individual trees hyperaccumulated S above-ground (BCF > 20; TF > 1), and this was reflected by lower concentrations of S and lower electrical conductivity in all four rooting zone soils by comparison with unplanted, control soil profiles (ANOVA, $p = 0.007$). By 9 years of age, *T. usneoides* had reduced the EC of the 0 – 100 cm depth of the substrate (ANOVA, $p < 0.05$), whilst simultaneously accumulating S, and to a lesser extent Ni, Co, and Pt. At a spacing of 1333 trees / ha, *T. usneoides* trees could remove an estimated 2.23 ± 0.30 mg Pt/ha, 3.02 ± 0.83 kg Ni/ha, 1.28 ± 0.90 kg Co/ha, and 1.28 ± 0.09 tons S/ha, excluding excreted salts. Excreted salts were visible but could not be quantified without confounding surface dust contamination. In conclusion, these findings indicate that *T. usneoides* can be used as a tool for the phytoremediation of sulphate-contaminated land, with plantings managed for root and canopy development.

Keywords: base metal refinery effluent, halophyte, metallophyte, phytoremediation, phytomining

2.2. Introduction

2.2.1. Phytoremediation

Precious metal mining and smelting are important sources of environmental contamination by heavy metals and salts (Lutts and Lefèvre, 2015, Nriagu, 1996). The recovery of precious metals from secondary resources, such as contaminated sites, has gained attention due to the growing demand for these critical elements (Fu *et al.*, 2020) as well as their toxicity in the environment (Nagajyoti *et al.*, 2010). However, conventional methods for the recovery of precious metals require high capital and operational investment, produce concentrated hazardous waste, and results in further disturbance of the environment (Weiersbye, 2007). The efficacy of recovery processes is also impacted by the presence of other contaminants such as salts. This highlights the need to investigate alternative technologies for the recovery of metals from saline sites.

Alternative approaches, such as phytoremediation involves the use of plants to sequester and / or degrade contaminants in the rhizosphere / *in planta*, as well as assimilate, volatilise, and / or excrete contaminants from a contaminated site (Capuana, 2011, Pulford and Watson, 2003, Van Oosten and Maggio, 2015). However, as primary and secondary salinization is expected to increase amid global climate change (Rozema and Flowers, 2008), the ability to use glycophytes for phytoremediation of metals from saline sites are reduced. Moreover, phytoremediation studies have focussed on the use of herbaceous plant species such as *Thlaspi caerulescens* (Zhao et al., 2003), *Berkheya coddii* (Robinson *et al.*, 1997a), and *Alyssum* spp. (Broadhurst and Chaney, 2016). However, the use of these plant species in the field is limited due to their small size and rate of growth. Thus, plant used for phytoremediation must be fast-growing, produce large biomass, tolerate geochemical site conditions, and accumulate elevated metals of interest (Ebbs and Kochian, 1997). This calls for the identification of tree halophytes which produce large biomass, are fast growing, and have the ability to desalinate metal-contaminated while simultaneously accumulating heavy metals (Van Oosten and Maggio, 2015).

2.2.2. The case for halophytes

Salts and heavy metals induce osmotic and ionic toxicity stresses in plants, negatively impacting the survival of salinity-intolerant species (Munns and Tester, 2008). This indicates a selective pressure for salinity-tolerance flora with various molecular, morphological, and/or physiological adaptations (Flowers and Colmer, 2008). Halophytes, which make up < 2% of terrestrial flora (Flowers and Colmer, 2008), have been studied to determine their phytoremediation potential of heavy metals. Members of the genus *Tamarix* have gained interest for its potential use for erosion control and for phytoremediation of contaminated sites in semi- and arid environments. Moreover, most *Tamarix* species, including *T. usneoides*, are considered to be exo-recretohalophytes, where salt glands (glandular trichomes), present in the leaf epidermis, actively excrete excess salt and metal ions to maintain ion homeostasis (Campbell and Strong, 1964, Wilson *et al.*, 2017). This phenomenon has also been reported in other plant species, such as *Spartina alterniflora* (Weis and Weis, 2004), indicating a ubiquitous salt and metal ion storage and / or excretion salt gland function between plant species for the maintenance of the plant's ion homeostasis network (Sanadhya *et al.*, 2015, Wilson *et al.*, 2017). This suggests that the presence of salt glands may confer salinity and heavy metal tolerance by maintaining ion homeostasis (Flowers and Colmer, 2015). Halophytes have developed various adaptations, such as the excretion of salts from vesiculated trichomes (salt glands) to maintain ion homeostasis in saline environments (Kadukova *et al.*, 2008, Wilson *et al.*, 2017). Santos *et al.* (2017) reported the excretion of various metal ions (e.g., aluminium (Al), Cr, Cu, Ni, Pb, and Zn) by the salt glands of *T. africana*.

Metal/loids have been reported to accumulate in the roots and shoots of *Tamarix* spp such as *T. nilotica* [nickel (Ni), cobalt (Co), and chromium (Cr) - (Fawzy *et al.*, 2006)], *T. hispida* [Ni, Cr, Co, and copper (Cu) - (Toderich *et al.*, 2010)], *T. smyrnensis* [cadmium (Cd) and lead (Pb) - (Kadukova *et al.*, 2008)], and *T. usneoides* [sulphur and selenium (Se) – (Kendall, 2010), and sulphur, gold (Au), Zn – (Dennis, 2008, Wilson *et al.*, 2022)]. Higher metal ions accumulated in roots of *T. aphylla* (Wafa'a, 2009), *T. gallica* (Sghaier *et al.*, 2016), and *T. nilotica* (Osman and Badawy, 2013), compared with shoots. In some of these cases, salinity enhanced metal/loid uptake and accumulation

by the *Tamarix* spp (Kadukova and Kalogerakis, 2007, Sghaier *et al.*, 2016) and other halophytes including *Sesuvium portulacastrum* (Khella, 2018), *Salicornia europaea, Suaeda maritima, Salsola soda*, and *Halimione portulacoides* (Milić *et al.*, 2012).

2.2.3. Metal allocation between plant organs

Determining the assimilation and distribution of metals between plant organs can help to inform the design of metal recovery from plants (Dinh *et al.*, 2022). *In planta* metal distribution is mediated by elemental (e.g., physicochemical properties) and plant (e.g., genotypic variation) factors (Tőzsér *et al.*, 2017). Nickel and Co have an affinity to bind to S ligands such as phytochelatins (Yadav, 2010), and metallothioneins (low molecular weight thiols – Zhi *et al.* (2020)), as well as amino acids (e.g., histidine – Amari *et al.* (2017)). The type and concentration of these ligands have been demonstrated to differ between plant species and plant organs – contributing to variation in metal content between plant organs and tissue types. Nickel and Co have similar physicochemical properties whereby an antagonistic interaction between these elements in plants has been reported (Keeling *et al.*, 2003). When present in combination, Ni may outcompete Co (or vice-versa) from binding sites and subsequent uptake by plant roots. This has been demonstrated by *Noccaea caerulescens* Tang *et al.* (2019) and *Alyssum* spp. Malik *et al.* (2000) whereby Co uptake significantly increased (1 320 mg/kg) when Ni was absent from the growing substrate.

The efficacy of the plant's ion homeostasis network mediates the degree of accumulation and distribution of metals between plant organs (Hanikenne *et al.*, 2021). For example, Ni is hyperaccumulated in the leaves of *Alyssum inflatum* (Ghasemi *et al.*, 2009) whereas non-hyperaccumulator species, such as *Rubus ulmifolius* (Marques *et al.*, 2009) limit the translocation of Ni in leaves by retaining Ni in roots. Metal hyperaccumulation as well as exposure to phytotoxic concentrations of certain elements (such as Ni) compromises the plant's Fe homeostasis network (Krämer *et al.*, 2007). This highlights the importance for hyperaccumulators, as well as adaptations of halophytes, to maintain ion homeostasis. Variation in the efficacy of ion homeostasis networks between plant species suggests that plant species differ in their ability to distribute metals between

plant organs (e.g., leaves, twigs, wood, and roots) and tissue types [e.g., above (outer bark, inner bark, sapwood and heartwood) and belowground (epidermis, cortex, stele) plant organs].

Sulphate (SO_4^{2-}) is the predominant form of sulphur (S) absorbed by roots through S-assimilatory pathways (Hawkesford and De Kok, 2006). Sulphate is then reduced in leaves to adenosine-5-phosphosulphate (APS), sulphite (SO_3^-), sulphide (S^{2-}), and then cysteine. Cysteine is then incorporated into glutathione (GSH) – a low-weight molecular thiol (antioxidant) involved in the detoxification of metals (Hasanuzzaman *et al.*, 2017). Thus, variation in the presence of S in plant organs may contribute to differences in the distribution of metals between plant organs due to the detoxification (e.g., compartmentalization) of metals. Furthermore, various studies have reported that sulphur (S) is co-localised with Co or Ni within the leaves of hyperaccumulators (Broadhurst *et al.*, 2009, Tappero *et al.*, 2007). Results obtained by Yamaguchi *et al.*, (2019) support these findings, where elevated S concentrations (where S may act as a counter ion for Co) in the leaves of *Clethra barbinervis* may have been implicated in Co accumulation. These authors suggested that differences in the localization of S (and more specifically SO_4^{2-}) relative to the distribution of Co and Ni in leaves indicates that different metal assimilatory pathways may exist.

2.2.4. Phytomining

Phytomining is the process whereby metals of economic importance are recovered from the biomass of plants with the ability to accumulate metals in particular plant organs (Chaney *et al.*, 1998). Although numerous plant species exhibit metal accumulation to varying degrees, hyperaccumulation is a rare and species-specific trait (Manara *et al.*, 2020). Hyperaccumulation differs between metals where the Ni and Co thresholds are generally considered to be > 1 000 mg/kg and > 300 mg/kg, respectively (Baker, 1981, Van der Ent *et al.*, 2013). Hyperaccumulators have been investigated for their phytomining potential (e.g., Ni - (Tognacchini *et al.*, 2020)). It has been generally assumed that plants used for phytomining must produce large biomass and/or be fast-growing, translocate metal(s) to shoots, and hyperaccumulate metal(s) in shoots which can be

harvested and precious metals recovered (Sheoran *et al.*, 2013). However, accumulation in fine root biomass may also be economically desirable, depending upon the growing medium.

2.2.5. Important considerations for phytoremediation trials

Root system architecture (RSA - the spatial partitioning of the root system) addresses the shape (location and the way in which the root system occupies the rhizosphere) and structure (describes root relationships, such as topographic features) of the plant's root system (Lynch, 1995). The RSA is influenced by biological (e.g. plant species) and physicochemical characteristics of the growing medium (Khan *et al.*, 2016). Physical characteristics, such as changes in soil hardness / compaction has been reported to restrict root growth and development (Håkansson *et al.*, 1988). Reduced root growth limits the plant's access to water and nutrients required for survival.

In the context of this study, the dilution effect is defined as the decrease in metal concentrations *in planta* over time (Jarrell and Beverly, 1981). This effect is attributed to the increase in bark: wood ratio as trees grow larger, whereby lower metal concentrations accumulates in bark compared with wood (i.e., sapwood and heartwood). As plants age, the relative contribution of bark to the overall biomass increases, consequently decreasing metal accumulation over time. Although some studies (Filipović-Trajković *et al.*, 2012) have reported elevated metal concentrations in the bark compared to roots and leaves, accumulation in bark as well as atmospheric metal deposition (where the irregular surface of bark increases the surface area for metal adsorption) must be considered when interpreting results.

Platinum, Co, and Ni are geochemically classified as siderophile elements with some chalcophile characteristics (Fairbridge, 1972). Based on these physicochemical properties, these elements interact with elements which are biogeochemically associated with iron (Fe) (Kabata-Pendias, 2011), influencing their bioavailability and assimilation of Pt, Ni, and Co by plants. Such metals include, but are not limited to, Cu (Lwalaba *et al.*, 2020), Zn (Boyd and Martens, 1998), and Pb (Khan *et al.*, 2019). The presence of these elements in contaminated sites must be considered when investigating plant species' phytoremediation potential relative to Pt, Ni and/or Co.

2.2.6. Rationale

This study forms part of a larger study investigating the ability to use *T. usneoides* to reduce the concentrations of ammonia (NH_3), sulphur (S), chloride (Cl^-) and some metal/loids including Pt, Ni, Co, and selenium (Se). *Tamarix usneoides* was selected to reduce electrical conductivity (EC) to survivable conditions for metal hyperaccumulating species including *Berkheya coddii* (Nemutandani *et al.*, 2006, Nethengwe, 2013) and plants to provide hydraulic control, namely *Searsia lancea*, and *S. pendulina* (Dye *et al.*, 2017).

2.2.7. Aim

The aim of this study was to determine the utility of 9-month-old *T. usneoides* trees in the desalination of soil contaminated by base metal refinery effluent through the extraction of sulphur, and precious metals Pt, Ni, and Co into harvestable biomass. This aim was achieved through assessing the fate of these elements between tree biomass (plant organs and tissue types) and soil profiles whereby soil chemical properties were compared between planted and unplanted pits. Specific objectives of the study were as follows:

1. Characterisation of soil chemical properties which are influenced by metallurgical effluent spillages, and in turn mediating the fate of metals between tree biomass and soil profiles;
2. Determination of S, Pt, Ni, and Co concentrations within harvested *T. usneoides* biomass (plant organs and tissue types) and soil pit profiles;
3. Establishing whether there is evidence for hyperaccumulation of elements of interest (i.e. potential for phytomining) through the derivation of the bioconcentration factor (BCF) and translocation factor (TF)
4. Investigating the effect of tree size (size categories based on tree measurements and allometric equations) on the amount of S, Pt, Ni, and Co extracted by tree replicates via applying the BCF and TF.

2.3. Materials and Methods

2.3.1. Study site

The study site is located within the Soweto Highveld Grassland vegetation type, with a mean annual precipitation of 662 mm (summer rainfall), MAPE of 2060 mm, with minimum and maximum temperatures of 6 °C and 27 °C, respectively, with a cool temperate climate and frequent frost occurrences (Mucina and Rutherford, 2006). Historically, the vegetation structure comprised grassland biome species. Soils associated with the study site are deep, reddish apedal and sandy loam plinthic soils, and typically comprise of Ea (black and red clay soil pattern), Ba (plinthic soil pattern), Bb (plinthic soil pattern) land types (Job *et al.*, 2019). The geological features associated with the site include intrusive Karoo Suite dolerites surrounded by bands of diamictite and arenite. The altitude ranges from 1 420 – 1 760 m. The site is located on a major dolomite aquifer which is recharged by the Blesbokspruit (Scott, 1995). The Blesbokspruit is a wetland of RAMSAR importance, located to the east of the study site. Both Cowles and Alexander Dams drain into the Blesbokspruit. Groundwater, as well as contaminants present within groundwater, naturally migrate along the path of least resistance (i.e., from higher to lower elevations). The study site has a higher elevation (1578 m above sea level) compared with both Cowles and Alexander Dam (1578 m), suggesting that surface and groundwater would flow towards the two dams (and associated wetland) and the Blesbokspruit. Historically, the Blesbokspruit has been polluted by mining activities such as dewatering, seepage from tailing storage dams, tailing reclamation, and aeolian erosion (Wittmann, 1981). Roychoudhury and Starke, (2006) recorded elevated Ni (36 mg/kg; ranging from 16.2 – 438 mg/kg) and Co (9.8 mg/kg; ranging from 3.6 – 123 mg/kg) concentrations in bulk sediments of the Blesbokspruit. Various anthropogenic activities along the Blesbokspruit may account for these elevated levels of Ni and Co concentrations. Surrounding land uses include mining-related activities (metal production and refining, and historic mining activity including TSFs and TSF footprints), agriculture, and urban sprawl.

The Impala Platinum Refinery Services (IRS)

The Impala Platinum Refining Services (IRS), located in Springs, Ekurhuleni East of Johannesburg, South Africa was created in 1998 to provide refining and smelting services through offtake agreements with Group companies (Implats, 2021). The IRS consists of the platinum (PMR) and base metal refineries (BMR), and smelters. Two effluent streams from the PMR, as well as any excess wastewater from the BMR, are pumped into ponds for tailing storage and further metallurgical processing (Figure 2.1; Figure S1.1). The ponds are situated approximately 2900 m west of the Blesbokspruit, an area of RAMSAR importance. The Blesbokspruit consists of a stream running through the towns Springs, Nigel, and Heidelberg, ultimately joining the Suikerbosrand River.

The storage ponds and Enhanced Evaporation Spray System (EESS)

The ponds consist of four, double high-density polyethylene (HDPE) lined ponds containing a combined total storage capacity of 110 000 m^3. These ponds store effluent from two streams produced by the PMR. The first effluent stream contains an acid stream (used to remove NH_3 and NO_x from scrubbers) whilst the second is an alkaline stream received from an effluent tank farm area (where the last step of the alkaline effluent treatment process takes place before being discharged into the ponds). These effluents are subsequently sent to crystallizers for treatment (waste concentration and disposal of sludge) where wastewater is discharged into the ponds. As water evaporates, precious metals are reclaimed from the sludge (Gunarathne *et al.*, 2022). The EESS (Figure 2.1), was built at the ponds in 1993 in order to enhance evaporation of water via aerial spraying, was and decommissioned in 2008, when the ponds were relined.

The ponds and EESS as a source of contamination

Effluents stored in the ponds contain varying concentrations of ammonia, platinum group metals (PGM), Ni, Co, Cl^-, Fe, sodium (Na), Se, SO_4^{2-}, and tellurium (Te) (Burger, 2015). The recovery and refining process is not 100 % effective and therefore, residual metal salts, both chlorides and sulphates, are discharged to the ponds (Figure S.1.1) (Crundwell *et al.*, 2011, Sinisalo and

Lundström, 2018). The pond effluent chemical properties and metal concentrations were measured from December 2004 to January 2012 (n = 10). The wastewater had an alkaline pH (average 9.62) and high electrical conductivity (EC: 13 467 mS/m), with SO_4^{2-} (37 548 mg/kg) being the major contributing salt, and elevated Pt averaging 10.24 mg/kg), Ni (11.56 mg/kg), and Zn (13.45 mg/kg) concentrations (Burger, 2015).

The combination of spillages associated with the ponds, residual contamination from historic mining activities (e.g., Old Geduld tailing storage facility (TSF)) (Figure 2.1), and previous evaporation spraying methods have resulted in the contamination of approximately 3.4 ha of soil, with an estimated volume of 30 000 m^3 and 60 cm average depth of significant contamination, surrounding the ponds. These spraying activities, commissioned in 1993 and decommissioned in 2008 (15 years), involved the abstraction of wastewater from the ponds, which was subsequently sprayed directly into the atmosphere to increase the rate of evaporation. The prevailing north-west to south-easterly wind direction resulted in the deposition of effluent onto soils surrounding the ponds. According to the Gaussian plume model, effluent metal concentration would have decreased as distance from the sprayers (Litalien and Zeeb, 2020).

Contaminants present in the study site soils may migrate vertically and horizontally, via aeolian erosion and rainwater or effluent leaching processes, along numerous flow pathways to surface and groundwater bodies. Groundwater, as well as contaminants present within groundwater, naturally migrate along the path of least resistance (i.e., from higher to lower elevations). The ponds (1578 m above sea level) are elevated above both Cowles and Alexander Dam (1578 m), suggesting that surface and groundwater would flow towards the two dams (and associated wetland) and the Blesbokspruit. Anthropogenic activities in Ekurhuleni, including gold mine tailings dam failures since the early 1900s (see (Sutton and Weiersbye, 2007)) have resulted in acid mine drainage and elevated concentrations of a range of metals, including Ni and Co, in drainage lines and sediments (Roychoudhury and Starke, 2006). These pollution levels provide an additional rationale to determining the Ni and Co phytoextraction potential, under saline conditions, of *T. usneoides*. Thus, the ecotoxicity of these metals as well as the growing demand for these critical and precious metals

(elucidated by current trading prices Co: USD $33.50 kg^{-1}; Ni: USD $14.99 kg^{-1}; and Pt: USD $24 958.94 g^{-1} (LME, 2022)), indicates the need to recover these precious metals while simultaneously remediating saline metal-contaminated sites.

2.3.2. EESS phytoremediation project

In 2007, the University of the Witwatersrand was requested to conduct a research project on phytoremediation of the salt and metal-contaminated land surrounding the ponds. Site contamination had been characterized by assessing chemical parameters, and concentration of elements present in effluent and the soils (C.J. Viljoen, unpublished data; I.M. Weiersbye, unpublished data) over a full hydrological cycle.

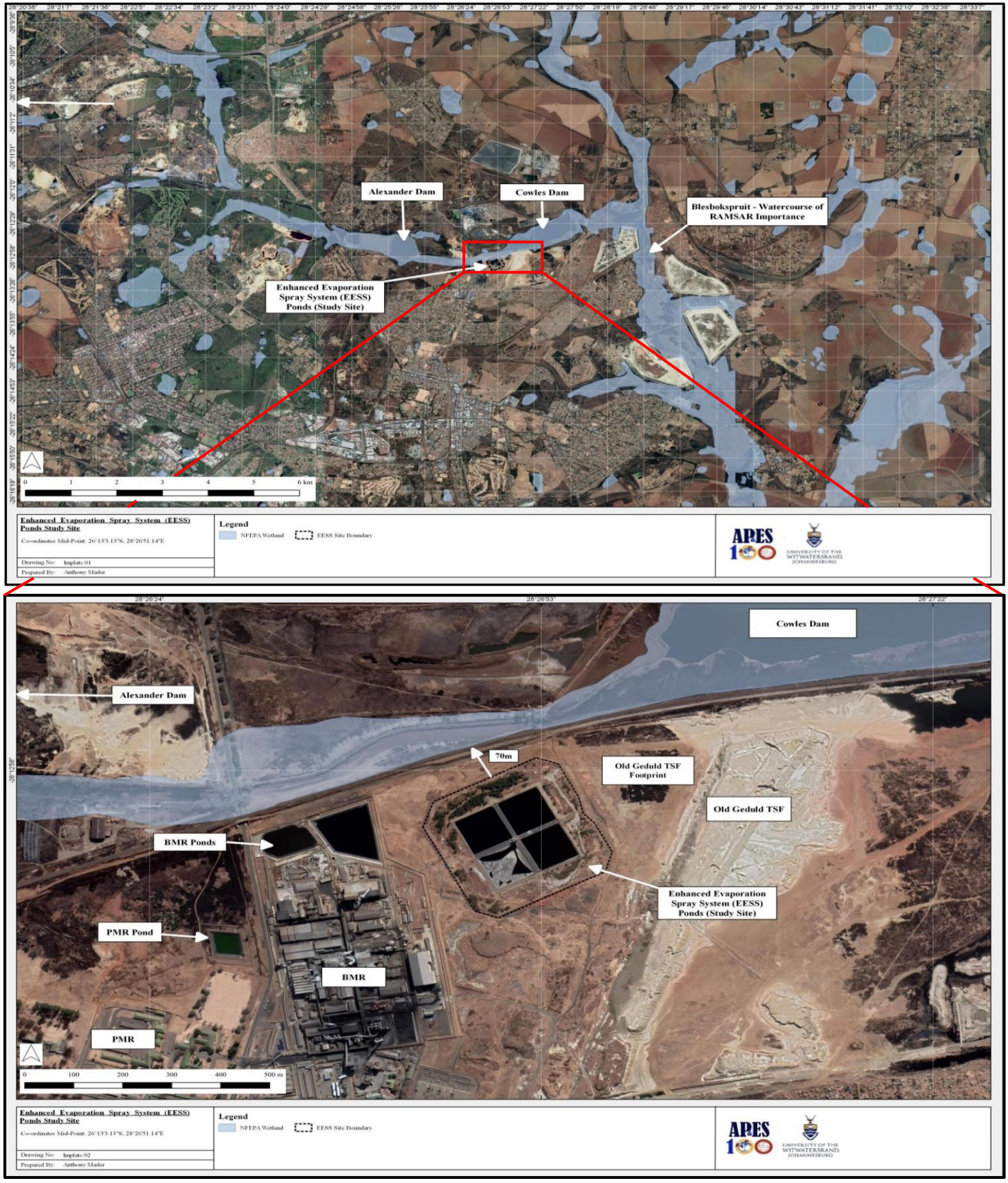


Figure 2.1. Impala Platinum Refinery Services (IRS) layout, comprised of the Enhanced Evaporation Spray System – EESS ponds (tailing storage facility), precious (PMR) and base metal refineries (BMR), and associated PMR and BMR ponds. Also shown are historical gold mining operations (Old Geduld tailings storage facility, TSF), and waterbodies - Alexander and Cowles' dams, and the Blesbokspruit wetland (located approximately 1.9 km east of the study siter, a watercourse of Ramsar Convention on Wetlands of International Importance), and a National Freshwater Ecosystem Priority Area (NFEPA) wetland. QGIS, version 3.10.14.

The study site (3.4 ha) was divided into planting blocks (Figure 2.2.A) based on the locations of tailings pipelines and roads. Site preparation in late 2008 comprised of ripping on contour to a depth of 600 mm and pitting of 300 mm x 300 mm. In January 2009, *T. usneoides* (accounting for 88% of all plants at the site) was planted in a broad band surrounding and abutting to the walls of the evaporation ponds, whereas *Searsia pendulina* and *S. lancea* were planted in two rings encircling *T. usneoides* trees. Planting involved treatment of the pits with Stockosorb® 660 hydrogel (Evonik, Essen, Germany). The trees were planted on contour at a density of 1333 trees / ha (2 m x 1.5 m espacement), where the age of the trees ranged from three-to-twelve months old with an approximate height of 20 - 50 cm. Trees received a single treatment in spade slots with 50 g of 2:3:2 soluble NPK starter fertiliser within two weeks of planting, and no further treatments. Please refer to Chapter 1 for more information on the study species, *T. usneoides*.

The aims of the phytoremediation trial were to test the tolerance to site conditions and efficacy of four species (I.M. Weiersbye, personal communication, 3rd February 2014):

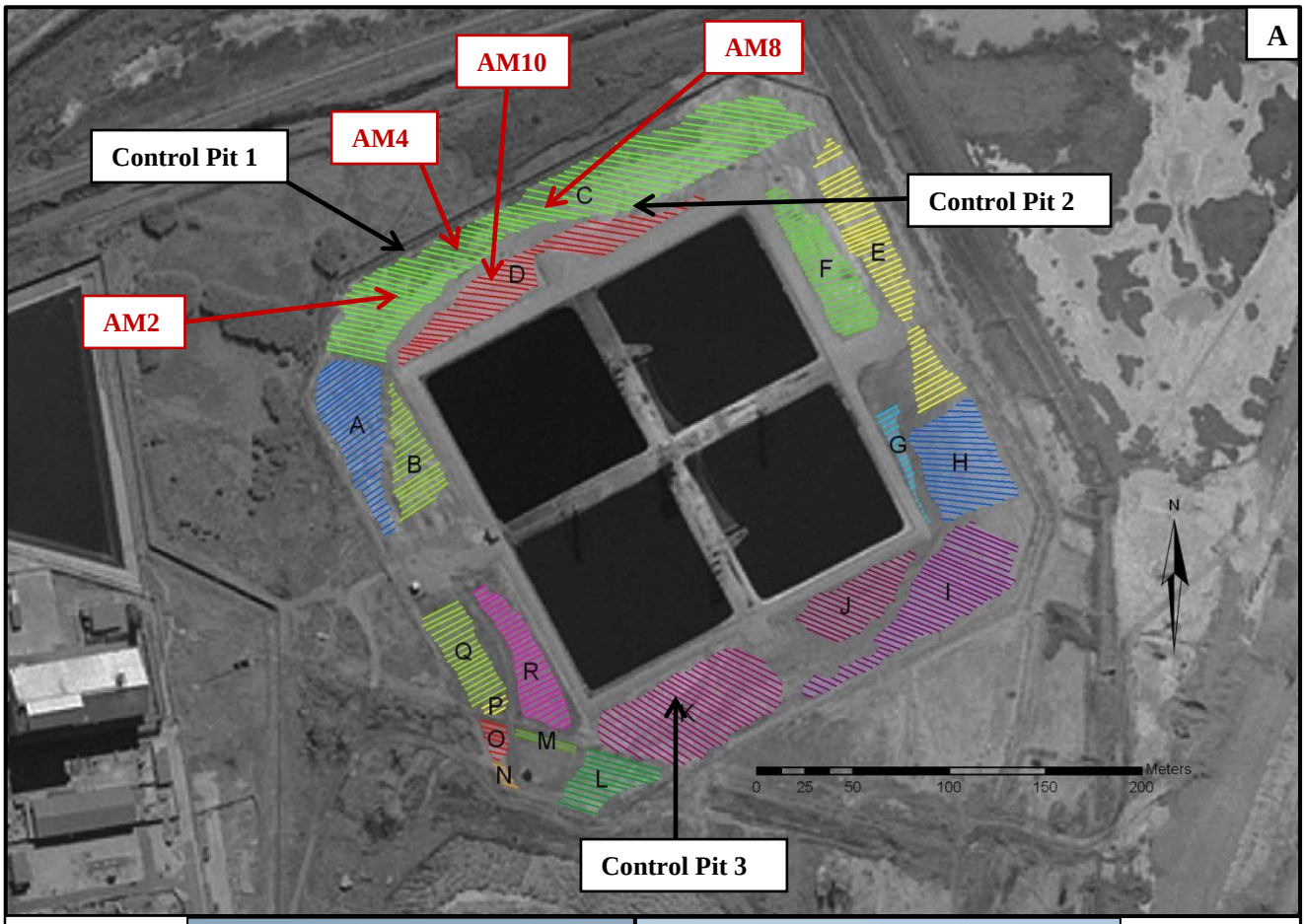
a) *T. usneoides* in terms of consumption of N, hyperaccumulation of S and soil desalination, and potentially accumulation of Se, Co, Ni and Pt in shoot or root under saline conditions,
b) *B. coddii* in terms of hyperaccumulation of Ni and Co in the shoot, subsequent to site desalination, and
c) *S. lancea* and *S. pendulina* in terms of water-use, to provide hydraulic control (thereby reducing leaching and groundwater contamination).

Tamarix usneoides was selected for the trial to provide some degree of hydraulic control, but primarily to reduce the concentrations of ammonia, sulphur, chloride and some metals (phytodesalination), creating survivable conditions for higher water-use trees to provide hydraulic control, *S. lancea* and *S. pendulina* (Dye *et al.*, 2008, Dye *et al.*, 2017), and a Ni and Co hyperaccumulating shrub, *Berkheya coddii* (Nethengwe, 2013, Slatter, 1998). *Tamarix usneoides* was also planted to provide fast-growing canopies in contact with the ground surface thereby mitigating any horizontal contaminant migration via aeolian erosion and run-off. It was predicted that *T.*

usneoides would die back as substrate salinity declined, and the glycophyte species *S. lancea*, *S. pendulina* and metallophyte *B. coddii* could then be planted over the area. However repeated spillages of effluents and destruction of trees by wildfires have prevented this progression, and to date, *T. usneoides* remains the species of preference on the site (I.M. Weiersbye, personal communication, 20 March 2022).

2.3.3. Genetic verification of Tamarix usneoides

The phytoremediation trial comprises clones of trees sourced from the Orange River near Upington and Augrabies region of the Northern Cape province of South Africa. Due to the presence of hybrids between *T. usneoides* and the invasive alien species *T. ramosissima* and *T. chinensis* in South Africa (Mayonde *et al.*, 2015), trees selected for this study were genetically verified as pure-breeding *T. usneoides* by G. Cron, H. Serepa and G. Mayonde (2015, unpublished data) according to the methods of (Mayonde *et al.*, 2016). Approximately 100 g of developing shoot tips were collected from 10 *T. usneoides* trees (differing in size), placed in a coffee filter bag and a Ziploc bag containing activated silica gel. Samples were stored in a cooler box and transported to the University of the Witwatersrand, Johannesburg, where they were stored at - 20 °C until samples were processed. Collected foliar samples underwent cleaning, DNA extraction, clean-up, purification using a modified standard technique, followed by restriction – ligation, pre-selective PCR, selective PCR, fingerprinting with the scoring of 102 alleles, and AFLP structure analysis alongside *T. chinensis* and *T. ramosissima*. A Bayesian model-based clustering analysis (Structure 2.3.4) was used to score the likelihood of hybridisation (Mayonde *et al.*, 2016). All trees used in this study (namely AM4, AM8, AM2, and AM10) were grouped as non-hybrid, pure-breeding *T. usneoides*.



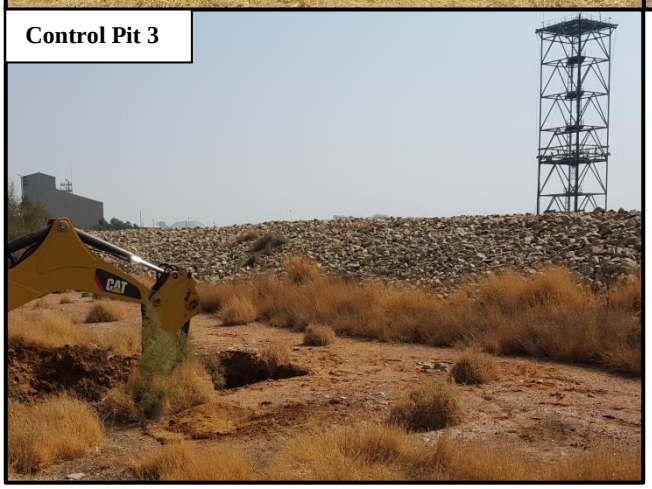


Figure 2.2. Plots surrounding ponds of the Enhanced Evaporation Spray System (EESS) showing locations of the four *Tamarix usneoides* trees used in this study: AM4 – very small, AM8 – small, AM2 – medium, AM10 – large. Site map constructed by D.J. Furniss and reproduced for non-publication purposes from D.J. Furniss, I.M. Weiersbye and B.J. Dawson. (b) Soil pits were excavated to characterize the composition and concentration of contaminants along the soil profile. No trees were present within a 10 m radius of selected control (unplanted) pits.

2.3.4. Allometric equations

Four replicate trees were selected based on height and canopy dimensions being representative of the range of trees on the site (Figure 2.3). Biomass parameters, *viz*. tree height (H_1), stem basal diameter (SBD, measured at 0.3 m above soil surface), stem diameter at breast height (DBH, measured at 1.2 m above soil surface), canopy diameter ($D_{1'}$ longest length of canopy), and canopy diameter (D_2, perpendicular to D_1) were measured (using a measuring stick and vernier calliper) to determine tree volume (Figure 2.3 - Williams *et al.* (2005)). Tree biomass was calculated using the following equation (Equation 2.1):

$$B_{T.\ usneoides} = V_{cone} - B_{over\text{-}cone} \qquad \textit{(Equation 2.1)}$$

where; $V_{cone} = \frac{\pi.D1.D2.Ht}{3}$

$B_{over.cone} = V_{cone} - \pi.\ SBD.\ DBH.\ H_{1,2}$

V_{cone} = *volume of cone*

$B_{over\text{-}cone}$ = *biomass of overestimated cone shape*

$H_{1,2}$ = tree height at 1.2 m above soil surface

Figure 2.3. Measuring of tree dimensions (canopy height, stem basal diameter, stem diameter at breast height, canopy diameter (D_1, longest length of canopy), and canopy diameter (D_2, perpendicular to D_1)) to determine tree biomass and size class categories using allometric equations. For reference, the author (in blue) is 1.76 m tall.

2.3.5. Harvesting of Tamarix usneoides trees

Aboveground biomass, *viz.* leafy shoots, twigs, stems, branches, trunks (wood) and flowers (if present), were harvested first, followed by belowground biomass, *viz.* coarse and fine roots from different soil depths. Roots were categorized into different sections based on soil depth layers – namely taproot, lateral roots, and fine roots (TR) (0 – 100 cm soil depth interval), red layer (RL) (100 – 210 cm soil depth interval), and rocky soil (RS) (210 – 340 cm soil depth interval). Subsamples of plant organs were further separated into different tissue types.

Above-ground biomass

Aboveground biomass of four trees was harvested from Plots C and D (Figure 2.2 and 2.3). A clean black tarpaulin was placed in the shade in close proximity to the tree being harvested. One tree branch was excised using a Zubat Silky Handheld Saw (330 mm) at a time to minimise plant material drying out before being weighed for fresh mass. Each branch was placed onto the black tarpaulin to prevent contamination by soil and any loss of plant material. The tarpaulin was cleaned between trees. *Tamarix usneoides* possesses scale-like leaves which are sessile and overlapping along fine stems. These are categorised as leafy shoots (Figure 2.4), non-leafy shoots, and woody material - including twigs (circumference < 1cm), wood (circumference > 1cm), and dead wood. Flowers were collected when present.

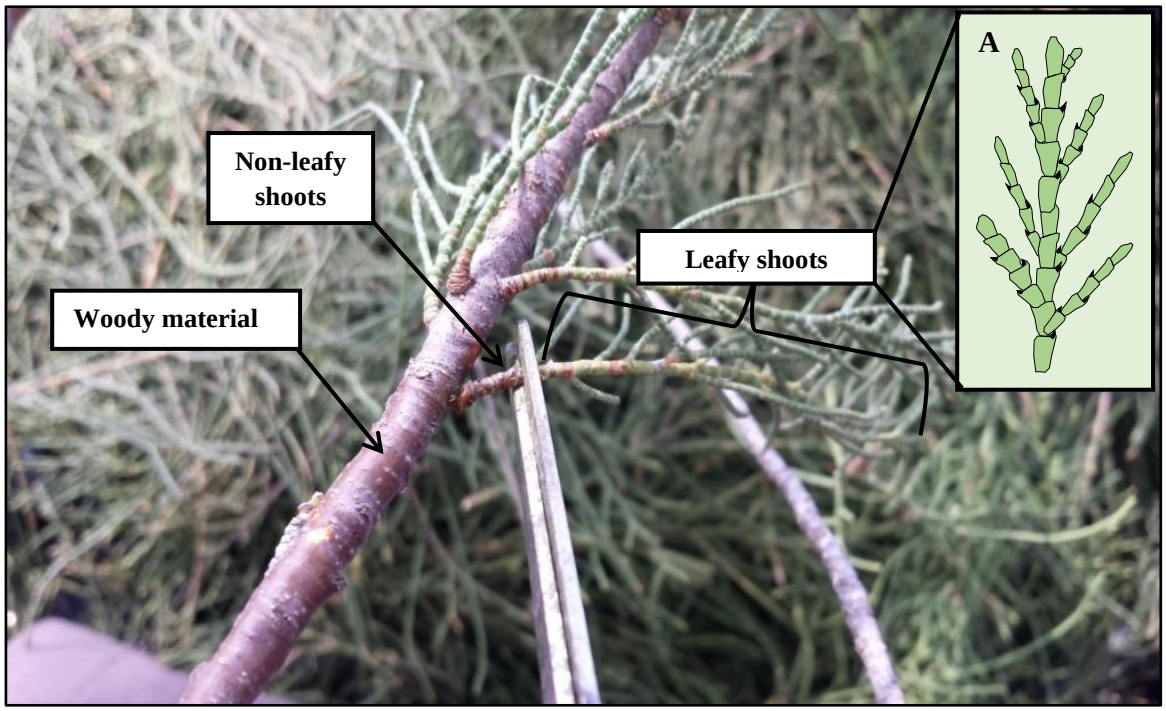


Figure 2.4. Separation of leafy shoots from non-leafy stems and woody material of *Tamarix usneoides.* Inset A: Leafy shoots scale-like leaves which are sessile and overlapping along fine twigs.

All plant material was placed in black plastic bag and immediately weighed. Plant materials were transferred to 3 mm mesh nylon 40 % shade net bags, which were stored in a shed on site and left to air dry to determine dry mass. These materials were not used for any further analyses. The tree trunk and branches were further separated into three tissue types, namely outer bark (OB, dead tissue derived from the phloem), inner bark (IB, the living phloem), and sapwood and heartwood combined (SH, the living and dead xylem, respectively, together with any pith present and medullary rays) (Figure 2.5). The OB, IB, and SH and medullary rays differ in form and function. These tissues differ in cell types, structure (Fahn and Arnon, 1963), function, and element composition and concentrations (Mesjasz-Przybylowicz *et al.*, 2016). Plant material was separated using a ceramic knife (Levo Ceramic Utility Knife) to prevent sample cross-contamination.

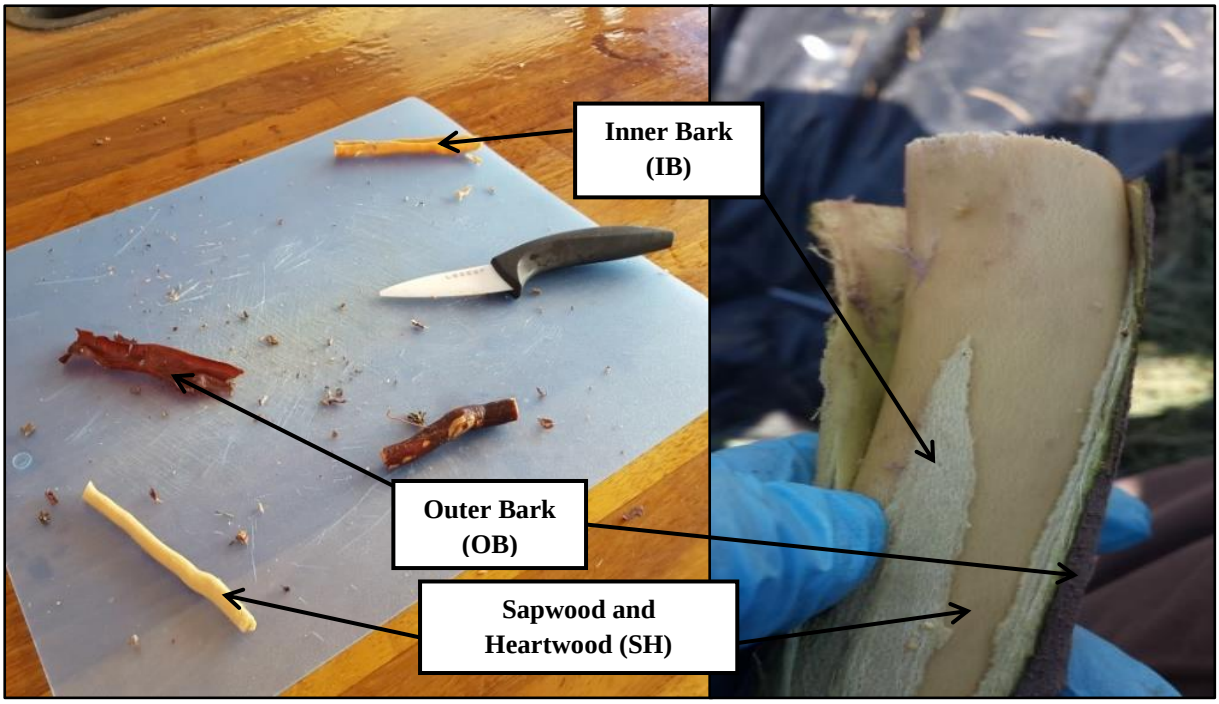


Figure 2.5. Separation procedure of twigs (circumference < 1 cm), branches and stem (circumference > 1 cm) into outer bark (OB), inner bark (IB), and sapwood and heartwood combined (SH) using a ceramic knife.

Chronological age of plant material impacts plant physiology and metal uptake dynamics (Rout and Sahoo, 2007). Sub-samples (> 100 g) of each plant organ and tissue type (e.g., twigs – OB, IB, and SH, wood – OB, IB, and SH) were collected from different areas of the tree to obtain an average representation of each tree for elemental analysis. Each sub-sample was washed well in tap-water to remove surface dust as well as the excreted salts, and then three times in distilled water. Each washed sub-sample was placed in a plastic Ziploc bag and stored in a chest freezer at - 20 °C until freeze-drying. The subsamples for elemental analysis were freeze-dried in a Labcon FreeZone 4.5 L Freeze Drying System (LABCONCO, Labconco Corporation, 8811 Prospect Avenue, Kansas City, USA) for 72 hours at – 85 °C. Tree trunk sections (Figure 2.6) were collected at 30 cm and 120 cm sections aboveground, relative to the height at which SBC and DBH were measured, respectively. The density of wood sections was determined using the water-displacement method (Olesen, 1971), whereby a wood block displaces an amount of water equivalent to its volume. During air-drying, plant material was weighed until there was no further loss of moisture. The final mass of plant tissue was recorded on 10/08/2016 (395 days from date of harvest).

Figure 2.6. Wood sections were collected for density and volume measurements. Wood section (on left) was cut at 1.1 – 1.3 magl ground (to preserve circumference where DBH was measured for allometric equations) whereas the wood section (on right) was cut at 0.2 – 0.4 m (to preserve circumference where SBC was measured at 0.3 magl).

Below-ground Biomass

Soil pit excavation

Soil pits containing the roots of AM8, AM2, AM4, and AM10 were excavated after harvesting of all above-ground biomass, on 13/07/2015, 20/07/2015, 24/07/2015, and 14/08/2015, respectively, by a soil surveyor (Red Earth cc, Pietermaritzburg, South Africa). A tractor-loader-backhoe (TLB – Caterpillar, CAT 428F Backhoe Loader, AEHQ6812-00) was used to excavate a total of seven soil pits, four pits containing the roots of each tree (i.e. planted), and three control (i.e. unplanted) pits (Figure 2.2). Control pits 1, 2, and 3 were selected in areas void of trees (> 10 m radius), at a distance of 10 m, 12 m, and 11 m, respectively, from the nearest tree. Soil pit dimensions differed slightly due to soil properties and safety considerations. The dimensions of each pit were approximately 3.5 m (depth) x 1.5 m (width) x 2 m (length), except for control pit three (2.4 m depth

x 1.5 m width x 2 m length). Each soil pit was excavated to taper off towards the bottom of the pit to maintain the pit's structural integrity.

Vegetation covering or surrounding each soil pit was carefully removed by hand with clippers and using a spade to avoid disturbing the surface soil. The soil classes were classified by Red Earth cc (13/07/2015 – 20/08/2015), and each soil pit was divided into three broad soil depth intervals based on depth and soil type and horizon, namely tap root, TR (0 – 100 cm surface soil depth interval), red layer, RL (100 – 210 cm subsoil depth interval), and rocky subsoil layer, RS (210 – 340 cm soil depth interval, which consisted of soil conglomerates). Soil from each layer was stockpiled approximately 5 m away from the pit in separate piles. Soil surrounding the root was first removed in order to allow the root to be retrieved as intact as possible. Rhizosphere soil samples surrounding the tap root and associated lateral and fine roots, were collected and placed in plastic Ziploc bags.

The retrieved taproot and laterals were photographed to observe root system architecture (RSA). Each layer of soil (TR, RL and RS) was sieved, using a square, stainless steel mesh sieve (1 m^2), to retrieve coarse (circumference > 1 cm) and fine (circumference < 1 cm) roots within each layer. Coarse and fine roots were placed in separate black plastic bags and immediately weighed. Coarse and fine root sub-samples (> 100 g where possible - as finer roots in RS layer were negligible) were collected from different parts of the root system to ensure a representative sample of the entire root system. The remaining roots were then transferred to nylon mesh (shade netting) bags and air-dried in a storage shed (as described above. The subsampled roots were washed well in tap water at the site and transported to the laboratory for further washing, and subsequently rinsed three times in distilled water and blot dried. It is accepted that this washing method is unlikely to remove all adsorbed contaminants from roots. The washed subsamples were stored in the cold room at 4 °C until further separation took place. Coarse and fine roots were separated into the epidermis (E), cortex (C), and stele using a ceramic knife, which was cleaned with distilled water between samples. The separated root tissues were weighed and stored at - 20 °C prior to freeze-drying, and then re-weighed for dry mass in order to calculate a fresh mass/dry mass ratio to apply to the total biomass.

Soil pit sampling

Soil (> 100 g) was collected at intervals down the profile from two opposing soil pit faces. Intervals sampled included surface litter, mineral crust (if present), 0 - 2 cm, 2 - 5 cm, 5 - 10 cm, 10 - 15 cm, 20 - 30 cm, 30 - 40 cm, 40 - 50 cm, 50 - 60 cm, 60 - 70 cm, 70 - 80 cm, 70 - 80 cm, 90 - 100 cm, 120 - 130 cm, 145 - 155 cm, 170 - 180 cm, 190 - 210 cm, 240 - 260 cm, 280 - 300 cm, and 320 - 340 cm (n = 38 soil samples per pit, excluding litter and crust). Soil samples were collected using 15 cm (length), sharp edged poly-vinyl chloride (PVC) tubes which were pushed into the face of the soil pit at right angles. The PVC tubes were soaked in acetic acid for 30 minutes prior to sampling to clean any contamination from the manufacturing process. The tubes were then rinsed well in distilled water and dried. The tubes were cleaned and dried between interval sampling to prevent interval cross-contamination. Before samples were collected, a PVC sampling trowel and back of the PVC tube were used to scrap the surface of the soil pit face to prevent any contamination from dust falling from above and/or traces of metals from the TLB steel claw during excavation.

It was assumed that the average number of residual roots remaining in the pit equalled the average number of roots exiting the pit. Residual roots were collected, weighed for fresh mass, dried, and re-weighed for dry mass. Residual root mass was added to the total but was not used for metal analyses. Three control pits were excavated in areas absent of any trees (radius < 10 m). Only one soil pit face was sampled at different intervals (mentioned above) for all control pits. Excavated soil layers from (unplanted) pits were not sieved through for roots. Soil samples were double bagged in plastic Ziplocs and placed in a cooler box and transported to the laboratory for measurement of ORP, EC and pH. Subsamples were removed for freeze-drying prior to metal analyses, and the remainder of the sample in the Ziploc bags were stored at - 20 °C. It was noted that the fresh soil samples emitted the scent of ammonia gas.

2.3.6. Characterizing soil contamination at the study site

Auger soil samples

Thirty-nine points within and surrounding the study site (*refer to Figure 2.9 for sampling locations*) were sampled with aplastic trowel and a soil auger to provide a quantitative 3-dimensional representation of the soil classes and chemical properties. The surface soil was first sampled with a hard plastic spatula or trowel for the surface litter layer, and at 0 - 0.2 cm, 0.2 – 2 cm and 2 – 10 cm depths. The subsequent auger samples were 10 – 20 cm, 50 – 60 cm, 100 – 110 cm, and 145 – 155 cm depths.

Sample preparation

Soil samples

Soil samples (n = 210) were homogenized using the cone-and-quarter method (Raab *et al.*, 1990). Individual soil samples were poured on clean plastic plates and shaped into a cone, using clean glass slides (Figure 2.7). All soil peds were broken down using clean glass slides. The soil was levelled using clean disposable plastic knives. The levelled soil sample was equally divided into four quarters. Two randomly chosen quarters were removed and bagged for archiving. The remaining two quarters were rearranged into another cone shape. This method was repeated until approximately 50 g of soil was obtained. Clean plastic plates were used between soil samples using distilled water and a new plastic knife was used for each soil sample. The soil subsample was placed in a sealed bottle, weighed to obtain wet mass, and stored at - 20 °C to reach eutectic temperature. The sample containers were then opened and sealed with Whatmann No. 1 filter paper secured with an elastic band and punctured with a pin. Soil samples were freeze-dried using a Labcon FreeZone 4.5 L Freeze Drying System (LABCONCO, Labconco Corporation, 8811 Prospect Avenue, Kansas City, USA) for 72 hours at – 85 °C, allowed to reach room temperature before removing from the chamber (to prevent condensation from the atmosphere), immediately sealed and re-weighed to obtain dry mass.

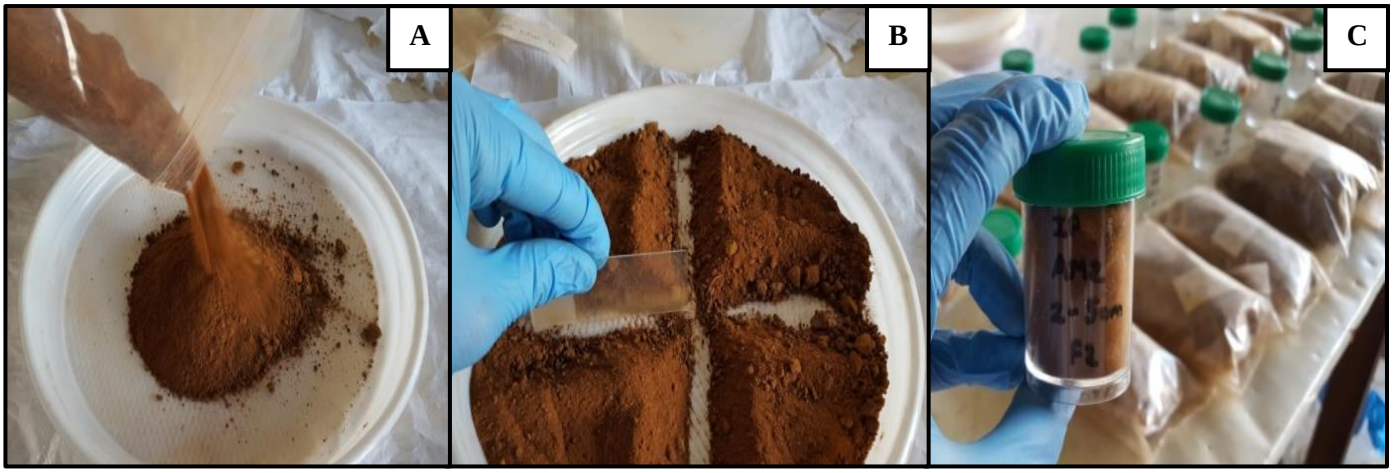


Figure 2.7. Bulk/cone and quarter method for soil homogenization. Sample was poured in a cone shape (A). Plastic plate and glass slide (B) were cleaned between samples whereas a new plastic knife was used for each soil sample. Samples were stored in sealed plastic specimen bottle for processing (C).

Analysis of soil chemical properties

The temperature (°C), pH (probe: WTW SenTix 41), electrical conductivity, EC (µS/cm) (probe: WTW Tetra Con® 325), and oxidation reduction (redox) potential (ORP or E_h , mV) (WTW SenTix ORP 0…100°C) were measured for each soil sample in a 1:2 soil : water solution. The pH probe was calibrated using two standard solution buffers at pH 4.00 and pH 7.00 at 25 °C degrees, the EC probe was calibrated using two standard solutions (1413 µS/cm). Twenty millilitres (20 mL) of distilled water were poured into each container containing ten grams (10 g) of homogenized soil. Each container was rigorously shaken for five minutes and left to stand for three minutes to facilitate the soil going into solution. All three probes were placed in the paste at the same time to record the EC, Eh pH and temperature simultaneously

Plant samples

Residual contaminants were removed from the surface of the plant tissue by washing the material vigorously under tap water followed by three rinses in distilled water (two minutes per rinse), in a borosilicate beaker. All glassware used had been acid-washed with 25 % nitric acid, HNO_3, for at least 24 hours to remove any adsorbed metals, and rinsed in deionised water. The excised ends of plant tissue were discarded to avoid potential contamination when samples were cut and collected in the field. The leafy shoots were cut on a clean plastic cutting mat, using a ceramic knife (Levo Clean-Cut Ceramic Knife) to prevent potential contamination from a steel blade. Shoots were cut into fine

sections and placed in a sealed plastic specimen bottle. Twigs and woody tissue were separated into OB, IB, and SH whereas coarse and fine roots were separated into epidermis E, C, and stele (Figure 2.5). All samples were weighed to three decimal places (Presica 125a SCS digital analytical balance), stored in a - 20 °C freezer, batch freeze-dried for 72 hours at – 85 °C, immediately sealed, and re-weighed for dry mass.

A hand-operated ceramic coffee grinder (Hario, Ceramic Coffee Grinder MSCS-2), with an adjustable particle size grinding capacity, was used to grind each sample (> 5 g) into a homogenous powder. Samples were ground using the finest grinding capacity and then re-ground. The coffee grinder was cooled to prevent heating the sample and loss of volatile elements, e.g., nitrogen (N), sulphur (S), mercury (Hg), arsenic (As) and selenium (Se). The coffee grinder was cleaned with distilled water and dried using compressed air between samples. Homogenized plant samples were stored in sealed plastic specimen bottles at – 20 °C until metal analysis.

2.3.7. Multi-elemental analysis

Three different analytical methods, namely Energy Dispersive X-Ray Fluorescence (ED-XRF), Inductively Coupled Plasma – Mass Spectrometry (ICP-MS), and Acetylene or nitrous oxide flame Atomic Absorption Spectrometry (AAS), were performed to determine trace concentrations of S, Ni, Co, and Pt in the plant and soil samples.

Energy Dispersive X-Ray Fluorescence (ED-XRF)

Energy dispersive X-Ray Fluorescence (ED-XRF) is a non-destructive analytical technique that has been used in numerous studies to determine the concentrations of a wide range of elements in different biological and non-biological samples. Exactly 5.0 g of each soil and plant sample, and Certified Reference Materials (CRM), were analysed for Na, Mg, Al, Si, **P, S, Cl, K, Ca, V, Cr, Mn, Fe, Co, Ni, Cu, Zn**, Ga, Ge, **As, Se, Rb, Sr, Mo,** Y, **Zr,** Nb, Ag, **Cd, Sn, Sb,** Te, I, Cs, **Ba,** La, Ce, Pr, Hf, Ta, **W,** Hg, Tl, **Pb, Bi, Th, and U** (Table S2.1) using a XEPOS ED-XRF (SPECTRO X-lab Pro, AMETEK Materials Analysis Division, Germany) using the TQ Powders Short method. The elements that are not in **bold** above, as well as platinum group metals (PGMs), including Pt,

palladium (Pd), rhodium (Rh), osmium (Os), ruthenium (Ru), and iridium (Ir), as well as gold (Au) are not detectable with this ED-XRF. Based on the level of agreement (LoA) between the analysed and certificate values of CRMs for metals of interest, samples were re-analysed.

Inductively Coupled Plasma – Mass Spectrometry (ICP-MS)

Sensitive and interference-free analytical techniques were required to determine Pt concentrations. Analyses were conducted by the Central Analytical Facility, University of Stellenbosch, South Africa. Plant samples were prepared according to Hansen *et al.* (2009): 0.2 g of dried plant samples were digested in a mixture of 1.5 mL 37 % HCl and 4 mL 65 % nitric acid (HNO_3) in polytetrafluoroethylene (PTFE) vessels in a microwave MLS 1200 mega digestion unit (MLS GmbH, Germany). Hydrofluoric acid, HF (0.5 mL at 48 %) was added to soil samples. The solution was left at ambient room temperature for 10 minutes. The digested samples were diluted to 50 mL using ultrapure water in a Nalgene flask. After dilution, samples were immediately analysed in triplicate using Inductively Coupled Plasma Mass Spectrometry (Agilent 8800 QQQ ICP-MS) calibrated with multi-elemental standards within the expected range. Certified reference materials (CRMs) were analysed alongside the samples. The CRMs were selected to cover similar biological matrices, namely leaves, shoots, roots, soil for the elements of interest. Percentage recoveries for the reported elements were within the US EPA guideline stipulation of 80% to 120% (*refer to Table 2.3*). The limit of detection for Pt was 0.01 mg/kg. Metal concentration, on a dry mass basis (mg/kg), was calculated as follows (Equation 2.2) (USEPA, 2007):

$$\textbf{Metal concentration (mg/kg)} = \frac{\mathrm{A \times V}}{F \times W}\ x\ DF \qquad (\textit{Equation 2.2})$$

A: mg/L of metal in processed sample
V: final volume of processed sample (mL)
F: concentration unit factor
W: Sample weight (g)
DF: Dilution factor for diluted samples

Acetylene or nitrous oxide flame Atomic Absorption Spectrometry (Flame AAS)

Acetylene or nitrous oxide flame atomic absorption spectrometry (Flame AAS) was Flame AAS was conducted by the School of Metallurgical and Chemical Engineering at the University of the Witwatersrand, to determine Pt concentrations in the 0 – 100 cm and 100 – 210 cm soil depth intervals of soil pits (n = 7). Soil depths of 0 – 2 cm, 2 – 5 cm, 5 – 10 cm, 10 – 15 cm, 15 – 20 cm, 20 – 30 cm, 30 – 40 cm, 40 – 50 cm, 50 – 60 cm, 60 – 70 cm, 70 – 80 cm, and 90 – 100 cm were combined in proportion (1 g per every 10 cm interval) to create a 0 – 100 cm soil depth interval, whereas 120 – 130 cm, 145 – 155 cm, 170 – 180 cm, and 190 – 210 cm were combined, proportionally, to create a 100 – 210 cm soil depth interval. The 210 – 340 cm soil depth was not analysed due to the low vertical mobility of Pt in soils and thus, Pt in this soil depth layer was considered negligible.

Soil samples were further homogenized using a pulveriser or mill. One gram (1 g) of each soil sample (n = 14) was digested using aqua regia (1 : 3 HNO_3 : HCl) under closed microwave digestion conditions. Samples were filtered to remove undigested particles and transferred to 100 mL volumetric flasks. Once transferred, 2 mL of 1 % lanthanum (La) solution was added, and the sample was made up to 100 mL with deionised water. Lanthanum (1 %) was added as the atomic absorption signal of Pt is depressed (due to signal interference) in the presence of other noble metals and acids. A 100 mL standard stock solution was prepared, without the addition of the digested soil sample, to have a matched matrix for analysis. Four standards with concentrations of 0.5, 2, 5, and 10 mg/L were prepared from the stock solution. Double sample spiking was carried out in which AM8 100 - 210 cm soil depth sample was spiked at 0.5 mg/kg and 5 mg/kg. The soil samples, spiked samples, and standards were analysed in triplicate on a Thermo Scientific iCE 3000 AAS (ThermoFischer Scientific, Waltham, USA). The flame emission was set to 266.00 nm with a slit width of 0.1 nm. Leaves and coarse roots (E, C, and stele) of the harvested AM10 tree were also digested as described above and analysed by Flame AAS to validate the results obtained from ICP-MS.

Certified Reference Materials (CRMs)

Certified Reference Materials (CRMs) comprised stream sediment sample (GSD-12; prepared and certified by the China National Analysis Centre for Iron and Steel), Standard Reference Material® 1570a (spinach leaves; prepared and certified by the National Institute of Standards and Technology), Standard Reference Material 1571 (orchard leaves; prepared and certified by the National Bureau of Standards), IPE sample 140 (dandelion roots; prepared and certified by Wageningen University, Environmental Sciences), and IPE sample 200 (maize shoots; prepared and certified by the Wageningen University, Environmental Sciences). Three CRMs were measured after every 10^{th} sample of soil or plant material. Randomly selected samples were further analysed in duplicate by ED-XRF (SPECTRO, AMETEK Materials Analysis Division, SPECTRO Analytical Instruments Inc., GmbH, Germany).

2.3.8. Efficacy of washing protocol

The aim of this study was to assess the allocation of Pt, Co, Ni and S between plant organs and tissue types. It is important to note that this did not include determining the phytoexcretion potential of *T. usneoides* (i.e., the amount and elemental composition of excreted salts). *Tamarix usneoides* has been reported to excrete a range of sulphur and chloride compounds and metals onto the leafy shoot surface (Wilson *et al.*, 2017). Metal ions have also been documented to accumulate in the leaf cuticular waxes and bark (OB) of various plant species due to both active excretion *in planta* (Krutul *et al.*, 2017) and atmospheric deposition *ex planta* (Böhm *et al.*, 1998). The study site is located in proximity to a smelter and platinum and base metal refineries, as well as mine tailings facilities in the area. Dust was visible on the tree foliage, and based on a review of the available literature, various contaminants may also have been emitted by the refinery, subsequently depositing onto the surface of the harvested trees. Due to the high likelihood of surface contamination confounding results (Ferrari *et al.*, 2006, IAEA, 2000), the scope of this field study did not include investigation of for the composition of salts excreted onto the leaf shoot surface via salt glands.

Therefore, plant material was washed in distilled water prior to elemental analysis in order to determine only the *in planta* metal concentrations (Wyttenbach and Tobler, 2002). The use of distilled water has been demonstrated to be reasonably effective in removing elements adsorbed to the surface of *Quercus ilex* (Alfani *et al.*, 1996, Ugolini *et al.*, 2013) and *Empetrum nigrum* (Monni *et al.*, 2001).

It must be noted that the presence of epicuticular waxes on the leaf surface also influences the level of surface contamination as demonstrated by Neinhuis and Barthlott (1998). Hexanes have been utilized as an extraction solvent for epicuticular waxes (Rashotte *et al.*, 2001), however, the use of hexane was discounted as hexane has been reported to remove a range of organic and inorganic elements, a variable which cannot be adequately controlled for. Moreover, soil particles may not be adequately digested during plant material digestions, limiting interference with digested plant material (Wyttenbach and Tobler, 2002). Thus, it was assumed that the presence of any residual dust particles that were not washed off did not significantly interfere with the analysis of plant material.

The factors mentioned above indicates the need to evaluate the efficacy of the plant washing protocol utilized in this study. Silicon (Si), iron (Fe), chromium (Cr), and tellurium (Te) are ubiquitous in the earth's crust and are relatively immobile elements. Lower elemental concentrations in plants (i.e., transfer factors) compared with the soil indicates a low level of surface contamination (Ferrari *et al.*, 2006). Thus, these elements were used as soil "tracer" elements to determine the efficacy of the washing procedure (i.e., distilled water) used in this study.

ED-XRF and level of agreement with CRM

Measurements obtained by ED-XRF may also be normalized by CRM analysis. This ensures measurement traceability (ability to relate an analytical measurement back to a national or international standard, such as a CRM) and reduces bias during normalization (Alvarez *et al.*, 2007).

2.3.9. Derivation of tree bioconcentration, and accumulation factors

Metal assimilation pattern was determined using the bioconcentration factor (BCF), where a BCF > 1 indicates active uptake based on the evidence of accumulation above soil levels *in planta* [*Equation 2.3* – Raskin *et al.*, (1994)] and the root-to-shoot translocation factor (TF), where a TF > 1

indicated enhanced root-shoot transport, with accumulation in shoots to a greater extent than in roots [*Equation 2.4* – Macnair, (2003)].

$$\mathbf{BCF}_{\mathbf{root\ or\ shoot}} = \frac{\text{metal concentration adsorbed and absorbed by trees(mg/kg)}}{\text{metal concentration in contaminated substrate (mg/kg)}} \quad (\textit{Equation 2.3})$$

$$\mathbf{TF} = \frac{\text{metal concentration in leaves (mg/kg)}}{\text{metal concentration in roots (mg/kg)}} \quad (\textit{Equation 2.4})$$

2.3.10. Coefficient of Industrial Pollution (CIP)

The CIP has been used to describe the relative level of soil contamination across large areas of land (Table 2.1) (Hakanson, 1980) and is calculated by dividing the concentrations of metals in site surface soil by their median background concentrations and taking the average. If there are too few spatial observations for the median to represent the regional geochemical background (as at this small site) the CIP is not a realistic index. *This exercise is therefore conducted for method demonstration purposes only*. The CIP was calculated for each auger sample. Two CIP values were calculated using the following equations (Equations 2.5 and 2.6):

$$CIP = [Ni/mNi) + (Co/mCo) + (S/mS)]/3 \quad (\textit{Equation 2.5})$$

$$CIP = [(Cr/mCr)+(Cu/mCu)+(Ni/mNi)+(Co/mCo)+(Se/mSe)]/7 \quad (\textit{Equation 2.6})$$

where: "[]" = measured concentration (mg/kg)

m = median

Table 2.1. Degree of contamination relative to the geochemical background based on the Coefficient of Industrial Pollution (CIP). Adapted from Hakanson, (1980).

Degree of contamination	Description
$x < 1.5$	Low
$1.5 \leq x < 4$	Moderate
$4 \leq x < 8$	High
$8 \leq x < 16$	Very High
$16 \leq x < 32$	Extreme
$x \geq 32$	Extremely High

2.3.11. Soil contamination maps and depth profiles

Maps of the soil contamination depth profiles and interpolated surface points were provided by I.M. Weiersbye and C.J. Davies (unpublished data, 2015). The maps were constructed using Esri ArcGIS Version 10.1, The Kernel smoothing kriging method was used to optimize fitted value smoothness for the interpolations where fitted values are governed by prior covariances modelled by a Gaussian process.

2.3.12. Guidelines for soil contamination levels and land-usages

Recommended and legislated permissible heavy metal concentrations (Table 2.2) differ between countries as well as proposed land use.

Table 2.2. Permissible concentrations (mg/kg) of nickel (Ni), cobalt (Co), copper (Cu), zinc (Zn), cadmium (Cd), and sulphur (S). N/A: data not shown. Please note that Agricultural soil screening values (SSV) are relatively complex to develop based on the receptors (crop species and nutrient requirements, type of livestock, impact on human health). Agricultural SSVs are site-specific.

	South Africa[(1)]				Australia[(2)]	USA[(3)]
Element/ land use	**All land uses protective of water resources***	**Informal residential***	**Standard residential***	**Industrial land-use***	**Commercial / industrial**	**Industrial**
Ni	91	620	1 200	10 000	3 000	20 000
Co	300	300	630	5 000	500	N/A
Cu	16	1100	2 300	19 000	5 000	41 000
Zn	240	9200	19 000	19 000	35 000	100 000
Cd	7.5	15	32	260	100	450
S	4000 (SO_4^{2-})	4000 (SO_4^{2-})	4000 (SO_4^{2-})	4000 (SO_4^{2-})	N/A	N/A

(1) National Environmental Management Act (NEMA): Waste Act, 2008 (Act No. 59 of 2008)
(2) National Environment Protection (Assessment of Site Contamination) Measure 1999 (as amended 2013)
(3) Preliminary Remediation Goals (PRG) from the US-EPA (Provoost et al., 2006)
**soil screening values (SSVs)*

2.3.13. Statistical analysis

Descriptive statistics were calculated (total, range, median, mean, standard deviation and skewness or kurtosis). Data were tested for absence of normal distributions using the Shapiro-Wilk test (Shapiro and Wilk, 1965), followed by a test for homogeneity of variance using the Fligner-Killeen test (Conover *et al.*, 1981). Non-normally distributed data were transformed using log, box-cox, square-root, or power transformations. Normally distributed data were analysed using a Students t-test (paired), or two-way analysis of variance, ANOVA (Fisher, 1946), to determine if there were significant differences between two or more groups. A multivariate ANOVA (MANOVA) was used to determine if the means of two or more vectors, as well as the interaction between these vectors, significantly differed. Kruskal-Wallis (KW) tests (Kruskal and Wallis, 1952) were performed where data transformations were unsuccessful. A post-hoc Tukey Honest Significant Difference (HSD) (Tukey, 1949) or a Dunn's test (Dunn, 1964) was performed on normally and non-normally distributed data, respectively, in order to show where significant differences occurred. All descriptive statistics and statistical analyses were performed using R Statistical program, R version 4.1.3 (“One Push-Up”).

A linear discriminant analysis (LDA) was used to predict group membership based on numerous continuous response variables, such as plant organs and tissue types relative to the BCFs for Pt, Ni, Co, and S plant metal concentrations data. These data were standardized to generate Z-scores. The LDA was used to determine standardized canonical discriminant function coefficients and demonstrate which predictor variables possessed the greatest factor loadings using the “MASS” package (Ripley, 2014) in R.

2.4. Results

2.4.1. Level of agreement (LoA)

The initial ED-XRF analysis (85.51 %) had a slightly higher average level of agreement (LoA) compared with the re-analysis (82.37 %) for 27 elements (Table S2.1). Iron (97.89 %), Cu (97.63 %), Sr (97.63 %), As (97.04 %), and S (95.06 %) had a higher LoA in GSD-12 (stream sediment) CRM. Level of agreement between initial and re-analysis differed significantly for Dandelion roots IPE 140 (ANOVA, $p < 0.001$) and maize leaf IPE 200 (ANOVA, $p < 0.001$) but did not differ significantly for orchard leaves IPE 1571 (ANOVA, $p = 0.071$). The highest LoA for Ni was obtained using the CRM dandelion root (Initial analysis = 71.61 %; re-analysis = 82.85 %) whereas the LoA for Ni in other CRMs averaged 41.06 %. The LoA for Co averaged 27.85 %. The LoA obtained for Ni significantly differed between plant organ (ANOVA, $P < 0.001$). Refer to Table S2.1 for LoA (%) between certified values and analysed values for the certified reference materials (CRMs) analysed using energy dispersive X-Ray Fluorescence (ED-XRF).

Platinum determination using acetylene/ nitrous oxide flame AAS

The 0.5 mg/kg Pt spiked samples had a higher LoA compared with the samples spiked with 5 mg/kg Pt (Table 2.3). The overall correlation coefficient was $R^2 = 0.76$ (Figure 2.8), in which point 3 was an outlier.

Table 2.3. Level of agreement (LoA) for Pt concentrations of spiked AM8 100 – 210 cm soil samples analysed in triplicate using acetylene / nitrous oxide atomic absorption spectrometry (Flame AAS). Note: AAS 1, 2, and 3 refers to returned values (triplicate: n = 3) using Flame AAS. Lanthanum (1%) was added to solutions to reduce interference with other metals within the samples. The LoA is shown in parentheses as a percentage (%).

Sample	**Spike concentrations**	
AM8 100 – 210 cm	**0.5 mg/kg**	**5 mg/kg**
AAS 1	0.469 (94 %)	0.676 (13.5 %)
AAS 2	0.472 (94 %)	0.679 (13.6 %)
AAS 3	0.471 (94 %)	0.680 (13.6 %)
Average	0.470 (94 %)	0.679 (13.6 %)
Level of Agreement (%)	94	13.6

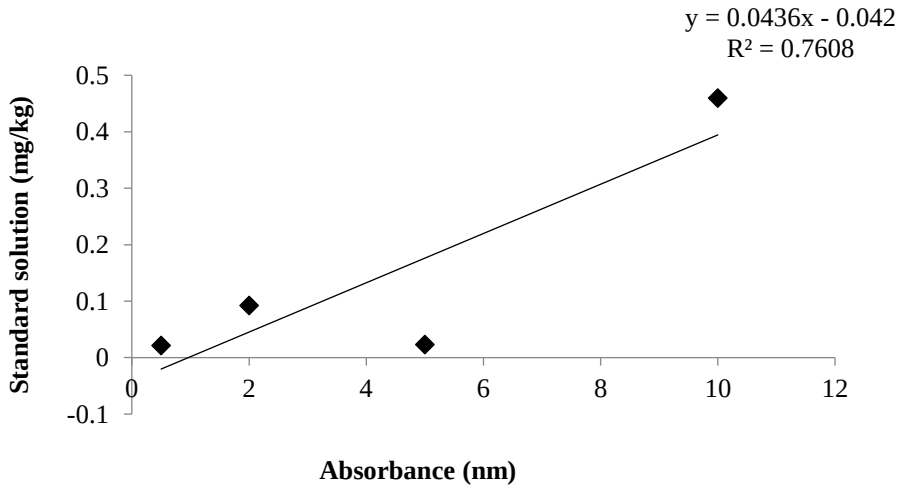


Figure 2.8. Standard solution (mg/kg) against absorbance (nm) for four standards with concentrations of 0.5, 2, 5, and 10 mg Pt/L.

Use of AAS in Pt determination

Results from Flame AAS indicate that interference was present – regardless of whether La was used to reduce/eliminate such interferences. The LoA between 0.5 mg Pt/kg and 5 mg Pt/kg spiked soil [AM8 (0 – 100 cm soil depth interval)] was 94 % and 14 %, respectively. This LoA suggests that the sensitivity of, and low detection limits associated with, Flame AAS should be used to determine Pt when analysing lower Pt ranges (≤ 0.5 mg/kg). As Pt concentrations present in soil

samples were below 0.5 mg/kg, the Flame AAS analytical technique (with the addition of La), effectively determined Pt concentrations in soil samples. Insignificant differences between Flame AAS and ICP-MS (Table 2.4) indicate that Flame AAS is an effective technique for Pt determination in soil samples containing low levels of the precious metals (i.e., ≤ 0.5 mg/kg).

Analytical instrument cross-validation (ICP-MS vs. Flame AAS)

Digested solutions from leaves and coarse roots (E, C, and stele) of AM10 were analysed using both ICP-MS and Flame AAS to compare the detection of Pt in the plant matrix. Platinum concentrations obtained for different *T. usneoides* organs and tissue types (refer to Table 2.14) did not significantly differ (t-test, p = 0.404) between the two analytical techniques (refer to Table 2.14).

Table 2.4. Cross-validation of Pt concentrations (µg/kg) obtained from acetylene/ nitrous oxide flame atomic absorption spectrometry (AAS) and inductively coupled plasma - mass spectrometry (ICP-MS). Note: C.roots = coarse roots.

***Tamarix usneoides* tree**	**Plant organ/ tissue type**	**ICP-MS**	**AAS**
AM10	Leaves	42	20
	C.Roots_ Epidermis	78.5	65.3
	C.Roots_ Cortex	86	127
	C.Roots_ Stele	16	13

2.4.2. Efficacy of washing procedure

With regards to the efficacy of the washing procedure, the transfer factors for Si (0.023), Fe (0.030), Cr (0.095), and Te (0.572) were relatively low [i.e. degree of transfer (uptake and translocation) of metals from soil into the plant].

2.4.3. Physicochemical properties of the study site soils

Physical description

The soils were classified by the soil surveyor, Red Earth cc. The 0 - 100 cm soil depth interval of each soil pit (n = 7) comprised of overburden, including relocated soil (soil, sandy, and/or waste) whereas the remaining soil depth intervals (100 – 340 cm) were predominantly red apedal B to yellow-brown apedal B parent soils (Table 2.5).

Table 2.5. Physical soil description of the soil depth intervals (0 – 155 cm) of thirty-nine (n = 39) auger points surrounding the ponds. Data commissioned from Red Earth cc for this study.

Soil depth interval (cm)	Material	Description
0 - 0.5	Crust	
0 – 2	Overburden (soil, sandy, and waste) and salts	Orthic A, red apedal B, yellow-brown apedal B, slag
2 - 10	Overburden (soil, sandy, and waste) and salts	Orthic A, red apedal B, yellow-brown apedal B, slag, and salts
10 - 20	Overburden (soil, sandy, and waste), soil and salts	Orthic A, red apedal B, yellow-brown apedal B, ash (20-30 cm), salts, G-horizon, soft plinthic B
50 - 60	Overburden (soil and waste), and soil	Orthic A, red apedal B, yellow-brown apedal B, Soft Plinthic B, ash, slag
90 - 100	Overburden (soil), and soil	Red apedal B (B1 and B2), yellow-brown apedal B, soft plinthic B
145 - 155	Soil	Red Apedal B and B2, yellow-brown apedal B, soft plinthic B

Coefficient of Industrial Contamination (CIP)

Based on auger samples collected from the unplanted areas of soils beyond tree canopies, the CIP relative to Ni, Co, and S concentrations was 2.00 ± SE 0.16, ranging from 0.95 to 6.08. The CIP indices differed between auger points (ANOVA, $p = 0.034$). The CIP for Ni, Co, and S falls within the moderate level of contamination relative to the background (Table 2.1). The mean CIP, relative to Ni, Co, Cr, Cu, As, and Se, was 1.74 ± SE 0.16 and ranged from 0.24 – 39.77. The CIP values significantly differed between sampled soil depths (KW, $p < 0.001$). The CIP values did not differ between sampled auger points (KW, $p = 0.488$). The CIP for a wider range of elements of interest also falls within the category of moderate level of contamination category with respect to the available

median background for this small site (Table 2.1). A stronger CIP trend and significant differences may have emerged from a wider selection of background values, at further distances from the ponds.

Contamination maps exhibit the extent of Ni, Co, and S contamination at the study site (Figure 2.10). Based on auger samples (i.e., 0 – 155 cm soil depth), Ni concentrations ranged from 300 – 750 mg/kg (primarily distributed in western and southern sections of the study site) whereas S concentrations were uniformly distributed throughout the study site and ranged from 100 – 1 000 mg/kg. Elevated concentrations of Ni and Co were present in the 0 – 20 cm soil depth interval. Elevated S concentrations were present within 0 – 100 cm soil depth interval (i.e. layer of soil explored by the tap root, lateral roots, and fine roots - TR).

Auger concentrations (0 – 110 cm and 145 – 155 cm)

Elemental concentrations significantly differed between the two soil depth groupings of 0 – 110 cm and 145 – 155 cm from each other (ANOVA, $p < 0.001$) (Table 2.6) (Figure 2.9). However, auger points did not significantly differ between sampled soil pits at comparable soil depth intervals, 100 – 210 cm, soil pits (t-test, $p = 0.802$) and 145 – 155 cm (t-test, $p = 0.947$).

Table 2.6. Elemental concentrations (mg/kg) at soil depths acquired from auger sampling (n = 40) of the 3.4-hectare area surrounding the refinery effluent ponds. Data acquired by ED-XRF. Data are from auger samples collected from open soil patches (i.e. not under trees).

Element	0 – 110 cm (mean ± SE)	145 – 155 cm (mean ± SE)
S	4 547 ± 642	2 152 ± 513
Cr	182 ± 5.5	131 ± 8.2
Mn	938 ± 58	584 ± 103
Fe	34 958 ± 748	41 136 ± 1 664
Co	22 ± 2.6	14 ± 4.3
Ni	245 ± 26	56 ± 6.9
Cu	75 ± 4.1	44 ± 4.1
Zn	136 ± 8.37	56 ± 6.9
As	41 ± 3.55	3.8 ± 0.4
Se	72 ± 7.61	38 ± 15
Cd	1.0 ± 0.04	1.2 ± 0.2
Pb	33 ± 3.7	16 ± 2.6

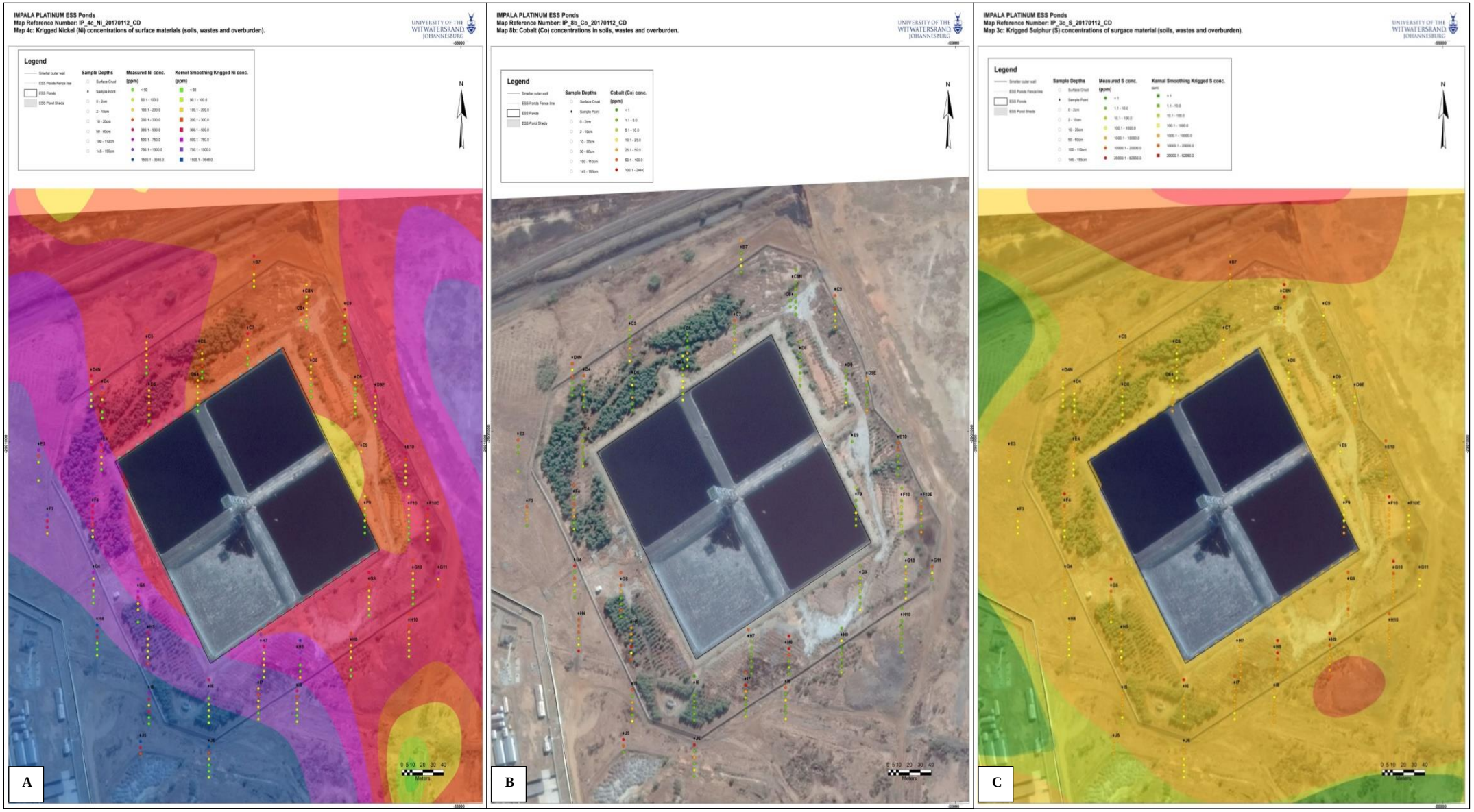


Figure 2.9. Maps of soil surface concentrations of nickel (A), cobalt (B), and sulphur (C) created using ArcGIS (Kernel smoothing kriging - ArcGIS Version 10.4, Esri). Data is derived from soil auger samples collected on soils that were not directly under trees (i.e. the unplanted patches). Maps reproduced with permission from I.M. Weiersbye and C.J. Davies (unpublished report).

Guideline and screening soil values for land-use from soil pit data

Sulphate exceeded the SSV for all land-uses in two of the unplanted, control soil pits, and approached the limit in the third control pit. Sulphate was well within all land-use limits in the *T. usneoides* planted pits (Table 2.7). Nickel soil concentrations in the 0 to 100 cm depth of both planted and unplanted soil pits exceeded the South African SSVs for all land-uses deemed to be protective of water-resources (ALUPWR), but not for informal residential, formal residential, or industrial land uses (Table 2.2) whereas Co Cd, Cu, and Zn concentrations did not exceed the SSVs for any land-uses set out by the NEMA Waste Act (Act No. 59 of 2008) [NEMWA], National Environment Protection (Assessment of Site Contamination) Measure 1999 (as amended 2013), or the Preliminary Remediation Goals (PRG) from the United States-Environmental Protection Agency (US-EPA) (Provoost *et al.*, 2006).

Table 2.7. Nickel (Ni), cobalt (Co), sulphur (S), cadmium (Cd), copper (Cu), and zinc (Zn) concentrations (mg/kg) in soil pits analysed at different soil depth intervals. Underlined values depict element concentrations that exceed the norms and standards for South Africa (= NEMA Waste Act, 2008: national norms and standards for remediation of contaminated land and soil quality, 2014). Concentrations exceeding the South African soil screening values, SSVs for All Land-Uses Protective of Water Resources (ALUPWR) are in (**bold** and underlined). Note that all values are within the SSVs stipulated for the current industrial land-use.

Soil pit	Soil Depth Interval	pH	Ni	Co	S	Cd	Cu	Zn
AM4	0 – 100 cm	6.65	**169**	20	1 665	0.96	**63**	188
	100 - 210 cm	5.34	49	8.7	628	0.94	**40**	45
	210 - 340 cm	5.74	46	20	383	1.17	**52**	33
AM8	0 – 100 cm	7.03	**178**	20	2 515	0.96	**66**	190
	100 – 210 cm	4.78	44	15	1 174	1.29	**38**	36.6
	210 – 340 cm	5.64	64	27	589	1.57	**56**	39.6
AM2	0 – 100 cm	5.88	**154**	23	2 090	1.09	**76**	159
	100 – 210 cm	5.25	43	11	518	0.79	**40**	33.7
	210 – 340 cm	5.68	49	21	342	1.00	**50**	31.2
AM10	0 – 100 cm	5.85	**150**	20	2 112	0.91	**73**	162
	100 – 210 cm	5.98	60	4.5	434	0.8	**40**	49
	210 – 340 cm	5.8	42	14	319	1.00	**44**	32
Control 1	0 – 100 cm	8.67	**250**	28	1 130	1.10	**113**	201
	100 – 210 cm	5.83	64	5.6	3 238	1.00	**42**	39
	210 – 340 cm	5.69	48	15	1 205	1.00	**47**	31
Control 2	0 – 100 cm	7.05	**114**	21	**4 339**	0.93	**61**	69
	100 – 210 cm	5.09	58	9.7	3 691	1.00	**36**	37

	210 – 340 cm	5.64	63	13	672	1.63	**47**	38
Control 3	0 – 100 cm	9.05	**126**	8	**17 107**	0.86	**51**	77
	100 – 210 cm	8.41	71	32	**28 730**	1.44	**51**	35
	210 – 340 cm	7.99	80	32	**25 200**	1.00	**76**	38

Soil chemical properties (pH, EC, and ORP)

The pH level (ANOVA, $p = 0.035$), E_h (ANOVA, $p < 0.001$), and EC (KW, $p = 0.032$) differed between the planted and unplanted soil pits (Table S2.2). The pH level and EC of planted soil pits (pH 6.0 and EC = 3 499 µS/cm) were lower compared with unplanted soil pits (pH 7.6 and EC = 9 644 µS/cm) whereas E_h was higher in planted pits (328 mV) than that of unplanted pits (221 mV). The pH level differed between soil depth intervals (0 – 100 cm, 100 – 210 cm, and 210 – 340 cm intervals) (KW, $p = 0.021$) but did differ significantly between soil pits (KW, $p = 0.207$). Electrical conductivity was significantly influenced by *T. usneoides* planting (ANOVA, $p = 0.007$), namely AM2 - Control 2 ($p = 0.041$), AM2 – Control 3 ($p = 0.009$), and AM4 – Control 3 ($p = 0.028$) (Tukey HSD post hoc test). Electrical conductivity did not significantly differ between soil depth intervals (ANOVA, $p = 0.354$) or experimental pits (ANOVA, $p < 0.05$). Oxidation reduction potential significantly differed between soil pits (ANOVA $p < 0.001$) Redox potential was not statistically discernible between soil depth interval (ANOVA, $p = 0.923$).

Across soil pits, pH level decreased in the order of 0 – 100 cm (7.16 ± 0.46) > 210 – 340 cm (6.03 ± 0.35) > 100 – 210 cm (5.80 ± 0.45). The E_h was lowest in the 0 – 100 cm profile (279 mV) compared with the 100 – 210 cm (285 mV) and 210 – 340 cm (294 mV) depths. The EC was lowest in the 210 – 340 cm interval (4 284 µS/cm) followed by 0 – 100 cm (5 858 µS/cm) and 100 – 210 cm (8 308 µS/cm). The surface EC of the study area ranged from 1000 – 10 000 µS/cm (Figure 2.10). Soil EC was elevated in soil pits areas absent of *T. usneoides* trees (i.e., south side and parts of the eastern side of ponds) and lower in areas where *T. usneoides* trees were established (north and west side of ponds), lower EC ranges, regardless of the soil depth.

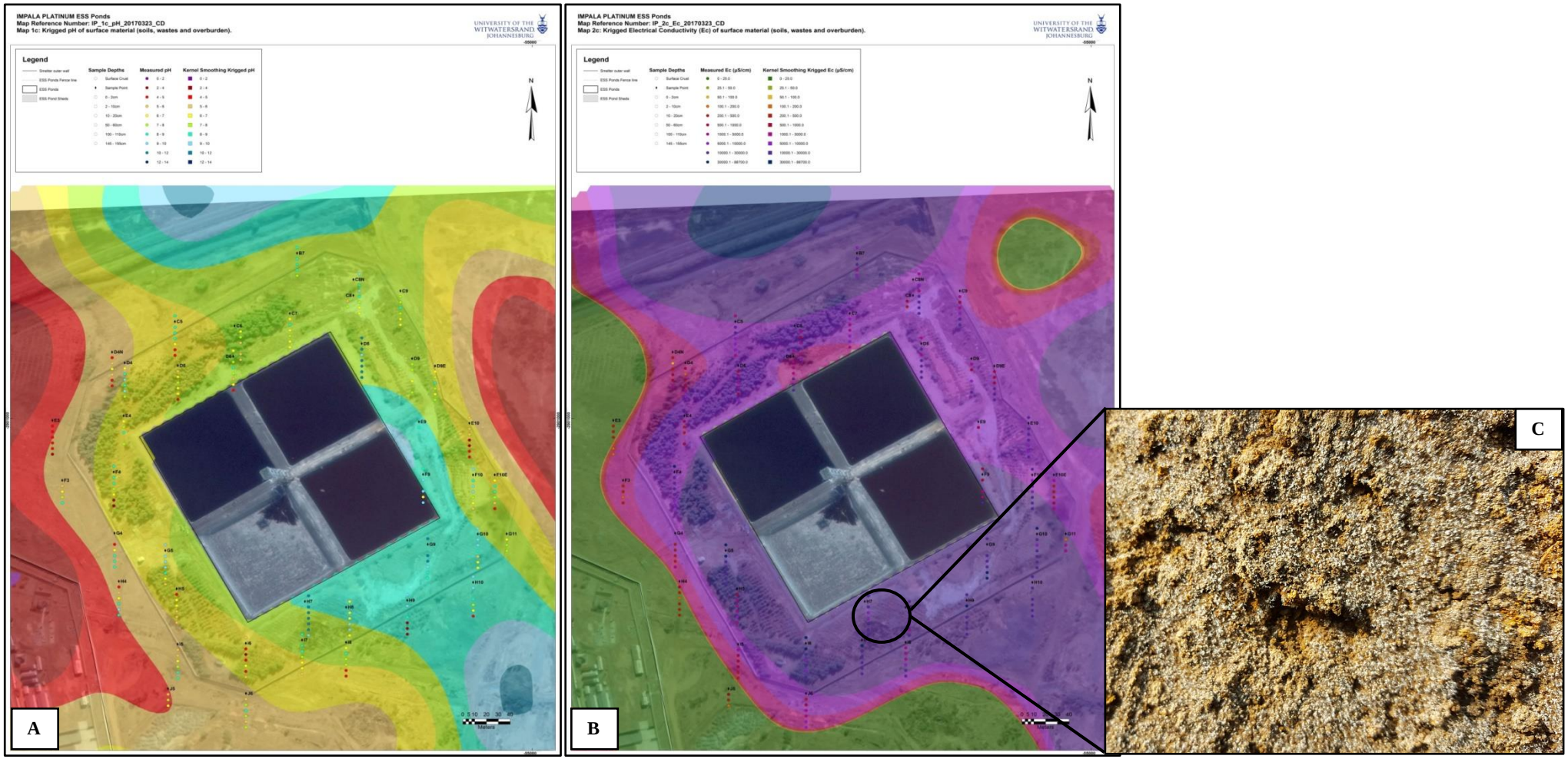


Figure 2.10. Maps showing the depth profiles and surface soil concentrations (krigged interpolations) of pH_{aq} (A) and electrical conductivity, EC (B). Salt crystal formation was observed on the profile (C) of control pit 3 at a depth of 0 – 100 cm. Maps reproduced with permission of I.M. Weiersbye and C.J. Davies, unpublished data.

Soil moisture content (%)

Soil moisture (%) differed between soil depth intervals (KW, $p < 0.001$), and between planted and unplanted soil pits (KW, $p < 0.001$) where lower (8.00 %) compared with control (9.71 %) pits (Figure 2.11). The highest moisture content was recorded within the 100 – 210 cm and 210 – 340 cm soil depth intervals (Figure 2.11.A, inset).

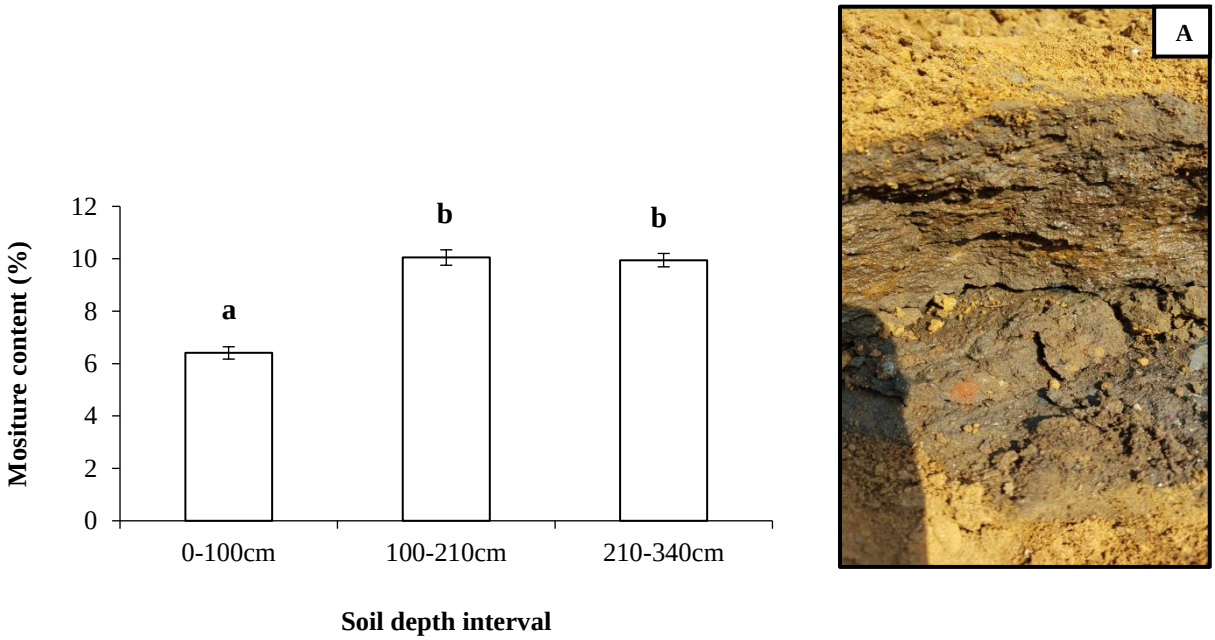


Figure 2.11. Soil moisture content (%) measured in soil pits grouped into intervals (0 – 100 cm, 100 – 210 cm, and 210 – 340 cm). Data are means + standard error. Bars with different letters depict significant differences (Tukey HSD post-hoc test, $p < 0.05$) (A) Inset showing observed saturated substrate in 0 – 100 cm soil depth interval of control pit 3.

Metal (Pt, Ni, and Co) and S concentrations

Platinum concentrations (0.348 ± SE 0.067 mg/kg) differed between soil pits (ANOVA, $p < 0.001$) (Table 2.8) but did not differ between soil depth intervals analysed (0-100 cm and 100-210 cm). Platinum concentrations differed between soil pits (ANOVA, $p < 0.001$), namely AM2 and AM10 ($p = 0.011$), AM4 ($p < 0.001$), AM8 ($p < 0.001$), Control pit 1 ($p = 0.010$), Control pit 2 ($p = 0.006$), and Control pit 3 ($p = 0.005$) (Tukey HSD post hoc test).

Nickel concentrations did not differ between planted (66.19 ± SE 3.72 mg/kg) and unplanted control (70.07 ± SE 7.85 mg/kg) pits, with a maximum concentration of 321 mg/kg. Ni concentrations (88.84 ± SE 4.35 mg/kg) were higher in the 0 – 100 cm soil depth interval compared with 100 – 210 cm (29.13 ± SE 1.13 mg/kg) and 210 – 340 cm (28.59 ± SE 1.18 mg/kg) (ANOVA, $p = 0.02$) (Figure 2.12).

Cobalt concentrations did not differ between planted (6.24 ± SE 0.51 mg/kg) and unplanted control (6.14 ± SE 0.89 mg/kg) pits (ANOVA, $p = 0.094$), with a maximum of 31.70 mg/kg (Table 2.8). Cobalt tended to be higher in the 0 – 100 cm (6.84 ± SE 0.63 mg/kg) and 210 - 340 cm profiles (6.73 ± SE 0.61 mg/kg) soil depth intervals compared with 210 - 340 cm (3.92 ± 0.60 mg/kg) (Figure 2.12).

Sulphur concentrations were higher (8 013 ± SE 1488 mg/kg) in unplanted pits compared with planted pits (1 533 ± SE 126 mg/kg) with a maximum concentration of 52 850 mg/kg. Sulphur concentrations were higher in 0 – 100 cm (3582 ± SE 536 mg/kg) and 100 – 210 cm (3743 ± 1283 mg/kg) soil depth intervals, than in the 210 - 340 cm soil depth (1310 ± 799 mg/kg) (Table 2.8) (Figure 2.12).

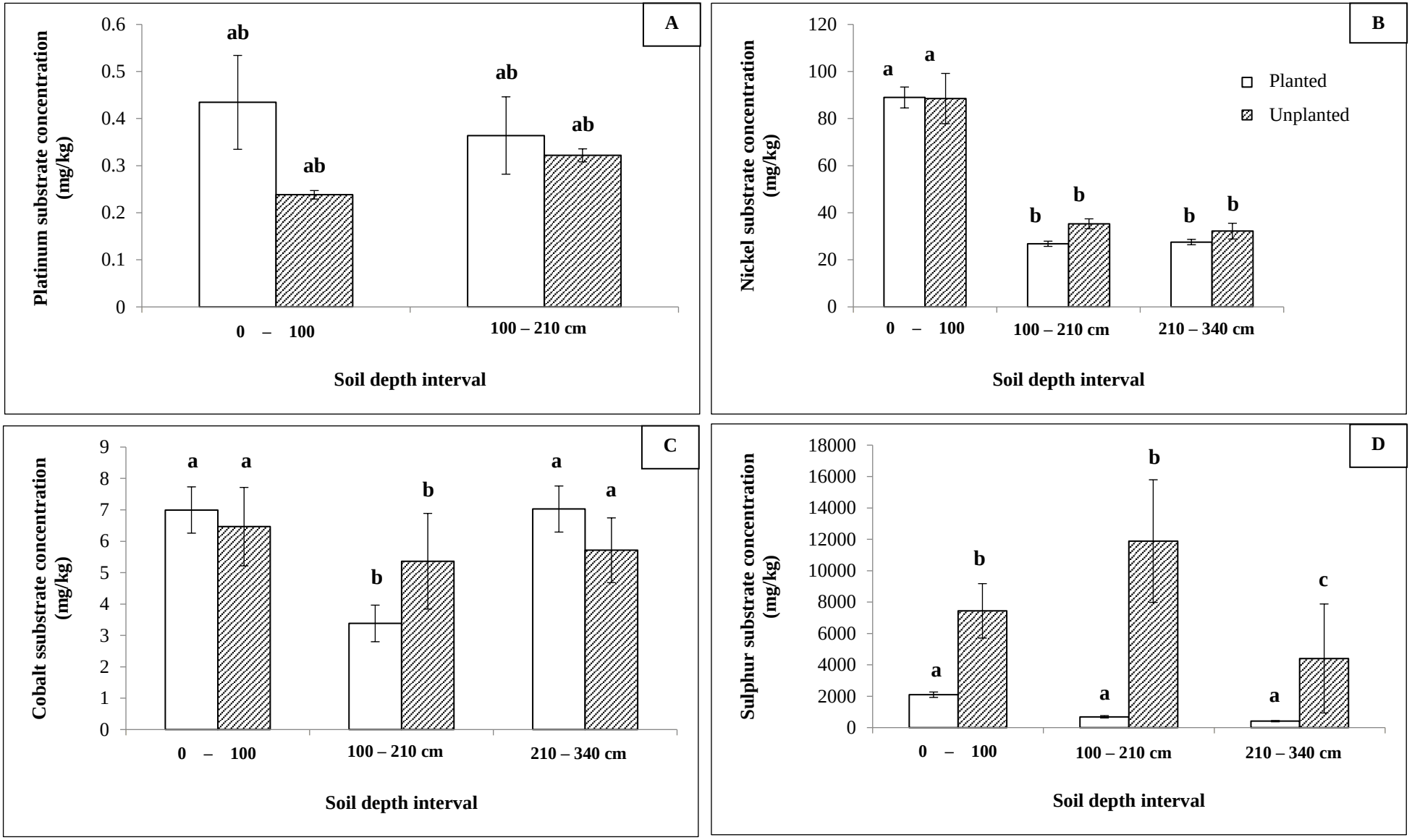


Figure 2.12. Platinum, Pt (A), nickel, Ni (B), cobalt, Co (C), and sulphur, S (D) concentrations in different soil depth intervals; 0 – 100 cm, 100 – 210 cm, and 210 – 340 cm from planted soil pits (presence of *T. usneoides* trees; n = 4) and unplanted soil pits (absence of *T. usneoides* trees within 10 m radius; n = 3). Two opposing walls of each soil pit were sampled at the same depths. Note: each figure has a different concentration range (y-axis values). *Co and Ni were normalized. Lowercase letters represent significant differences between elemental concentrations present in planted and unplanted pits. *Platinum concentrations were determined (only in 0 – 100 cm and 100 – 210 cm soil depth intervals) using acetylene/ nitrous oxide atomic absorption spectrometry (Flame AAS) whereas Co, Ni, and S concentrations were determined using ED-X-Ray Florescence (ED-XRF).

Table 2.8. Statistical analyses (ANOVA) of platinum (Pt), nickel (Ni), cobalt (Co), and sulphur (S) concentrations between sampled soil pits and soil depth intervals (0 – 100 cm, 100 – 210 cm, and 210 – 340 cm). NS = Not significantly different. A Tukey HSD post-hoc test was calculated to determine where the significant difference occurred between each soil level. Sig. code: * represents the degree of significance.

Element	Level		Statistical test	Statistic	df	p =	Sig. code
Platinum	Soil pits		ANOVA	F = 16.020	6, 7	< 0.001	***
			Tukey HSD	AM2 - AM10 (p = 0.011)			
				AM2 - AM4 (p < 0.001)			
				AM2 - AM8 (p < 0.001)			
				AM2 – Control 1 (p = 0.010))			
				AM2 – Control 2 (p = 0.006)			
				AM2 – Control 3 (p = 0.005)			
	Soil interval	depth	ANOVA	F = 0.003	1, 12	0.885	NS
Nickel	Soil pits		Kruskal-Wallis	χ^2 = 1.905	6	0.928	NS
	Soil interval	depth		χ^2 = 13.380	2	0.001	***
Cobalt	Soil pits		ANOVA	F = 0.585	6,14	0.737	NS
	Soil interval	depth		F = 2.396	2,18	0.039	*
Sulphur	Soil pits		ANOVA	F = 7.252	6, 14	0.001	***
				AM10 - Control 3 (p = 0.001);			
				AM2 _ Control 3 (p = 0.002);			
				AM4 - Control 3 (p = 0.002);			
				Control 3 - AM8 (p = 0.007);			
				Control 3 - Control 1 (p = 0.016);			
				Control 3 - Control 1 (p = 0.037)			
	Soil interval	depth	ANOVA	F = 1.297	2,18	0.298	NS

Other ecotoxicologically important elements

Substrate concentrations of Cu, As, Sr, and Zn decrease as soil depth increases (Figure 2.13). Elevated concentrations of Cu (72 ± 7 mg/kg), As (22 ± 5.4 mg/kg), Sr (49 ± 12 mg/kg), Zn (149 ± 21 mg/kg), and were present in the 0 - 100 cm soil depth interval, compared with 100 - 210 cm [Cu: 53 mg/kg; Zn: 39 mg/kg; As: 3.5 mg/kg; and Sr: 10 mg/kg] and 210 - 340 cm [Cu: 53 mg/kg; 35 mg/kg; As: 2.46 mg/kg; and Sr: 14 mg/kg] soil depth intervals (Figure 2.13). Lead and Cd occurred in similar concentrations throughout each soil depth interval. The highest Fe concentration occurred in 210 –

340 cm (63 582 ± 4 182 mg/kg) compared with 0 – 100 cm (38 692 ± 2 511 mg/kg) and 100 – 210 cm (46 171 ± 1 968 mg/kg) (Table 2.9).

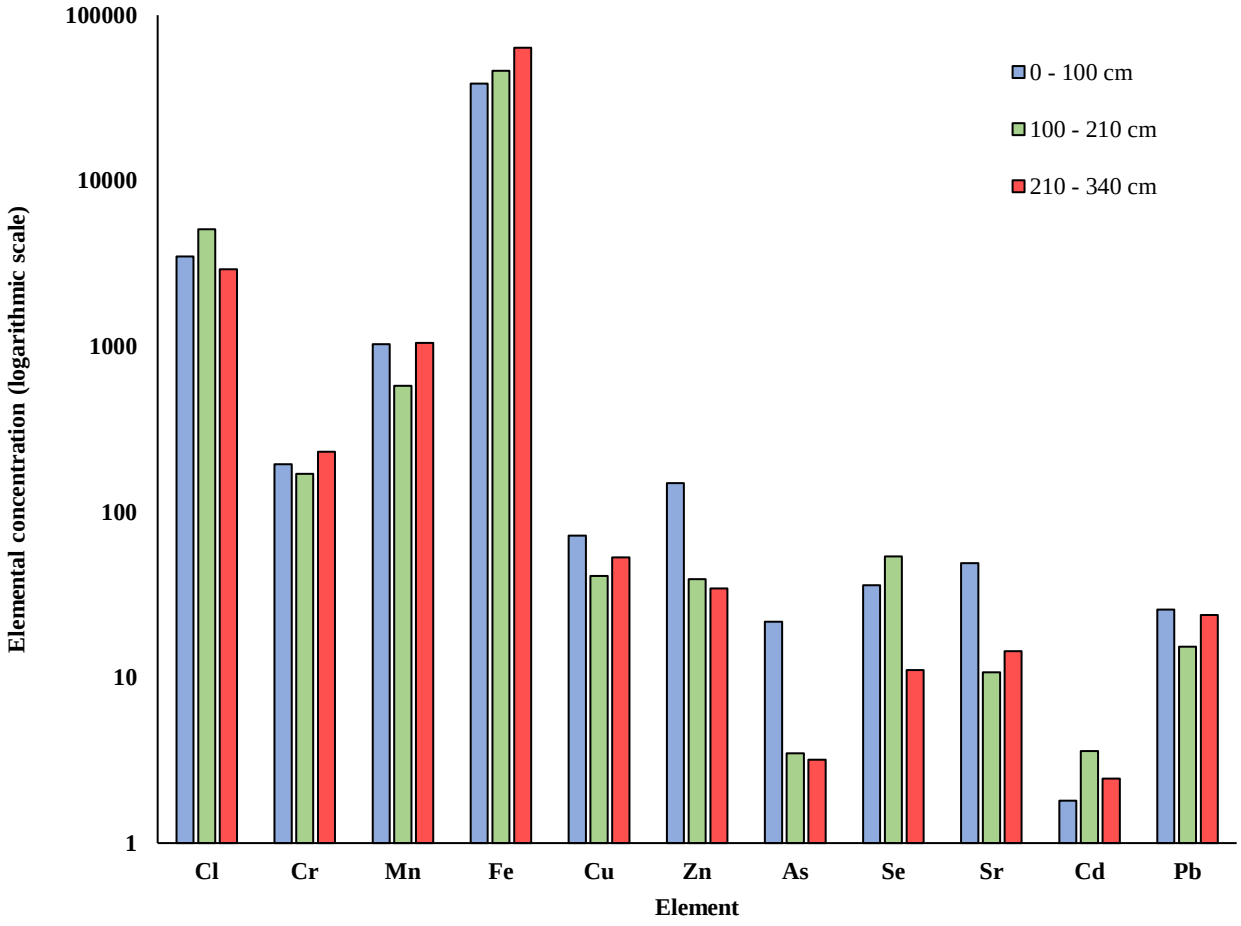


Figure 2.13. Concentrations of ecotoxicologically important metal/loids and halogens (Cl) present within different soil depth intervals (0 – 100 cm, 100 – 210 cm, and 210 – 340 cm) of seven soil pits [four planted pits and three control (unplanted) pits] excavated from the study site. Element concentrations were plotted on a logarithmic scale. Cl: chlorine, Cr: chromium, Mn: manganese, Fe: iron, Cu: copper, Zn: zinc, As: arsenic, Se: selenium, Sr: strontium, Cd: cadmium, and Pb: lead.

Table 2.9. Statistical analyses of ecotoxicologically important elements between planted soil pits and unplanted control pits, and soil depth intervals (0 – 100 cm; 100 – 210 cm; 210 – 340 cm) for ecotoxicologically important elements.

Element	Level	Statistical test	Statistic	df =	p =	Sig. code
Cr	Soil pits	ANOVA	F = 0.087	6, 14	0.997	NS
	Soil depth interval	ANOVA	F = 18.390	2, 18	< 0.001	***
		Tukey HSD	0 – 100 cm_210 – 340 cm ($p < 0.05$), 100 - 210 cm_210 - 340 cm ($p < 0.001$)			
Mn	Soil pits	ANOVA	F = 0.448	6, 14	0.835	NS
	Soil depth interval	ANOVA	F = 5.954	2, 18	0.01	*
		Tukey HSD	0 – 100 cm_210 – 340 cm ($p = 0.024$), 100 – 210 cm_210 - 340 cm ($p = 0.018$)			
Fe	Soil pits	ANOVA	F = 0.382	6, 14	0.878	NS
	Soil depth interval	ANOVA	F = 18.640	2, 18	< 0.001	***
		Tukey HSD	0 – 100 cm_100 – 210 cm ($p = 0.004$), 0 – 100 cm - 210 – 340 cm ($p < 0.001$)			
Cu	Soil pits	ANOVA	F = 0.213	6, 14	0.967	NS
	Soil depth interval	ANOVA	F = 14.150	2, 18	< 0.001	***
		Tukey HSD	0 – 100 cm_100 – 210 cm ($p < 0.001$), 0 – 100 cm_210 – 340 cm ($p = 0.026$)			
Zn	Soil pits	Kruskal-Wallis	$\chi^2 = 2.139$	6	0.907	NS
	Soil depth interval	Kruskal-Wallis	$\chi^2 = 14.345$	2	< 0.001	***
As	Soil pits	ANOVA	F = 0.506	6, 14	0.794	NS
	Soil depth interval	ANOVA	F = 25.150	2, 18	< 0.001	***
		Tukey HSD	0 – 100 cm_100 – 210 cm ($p < 0.001$), 0 – 100 cm_210 – 340 cm ($p < 0.001$)			
Se	Soil pits	Kruskal-Wallis	$\chi^2 = 9.926$	6	0.128	NS
	Soil depth interval	Kruskal-Wallis	$\chi^2 = 7.240$	2	0.027	*
Sr	Soil pits	Kruskal-Wallis	$\chi^2 = 1.056$	6	0.983	NS
	Soil depth interval	Kruskal-Wallis	$\chi^2 = 16.794$	2	< 0.001	***
Cd	Soil pits	Kruskal-Wallis	$\chi^2 = 5.443$	6	0.488	NS
	Soil depth interval	Kruskal-Wallis	$\chi^2 = 3.522$	2	0.172	NS
Pb	Soil pits	ANOVA	F = 1.023	6, 14	0.45	NS
	Soil depth interval	ANOVA	F = 4.573	2, 18	0.025	*
		Tukey HSD	0 – 100 cm_210 – 340 cm ($p = 0.030$)			

2.4.4. Dimensions of the harvested Tamarix usneoides trees

Allometric equations

Numerous parameters were measured in order to determine the volume and mass of trees using a non-destructive technique. For the purposes of deriving allometric relationships four trees of different heights were selected. The volume of each tree significantly differed (ANOVA, $p < 0.001$)

between size class intervals (Table 2.10), namely between AM10 – AM2 (p = 0.007), AM10 - AM8 (p = 0.002), and AM10 - AM4 (p = 0.001). Stem basal diameter (SBD) and stem diameter at breast height (DBH) were the strongest predictors for tree dry mass compared with tree height, canopy diameter (D_1, D_2), and canopy cover (Table 2.11).

Table 2.10. Total estimated aboveground volume (m^3) of the four *Tamarix usneoides* trees, varying in size, harvested from the study site.

Tree	Size class	Volume (cone)	B (overcone)	Volume [Total (m^3)]
AM4	Very Small	7.78	- 50 238	50 245
AM8	Small	20.95	- 93 349	93 370
AM2	Medium	43.28	- 475 043	475 086
AM10	Large	162.46	- 2 785 896	2 786 058
Total		234.47	- 3 404 525	3 404 760

Table 2.11. Linear regression was used to determine the correlation and strength of predictors in determining the dry mass of *Tamarix usneoides* trees subsequently harvested from the study area. Note, H: height, D1: Diameter, D2: diameter perpendicular to D1, SBD: stem basal diameter measured 30 cm aboveground, DBH: stem diameter measured at 120 cm aboveground. Significance codes: < 0.001 (***); 0.001 (**); 0.01 (*); > 0.5 (NS = not significant).

Equation	Coefficient	Estimation	Standard Error (SE)	Adjusted	p-value	Sig. code
Dry mass (kg) = aH+c	a	16.152	2.362	0.939	0.021	*
	c	- 49.523	10.498		0.042	*
Dry mass (kg) = aD1+c	a	15.181	2.062	0.947	0.018	*
	c	- 27.156	6.885		0.059	NS
Dry mass (kg) = aD2+c	a	16.040	1.778	0.964	0.012	*
	c	- 27.596	5.667		0.040	*
Dry mass (kg) = aSBD+c	a	0.148	0.009288	0.988	0.004	**
	c	- 24.574	3.042372		0.015	*
Dry mass (kg) = aSD+c	a	0.172	0.005018	0.998	0.001	***
	c	- 7.143	0.971789		0.018	*

Plant mass

Wet and dry mass increased as tree size increased (Table 2.12). Plant biomass (DM) significantly differed between trees (ANOVA, $p = 0.044$) and plant organs (ANOVA, $p = 0.01$).

Table 2.12. Wet (WM) and dry mass (DM) of above- and belowground biomass of the four *Tamarix usneoides* trees harvested from the study site.

Trees	**AM4 (Very small)**		**AM8 (Small)**		**AM2 (Medium)**		**AM10 (Large)**	
Tissue Type	**WM (g)**	**DM (g)**	**WM (g)**	**DM (g)**	**WM (g)**	**DM (g)**	**WM (g)**	**DM (g)**
Leaves	1 953	776	3 925	1 744	18 048	6 720	39 021	12 168
Twigs	612	295	913	582	24 379	16 283	32 998	17 152
Wood	1 796	940	4 565	2 435	107 450	47 171	115 565	51 821
Coarse Roots	2 255	1 072	2 406	973	9 795	4 177	24 698	9 941
Fine Roots	41.6	23.8	22	15	30	19	106	67.2
Flowers	NP	NP	NP	NP	8.8	3.3	22	7.4
TOTAL (g)	6 658	3 106	11 830	5 749	159 711	74 373	212 410	91 156
TOTAL (kg)	6.66	3.11	11.83	5.75	160	74	212	91

Moisture content (%)

Moisture content (%) differs between trees or plant organ (KW, $p < 0.05$) (Table 2.13).

Table 2.13. Moisture content (%) of different above- and belowground plant organs of the four *Tamarix usneoides* trees, varying in size, harvested from the study site. Roots were collected from 0 – 100 cm (TR), 100 – 210 cm (RL), and 210 – 340 cm (RS). NP: not present.

Plant organ	**AM4 (Very small)**	**AM8 (Small)**	**AM2 (Medium)**	**AM10 (Large)**
Leaves	39.73	44.44	37.23	31.18
Twigs	48.23	63.73	66.79	51.98
Wood	52.33	53.35	43.90	44.84
Flowers	NP	NP	37.08	33.29
Coarse Roots - TR	47.52	40.45	40.25	40.22
Fine Roots -TR	57.24	68.05	64.16	63.38
Coarse Roots - RL	36.68	40.45	42.65	40.25
Fine Roots - RL	57.24	68.05	64.16	63.38
Coarse Roots - RS	47.52	40.45	42.65	40.25
Fine Roots - RS	NP	68.05	64.16	63.38
Average	48.31	54.11	50.30	47.22

Density of tree trunks (including outer bark)

Tree trunk sections were harvested from 0.2 - 0.4 m and 1.1 - 1.3 m aboveground (positions where SBD and DBH was measured, 0.3 and 1.2 m, respectively). Tree trunk density (including OB) significantly differed between the four *T. usneoides* trees (ANOVA, $p = 0.001$) but did not differ significantly between SBD (0.3 m aboveground) and DBH (1.2 m aboveground) (ANOVA, $p = 0.837$) (Table S2.3).

Tap root system (TR)

Taproot (and associated lateral and fine roots) differed in size, mass (Table 2.12), and number of co-dominant tap roots (Figure 2.14). *Tamarix usneoides* had multiple co-dominant tap roots (Figure 2.14 – depicted by numbers). The taproot of AM4 (Figure 2.14) had grown horizontally. Smaller trees had fewer co-dominant tap roots.

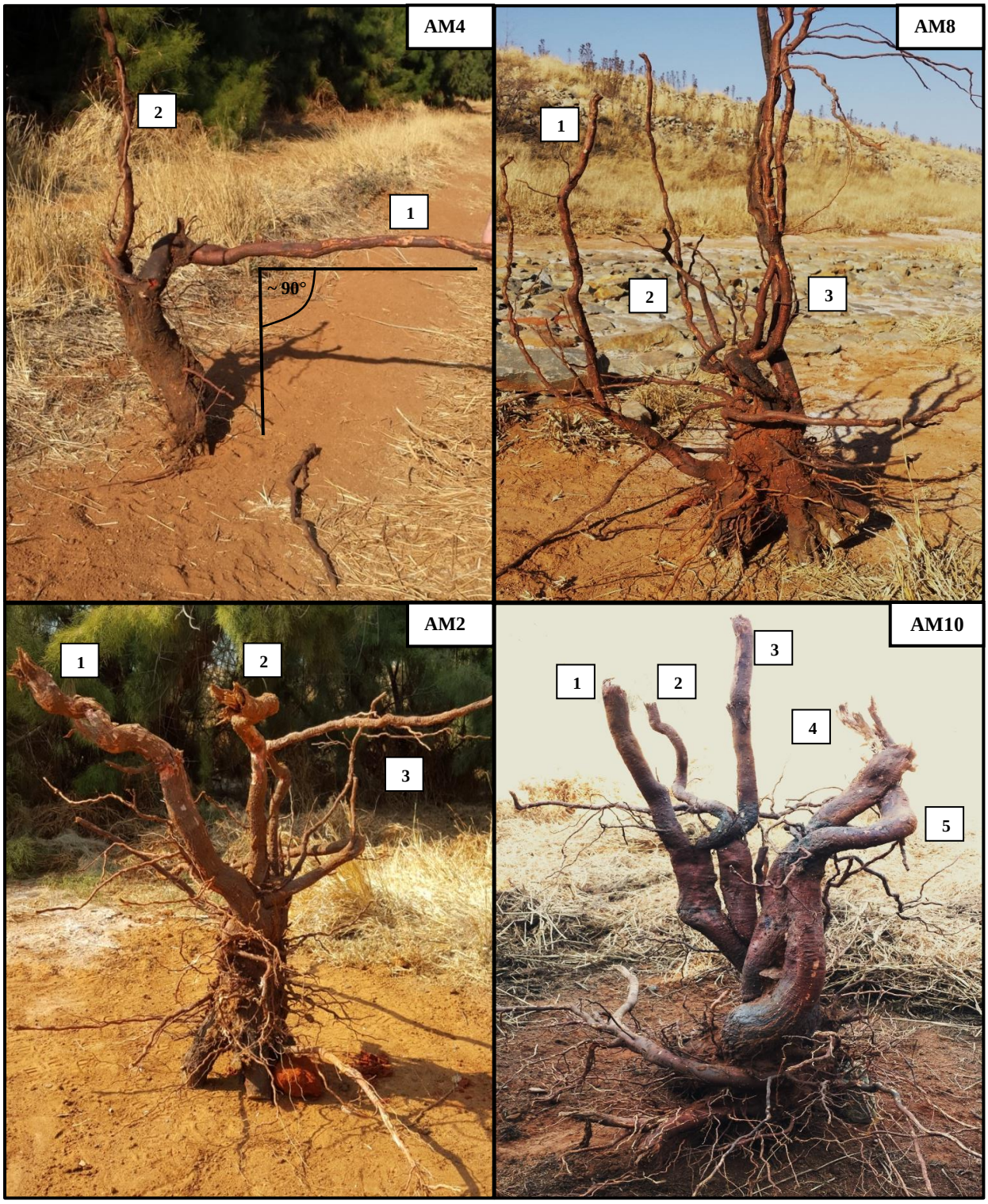


Figure 2.14. Taproot system of four *Tamarix usneoides* trees varying size classes, *viz.* very small (AM4), small (AM8), medium (AM2) and large (AM10) harvested from the study site. Numbers in blocks represent co-dominant tap roots. Right angle (90 °) on AM4 represents approximate angle of tap root growth.

2.4.5. Platinum, Ni, Co, and S concentrations in Tamarix usneoides

Flowers contained the highest concentration of Co (7.15 ± 2.05 mg/kg) and Ni (180 ± 50 mg/kg) whereas wood and twigs contained the lowest concentration of Co (1.24 ± 0.30 mg/kg) and Ni (50.46 ± 14.56 mg/kg), respectively (Figure 2.15; Table 2.14). Higher concentrations of Pt were accumulated in coarse roots (0.085 ± 0.028 mg/kg) compared with other plant organs. Higher concentrations of Co (3.15 ± 0.52 mg/kg) and Pt (0.06 ± 0.01 mg/kg) accumulated in belowground biomass (coarse and fine roots) compared with Co (1.96 ± 0.33 mg/kg) and Pt (0.039 ± 0.005 mg/kg) in above ground plant biomass (*viz.* leaves, twigs and wood). Similar concentrations of Ni were accumulated in belowground (59.38 ± 5.04 mg/kg) and aboveground (59.58 ± 8.77 mg/kg) biomass.

Overall, the four trees contained metals in the order of: Ni (59.46 ± 4.67 mg/kg) > Co (2.65 ± 0.34 mg/kg) > Pt (50 ± 6 µg/kg). Nickel levels significantly differed from Co (ANOVA, $p < 0.001$) and Pt (ANOVA, $p < 0.001$). Platinum, Ni, and Co concentrations in above-ground tissue types were in the order of Ni > Co >> Pt. In belowground tissue types, Pt, Ni and Co concentrations were in the order of; epidermis > cortex > stele (Figure 2.16). Sulphur concentrations differed between plant organ, tissue type, AG / BG (Table 2.15). However, S concentrations did not differ between tree individuals. Elevated S hyperaccumulated in the leaves (39 900 ± 746 mg/kg) of *T. usneoides* trees followed by coarse roots (31 683 ± 1 798 mg/kg) > twigs (25 083 ± 951 mg/kg) > wood (23 005 ± 2052 mg/kg) > flowers (22 300 mg/kg; n = 2) (Table S2.4).

Table 2.14. Platinum (Pt), nickel (Ni), cobalt (Co), and sulphur (S) concentrations in different plant parts (leaves, twigs, wood, flowers, coarse and fine roots) and aboveground (outer bark, inner bark, and sapwood and heartwood) and belowground (epidermis, cortex, and stele) tissue types of *T. usneoides* trees harvested from the study site. Plant samples were analysed using Inductively Coupled Plasma – Mass Spectrometry (ICP-MS). Platinum determination was cross validated using acetylene/ nitrous oxide flame atomic absorption spectrometry. Coarse roots were collected from the 0 – 100 cm (taproot - TR), 100 - 210 cm (RL), and 210 – 340 cm (RS) soil depth intervals. Note, roots of AM4 were absent in RL and RS soil depth intervals. Sulphur was analysed using ED-

Plant organ	Tissue type	Tree	Platinum (µg/kg)*	Nickel (mg/kg)	Cobalt (mg/kg)	Sulphur (mg/kg)
Leaves		AM4	60	37	1.2	39 150
		AM8	44	48	1.6	42 360
		AM2	71	71	2.8	38 400
		AM10	42	58	2.2	39 690
		Average (leaves)	54	54	1.9	39 900
Twigs	Outer bark (OB)	AM4	51	59	3.2	27 730
		AM8	32	57	1.9	29 160
		AM2	68	66	3.5	23 800
		AM10	90	58	3.1	19 510
	Inner bark (IB)	AM4	48	44	1.5	26 450
		AM8	21	36	1.1	27 470
		AM2	50	47	2.2	22 730
		AM10	20	52	1.4	27 880
	Sapwood and heartwood (SH)	AM4	18	65	1.2	27 390
		AM8	14	23	0.7	18 240
		AM2	20	66	1.4	24 770
		AM10	17	32	0.7	25 870
		Average (twigs)	37	50	1.8	25 083
Wood	Outer bark (OB)	AM4	29	30.00	1.3	11 380
		AM8	68	38.00	1.3	13 110
		AM2	28	34.00	1.2	28 860
		AM10	110	190.00	3.8	23 930
	Inner bark (IB)	AM4	3	16.00	0.4	26 000
		AM8	3	13.00	0.8	26 730
		AM2	33	84.00	1.6	30 930
		AM10	32	43.00	1.1	33 655
	Sapwood and heartwood (SH)	AM4	3	12.00	0.2	12 760
		AM8	15	17.00	0.3	22 610
		AM2	20	86.00	2.4	18 930
		AM10	3	45.00	0.6	27 160
		Average (wood)	29	51	1.2	23 005
Flowers	Flowers	AM2	100	230	9.2	21 300
		AM10	69	130	5.1	23 300
		Average (flowers)	85	180	7.2	22 300
Coarse roots	Epidermis - TR	AM4	140	160	18	18 010
		AM8	370	67	4.1	23 540
		AM2	130	120	5.1	23 340
		AM10	79	87	11	17 060
	Cortex - TR	AM4	22	55	0.7	38 650
		AM8	61	44	1.2	43 270
		AM2	43	94	1.2	44 720
		AM10	41	66	1.1	43 380
	Stele - TR	AM4	21	19	0.5	25 030
		AM8	30	23	0.7	32 710

	AM2	68	57	1.3	34 180
	AM10	16	41	0.8	24 940
	Average (TR)	85	69	3.8	30 736
Epidermis - RL	AM8	210	46	8.4	22 620
	AM2	36	35	3.0	17 550
	AM10	28	28	3.2	26 930
Cortex - RL	AM8	30	44	2.2	43 180
	AM2	30	86	1.8	47 720
	AM10	63	87	1.7	44 940
Stele - RL	AM8	22	19	0.7	32 710
	AM2	34	45	0.6	33 850
	AM10	25	48	0.9	33 920
	Average (RL)	53	49	2.5	33 713
Epidermis - RS	AM8	23	21	9.4	11 750
	AM2	99	100	2.9	21 340
	AM10	29	30	2.9	23 090
Cortex - RS	AM8	23	32	5.3	34 930
	AM2	52	92	1.4	46 410
	AM10	86	61	2.2	39 590
Stele - RS	AM8	14	16	1.1	32 210
	AM2	34	67	1.2	30 970
	AM10	41	52	1.0	37 940
	Average (RS)	45	52	3.0	30 914

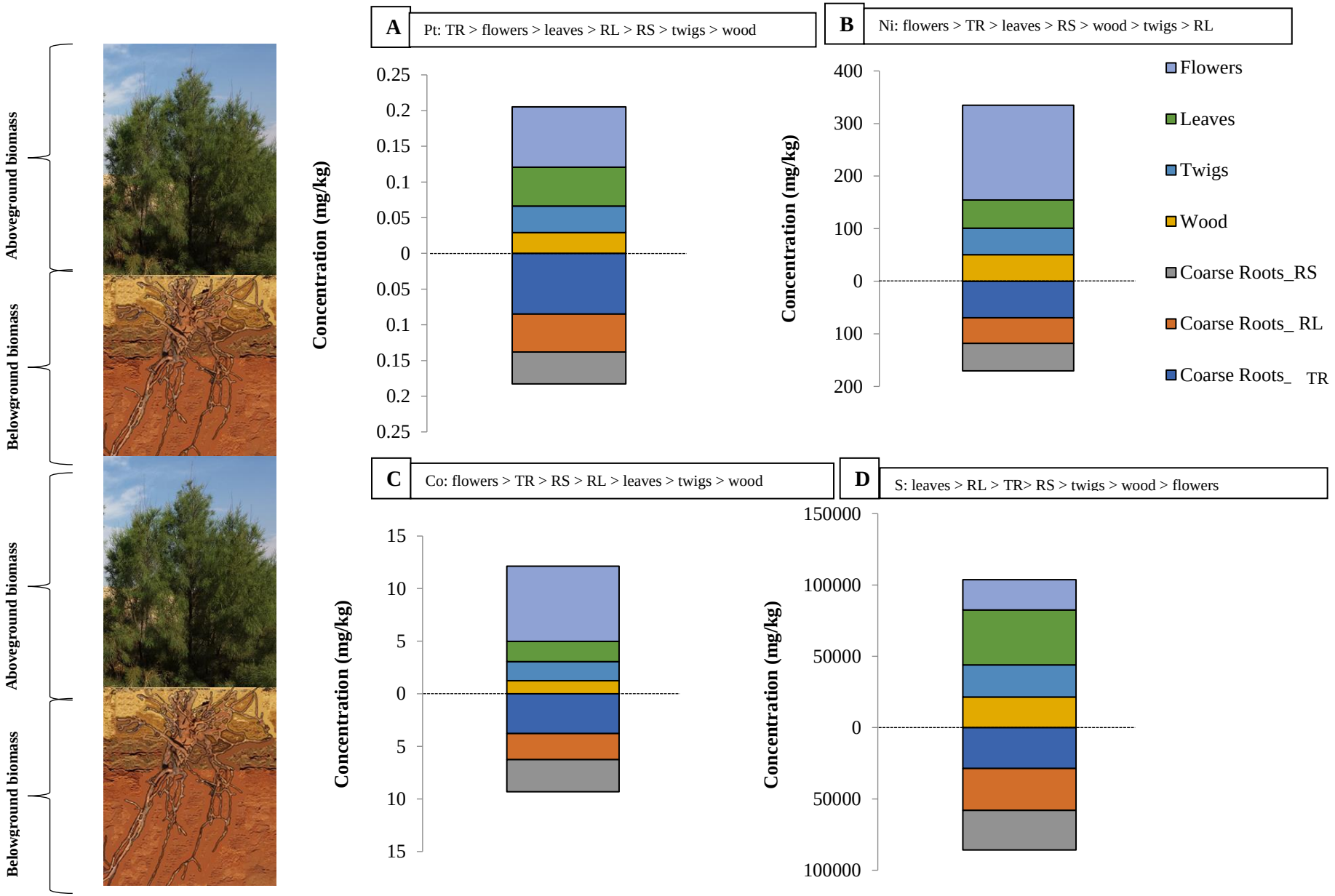


Figure 2.15. Platinum, Pt (A), nickel, Ni (B), cobalt, Co (C) and sulphur, S (D) concentrations accumulated in different aboveground (leaves, wood, twigs, and flowers) and belowground (coarse roots TR, RL, and RS) plant parts of four *Tamarix usneoides* trees, harvested from the study site. Note: dashed line represents soil surface. Caption next to A, B, C, and D represent accumulation series for that particular element.

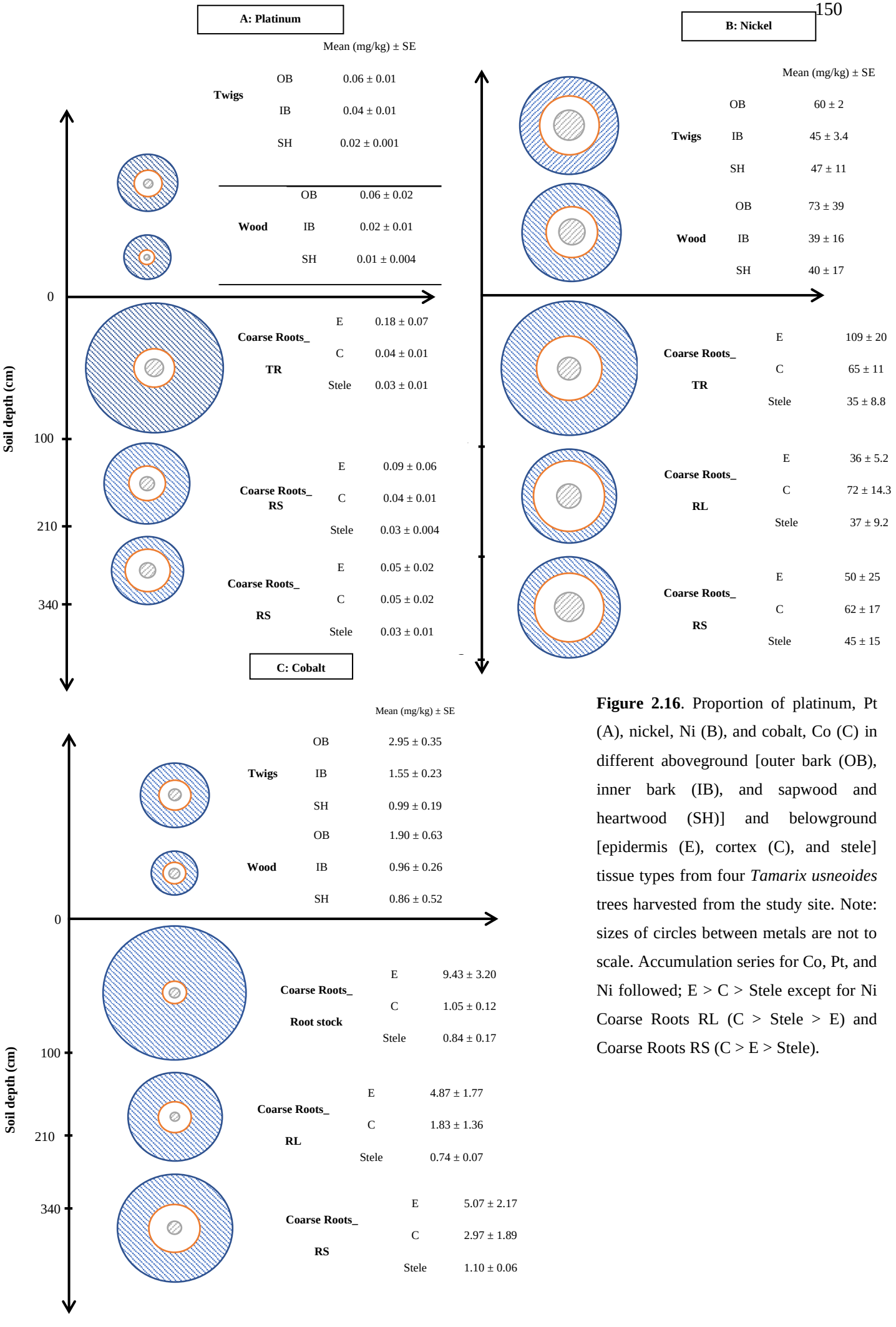


Figure 2.16. Proportion of platinum, Pt (A), nickel, Ni (B), and cobalt, Co (C) in different aboveground [outer bark (OB), inner bark (IB), and sapwood and heartwood (SH)] and belowground [epidermis (E), cortex (C), and stele] tissue types from four *Tamarix usneoides* trees harvested from the study site. Note: sizes of circles between metals are not to scale. Accumulation series for Co, Pt, and Ni followed; E > C > Stele except for Ni Coarse Roots RL (C > Stele > E) and Coarse Roots RS (C > E > Stele).

Table 2.15. Statistical analyses of platinum (Pt), nickel (Ni) and cobalt (Co) concentrations in different *Tamarix usneoides* plant organs (*viz.* leaves, twigs, wood, coarse roots, and fine roots), tissue type of aboveground (outer bark, inner bark, and sapwood and heartwood) and belowground (epidermis, cortex, and stele), and tree individuals (AM4, AM8, AM2, and AM10). Note: AG/BG represents aboveground (AG) versus belowground (BG) biomass. NS = not significant. "*" represent degree of significant difference relative to initial soil concentration, where p-value: * = 0.01, ** = 0.001; and *** = < 0.001.

Element	Level	Statistical test	Statistic	df	p =	Significant code
Pt	Plant organ	ANOVA	F = 4.069	4;55	0.006	***
		TukeyHSD	Wood-Coarse Roots (p=0.006)			
	Tissue type	ANOVA	F = 5.761	5;54	< 0.001	***
		TukeyHSD	Stele - Bark (p = 0.032), Epidermis - Cortex (p = 0.026), Epidermis - Stele (p < 0.001)			
	Trees	ANOVA	F = 1.199	3;56	0.319	NS
	AG/BG	ANOVA	F = 4.377	1;58	0.041	*
Ni	Plant organ	ANOVA	F = 3.080	4;55	0.023	*
		TukeyHSD	Flowers - Coarse Roots (p = 0.043), Wood - Flowers (p = 0.009)			
	Tissue Type	ANOVA	F = 3.284	0.012	*	
		TukeyHSD	Stele -Flowers (p = 0.007)			
	Trees	ANOVA	F = 8.232	3;56	< 0.001	***
		TukeyHSD	AM8 - AM10 (p = 0.009), AM4 - AM2 (0.020), AM8 - AM2 (p < 0.001).			
	AG/BG	ANOVA	F = 0.085	1;58	0.772	NS
Co	Plant organ	ANOVA	F = 3.462	4;55	0.014	*
		TukeyHSD	Wood-Flowers (p = 0.018)			
	Tissue Type	ANOVA	F = 18.490	5; 54	< 0.001	***
		TukeyHSD	Epidermis - Bark (p = 0.014), Bark - Stele (p = 0.001), Epidermis - Cortex (p < 0.001), Cortex - Flowers (p = 0.005), Cortex - Stele (p = 0.047), Epidermis - Stele (p < 0.001), Stele - Flowers (p < 0.001).			
	Trees	ANOVA	F = 0.901	3;56	0.447	NS
	AG/BG	ANOVA	F = 2.017	1;58	0.161	NS
S	Plant organ	KW	χ^2 = 14.563	4	0.006	**
	Tissue Type	KW	χ^2 = 46.571	13	< 0.001	***
	Tree	KW	χ^2 = 1.344	3	0.719	NS
	AG/BG	KW	χ^2 = 1.344	1	0.036	*

Bioconcentration factor (BCF)

Platinum, Ni, Co, and S were bioaccumulated by *T. usneoides* in the following series: S (25) >> Ni (2.1) > Co (1.2) > Pt (0.4) (Table 2.16). Platinum, Ni and Co followed similar bioconcentration series, in which BCF: flowers > coarse roots > twigs > wood > leaves, whereas S accumulated in washed leaves > coarse roots > flowers > twigs > wood. The BCF significantly differed between plant organs relative to Pt, Ni, and Co (ANOVA, $p < 0.001$) but not S (ANOVA, $p = 0.932$). Cobalt BCF significantly differed from Ni (ANOVA, $p = 0.009$) (Table 2.17).

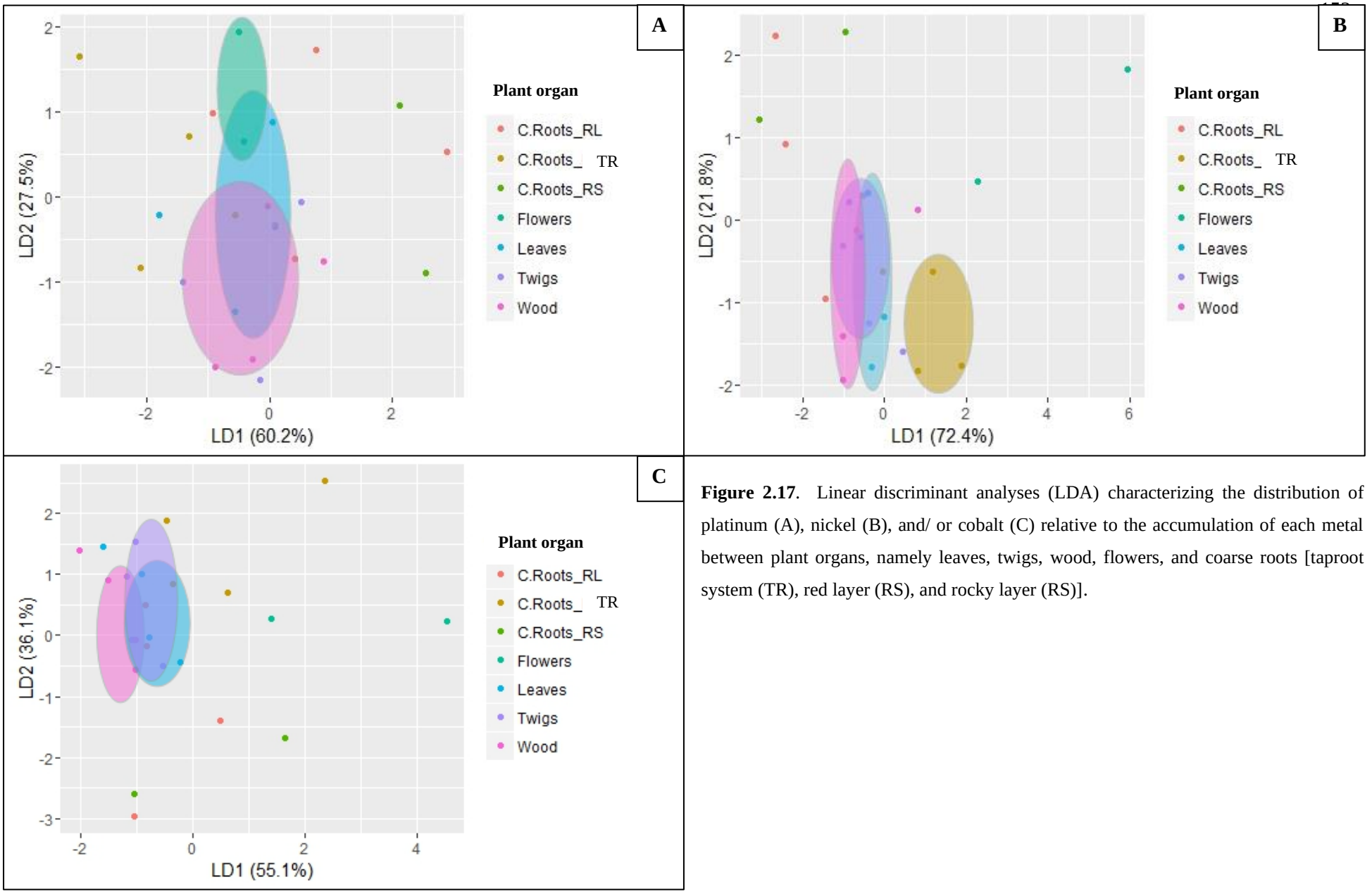


Figure 2.17. Linear discriminant analyses (LDA) characterizing the distribution of platinum (A), nickel (B), and/ or cobalt (C) relative to the accumulation of each metal between plant organs, namely leaves, twigs, wood, flowers, and coarse roots [taproot system (TR), red layer (RS), and rocky layer (RS)].

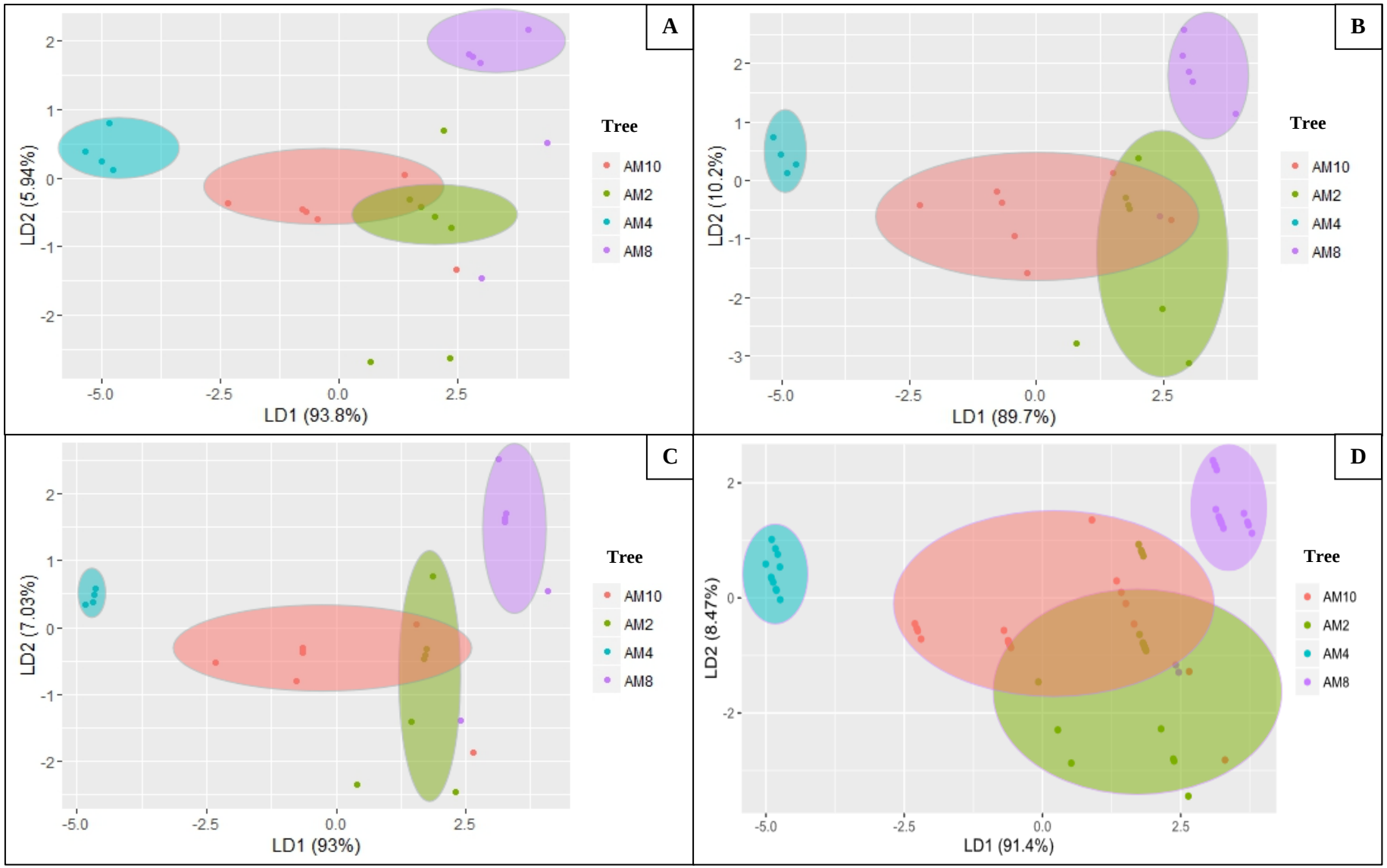


Figure 2.18. Linear discriminant function analyses (LDA) characterizing the distribution of platinum (A), nickel (B), cobalt (C), and sulphur (D) between harvested *Tamarix usneoides* trees.

Table 2.16. Bioconcentration factor (BCF) of platinum (Pt), nickel (Ni), cobalt (Co) and sulphur (S) in different plant organs and tissue types of four *Tamarix usneoides* trees of different size classes (AM4 – very small, AM8 – small, AM2 – medium, AM10 – large). Note: Coarse (circumference > 1 cm) and fine root (circumference < 1 cm) were harvested from 0 – 100 cm (taproot), 100 – 210 cm (RL), and RS (210 – 340 cm). Values in bold represent BCF > 1.

Plant organ	Tissue type	Tree	Pt	Ni	Co	S
Leaves		AM4	0.10	0.68	0.25	**29**
		AM8	0.06	0.73	0.52	**20**
		AM2	**1.54**	**1.03**	0.76	**24**
		AM10	0.17	0.95	**1.02**	**25**
	Outer Bark	AM4	0.05	**1.47**	0.96	**14**
		AM8	0.08	**1.36**	0.91	**15**
		AM2	**1.47**	**1.58**	**1.01**	**14**
		AM10	0.37	**1.85**	**2.26**	**11**
Twigs	Inner bark (IB)	AM4	0.08	**1.03**	**1.35**	0.3
		AM8	0.03	0.75	0.59	**13**
		AM2	**1.08**	**1.16**	**1.05**	**14**
		AM10	0.08	**1.24**	**1.04**	**16**
	Sapwood and Heartwood	AM4	0.03	**1.18**	0.44	**18**
		AM8	0.02	0.54	0.33	**9.0**
		AM2	0.43	**1.32**	0.41	**14**
		AM10	0.07	0.78	0.50	**15**
	Outer Bark	AM4	0.05	0.78	**1.13**	**7.7**
		AM8	0.10	**1.05**	0.87	**5.5**
		AM2	0.61	0.89	0.71	**16**
		AM10	0.45	**3.80**	**2.52**	**13**
Wood	Inner Bark	AM4	0.01	0.51	0.62	**17**
		AM8	0.01	0.38	0.85	**11**
		AM2	0.71	**1.82**	0.67	**18**
		AM10	0.13	**1.59**	0.78	**17**
	Sapwood and Heartwood	AM4	0.01	0.43	0.48	**8.5**
		AM8	0.02	0.49	0.44	**9.8**
		AM2	0.43	**1.64**	0.91	**11**
		AM10	0.01	**1.11**	0.69	**15**
Flowers		AM2	**2.16**	**4.64**	**2.07**	**13**
		AM10	0.28	**3.11**	**2.57**	**14**
Coarse Roots	Epidermis - TR	AM4	0.31	**1.88**	**1.99**	**8.7**
		AM8	0.39	**1.26**	**1.27**	**7.8**
		AM2	**2.63**	**2.13**	**1.67**	**9.6**
		AM10	0.28	0.89	0.37	**15**
	Cortex - TR	AM4	0.05	0.89	0.71	**20**
		AM8	0.06	0.61	0.37	**15**
		AM2	0.87	**1.32**	0.32	**19**
		AM10	0.14	**1.19**	0.82	**12**
	Stele - TR	AM4	0.05	0.37	0.39	**13**
		AM8	0.03	0.37	0.35	**12**
		AM2	**1.38**	0.91	0.77	**14**
		AM10	0.06	0.80	0.36	**9.9**
Coarse Roots	Epidermis - RL	AM8	0.47	**3.16**	**2.14**	**17**
		AM2	0.83	**2.10**	**2.17**	**28**
		AM10	0.14	**14.04**	**5.98**	**39**
	Cortex - RL	AM8	0.07	**2.14**	0.44	**33**
		AM2	0.69	**3.51**	0.63	**81**
		AM10	0.31	**2.83**	3.15	**97**
	Stele - RL	AM8	0.05	**1.26**	0.50	**26**
		AM2	0.79	**2.58**	0.65	**57**
		AM10	0.12	**3.99**	**2.29**	**14**
Coarse Roots	Epidermis - RS	AM2	NA	**6.16**	**1.09**	**54**
		AM10	NA	**10.24**	**2.72**	**30**
	Cortex - RS	AM8	NA	**1.05**	0.47	**53**
		AM2	NA	**4.22**	0.35	**119**
		AM10	NA	**1.99**	**1.84**	**71**
	Stele - RS	AM8	NA	0.84	0.28	**47**

		AM2	NA	**3.67**	0.37	**81**
		AM10	NA	**7.36**	**4.08**	**10**
	TR	AM8	0.08	0.62	0.46	**11**
		AM2	**1.46**	**1.82**	**1.13**	**12**
		AM10	0.12	**2.53**	**3.62**	**3.4**
Fine Roots	RL	AM4	0.03	**1.92**	**1.16**	**48**
		AM8	0.34	**2.49**	**1.50**	**23**
		AM2	0.99	**4.84**	**2.58**	**56**
		AM10	0.11	0.62	**1.31**	**48**

Table 2.17. MANOVA statistical analysis of platinum (Pt), nickel (Ni), cobalt (Co), and sulphur (S) bioconcentration factors relative to plant organ (leaves, twigs, wood, flowers, coarse and fine roots), aboveground (outer bark, inner bark, and sapwood/heartwood) and belowground (epidermis, cortex, and stele) tissue type, tree size class, aboveground (leaves, twigs, wood, and flowers) versus belowground (AG vs BG) biomass of *Tamarix usneoides*.

Element	Level	Statistic (F =)	df =	p =	Sig code
Pt	Plant organ	20.074	5, 9	< 0.001	***
	Tissue type	20.278	4, 9	< 0.001	***
	Trees	13.31	3, 9	< 0.001	***
	AG vs BG	2.798	1, 56	0.100	NS
	Plant organ: Tissue type	1.749	3, 9	0.226	NS
	Plant organ: Trees	2.293	13, 17	0.108	NS
	Tissue type: Trees	3.257	11, 17	0.043	*
	Plant organ: Tissue type: Trees	2.613	9, 17	0.084	.
Ni	Plant organ	2.291	5, 17	0.092	.
	Tissue type	1.167	4, 17	0.216	NS
	Trees	3.858	3, 17	0.028	*
	AG vs BG	7.719	1, 64	0.007	***
	Plant organ: Tissue type	0.005	3, 17	0.999	NS
	Plant organ: Trees	0.357	13, 17	0.967	NS
	Tissue type: Trees	0.514	11, 17	0.868	NS
	Plant organ: Tissue type: Trees	0.195	9, 17	0.992	NS
Co	Plant organ	1.624	5, 17	0.207	NS
	Tissue type	3.741	4, 17	0.023	*
	Trees	3.999	3, 17	0.025	*
	AG vs BG	0.929	1, 64	0.339	NS
	Plant organ: Tissue type	0.565	3, 17	0.646	NS
	Plant organ: Trees	0.245	13,17	0.646	NS
	Tissue type: Trees	0.722	11,17	0.993	NS
	Plant organ: Tissue type: Trees	0.106	9, 17	0.704	NS
S	Plant organ	2.097	5, 17	0.116	NS
	Tissue type	1.939	4, 17	0.150	NS
	Trees	1.826	3, 17	0.181	NS

AG vs BG	7.598	1, 64	0.008	***
Plant organ: Tissue type	2.087	3, 17	0.140	NS
Plant organ: Trees	0.321	13, 17	0.978	NS
Tissue type: Trees	0.862	11, 17	0.589	NS
Plant organ: Tissue type: Trees	1.026	9, 17	0.459	NS

Translocation factor (TF)

Translocation factors, TF > 1 for AM4 (Pt and S), AM 8 (Ni and S), AM2 (Pt, Co, and S), and AM10 (Pt, Ni, and S) (Table 2.18). Tree individuals significantly differed in their ability to translocate Co whereas no significant difference was present for Pt and Ni TFs (Table 2.19). Selenium (Se) had a TF > 1 throughout.

Table 2.18. Translocation factor (TF) of platinum (Pt), nickel (Ni), cobalt (Co), sulphur (S) and selenium (Se) from belowground biomass to aboveground biomass by four *Tamarix usneoides* harvested from the study site. Note: AB = aboveground biomass (leaves, twigs, wood, and flowers) whereas BG = belowground biomass (coarse and fine roots harvested from different soil depth levels; TR = 0 – 100 cm, RL = 100 – 210 cm, and RS = 210 – 340 cm).

Tree ID	TF	Platinum	Nickel	Cobalt	Sulphur	Selenium
AM4	AG/BG	0.64	0.61	0.28	0.85	0.95
	Leaves/BG	**1.27**	0.6	0.26	**1.51**	**2.09**
AM8	AG/BG	0.3	0.95	0.32	0.78	**1.12**
	Leaves/BG	0.46	**1.37**	0.48	**1.35**	**2.53**
AM2	AG/BG	0.84	**1.03**	**1.15**	0.81	**1.34**
	Leaves/BG	**1.23**	0.85	**1.06**	**1.24**	**2.77**
AM10	AG/BG	**1.23**	**1.29**	0.83	**1.20**	**2.27**
	Leaves/BG	**1.08**	0.98	0.80	**1.86**	**4.86**

Table 2.19. Statistical analyses for platinum (Pt), nickel (Ni), and cobalt (Co) translocation factors (TF).

Element	Level	Statistical Test	Statistic	df =	p =	sig. code
Pt	Tree	ANOVA	F = 3.173	3;4	0.147	NS
Ni	Tree	ANOVA	F = 3.446	3;4	0.132	NS
Co	Tree	ANOVA	F = 67.220	3;4	< 0.001	***
		Tukey HSD	AM2 - AM10 (p = 0.039), AM4 - AM10 (p = 0.004), AM8 - AM10 (p = 0.011), AM4 - AM2 (p < 0.001), AM8 - AM2 (p = 0.001) [NS: AM8 - AM4]			

2.4.6. Amount of metal allocated between plant organs

Plant organs of *T. usneoides* contained 86 – 740 µg of Pt, 143 – 1 702 mg of Ni, 8.88 – 68.1 mg of Co, and 124 – 313 g of S, excluding excreted salts (Table S.2.4). *Tamarix usneoides* are planted at a spacing of 1333 trees / ha. Therefore, the *Tamarix usneoides* trees remove an estimated 2.23 ± 0.30 mg Pt/ha, 3.02 ± 0.83 kg Ni/ha, 1.28 ± 0.90 kg Co/ha, and 1.28 ± 0.09 tons S/ha (Table 2.20). The amount of S (ANOVA, $p < 0.001$), Pt (ANOVA, $p < 0.05$), Ni (ANOVA, $p < 0.001$), and Co (ANOVA, $p < 0.001$) assimilated by *T. usneoides* differed between plant organs.

Table 2.20. Amount of platinum (Pt) (µg), nickel (Ni) (mg), cobalt (Co) (mg), and sulphur (S) (g) removed per hectare by *T. usneoides* planted at a spacing of 1 333 stems / ha. Amount of metal removed per hectare is based on trees varying in sizes, namely very small (AM4), small (AM8), medium (AM2), and large (AM10) stands. These calculations were based on *T. usneoides* biomass harvested on a particular date (i.e. single point in time).

Tree	Pt (mg)	Ni (kg)	Co (g)	S (tons)
Large	2.44	3.78	0.13	1.46
Medium	2.98	4.99	0.18	1.41
Small	1.80	1.42	0.07	1.17
Very Small	1.69	1.88	0.10	1.09

2.5. Discussion

2.5.1. Reliability of metal analyses performed (ED-XRF, ICP-MS, and Flame AAS)

ED-XRF

Energy- dispersive XRF (ED-XRF) measurements for most elements of interest on CRMs had an LoA > 70 % (Tables 2.3 and 2.4), a finding supported by other studies (e.g. Jørgensen *et al.* (2005)). However, the low LoA for Co soil and plant samples may be attributed to (i) emission line overlap, (ii) critical penetration depth, and/or (iii) type and nature of ED-XRF procedure used. Interference caused by emission line overlap occurs between two elements with similar physicochemical properties such as Co and Ni, as well as Co and Fe [common peak overlap whereby the Fe Kβ-line interferes with the Co Kα-line) (Potts and Webb, 1992). Thus, the determination of Co concentration is impeded when Fe is present in high concentrations (Parsons *et al.*, 2013). Thus, elevated levels of Fe in the soil (Figure 2.13) and plant samples may have interfered with Co detection and quantification.

The critical penetration depth, which allows for the effective reception of emitted radiation of elements by the detector, is influenced by matrix mineralogy and the amount of energy emitted by a particular element (Parsons *et al.*, 2013). Low *Z* elements ($Z \leq 14$), ranging from hydrogen to silicon, emit lower energy emission lines when struck by incident radiation. These lower *Z* elements have a lower critical penetration depth compared with higher *Z* elements. The mass attenuation coefficient (i.e., how easily the sample can be penetrated by incident or emittance radiation based on sample volume) depends on the analyte (low or high *Z* element), photon energy, as well as density-dependent differences in sample matrices (soil, plant shoots, or plant roots), resulting in large variations in critical depth penetration (Clark *et al.*, 1999). Thus, the volume and type of ED-XRF technique used (TQ Powders Short) may have reduced the accuracy of Co measurement. However, should this have been the case, discrepancies of other elements of interest (e.g., Ni), from the same sample, would have been obtained.

The significant differences obtained from the LoA for Co may be attributed to the degree of similarity in the type and nature of the procedure (i.e., TQ Powder Short) used in this study. This procedure did not involve any complete digestion of the sample. This may have increased the probability of incident rays striking the element whereby differences in soil particle sizes, residual concretions, or aggregations, between samples differing in density and mineralogy, may have emitted scattered rays. Samples may also agglomerate, due to heat, moisture, and/or static charge, thereby inhibiting grinding particles to a particular size (Živanović, 2011). Although sample preparation (e.g., grinding procedure) may contaminate samples, the potential for cross-contamination was mitigated using ceramic knives and ceramic burr coffee grinders which were cleaned between samples. Therefore, only constituents of ceramic equipment, *viz.* Al (as Al_2O_3), zirconium, Zr (as ZrO_2), and/or silicon, Si (as SiO_2), may have been introduced into samples.

However, ED-XRF was successful in measuring elements (refer to Table 2.3) in soil and plant matrices. The accuracy of these measurements (level of agreement > 70 %). Therefore, based on current data, and that of previously published studies, ED-XRF is a reliable technique that can be used to accurately determine element concentrations when elements with interfering emission line overlap are in low and/or moderate concentrations (Parsons *et al.*, 2013).

2.5.2. Physical and chemical soil properties

Physical substrate description

Sampled augering pits indicated a uniform overburden layer (transported soil and waste) within the 0 – 100 cm soil depth layer followed by soil (100 – 340 cm). Overburden substrate has been demonstrated to impact plant root growth processes such as root penetration, anchorage, and sorption of nutrients due to limited access to elements required to maintain ion homeostasis and for plant survival (Correa *et al.*, 2019, Håkansson *et al.*, 1988).

CIP

Sulphate concentrations in two unplanted pits exceeded the SSV for all land uses whereas SO_4^{2-} were within the SSV limits (Table 2.2). Sulphur concentrations in unplanted pits, compared

with planted pits, may be attributed to the presence of plant species (i.e., *T. usneoides*) in planted pits. This may be attributed to the halophytic nature of *T. usneoides* whereby elevated levels of salts are assimilated *in planta*. The presence of *T. usneoides* may have effectively reduced the S concentrations in planted pits, highlighting the efficacy of halophytes in desalinating sites with elevated salt loads (i.e. candidate for phytodesalination). Nickel, Co, and S concentrations did not significantly differ between auguring points (0 – 100 cm) and unplanted pits, whereas element concentrations in the unplanted and planted pits did. This indicates that the concentration of elements within sampled unplanted pits and auger points represent the level of contamination of the study site – in the absence of *T. usneoides* trees. It may therefore be assumed that the concentration of elements within the 0 – 100 cm soil depth interval did not significantly differ before the introduction (planting) of *T. usneoides.* The concentration of elements within the lowest auger depth (145 – 155 cm) did not differ from concentrations measured within the 100 – 210 cm soil depth. This suggests that a maximum sampling depth of 155 cm would be sufficient to determine the level of contamination at the study site – reducing overall costs and time of future sampling, collection, storage, and analysis.

Relationship between pH, EC and ORP and sulphur and metal concentrations in the soil profile

Platinum, Ni, and S concentrations decreased with soil depth whereas Co was relatively uniform along the soil profile, indicating vertical mobility (leaching downwards). A review of available literature suggests that limited studies have assessed the degree of Pt (> 10 cm depth) mobility down soil profiles. Other studies have reported that the major depth of Ni and Co contamination occurs within 0 – 100 cm depth intervals in wetland systems impacted by mining activities (Lusilao-Makiese *et al.*, 2015, McCarthy and Venter, 2006) and 0 – 10 cm of soils in proximity to a copper smelter (Ettler *et al.*, 2011). However, the elevated concentrations of Pt, Ni, and S in the 0 – 100 cm soil depth profile of this study are supported by previously published studies – e.g., Pt (Mihaljevič *et al.*, 2013, Rauch and Fatoki, 2013), Ni (Izosimova, 2005, Kashem *et al.*, 2007, Kashulina, 2017), and Co (Gray and Eppinger, 2012, Kashulina, 2017). Elevated element concentrations in the 0 – 100 cm soil depth interval may be attributed to (1) surface sources of pollution (e.g., spillages, atmospheric disposition deposition of sulphur and metal ions, vehicle

emissions, etc) (Ettler *et al.*, 2011), (2) the solubility and vertical mobility of some elements, especially sulphate and metal ions under acidic pH conditions down the soil profile (Mihaljevič *et al.*, 2013), (3) binding on substrate materials, such as organic matter, (4) tree transpiration and hydraulic lift, and (5) other biological factors, including root transport of elements, as well as interactions between these factors.

Smelters have been reported to contaminate areas surrounding these facilities. For example, Everhart *et al.*, (2006) demonstrated that emissions from a Ni smelting facility introduced elevated Ni concentrations, ranging from 63 – 22 000 mg Ni/kg in areas surrounding the smelter. This suggests that emissions from the IRS smelters may have contributed to elevated S and metal concentrations in 0 - 100 cm soil depth intervals whereby the degree of contamination decreases as soil depth increases.

Previous spraying methods and effluent spills from broken pipelines contributed to elevated sulphate and metal ion concentrations on the land surface surrounding the ponds. Some deposited ions migrated down the soil profile – whereby the rate of migration is mediated by the physicochemical properties of the element (e.g., speciation) and environmental factors (e.g., sorption processes, precipitation, etc.). Mihaljevič *et al.* (2013) calculated that Pt migrates down the soil profile (of parks and under tree crowns in various cities) at a rate of 1.1 – 2.2 cm/year, whereas other studies have calculated the migration of Pb at 1.36 cm/year in Oxisol soils impacted by a Cu smelter (Ettler *et al.*, 2011). Nickel is a relatively immobile heavy metal in soil, compared with Co - which exhibited the highest degree of mobility compared with Cd and Zn in a study conducted by Anderson and Christensen, (1988). This degree of mobility, and presence of few binding sites, may account for the relatively uniform distribution of Co throughout the sampled soil depth layers (Table 2.6 and 2.7) whereas the presence of Ni in the 0 – 100 cm soil depth soil layer is supported by a low degree of mobility in substrate.

The mobility and bioavailability of metals in the soil are mediated by precipitation-dissolution, sorption-desorption, complexation-dissociation, and, redox reactions (Violante *et al.*, 2010). The pH and E_h significantly differed between soil depths. The relationship between pH and E_h

largely governs metal mobility and mineral formation and therefore could influence Pt, Ni, Co, and S distribution along the soil profile (Table 2.7). Nickel, as Ni^{2+}, is the predominant form under acidic soil conditions, whereas Ni is complexed with ligands under alkaline conditions (Sposito, 2008). Although lower pH values were strongly associated with higher Ni concentrations, low positive correlation factors were obtained. However, the positive correlation indicated that elevated pH levels (> 7) result in Ni retention. Thus, the variation in Ni and Co between soil depth layers may be attributed to differences in pH and E_h as well as the interaction between these parameters. This significant difference may have contributed to differences in Ni concentrations along the soil depth intervals as 0 - 100 cm had a higher pH (7.2) and lower E_h (279 mV) compared with 100 – 210 cm (pH level: 5.8; E_h: 285 mV) and 210 – 340 cm (pH level: 6; E_h: 294 mV) (Table S2.4). Thus, the higher pH level and lower E_h may have reduced Ni mobility and bioavailability in the 0 – 100 cm soil depth interval. Additionally, as overburden substrate generally possesses little organic matter (along with pH > 7), Ni may have formed irreversible complexes such as precipitates, further contributing to the retention of Ni within this soil depth interval (Smolders *et al.*, 2009, Wendling *et al.*, 2009). This may also account for elevated concentrations of As, Cr, Cu, Mn, Se, and Zn in the 0 – 100 cm soil depth interval. As Ni has a high affinity for S, Ni-S compounds may have been taken up and translocated (in the form, $NiSO_4$) to the leaves (TF > 1) resulting in elevated Ni concentrations in *T. usneoides* leaves.

Lower pH levels were present in the planted pits compared with control (unplanted) pits (with the exception of deep soils which were mostly acidic throughout). Another potential mechanism resulting in the amelioration of pH by *T. usneoides* involves the release of root exudates – decreasing pH while increasing the cation: anion uptake ratio (Bali *et al.*, 2020, Chen *et al.*, 2017). The presence of *T. usneoides* may have also influenced E_h (e.g. via the release of low and high molecular wight exudates) accounting for elevated E_h in planted pits (328 mV) compared with unplanted (221 mV) pits. The decrease in pH and increase in E_h enhances the mobility and bioavailability of metals in soil, creating an environment conducive to metal uptake by plants (Weng *et al.*, 2004). Thus, elevated pH levels, particularly in the 0 – 100 cm soil depth layer, may have increased the retention of metals in

unplanted pits. The difference in pH measured from the auger samples in the areas where *T. usneoides* trees were present (north and west side of ponds) compared with the absence of any vegetation (south and east sides of ponds) is simply an artefact of the original pollutant emissions. The prevailing wind direction resulted in most EESS spray depositing in these areas, and pollution was exacerbated by effluent pipe bursts which drained through these areas (C. Burger, I.M. Weiersbye, personal communication, 2015).

Salinity has been demonstrated to increase metal bioavailability (Acosta *et al.*, 2011). This is attributed to the (i) complexation of salt-derived anions with metals, and (ii) competitive ion adsorption processes between salt-derived cations and metal ions for binding sites (Acosta *et al.*, 2011). Platinum, Co, and Ni, classified as soft borderline Lewis acids (Pearson, 1968), bind with salt-derived anions (such as SO_4^{2-}) which increases the solubility and potential toxicity of these elements in aqueous solution. The competition between salt-derived cations and metals for binding sites also increases the bioavailable fraction of the metal in solution due to the displacement of cations from binding sites (Irving and Williams, 1953). Thus, complexation between Pt, Ni, and/or Co with S (major salt constituent present in planted pits) may have increased the bioavailability of these metals and uptake by *T. usneoides*. This is supported by elevated S concentrations in leaves of *T. usneoides* trees, highlighting a potential metal inclusion pathway (i.e., S - related assimilation pathways) (Hawkesford and De Kok, 2006). Although the species of Pt, Ni, or Co were not determined in this study, these siderophilic elements have an affinity for S complexation, and more specifically for SO_4^{2-} binding (El-Naggar *et al.*, 2018). Thus, metal ions may have complexed with ligands, increasing their affinity for root binding sites and *in planta* S assimilation pathways (Hawkesford and De Kok, 2006). The presence of these metal salts may have increased their solubility and bioavailability. These findings are supported by Zhang *et al.* (2020) whereby salinity increased the assimilation of Cd by two halophytes, *viz. Suaeda glauca and Limonium aureum.*

Sulphur concentration differed between soil depths in planted pits, as well as between planted and unplanted pits. The distribution of salts along the soil profile could be associated with *T. usneoides*' mode of salt transport, accumulation, and excretion. *Tamarix usneoides* has been reported

to hyperaccumulate and excrete Cl and SO_4^{2-} salts, and accumulate a range of metal/loids in its tissues (Dennis, 2008, Wilson, 2019). Thus, elevated concentrations of salts (and their constituents) may have been extracted from the study site whereby excess salts were excreted by salt glands (Wilson *et al.*, 2017) onto the plant's surface. During rainfall events, these salts are deposited onto the surface of the soil and may increase soil salt concentration within this soil depth layer. Moreover, the difference in soil moisture between soil depths (Figure 2.11) may have resulted in the precipitation of SO_4^{2-} out of solution. In soils with high Ca concentrations, gypsum precipitation occurs. Gypsum is the main form of SO_4^{2-} excreted by *T. usneoides* (Dennis, 2008, Wilson *et al.*, 2017). This may have resulted in the observed S distribution gradients down the soil profiles of excavated soil pits.

Salts may also be dispersed by the wind, a process known as haloconduction whereby salts excreted onto the leaf surface are mobilized by wind and deposited in surrounding areas (Yun *et al.*, 2019). Haloconduction results in the deposition of salts away from the contaminated site, enabling the dispersal of salts over large areas which may have also contributed to elevated S concentrations in 0 – 100 cm soil depth intervals outside the study area (Table 2.7). Authors have hypothesized that the increase in salinity of surrounding soils, via the deposition of excreted salts, may be a competitive advantage for salinity-tolerant flora, creating a selective pressure for halophytes due to the inability of glycophytes to survive and compete for resources (e.g.., nutrients and water) under saline conditions (Erfanifard and Khosravi, 2019). Therefore, *T. usneoides*' ability to hyperaccumulate and excrete excess salts (via salt glands) may contribute to elevated salts (and their constituents) within the surface soil depth layer while decreasing the overall salt concentration in planted pits compared with unplanted pits. This is supported by the significant difference in salt concentrations (EC and S elemental concentrations) between planted and unplanted pits.

Moisture content was significantly lower in the 0 – 100 cm soil depth interval, compared with the 100 - 210 cm and 210 - 340 cm soil depth intervals (Figure 2.11). Elevated Ni concentrations in the 0 – 100 cm soil depth interval may also be attributed to the evaporative recycling and emplacement model (Mann *et al.*, 2005). The degree of evaporation decreases as soil depth increases. Mobile (Ag, Cd, and Zn) and non-mobile elements (such as Au and Pb) are present in soils as free,

complexes, adsorbed, and/ or water-soluble forms. During, and subsequent to, rainfall events, water may vertically migrate down the soil profile, filling pores and dissolving a fraction of water-soluble heavy metals, until the gravitational pull is equivalent to the soil's surface tension. Evaporation process decreases the volume of water, forming a zone of evapotranspiration. This zone has elevated concentrations of solutes in which elements may be strongly adsorbed and precipitate out of solution (Mann *et al.*, 2005). Thus, elements are concentrated within the zone of evapotranspiration (significantly lower moisture content) thereby accounting for the elevated Ni concentration in the 0 – 100 cm soil depth interval (refer to Figure 2.11; Table 2.7). The significantly lower moisture content may also prevent Ni from forming water-soluble complexes thereby restricting Ni mobility and distribution to lower soil depth intervals. The evaporative recycling and emplacement model (Mann *et al.*, 2005) may also account for the significantly elevated salt load (based on EC and elemental S concentrations) within the 0 – 100 cm soil depth interval - which formed observable precipitates (salt crystal formation; Figure 2.11.A).

2.5.3. Tree growth

Allometric equations, which correlate aboveground tree biomass to stem dimensions by measuring certain tree components, have been used worldwide to estimate forest (Segura and Kanninen, 2005) and savanna (Nickless *et al.*, 2011) tree biomass. Determining the tree biomass, using this non-destructive technique, may be applied in different fields, including studies investigating the phytoremediation potential of trees, species-specific habitat requirements, carbon distribution within a study site, as well as tree survival auditing programs (Houghton *et al.*, 2001).

Allometric equations were successfully used to categorize trees into different size classes. Stem basal diameter (SBD) and stem diameter (DBH) measurements were significant predictors in determining tree biomass, results supported by Segura *et al.,* (2005) and Nickless *et al.,* (2011). Segura *et al.,* (2005) demonstrated that accurate results can be obtained from studies utilizing stem diameter, to predict tree biomass and that results are comparable to studies measuring multiple parameters. The biological implication of these findings suggests that the aboveground biomass of

trees, used for phytoremediation, can be measured using a non-destructive technique without negatively impacting the efficacy of phytoremediation trials (i.e., harvesting trees to determine biomass).

Root system size and architecture of *T. usneoides* differed between trees varying in size. Tree AM4 had (i) lower root system mass, (ii) lower aboveground biomass (compared with other harvested *T. usneoides* trees), and (iii) an approximately 90 ° angle tap root(s) (Figure 2.14). During the construction of the ponds, the deposition of overburden material (within the 0 – 100 cm soil depth interval), has been reported to reduce gaseous exchange and inhibit root penetration. This is attributed to the elevated bulk density of overburden material compared with soil This leads to reduced root growth and development (i.e., physiological and morphological malformation) by restricting uptake of nutrients required for growth (Olubanjo and Yessoufou, 2019). Although the degree of soil compaction and gaseous exchange was not measured in this study, the presence of overburden in AM4 soil pit may have impeded vertical root penetration, limiting AM4 root growth and access to soil layers containing elevated nutrients (i.e., 100 – 340 cm soil depth interval).

In studies conducted by other researchers, soil compaction reduced the biomass of *Forsythia ovato* roots (Alberty *et al.*, 1984) and inhibited vertical root penetration and total root development by *Gleditsia triacanthos* (Gilman *et al.*, 1987) and *Zea mays* (Olubanjo and Yessoufou, 2019). Rokich *et al.,* (2001) concluded that environmental conditions, such as the presence of overburden layers, impeded root penetration and vertical root growth whilst increasing lateral root formation by numerous tree species (*Banksia attenuata, B. menziesii, Scholtzia involucrata, Bossiaea eriocarpa, Gonocarpus pithyoides, Boronia racemosa, Hibbertia racemose,* and *Gompholobium tomentosum*). The presence of an overburden layer also resulted in significantly shorter tap roots as well as the formation of co-dominant taproots compared with one individual tap root (growing in native soils) within the same species. This may account for multiple co-dominant tap roots of *T. usneoides* trees harvested from site. Harvested *T. usneoides* trees formed multiple co-dominant tap roots (Figure 2.14). This may be attributed to the presence of the overburden layer throughout the site – findings supported by Rokich *et al.,* (2001). The biological implication of this finding suggests that substrate

conditions prior to planting, such as the degree of substrate compaction, should be conducive to the need for plant roots to penetrate the substrate. Moreover, root morphology and rooting depth are important characteristics required for effective phytoremediation. Root systems comprise of an individual tap root have reduced root-contaminant contact compared with multiple roots and root systems containing lateral roots and root hairs (i.e., increase in root surface area). The root systems of all harvested trees comprised of multiple (> two) 'co-dominant' tap roots compared with a single dominant taproot (Figure 2.14). Although AM4 had reduced root growth and development, the development of multiple taproots in AM8, AM2, and AM10 may have increased the surface area for element uptake.

Heavy metals have been demonstrated to negatively impact root growth and development [see Nagajyoti *et al.* (2010) for review]. However, substrate element concentrations, relative to Ni, Co, Cd, Cr, Cu, Fe, Mn, Pb, and Sr, did not significantly differ between the excavated planted pits. Thus, reduced above- and belowground AM4 biomass may be attributed to the physical characteristics of the substrate that differed between planted pits, compared to the phytotoxicity of metals present in the substrate. However, approximately 67 % of AM4 root biomass was present within 0 – 100 cm. Significantly elevated concentrations of Ni, Cu, Zn, As, Sr, and Pb were present in the 0 – 100 cm soil depth interval, compared with 100 – 210 cm and 210 – 340 cm soil depth intervals. The implication of these results indicates that the root system of AM4 was predominantly exposed to significantly elevated concentrations of elements, which may have partly contributed to the reduced root system growth of AM4. However, further studies are required to investigate the phytotoxicity of these ecotoxicologically important metals of *T. usneoides* root growth and development.

2.5.4. Metal extraction and translocation

The discussion below is based on each (i.e., one) T. usneoides specimen (N = 4). Elevated Ni and Co concentrations were bioaccumulated in coarse roots > aboveground biomass (Table 2.14). Nickel and Co BCFs decreased in a tree-size dependent fashion (AM10 > AM2 > AM8 ≥ AM4) whereas AM2 had the highest Pt BCF. However, Pt BCF was not impacted by the size (i.e., AM2 >

AM8 > AM10 > AM4) (Table 2.16) and was independent of Pt concentrations between planted pits as Pt levels increased in the series: AM2 (46 µg/kg) < AM10 (243 µg/kg) < AM4 (608 µg/kg) < AM8 (699 µg/kg). Platinum and Co BCF > 1 in leaves of AM2 and AM10, respectively. The BCF significantly differed between plant organs (Pt), tissue type (Pt and Co), and harvested trees (Pt, Ni, and Co) (Table 2.20 and 2.21).

Plant organs

Elevated concentrations of Ni accumulated in different plant organs compared with Pt and Co (Table 2.14; Figure 2.15 and 2.16). Although the bioavailable fraction of Pt, Ni, and Co was not determined in this study, it is expected that – based on total metal content in the substrate – an elevated bioavailable fraction of Ni would be present at the study site compared with Pt and Co. This may also account for elevated Ni BCF compared with Co >> Pt. Nickel and Co, elements that share similar physicochemical properties, have been demonstrated to interact antagonistically (Tang *et al.*, 2019). This antagonistic interaction has been documented in metal uptake dynamics by *Berkheya coddii* (Rue *et al.*, 2020) and *Alyssum bertolonii* (Gabbrielli et al., 1991) whereby Ni outcompeted Co for similar binding sites and transporters on the surface of root cells. In the latter study, elevated Ni was accumulated by *A. bertolonii* whereby elevated Co (3 149 mg/kg) was present in the growing medium compared with Ni (2 053 mg/kg).

Tamarix usneoides trees were exposed to substrate containing Ni: Co concentration ratios as follows; 7.1 : 1 (AM4), 6.6 : 1 (AM8), 5.6 : 1 (AM2), and 7.4 : 1 (AM10). Rue *et al.*, (2020) reported an elevated accumulation of Ni (357 mg/kg), compared with Co (27 mg/kg), in *Berkheya coddii* when treated with Ni : Co in a 1 : 1 ratio. This result was supported by other studies such as Polechońska and Samecka-Cymerman, (2018) whereby Ni reduced the bioconcentration of Co by *Hydrocharis morsus-ranae*. In this study, the *in planta* translocation of Ni and Co by *T. usneoides* differed (Table 2.18 and 2.19), suggesting an antagonistic interaction between these elements at the study site.

Although Ni and Co share similar physicochemical properties, Ni and Co assimilation pathways have been demonstrated to differ in plants. For example, Yamaguchi *et al.*, (2015)

demonstrated differential accumulation mechanisms for Ni (vacuole sequestration) and Co (exocellular sequestration) by *Clethra barbinervis*. It has also been hypothesized that as Ni is an essential micronutrient [incorporated into enzymes (such as urease) and involved in nitrogen metabolism], plants have efficient Ni ion homeostasis networks to control *in planta* assimilation of Ni to avoid phytotoxicity (Merlot, 2020). However, although some benefits have been determined for Co (see Akeel and Jahan, (2020) for more information), an essential role of Co in non-legume plants is yet to be determined. This suggests that Co may utilize general and/or Ni-specific binding sites (due to similar physicochemical properties) for uptake and *in planta* translocation by *T. usneoides*. Therefore, elevated Ni accumulation by *T. usneoides* may be attributed to elevated Ni concentrations in the substrate (along with lower Co concentrations) and competitive ion adsorption. Elevated Co concentrations in the roots of *T. usneoides* compared with aboveground biomass, supported by TF < 1, indicates that Co was retained in the roots of *T. usneoides,* results supported by Lwalaba *et al.* (2017b).

Linear discriminant function (LDA) analyses were performed to determine if plant organs, as well as substrate parameters (pH, EC, and E_h) characterized/ predicted Pt, Ni, or Co (explanatory variables) accumulation *in planta* (Figure 2.17). The performed LDA discriminated group membership whereby leaves, twigs, and wood groups overlapped whereas coarse roots formed a distinct group (Figure 2.17). Grouping overlap indicated that leaves, twigs, and wood share similar accumulation traits/ characteristics. This suggests that Pt, Ni, and Co accumulation is significantly influenced by aboveground plant organs of *T. usneoides* (Figure 2.17). Separate grouping for coarse roots indicates variation in the accumulation characteristics of above- and belowground plant organs, as well as between roots from different soil depth intervals (i.e., RL and RS).

Sulphur concentrations decreased in the series: leaves > coarse roots > twigs > wood. Elevated S in leaves may indicate an effective metal tolerance strategy. Sulphur is incorporated into glutathione, GSH, a precursor for phytochelatin synthesis – molecules directly involved in the detoxification of metals and ROS scavenging (Freeman *et al.*, 2004). Levels of glutathione was demonstrated to significantly differ between shoots and roots of *Brassica juncea* when plants were

exposed to metal (Zn, Cu, and/or Cd) treatments (Kutrowska *et al.*, 2017). Freeman *et al.*, (2004) reported that concentrations of glutathione present in shoot tissue were strongly, positively correlated with the ability of various *Thlaspi* species to hyperaccumulate Ni. Sulphur concentrations have also been demonstrated to positively correlate with Ni concentrations in leaves although linear models performed in this study were not strongly correlated (results not shown). Elevated BCF > 20 for S did not significantly differ between harvested trees, plant organs, and/or tissue type. This suggests that S uptake, bioconcentration, and translocation is a characteristic of this halophyte. However, the positive correlation recorded by numerous other authors suggests that salinity-based tolerance mechanisms confer cross-tolerance to heavy metal stress. Although glutathione content was not determined in this study, the presence of S concentrations may account for elevated Ni concentrations in leaves compared with other plant organs. The documented difference in GSH content between roots and shoots of other plant species (Kutrowska *et al.*, 2017) may account for group membership overlap between aboveground plant organs. *Tamarix usneoides* also translocated elevated Se into leaves (TF > 1), findings supported by (Kendall, 2010).

Retention of metals in roots is characterized as BCF > 1 in roots and TF < 1. Results from this study (i.e., BCF > 1 and TF < 1) indicates that AM10 (Co), AM2 (Ni), and AM4 (Ni and Co) retained metals in roots. The retention of metal(s) in roots was confirmed by the LDA whereby above- and belowground plant organs possess different traits for accumulation (i.e., groupings did not overlap). Cobalt has been demonstrated to bond with negatively charged binding sites present on root cell walls whereas other studies reported the formation of a suberized exodermis, as well as the inhibition of metal translocation by the Casparian strip (limiting metal xylem loading processes) (MacFarlane and Burchett, 2000). These exclusion mechanisms restrict root-to-shoot metal translocation, reducing the introduction of metal ions into the physiological functioning of sensitive plant organs, such as leaves. This finding is supported by the retention of Co in roots by *Allium sativum* (Soudek *et al.*, 2011), *Panicum antidotal, Pennisetum purpureum*, and *Helianthus annuus* (Lotfy and Mostafa, 2014), *Lycopersicon esculentum* and *Triticum aestivum* (Bakkaus *et al.*, 2005), *Brassica oleracea* (Chatterjee and Chowdhury, 2003), as well as the halophytes *Salicornia europaea, Suaeda maritima, Salsola*

soda, and *Halimione portulacoides* (Milić *et al.*, 2012). The implication of these findings is the use of *T. usneoides* as a potential candidate for the phytostabilization (BCF > 1; TF < 1) of Ni and Co in saline metal-contaminated sites.

Although Pt essentiality has yet to be determined, the significant difference between Pt BCF by harvested trees suggests that AM2 possesses effective Pt assimilation mechanisms, compared with AM10 > AM8 > AM4. However, further studies will be required to confirm this finding. Platinum BCF also significantly differed between plant organs in which only AM2 bioconcentrated Pt (BCF > 1) in leaves, twigs, flowers, and coarse and fine roots (taproot) (Table 2.16). This Pt accumulation series also suggests that Pt BCF is influenced by mechanisms other than tree size. Thus, future studies should investigate the Pt accumulative mechanisms of AM2 as well as the harvested tree's potential for Pt hyperaccumulation. Thus, AM2 may be a potential candidate for Pt phytoextraction from saline metal-contaminated sites. Therefore, the Pt phytoextraction potential of AM2 should be investigated under controlled conditions (refer to Chapter 4).

Tissue types

Outer bark (OB) vs inner bark (IB) and sapwood and heartwood (SH)

Elevated concentrations of metals in OB of *T. usneoides* (Table 2.14) may be attributed to a combination of factors including (i) atmospheric deposition of metal ions onto the bark surface (Böhm *et al.*, 1998), (ii) re-translocation of elements from assimilation plant organs (Zimka and Stachurski, 1992), dilution effect (Zárubová *et al.*, 2015), and / or (iii) variation on tissue affinity of metal binding.

Elevated concentrations of Pt, Ni, and Co were present in OB compared with other woody tissue in the following series: OB > IB > SH (Figure 2.18; Table 2.14). This finding is supported by numerous studies whereby the analysis of bark of various plant species have been successfully utilized for environmental pollution monitoring of a range of elements including Al, Co, Cr, Hg, Na, Ni, Se, U, and Zn by *Quercus robur* and *Q. petraea* (Böhm *et al.*, 1998), Ni, Cu, Zn, As and Pb by *Pinus sylvestris* (Harju *et al.*, 2002), and Al, Fe, titanium (Ti), and scandium (Sc) by *Olea europaea*

(Pacheco *et al.*, 2002). The determination of metal concentrations in tree bark provides information on the level and type of air pollution at the study site (Joshi *et al.*, 2016). These data are vital in the compilation of contaminant risk assessments and to formulate measures to mitigate the dispersal of contaminants from a study site and reduce risk to human health in the surrounding area. Therefore, the OB of *T. usneoides* can be utilized for environmental monitoring of trace elements emitted by the refinery.

Outer bark serves as a physical barrier against direct IB and SH contamination, accounting for elevated Ni concentrations in in OB compared with IB > SH (Oliva and Mingorance, 2006). Moreover, toxic elements and by-products are radially transported into the pith of tree stems – resulting in the formation of heartwood (Stewart, 1966). The toxicity of transported elements results in the death of parenchyma cells. As these cells form the first cylinder of the heartwood (i.e. barrier restricting the transport of elements into the pith), the death of these cells results in increased transport of toxic ions across this layer. The sapwood-heartwood boundary moves outwards as tree diameter increases. This phenomenon was demonstrated by Kuroda *et al.* (2018) whereby caesium (Cs) was injected into the outer section of the sapwood. Caesium was actively transported, via xylem parenchyma cells and diffusion through cell walls moving into heartwood by diffusion. Similar patterns of distribution may have occurred for Pt, Ni, and Co whereby elements may have been radially distributed towards to pith of *T. usneoides* for storage (Martin *et al.*, 2003).

Elevated concentrations of metals have been documented to accumulate in the flowers of *Stevia rebaudiana* (Ni, Fe, and Al) (Hajar *et al.*, 2014), *Raphanus sativus* (Cu) (Hladun *et al.*, 2015), as well as *Cucurbita pepo* (Zn > Cu > Ni > Pb) (Xun *et al.*, 2017). However, the accumulation of metals in floral parts negatively impacted the reproductive success of *Cucurbita pepo* relative to pollen viability, seed mass, and pollination dynamics (i.e., reduced pollinator visitation) (Xun *et al.*, 2017). The impact associated with the accumulation of Pt, Ni, and Co (Table 2.14) in floral parts of AM2 and AM10 requires further investigation. However, although *T. usneoides* are typically propagated using cuttings (Wilson, 2010), the aim of other phytoremediation trial may include the restoration of biodiversity in mine-impacted sites. Therefore, future studies should investigate the

impact of metal accumulation in floral parts of *T. usneoides* trees if selected for use in phytoremediation trials with the aim of restoring biodiversity.

Inner bark (IB) vs sapwood and heartwood (SH)

Elevated concentrations of Pt, Ni, and Co were present in IB compared with SH. This finding may be attributed to the radial transportation of elements, along with water, from xylem to phloem resulting in the redistribution of heavy metals in the stem (Hagemeyer and Hübner, 1999). In a study conducted by Hagemeyer and Hubner, (1999), Pb retained in the SH had the potential to be remobilized and subsequently redistributed to the IB of the wood of *Pinus sylyestris*. These results may also be attributed to varying cation binding capacities of IB and SH (Lev-Yadun and Aloni, 1995, Momoshima and Bondietti, 1990). The lower metal concentrations present in IB and SH, compared with leaves, indicate that leaves should be harvested if metals are to be reclaimed. Although elevated metal concentrations accumulated in OB, bark stripping would compromise the growth and survival of trees (Nagaike, 2020) – negatively impacting the efficacy of phytoremediation trials.

With regards to root C and stele, the Casparian strip may have selectively excluded the transport of metals to the root stele and subsequent xylem loading. This decrease in xylem loading may have accounted for lower concentrations of metals in the stele compared with E and C (MacFarlane and Burchett, 2000). Platinum, Ni and Co accumulation series in coarse roots differed between 0 – 100 cm and 100 – 340 cm. Metals accumulated between coarse root tissue types (0 – 100 cm soil depth layer) in the series, E > C > stele whereas Pt and Ni accumulated in the series, C > E > stele in 100 – 340 cm soil depth layer (Table 2.14). Also, elevated metal concentrations accumulated in coarse root E, compared with C and stele (Table 2.14), may be attributed to sorption of Pt, Ni, and Co directly onto the root cell surface as the root epidermis was in direct contact with soil contaminants.

Although it is unlikely that all contaminants, deposited onto the surface of plant material, were removed during the washing process, the low transfer factors of Al, Si, Fe, Cr, and Te, indicate that (i) plant surface contamination was negligible, and (ii) the washing procedure was effective in

removing contaminants potentially adsorbed to the surface of plant material (Ferrari *et al.*, 2006, Wyttenbach and Tobler, 2002). These findings are supported by Sasmaz and Yaman (2006). Thus, future studies can utilize this washing procedure to reduce any source of error which may occur from plant surface contamination in the field.

Tree size

Platinum, Ni, and Co (excluding S) concentrations significantly differed between harvested *T. usneoides* trees varying in size (Table 2.14 and 2.15). Typically, larger trees of the same plant species have elevated rates of evapotranspiration (ET) due to increased surface area and volume (Rötzer *et al.*, 2017). Metals, present in solution, are taken up by the plant and translocated to aboveground biomass via transpirational pull. Thus, although elevated rates of evapotranspiration by larger trees (e.g., AM10 and AM2) may contribute to higher *in planta* metal concentrations (Dye *et al.*, 2018, Dye *et al.*, 2017), further studies will be required to confirm this finding. As ET depends on a plant's surface area, water availability, and climatic conditions, lower element concentrations present in AM4 may be attributed to the inability of the plant's root system to access deeper soil depths and the water table.

The dilution effect (Jarrell and Beverly, 1981) was not evident in this study relative to Pt, Ni, and Co, which followed the series: AM2 > AM10 > AM4 > AM8 (Table 2.14). As plants age, physiological activity decreases. This was demonstrated by (Tu *et al.*, 2004) whereby arsenic accumulation by *Pteris vittata* decreased as plant age increased due to reduced physiological activity. However, larger trees may compensate for the reduction in physiological activity due to an increase in number of metal storage sites (i.e. cells and associated storage sites). Moreover, *T. usneoides* investigated in this study were of similar ages (approximately 9 years) at the time of harvest. Thus, it was unlikely that the dilution effect contributed to the variation in metal accumulation by trees ranging in sizes.

Variation in elemental assimilation between harvested T. usneoides trees

Results from the LDA indicate that the predictor variables (Pt, Ni, Co, and S) were able to discriminate between different groups. Results from LDA (along with LD1 > 90 %, refer to Figure

2.17) suggest that the individual characteristics of harvested *T. usneoides* trees impact / influences Pt, Ni, Co, and S BCF. The overlapping of AM2 and AM10 groups indicated that these two trees share common traits with regards to Pt, Ni, Co, and S bioconcentration. Trees AM10 (Ni) and AM2 (Pt) bioaccumulated (BCF > 1) and translocated (TF > 1) Ni and Pt, respectively, into leaves. Platinum was also bioaccumulated in the leaves of AM10 and AM4 whereas Co and Ni were bioaccumulated in the leaves of AM2 and AM8, respectively. Elevated concentrations, and BCFs, for Pt, Ni, and Co in AM2 and AM10, may be attributed to geochemical conditions (i.e. differences in pH, EC, E_h and element composition and concentrations which differed between planted pits) (Assunção *et al.*, 2003), plant factors (e.g. tree size), as well as the interplay between these factors. Sulphur allocation did not significantly differ between *T. usneoides* trees (Table 2.15). This may be attributed to the halophytic properties of this plant species whereby S is hyperaccumulated to similar degrees independent of the tree size. This indicates that the regulation of S by *T. usneoides*, via the hyperaccumulation of S between plant organs, may play a role in maintaining the species ion homeostasis network (Sanadhya *et al.*, 2015, Wilson *et al.*, 2017).

Results from this study suggest that AM2 and AM10, have the ability to bioaccumulate (BCF > 1) and translocate (TF > 1) elevated levels of Pt, Ni, and/or Co, compared with other harvested *T. usneoides* trees, namely AM4 and AM8. Cobalt distribution in plants is species (Lago-Vila *et al.*, 2015). Cobalt translocation was below 1 (TF < 1) for AM4, AM8, and AM10 (Table 2.18), suggesting that the accumulation and translocation of Co is not influenced by tree size but rather by individual tree / site characteristics. Further studies under controlled conditions, such as plant tissue culture or pot trials, should be conducted as each harvested tree was exposed to different abiotic (e.g., element composition and concentration, substrate conditions such as depth of overburden layer) and biotic factors. Therefore, these factors along with genotypic and phenotypic variation between harvested trees contributed to assimilation by different *T. usneoides* trees.

2.5.5. Phytoremediation potential of T. usneoides

Nickel and Co concentrations accumulated by *T. usneoides* were above the standard reference plant concentrations (Ni: 1 - 10 mg/kg; Co: 0.05 - 0.50 mg/kg) (Misra and Mani, 1991) whereas Pt was within this range (0.02 – 256 µg/kg) (Rauch and Fatoki, 2013). Baker and Whiting, (2008) previously characterized the criteria for metal hyperaccumulation [see Van der Ent *et al*., (2013) for review]. Although BCF > 1 and TF > 1 for Pt, Ni, and Co for certain harvested trees, the *in planta* metal concentration did not exceed the hyperaccumulation threshold for Ni (> 1 000 mg/kg), Co (> 300 mg/kg), or Pt (hyperaccumulation threshold yet to be determined – please refer to Chapter 4) (Figure 2.19) (Baker *et al*., 2020). Although *T. usneoides* did not hyperaccumulate Pt, Ni, or Co, elevated concentrations of these metals were accumulated by *T. usneoides* trees under saline conditions. The biological implication of these findings indicates that *T. usneoides* is a potential candidate for the phytodesalination and phytoremediation of heavy metals from saline metal-contaminated substrate.

Salinity tolerance confers heavy metal tolerance

Halophytes have been identified as an emerging biotechnology for the desalination of heavy metal-contaminated sites due to a range of molecular (generation of osmoprotectants), cellular (vacuolar storage, apoplastic vs. symplastic metal transport), and physiological (storage and excretion via salt glands) mechanisms. These mechanisms are involved in regulating the plant's metal homeostasis network to tolerate elevated levels of salinity (Sanadhya *et al.*, 2015, Wilson *et al.*, 2017). *Tamarix spp.* can differentiate, separate, and excrete a range of salts and metal ions via salt glands (Hagemeyer and Waisel, 1988, Sookbirsingh *et al.*, 2010). Platinum, Ni and Co may have been translocated to, and excreted via salt glands present in the leaves of *T. usneoides*, accounting for elevated metal concentrations in leaves. These salt glands enable *T. usneoides* to take up, hyperaccumulate (S in leaves: BCF > 20 and TF > 1), and hypertolerate elevated concentrations of salts (salt derived anions bound with heavy metals) in which excess salts may be excreted onto the surface of the leaves (Dennis, 2008, Wilson *et al.*, 2017). Some studies have demonstrated the use of exo-recretohalophytes for phytodesalination (via haloconduction) of saline metal-contaminated sites.

Yensen and Biel, (2008) estimated that 5 – 50 tons / ha/ year of salt could potentially be dispersed if 50 % of salts excreted on the surface were to be dispersed via wind. As salts are typically macro- and / or micro-nutrients, the dispersal of salts at low concentrations may improve growth conditions of areas that are salt-deficient. Recretohalophytes are therefore advantageous for phytodesalination, compared with salt accumulators, in this regard based on the mode of salt removal (i.e., haloconduction). Thus, salts excreted onto the leaf surface may have been mobilized and dispersed (via wind) into surrounding areas – accounting for elevated salt concentrations away from the planted pits. Therefore, *T. usneoides* is a potential candidate for the phytodesalination of saline sites via haloconduction. However, *T. usneoides* trees may be considered small-scale sources of salt contamination whereby salts extracted from different soil depths are transported to the surface and dispersed onto surrounding areas via haloconduction.

Tolerance

Although tolerance was not measured and quantified in this study, no sign of metal phytotoxicity (chlorosis and necrosis due to elemental concentrations) was observed. Leaf senescence was observed in lower parts of the tree which may be attributed to maturational developmental processes and/or mechanical damage. *Tamarix usneoides* may be termed a metallophyte (Lambinon and Auquier, 1963) due to its ability to tolerate and complete its lifecycle (observed as production of flowers) on metalliferous soils (namely secondary heavy metal sites) (Baker *et al.*, 2010).

2.5.5. Removal of Pt, Ni, Co, and S from the area surrounding ponds

Few studies have investigated the phytomining potential of plant species relative to PGEs and Ag (Dinh *et al.*, 2022). A review of the available literature suggests that various Ni hyperaccumulators are potential candidates for Ni phytomining. For example, estimated Ni removal ranges from 27 kg/ha (*Alyssum serpyllifolium* - (Morais *et al.*, 2015)), 36 kg/ha (*Noccaea goesingensis*), 55 kg/ha (*Odontarrhena chalcidica*) (Rosenkranz *et al.*, 2019), to 72 kg/ha (*Alyssum bertolonii* - (Robinson *et al.*, 1997b)). Plant species for effective Co phytomining is limited (Chaney

et al., 2014). One factor contributing to this is the antagonistic interaction between Ni and Co, whereby Ni displaces Co for root sorption.

Although these plant species have been documented to remove economically significant amounts of metals, most hyperaccumulators are herbaceous plant species with low biomass (Luo *et al.*, 2016). Low biomass production limits vertical root growth and consequently, root access to lower soil depth intervals. This may limit the efficacy of phytoremediation (and phytomining trials). This indicates the need to identify fast-growing, tolerant plant species which accumulate elevated metal concentrations (Ebbs and Kochian, 1997). In this study, the allocation of Pt, Ni, and Co by *T. usneoides* was investigated. However, based on current trading prices (LME, 2022), the harvesting of *T. usneoides* trees for Ni, Co, or Pt would not be economically viable. Thus, from an anthropocentric standpoint, the use of *T. usneoides* for Ni, Co, or Pt phytomining would not be economically feasible. Site-specific conditions may have limited the assimilation and allocation of metals by *T. usneoides*. It is therefore proposed that further studies should be conducted to determine the phytoextraction potential of *T. usneoides* under hydroponic conditions (i.e. simulating base metal refinery effluent).

Based on the (a)biotic conditions at the time of tree harvest, it was estimated that harvested 9-year old *T. usneoides* contain at least 1 088 – 1 461 kg of S/ha. Because *T. usneoides* is continuously excreting and shedding salts, and these were excluded from the measurements, the actual phytoextraction capacity over time is beyond the scope of this study. Elevated S allocation by *T. usneoides* is supported by studies conducted by (Dennis, 2008) and (Kendall, 2010). This indicates that *T. usneoides* can effectively desalinate metal contaminated sites by significantly reducing salt load. A review of the available literature focuses on the desalination of land relative to Na^+ and Cl^-. For example, the halophyte, *Halogeton glomeratus*, has been reported to remove an estimated 2 105 kg/ha of salt (Li *et al.*, 2019) whereas lower NaCl yields have been reported for *Sesuvium portulacastrum* (approximately 1 000 kg/ha) (Rabhi *et al.*, 2010) and *Sesuvium edmonstonei* (251 kg Na/ha) (Islam *et al.*, 2022). This highlights the use of halophytes, such as *T. usneoides*, as a tool for phytodesalination.

Furthermore, the release of salts and metal ions into the environment is expected to increase (Lutts and Lefèvre, 2015, Nriagu, 1996), limiting the use of glycophytic hyperaccumulators for metal recovery. Results from this study indicate that *T. usneoides* has the potential to significantly reduce the salinity of metal-contaminated sites – advancing the survival conditions of glycophytes used for phytomining.

2.5.6. Other ecotoxicologically important elements

Tamarix usneoides accumulated (BCF > 1) a range of metals and metalloids in the following series: Ni > Co > Zn > Mo > Cu ≥ As, whereas *T. usneoides* excluded (BCF < 1) certain metals in the series: Fe > Te > Cr > Pb. These findings are supported by results obtained by Dennis *et al.,* (2008). Sulphur and Se share similar physicochemical properties and *T. usneoides* is known to hyperaccumulate Se from selenate, and S (Kendall, 2010). Based on these similar properties, S and Se may utilize similar biochemical and metabolic pathways. Although pathways involved in S and Se uptake, accumulation, and translocation were not studied, elevated BCF and TFs may be attributed to low and high affinity transporters present in root cells. Also, the significant difference between S and Se TFs may suggest that, although there were elevated soil concentrations of S (4 282 ± 694 mg/kg) compared with Se (32 ± 4.6 mg/kg), the elevated presence of S may have reduced TF_{Se}. The biological implication of this finding, in the context of this study, indicates that competitive ion absorption between element pairs with similar physicochemical properties, such as S and Se, and Ni and Co, may have contributed to the observed patterns of uptake and *in planta* translocation. Moreover, calcium (Ca) has been demonstrated to compete with Co for binding sites (i.e., competitive ion adsorption) (Lwalaba *et al.*, 2017a). This reduces the uptake and subsequent phytotoxicity of Co. Calcium soil concentrations for AM10 (3 276 mg/kg) and AM4 (2 164 mg/kg) were higher compared with AM2 (1 900 mg/kg). Although total element concentration is not a true indicator of the bioavailable fraction of Ca in the soil, Ca (as well as Ni) may have outcompeted Co for plant uptake and subsequent translocation. This finding highlights the importance of determining the composition and concentration of minerals as well as elements present in the substrate of the study site.

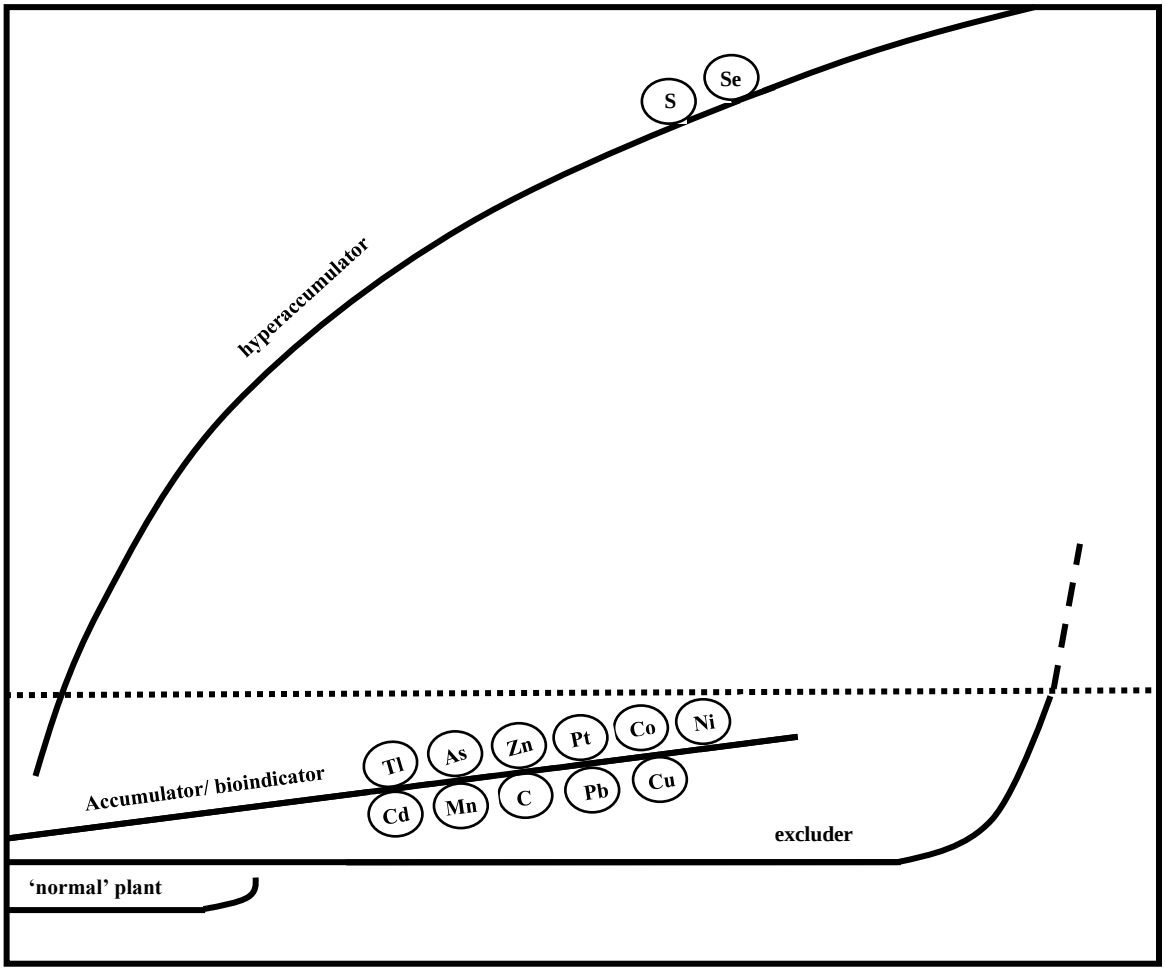


Figure 2.19. Uptake and accumulation of elements in plant leaves by *Tamarix usneoides* demonstrating varying degrees of tolerance. 'Normal' plants can only tolerate low levels of metals in soil and are highly susceptible to metal phytotoxicity. Metal excluders possess avoidance strategies, tolerating and surviving over a range of metal concentrations under a particular threshold. However, once this threshold is exceeded, unregulated levels of metals may enter the plant resulting in deleterious effects. Accumulators/ bioindicators are plants which accumulate (below the hyperaccumulation threshold) metal concentrations, in the leaves, reflecting the metal concentrations in the soil. Metal accumulators provide qualitative information about soil quality. Hyperaccumulators can (hyper)tolerate elevated soil and *in planta* metal concentrations by deploying efficient and effective metal ion detoxification strategies. Note: Dotted baseline represents hyperaccumulator threshold concentration. Also, latent hyperaccumulator line depicts inability of obligate hyperaccumulators to occur in soils with low metal concentrations. Element symbols represent the strategy of *T. usneoides*, harvested from the study site, with regards to certain elements accumulated in leaves. Adapted from Baker, (1981); Mertens *et al.,* (2005); van der Ent and Baker, (2013); Lange *et al.,* (2017).

2.6. Conclusion

Tamarix usneoides on the study site was found to hyperaccumulate S and Se (data not shown), and accumulate Pt, Ni, and Co. The site substrate was too heterogenous overall to allow for unambiguous conclusions, although tree size and genotype appear to influence metal extraction, whereby AM2 and AM10, are the best candidate trees for the phytoextraction of these metals. Elevated *in planta* Ni concentrations (BCF > 1 and TF > 1), compared with Pt and Co may be attributed to elevated concentrations of Ni (i) in the substrate, (ii) *in planta* mobility compared with other metals, as well as (iii) competitive ion absorption in which Ni may have outcompeted / displaced Co for binding sites and subsequent assimilation by *T. usneoides*. The elevated S with BCF > 20, TF > 1, indicates significant removal of S from the study site. The reduction in EC in planted pits, compared with unplanted control pits, indicates that *T. usneoides* is a suitable candidate for phytodesalination, with simultaneous accumulation of Pt, Ni, and Co under saline conditions.

Further studies need to be conducted to determine the degree of Pt, Ni, and Co uptake, translocation, and tolerance (relative to measuring physiological parameters over time) under controlled conditions, such as plant tissue culture (refer to Chapters 3 and 4). This is also required as each harvested tree was exposed to highly dissimilar substrate physicochemical properties [i.e., variation in physical (e.g. depth of overburden layer) and chemical (e.g. element composition and concentration)] which may all have contributed to differences in tree size, metal accumulation (BCF), and metal translocation (TF). Tree size influenced the accumulation of metals between plant organs whereas the dilution effect was not observed in this study.

The medium and large individual 9-year *Tamarix usneoides* accumulated S above-ground (BCF > 20; TF > 1). These results were supported by the lower concentrations of S and EC in the rooting zone soils compared with control (unplanted) soil profiles. This indicates that *T. usneoides* can be used as a tool for the decontamination of sulphate-contaminated soils under site conditions investigated in this study. The results of this study demonstrate that by 9 years of age, *T. usneoides* has significantly reduced the EC of the 0 – 100 cm depth of the substrate, whilst simultaneously accumulating, to a lesser extent, Ni, Co, and Pt). In conclusion, the managed growing and routine

harvesting of *T. usneoides* biomass can ameliorate substrate contamination over time and create conditions which are more conducive to the survival of glycophytes.

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Chapter 3

Development of a protocol for *in vitro* mass propagation of *Tamarix usneoides* – an exo-recretohalophyte used for phytoremediation

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Aspects of this chapter will be submitted for publication in ***Plant Cell, Tissue and Organ Culture (PCTOC)***

[**]Dr Hayden T. Wilson mentored the primary author with regards to the plant tissue culture protocol during the primary author's honours research. Tissue culture data from Dr. Wilson's Honours, MSc, or PhD has not been included in this Chapter.

3.1. Abstract

Factors impacting the rhizogenesis of explants were investigated to develop a protocol for the mass propagation of the recretohalophyte, *Tamarix usneoides*. *Tamarix usneoides* is utilised in dust control and phytoremediation of acid mine drainage in southern Africa. This highlights the importance of screening and mass propagating *T. usneoides* genotypes with an elevated capacity to tolerate and accumulate salts and metal ions. Experimental conditions were selected based on the different stages of plant tissue culture, namely donor plant, disease control, explant, and culture conditions. Explant establishment *in vitro* was influenced by donor plant factors, namely growing conditions [explant establishment of donor plants cultivated in non-contaminated areas (89 %) > contaminated areas (22%); (KW, $p < 0.05$)], physiological age of the donor plants [5 month old donor plants > 10 – 24 month old donor plants; ANOVA, $p < 0.05$], genotype (KW, $p < 0.001$), season of propagation (highest in winter compared with other seasons; KW, $p < 0.001$), length of explants (40 mm > 25 mm; KW, $p < 0.05$), and volume of growth vial (50 mL > 15 mL; KW, $p < 0.05$) also influenced explant establishment. Conversely, the chronological age of explants, pH level, strength of plant growth medium, and auxin pulse treatments did not affect explant establishment. A standardised and rapid *in vitro* protocol was developed for the mass micropropagation of *T. usneoides* explants. This study identified variables that influence explant establishment *in* vitro whereby limited studies have been conducted on the impact of donor plant growing conditions and genotype on the i*n vitro* morphogenetic response of explants. This highlights a crucial gap in research and the need for further study.

Keywords: Micropropagation, Tamaricaceae, genotype, growing conditions

3.2. Introduction

Salinisation, in combination with heavy metal assimilation, negatively impacts the growth and survival of both flora and fauna (Amezketa, 2006). This creates precedence for the use of halophytic species for the (i) decontamination of soils (Rabhi *et al.*, 2010), and / or (ii) use as new salt-tolerant crops (Panta *et al.*, 2014). The predicted increase in the combination of these stresses (Lutts and Lefèvre, 2015) highlights the need to develop mass propagation protocols for halophytes with elevated phytoremediation potential.

Tamarix usneoides E. Mey ex Bunge 1843 (Tamaricaceae) is an indigenous, evergreen recretohalophyte tree (Baum, 1978). Like other recretohalophyte species, *T. usneoides* possesses ion uptake, translocation, and excretion mechanisms to maintain cellular homeostasis (as evidenced by the presence of foliar salt glands). This trait, and observations that *Tamarix* species naturally colonise gold and uranium mine tailings in South Africa (Weiersbye *et al.*, 2006a), make this study species of particular importance for phytotechnologies. *Tamarix ramosissima* and *T. chinensis* were planted on mine sites in the early 1900s for dust control (Thatcher, 1979). The presence of *Tamarix* hybrid swarms in the Cape provinces were noted in the mid-1900s and hybridisation between *T. ramosissima, T. chinensis* and *T. usneoides* has been verified using DNA barcoding and microsatellites or short sequence repeats (SSR) (Mayonde *et al.*, 2015, Mayonde *et al.*, 2019b, Mayonde *et al.*, 2016). However new evidence shows that barcoding cannot discriminate *T. ramosissima* and *T. chinensis,* can give misleading results (Terrones Contreras *et al.*, 2022). Since 2002, *T. usneoides* cuttings from wild populations in the Northern Cape Province have been planted on areas impacted by acid mine drainage (Dye *et al.*, 2008, Weiersbye *et al.*, 2006a). Only this species has desirable traits for phytotechnologial applications, i.e. indigenous with an evergreen habit (Baum, 1978), dense foliar salt glands (Wilson *et al.*, 2017), tolerance to acid mine drainage (Dye *et al.*, 2022, Dye *et al.*, 2014), and relatively low water-use (Dye *et al.*, 2022, Krug, 2017) with phytodesalination ability (Webb *et al.*, 2018). Therefore, there is considerable justification for mass propagation of *T. usneoides.*

Numerous types of studies (*in vitro*, hydroponic, pot trials, or field studies) are conducted to investigate the phytoremediation potential of plant species and their genotypes (i.e. implications for clone propagation). However, these studies are limited by the inability to quantify abiotic and biotic factors which fluctuate through space and time. Differences in experimental parameters limit the (i) repeatability of study methodology, (ii) verification of obtained results, (iii) quantification of numerous variables, as well as (iv) standardisation of experimental conditions – hindering the ability to compare data and results between different studies (Golan-Goldhirsh *et al.*, 2004, Watson *et al.*, 2003b). A potential solution lies in the development of protocols for the propagation of plants under standardised and quantified abiotic and biotic conditions, namely, plant tissue culture (PTC) – enabling the effect of experimental variables to be distinguished from controlled variables. Plant tissue culture involves the *in vitro* culturing of plant material excised from a donor plant, which is subsequently exposed to a set of known (a)biotic factors and is divided into five stages (Table 3.1) (García-Gonzáles *et al.*, 2010).

A number of *in vitro* studies have been conducted on *Tamarix* spp (English language publications only are listed in Table 3.2). Those studies investigated the function and/or efficacy of salt-tolerance structures (*T. usneoides* – Wilson *et al.* (2017); *T. gallica* – Lucchesini *et al*., (1993); *T. hispida* - Wang *et al.*, (2018); propagation of explants for potential use in biocontrol studies (*T. ramosissima* - Yu and Tserendagva, (2012)), the potential production of pharmaceutical compounds (*T. nilotica* - Al-Qurainy et al., (2015)), and screening of genotypes for tolerance and recovery of gold (*T. usneoides* - Wilson *et al.* (2022)) (Table 3.2). These studies highlight the importance of developing protocols for the mass micropropagation of *T, usneoides*. In order to optimise the efficacy of *in situ* phytomining or phytoremediation, clones are required to be mass propagated with little polymorphism. This highlights the advantage of using explants from genetically verified donor plants as plantlets derived from seeds have a heterozygous nature, and therefore are not favoured for propagation of clones with desirable traits (Upadhyay *et al.*, 2014). However, in order to optimise PTC protocols, factors affecting explant rhizogenesis (i.e. establishment) must be investigated.

Table 3.1. Stages of plant tissue culture affecting explant establishment.

Stage of plant tissue culture	Variables influencing explant establishment
Stage 0 - Donor plant status	
The donor plant provenance, genotype, physiology (e.g. plant age/ topophysis[7]), plant growing conditions, and the time of explant excision are important factors contributing to the success of explant introduction and establishment *in vitro* (Kartsonas and Papafotiou, 2007).	**Genotype:** Numerous studies have concluded that differences in morphogenetic[8] responses of the same plant species to *in vitro* culture may be attributed to plant provenance/genotype (Kim *et al.*, 1998). Furthermore, genotype-specific responses may also occur (mediated by donor plant status), leading to somaclonal variation[9] (Miguel and Marum, 2011). Thus, different plant species, and more specifically different genotypes of the same species, may require different culturing environments (temperature, photoperiod, and nutrient media composition).
	Donor plant growing conditions: Standardising environmental growing conditions (e.g. use of greenhouses) enables variables such as temperature, relative humidity, irrigation, and positioning of plants within the growing environment to be controlled. The regulation of these variables has been found to improve the success of *in vitro* explant establishment (Pierik, 1997). By way of example: • The nutrient status of donor plants (equivalent to the nutrient status of the explant excised from that particular donor plant) is influenced by season and the year (Romano *et al.*, 2002). The bioavailability of elements within the donor plant's growing medium differs between seasons due to changes in environmental (such as temperature) and plant (such as increased rates of evapotranspiration (ET) and the release of organic compounds into the soil) factors, mediating element uptake by the donor plant and contributing to the

[7] Positional effect of explants on the donor plant before excision.
[8] Refers to the development of morphological/ physiological features. Morphogenetic/ physiological characteristics measured in this study include shoot length, root length, number of shoots and roots, secondary (lateral) rooting, and presence of root hairs.
[9] Defined as phenotypic variation in explants of the same genotype.

nutrient status of the donor plant;

- During the growing season of *Ulmus villosa*, cuttings had significantly[10] elevated levels of total carbohydrates and peroxidase activity accompanied by low nitrogen levels.

These examples highlight the potential differences in the morphogenetic response of explants when excised from donor plants during different seasons. This may also limit the repeatability of explant response in culture due to variation in donor plant and environmental factors between seasons and years. Furthermore, donor plants are routinely treated with an insecticide and/or fungicide in order to prevent the introduction of contaminated plant material into culture (García-Gonzáles *et al.*, 2010). Thus, the success of *in vitro* plantlet establishment is mediated by the spatial and/or temporal (such as the seasonal differences and the age of donor plants) differences in the growing conditions of donor plants.

Physiological age of donor plant: The physiological age[11] of the donor plant has received much attention in recent decades (Basto et al., 2012, Dumas and Monteuuis, 1995). Physiological maturation increases with increasing chronological age (cyclophysis) whereas some areas of an individual plant (e.g. top of plant) react differently compared with other zones (e.g. inner parts of the individual) of the same individual due to variation in exposure to environmental conditions (i.e. topophysis). Explants excised from younger donor plants generally have elevated rates of root formation compared with explants from older plants. Thus, the *in vitro* establishment response (rate and percentage of explant establishment) may be reduced when explants are excised from physiologically older donor plants.

Stage I - In vitro introduction of explants

Tissue culture provides a controlled, aseptic growing environment where certain variables, such as light, temperature, relative humidity, the nutrient composition of the plant growth medium, as well as the volume of the plant growth medium, are standardised. Tissue culture enables the results obtained under these controlled conditions to be attributed to the

[10] $p < 0.05$

[11] Refers to plant tissue in a particular stage of development which has undergone more cell divisions and differentiation. For example, physiologically older tissues have undergone more cell divisions and differentiation compared with developing / younger plant tissue.

response of an explant to a particular independent variable, such as the addition of certain heavy metals to the growing media in order to determine the plant's physiological response to varying concentrations of that particular heavy metal(s) (Bhojwani and Dantu, 2013). The standardisation of these environmental variables, along with the aseptic practice, allows for increased study repeatability and result verification. However, it must be noted that the controlled environment may limit/ affect the physiological (e.g. morphological) functioning of explants *in vitro*. Previously published studies have demonstrated the reduction in shoot length growth, cellular elongation, as well as changes in stomatal size, density, and aperture whereas the number of shoot branches has been reported to increase under tissue culture conditions (Chen *et al.*, 2006).

Stage II - Explant propagation

The success of explant establishment has been demonstrated to depend on the culture technique (explant type, size, and age) which is influenced by the species being cultured as well as the genotype (García-Gonzáles *et al.*, 2010).

Explant type: The type of explant used depends on the aim of the experiment such as the use of established plantlets[12] to determine the tolerance, uptake, and translocation of metal species by plantlets (pertinent to this study). A number of different types of explants can be cultured including single cell, organ, callus, embryo, or protoplast culture (García-Gonzáles *et al.*, 2010). Santa-Maria *et al* (2009) demonstrated that the optimal culturing technique for sweet potato is via organ culture, whilst somatic embryogenesis was used to micropropagate coffee plants (Santana-Buzzy *et al.*, 2007).

Explant size: The size of the explant influences the success of *in vitro* establishment. Smaller explants may have lower establishment rates, compared with larger explants, as smaller explants typically have lower levels of phytohormones (e.g. less auxin per unit of plant tissue) and nutrient reserves (Pierik, 1997). This may lower the rate and number of explants establishing *in vitro*. Differences in the establishment and physiological growth of plantlets varying in size may also be attributed to the chronological age of the explant, as well as topophysis (Mencuccini *et al.*, 2005).

Chronological age of explants: Maturation refers to the chronological[13], ontogenic[14] , and physiological ageing of the plant tissues. With regards to the chronological age of the donor plant and axillary bud culture, buds isolated closer to

[12] In the context of this study, a plantlet refers to an established shoot explant (i.e. explant comprising of both shoots and roots).
[13] Chronological ageing refers to the time since a particular plant tissue differentiated.

the apical meristem (top of donor plant) are generally younger than those located and isolated near the base of the plant stem/ branch. Chronological aging is influenced by environmental conditions. Numerous studies have highlighted an elevated physiological functioning in chronologically younger explants. Rout and Sahoo (2007) concluded that the ability of a certain plant species to tolerate heavy metal stress is mediated by plant age. Also, as donor plant age increases, levels of endogenous indole-3-acetic acid (IAA) decreases thereby limiting the explant's ability to establish *in vitro*. These stages of ontogenic aging may be observed as differences in physical (such as the number and development of salt glands) and chemical (such as organic compounds) mechanisms involved in metal tolerance, uptake, and translocation, as well as plant defences (Barton and Koricheva, 2010).

Plant growth media strength: Plant growth medium was developed, and revised, to meet the requirements of plant material *in vitro*. Growth media generally consists of inorganic nutrients, vitamins, carbon source(s), as well as growth regulators. Murashige and Skoog (MS) standard plant growth medium consists of inorganic salts and is widely used for a range of plant species (Table S3.1) (Murashige and Skoog, 1962). The development of a defined MS standard plant growth medium enabled numerous plant species to be cultured *in vitro* and thus, is the most widespread nutrient medium used in plant tissue culture (Murashige and Skoog, 1962, Thorpe, 2007). Some studies have modified the strength/ concentration of MS media, as well as the addition of constituents such as vitamins, which further increased the range of plant species that could be optimally established and grown using MS medium (Gatti, 2008). It must be noted that the widespread use of MS media allows the *in vitro* response of numerous plant species to be directly compared as well as method repeatability and optimisation, and the standardisation of numerous variables.

Phase of growth medium: The addition of gelling agents to plant growth medium provides support for explants thereby standardising growing conditions. For example, the orientation of the explant is standardised (i.e. the amount of light reaching certain proportions of each explant is standardised) (Thorpe, 2007). Liquid (hydroponic) plant growth medium has been studied and used in numerous plant growth and nutrition experiments. Although semi-solid

[14] Ontogenic age refers to the growth and development of the same plant tissue, such as leaves, passing through a series of recognizable and predictable stages of the same organ. Barton, K. E. & Koricheva, J. 2010. The ontogeny of plant defense and herbivory: characterizing general patterns using meta-analysis. *The American Naturalist,* 175, 481-493.

	plant growth media has a homogenous nutrient composition, liquid medium may have elevated levels of bioavailable and bioaccessible elements to plantlet root systems. The production of root hairs, which increases surface area for element uptake, and root system architecture (RSA)[15], are influenced and impeded by a solid phase medium. Thus, a liquid medium is advantageous where the ability of established explants to take up metals under controlled conditions is being investigated (such as the present experiments) (Hooda, 2007).
Stage III - Explant establishment	
Adventitious root formation (ARF), mediated by genetic, molecular, cellular, and physiological processes, involves the formation of roots from differentiated explant tissue such as stems (Pacurar *et al.*, 2014). In this study, ARF was used as a proxy for plantlet establishment. Adventitious root formation consists of three physiological processes, including the induction phase (activation/ expression of ARF genes), initiation phase (dedifferentiation of cells at the site, or close to the site, of excision resulting in the formation of internal root meristems), and the expression phase (growth of root primordia and emergence). Secondary root formation (lateral rooting) and root hair production increase surface area for nutrient absorption (Pijut *et al.*, 2011). The two main types of adventitious roots are preformed and wound-induced roots. Preformed roots, present during stem development,	**Auxin treatments:** Numerous studies have demonstrated that the addition of phytohormones to the plant growth medium promotes the explant's *in vitro* morphogenetic response (Table S3.2) (Tilkat *et al.*, 2009). Phytohormones, especially auxins such as indole-3-butyric acid (IBA), naphthaleneacetic acid (NAA), and indole-3-acetic acid (IAA), are common additives to the culture medium to promote a desired morphogenetic response. Nissen and Sutter (1990) demonstrated that IBA is significantly more stable compared with IAA when exposed to elevated temperatures (conducive to autoclaving) and light intensity and has elevated stability in MS solution and resistance to enzymatic degradation. Indole-3-butyric acid has also been implicated in significantly improving ARF, compared with IAA, when added to the plant growth medium (Ludwig-Müller *et al.*, 2005). Studies have also suggested that auxin pulse treatments (addition of auxin to plant growth medium for a certain period of time, such as 24 hours) promote the ARF induction phase. Numerous studies have demonstrated the effect of IBA on ARF induction in which the auxin concentration, exposure period, and plant species differed (Table S3.2).

[15] RSA refers to the spatial arrangement of the plant's root system within a solid phase medium such as soil or gelled MS growth medium. The RSA also determines the plant's access to nutrients within the solid phase medium.

remain dormant until the stem (such as a cutting or explant) is exposed to optimal growing conditions (Pijut *et al.*, 2011). Wound-induced roots involve the formation of a protective suberised cell layer protecting the explant from biotic infections. Subsequently, cells divide forming dedifferentiated parenchyma cells or callus, followed by the differentiation of cells into roots facilitated by phytohormones. The main factors influencing ARF are plant genotype and the endogenous levels of phytohormones (Pacurar *et al.*, 2014).

3.2.1. Factors influencing rhizogenesis in vitro

The donor plant mineral nutrition, genotype (Miguel and Marum, 2011), physiological age (Basto *et al.*, 2012, Dumas and Monteuuis, 1995), plant growing conditions, and time of explant excision (Ramanayake and Yakandawala, 1997) have been reported to be important factors mediating explant establishment (including rhizogenesis). Although limited studies have investigated the impact of donor plant growing conditions on *in vitro* explant establishment, the presence of heavy metal (Gatti, 2008), salinity (Munns and Tester, 2008), and drought (Tadayyon *et al.*, 2018) stressors have been demonstrated to impact explant establishment by altering plant mineral nutrition. For example, the presence of heavy metals such as copper (Cu), may disrupt the assimilation of essential cationic micronutrients (e.g. calcium) due to competitive ion adsorption in plants, modifying plant mineral nutrition (Schwambach *et al.*, 2005). This mediates explant rhizogenesis as mineral nutrition of explants is equivalent to the nutrient status of the donor plant (De Almeida *et al.*, 2017, Schwambach *et al.*, 2005). As the bioavailability of elements within the donor plant's growing media varies spatially and temporally, the physicochemical properties of the growth media, as well as season of explant harvest, must be considered as an important variables influencing plant nutrition, carbon status, and water content and therefore, explant rhizogenesis (Eviner *et al.*, 2006). Maturation refers to the chronological, ontogenic, and physiological ageing of the plant tissues (Rasmussen *et al.*, 2015), whereby buds isolated closer to the apical meristem of the donor plant are generally younger than those isolated from the base of the plant (Munné-Bosch, 2007). Numerous studies have highlighted an elevated physiological functioning (e.g. transpiration, abiotic stress response, photosynthesis, etc) by chronologically younger explants. The physiological age of the donor plants also influences the morphogenetic response of explants *in vitro*, where explants excised from younger donor plants generally have elevated rates of root formation compared with explants from older plants (Amri *et al.*, 2010).

Table 3.2. *In vitro* propagation of *Tamarix* spp.

***Tamarix* spp**	**Aim of study**	**Uses of *Tamarix* species**	**Micropropagation conditions**	**Outcome**	**Reference**
T. usneoides	To describe the ultrastructure of vesiculated trichomes (salt glands), and screen genotypes *in vitro* for tolerance to pH, gold compounds and gold recovery.	Phytoremediation (phytodesalination) and phytomining of gold (rhizofiltration)	3 cm explants (apical shoots) were excised from donor plants grown under greenhouse conditions. Explants were surface sterilised in 2 % sodium hypochlorite for 15 minutes and subsequently rinsed three times in ultrapure water. Explants were cultured in 125 mL growth vials containing 2 mL liquid, quarter-strength MS Medium (1.1 g/L), where pH was adjusted to 4.6, 5.6, or 7.6 prior to autoclaving. Cultures were incubated under controlled conditions (Temperature: 25 °C); photoperiod: 16 hr light/ 8 hr dark).	Physiological growth parameters and efficacy of *in vitro* protocol were not reported.	Wilson *et al.*, (2017) and Wilson *et al.* (2022)
T. gallica	Establish an *in vitro* protocol for the micropropagation of *T. gallica* by shoot proliferation from axillary buds.	Not reported	1-year old branches were excised, in summer, from 50-year-old, actively growing trees. 5 cm portions were washed in Tween20 (0.1 % (v/v)) supplemented with ascorbic acid (80 mg/L) for 1hr. Explants (1.0 - 1.5 cm in length with 4 - 6 nodes) were surface sterilised in sodium hypochlorite (1.05 %) for 10 minutes and subsequently rinsed three times in distilled water. Explants were placed vertically into 30 mi polystyrene vials containing 5 mL LS Medium. LS Medium was supplemented with sucrose (30 g/L), 200 mg/L reduced glutathione, and 7 g/L Difco Bacto-agar was used as a basal medium, and pH was adjusted to 5.8 (before autoclaving). Vials were incubated at 22 °C ± 1 °C under 16hr photoperiod with cool, white fluorescent lights. Initial shoot proliferation was induced on basal medium supplemented with 3.3uM	The addition of IBA at 0.5 or 1.5 µM IBA increased lateral shoot formation and rate of rooting. Repetitive sub-culturing on 1.0 µM IBA resulted in elevated *in vitro* morphogenetic response (namely well-developed roots with an elevated number of axillary shoots) and acclimation under greenhouse conditions. Shoot length as well as root length and the number of roots (which both significantly) increased in an IBA-dose-dependent response whereas elevated IBA concentrations (i.e. 0.5 and 1.0 µM) resulted in a significantly higher number of shoots compared with control (i.e. no IBA addition).	Lucchesini *et al.*, (1993)

			BA, and subsequently cultured on basal medium supplemented with BA (0.0, 1.1, or 3.3 µM), 3.3uM BA combined with GA (0.0, 0.58, or 2.9 µM), or IBA (0.0, 0.5, or 1.0 µM).		
T. hispida	Investigate the physiological, salt-tolerance function of aquaporins (AQP)	Phytoremediation, namely rhizofiltration (e.g. removal of Zn from wastewater – Khademi *et al.,* 2015)	*T. hispida* seedlings were cultured on half-strength MS, solid-phase medium under controlled conditions (temperature: 24 °C, photoperiod: 14 L/10 D). Plants were transformed (using the transient transformation method), grown on half-strength MS supplemented with 120 mM NaCl or 175 mM mannitol for 24 hours.	Used an *in vitro* system for the propagation of *T.* hispida which aided in investigating salt-tolerance function (as well as cloned and characterised) of an AQP gene from *T. hispida*.	Wang *et al.,* (2018)
T. tetrandra	Develop a protocol for optimal plant growth and ellagitannin tetramers production	Medicinal use [see Bahramsoltani, *et al.,* (2020) for review]	Explants (stems), 1 - 2 cm with several nodes, were cultured in glass tubes (1.8 x 18 cm) containing solid-phase Linsmaier and Skoog Medium (LS), incubated at 25 °C in the dark or 12 hr/day photoperiod (for IAA/BA and IAA/KIN combinations). LS medium was supplemented with sucrose (30 g/L) and different auxin (IAA, BA, KIN, NAA, 2,4-D) combinations at different concentrations.	*T. tetrandra* explants were successfully cultured where shoot cultures produced various ellagitannins. The first study where *T. tetrandra* shoot cultures produced tetrameric and pentameric ellagitannins. This study offers large-scale mass propagation within a short time and may be a rapid introduction of new genotypes with desirable traits (e.g. elevated salt tolerance).	Orabi *et al.,* (2011)
T. nilotica	Develop micropropagation protocol for the mass propagation of *T. nilotica* for conservation and pharmaceutical-compound production.	Medicinal (production of pharmaceutical compounds) [see Bahramsoltani *et al.,* (2020) for review]	Cuttings (15 - 20 cm in length) were harvested from young (10-year-old trees) and further excised to 1.0 cm explants for culture. Explants were washed (tap water), decontaminated with ethanol (70 % v/v) for 1 minute, and dipped two times in bleach solution (Chlorox, 3 – 4 % chloride) supplemented with six drops of Teepol. Explants were rinsed with a sterilised fungicide (Carbendazim) for 10 minutes and subsequently washed three times in sterile water (three five-minute washes). Explants were placed in phytajars (15	Elevated morphogenetic response was induced by explants cultured on MS medium supplemented with TDZ (1.9 µM) followed by 2.5 µM BA, KIN, and 2ip. The number of shoots decreased in a cytokinin-dose-dependent response. IBA pulse treatments (100 µM IBA for 5, 10, or 15-day exposure periods) were required to initiate root growth. Root induction decreased as growth regulator concentration increased whereas	Al-Qurainy *et al.,* (2015)

			explants/ jar) containing 50 mL, solid-phase MS Medium supplemented with different growth regulators (BA, KIN, TDZ, or 2iP). Cultures were incubated under controlled conditions (Temperature: 25 ºC ± 1 ºC, photoperiod: 16 hr). Physiological growth measurements (namely shoot morphological features and number of shoots per explant) were measured.	auxin pulse treatment for 10-days resulted in elevated rooting %, root length, and shoot length. Ten-day-old, rooted plantlets were successfully acclimated in sand and soil under greenhouse conditions.	
T. ramosissima	Establish micropropagation protocol	*Tamarix ramosissima* Ldb. is rare, endemic plant in Gobi Desert. There is very limited information on work on tissue culture propagation of T. ramosissima Ldb. Thus, this aim of this research was to establish micropropagation of this plant and transfer to *ex vitro*	Explants were harvested from five-year-old plants. Explants were cultured on quarter-strength MS Medium supplemented with BA and NAA. Rooting was induced by culturing explants on quarter-strength MS supplemented with 2, 4-D, NAA, IBA, Kinetic, or Zeatin.	Elevated morphogenetic response (namely rooting) was induced by MS medium supplemented with NAA (0.57 µM) and IVA (0.49 µM). Elevated shoot length was induced by MS supplemented with NAA (0.57 µM) however, this treatment resulted in lower root growth and increased callus formation – compromising the explant's acclimation process. MS supplemented with IBA (0.49 µM) promoted elevated morphogenetic responses.	Yu and Tserendagva, (2012)

Note: indoleacetic acid: IAA; indolebutyric acid: IBA; α-napthaleneacetic acid: NAA; 2-4, dichlorophenoxyacetic acid: 2,4-D; benzyl adenine: BA; kinetin: KIN; Thidiazuron: TDZ; isopentynyl: 2iP.

Supplementing the plant growth medium with phytohormones can promote the explant's *in vitro* morphogenetic response (Tilkat *et al.*, 2009). Although phytohormones, especially auxins such as indole-3-butyric acid (IBA) are common additivities to culture media to enhance rhizogenesis, the levels of endogenous phytohormones, such as indole-3-acetic acid (IAA), differs between explant type and size (Ćosić *et al.*, 2015). The supplementation of IBA, compared with IAA, has been demonstrated to be (i) more stable during culture preparation (e.g. elevated temperatures during autoclaving) and (ii) significantly improves rhizogenesis, compared with IAA (Ludwig-Müller *et al.*, 2005, Nissen and Sutter, 1990). It must, however, be noted that the type of explant used in PTC depends on the aim of the study. For example, rhizofiltration studies involve the sorption of ions of interest by plant roots, and thus, *in vitro* plantlets in liquid media are a viable explant type for studies investigating phytoextraction. Hence, the aim of this research was to develop an efficient, reliable, and repeatable direct organogenesis *in vitro* procedure for *T. usneoides* explant establishment.

3.3. Materials and methods

The following PTC protocol was implemented for all pilot studies unless otherwise stated.

3.3.1. Plant tissue culture propagation for pilot studies

Stem cuttings of eight individuals were acquired as follows. Five genotypes of *T. usneoides* (G1 to G5) were collected from trees growing on a metal contaminated site in the North West Province of South Africa (SA) (AngloGold Ashanti Ltd and the University of the Witwatersrand, Johannesburg: The Mine Woodlands Project). These trees were grown from cuttings, i.e. are clones, of trees in the Northern Cape province. Stem cuttings from three genotypes of *T. usneoides* (G6 to G8) were collected from the Northern Cape, their natural home range (non-contaminated sites) (Table 3.3). Donor plants were sourced from clones of trees from the Orange River near Upington and Augrabies region of the Northern Cape province of South Africa. Due to the presence of hybrids in the source populations, all the trees used in this study were verified as *T. usneoides* in a parallel study using DNA barcoding and SSR (Mayonde *et al.*, 2015, Mayonde *et al.*, 2019a).

3.3.2. Explant culture

Donor plants of the eight *T. usneoides* genotypes were grown in a greenhouse (temperature ranged from 15 - 30 °C, irrigated by overhead water systems using potable, municipal water for a period of 15 minutes every 24 hours) located at the University of the Witwatersrand, SA. Genetically verified *Tamarix usneoides* cuttings were propagated according to Wilson *et al.* (2017). Plants were pre-treated with a systemic fungicide, Bravo 720 (2 mL/L) (active ingredient: Chlorothalonil at 720 g/L, Syngenta, Basel, Switzerland), and an insecticide, Malasol (1.75 mL/L) (active ingredient: Mercaptothion at 500 g/L, Efekto, Isando, SA) in weekly rotations for at least 45 days before explants were harvested. Cuttings were treated with a systematic fungicide, Previcur (1.5 mL/L) (active ingredient: Propamocarb 600 g/L, Bayer Cropscience Pty Ltd, Melbourne, Australia) once every three months. Nodal cuttings of 40 mm in length with at least two axillary buds, were excised from the donor plants, surface decontaminated for 20 minutes in 2 % sodium hypochlorite, and rinsed three

times in ultrapure water (Millipore Direct-Q UV water purification system, Merck Millipore, Darmstadt, Germany). After removal of the bleached basal areas, the explants were cultured in sterile 25 % (quarter strength) Murashige and Skoog (MS) liquid basal medium with vitamins (Sigma Aldrich, Saint Louis, MO, USA) (Murashige and Skoog 1962), adjusted to pH 5.5 or 7.5 by the addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH), respectively. Each explant was grown in 15 mL or 50 mL borosilicate glass tubes that had been sterilised in nitric acid (0.1 M) followed by rinsing in deionised water. All glassware, culturing equipment, and plant growth medium was autoclaved at 121 ˚C and 1 KPa for 20 minutes. The vials contained 0.2 mL of medium. Explants were incubated at 25 °C ± 0.2 °C with a photoperiod of 16L8D (Osram 36W, cool white) for six weeks, re-randomised every second day, and topped up with 0.2 mL of medium every third day. Establishment, referring to the development of primary and secondary (lateral) root systems, as well as root hairs, was measured as the morphologically observable initiation of root growth.

3.3.3. Pilot studies

Pilot studies were conducted (Table 3.3), based on variables of different stages of PTC (Table 3.1) reported to impact the success of explant establishment).

3.3.4. Statistical analysis

Data were analysed using R statistical software [R version 4.1.3 (“One Push-Up”)]. Descriptive statistics (mean, median, standard deviation, standard error, minimum, maximum, skewness, and kurtosis) were calculated. Data were tested for non-normal distributions using the Shapiro-Wilk test (Shapiro and Wilk, 1965), followed by the Fligner-Killeen test for equality of variances (Conover *et al.*, 1981). Non-normally distributed data were normalised using logarithmic, square-root, power, or box-cox transformations. Normally distributed data were tested using analysis of variance (ANOVA) (Fisher, 1946) followed by Tukey’s Honest Significant Difference (HSD) post hoc test (Tukey, 1949) to determine which sample means were statistically different. Kruskal-Wallis (KW) test was used to test non-normally distributed data (after transformations) for statistical

differences (Kruskal and Wallis, 1952). Pearson's chi-squared (χ^2) statistical test was used to test for statistical differences between categorical dependent and categorical independent variables.

Table 3.3. Studies conducted, relative to applicable stages of plant tissue culture, to develop and optimise the *in vitro* establishment (rhizogenesis) of *Tamarix usneoides* explants. Note, some samples overlap between investigated variables.

Variable	Description	Sample size (n =)	
Donor plant growing conditions (contaminated vs. non-contaminated sites)	Donor plant material was harvested and collected from genetically verified trees growing in a phytoremediation trial on (i) a contaminated site (CS, polluted soils at the East Pay Dam footprint, AngloGold Ashanti Ltd Vaal River Mining Operations, Orkney, North West Province of South Africa – Genotypes 1 – 5 (Mayonde *et al.*, unpublished data), and non-contaminated sites (NCS, namely *T. usneoides'* natural home range) (Mayonde *et al.*, 2019a) as follows: Genotype 1: LH31 Genotype 2: LH38 Genotype 3: LH40 Genotype 4: LH47 Genotype 5: LH48 Genotype 6: 28°41'36.12"S; 20°30'30.24"E, Marchand, Northern Cape, South Africa Genotype 7: 28°41'25.02"S; 17°35'13.86"E, Vioolsdrif, Northern Cape, South Africa; and Genotype 8: 30° 1'5.70"S; 17°52'48.90"E, Kamieskroon, Northern Cape, South Africa	**CS** $n_{G1} = 90$ $n_{G2} = 90$ $n_{G3} = 88$ $n_{G4} = 90$ $n_{G5} = 89$	**NCS** $n_{G6} = 102$ $n_{G7} = 94$ $n_{G8} = 95$
Plant genotype	Cuttings were harvested from eight genetically verified *T. usneoides* trees originating from the Northern Cape, with one set planted on contaminated land. As growing condition was investigated, differences within donor plant site groups were determined to assess the impact of genotype on explant establishment, i.e., assessed genotypes collected from CS or NCS growing conditions). Thus, for this variable - establishment efficiency was not compared across donor plants.	**CS** $n_{G1} = 90$ $n_{G2} = 90$ $n_{G3} = 88$ $n_{G4} = 90$	**NCS** $n_{G6} = 102$ $n_{G7} = 94$ $n_{G8} = 95$

		n_{G5} = 89
Re-culturing of G6	Donor plants of known genotype and physiological characteristics, namely G6, were re-cultured in 15 mL growth vials. At the time of re-culture, donor plants were 20 months older compared with the initial PTC study.	n = 22
Explant size	Explants varying in length, *viz*- 40 mm or 25 mm, were cultured to investigate the effect of length on explant establishment.	n_{40mm} = 450 n_{25mm} = 130
Strength of MS plant growth medium	Explants were cultured in 0.2 mL 25, 50, or 100 % MS standard plant growth medium with vitamins (refer to Table S3.1 for MS constituent composition and concentrations).	$n_{25\%}$ = 49 $n_{50\%}$ = 30 $n_{100\%}$ = 30
Volume of growth vial	Explants were cultured in 50 mL growth vials or 15 mL growth vials to investigate the effect of growth vial volume and shape on explant establishment. 15 mL growth vials enable explants to maintain upright position (i.e. apex of explant was not in contact with the wall of the growth vial) as well as constant exposure to 0.2 mL plant growth medium.	$n_{15mL\ vials}$ = 130 $n_{50mL\ vials}$ = 450
Auxin pulse treatment	Explants were treated with indole-3-butyric acid (IBA) at different concentrations (0.01, 0.1, or 1 mg/L). The control for this pilot study was no addition of IBA. IBA was solubilised in NaOH before being added to 25 % MS plant growth medium at pH 5.5 and autoclaved. Explants were exposed to one of three IBA treatments for 24 hours. The auxin pulse period and concentration were selected based on the range of pulse periods and concentrations used for optimal *in vitro* rooting by a range of plant species (Table S3.2). The solution was removed from each growth vial, via the use of an aseptic glass sucker, and replaced with 0.2 mL 25 % MS solution.	$n_{0.01mg/L}$ = 10 $n_{0.1mg/L}$ = 10 $n_{1mg/L}$ = 10 $n_{Control}$ = 10
Open bench trial	Open bench trials are used to equilibrate relative humidity (RH) conditions present in natural environments. The RH within each growth vial is high (up to 99.4 % RH has been reported – e.g. Chen, (2004), compared with ambient humidity (~59 %	n = 57

	RH in Johannesburg, South Africa – where this study took place). This lowers the explant's water potential due to the "unnatural" evapotranspiration (ET) rate which may impact certain physiological processes. The rate of evaporation was calculated [rate of evaporation = volume of 25% MS liquid medium / time] to determine the adequate replacement of growth media to avoid explant cavitation (and subsequent death). Explants (25 mm) were treated with an antibiotic antimycotic at 10 mL/L (filter sterilised) working concentration or sodium hypochlorite (1, 2, 3, 5, or 7 mL/L), which was added to the MS medium, to reduce infections associated with open culture systems. A clean and transparent plastic sheet was placed across the length and width of the PTC growth bench to reduce the transmission of any airborne pathogens within the growth room.	
Effect of pH	The effect of pH was investigated by exposing explants to a range of pH levels. Genotypes were cultured in media at pH 4.6, 5.5, or 7.5.	$n_{pH\ 5.5} = 225$ $n_{pH\ 5.6} = 95$ $n_{pH\ 7.5} = 225$ $n_{pH\ 7.6} = 99$ $n_{pH\ 4.6} = 97$
Donor plant growing conditions	The pH, E_h, and moisture content of two different donor plant growth media, namely silica sand or 2:1 compost: silica sand mixture, were measured. Soil samples were collected from 10 different plant bags of the same sizes. Fifty grams (50 g) of each soil type was weighed (to obtain wet mass) and placed in an oven at 100 °C for 24 hours. The soil was weighed every 60 minutes until the mass of the soil was constant. The final mass was recorded as the dry mass. Twenty millilitres (20 mL) of distilled water were poured into each container containing ten grams (10 g) of homogenised soil. Each container was rigorously shaken for five minutes and left to stand for three minutes to facilitate the soil going into solution. The pH (probe: WTW SenTix 41), electrical conductivity (EC. probe: WTW Tetra Con® 325, Manufacturer), and oxidation reduction potential (E_h, WTW SenTix ORP 0…100 °C) were measured by placing all three probes in the paste at the same time.	n = 20
Season and year	In South Africa, dates for each season generally range from 1st March – 31st May (Autumn), 1st June – 31st August (Winter), 1st September to 30th November (Spring), and 1st December – 28 / 29th February (Summer) (Mucina and Rutherford, 2006). Explants were isolated and cultured during different seasons over a four-year period (2013 - 2016).	$n_{Autumn} = 166$ $n_{Spring} = 222$ $n_{Winter} = 741$ $n_{Summer} = 52$

Physiological age of donor plant	To determine the effect of the physiological age of the donor plant, explants were isolated from donor plants of varying ages (5, 10, 14, 18, 20, 21, 22, and 24 months old) and cultured.	$n_{5\ months} = 291$ $n_{20\ months} = 30$ $n_{10\ months} = 450$ $n_{21\ months} = 12$ $n_{14\ months} = 57$ $n_{22\ months} = 22$ $n_{18\ months} = 192$ $n_{24\ months} = 109$
Chronological age of explants	The stem of the donor plant was divided into three sections (categories) based on their chronological age, *viz* - developing (top third), intermediate (middle third), and the developed (bottom third) plant tissue (refer to inset, Figure 3.4). Explants were not excised from recently formed branches/ stems within the intermediate and developed sections of the donor plants. As each donor plant differed in length, the division of each section was based on the length relative to each donor plant. Explants were cultured in 50 mL growth vials.	$n_{Developing} = 150$ $n_{Intermediate} = 150$ $n_{Developed} = 150$

3.4. Results and Discussion

Overall, the percentage of *T. usneoides* explant establishment ranged from 0 % (open bench trials) to 89 % explant establishment (G6 - 8). Aspects contributing to explant death or establishment (Table 3.1) are detailed below.

3.4.1. Donor plant growing conditions and genotype

Donor plant growing conditions impacted plantlet establishment (KW, $p < 0.001$) and root length (KW, $p < 0.001$) (Figure 3.1). With regards to donor plants harvested from the contaminated site (CS: G1 - 5), G5 explants initiated rooting within the first week, achieved the highest percentage of establishment (46.67 %), and produced the longest roots (77.80 mm) followed by G2 (establishment - 26.67 %; 44.21 mm root length increment), whereas G3 explants had the lowest establishment (7.78 %) and root length (38.86 mm). Explant mortality increased in the fifth week of the culture growth period (7.78 plantlets across all clones). Explants excised from donor plants growing in non-contaminated sites (NCS, G6 - 8) had a significantly higher establishment percentage (i.e., 89.25 %) compared with G1 - 5 (i.e. 22 % establishment). Roots were initiated within the first (G6) and second week of culture (G7 and G8), where root growth was initiated at least 1 week before shoot growth increment was observed. After a six-week growth period, root length ranged from 28.36 to 98.96 mm where the number of roots produced, the degree of root branching, and root growth increased in a time-dependent fashion.

Numerous studies have demonstrated that mineral nutrition impacts rhizogenesis and growth (e.g. Schwambach *et al.*, (2005)). Exposure to excess amounts of micronutrient metals impacts plant nutrient status, compromising the assimilation and metabolism of macro- and micronutrients required for plant growth and survival (Hussain *et al.*, 2020, Nagajyoti *et al.*, 2010). Thus, as explants share similar nutrient content to the donor plant (Mitić *et al.*, 2009), the growth of trees on contaminated sites may have negatively impacted the nutrient status of donor plants, and consequently, explants that were cultured *in vitro*. This may have reduced root initiation and development by explants harvested from CS (G1 - 5) due to the root growth potential of donor plants, resulting in a lower establishment

percentage (Figure 3.1). This presents a gap in research that should be considered in future studies. The biological implication of these results suggests that the *in vitro* morphogenetic response of explants is influenced by the growing conditions as well as donor plant genotype, where elevated establishment may be achieved from donor plants sourced from non-contaminated home ranges compared with those transplanted to contaminated sites.

Genotype influenced *T. usneoides* explant establishment within each growing conditions group (KW, $p < 0.05$). Results from this study are in agreement with others (e.g. *Salix* spp) which have reported genotype to be an important factor contributing to explant establishment and morphogenetic response *in vitro* (Watson *et al.*, 2003a). Thus, to obtain the required number of established plantlets, the number of explants cultured per genotype would have to be increased. Variability in explant establishment capacity between genotypes indicates that selective breeding for improved traits is feasible.

3.4.2. Donor plant growing media

River sand and silica sand growing media did not differ relative to pH (7.21 and 6.73, respectively), E_h (-18.34 mV and 11.57 mV, respectively), and moisture content (3.8 and 4.1 %, respectively). This suggested that factors other than plant growing medium affected differences in explant establishment. However, future studies should determine the EC (i.e. proxy for salinity) of the donor plant growing media as salinity mediates the growth and survival of obligate halophytes. Furthermore, donor plant growth media may require supplementation with salts to optimize the growth of donor plants used for PTC.

3.4.3. Season and year

Plantlet establishment significantly differed between seasons (KW, $p < 0.001$) and year (KW, $p < 0.001$). A total of 1 193 explants were cultured in this study. Elevated numbers of explants established in winter (48 % of cultured explants, n = 741) compared with Spring (15 % of cultured explants, n = 222), Summer (9 % of cultured explants, n = 64), and Autumn (2 % of cultured explants,

n = 166). A total of 260 explants (89 % of cultured explants, n = 291) successfully established in 2013, compared with 98 explants (21 % of cultured explants, n = 450) in 2016. Differences in explant establishment between seasons are supported by studies conducted on *Quercus euboica* (Kartsonas and Papafotiou, 2007), *Ceratonia siliqua* (Romano *et al.*, 2002), and F*icus religiosa* (Siwach *et al.*, 2011). These findings were attributed to the season at which the plant species has access to water in their natural environment as well as the status of the donor plants relative to their nutrient and phytohormone levels.

Winter, in South Africa, occurs from June to August (Mucina and Rutherford, 2006). Of the sample genetically analysed, pure *T. usneoides* were found to naturally occur in areas within the Northern Cape province, and more specifically in Upington and the Richtersveld - areas located in near proximity to the Orange River (Mayonde *et al.*, 2016). These areas are located mainly within the Lower Gariep Alluvial and Noms Mountain Desert vegetation types, which are typically characterised as arid / semi-arid environments with unimodal, winter-rainfall climate and mean annual precipitation (MAP) ranging from 40 to 131 mm, and MAPE of 2 888 mm (Mucina and Rutherford, 2006). Mean annual temperatures throughout sampled areas range from - 3.7 °C (minimum in July) to 50 °C (maximum in January) (such as the Bushmanland Basin Shrubland vegetation type) along with high solar radiation (Mucina and Rutherford, 2006). These include areas with high salt content. As higher numbers of explants established during winter (KW, $p < 0.05$), this may suggest that *T. usneoides*' PTC period coincides with the season where natural populations of *T. usneoides* are not as stressed due to access to rainwater and cooler temperatures (i.e. June to August) whereby trees have lower rates of evapotranspiration and salts (excreted via salt glands) on the surface of leaves are washed off. This is supported by the optimal partitioning theory, where plants may allocate net primary productivity (NPP) to the organ that acquires the most limiting resource – in this case, water (Zhang *et al.*, 2019). This theory suggests that plants experiencing physiological drought due to MAPE >> MAP and saline conditions (i.e. evapotranspiration > precipitation resulting in the movement of salts to shallow soil layers), such as *T. usneoides* in their natural environment, should allocate more carbohydrates to their roots to maximise water uptake (van Wijk, 2011). Thus, *T. usneoides* may have

allocated elevated concentrations of carbohydrates, resulting in higher *in vitro* establishment during the winter months where *T. usneoides* have access to water in its natural environment.

Vernalisation refers to the enhancement of a plant's ability to flower after being exposed to cold stress over a certain period (Sung and Amasino, 2004). To increase the plant's ability to flower, phytohormones including auxins (involved in root initiation and development) increases. Thus, as the explant shares similar characteristics, including nutritional and hormone status, as the donor plant, explants may have an elevated capacity to root during the colder months due to vernalisation (i.e. associated increase in endogenous phytohormone levels).

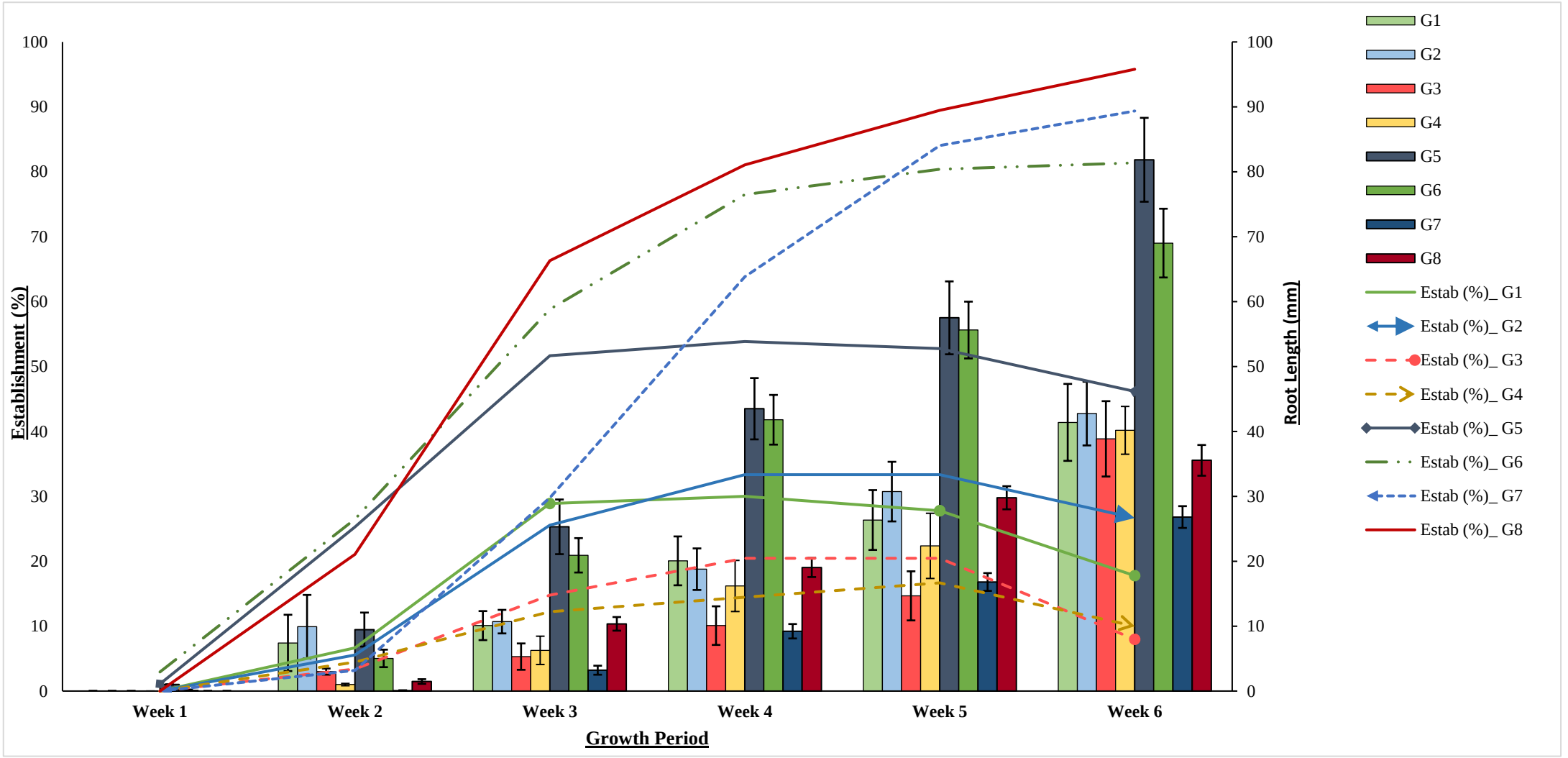

Figure 3.1. Establishment (%) and root length (mm), of established plantlets of *Tamarix usneoides* genotypes (G1-8) cultured *in vitro* over six-week period where donor plant material was harvested from different provenances [namely contaminated (G1 – 5) and non-contaminated site (naturally home ranges (G6 - 8)]. Number of explants cultured: $n_{G1} = 90$; $n_{G2} = 90$; $n_{G3} = 88$; $n_{G4} = 90$; $n_{G5} = 89$; $n_{G6} = 102$; $n_{G7} = 94$; $n_{G8} = 95$. Plantlet mortality accounts for decrease in establishment percentage.

3.4.4. Physiological age of donor plant

The establishment of explants *in vitro* decreased as the physiological age of the donor plant increased (ANOVA, p = 0.030) (Figure 3.2). Explants excised from 5-month-old donor plants had higher establishment percentages compared with older donor plants.

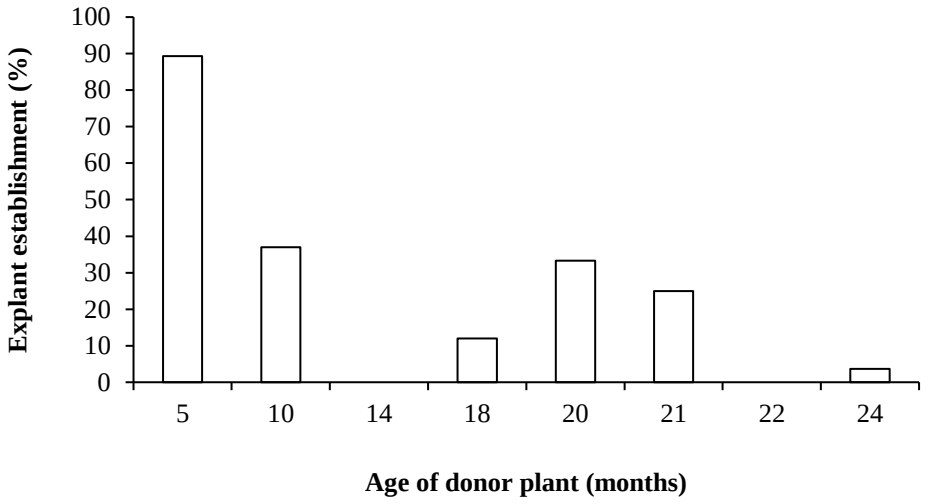

Figure 3.2. Explant establishment (%) relative to the physiological age of *Tamarix usneoides* donor plants used for explants. Note: Number of explants cultured for 5, 10, 14, 18, 20, 21, 22, and 24 months, were 291, 450, 57, 192, 30, 12, 22, and 109, respectively.

The lower explant establishment, relative to the re-culturing of G6, may be attributed to the age of the donor plant which was approximately 20 months older (when G6 was 're-cultured': 0 % establishment) compared with when G6 explants were initially cultured from 5-month-old donor plants (96 % establishment) in 2013. The reduction in establishment percentage as a function of the physiological age of the donor plant has been reported in various studies (Andreu and Marín, 2005). For example, rooting by *Passiflora edulis* (Becerra *et al.*, 2004) and *Tectona grandis* (Husen and Pal, 2006) decreased as the age of the donor plant increased. Moreover, Amri *et al.* (2010) demonstrated that 71% of explants excised from juvenile African Blackwood (*Dalbergia melanoxylon*) donor plants rooted compared with only 24% of explants from mature (24 %) donor plants. This finding may be attributed to factors including, but not limited to, decreased physiological activity (i.e. change in

phytohormone profile). Thus, physiologically older donor plants may be less suitable for successful ARF propagation, compared with younger donor plants, due to changes in phytohormone activity (increased auxin concentrations in juvenile cuttings) (Osterc and Štampar, 2011).

3.4.5. Re-culturing the most prolific rooting individual, "Genotype 6"

After four weeks of culture, all G6 explants died. Plantlet death was characterised by senescence within the second week of culture, along with the discoloration of the plant growth medium suggestive of exudation of polyphenolics. As per the following section on the *physiological age of donor* plants – the inability of G6 explants to establish was attributed to the physiological age of the donor plant which was twenty (20) months older than when explants were initially harvested and used in PTC.

The decrease in the morphogenetic response of explants cultured *in vitro* may be attributed to elevated levels of endogenous auxins and degree of auxin activity in younger donor plants, as well as decreased physiological activity (e.g. photosynthetic activity and nutrient metabolic activity) in older donor plants (Amri *et al.*, 2010, Bouma *et al.*, 2001). Therefore, younger (< 5 months old) *T. usneoides* donor plants should be used to optimise explant percentage establishment.

3.4.6. Explant length

Explant length (40 mm or 25 mm) influenced the time taken for explants to root (KW, $p = 0.022$) and root length (KW, $p < 0.005$) but did not influence the percentage of plantlet establishment (Figure 3.3).

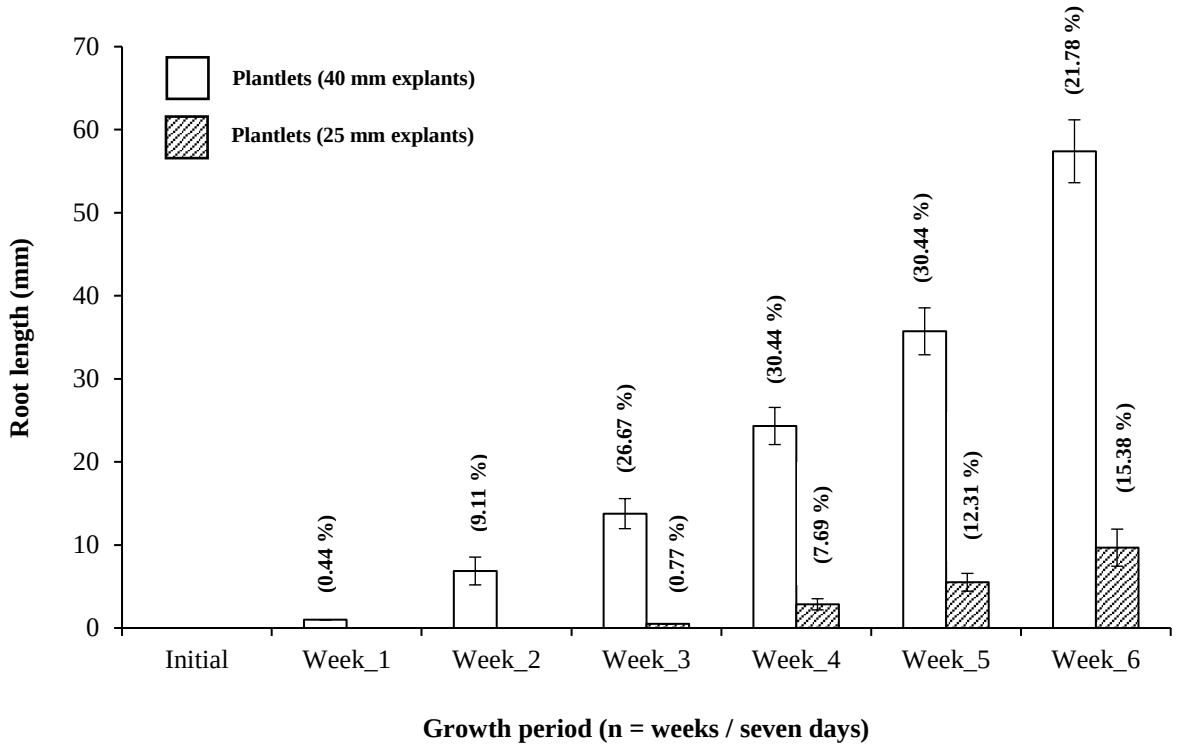

Figure 3.3. Root length of established (in parenthesis) plantlets varying in initial explant length, 40 mm (n = 450) or 25 mm (n = 130) over seven-day periods (week). Establishment is represented as a percentage in parenthesis.

This may be attributed to larger plantlets having higher concentrations of nutrients and endogenous phytohormones required for root growth than smaller plantlets (Pierik, 1997). Moreover, larger explants have a larger surface area for photosynthesis. Thus, longer *T. usneoides* explants (40 mm) should be used in PTC experiments.

3.4.7. Chronological age

Chronological age did not influence explant establishment percentage or plantlet root length. Although the establishment percentage of explants did not differ, morphogenetic response (such as root length) decreased as chronological age increased (young > intermediate > older) (Figure 3.4). Results from this study indicated that the same percentage establishment can be achieved regardless of the chronological age of the donor plant. Thus, if the availability of donor plants is limited, explants can be excised from the entire donor plant as such *T. usneoides* explants.

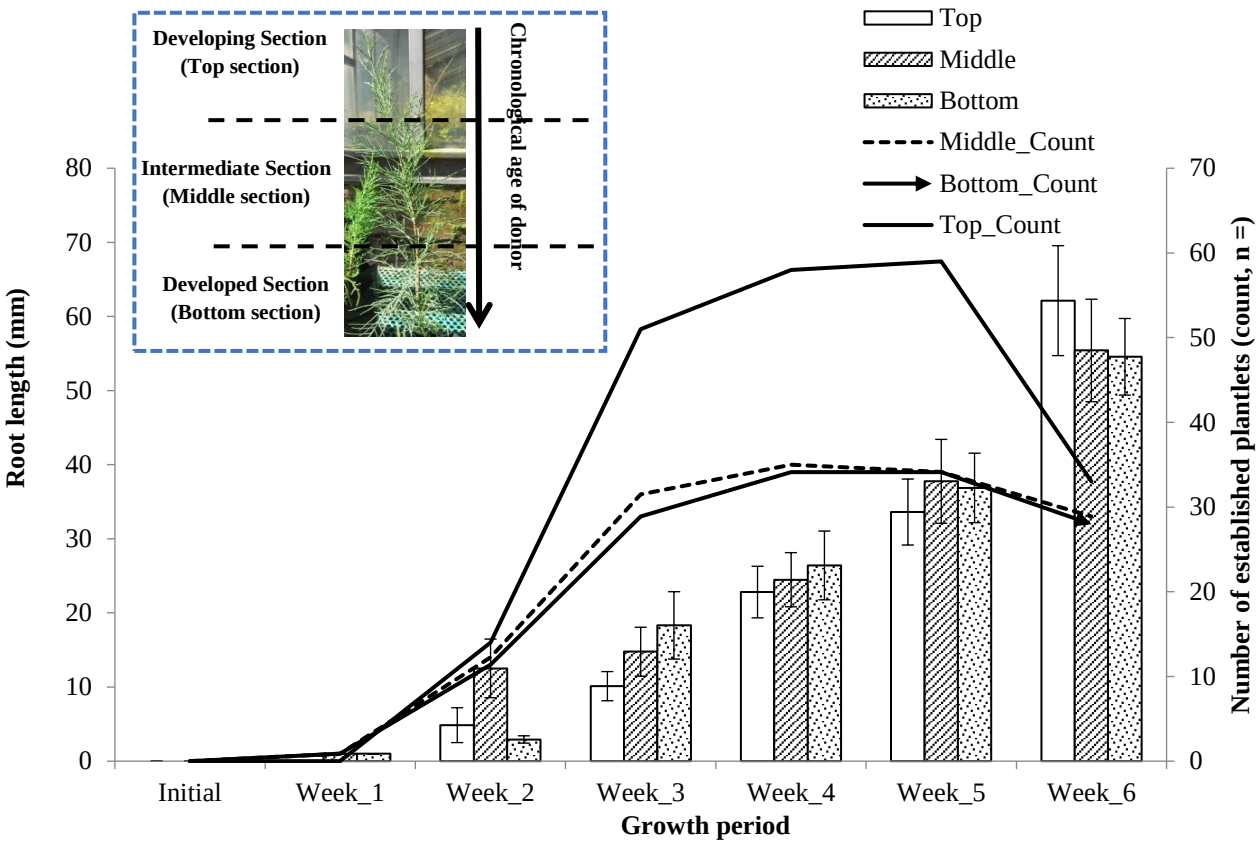

Figure 3.4. Number established and root length of plantlets from explants of different chronological ages. Chronological age categories were comprised of developing explants (excised from the *top* third of donor plants), intermediate explants (excised from the *middle* third of donor plants) and developed explants (excised from the *bottom* third of donor plants)*

3.4.8. Strength of Murashige and Skoog plant growth medium

The strength of the MS medium (25, 50, or 100 %) did not impact plantlet establishment, shoot length, number of shoot branches, root length, or number of roots. Thus, 25 % MS strength was used and therefore, future studies should use 25 % MS strength for comparative purposes. These findings suggest that *T. usneoides* explants can establish in a range of nutrient conditions. However, it must be noted that full-strength MS growth medium formed salt = precipitates at the base of the growth vial, and these may influence the outcome of metal uptake trials due to interferences between ions, including nutrients.

3.4.9. Volume of growth vial

The volume of the culture vessel impacted explant establishment (KW, $p < 0.005$). Explant establishment and morphological growth (e.g. shoot growth, shoot branching, root branching, and the number of roots) was higher in 50 mL growth vials than in 15 mL growth vials (Figure 3.5). This effect may be attributable to the amount of growing space (García-Gonzáles *et al.*, 2010). Thus, 50 mL growth vials should be used.

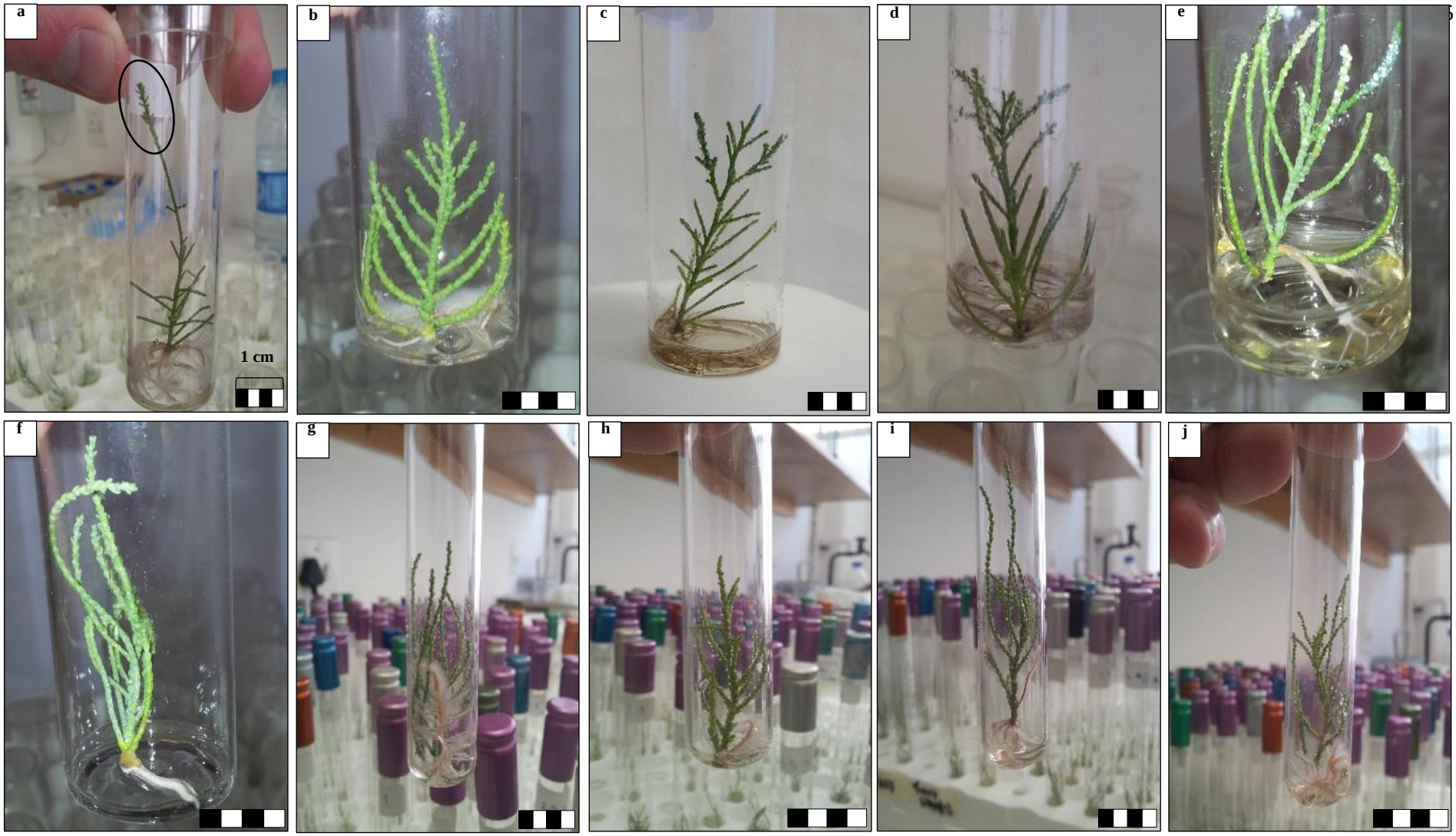

Figure 3.5. Established (i.e. rooted) *Tamarix usneoides* explants differing in donor plant growing conditions (a and f; b and h), genotype (b and c), vial volume (d and g), and explant length (a and j). The pH level impacted the position of root growth (i.e. adventitious root formation, ARF), where wound ARF (f), compared with stem ARF (e), resulted in explant mortality. The degree of shoot growth (a, encircled) was only observed in 50 mL growth vials. Upright shoot positions were maintained in 15 mL growth vials (g - j) whereas the apex of some explants in 50 mL growth vials was in contact with the wall of the growth vial which generally resulted in shoot senescence. Explant transpiration was observed as droplets on the surface of shoots.

3.4.10. Auxin treatments

Auxin (IBA) pulse treatments did not influence explant establishment, root length, or number of roots produced by plantlets. The highest percentage of plantlet establishment was induced by 0.1 mg/L IBA treatment (30 %) compared with the lowest establishment percentage, control (20 %). Low explant percentage establishment may be attributed to exogenously applied auxins having less of an effect on larger explants due to higher concentrations of endogenous auxins (Osterc and Štampar, 2011). The auxin concentration (0.1 mg/L) which contributed to the highest percentage of establishment of *T. usneoides* explants is supported by a study conducted by Nakhooda *et al.* (2012) in which *Eucalyptus grandis* explants exposed to an IBA concentration of 0.1 mg/L, had high establishment percentages (87 %) (Table S3.2).

Explant establishment, and in particular root formation, may be attributed to elevated levels of endogenous auxins being present in younger donor plants. Although developing (younger) and larger explants may have elevated levels of auxins, compared with developed (older) and smaller explants, the degree of auxin activity, present as conjugated forms *in planta*, is mediated by genetic (Zhao, 2010), molecular (Hayward *et al.*, 2009), cellular and whole plant levels (including the physiological age of donor plants - Osterc and Štampar (2011)). Low percentage establishment of explants may also be attributed to the duration of the auxin pulse. Although *T. usneoides* explant treatment with IBA may have contributed to root formation, the unquantified effect of an additional variable (e.g. supplementation of exogenous auxin) on studies investigating certain phenomena, such as metal uptake dynamics and *in planta* metal translocation dynamics, requires further investigation

3.4.11. Open bench trials

No explant establishment was observed over the six-week growing period under open bench experimental conditions. All explants developed browning at the tip of each shoot ‘branch’ which was accompanied by plantlet tissue desiccation. Once, this ‘drying out’ effect was observed, explants could not recover. Evapotranspiration rate of liquid growth media was 0.53 mL / 24 hours. A white

precipitate (salts from MS growth medium) was observed at the base of culture vials. No symptoms of infections (such as fungal hyphal growth at the base of the explant excision) were observed. Thus, the expected increase in rate of evapotranspiration by explants in open bench trials may have lowered the osmotic potential, resulting in explant mortality.

3.4.12. Impact of pH

Establishment of *T. usneoides* G1 - 5 *in vitro* was not affected by pH levels (pH 5.5 or 7.5) tested in this study. *Tamarix usneoides* G6, G7 and G8 exhibited high establishment percentages (> 86 %) in a range of pH levels including pH 4.6 (86.60 % establishment), pH 5.6 (89.47 % establishment), and pH 7.6 (89.90 % establishment). Establishment was observed in week 1 by G6 explants (in pH 4.6 and 5.6), G7 (in pH 4.6), and G8 (for all pH levels). Although pH impacted the length of root growth (KW; $p < 0.001$), pH level did not influence measured morphologic parameters, namely shoot length growth and the degree of shoot branching. The range of pH levels tested (4.6, 5.6 and 7.6) affected the position of root formation and growth for G6, G7 and G8.

The pH level influences physiological processes, such as the assimilation of nutrients, where levels between pH 4.0 and pH 5.8 are generally associated with an increase in micronutrient bioavailability (Esposito *et al.*, 2002). These results are also supported by Zhang and Furusaki, (1997) where the biomass production of strawberry cultures was not influenced by pH of the growing medium – in contrast with Naik *et al.,* (2010), where growth at pH 4.5 resulted in higher biomass production by *Bacopa monnieri in vitro*. Collectively these results indicate that *T. usneoides* has the capacity to tolerate and establish in a range of pH levels *in vitro*. , which has been supported by the introduction of *T. usneoides* to gold and uranium tailings dams and adjacent polluted areas (i.e. for treatment of AMD (Weiersbye *et al.*, 2006b).

3.5. Summary

Results obtained from these studies (based on the applicable stages of PTC) have been used to optimise and rationalise the development of the following protocol for the establishment of *Tamarix usneoides* explants *in vitro* (Table 3.4).

Table 3.4. Protocol developed for the *in vitro* establishment of *Tamarix usneoides* – an important recretohalophyte utilised in phytotechnologies in southern Africa. PTC: plant tissue culture. *Refer to Table 3.1.

Stage of PTC*	Protocol	Rationale and comments
Donor Plant Status (Stage 0)	Cuttings must be sourced from genetically verified *T. usneoides* (Mayonde *et al.*, 2016), ideally growing on non-contaminated land. During transport, cuttings were stored in sealed ziplock bags containing 50 mL distilled water. Cuttings (circumference: 44 – 65 mm) were excised to 150 mm in length using aseptic techniques. The bottom end of each cutting was excised at a 45 ° angle (to increase surface area for nutrient and phytohormone uptake), moistened in distilled water, and dipped in Seradix B No. 2 (Bayer CropScience AG, Germany) to form a thin grey layer on the area of excision (all residual rooting hormone must be removed). Cuttings were grown according to Wilson *et al.,* (2017), where each cutting was placed in a labelled 5 L black plastic planting bag containing moistened 75 : 25 sand : sterilised compost. Cuttings were placed in a greenhouse (25 °C ± 10 °C) and irrigated every day at 07h00 for 15 minutes. Cuttings were pre-treated using a systemic fungicide [Bravo 720 (2 mL/L), active ingredient: Chlorothalonil at 720 g/L)] and an insecticide [Malasol (1.75 mL/L), active ingredient: Mercaptothion at 500 g/L]. During growth, treatments involved weekly rotations of Bravo 720 and Malasol at least 45 days before culturing took place. Cuttings were also treated with Previcur (1.5 mL/L. active ingredient: Propamocarb 600 g/L) once every three months.	[1]Donor plant growing conditions and genotype impacted explant establishment, where G1 - 5 (cuttings obtained from the contaminated site) had \lower establishment (22 %) compared with G6 - 8 (cuttings obtained from *T. usneoides'* natural home range). [2]Low levels *in vitro* contamination (< 1 % of total) were observed during the experiment, suggesting the combination of pesticides was effective. The fungicides were applied to reduce any endogenous fungal loads whereas the insecticide was used to reduce any insect-related damage, as well as infections by pathogens transmitted by insects.
Stage I - In vitro introduction of explants	Explants (shoots), measuring 45 mm in length and consisting of two or more axillary buds, were excised. Explants must only be excised from donor plants under five months old[3]. The position where explants are excised from the donor plant is irrelevant[4]. Optimal culturing of *Tamarix usneoides* explants is during winter months (in South Africa, namely June-to-August)[5]. Explants were placed in 40 mL cleaned Falcon tubes containing distilled water and	[3]Explants excised from donor plants above five months old had a reduced establishment percentage (refer to Figure 3.2). [4]Chronological age of explants did not influence explant establishment (Figure 3.4).

	one drop of Tween 20 to maintain explant hydration during transport to the Laminar Air Flow, LAF. Quarter-strength (25 %) MS standard plant growth media was used (Murashige and Skoog, 1962)[6]. Medium pH was altered to pH 4.6 - 7.5[7] by the addition of hydrochloric acid (HCl) or sodium hydroxide (NaOH) or to decrease or increase the pH, respectively, and adjusted to constant molarity for sodium (Na) and chloride (Cl). Medium was autoclaved at 121 °C and 1 KPa for 20 minutes. Explants were surface decontaminated in a nitric acid (0.1 M) sterilised and rinsed 500 mL beaker containing 2 % sodium hypochlorite (NaClO), and two drops of Tween 20, for 20 minutes. Explants were agitated every 5 minutes by stirring the NaClO solution. After 20 minutes, sterilised explants were rinsed three times in ultrapure water. Five millimetres of the base of each explant was excised to avoid any transfer of explants with bleached (white) areas of excision into culture vials, as well as to maintain an explant length of 40 mm.	[5]Explants cultured during winter months had the highest establishment percentage. This may be attributed to the optimal partitioning theory where NPP was allocated to roots that typically only have access to a low amount of water during winter months in the plant species' natural home range. [6]Although the strength of MS growth media did not influence explant establishment, the use of 100 % MS resulted in salt precipitate formation at the bottom of growth vessels. [7]pH level did not influence *T. usneoides* explant percentage establishment.
Stage II - Explant propagation **and** ***Stage III - Explant establishment***	Each explant was transferred to nitric acid (0.1 M) sterilised and rinsed 50 mL growth vial[8] in which 0.2 mL 25 % MS liquid plant growth media, using ultrapure water, was dispensed into each growth vial. No exogenous auxin was added to growth media[9]. Growth lids were sealed with parafilm to reduce evapotranspiration and explant desiccation. Vials were placed in a growth room under controlled conditions [25 °C ± 0.2 °C with a photoperiod of 16L8D under two fluorescent tubes (Osram 36W, cool white)] for six weeks for explants to establish. A further 0.2 mL 25 % MS plant growth media was added to each growth vial every third (3^{rd}) day, under aseptic conditions. Growth vials were randomly re-positioned every second day to prevent plantlets from being exposed to any potential environmental gradient within the growth room.	[8]Higher explant establishment and physiological growth occurred in 50 mL growth vessels compared with 15 mL growth vessels. [9]Auxin pulse treatments did not increase *T. usneoides* explant establishment.

3.6. References

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Chapter 4

Recovery of platinum (Pt), cobalt (Co), and nickel (Ni) by *Tamarix usneoides* from solution

Aspects of this chapter will form part of a manuscript which has been prepared and submitted to the journal ***Plant Physiology and Biochemistry*** for consideration of publication.

4.1. Abstract

Global demand for platinum (Pt), as well as cobalt (Co) and nickel (Ni), has consequently increased the production of metallurgical effluent containing economically significant concentrations of these previous metals. Increased mining and subsequent application of these metals in various industries have led to elevated levels of these metals in the environment - negatively impacting the growth and survival of flora and fauna. Based on the ecotoxicity and demand for these critical elements, present at low concentrations within effluent, methods of metal recovery have gained a growing interest – namely (i.e., phytomining) and rhizofiltration. Therefore, the pH dependency of metal tolerance and extraction capacity by five *Tamarix usneoides* genotypes was investigated *in vitro*. Six-week-old plantlets were exposed to platinum (as $PtSO_4$), cobalt (as $CoSO_4$), or nickel (as $NiSO_4$) in nutrient media supplemented with 0 (control), 25, 50, or 100 mg/L metal at pH 5.5 or 7.5 for 14 days. Elevated metal concentrations (Ni > Co >> Pt) were accumulated in roots (combined measure of metals adsorbed on the root surface and assimilated *in planta*) compared with shoots whereby bioconcentration factor (BCF) > 1 (excluding Pt) and translocation factor (TF) < 1. Metal BCF (Ni > Co >> Pt; KW, $p < 0.05$) and TF (Co > Ni >> Pt; KW, $p < 0.05$) increased in a dose-dependent fashion and were not influenced by pH level. Cobalt and Ni (≤ 50 mg/L) uptake dynamics did not differ suggesting similar uptake dynamics when treated separately. Platinum (defined in this study as Pt > 1 – 4 mg/kg), Ni (> 1 000 mg/kg), and Co (> 300 mg/kg) were hyperaccumulated in roots ("rhizo-hyperaccumulation") across treatments with possible Co-hyperaccumulation in shoots by two genotypes. Plantlet genotype influenced Co uptake dynamics (accumulation, BCF, and recovery by shoots; KW, $p < 0.05$) but not Ni or Pt. Tolerance indices did not differ [Co (97 %) > Pt (82 %) > Ni (77 %)] between pH, metal, treatment concentration, or the interplay between these factors. Metal treatments did not impact measured morphological parameters excluding shoot length increment which increased in response to Ni treatments (KW, $p < 0.05$). Plantlet survival differed between pH and metals [Pt (90 %) > Ni (81 %) > Co (62 %)] (KW, $p < 0.05$). *Tamarix usneoides* plantlets exhibited tolerance to Ni, Co, and Pt at 1, 10, and 10,000 times the average soil crustal abundance, respectively, under both moderately acidic (pH 5.5) and slightly alkaline (pH 7.5) conditions. Results demonstrate that *T. usneoides* has the potential for recovery of Ni and Co (and Pt

to a lesser degree) from effluents. Variability in Co accumulation capacity between genotypes indicated that selective breeding for improved rhizofiltration and phytoextraction traits is feasible. However, whether phytoextraction of Pt, Ni, and Co can be achieved from more complex media and soils requires further study.

Keywords: halophyte, metallurgical effluent, phytomining, recretohalophyte

4.2. Introduction

4.2.1. Demand for Pt, Co, and Ni

The intensification of platinum (Pt) mining and processing augments the production of solid and liquid (e.g., metallurgical effluent) waste containing residual precious metals including cobalt (Co) and nickel (Ni) (Rachidi *et al.*, 2021, Tutu *et al.*, 2008). The current trading prices of these strategic elements have created a growing interest in the recovery of these critical elements from effluent [elucidated by their current trading prices; Pt: USD $28 16.58 g^{-1}; Co: USD $70.48 kg^{-1}; and Ni: USD $21.81 kg^{-1} (LME, 2022)]. Associated with these increased mining and processing activities, as well as subsequent application in various industries (Table S1.1), has been the mobilisation of elevated concentrations of these metals into the environment.

The presence of these metals in the environment negatively impacts the growth and survival of fauna and flora, highlighting the need to recover these metals from secondary sources (e.g., metallurgical effluent and e-waste) and the environment. Existing methods of precious metal recovery, such as bioleaching, chemical precipitation, reverse osmosis, and ion exchange processes, are costly processes that also produce toxic waste as by-products (Baniasadi *et al.*, 2019, Junior *et al.*, 2019). These disadvantages favour the development and application of biotechnologies such as biosorption (Escudero *et al.*, 2019). Living (e.g., plants) and non-living (e.g., dried plant material) biological materials have been used for the remediation and recovery of heavy metals, metalloids, and dyes from effluent and e-waste originating from various sources. The use of plants for the recovery of metal ions from effluent presents an economically feasible and effective biotechnology for the potential recovery of Pt, Co, and Ni from metallurgical effluent. This biotechnology is advantageous in comparison with non-living biosorbents due to a, (i) higher selectivity for metal ions of interest compared, (ii) lower capital and operational costs, and (iii) lower waste production methods compared with conventional methods (e.g., ion exchange, precipitation, and bioleaching) (Bashir *et al.*, 2019). Living biosorbents, such as plants, may also release solubilising agents (i.e. lixiviants) into the

rhizosphere, increasing the bioavailability and sorption of metals of interest (Ayangbenro and Babalola, 2017, de Freitas *et al.*, 2019).

4.2.2. The case for rhizofiltration

Rhizofiltration, a biosorption-based phytotechnology, involves the use of roots of aquatic or terrestrial plants for the sorption (adsorption and absorption), concentration, and/or precipitation (e.g. on root surface) of precious metals as well as contaminants of environmental concern [such as metals (e.g. cadmium (Cd)), metalloids (e.g. As and Se), and radionuclides (e.g. strontium (^{90}Sr) and uranium (^{234}U)] from waste-, surface-, and/ or extracted groundwater (Table S4.1) (Benavides *et al.*, 2018, Selvi *et al.*, 2019, Woraharn *et al.*, 2021). The sorption of metals by plant root biomass involves various physicochemical interactions (*viz.* – electrostatic, ion chelation, precipitation, physisorption, or complexation interactions), between metal ions and functional groups present on the surface of root cells (Bashir *et al.*, 2019).

Sorption methods have successfully been utilised for precious metal recovery (Mosai *et al.*, 2020). Although 'traditional' sorption methods (such as bentonite, activated carbon, and chitosan) have been widely studied and utilised, research has demonstrated the ability to increase sorption efficacy by adding / altering functional groups present on the surface of these adsorbents (Bakatula *et al.*, 2014a, Bakatula *et al.*, 2014b, Cantuaria *et al.*, 2016, Escudero *et al.*, 2019, Karnib *et al.*, 2014, Roosen *et al.*, 2016). For example, Bakatula *et al.*, (2014b) concluded that bentonite modified with L-histidine had an elevated heavy metal adsorption rate compared with natural bentonite, and was also influenced by pH. This suggested that the modification of binding sites at a root surface level mediates the rate and efficacy of biosorption processes

Plant species selected for rhizofiltration must have (i) a fast growth rate and large root system (i.e. elevated biomass production and the number of metal-binding / storage sites), (ii) an elevated tolerance to a range of metals and salts present in wastewater, (iii) elevated bioconcentration (BCF > 1) in roots and low translocation factor (TF < 1) (Capuana, 2011, Di Lonardo *et al.*, 2011, Wang *et al.*, 2017). The biological implication associated with selecting plant species with the ability to

(hyper)accumulate metals within the roots includes limiting the release of recovered precious metals or contaminants into the environment.

The use of terrestrial plant species for rhizofiltration has gained growing interest due to terrestrial plant species generally possessing greater shoot and root growth characteristics compared with aquatic plant species currently utilised for rhizofiltration. A growing body of research has demonstrated the ability to use terrestrial plant species for hydroponic metal rhizofiltration studies. For example, pea plant (*Pisum sativum*), lupine (*Lupinus angustifolius*), and cucumber (*Cucumis sativus*) were studied under hydroponic conditions to determine the impact of PGE (namely Pd and Rh), Fe, Ni, Pb, and Cu on metal uptake and phytotoxicity (Egorova *et al.*, 2019). Other studies such as Niu *et al*., (2007) investigated the ability of the terrestrial plant species, *viz* - *Brassica juncea* (Indian Mustard), *Helianthus annuus* (sunflower), *Medicago sativa* (alfalfa), as well as *Ricinus* communis (Castor Oil Bush), to recover Cd and Pb via rhizofiltration from an aqueous solution, whereas Boominathan and Doran, (2002), investigated and demonstrated the recovery of Ni from aqueous solution by the roots of *Alyssum bertolonii*, *Nicotiana tabacum*, and *Mesembryanthemum crystallinum*. Numerous examples have been tabulated in Table S.4.1. Although many plant species exhibit accumulation of Pt, Ni, or Co in roots or shoots to some extent, hyperaccumulation is a rare and species-specific trait with some genotypic variation within or between populations (Manara *et al.*, 2020).

4.2.3. Metal uptake, translocation, and tolerance

Hyperaccumulation differs between metals, where Ni and Co are proposed to be 1000 mg/kg and 300 mg/kg, respectively (Baker, 1981), whereas the threshold for Pt has not been defined [see Van der Ent *et al.*, (2013) for review]. These thresholds are based on various factors associated with the active uptake of metals (Baker, 1981, Van der Ent *et al.*, 2013). Rhizofiltration studies utilise plant roots to extract and recover elevated levels of metals of interest, whereby metals may accumulate in roots to the degree of hyperaccumulation (“rhizosphere hyperaccumulators”). In the context of this study, “rhizo-hyperaccumulation” refers to metal assimilation within the root system whereby the

concentration exceeds the defined hyperaccumulation threshold. Rhizo-hyperaccumulation is an excluder strategy whereby metals are assimilated and retained within the root system to restrict translocation to shoots (metal-sensitive plant organs). Parameters used to indicate rhizo-hyperaccumulation includes metal bioconcentration factor (BCF) > 1 in roots, TF < 1 (Macnair, 2003), and tolerance indices (TI) > 60 % (Lux *et al.*, 2004, Wilkins, 1978). Hyperaccumulators have been used to recover a range of elements from different matrices, such as land or water containing residual concentrations of metals and metalloids (Baker *et al.*, 2020, Talebi *et al.*, 2019). Although hyperaccumulators are generally used to remediate contaminated sites, from an anthropocentric standpoint, hyperaccumulators may allow for phytomining (Chaney and Baklanov, 2017, Rosenkranz *et al.*, 2019).

Phytomining, and more specifically rhizomining (defined as the use of plant roots for the hyperaccumulation and effective recovery of metals), is the process whereby plants are used to extract and recover metals of economic value, such as Pt, from contaminated land (e.g. shallow surface ore-bodies) or water where conventional methods of secondary mining are not feasible (Chaney *et al.*, 1998, Chaney and Baklanov, 2017). Plants preferred for phytomining should produce high biomass or be fast-growing, tolerate a range of contaminants present in the medium, and accumulate metal(s) to economically viable levels (Chaney *et al.*, 2021, Sheoran *et al.*, 2013). The extracted desired metal, in some cases, present within the plant as nanoparticles, may be recovered from plant tissues and used in various industries. For example, *Odontarrhena chalcidic* was demonstrated to hyperaccumulate Ni from galvanic sludge-derived technosols, containing 85-150 g Ni/kg, where the highest uptake was 26.8 g/kg. It was estimated that approximately 1.46 kg Ni/ha can be recovered from this growth medium (sludge) at a planting density of 1 plant / 0.25m^2 (Tognacchini *et al.*, 2020).

4.2.4. Plant tissue culture in metal uptake studies

Plant tissue culture refers to the aseptic culture of explants (cells, tissues, or organs excised from a donor plant) under defined/ known physical (temperature, humidity, and photoperiod) and chemical (plant growth media constituents) conditions *in vitro* (Phillips and Garda, 2019). Plant tissue

culture has gained considerable interest due to the technique's numerous applications, such as screening plants for phytoremediation potential, abiotic stress tolerance, mass propagation, conservation of endangered species, biopharmaceuticals (production of secondary metabolites), hybridisation, and screening transgenic genotypes (Espinosa-Leal *et al.*, 2018, Shahzad *et al.*, 2017). The basis of tissue culture relies on plant cell totipotency (retention of a cell's genetic capacity to differentiate into any other cell type making up that organism) and cell plasticity (change in the structure associated with environmental cues modulated by gene expression) (Campbell *et al.*, 2019, Fehér, 2019, García-Gonzáles *et al.*, 2010). The physiological functioning of explants is influenced by chronological age (Rout and Sahoo, 2007) whereby younger explants have elevated morphological and physiological responses *in vitro* compared with older explants. It would therefore be expected that elevated metal ions would be taken up and accumulated by chronologically younger explants.

Numerous hydroponic studies have been conducted to determine the impact of a range of heavy metals at varying concentrations on the growth and metal uptake dynamics of various plant species genotypes (Table S4.1). Hydroponic studies have also been successfully used for the rapid screening of plant species for rhizofiltration and phytoextraction potential relative to a wide range of elements including aluminium (Al), Cd (Woraharn *et al.*, 2021), caesium (Cs), chromium (Cr), Co, Cu, gold (Au) (Wilson *et al.*, 2022), Ni, Pb, and Zn, as well as the metalloid, As (Benavides *et al.*, 2018, Yongpisanphop *et al.*, 2017). Stress indices such as morphological growth parameters, *viz.* shoot and root length, number, and branching, and plant dry matter production is widely used as proxies to evaluate tolerance to metal exposure (Schwambach *et al.*, 2005). As nutrition plays a vital role in mediating root growth explant exposure to essential (Ni) and non-essential (Pt and Co) can be used to distinguish the relationship between such metals and the (physiological and morphological) growth of explants under controlled conditions (Beck-Pay and Laing, 2019). The inhibition of these growth parameters at various metal concentrations is attributable to the limits of plant metal tolerance (Borghi *et al.*, 2007). Morphological growth parameters were used as a proxy for metal tolerance due to the (i) susceptibility of these parameters to metal phytotoxicity as the increase in growth, increases

the surface area for nutrient sorption (adsorption and absorption processes) and, (ii) wide use of these parameters in metal tolerance studies (Li *et al.*, 2009, Nagajyoti *et al.*, 2010, Pijut *et al.*, 2011).

These types of studies may also be used to determine the distribution of heavy metals between plant parts, namely shoots and roots, enabling the feasible recovery of heavy metals from the most economically significant plant parts (Chaney and Baklanov, 2017). The use of clonal material and tissue culture is advantageous as it reduces the time taken to conduct experiments and allows (i) the response of the plant to be distinguished from other biological variables (e.g. symbiotic and/or pathogenic microorganisms), (ii) different genotypes to be assessed for their ability to survive metal stress, (iii) the sample size of genetically identical explants from the same donor plant to be increased, and (iv) genotypes with appropriate responses to be mass propagated (Doran, 2009, Phillips and Garda, 2019).

The recretohalophyte *T. usneoides* was selected for this study based on the species' ability to tolerate and (hyper)accumulate a range of elements found in Pt metallurgical effluent and contaminated sites, namely heavy metals and various salt constituents including SO_4^{2-}, Cl, calcium (Ca), magnesium (Mg), and sodium (Na) (Almécija *et al.*, 2017, Erasmus *et al.*, 2020, Wei *et al.*, 2020, Wilson *et al.*, 2021, Yun *et al.*, 2019).

The aim of this study was to determine the range in metal tolerance indices and metal recovery by five genotypes of *T. usneoides in vitro*. Plantlets were exposed to doses of Pt, Co, or Ni in liquid growth medium, at pH 5.5 or 7.5.

4.3. Materials and Methods

4.3.1. Provenances and genetic fingerprinting

Stem cuttings from five genotypes of *Tamarix usneoides* (LH31, LH38, LH40, LH47, and LH48) were collected from a site (phytoremediation trial) on a mine property in the North West Province, South Africa (SA) [AngloGold Ashanti Ltd and the University of the Witwatersrand, Johannesburg: The Mine Woodlands Project, (Dye and Weiersbye, 2010)]. That trial comprises clones of trees sourced from the Orange River near Upington and Augrabies region of the Northern Cape province of South Africa. Due to the presence of hybrids between *T. usneoides* and the invasive *T. ramosissima* in southern Africa (Mayonde *et al.*, 2019), a parallel study was conducted to verify that all genotypes used in this study were pure-breeding *T. usneoides*. Foliar samples were collected which underwent cleaning, DNA extraction, clean-up, and purification using a modified standard technique. This was followed by restriction - ligation, pre-selective PCR, and selective PCR, fingerprinting with scoring of 102 alleles and AFLP structure analysis alongside *T. chinensis* and *T. ramosissima* using a Bayesian model-based clustering analysis (Structure 2.3.4) (Mayonde *et al.*, 2019), scores the likelihood of hybridisation. All the trees used in this study were grouped as non-hybrid, pure-breeding *T. usneoides*.

4.3.2. Explant culture

Donor plants were grown under greenhouse conditions at the University of the Witwatersrand, Johannesburg, SA. Plants were pre-treated with a systemic fungicide, Bravo 720 (2 mL/L) (active ingredient: Chlorothalonil at 720 g/L, Syngenta, Basel, Switzerland), and an insecticide, Malasol (1.75 mL/L) (active ingredient: Mercaptothion at 500 g/L, Efekto, Isando, SA) in weekly rotations for at least 45 days before explants were harvested. Nodal cuttings of 40 mm in length with at least two axillary buds were excised from the donor plants, surfaces of explants were decontaminated for 20 minutes in 2 % sodium hypochlorite and rinsed three times in ultrapure water (Millipore Direct-Q UV water purification system, Merck Millipore, Darmstadt, Germany). After removal of bleached basal areas, explants were cultured in sterile 25 % (quarter strength) Murashige

and Skoog (MS) liquid basal medium with vitamins (Sigma Aldrich, Saint Louis, MO, USA) (Table 4.1) (Murashige and Skoog, 1962), adjusted to pH 5.5 or 7.5 by the addition of 0.1 M hydrochloric acid (HCl) or 0.1 M sodium hydroxide (NaOH), respectively. Each explant was grown under axenic conditions in 0.2 mL of MS medium, topped up every third day, within 50 mL borosilicate glass tubes that had been cleaned in 0.1 M nitric acid and rinsed in ultrapure water. The plant material was incubated at 25 °C ± 0.2 °C with a photoperiod of 16L8D (Osram 36W, cool white fluorescent bulbs) for six weeks, and re-randomised every second day.

4.3.3. Chronological age of explants

Although results obtained in Chapter 3 indicated that explant age did not impact explant growth, the developmental status of explants may impact metal uptake, translocation, and tolerance and hence was investigated further. In order to determine the effect of chronological age on explant establishment, morphological growth, as well as metal tolerance and recovery, explants were excised from one of three sections of the donor plant. The chronological age of explants was assumed to increase from the top to bottom of the donor plant (Figure 4.1) and was categorised into the top (young / developing plantlets), middle (intermediate plantlets), and bottom (developed/ older plantlets) sections (thirds). Explants were not excised from recently formed and/or developing branches from the middle and bottom sections of the donor plant. As each donor plant differed in length, the division of each third/ section was based on the length of each donor plant.

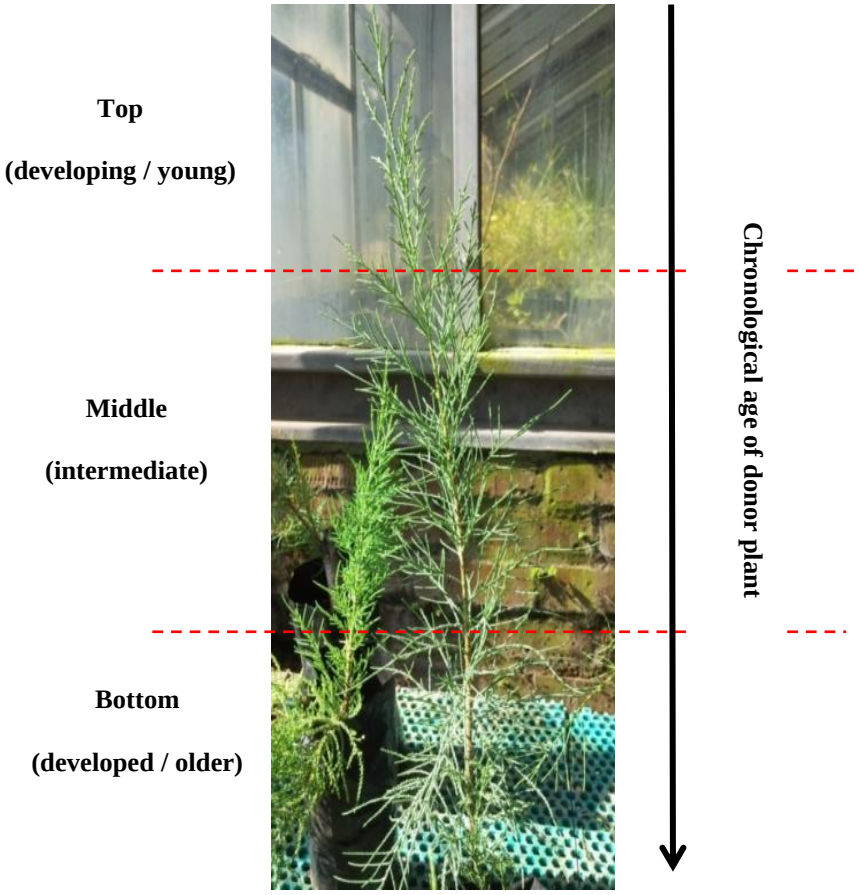

Figure 4.1. Standardised procedure in which the length of the stem, relative to each donor plant, was divided into three equal sections (top, middle, and bottom) based on the chronological age of the donor plant. Thirty explants were harvested from each section (n = 90) per genotype (N = 451).

Table 4.1. Constituents of Murashige and Skoog (MS) standard plant growth medium (Murashige and Skoog, 1962). Note: Although presented in chapter 3 the addition of the metal compounds altered sulphate (SO_4^{2-}) levels. Bold and underlined compounds represent compounds with sulphur constituents.

Compound	Compound formula	Concentration (mg/L)	Molar mass (g/mol)
Major salts			
Ammonium nitrate	NH_4NO_3	1650.00	80.05
Calcium chloride	$CaCl_2.2H_2O$	440.00	36.03
Magnesium sulphate	$MgSO_4.7H_2O$	370.00	126.11
Potassium phosphate	KH_2PO_4	170.00	136.09
Potassium nitrate	KNO_3	1900.00	101.10
Minor salts			
Boric acid	H_3BO_3	6.20	61.83
Cobalt chloride	$CoCl_2.5H_2O$	0.025	90.08
Cupric sulphate	$CuSO_4.4H_2O$	0.025	90.08
Ferrous sulphate	$FeSO_4.7H_2O$	27.80	126.11
Manganese sulphate	$MnSO_4.4H_2O$	22.30	72.06
Potassium iodide	KI	0.83	166.00
Sodium molybdate	$NaMoO_4.2H_2O$	0.25	36.03
Zinc sulphate	$ZnSO_4.7H_2O$	8.60	126.11
Na2EDTA	$C_{10}H_{14}N_2Na_2O_8$	37.20	45.98
Vitamins and organics			
i-inositol	$C_6H_{12}O_6$	100.00	180.16
Niacin	$C_6H_5NO_2$	0.50	123.11
Pyridoxine.HCl	$C_8H_{11}NO_3$	0.50	205.64
Thiamine.HCl	$C_{12}H_{17}ClN_4OS.HCl$	0.10	337.27
IAA	$C_{10}H_9NO_2$	1.00 – 30.00	175.18
Kinetin	$C_{10}H_9N_5O$	0.04-10.00	215.21
Glycine	$C_2H_5NO_2$	2.00	75.07
Ethylenediamine	$C_2H_4(NH_2)_2$	1000.00	60.10

After six weeks of growth, residual pure MS medium was aspirated, and replaced with 2.0 mL of a particular metal concentration treatment in MS medium. The control solution for this study was the addition of 2 mL 25 % MS medium [without the addition of Ni and Pt, but with a basal concentration of Co (as cobalt chloride hexahydrate – $CoCl_2.6H_2O$) – 15 µg/L] (Figure 4.2). It must be noted that SO_4^{2-} concentration increased as metal treatment concentration increased. Plantlets were harvested after 14 days of growth in solution and rinsed three times in separate acid-etched glass beakers containing ultrapure water to remove unbound residual metals and MS medium from the root

and shoot surface. Plantlets were blotted dry, and shoots were immediately separated from roots using a plastic knife, roots and shoots were subsequently weighed, freeze-dried for 72 hours (LABCONCO, Labconco Corp., USA), and re-weighed to obtain dry mass. Samples were sealed and stored at – 20 °C until dissolution for metal analyses.

4.3.4. Metal treatments

The compounds ($PtSO_4$, $CoSO_4$. $7H_2O$, and $NiSO_4.6H_2O$) selected for this study were chosen as they are present in smelter and refinery effluents (Crundwell *et al.*, 2011). Selected concentrations were based on average crustal abundances (Wedepohl, 1995), multiplied 1x (Ni), 10x (Co), and 1000x (Pt); and published tolerance limits for crop plants (Alloway, 2012). Stock solutions of $PtSO_4$ (City Chemical LLC), $CoSO_4.7H_2O$ and $NiSO_4.6H_2O$ (both Saarchem, uniVAR®) were added to 25 % MS medium to obtain three different metal concentrations, *viz.* 25, 50, and 100 mg/L [Pt (128, 256, and 513 µM), Co (424, 848, and 1 697 µM), and Ni (426, 852, and 1704 µM)] in addition to the basal medium (control) concentration of 15 µg Co/L (Table 4.2). The sulphur (S) contribution of supplemented metal treatments (as metal-SO_4^{2-}) across metal concentrations was not normalised across treatments as it did not exceed the concentrations of S in standard 100 % MS plant growth medium (see chapter 3).

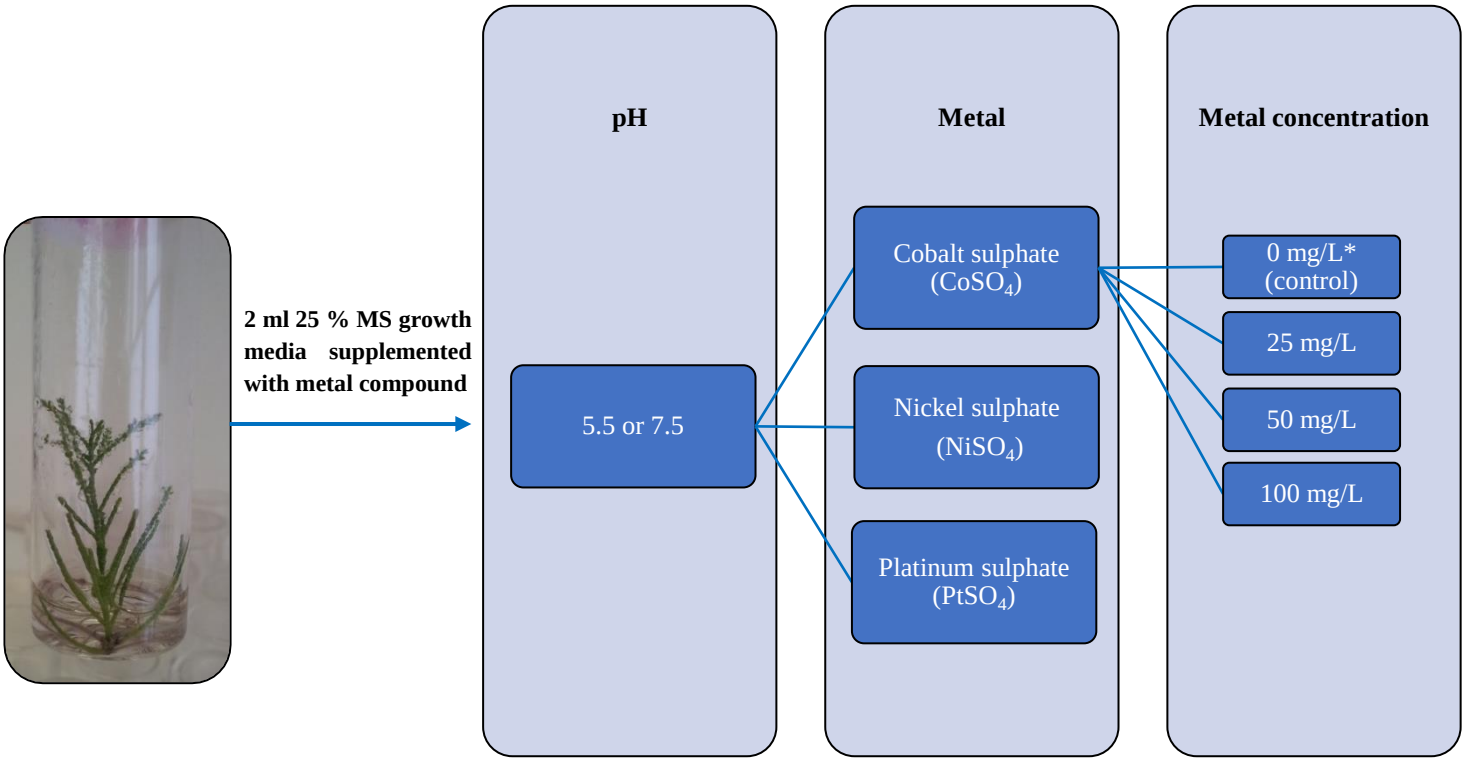

Figure 4.2. Experimental design and protocol to investigate metal ($PtSO_4$, $NiSO_4$, or $CoSO_4$) tolerance and extraction capacity by five *Tamarix usneoides* genotypes *in vitro* (n = 87). Explants were treated with 25 % MS medium supplemented with a metal (Pt, Ni, or Co) at a metal concentration (0*, 25, 50, or 100 mg/L) at pH 5.5 or 7.5 for 14 days. *Refer to Table 4.2. for metal molarity*. *Basal concentration of Co (as $CoCl_2.6H_2O$) present in control (i.e., 25 % MS medium) was 15 µg/L (Table 4.1).

4.3.5. Measurements conducted on plantlets

The following morphological growth parameters were recorded at weekly intervals, without handling the plantlet: shoot length increment (SL), shoot branching (SB), degree of shoot branching (DSB), root length growth increment (RL), number of roots (NR), degree of root branching (RB, i.e. secondary root growth), position of root initiation/growth, as well as the presence of root hairs, calli, phenolics, and infections. In the context of this study, these measured morphological growth parameters (namely SL, SB, RL, RB, NR, dry mass, moisture content, plantlet mortality, etc) were used as a proxy for physiological growth by plantlets *in vitro*.

Table 4.2. Molarity (µM) of metals in 2 mL 25 % MS plant growth medium.

	Media volume used in metal uptake experiments: 2 mL			**1L stock solution**		
Metal / metal concentrations	**Pt (µM)**	**Co (µM)**	**Ni (µM)**	**Pt (µM)**	**Co (µM)**	**Ni (µM)**
25 mg/L	2.6	8.5	8.5	128	424	426
50 mg/L	5.1	17	17	256	848	852
100 mg/L	10	34	34	513	1 697	1 704

4.3.6. Important considerations for MS medium preparation

To distinguish the impact of each metal treatment on plantlet growth, metal tolerance, and extraction efficiency, the SO_4^{2-} contribution of each treatment was considered (Table 4.3). *Tamarix usneoides* plantlets have been successfully established and grown *in vitro* using full strength (100 %) MS media (see chapter 3, data not shown here). Thus, the contribution of SO_4^{2-} was calculated to determine whether the S constituent of each metal compound (at the highest concentration – 100 mg/L) added to 25 % and 50 % MS medium would exceed the S content at 100 % MS medium. This was carried out to ameliorate any potential impact caused by the SO_4^{2-} constituent and thus attribute the growth, metal tolerance, and extraction efficiency of metals and not the SO_4^{2-} constituent of each metal treatment (Table 4.1 and Table 4.3).

The S contribution of added metal compounds ($PtSO_4$, $NiSO_4$, or $CoSO_4$) at 25 % or 50 % MS strength did not exceed the sulphur contribution present in 100 % MS medium (Table 4.3). Full-strength (100 %) MS medium did not impede *in vitro* plantlet physiological growth (*refer to Chapter 3*). Quarter-strength (25 %) MS medium was selected for this study as previous studies determined optimal plantlet growth at 25 % MS, enabling (i) a standardised tissue culture protocol, and (ii) comparisons to be drawn with previous studies investigating different metals.

Table 4.3. Sulphur contribution (percent composition) of Murashige and Skoog (MS) standard plant growth medium supplemented with metal compounds at different MS strengths (25, 50, or 100 %).

Strength of MS	Sulphur contribution (%)	Sulphur contribution of metal complexes			Sulphur contribution with any added metal complex
		$PtSO_4$ (100 mg/L)	$NiSO_4$ (100 mg/L)	$CoSO_4$ (100 mg/L)	
Full strength (100 %)	5.10	0.012	0.012	0.012	5.10
Half strength (50 %)	2.54	0.006	0.006	0.006	2.55
Quarter strength (25 %)	1.27	0.003	0.003	0.003	1.27

4.3.7. Harvesting and sample preparation

Plantlets (n = 87) were harvested after 14 days of growth in each solution and rinsed three times in separate acid-etched glass beakers containing ultrapure water to remove unbound residual metals and MS medium from the root and shoot surface. Plantlets were blotted dry and shoots were immediately separated from roots using a plastic knife. The roots and shoots were weighed, freeze-dried for 72 hours (LABCONCO, Labconco Corp., USA), and re-weighed to obtain dry mass. Samples were sealed and stored at - 20 °C until dissolution for metal analyses.

4.3.8. Metal determination

Analyses were conducted by the Central Analytical Facility, University of Stellenbosch, SA. Plant samples were prepared according to Hansen *et al*., (2009). Freeze-dried, homogenised plant samples were digested in a mixture of 1.5 mL 37 % HCl and 4 mL 65 % nitric acid (HNO_3) in polytetrafluoroethylene (PTFE) vessels. Hydrofluoric acid, HF, (0.5 mL at 48 %) was added to samples that did not fully digest. The solution was left at ambient room temperature for 10 minutes. Samples were subsequently microwave digested (Microwave MLS 1200 mega, MLS GmbH, Germany) followed by sample dilution to 50 mL using ultrapure water in a Nalgene flask. After dilution, samples were immediately analysed using Inductively Coupled Plasma Mass Spectrometry (Agilent 8800 QQQ ICP-MS) calibrated with multi-elemental standards within the expected range. Control samples spiked with metal standard solutions (Pt, Co, and Ni) at 25, 50, or 100 mg/L and three replicates of each standard reference material (SRM) spiked with either 0.079 mg Pt/kg, 0.101

mg Co/kg, or 0.104 mg Ni/kg were analysed in duplicate to verify metal recoveries. Percentage recoveries were within the US EPA guideline stipulation of 80 % to 120 % (Table 4.4). Interferences in plant samples were corrected using a mathematical correction based on the single ratio measurement. Certified reference materials (CRMs) were analysed alongside the samples. The CRMs were selected to cover similar biological matrices, namely leaves, shoots, roots, and elements of interest (Table 4.4).

Metal concentration, on a dry mass basis (mg/kg), was calculated as follows (USEPA, 2007):

Metal concentration (mg/kg) = $\frac{\mathrm{A \times V}}{F \times W} \; x \; DF$ (*Equation 1*)

where:

A: mg/L of metal in processed sample (from read-out)

V: final volume of processed sample (mL)

F: concentration unit factor

W: Sample weight (g)

DF: Dilution factor for diluted samples

It must be noted that the total metal removal from liquid culture is a combined measure of metals adsorbed on the root surface, assimilated *in planta*, and excreted on the plant surface from the foliar salt glands.

4.3.8.1. ICP-MS accuracy

Certified Reference Materials (CRMs) were spiked in triplicate (Table 4.4), i.e., three replicates of each CRM (orchard leaves, spinach leaves, maize leaves, or dandelion roots) were spiked with either 0.079 mg Pt/kg, 0.104 mg Ni/kg, or 0.101 mg Co/kg. Accuracy was within US EPA Guidelines (15 – 20 %) above or below 100 % (Table 4.4).

Table 4.4. Accuracy of the ICP-MS data for Pt, Ni, and Co inferred from percentage recoveries from Certified Reference Materials (CRM) and spiked materials. Spiking consisted of the addition of a single metal and not the combination of metals. Method accuracies are presented in parenthesis.

Metal	**Platinum (Pt)**	**Nickel (Ni)**	**Cobalt (Co)**
Spike concentration (µg/kg dry mass)	78.7	104	101
Certified Reference Material (CRM)	**Concentration (µg/kg) detected, (% recovery)**		
Orchard Leaves:			
National Bureau of Standards (NBS) Standard Reference Material (SRM) 1571	75 (96 %)	99 (96 %)	106 (105 %)
(n = 3)			
Spinach Leaves:			
National Institute of Standards and Technology (NIST) SRM 1570a (n = 3)	85 (108 %)	97 (93.0 %)	96 (94 %)
Maize Shoots:			
Wageningen University, Environmental Sciences Reference Material IPE sample 200 (n = 3)	77 (98 %)	102 (98 %)	90 (88 %)
Dandelion Root:			
Wageningen University, Environmental Sciences Reference Material IPE sample 140 (n = 3)	84 (107 %)	96 (92 %)	93 (92 %)
Method accuracy (%)	**102 %**	**95 %**	**95 %**

4.3.9. Derivation of plantlet tolerance, bioconcentration, and accumulation factors

Determination of metal tolerance index (TI) was calculated [*Equation 2* - calculated relative to root length, according to Wilkins, (1978)] whereas the mass tolerance index (MTI, *Equation 3*), relative to dry mass, was used to (i) support the TI and (ii) determine the impact of Pt, Co, and Ni on explant dry matter production (Laureysens *et al.*, 2004). Metal uptake was determined using the BCF,

where a BCF > 1 indicates accumulation *in planta* [*Equation 4* – Raskin *et al.*, (1994)] and the root-to-shoot translocation factor (TF), where a TF > 1 indicated accumulation in shoots [*Equation 5* – Macnair, (2003)]. Metal recovery (%) [*Equation 6*] was calculated according to Jiang *et al.*, (2015).

$$\textbf{TI (\%)} = \frac{\text{Root growth in metal solution}}{\text{Root growth in control solution (0 mg/L)}} \times 100 \qquad (\textit{Equation 4.2})$$

$$\textbf{MTI} = \frac{\text{Dry mass of explant grown in metal solution}}{\text{Dry mass of explant grown in control solution (0 mg/L)}} \qquad (\textit{Equation 4.3})$$

$$\textbf{BCF}_{\textbf{root or shoot}} = \frac{\text{metal concentration adsorbed/absorbed by plant part (root or shoot) (mg/kg)}}{\text{metal concentration in media (mg/L)}} \qquad (\textit{Equation 4.4})$$

$$\textbf{TF} = \frac{\text{metal concentration in shoots (mg/kg)}}{\text{metal concentration in roots (mg/kg)}} \qquad (\textit{Equation 4.5})$$

$$\textbf{Metal Recovery \%} = \frac{\text{amount of metal present in plant part (shoot or root)(g)}}{\text{amount of metal present in media (g)}} \times 100 \qquad (\textit{Equation 4.6})$$

4.3.10. Data analysis

Descriptive statistics were calculated. Data were tested for normality using the Shapiro-Wilkinson test, followed by a test for homogeneity of variance using the Fligner-Killeen test. Non-normally distributed data were transformed using log, box-cox, square-root, or power transformations. Normally distributed data were analysed using a t-test or two-way analysis of variance (ANOVA) to determine significant differences between two or more groups whereas a multivariate ANOVA (MANOVA) was used to determine if the means of two or more vectors, as well as the interaction between these vectors, significantly differed. Kruskal-Wallis (KW) tests were performed where data transformations were unsuccessful. A post hoc Tukey Honest Significant Difference (HSD) or a Dunn's test (Dunn, 1964) was performed on normally and non-normally distributed data, respectively. All descriptive statistics and statistical analyses were performed using R Statistical program, R version 4.1.3 (“One Push-Up”).

4.4. Results

4.4.1. ICP-MS Method Accuracy

ICP-MS accuracy > 94 % was achieved for all of the investigated metals *viz*. Pt (102 %), Ni (95 %), and Co (95 %). Accuracy was within stipulated US EPA guidelines where percentage ranged from 8.2 % - 11.2 %, indicating that method accuracy (> 94 %) of ICP-MS was consistent for the different metals (Pt, Co, and Ni), the sample matrices [plant species (orchard, spinach, maize, and dandelion) and the tissue types (leaves vs. roots)]. Metal concentrations in the control samples were below the limits of detection for ICP-MS (Pt: 5.7 µg/kg; Co: 46 µg/kg; Ni: 527 µg/kg).

4.4.2. Baseline growth parameters

Plantlet survival was significantly influenced by pH level (Chi-Squared test; $p < 0.05$) and metal compound (Chi-Squared test; $p < 0.05$) where survival was recorded in the order; Pt (90 %) > Ni (81 %) > Co (62 %). Plantlet SL increased (KW, $p < 0.05$) at a rate of 0.84 mm/week ± 0.09 mm as growth period increased and differed between genotype (KW, $p < 0.05$) but not chronological age (KW, $p = 0.103$) or pH levels (KW, $p = 0.561$). Plantlet SB was influenced by genotype (ANOVA, $p < 0.001$) and pH level (ANOVA, $p = 0.009$) but not chronological age (ANOVA; $p = 0.142$). Root length increased between growth weeks (KW, $p < 0.05$) (Figure 4.3) and plantlet genotype (KW, $p < 0.001$) (Table S4.2). However, RL was not impacted by chronological age or pH level. The NoR did not differ between plant genotype, chronological age, or pH level. Plantlet RB differed between growth periods (χ^2 test; $p < 0.001$) but not plant genotype or pH level. Second (Figure 4.4.E) and 3rd-degree (Figure 4.4.F) root branching was initiated within the third and fourth week of culture, respectively. After the experimental period, 61 % and 37 % of total plantlets tested initiated 3rd-degree and 2nd-degree RB, respectively. Calli, produced by plantlets, differed in colour (white, yellow, brown, or red).

During the six-week growth period, senescence of explants that did not establish was observed. Plantlets started yellowing from the area of the excision towards the apex, followed by browning (Figure 4.5). Approximately 38 % of non-established explants browned from the base up

whereas 7 % of plantlets yellowed from the base up after the growing period. Senescence was accompanied by the discolouration of the growth media (potential phenolic exudation), including yellow (1.1 % - Figure 4.5.B), brown (18.5 % - Figure 4.5.C), dark brown (10.5 % - Figure 4.5.D), and 'pinkish' precipitates (2.6 %) of all these explants did not establish. No infections were observed during the experimental period. The initial observable (superficial) stages of plantlet death included plantlet senescence (yellowing of shoot tip and/or base of explant), followed by shoot browning, inhibition of growth, no observable guttation, as well as the discolouration of media (release of phenolics) observed as the yellowing and browning of medium (Figure 4.4).

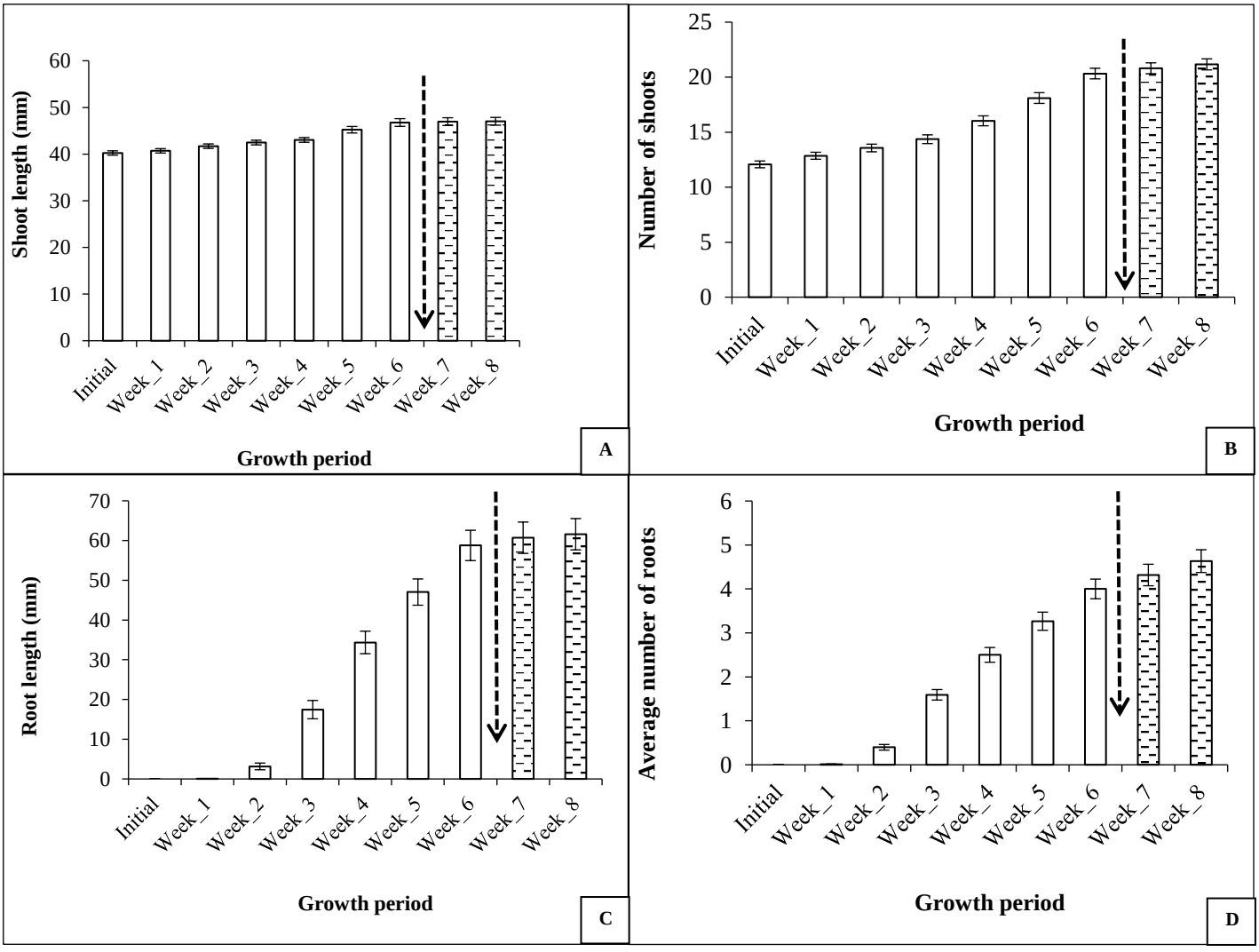

Figure 4.3. Average baseline growth parameters of five *Tamarix usneoides* genotypes of plantlets (n = 98 plantlets) during the experiment period. Explants were established in 25 % MS medium *in vitro* for six weeks followed by a two-week metal exposure period. A: shoot length (mm); B: shoot branching; C: root length increment (mm); D: number of roots. Note: explants were exposed to metal treatments after week 6 (represented by arrow and hatched bars).

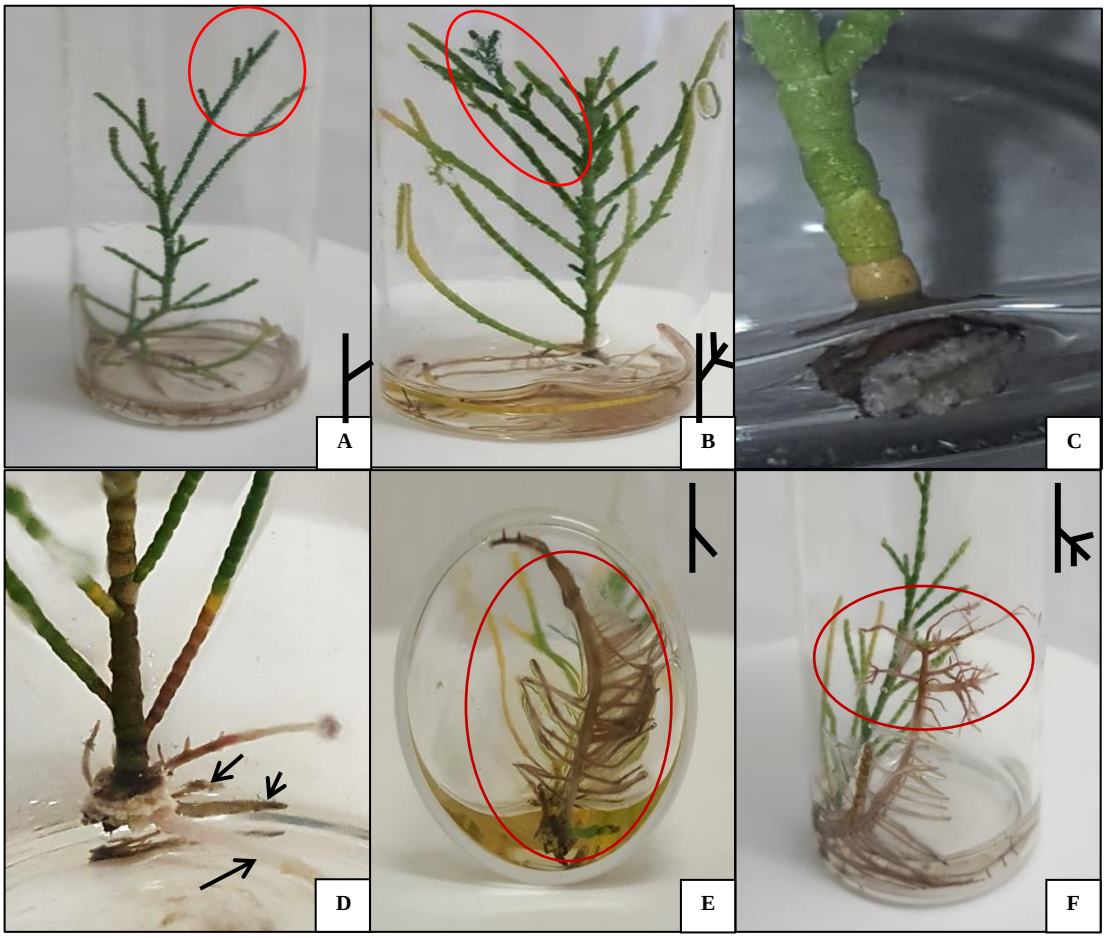

Figure 4.4. Measured morphological growth parameters. (A) second and (B) third-degree shoot branching. Shoot branching was observed as a developing branch situated on a new, recently (after plantlet was cultured) formed branch (encircled). (C) callus production where no rhizogenesis (i.e., initiation of rooting) was observed, and (D) root growth from calli where roots which were produced from calli browned. Note: these plantlets are of the same tissue culture age (Week 5); (E) second- and (F) third-degree rooting by *Tamarix usneoides in vitro*.

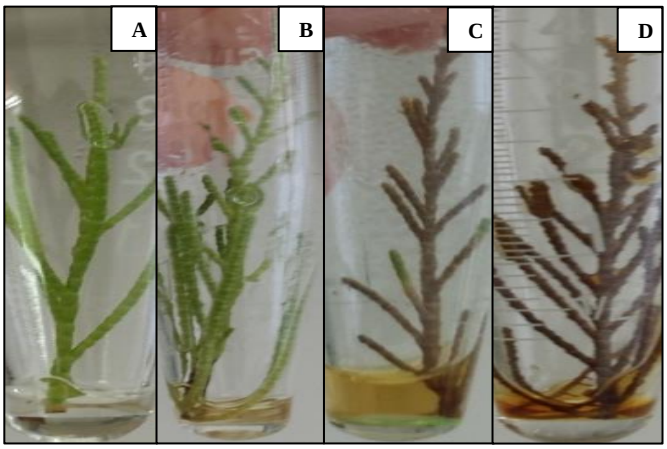

Figure 4.5. Discolouration of 25 % MS media by *Tamarix usneoides* plantlets (possible exudation of phenolics) cultured *in* vitro. Note - A: clear (no discolouration); B: very light brown (minimal discolouration); C: brown (moderate discolouration); D: dark brown (elevated discolouration).

After the experimental period, plantlet dry mass and moisture content (MC) was higher for shoots compared with roots (KW, $p < 0.001$) whereas pH level, metal, or metal concentration did not influence MC.

4.4.3. Impact of metal treatments on growth parameters

The rate of SL was impacted by metal treatments (KW, $p < 0.05$) (Figure 4.6A) with plantlets exposed to Ni treatments increasing their rate of SL. Whereas, the rate of SL, RL, and RB decreased in a dose-dependent fashion when material was exposed to the Co treatments (Figure 4.6A). Plantlet shoot (SL and SB) and root (RL) growth differed between genotypes (KW, $p < 0.05$). Plantlets exposed to 50 mg Ni/L had the highest rate of SL (0.023 mm/day) whereas the lowest rate of SL (0.002 mm/day) was observed for plantlets exposed to 50 mg Pt/L treatments. The rate of RL was higher at 100 mg Pt/L treatments (0.421mm/day) compared with other metal treatments (lowest at 100 mg Co/L treatment - 0.075mm/day) and the control. The SB was highest at 50 mg Pt/L (0.087 shoots/day) and lowest at 50 mg Ni/L (0.023 shoots/day). Elevated NoR was observed for the 25 mg Co/L (0.50 roots/day), 25 mg Pt/L (0.50 roots/day), and 50 mg Pt/L (0.39 roots/day) treatments, compared with the control (0.36 roots/day) (Figure 4.6D). Thirty (n = 30) plantlets (34 % of total plantlets tested) increased the degree of shoot branching (i.e., 2^{nd} to 3^{rd}-degree branching) during the metal exposure period.

Overall, SL (excluding Co and Pt), SB, RL, and RB were not inhibited by metal treatments. Symptoms of metal phytotoxicity, namely senescence, shoot browning, abscission, discolouration of growth media, and root blackening were observed for the 100 mg Co/L metal treatments (Figure 4.7.F, G, and H). Guttation, observed as tiny droplets on the surface of shoots, was present on all plantlets exposed to metal treatments as well as the control (Figure 4.7.E and I).

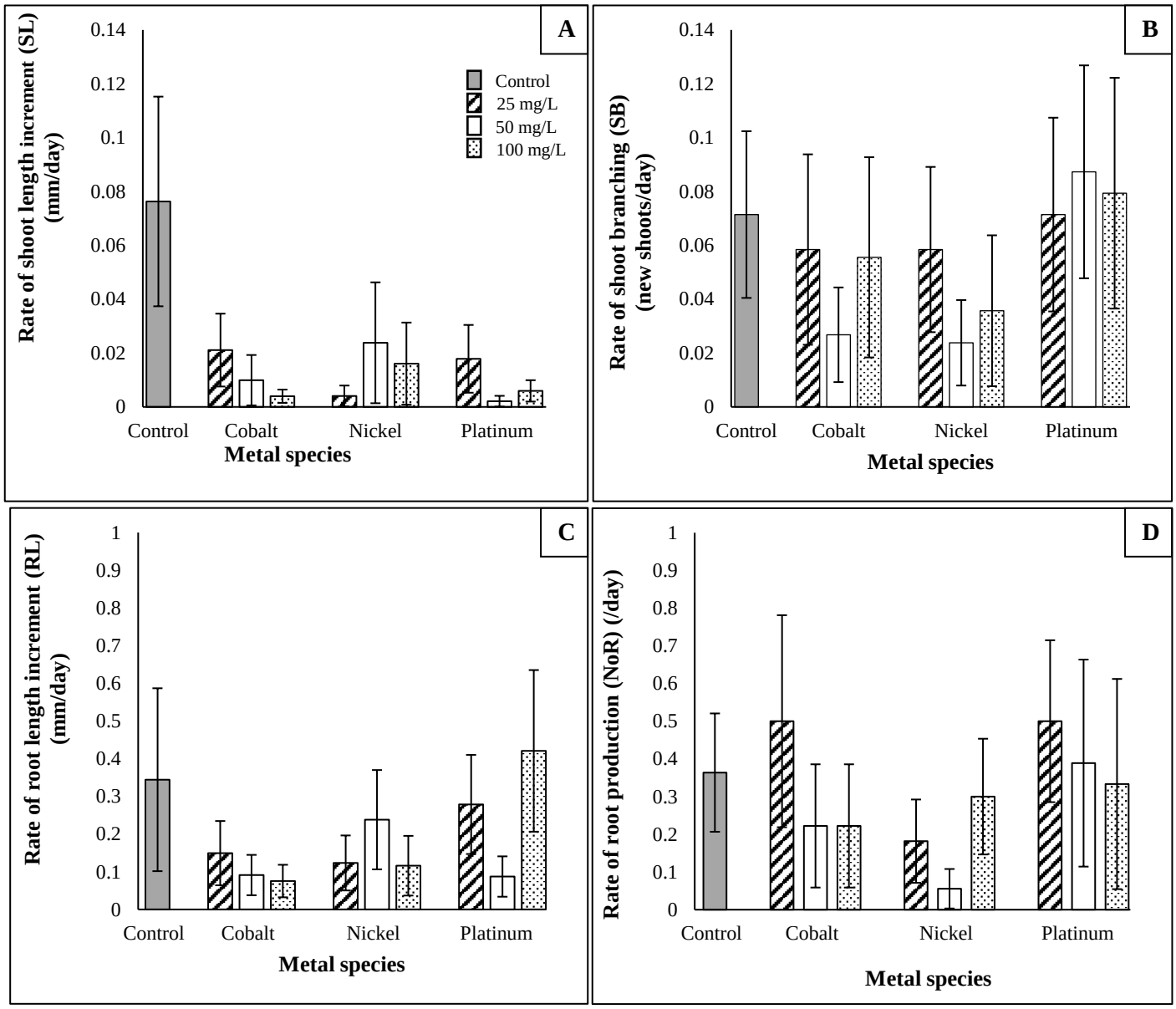

Figure 4.6. Impact of metal treatments [Pt, Ni or Co at 0 (control)*, 25, 50 or 100 mg/L] on the rate of morphological growth by *Tamarix usneoides* plantlets *in vitro* over a two-week exposure period. A: shoot length growth increment (SL); B: number of shoot branches (SB); C: root growth increment (RL); D: number of roots (NoR). Note, *y-axes* are scaled differently. *Basal concentrations of Co (as $CoCl_2.6H_2O$) in control - 15 µg Co/L.

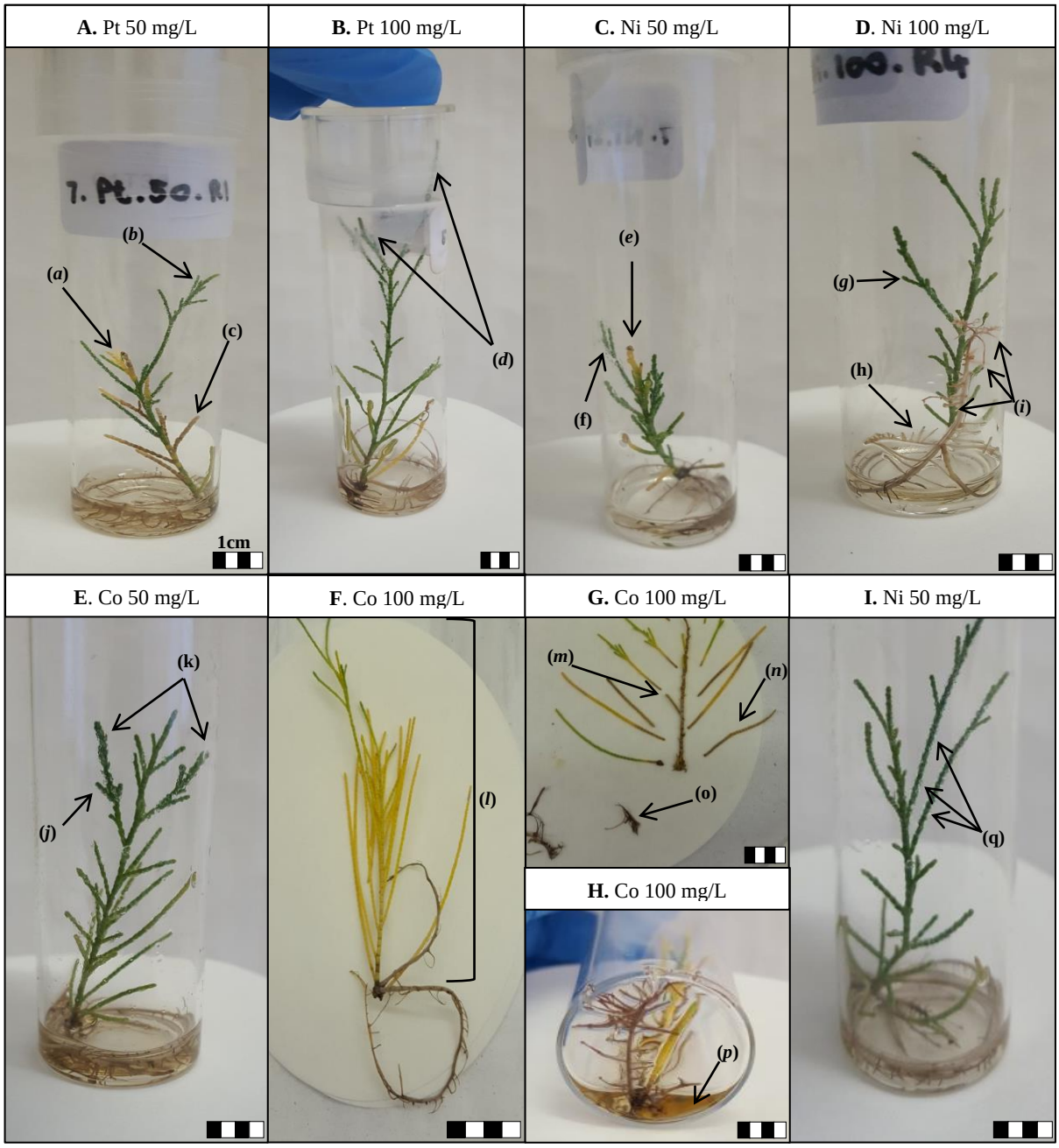

Figure 4.7. Plantlets were exposed to respective metal treatment for two weeks where phytotoxic symptoms were only observed at metal concentrations ≥ 50 mg/L. **A:** (a) Apex senescence where the growth of initial apex was inhibited, (b) shoot length increment of new (potentially dominant) shoot and associated shoot branching, and (*c*) browning from the area of excision (base) towards the apex; **B:** *(d)* Increased SL with no observed morphological phytotoxic symptoms; **C:** (*e*) Senescence of apex and (*f*) increased SL / shoot co-dominance; **D:** Second degree (*g*) shoot and (*h*) root branching along with (*i*) third-degree rooting; **E:** (*j*) Second-degree shoot branching, and (*k*) growth of new shoots; **F:** (*l*) Senescence of shoots as well as root blackening. Plantlet mortality was characterised by observable phytotoxic symptoms and inhibition of measured morphological growth parameters; **G:** (*m*) Abscission (i.e. shoot branches abscised during plantlet harvesting), (*n*) shoot senescence which initially occurred from the base of plantlets towards the apex, and (*o*) root blackening (i.e. Co 'stained' root system dark purple/ black), along with discolouration of growth medium; **H:** (*p*) discolouration of plant growth medium; **I:** (*q*) Guttation observed as droplets on the surface of plant tissue.

4.4.4. Metal assimilation

Shoot length increment (SL, KW, $p < 0.05$) was influenced by the various metal treatments (i.e. metal and metal concentrations) whereas the rate of shoot production (SB), root length increment (RL), and root production (RB) were not influenced (Figure 4.3). Elevated amounts of metal ions were accumulated in roots compared with shoots where metal concentrations ranged from 985 – 8 622 µg Pt/kg in roots compared with 18.6 – 317 µg Pt/kg in shoots; 942 – 5 234 mg Ni/ kg in roots compared with 55 – 280 mg Ni/kg in shoots; and 2.72 – 1 753 mg Co/kg in roots compared with 0.2 – 256 mg Co/kg in shoots, across pH levels and metal concentrations (Table 4.5 and 4.6). The highest levels of Co, Ni, and Pt were assimilated in the roots of plantlets treated with metals at 50 mg Co/L (17 µM), 100 mg Ni/L (34 µM), and 100 mg Pt/L (10 µM), respectively, whereas the highest metal accumulation into the shoots occurred in plantlets exposed to 50 mg Co/L, 100 mg Ni/L, and 50 mg Pt/kg.

Bioaccumulation differed between metals (Ni > Co >> Pt; KW, $p < 0.05$), metal concentration (100 mg/L > 50 mg/L > 25 mg/L; KW, $p < 0.05$), and plantlet part (roots >> shoots; KW, $p < 0.05$; Table 4.5) but was not influenced by pH level (Figure 4.8). Metals were ad/absorbed in a dose-dependent fashion by roots where the BCF ranged from 28 – 78 (Ni) > 0.002 – 38.72 (Co) > 0.04 – 0.10 (Pt) (Table 4.5). The BCF in shoots for 2.21 – 4.64 (Ni) > 0.002 – 5.127 (Co) >> 0.001 - 0.006 (Pt) increased in a dose-dependent fashion (excluding 100 mg Co/kg treatments). Metal concentrations in the control samples were below the limits of detection for ICP-MS (Pt: 5.7 µg/kg; Co: 46 µg/kg; Ni: 527 µg/kg). Elevated Co concentrations were accumulated in shoots of three individuals: LH31 (306 mg Co/kg; n = 2) and LH47 (280 mg Co/kg) when plantlets were treated with 50 mg Co/L (Table S.4.3). Nickel and Co assimilation in shoots, BCF, TF and TI did not differ between plantlets treated with either metal at the 25 mg/L or the 50 mg/L treatments.

4.4.5. Metal translocation

The translocation factor (TF) was influenced by the different metals [Co (0.17) > Ni (0.11) >> Pt (0.03); KW, $p < 0.05$) but not the pH level (Table 4.5). The TF increased in a dose-dependent

fashion (Table 4.5), and was less than 1 across all metal treatments, ranging from 0.02 – 0.03, 0.07 – 0.14, and 0.11– 0.26 for Pt << Ni < Co, respectively.

4.4.6. Metal tolerance

The TI did not differ between metal type, concentration, pH, or the interplay between these factors (Table 4.5). Tolerance indices differed between genotypes (ANOVA; $p = 0.005$), namely between LH48 (Clone 5) and LH31 (Clone 1) (Tukey HSD; $p < 0.05$), as well as LH48 (Clone 5) and LH47 (Clone 4) (Tukey HSD; $p = 0.006$) (Table S4.2 and S4.3). Genotype LH48 (Clone 5) exhibited the highest tolerance indices for Co (108 %) and Ni (117 %), as well as similarly elevated TI for Pt (103 %), compared with the other clones (Table S4.2). Genotype LH38 (Clone 2) had the highest TI for Pt whereas LH47 (Clone 4) had the lowest TI for Co and Pt whilst LH31 (Clone 1) had the lowest TI for Ni (Table S4.2). The MTI ranged between 94 – 104 %, 92 – 108 %, and 86 – 129 % for Co, Ni, and Pt, respectively, across the pH levels and metal concentrations (Table 4.7). The pH level impacted the MTI (KW, $p < 0.05$) where the lower pH of 5.5 resulted in elevated MTI for Ni and Pt treatments whereas higher the pH level of 7.5 resulted in elevated MTI for Co treatments (Table 4.7).

4.4.7. Metal removal

Metal removal (%) varied with the metal type (Ni > Co >>> Pt; KW, $p < 0.001$), the concentration (KW, $p < 0.001$), and plant part (roots >> shoots; KW, $p < 0.05$) but not the pH level (Table 4.5). Platinum removal ranged from 0.008 – 0.009 % in roots compared with 0.0018 – 0.0022 % in shoots. Nickel recovery ranged from 4.4 – 5.7 % in roots and 4.0 – 6.2 % in shoots whereas Co recovery ranged from 5.1 – 7.0 % in roots compared with 4.7 – 5.4 % in shoots (Table 4.5). Metal removal differed between the metal types (KW, $p < 0.05$), the treatment concentration (KW, $p < 0.05$), and the genotype (KW, $p < 0.05$).

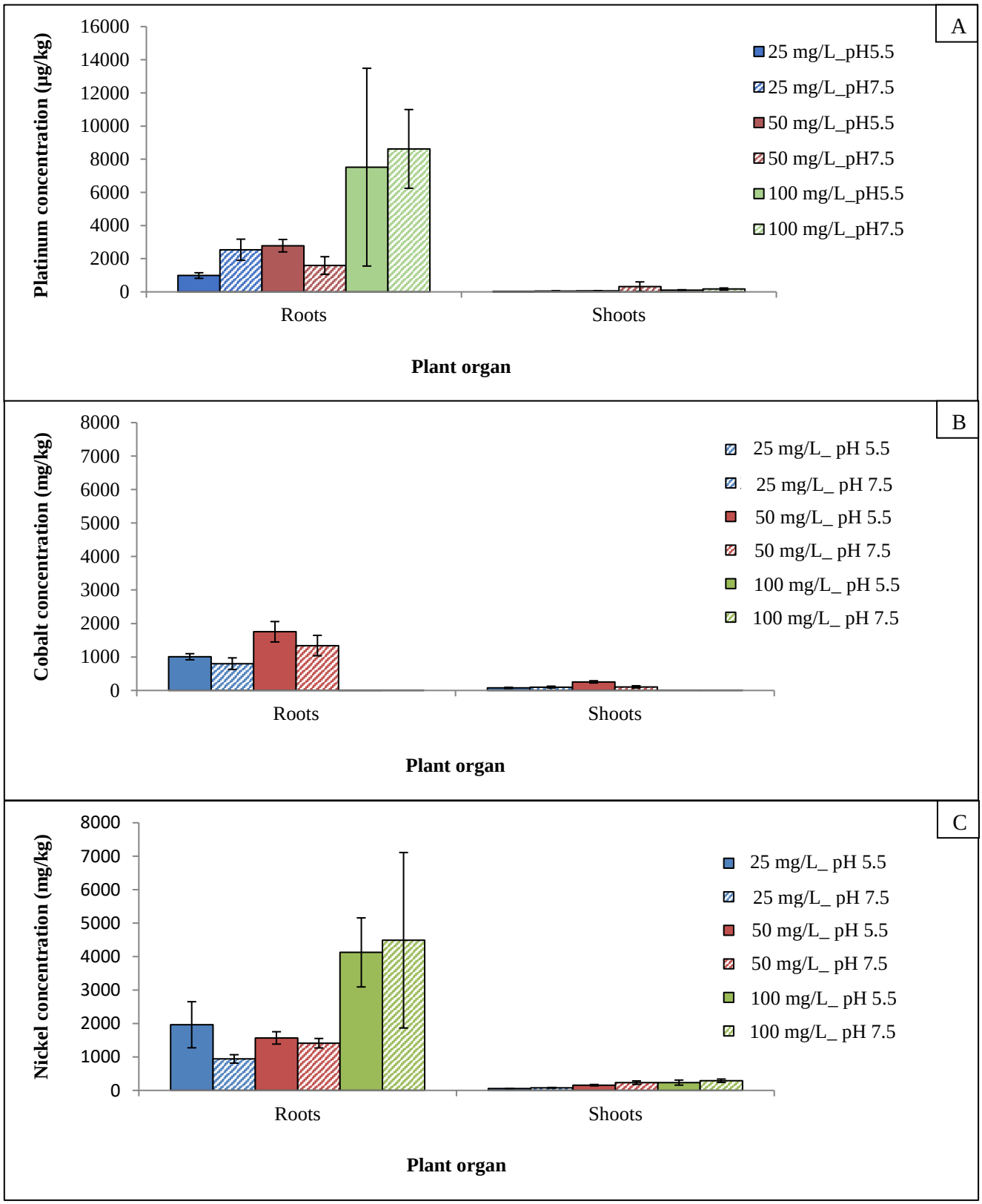

Figure 4.8. Platinum (A), cobalt (B), and nickel (C) concentrations in harvested root and shoot plant parts of *Tamarix usneoides* plantlets treated with 25 % MS medium supplemented with different metals (Pt, Ni, or Co) at 0*, 25, 50, or 100 mg/L at either pH 5.5 or 7.5, *in vitro*. *Note: y-axes scales differ between graphs*. *Basal concentrations of Co (as $CoCl_2.6H_2O$) in control - 15 µg Co/L.

Table 4.5. Metal concentrations, bioconcentration factor (BCF), translocation factor (TF), tolerance index (TI), and metal removal by *Tamarix usneoides* plantlets treated with 25 % MS supplemented with Pt, Ni or Co at 0*, 25, 50, or 100 mg/L at pH 5.5 or 7.5 *in vitro* for a 14-day exposure period. Total metal removal from liquid culture is a combined measure of metals adsorbed on the root surface, assimilated in planta, and excreted on the plant surface from the foliar salt glands. *Control samples contained basal Co concentration of 1.5 µg/L. Total removal by whole plant is presented in parentheses. DL: Detection Limit; N/A: Not Applicable.

Metal	Treatment concentration (mg/L)	pH	Plant part	(n =)	mg/kg dry mass ($\bar{x}$ ± SE) [All Pt concentrations are expressed as µg/kg]	BCF ± SE	TF ± SE	TI ± SE (%)	Metal removal (%) between plant organs [*Whole Plant*]	
Control	0*	5.5	Root	(5)	< DL	N/A	N/A	N/A	N/A	
			Shoot	(5)	< DL	N/A	N/A	N/A	N/A	
		7.5	Root	(5)	< DL	N/A	N/A	N/A	N/A	
			Shoot	(5)	< DL	N/A	N/A	N/A	N/A	
Pt (µg/kg)	25	5.5	Root	(5)	986 ± 168	0.039 ± 0.007	0.02 ± 0.01	111 ± 33	0.008 ± 0.001	[0.0022 ± 0.0007]
			Shoot	(5)	18.6 ± 9.7	0.0007 ± 0.0003			0.0013 ± 0.0008	
		7.5	Root	(5)	2 542 ± 636	0.102 ± 0.025	0.03 ± 0.02	51 ± 7.5	0.010 ± 0.002	[0.0029 ± 0.0004]
			Shoot	(5)	47.7 ± 16.9	0.0019 ± 0.0007			0.0023 ± 0.0005	
	50	5.5	Root	(4)	2 783 ± 376	0.056 ± 0.008	0.03 ± 0.00	59 ± 12	0.009 ± 0.001	[0.0032 ± 0.0003]
			Shoot	(4)	67 ± 6.3	0.0013 ± 0.0001			0.0027 ± 0.0005	
		7.5	Root	(5)	1 587 ± 534	0.032 ± 0.011	0.02 ± 0.01	98 ± 16	0.007 ± 0.002	[0.0020 ± 0.0004]
			Shoot	(5)	317 ± 284	0.0063 ± 0.0057			0.002 ± 0.001	
	100	5.5	Root	(4)	7 521 ± 5 964	0.08 ± 0.06	0.04 ± 0.02	77 ± 20	0.0064 ± 0.0007	[0.0024 ±

				Shoot	(4)	98 ± 31	0.0009 ± 0.0003			0.0016 ± 0.0005	0.0005]
			7.5	Root	(5)	8623 ± 2374	0.09 ± 0.02	0.03 ± 0.01	97 ± 16	0.011 ± 0.002	[0.0029 ± 0.0005]
				Shoot	(5)	171 ± 62	0.0017 ± 0.0006			0.0021 ± 0.0006	
		25	5.5	Root	(5)	1 962 ± 770	79 ± 31	0.04 ± 0.01	99 ± 24	6.53 ± 0.78	[3.84 ± 0.37]
				Shoot	(5)	55 ± 4	2.2 ± 0.2			3.55 ± 0.44	
			7.5	Root	(6)	942 ± 137	38 ± 5.5	0.09 ± 0.02	62 ± 6.6	5.05 ± 0.63	[4.44 ± 0.28]
				Shoot	(6)	79 ± 10	3.1 ± 0.4			4.35 ± 0.28	
		50	5.5	Root	(4)	1 569 ± 213	31 ± 4.3	0.10 ± 0.02	51 ± 4.2	5.15 ± 0.92	[5.43 ± 1.05]
Ni				Shoot	(4)	156 ± 24	3.1 ± 0.5			5.27 ± 1.30	
			7.5	Root	(4)	1 411 ± 163	28 ± 3.6	0.17 ± 0.04	97 ± 27	3.85 ± 0.30	[6.79 ± 0.79]
				Shoot	(4)	232 ± 59	4.6 ± 1.2			7.06 ± 0.86	
		100	5.5	Root	(5)	3 450 ± 1 143	35 ±11	0.14 ± 0.08	95 ± 34	3.50 ± 1.05	[3.86 ± 0.63]
				Shoot	(5)	253 ± 70	2.5 ± 0.7			3.81 ± 0.70	
			7.5	Root	(5)	5 234 ± 3 398	52 ± 35	0.15 ± 0.05	74 ± 15	5.24 ± 1.50	[5.08 ± 1.46]
				Shoot	(5)	280 ± 67	2.8 ± 0.7			4.72 ± 1.50	
Co		25	5.5	Root	(6)	968 ± 92	39 ± 3.7	0.09 ± 0.02	109 ± 41	8.08 ± 1.4	[4.71 ± 0.32]
				Shoot	(6)	80 ± 15	3.2 ± 0.6			4.12 ± 0.51	

	7.5	Root	(6)	801 ± 189	32 ± 7.6	0.14 ± 0.04	96 ± 25	5.98 ± 0.96	[5.65 ± 0.92]
		Shoot	(6)	100 ± 30	4 ± 1.2			5.33 ± 1.10	
50	5.5	Root	(4)	1 753 ± 178	35 ± 3.6	0.15 ± 0.03	92 ± 23	4.33 ± 1.53	[7.72 ± 1.97]
		Shoot	(4)	256 ± 42	5.1 ± 0.8			7.76 ± 2.20	
	7.5	Root	(5)	1 340 ± 341	27 ± 6.8	0.13 ± 0.05	64 ± 13	5.65 ± 1.92	[3.83 ± 1.20]
		Shoot	(5)	107 ± 37	2.1 ± 0.7			3.46 ± 1.27	
100	5.5	Root	(4)	7.9 ± 4.8	0.08 ± 0.05	0.06 ± 0.02	84 ± 40	0.008 ± 0.002	[0.005 ± 0.002]
		Shoot	(4)	0.2 ± 0.1	0.0021 ± 0.0009			0.005 ± 0.003	
	7.5	Root	(5)	2.7 ± 0.5	0.027 ± 0.005	0.43 ± 0.18	138 ± 31	0.006 ± 0.001	[0.013 ± 0.005]
		Shoot	(5)	1.3 ± 0.6	0.013 ± 0.006			0.014 ± 0.006	

Table 4.6. Degree of platinum (Pt), nickel (Ni), and cobalt (Co) accumulation in roots compared to shoots.

Metal	Concentration (mg/L)	pH level	Degree of accumulation in roots compared with shoots
Cobalt (Co)	25	5.5	12
		7.5	8
	50	5.5	7
		7.5	13
	100	5.5	37
		7.5	2.2
Nickel (Ni)	25	5.5	36
		7.5	12
	50	5.5	10
		7.5	6
	100	5.5	14
		7.5	19
Platinum (Pt)	25	5.5	53
		7.5	53
	50	5.5	41
		7.5	5
	100	5.5	77
		7.5	51

Table 4.7. Mass tolerance index (MTI) and plantlet mortality of *Tamarix usneoides* plantlets treated with Pt, Ni, or Co at 0*, 25, 50, or 100 mg/L at pH 5.5 or 7.5 *in vitro* for a 14-day exposure period. Plantlet mortality included observable morphological growth parameters (e.g., senescence and browning), inhibition of measured growth parameters, and discolouration of the growth medium.

pH level	Metal	Concentration [mg/L]	Molarity (µM)	Number of samples (n =)	Dry mass (mg) ± SE		MTI (%)	Mortality %
pH 5.5	Pt	25	128	5	Root	4 ± 0	100	0
					Shoot	29 ± 2		
		50	256	5	Root	3 ± 1	129	20
					Shoot	40 ± 5		
		100	513	4	Root	7 ± 2	122	25
					Shoot	34 ± 0		
	Ni	25	426	6	Root	3 ± 1	107	17
					Shoot	33 ± 4		
		50	852	4	Root	4 ± 1	107	25
					Shoot	33 ± 3		
		100	1 704	4	Root	2 ± 1	108	25
					Shoot	34 ± 3		
	Co	25	424	5	Root	4 ± 0	94	0
					Shoot	28 ± 3		
		50	848	4	Root	3 ± 1	95	50
					Shoot	29 ± 3		
		100	1 697	4	Root	4 ± 1	125	50
					Shoot	38 ± 4		
	Control*		0*	5	Root	4 ± 1	NA	0
					Shoot	29 ± 5		
pH 7.5	Pt	25	128	5	Root	2 ± 0	87	0
					Shoot	29 ± 4		
		50	256	5	Root	9 ± 3	91	0
					Shoot	24 ± 6		
		100	513	5	Root	3 ± 0	86	0
					Shoot	28 ± 3		
	Ni	25	426	6	Root	3 ± 0	92	17
					Shoot	30 ± 3		
		50	852	5	Root	3 ± 0	104	0
					Shoot	35 ± 4		
		100	1 704	6	Root	6 ± 2	102	33
					Shoot	31 ± 2		
	Co	25	424	6	Root	5 ± 1	104	0
					Shoot	33 ± 5		

	50	848	5	Root Shoot	5 ± 1 33 ± 3	104	20
	100	1 697	5	Root Shoot	4 ± 0 32 ± 6	101	20
Control*	0*		5	Root Shoot	3 ± 1 33 ± 4	NA	0

*Basal Co concentrations at 1.5 µg/L (0.026 µM) present in control plant growth medium.

4.5. Discussion

4.5.1. Baseline measurements

The relative maintenance of SL over the first two weeks (Figure 4.3 and S4.1) may be linked with explants initiating root production. That is the explants were initially utilising energy (and other resources) in the formation of roots (plant's structure for nutrient uptake) before initiating shoot growth. However, once explants established, the uptake of nutrients by roots may account for the significant increase in SL increment as the growth period increases. Plantlet SL (as well as RL and RB) was not impacted by pH level – results in agreement with other studies, e.g., Nason *et al.,* (2018) and Rudolph-Mohr *et al.,* (2017). For example, Martins *et al.,* (2013) observed no difference in shoot length growth between *Plantago almogravensis* and *P. algarbiensis in vitro,* across a range of pH levels. This may be attributed to the ability of plant species to actively alter medium pH, by exuding hydroxyl (OH^-) and bicarbonates (HCO_3^-) into the medium, providing optimal and similar pH growing conditions that result in similar growth patterns. In this regard, *T. usneoides* explants may have actively altered the pH of the growth medium resulting in no significant differences in SL, RL, RB, and NR between pH 5.5 and 7.5 (net excretion or uptake of protons, hydroxyls, and/or organic compounds, such as phenolics) (Custos *et al.,* 2020). However, such alterations in medium pH were not investigated in this study.

The low establishment rate may be attributed to numerous factors including plant genotype, season and year of excision from donor plant, physiological age of donor plants, endogenous phytohormone synthesis (such as auxin concentrations), and growth conditions – *viz.* media constituents and vial type and size (*refer to Chapter 3*) (Phillips and Garda, 2019). Hyperhydricity, the formation of water-saturated plant organs, may also contribute to low plantlet establishment and non-significant differences in moisture content (MC). Hyperhydricity is common in tissue culture propagation processes due to culturing conditions, namely the high relative humidity (RH) with low plantlet transpiration (Gao *et al.,* 2018a). Hyperhydricity also induces morphological, anatomical, and physiological changes in plantlets such as the production of smaller grana in chloroplasts (Phillips and Garda, 2019). This reduces light-dependent reactions and hence, a reduction in photosynthesis. Thus,

as morphogenetic responses in tissue culture differs between genotypes, plantlets that did not establish may have died due to the combination of stresses including the reduction in metabolic activity. Although plantlet hyperhydricity can be reduced by increasing vessel ventilation and the levels of osmoregulating chemicals, previous studies using the same culturing conditions (as this experiment) resulted in elevated levels (89 %) of *T. usneoides* plantlet establishment. Also, vessel ventilation introduced novel variables (e.g., precipitation of salts at the base of the growth vessel) and resulted in a 0 % establishment percentage (*refer to Chapter 3*) and thus, was not a feasible option.

Elevated rates of SB (Figure 4.3 and S4.2), recorded in this study, are supported by results obtained by Bairu *et al*., (2009). Shoot branching is mediated by the interaction between plant species/genotype, phytohormones (chemical messengers), nutrient status, and numerous other signals regulated by the environment (de Jong *et al.*, 2019, Hamama *et al.*, 2019, Lv *et al.*, 2018). Root growth was initiated at least one week prior to increases in SL increments and initiation of SB (Figure 4.3). This strongly suggests that cytokinin was synthesised by roots and translocated within the second week of plant growth *in vitro*. Auxin-mediated apical dominance may have occurred within the first week of growth as the site of cytokinin synthesis, namely the roots, was not yet produced. Hayward *et al.,* (2009) demonstrated that SB by *Arabidopsis* spp. may have been inhibited/ promoted by a feedback loop involving the interaction between auxins (involved in apical dominance and adventitious root production), strigolactone (a carotenoid-derived tarpenoid lactone), and cytokinins.

In this study, chronologically older plantlets retained their morphogenetic competence due to plant plasticity (cell and phenotype), results supported by Graner *et al.*, (2019). However, other studies such as those of Tangahu *et al.,* (2011) demonstrated that chronologically younger plantlets had an elevated morphological response compared with older plantlets. These authors' findings may be attributed to the variation in tissue culture morphogenetic responses, which differ between plant species, plant genotype, and culture conditions (Phillips and Garda, 2019). The biological importance of these results indicates that viable *T. usneoides* plantlets can be excised from any part of the donor plant, irrespective of chronological age, and have similar establishment and morphogenetic responses (namely rooting). The non-significant impact of pH on RL increment growth supports the potential

use of *T. usneoides* to remediate contaminated sites and stabilise erosion of other types of contaminated substrate regardless of pH levels (Salas-Luévano *et al.*, 2017, Wu *et al.*, 2019). This finding is supported by results obtained in Chapter 2 where *T. usneoides* trees grew at the study site where pH levels ranged from 4.77 to 8.17 (pH 6.45).

Roots were predominantly produced from the stem (87 %) rather than from calli (situated around the necrotic plate – 13 %, Figure 4.4.C and Figure 4.4.D). Callus, amorphous tissue induced by biotic and/or abiotic stress(es) such as wounding, has been demonstrated to differ in colour, appearance, as well as morphogenetic capacity. Findings from this study demonstrated that calli characteristics, relative to differences in root formation, differed between plantlets of the same genotype (i.e., somaclonal variation) (e.g., Figure 4.4.C and Figure 4.4.D). Browning of roots produced from calli may be attributed to an insufficient vascular connection between the root and shoots (Miguel and Marum, 2011). The high percentage (87 %) of root growth from plantlet stems suggests that *T. usneoides* is a viable candidate for use in *in vitro* metal uptake studies.

Root branching (2^{nd} – and 3^{rd} - degree branching – Figure 4.4.E and 4.4.F) and production of root hairs increases the surface area for nutrient absorption (Yu *et al.*, 2019). Root hairs, as well as lateral roots, reduce the distance required for nutrients to be transported to the roots within the medium. This increased the rate and degree of nutrient absorption and thus contributed to SL and RL growth by *T. usneoides* plantlets *in vitro* (Duque and Villordon, 2019).

As the exudation of phenolics has been associated with heavy metal stress, the release of phenolics and other organic compounds may have increased the bioavailability of essential nutrients within the low nutrient concentrated medium (25 % MS growth media) used in this study (Chen *et al.*, 2017). Phenolics have also been demonstrated to (i) protect plants from microbial infections (Gao *et al.*, 2018b) and (ii) scavenging of ROS (thereby reducing the deleterious impacts associated with oxidative stress). Although not determined in this study, the presence and role of phenolics on morphological growth, protection from pathogenic activity, and change in pH level should be investigated in future studies.

4.5.2. Metal treatments

4.5.2.1. Morphological growth

Morphological parameters were used as a proxy for growth and metal tolerance due to the (i) susceptibility of these parameters to metal phytotoxicity, (ii) root and shoot growth increment, increases the surface area for nutrient sorption (adsorption and absorption processes), and, (iii) wide use of these parameters in metal tolerance studies (Li *et al.*, 2009, Nagajyoti *et al.*, 2010, Pijut *et al.*, 2011). The Co-induced phytotoxic symptoms (Figure 4.7) and plantlet mortality may be linked to the elevated *in planta* mobility of Co (TF = 0.17) compared with Ni (TF = 0.11) and Pt (TF = 0.03). The elevated translocation to plantlet shoots may have impacted sensitive plant tissue, such as the photosynthetic tissue and consequently the growth (*viz.* SL and RL in a dose-dependent fashion; Figure 4.3) and survival of plantlets exposed to Co treatments (Lwalaba *et al.*, 2019, Lwalaba *et al.*, 2020). These findings are supported by results obtained by Jayakumar *et al.*, (2009) and Javed and Anis (2015) where increasing Co treatment concentrations impacted morphological growth of *Erythrina variegate* (Indian Coral tree) and *Glycine max* (Soybean), respectively. Li *et al.*, (2009) also reported reduced shoot growth and biomass production when plants were exposed to Co. Therefore, results from this study indicate that *T. usneoides* plantlets exceeded their Co toxicity threshold (i.e., > 50 mg/L) (Figure 4.7 (H) and (G)). Root damage, associated with underlying loss of membrane integrity, is also known to reduce metal ion assimilation and translocation (e.g. excess metal influx and efflux), which may have resulted in the lower Co concentrations in plantlets treated with 100 mg Co/L (Table 4.5) (Rizvi and Khan, 2017).

Increased SL by plantlets exposed to Ni treatments (Figure 4.3) may be associated with the role of Ni as an essential micronutrient in plants. For example, Ni is incorporated into the enzyme, urease (Merlot, 2020). Elevated Ni treatment concentrations ($\geq$ 25 mg Ni/L) may have promoted the hydrolysis of urea into carbon dioxide (CO_2) and ammonia (NH_4^+) (Hassan *et al.*, 2019), promoting SL growth. Other growth parameters, namely SB, RL, and NoR, were not impacted by metal treatments. This was supported by increased plantlet growth under metal treatment exposure. These findings may also account for the elevated TI (calculation based on RL) from this study.

This dose-dependent experiment indicated that *T. usneoides* exhibits hormesis [*sensu* Agathokleous *et al.*, (2019)] (e.g. SB - Pt) stress responses to metal treatments (Figure 4.6). This suggests that *T. usneoides* exhibits a level of biological plasticity at a morphological scale which was observed as the "stimulation" of growth at 25 mg/L (exceeding the rate of growth by control samples), optimal growth at 50 mg/L, which subsequently decreased during plantlet exposure to 100 mg/L metal treatments. This hormesis stress response by *T. usneoides* may also contribute to elevated metal sorption by plantlet roots at 50 mg/L compared with 25 mg/L. For example, the stimulation of root growth at concentrations > 25 mg Ni/L (8.52 μM) increased RL and subsequent root surface area for metal binding and storage. These results are supported by Gawrońska *et al*. (2018b) where low Pt concentrations (< 2.5 μM) induced a hormesis stress response by *Arabidopsis thaliana*. The observed hormetic stress response supports the TI obtained from this study.

Exposure to ionic and osmotic stress induces the plant's antioxidant system. This includes the release of phenolic acids to ameliorate the generation of reactive oxygen species (ROS) (Chen *et al.*, 2019). In this study, the roots of *T. usneoides* plantlets, treated with metal doses at ≥ 50 mg/L (and more specifically 100 mg Co/L) released dark brown compounds, possibly phenolics, into the growth medium (Figure 4.6). The chelation of metals by phenolics in culture solutions can result in their precipitation, reducing metal availability for root uptake. Plantlet exposure to elevated Co concentrations may have also damaged plantlet root systems (observed as root browning and lack in subsequent root growth and degree of root branching – Figure 4.7) resulting in the reduction of active Co uptake and accumulation by *T. usneoides* roots and subsequent plantlet death. These results are in accordance with Bali *et al*., (2010) who demonstrated that although *Medicago sativa* and *Brassica juncea* plants were necrotic, due to the phytotoxic effects of elevated Au concentrations used in the study, Au was still accumulated by those plant species. Therefore, the passive uptake, via capillary action, of Co by *T. usneoides* roots may have predominantly contributed to the measured concentrations, and subsequent BCF, present in the roots of plantlets treated with 100 mg Co/L.

4.5.2.2. Metal assimilation

Tamarix usneoides plantlets exhibited accumulation of Ni > Co >>> Pt, with significantly elevated concentrations (BCF) of Ni, Co, and Pt in roots compared with shoots (Tables 4.5 and 4.6). This highlighted the ability to use *T. usneoides* roots to adsorb and absorb these precious metals from wastewater or effluent for subsequent harvest and recovery, i.e. phytomining (Dinh *et al.*, 2022). The retention of metals in roots (i.e., BCF > 1; TF < 1) also reduces the biotransfer of these metals into the food chain as elevated concentrations of metals were not assimilated into the potentially edible parts of the plant (i.e. shoots) and are therefore not accessible to foliar herbivores (Correia *et al.*, 2018, Naikoo *et al.*, 2021). Furthermore, the observable retention of metals in the roots (Tables 4.5 and 4.6) is considered a metal tolerance strategy by which metal xylem loading for translocation to the shoots is restricted, limiting the phytotoxic impact of metals on the photosynthetic tissues (Marrugo-Negrete *et al.*, 2015, Tappero *et al.*, 2007a).

Results from this study show that *T. usneoides* accumulated 5 – 76 times more Pt in roots compared with shoots (Table 4.6) (Khan *et al.*, 2000; Khan, 2005). This finding is supported by the retention of Pt by *Arabidopsis thaliana* (Gawrońska *et al.*, 2018a) and *Cucumis sativus* (Verstraete *et al.*, 1998); Ni by *Salix viminalis* (Drzewiecka *et al.*, 2012); and Co by *Allium sativum* (Soudek *et al.*, 2011) in roots from solution. *Tamarix usneoides* has also been demonstrated to accumulate the noble metal, Au, within the root, and to a lesser extent in the shoot. It was further demonstrated that an elevated rate of translocation of these metals to the shoot occurred from solution *in vitro* compared with complex solid matrices (Dennis, 2008, Wilson *et al.*, 2021). Thus, the assimilation, translocation, and accumulation of Pt and Au by *T. usneoides* plantlets *in vitro* exhibit similar patterns in metal assimilation and tolerance. The retention of Pt in roots, across metal concentrations and pH levels, is supported by various other studies (Bednarova *et al.*, 2014, Leśniewska *et al.*, 2004, Lyubomirova and Djingova, 2014, Mikulaskova *et al.*, 2013, Oke *et al.*, 2013). These findings are supported by results obtained from field studies (*please see Chapter 2*). Collectively these findings indicate that *T. usneoides* can be used as a tool for phytostabilisation, i.e., sequestering and retaining Pt, Ni, and Co within the roots/ rhizosphere-soil interface (Salas-Luévano *et al.*, 2017, Shackira and Puthur, 2019,

Wu *et al.*, 2019). Plants used for phytostabilisation create vegetative 'caps', reducing aeolian erosion and thus, the migration of harmful contaminants (Mendez and Maier, 2008, Shackira and Puthur, 2019). The observed pattern of Pt uptake and allocation, in a dose-dependent fashion, may be related to the catalytic properties of Pt (and PGEs in general) increasing processes involved in Pt assimilation between plant parts (Gawrońska *et al.*, 2018a).

The metal concentrations in plantlets grown in the three metal solutions at pH 5.5 or pH 7.5 did not differ (Figure 4.8). This may be attributed to the alteration of solution pH at the root/medium interface by the net excretion of protons or hydroxyls by plantlet root cells resulting in acidification / alkalinisation, respectively (Custos *et al.*, 2020). Plantlet roots may have therefore been exposed to similar pH levels reducing differences in pH-dependent processes (e.g., the influence of pH on metal bioavailability). However, further studies are required to support this finding where pH levels should be recorded before, during, and after metal treatments, across experimental (controlled and field) conditions. Terrestrial plant species have gained attention due to the size of root systems (i.e., longer roots with larger surface areas which contribute to increased metal sorption) compared with aquatic plants (Rezania *et al.*, 2016). The low metal removal percentages may be attributed to the relatively smaller root systems of plantlets used in this experiment (Table 4.5). However, the demonstrated sorption of metals by plantlet roots indicated the potential use of *T. usneoides* for the removal of these previous elements in a scalable system.

The low TF (< 1) (Table 4.5) may be linked with the tissue culture conditions (e.g., relative humidity) which limit the evapotranspirational pull of metals from the roots, via the transpiration stream, and into the shoots (Amari *et al.*, 2017, Hao *et al.*, 2012, Peralta-Videa *et al.*, 2002). Robinson *et al*., (2003) attributed the translocation and assimilation of Ni in shoots of the Ni-hyperaccumulator *Berkheya coddii* to evapotranspiration processes.

4.5.2.3. Rhizo(hyper)accumulation

The hyperaccumulation threshold of elements has been defined based on the active accumulation (i.e. in leaves) of two-to-three orders of magnitude greater than *in planta* metal concentrations of plants growing on normal soils and one order of magnitude of plants growing on metalliferous soils (Baker, 1981, Van der Ent *et al.*, 2013). Generally, Pt concentrations range from 0.001 – 0.160 mg/kg in unpolluted soils (Mihaljevič *et al.*, 2013) and 0.04 – 0.44 mg/kg in polluted soils (Erasmus *et al.*, 2020, Savignan *et al.*, 2021). Plants growing in polluted areas (e.g., along the roadside or in mining-impacted areas) can accumulate Pt in the range of 0.002 – 0.642 mg/kg. Based on the criteria used to define hyperaccumulation thresholds, the Pt hyperaccumulation threshold was defined as the accumulation of > 1 mg/kg and > 4 mg/kg (dry mass) by plants in unpolluted and polluted soils, respectively. In this respect, Pt was accumulated in the roots to the degree of hyperaccumulation (i.e., Pt "rhizo-hyperaccumulation") in a dose-dependent fashion. Cobalt and Ni were rhizo-hyperaccumulated across *T. usneoides* genotypes with potential Co hyperaccumulation in shoots linked to genotype; LH31 (306 mg/kg; BCF = 6.1) and LH47 (280 mg/kg; BCF = 5.6). Genotypic variation (Table S4.2) in metal (hyper)accumulation has been reported in various studies (Table S4.1). This study has indicated that *T. usneoides* holds potential for the rhizofiltration and subsequent recovery of Co (≤ 50 mg/L), Ni, and to a lesser degree Pt, from effluents. In this regard, Erasmus *et al*., (2020) determined that Pt concentrations present in a Pt mine settling pond were 0.47 µg/L. Although Pt was accumulated to a lesser degree compared with Co and Ni, results from this study support the application of *T. usneoides* to recover (sorb and concentrate) metals from sources such as Pt mine settling ponds.

Previous studies have demonstrated the ability of *T. hispida* and *T. nilotica* to hyperaccumulate Zn, whilst Co was shown to accumulate in the roots of *T. nilotica* (Fawzy *et al.*, 2006, Sookbirsingh *et al.*, 2010). This study demonstrated that *T. usneoides* has a higher accumulation capacity for Co from liquid medium in comparison with *T. nilotica*, based on the bioavailable fraction of Co present at the study site (Fawzy *et al.*, 2006). Numerous studies have attributed variability in metal tolerance and accumulation, as well as growth, to genotypic variation (Gawrońska *et al.*,

(2018a)). The variability in response by *Salix alba* genotypes to Cd (Iori *et al.*, 2015), Co tolerance (Lwalaba *et al.*, 2020), Ni tolerance by *B. juncea* (Ansari *et al.*, 2015), uptake and accumulation of lead (Pb), copper (Cu) and zinc (Zn) accumulation by *O. sativa* (Zhou *et al.*, 2015)], and the degree of Ni accumulation and root-to-shoot translocation by *O. sativa* (Wang *et al.*, 2019) was attributed to genotypic variation. The genotypic variation in the assimilation of Ni and Co by roots (Table 4.3 and S4.2), combined with the range in TFs and shoot concentrations, highlights the importance of screening multiple genotypes under controlled conditions prior to the implementation of phytoremedial programmes.

Antagonistic interactions between Ni and Co for uptake by roots have been demonstrated in numerous studies (e.g., Deng *et al.*, (2021)). Yamaguchi *et al.*, (2015) concluded that *Clethra barbinervis* was unable to distinguish between Ni and Co during uptake, resulting in competitive inhibition (i.e., competitive ion adsorption) of Co by Ni. Other authors have suggested that Co is absorbed by non-specific transporters, located in the membranes of the root cells, as no Co-specific transporters are expected to occur in plants (i.e., Co is classified as a non-essential element) (Kabeya *et al.*, 2018, Tang *et al.*, 2019). The experimental design of the current study has highlighted the uptake parameters of these elements without antagonism or competitive inhibition. When Ni and Co were assessed in isolation at the same doses, metal assimilation, BCF, TF, and TI parameters of *T. usneoides* did not differ between plantlets exposed to Ni or Co at 25 or 50 mg/L. This indicated similar patterns of sorption, transport, assimilation, and tolerance to either metal when treated separately up to 50 mg/L. However, an assessment of antagonism or competitive inhibition would be required to confirm that common assimilation and translocation mechanisms for Ni and Co are present in *T. usneoides*. Thus, the selective recovery of Ni or Co from Ni-Co effluent should be explored in future studies.

Most species of *Tamarix*, including *T. usneoides*, are considered to be exo-recretohalophytes, where glandular trichomes in the leaf epidermis actively excrete excess ions to maintain metal homeostasis (Campbell and Strong, 1964, Wilson *et al.*, 2017). Studies have demonstrated the ability of *T. symrnensis*, *T. ramosissima*, *T. aphylla*, and *T. usneoides*, to excrete a range of ions, including

Na, Cl, S, Ca, Mg, Cd, lithium (Li), and Pb, which was related to the composition of elements in the root environment (Hagemeyer and Waisel, 1988, Kadukova *et al.*, 2008, Manousaki and Kalogerakis, 2011, Wilson *et al.*, 2017). Other halophyte species, such as *Atriplex halimus*, *A. murale* (elevated Co concentrations on areas near leaf trichomes), and *A. canescens*, also utilise salt glands to excrete excess metals and halogens from the leaves, preventing the toxic build-up of these ions *in planta* (Manousaki and Kalogerakis, 2009, Tappero *et al.*, 2007b, Wilson *et al.*, 2017). Collectively these studies suggest a ubiquitous metal storage and/or excretion salt gland function between plant species, where the presence of salt glands has been suggested to confer salinity and heavy metal tolerance by maintaining ion homeostasis (Moray *et al.*, 2016, Wei *et al.*, 2020). In this regard, *T. usneoides* demonstrated the ability to tolerate, take up and accumulate Pt, Ni, and Co to varying levels from liquid media however, further studies are required to determine the role of salt glands in the excretion of Pt, Co, and Ni. Thus, salt gland excretory processes and similarities in the coordination chemistry of $NiSO_4$ and $CoSO_4$ may account for the similar patterns of Ni and Co assimilation, BCF, TF, and TI by the *T. usneoides* plantlets, indicating the potential inability of this species to differentiate between Co and Ni ($\leq$ 50 mg/L) – results supported by Yamaguchi *et al.*, (2015).

Metals were accumulated by plantlets in a dose-dependent fashion (Table 4.5). Based on the experimental design, increasing metal treatment concentrations consequently introduced increasing amounts of sulphur (as SO_4^{2-}) into solution (25 < 50 < 100 mg/L = ~1.27 < 2.55 < 5.10 mg SO_4^{2-}/L, respectively) (Table 4.3). Although a relatively low concentration of SO_4^{2-} was introduced into the *in vitro* environment, the effect of SO_4^{2-} on metal uptake should not be discounted.

Salinity increases metal bioavailability due to the complexation of salt-derived anions with metals as well as the competition between salt-derived cations with metal ions for binding sites (Acosta *et al.*, 2011). Thus, compounds used in this study (i.e., $NiSO_4$) may have increased the bioavailability of the particular metal via SO_4^{2-} - assimilatory pathways present in the halophyte, *T. usneoides* (Hawkesford and De Kok, 2006). This was demonstrated by Wilson *et al.*, (2021) who investigated the ability of *T. usneoides* to accumulate different Au species, namely gold chloride, gold potassium cyanide, and gold sodium thiosulphate. Elevated concentrations of Au were accumulated in

the roots compared with shoots, and in the series: gold potassium cyanide > gold chloride > gold sodium thiosulphate. Those results demonstrate the impact of the salt-derived anion constituent in metal uptake dynamics.

Based on the physicochemical properties of the investigated metals, Pt has a heavier relative atomic mass (195.08), compared with Ni (58.69) and Co (58.93). In this regard, less SO_4^{2-} was added to the solution to obtain the respective metal treatment concentrations. The increased concentration of SO_4^{2-}, present in Ni and Co treatments, may have increased the metal's bioavailability compared with Pt treatments.

4.5.2.4. Tolerance Index (TI_{ROOT}) and Mass Tolerance Index (MTI)

Plant tolerance may be classified as ranging from high (TI > 60 %), moderate (TI 35 % - 60 %), or low (TI < 35 %), to marginal tolerance or intolerance (Lux *et al.*, 2004). Overall, *T. usneoides* plantlets proved tolerant to Co (97 %) > Pt (82 %) > Ni (77 %) (Table 4.5). Numerous factors, including metal complexation and compartmentalisation (Leitenmaier and Küpper, 2013), metal precipitation (Robinson *et al.*, 2003), and/or adsorption by roots (Fourati *et al.*, 2016) may have contributed to elevated TI (> 60 %) (Table 4.5). It has been demonstrated that halophytes are better adapted to cross-tolerate a range of stresses, compared with glycophytes (Fourati *et al.*, 2016). This is attributed to various salt tolerance and avoidance strategies which play a vital role in metal detoxification by halophytes (Flowers and Colmer, 2008, Van Oosten and Maggio, 2015). In this regard, the elevated Co TI (Table 4.5) may be linked to the low root growth of the control plantlets (as TI calculations were based on root length) supporting the possible stimulation of plant morphological growth (RL in this case) by the presence of metals in the culture medium (i.e., a hormesis stress response) (Figure S4.4) (Agathokleous *et al.*, 2019).

A study conducted by Li *et al.*, (2009) demonstrated that Co significantly reduced dry mass and rate of transpiration by *Zea mays*. Those authors attributed the decrease in dry mass to competitive ion adsorption, whereby the assimilation of nutrients required for biomass production was outcompeted by the investigated metals. Based on the MTI (Figure 4.7), regardless of genotype or pH

conditions, *T. usneoides* plantlets were highly tolerant to Pt, Ni, and Co up to a maximum tolerance index of 129 % (Lux *et al.*, 2004), suggesting that mass was maintained and/or enhanced during the metal exposure experiment. The high relative humidity (RH) in culture vials is known to reduce plantlet water potentials, and therefore the translocation of metals to shoots. This highlights the importance of conducting greenhouse and field trials to verify results obtained from *in vitro* experiments.

Plant tissue culture allows the determination of a single variable's attribution to a particular morphogenetic response *in vitro* (Chapter 3). Hence, it must be noted that the morphogenetic response of plants, of the same species or genotype, *ex vitro/ in situ* may differ as a plant is never exposed to a single stress but rather a combination of stresses, at varying degrees, at any given point in time or space. Thus, field trials (*refer to Chapter 2*) were conducted to verify results obtained from this tissue culture experiment (García-Gonzáles *et al.*, 2010, Watson *et al.*, 2003). This study highlights the importance of considering results obtained from *in vitro* and *in situ* experiments in combination when interpreting data obtained under a single experimental design. Based on a review of available literature, the interpretation and comparison of results obtained from various studies may be limited due to the experimental conditions which differed between studies. Such results are influenced by abiotic (e.g., physicochemical properties of soil, seasonality, and humidity) and biotic (plant exudates, plant-microorganism interactions, and/or rhizosphere conditions) variables, as well as the combinations thereof, which cannot be controlled for between studies (Liu *et al.*, 2020, Rosenfeld *et al.*, 2018).

4.6. Conclusion

Tamarix usneoides is tolerant to Ni, Co, and Pt in solution at one, ten, and ten thousand times the average soil crustal abundance, respectively. Based on metal root-binding affinity or uptake, the species holds the potential to recover these metals from effluent under moderately acidic (pH 5.5) and alkaline (pH 7.5) conditions and across a wide metal concentration range (25, 50, and 100 mg/L). More specifically the genotypes LH31, LH47, and LH48 (Table S4.2) hold the most potential for rhizomining and recovery of Co, Ni, and Pt (to a lesser degree) from effluents. The variation in BCF, TF, and TI between the tested genotypes indicated that selective breeding for improved rhizofiltration and phytoextraction traits is feasible. These results may have further implications for the recovery of precious metals from saline effluent, as well as other secondary sources (e.g., e-waste effluent), – a growing challenge whereby halophytes have been utilised as an emerging biotechnology for the phytoextraction and desalination of effluent. The variability in accumulation capacity between genotypes indicated that selective breeding for improved rhizofiltration and phytoextraction traits is feasible and can be carried out by mass propagation of prolific *T. usneoides* genotypes by plant tissue culture.

4.7. References

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Chapter 5

Conclusion

5.1. Overview

The aim of this study was to investigate the uptake, translocation, and tolerance of Pt, Ni, and Co by *Tamarix usneoides*, an indigenous exo-recretohalophyte, from liquid medium (*in vitro*) and soil contaminated by base metal refinery effluent and overspray from the enhanced evaporation spray system (*in situ*). To achieve the aims and objectives set out in this thesis, two studies differing in experimental conditions were conducted to build a network of cumulative evidence. The aim of building this network was to create consilience (Saad, 2020) with regards to the ability of *T. usneoides* to allocate Pt, Ni, and Co (and S) under different experimental conditions. Results from previously published studies, investigating the phytoremediation potential of various plant species with similar / different modes of salt (i.e. halophytes and glycophytes) and metal (e.g., excluders, accumulators, and hyperaccumulators – Tables S1.3 and Table S4.1) traits were used to support and / or challenge the obtained results.

No previously published studies have investigated the ability of *T. usneoides* to allocate Pt, Ni, or Co, across a wide metal concentration range from moderately acidic (pH 5.5) or slightly alkaline (pH 7.5) liquid media. Furthermore, no previous studies have determined the fate of metals and S in *T. usneoides* trees (plant organs or tissue types) or soils at the study site. The scope of this PhD was also novel in the context of previous and current research being undertaken to investigate the underlying biology and ecology of *T. usneoides* (Tables S1.2). This research also presented the use of *T. usneoides* for phytoextraction of S and Ni and phytostabilisation of Co and Pt. Estimates of *T. usneoides* biomass, based on allometric equations, are also presented. To achieve the research aims, specific objectives (outlined in each chapter) were achieved as follows:

The aim of Chapter 2 was addressed by (i) characterising soil chemical properties which mediate the fate of elements between *T. usneoides* and soil profiles, (ii) concentrations, bioconcentration (BCF), and translocation factors (TF) of S and metals (Pt, Ni, and Co) were determined within *T. usneoides* biomass (plant organs and tissue types) and soil pits, (iii) determine whether S and metals hyperaccumulated in *T. usneoides* biomass, and (iv) investigated the effect of tree size on the amount of S and metals extracted by *T. usneoides* trees harvested from the study site.

Elements were allocated in trees in the order: S (39 900 ± 746 mg/kg) > Ni (59.46 ± 4.67 mg/kg) > Co (2.65 ± 0.34 mg/kg) > Pt (50 ± 6 µg/kg), indicating that S (BCF > 20; TF > 1) was hyperaccumulated in leafy shoots of *T. usneoides*. Although Pt (BCF > 1; TF > 1), Ni (BCF > 1), and Co (BCF > 1) were bioaccumulated in the leafy shoots of certain tree replicates, these metals were not accumulated to the degree of hyperaccumulation in tree biomass. Soil chemical properties (pH, electrical conductivity, and redox potential) differed (ANOVA, $p < 0.01$) between planted and unplanted pits. The pH level and EC were lower in planted pits (pH 6.0; EC = 3 499 µS/cm) compared with unplanted (pH 7.6; EC = 9 644 µS/cm). The lower EC, along with S hyperaccumulation supports the potential use of *T. usneoides* for phytoextraction of S and Ni in shoot tissues and Co and Pt in roots. At a spacing of 1333 trees / ha, *Tamarix usneoides* trees could remove an estimated 2.23 ± 0.30 mg Pt/ha, 3.02 ± 0.83 kg Ni/ha, 1.28 ± 0.90 kg Co/ha, and 1.28 ± 0.09 tons S/ha, excluding excreted salts. Large and medium tree replicates allocated higher element concentrations in tissues compared with small and very small trees, indicating a trend for higher element concentrations in tissues with tree size. Thus, *T. usneoides* can decontaminate soils polluted by base metal refinery effluent, creating conditions conducive to the survival of the glycophytes *Searsia lancea* and *S. pendulina* (Dye *et al.*, 2017) and *Berkheya coddii* (Nethengwe, 2013).

The aim of Chapter 3 was to develop a standardised and rapid *in vitro* mass propagation protocol for *T. usneoides*. This aim was achieved by identifying factors, relative to the different stages of plant tissue culture (PTC), influencing explant establishment. Low initial explant establishment (i.e., root production) percentages were identified as a challenge compromising the sample size required for metal uptake studies (Chapter 4). The development of a reproducible PTC procedure would also enable the mass screening and propagation of *T. usneoides* genotypes for desirable traits (e.g. improved rhizofiltration and phytoextraction traits). Variables which influenced explant establishment included donor plant growing conditions (KW, $p < 0.05$), physiological age of the donor plants (ANOVA, $p < 0.05$), genotype (KW, $p < 0.001$), season of explant propagation (KW, $p < 0.001$), length of explants (KW, $p < 0.05$), and volume of growth vial (KW, $p < 0.05$) whereas pH, chronological age of explants, strength of plant growth medium, and auxin pulse treatments did not.

Based on these results, a standardised *in vitro* PTC protocol was developed for the mass propagation of *T. usneoides* explants. Limited studies have been conducted on the impact of donor plant provenance on the *in vitro* morphogenetic response of explants. This highlighted a crucial gap in research and the need for further investigation. For instance, these factors influencing explant establishment should be considered during the development of PTC protocols. The developed PTC protocol can be (i) reproduced, (ii) adapted for other halophytes, (iii) used for comparative research (e.g., investigating different metal species), and (iv) used for the mass propagation of identical genotypes with desirable traits (e.g., elevated phytodesalination or phytoextraction capacity).

The aim of Chapter 4 was to determine the ability of five *T. usneoides* genotypes to recover Pt, Ni, and Co from 25 % MS growth medium supplemented with the metals at 0 (15 µg Co/L), 25, 50, or 100 mg/L at pH 5.5 or 7.5. *Tamarix usneoides* explants were successfully established using the PTC protocol developed in Chapter 3. *Tamarix usneoides* exhibited genotypic variation in the accumulation of Co but not Ni or Pt, with shoot (stems and leaves combined) levels of Co reaching the range typical of Co hyperaccumulating plants (> 300 mg/kg) in the aboveground biomass of two of the five genotypes assessed. Roots of plantlets hyperaccumulated Pt (defined in this study as Pt > 1 - 4 mg/kg), Ni (> 1 000 mg/kg), and Co (> 300 mg/kg) across treatment concentrations (excluding 100 mg Co/L treatment - where 100 mg Co/L treatments proved phytotoxic, demonstrating a Co-specific hormetic response) and pH levels. The BCF and TF for all three metals increased in a dose-dependent fashion. The mean maximum plant/media BCF (Ni > Co >> Pt) was 52, 39, and 0.1 respectively, whereas the mean maximum shoot/root TF (Co > Ni > Pt) was 0.04, 0.17, and 0.43 for Pt, Ni and Co, respectively. Whether phytoextraction of Pt, Ni, and Co can be achieved from geochemically complex substrate was investigated in Chapter 2. This *in vitro* study indicated that *T. usneoides* is tolerant to Ni, Co, and Pt at 1, 10 and 10,000 times the average soil crustal abundance, respectively, and supports findings obtained from previous studies showing that *T. usneoides* can tolerate and (hyper)accumulate salts and metals across a wide pH range (Dennis, 2008, Kendall, 2010, Weiersbye and Witkowski, 2007, Wilson *et al.*, 2022). These results may have further implications for the recovery of precious metals from saline effluent – a growing challenge expected to increase due to

the growing demand for these precious metals. The variability in Co accumulation capacity between genotypes indicated that selective breeding for improved rhizofiltration and phytoextraction traits is feasible and can be carried out by mass propagation of prolific *T. usneoides* genotypes by PTC. Based on metal root-binding affinity or uptake, *T. usneoides* holds the potential to recover Pt, Ni, and Co, in a dose-dependent fashion, from relatively saline effluent under moderately acidic to alkaline conditions, across a wide metal concentration range.

5.2. Comparison between results obtained from *in situ* and *in vitro* studies

Field, pot, hydroponic, and *in vitro* experiments are utilized to determine the phytoremediation potential of plant species and their genotypes. However, these types of studies differ in their experimental (a)biotic conditions – limiting the ability to distinguish plant responses (e.g., metal uptake, translocation, and tolerance) to metal treatments (Figure 5.1). Moreover, due to the geochemically complex, site-specific nature of contaminated substrate, desirable plant traits such as metal hyperaccumulation, distinguished under hydroponic conditions (or even *in vitro* as reported in Chapter 4) may not be observed / distinguished during field trials. For example, Zabłudowska *et al.*, (2009) concluded that the pattern of arsenic (As) accumulation, distribution, and *in planta* speciation differed between hydroponic and field experiments. Similarly, a study conducted by McBride and Zhou, (2019) investigated the bioaccumulation of Cd and Zn by *Phytolacca americana* across experimental conditions (*viz* – hydroponic, pot, and field conditions) and showed that elevated concentrations were accumulated by the study species under hydroponic conditions, compared with greenhouse and field experiments. Variation in metal bioaccumulation under different experimental conditions was attributed to elevated metal bioavailability in hydroponic trials compared with pot (under greenhouse conditions) and field experiments, as well as the bioavailability of competing elements (Figure 5.1). The influence of biogeochemical factors (e.g. type of substrate used), limiting the availability of metals and therefore, phytoremediation potential of plants, has also been reported in various studies (Rosenfeld *et al.*, 2018).

To strengthen any scientific investigation, all limitations must be considered and addressed when interpreting the obtained results. In this regard and by way of an example, one of the limitations

associated with the PTC included hyperhydricity, a phenomenon resulting in morphological and physiological (e.g., evapotranspiration) changes in explants cultured *in vitro* (Chapter 3). This limitation was addressed by the initiation of an open bench study, however, no explants established under these conditions. The elevated rate of evaporation resulted in the accumulation of salts at the base of the growth vial – an additional, unquantified variable that may have interfered with metal ion uptake by plants. Future studies should therefore investigate the ventilation of the culture vessel in order to account for the role of evapotranspiration in such metal uptake experiments (Lazzarini *et al.*, 2019).

Limitations associated with *in situ* experiments included the low sample size (N = 4). Due to logistic and financial constraints, along with the need to retain the efficacy of the phytoremediation trial, this limited sample size was considered adequate and representative of different size classes (namely very small, small, medium, and large tree sizes). Furthermore, these four trees were used as replicates. Plant BCF, TF, and TI (Chapter 2) calculations were based on the total metal content of analysed soil samples and not the bioavailable fraction of heavy metals present in the contaminated soil. This may have resulted in the underestimation of BCF, TF, and TI (Figure 5.1). Collectively these may account for the observed differences in metal uptake between the *in situ* and *in vitro* studies where the treatment metals (present as metal-sulphate salt compounds) may have had an elevated bioavailability compared with the species of metals (not determined in this study) present *in situ*. It is also important to appreciate that the results from the *in situ* experiment represent a 'snapshot' of the conditions present at the study site; that is the soil and plant samples data were obtained at a single point in time and space. This study, therefore, measured the efficacy of the phytoremediation trial at a single point in space and time, compared with the efficacy of the trial over time. To an extent this limitation was addressed by including the control soil pits (i.e., soil pits absent of *T. usneoides* trees - unplanted) providing insight into the efficacy of the phytoremediation trial relative to unplanted areas.

This study has created some consilience in this research area as the results obtained from the *in vitro*, hydroponic study (Chapter 4); namely *T. usneoides*' pattern of metal (i) accumulation [Ni > Co >> Pt (BCF > 1)], (ii) translocation [(TF < 1)], (iii) tolerance to a range of metal concentrations

and geochemical conditions (e.g., pH range), are supported by the *in-situ* study (Chapter 2). In terms of limitations of these two studies the factors influencing metal bioavailability which include the actual species of Pt, Ni, and Co present at the study site did not form part of this research (Figure 5.1). Furthermore, a known variable, differing between *in situ* and *in vitro* experiments, includes the presence of competing metal ions – namely Ni and Co (i.e., competitive ion adsorption).

Although Ni had an elevated *in planta* mobility, compared with Co, results from this study suggested that *T. usneoides* triggers similar processes and patterns in the accumulation and distribution of Ni and Co under *in vitro* conditions although these differ from the results obtained from the *in situ* study. This may be attributed to the (i) bioavailability of Ni and Co under *in vitro* conditions (with an elevated bioavailable fraction of total metal content under hydroponics was higher compared with field conditions), and (ii) presence of competing elements, namely competitive ion adsorption (whereby Ni may have outcompeted Co for uptake – antagonistic relationship) under field conditions (Moreno-Jiménez *et al.*, 2010, Rosenfeld *et al.*, 2018), and / or (iii) the elevated, initial Ni concentrations at the study site compared with Co (Chapter 2).

Thus, both the *in situ* and *in vitro* experiments demonstrated that *T. usneoides* has the ability to accumulate, translocate, and tolerate Ni, Co, and Pt under various experimental conditions. This emphasises *T. usneoides*' ability to be used as a tool for phytostabilisation, phytoextraction (i.e., rhizofiltration – rhizo-hyperaccumulation of Pt, Ni, and Co), and phytodesalination. These conclusions are supported by the results obtained by McBride and Zhou, (2019) where the elevated accumulation of metals occurring under hydroponic conditions correlated with the field studies on *Phytolacca americana*.

FACTORS INFLUENCING COMPARABILITY BETWEEN STUDIES DIFFERING IN EXPERIMENTAL CONDITIONS

Abiotic Factors

Field conditions (in situ)

Hydrogeochemical factors (e.g., element composition and concentration, pH, EC, and E_h) influence the fate of metals (metal speciation, bioavailable fraction, and type of metal available) in the environment. Comparisons between field and hydroponic studies, from studies such as Moreno-Jiménez *et al.*, (2010)**,** may have a limited interpretation as plants were exposed to differing metalloid species (i.e., NaH_2AsO_4 in hydroponics and an unquantified As species compound in the field trial). The composition of metals may also interfere (antagonistic interaction) with other metals for plant uptake (competitive ion adsorption). Therefore, quantifying the composition of elements at the field study enables researchers to identify any metals, sharing similar physicochemical properties, which may interact antagonistically, therefore contributing to the interpretation of obtained results. Under field conditions, abiotic factors fluctuate through space and time. This limitation is addressed by conducting *in vitro* studies where a plant species is exposed to a treatment with known abiotic factors (e.g., pH, element composition, temperature, etc.) – allowing plant responses to be distinguished. The use of other measurements (e.g., stem diameter) should also be investigated.

Hydroponic conditions (in vitro)

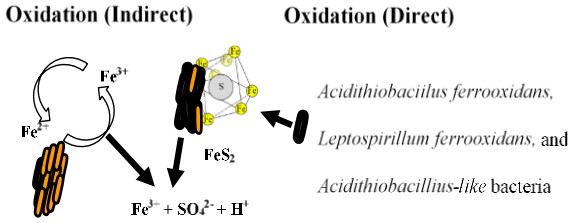
However, abiotic factors such as relative humidity may induce hyperhydricity – impacting the explant's morphological and physiological (e.g., evapotranspiration, ET) features. These features, including ET, are involved in metal uptake via the plant's transpiration stream. Thus, TF obtained from *in vitro* may be underestimated based on a low ET rate. Moreover, plants are never exposed to a single stress but rather a combination of stresses, at varying degrees, at any given point in time and space, limiting the applicability of utilizing *T. usneoides* in field trials.

Therefore, addressing these limitations, by conducting different types of studies, are required to validate results

Biotic Factors

Field conditions (in situ)

Microorganisms (e.g., plant-growth promoting bacteria and arbuscular mycorrhizal fungi) influence plant growth (by altering soil metal bioavailability) and the fate of metals in the environment (i.e., speciation of metals influencing their bioavailability, mobility, and phytotoxicity) (Ma, 2019). For example, microbial-assisted ARD generation results in iron (Fe) speciation and decrease in pH (see inset below) (Ayangbenro *et al.*, 2018, Tutu *et al.*, 2008). Plant growth promoting bacteria have been reported to enhance plant biomass production and plant's phytoremediation potential [see Ma, (2019)]. Moreover, the mobilization mechanism of some microbes may contribute to the plant's phytoextraction (via increased metal bioavailability) and/or phytostabilization (precipitation of metal-immobilizing bacteria) potential. Ma (2019) also identified a gap in research with regards to the characterization and understanding of the role of soil microbe communities, which vary through space and time, under field studies.



[Microbial-assisted acid rock drainage (ARD) generation]

Hydroponic conditions (in vitro)

Under these experimental conditions, the presence of pathogens (and their influence on distinguishing plant responses) can be controlled. These include the (i) pre-treatment of donor plants with pesticides (e.g., insecticide or fungicide); and / or (ii) aseptic culture conditions (including surface sterilization with NaClO, addition of antibiotics, etc.). Although PTC conditions enable the plants response to be distinguished from the influence of microbes – extrapolation of results obtained from *in vitro* studies must be validated using field studies.

Figure 0.1. Factors, limiting the ability to distinguish the plant's response to metals under differing experimental conditions. These factors must be considered when comparing results obtained from previous studies differing in experimental conditions. Arrow represents the interplay between factors. Please note that this discussion is not exhaustive.

5.3. Direction for future studies

Based on the findings of this thesis, future studies should focus on assessing the following variables. The genetic, molecular, cellular, and physiological mechanisms, as well as the interplay between these levels, were not distinguished in this study/thesis. Salt glands have been identified to confer salt and/or metal tolerance in other plant species via the storage and/or excretion of salts and their metal ion constituents (Dassanayake and Larkin, 2017, Kiani-Pouya *et al.*, 2017, Wei *et al.*, 2020). This may enable plants to selectively excrete excess metal ions via salt glands, contributing to the ability of *T. usneoides* to accumulate, translocate, and tolerate Pt, Ni, and Co under *in situ* and *in vitro* conditions. Future studies should investigate whether this salt removal mechanism is selective or ubiquitous and whether this excretory mechanism confers salt and / or heavy metal tolerance (i.e., mediate *T. usneoides'* metal homeostasis network).

One of the biological implications of this study is that soil amendments [e.g., Ethylenediaminetetraacetic acid (EDTA) or growth-promoting rhizobacteria (Abbaszadeh-Dahaji *et al.*, 2019)] may be used to increase the bioavailability of Pt, Ni, and Co, thereby increasing the uptake of these metals by *T. usneoides* roots. However, further studies would be required as such soil amendments may also increase the mobility and toxicity of these metals – impacting the receiving environment, as well as associated fauna and flora (Grčman *et al.*, 2001). Findings from Chapter 2 and Chapter 4 indicate that the study species tolerate metals to the degree of hyperaccumulation in roots (i.e., rhizo-hyperaccumulation). Therefore, studies differing in bio- and hydrogeochemical conditions should be conducted to validate results obtained from a particular study – increasing the network of cumulative evidence across experimental conditions.

As previously reported by Chen *et al.* (2017) and Bali *et al.* (2020), the exudation of compounds by roots has been demonstrated to influence the biological and physicochemical conditions of the rhizosphere (including the bioavailability of metals). Determining the composition and concentration of exudates may highlight (i) a potential *T. usneoides* tolerance mechanism to a range of pH levels, and (ii) may contribute to the alteration of metal bioavailability and subsequent

uptake by *T. usneoides.* The antagonistic interaction between metals with similar physicochemical properties has been reported in various studies (Polechońska and Samecka-Cymerman, 2018, van der Ent *et al.,* 2018, Yamaguchi *et al.,* 2017). Understanding whether such an interaction influences metal uptake by *T. usneoides* will impact the application of *T. usneoides* for the phytostabilization and phytodesalination of Ni / Co contaminated sites. Moreover, undertaking such a study would enable researchers to validate results obtained in Chapter 2, namely the elevated accumulation of Ni compared with Co. Thus, established *T. usneoides* plantlets should be exposed to binary (i.e., 25 % MS medium supplemented with Ni and Co) metal across differing Ni : Co ratios. S is incorporated into a range of biological compounds (e.g., glutathione and phytochelatins) involved in metal detoxification (Xiao *et al.,* 2020). *Tamarix usneoides* has been demonstrated to hyperaccumulate a range of salts including SO_4^{2-} (Chapter 2) (Dennis, 2008). Thus, the role of S in mediating metal uptake, translocation, and degree of tolerance by *T. usneoides* should be explored further.

Soil microbes (e.g. arbuscular mycorrhizal fungi (AMF))) influence metal bioavailability (Ma *et al.*, 2019) (Figure 5.1). Spruyt *et al.,* (2014) demonstrated that approximately 10% of *T. usneoides* roots, sampled from the study site (*refer to Chapter 2*), were predominantly colonised by *Claroideoglomus* arbuscular mycorrhizal species. Other *Tamarix* spp, such as *T. gallica*, have also been demonstrated to be mycorrhizal. Although the characterisation of AMF did not form part of this thesis, future studies should determine the composition and contribution of these microbes to the remediation potential of *T. usneoides*. This topic is currently under investigation by the EEPP (Table S1.2). Due to the application of pesticides to donor plants (prior to explant harvest and culture), as well as aseptic practices involved in PTC, determining the role of microbial activity *in vitro* is limited. Therefore, soil microbes at the study site may have mediated the fate of Pt, Ni, and / or Co in the environment and consequently, influenced the uptake and distribution of these metals by *T. usneoides* trees. Future studies should characterize the composition of microbial communities present at the study site and determine whether previously published studies have demonstrated the ability of characterized microbes to alter metal bioavailability.

The difference in the bioavailable fraction of metals under *in situ* and *in vitro* conditions should be determined. The potential differences in metal (Pt, Ni, and Co) speciation *in situ* and thus, the bioavailability of metals influence BCF, TF, and TI, limiting the comparability of these parameters between studies differing in experimental conditions. This is a gap in knowledge and research in the field of phytoremediation whereby the aims of future studies should include standardizing certain factors (e.g., exposure to similar metal species) under different experimental conditions.

5.4. Concluding remarks

This study has created consilience in this research area and highlighted the ability of *T. usneoides* to take up [Ni > Co >> Pt (BCF > 1)], translocate [TF < 1 *in vitro* / TF > 1 *in situ*], and tolerate Pt, Ni, and Co across a wide metal concentrations and pH range, under different experimental conditions. The biological implications of this work, as well as the novelty of this work (Table S1.2) which contributes to both the scientific, and non-scientific, community, are described below.

Hydroponic and field studies should be conducted in combination to (i) validate results obtained from either type of study, and (ii) to distinguish the degree to which the study species can accumulate, translocate, and / or tolerate metals of interest. *T. usneoides* can take up and translocate Pt, Ni, and Co from geochemically complex sites (Chapter 2) – supporting results obtained *in vitro* (Chapter 4) whereby *T. usneoides* plantlets have the ability to rhizo-hyperaccumulate metals (i.e., application for rhizomining). Hydroponic and field studies demonstrated that *T. usneoides* can tolerate varying concentrations and compositions of elements whilst simultaneously (hyper)accumulating metals and salts.

A reproducible PTC protocol was developed for the establishment of *T. usneoides* explants *in vitro*. This protocol may be used to screen plant species (and their genotypes) for their phytoremediation potential and the subsequent mass propagation of genotypes with desirable traits. *In vitro* screening of plants prior to planting in field trials may also reduce plant mortality due to unknown plant properties, i.e. the degree of metal tolerance, and whether edible plants (such as

Amaranthus spp.) can hyperaccumulate toxic concentrations of metal ions into edible plant organs (e.g., leaves) must be investigated prior to utilizing such plants for phytoremediation. This is due to the introduction of toxic metals into the food chain - impacting faunal health and survival (Naikoo *et al.*, 2021). This protocol may also be adapted for the establishment of other recretohalophytes.

Although Ni has an elevated *in planta* mobility compared with Co, results from this hydroponic study suggest that *T. usneoides* exhibits similar patterns of accumulation and translocation for Ni and Co when treated with either metal (i.e., not in combination) under similar experimental conditions. This may be attributed to Ni and Co having similar physicochemical properties, compared with Pt. This finding suggested that differences in Ni and Co TFs from *T. usneoides* trees harvested from the site may be attributed to the antagonistic effect of Ni and Co uptake via competitive ion. Another biological implication of this finding is that *T. usneoides* may be effectively used for the phytoremediation of Ni or Co on Ni-polluted or Co-polluted soils where the competing metal (i.e., Co or Ni, respectively) is absent.

The patterns of metal accumulation and translocation, under *in situ* and *in vitro* (rhizo-hyperaccumulation where BCF > 1 and TF < 1) conditions highlight the use of *T. usneoides* as a potential candidate for phytostabilization and phytoextraction (under hydroponic conditions). The hyperaccumulation of sulphur from the field study suggested a potential application of *T. usneoides* for the desalination of saline metal-contaminated sites whilst simultaneously accumulating Ni > Co >> Pt. Medium and larger trees have an elevated ability to effectively desalinate and accumulate elevated metal concentrations from the saline metal-contaminated study site. Thus, *T. usneoides* can be utilized as an effective tool for phytoremediation of cross (salt and metal)-contaminated sites, a phenomenon which is expected to increase due to the simultaneous salinization and heavy metal pollution of land and water by anthropogenic processes (Shang *et al.*, 2020).

The hyperaccumulation threshold of other elements has been defined based on the active accumulation (i.e. in leaves) of two-to-three orders of magnitude greater than *in planta* metal concentrations of reference plants growing on non-polluted and polluted sites (Baker, 1981, Reeves,

2006, Van der Ent *et al.*, 2013). The Pt hyperaccumulation threshold could therefore be defined as 1 - 4 mg/kg in unpolluted and polluted soils, respectively. Thus, roots of *T. usneoides* plantlets hyperaccumulated Pt in a dose-dependent fashion at pH 5.5 (i.e. Pt rhizo-hyperaccumulation). The biological implication of this finding involves the use of *T. usneoides* as an effective tool for the removal of Pt (i.e., phytoextraction) from effluent and therefore, the potential for Pt rhizomining from effluent containing residual Pt concentrations.

Tamarix usneoides is tolerant to Ni, Co, and Pt at 1, 10, and 10,000 times the average soil crustal abundance, respectively, holding potential for effective recovery of Ni and Co (and Pt to a lesser degree) from effluents under moderately acidic- to alkaline-conditions. Genotypes LH31, LH47, and LH48 hold the most potential for rhizomining. Variability in Co accumulation capacity between genotypes indicated that selective breeding for improved traits is feasible. The combination of *in situ* and *in vitro* studies has improved our understanding of the previously undetermined patterns of Pt, Ni, and Co accumulation (between plant organs), translocation, and tolerance by *T. usneoides* under field (geochemically complex substrate) and *in vitro* experimental conditions. The undertaking of both *in situ* and *in vitro* experiments has also improved our understanding and ability to (i) validate results obtained from a single type of study; (ii) compare studies differing in experimental conditions by distinguishing the plant's response to specific, quantified factors, and (iii) how certain limitations can be addressed in future studies.

Based on the results obtained in the *in vitro study* (Chapter 4), *T. usneoides* plantlets exhibited elevated tolerance to the investigated metals across a wide metal concentration range (25 – 100 mg/kg) under both moderately acidic (pH 5.5) and slightly alkaline (pH 7.5) *in vitro* conditions. Results demonstrate that *T. usneoides* has the potential to effectively recover Ni and Co (and Pt to a lesser degree) from effluents. Moreover, the similarity in Ni and Co BCF, TF, and TI patterns indicate similar patterns of sorption, transport, assimilation, and tolerance to either metal when treated separately. Moreover, the variability in Co accumulation capacity (i.e., Co was actively accumulated in the shoots of two genotypes) between genotypes indicated that selective breeding for improved rhizofiltration and phytoextraction traits is feasible. However, whether phytoextraction of Pt, Ni, and

Co can be achieved from more complex media and soils requires further study. Results obtained from the *in situ* study (Chapter 2) demonstrate that *T. usneoides* significantly reduced the EC (elevated removal of salts including S salts) of the 0 – 100 cm depth of the substrate, whilst simultaneously accumulating Ni and Co (and Pt to a lesser degree). In conclusion, the managed growing and harvesting of *T. usneoides* can create conditions which are conducive to the survival of certain glycophytes, namely *S. lancea*, *S. pendulina*, and *Berkheya coddii*.

Tamarix usneoides is thus able to assimilate, translocate, and tolerate Ni, Co, and Pt (to a lesser degree) when exposed to metals across a wide pH and metal concentration range, under differing (field and *in vitro*, hydroponic) experimental conditions. This opens the possibility for the species to be used in a range of phytotechnologies.

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Supplementary Data

Chapter 1

Supplementary Table 1.1: Use of platinum (Pt), nickel (Ni), and cobalt (Co) in various industries.

Metal	Industry	Use in industry	Reference
Pt	Automotive	Used in combination with other PGMs, as an active component of vehicle catalytic converters. Pt catalyses the oxidation of carbon monoxide (CO) and unburned hydrocarbons into carbon dioxide (CO_2) and water (H_2O) whilst catalysing the reduction of nitrous oxide (NO_x) into nitrogen and oxygen (O_2) in which the hydrocarbons, CO and hydrogen are used as reducing agents.	(Bali *et al.*, 2010, Cowley, 2011)
		Pt is also used as a catalyst in fuel cells to reduce O2, as well as in the petroleum industry in catalytic reforming to refine cycloalkanes into high-octane petrol.	(Crundwell *et al.*, 2011)
	Medicine	Pt nanoparticles incorporated into antitumor drugs (cisplatin, carboplatin, and oxaliplatin) in cancer therapy (bind to DNA thereby disrupting replication processes).	(Alonso *et al.*, 2012)
	Jewellery	Pt for fine detailed jewellery castings.	(Zereini and Wiseman, 2015)
Ni	Construction and metallurgy	Ni is used to produce alloy (when combined to Fe, Cu, Cr and/or Zn) and extensively used to produce stainless steel. Ni is also used in the aerospace industry.	(Easton *et al.*, 1992)
	Electroplating	Used as a catalyst.	(ATSDR, 2005, Yusuf *et al.*, 2011)
	Electronics	Major component of electric batteries.	
Co	Metallurgy	Majority (26% of total demand) of cobalt production is used to make Co-based super-alloys (in Ni-based and Fe-based super-alloys). Cobalt is also used in other alloys such as low-expansion alloys, corrosion-resistant alloys, and in magnetic alloys. Cobalt is also used in cemented carbides as a ductile metallic binder. Used in gas turbines and engines of aeroplanes. Radioisotopes of Co are used in industrial radiography (I.e. to determine quality of welds). Cobalt is also added to Ni alloys to improve the alloy's stability at high temperatures. Platinum and Co (23% Co) is combined to produce corrosion- and heat- resistant, isotropic permanent magnets.	(Michel *et al.*, 1991); (Crundwell *et al.*, 2011, Hamilton, 1994, Hawkins, 2001)
	Medicine	Cancer treatment (use of the synthetic radioactive isotope, ^{60}Co). Vitallium (Co-Cr-Mo alloy) used in prosthetics as components of knee and hip replacements. ^{57}Co is used in medical research for radiolabelling vitamin B12. Also, used for sterilization of equipment and medical waste.	
	Electroplating	Cobalt sulphate ($CoSO_4 \cdot 7H_2O$) is used as an electrolyte in Co electroplating in operating conditions of pH 1-4, concentrations of 332g/L, at temperatures of 20-50°C. Cobalt is also used in wear-resistant coatings.	

Food	Crop seed and germplasm irradiation (radiation in the form of heavy-ion beam which controls and reduces food spoilage and foodborne pathogens). Cobalt is also used for food sterilization in the process of cold pasteurization.
Dentistry	Cobalt alloys used in dental prosthetics.
Jewellery	Cobalt is alloyed with 95% Pt for fine detailed jewellery castings.
Electronics	Lithium cobalt oxide ($LiCoO_2$) is used in the cathodes of lithium-ion batteries. Also improves nickel battery's capacity for oxidation thereby, increasing the battery's power output. Nickel metal hydride and Ni-Cd batteries also contain large amounts of Co for this increase in oxidation capacity. Other uses include semiconductors and gas sensors.
Chemistry	Used as oxidation catalysts (such as cobalt carboxylates which are used to catalyse the oxidation of drying oils). Co is used as a catalyst in the Fischer- Tropsch process which coverts CO and H_2 into liquid hydrocarbons. Cobalt-molybdenum is used in the removal of sulphur impurities from petroleum. Also used in adhesives.
Nuclear warfare	Production of cobalt bombs- ^{59}Co may be used in nuclear warheads which trans-mutates into ^{60}Co due to the input of energy into the system on impact. ^{60}Co also has indirect effects as it produces a fine dust.

Supplementary Table 1.2. List of postgraduate degrees conducted at the University of the Witwatersrand between 2006 and 2022 on the biology or ecology of *Tamarix usneoides*. MWP: Mine Woodlands Project, Ecological Engineering & Phytotechnology Programme, School of Animal, Plant & Environmental Sciences, University of the Witwatersrand (Programme leader: I.M. Weiersbye). BCP: Alien Plant Biocontrol programme, School of Animal, Plant & Environmental Sciences, University of the Witwatersrand (Programme leaders: M. Byrne and S. Newete). Research conducted for non-degree purposes is not listed.

Programme	Year	Author (Degree)	Supervisor / Advisor	Title
MWP	2008	A. Dennis (BSc Hons)	I.M. Weiersbye	Salt glands and elemental concentrations in leaves of *Tamarix* species (Tamaricaceae) growing on seepage from Highveld gold mines
MWP	2010	L. Kendall (BSc Hons)	E. Cukrowksa, I.M. Weiersbye	Phytoextraction of selenium and sulphur by the halophyte tree *Tamarix usneoides*
MWP	2010	H. Wilson (BSc Hons)	J. Botha, I.M. Weiersbye	Optimisation of a protocol for propagation of nodal cuttings of *Tamarix usneoides* E. Mey ex Bunge in autumn
MWP	2010	G. Mayonde (BSc Hons)	M. Byrne, G. Cron / I.M. Weiersbye	Molecular phylogenetic investigation for identification of *Tamarix* species and hybrids in South Africa
MWP	2011	L. Buckham (MSc)	M. Byrne, I.M. Weiersbye	Contrasting growth traits and insect interactions of two *Tamarix* species and a hybrid (Tamaricaceae) used for mine rehabilitation in South Africa
MWP	2011	A. Spruyt (BSc Hons)	C. Straker / I.M. Weiersbye	Arbuscular mycorrhizal fungi associated with *Tamarix usneoides*
MWP	2011	S. Buck (BSc Hons)	C. Straker / I.M. Weiersbye	Status and molecular identification of arbuscular mycorrhizal fungi (AMF) associated with planted and wild populations of *Tamarix usneoides* E. Mey. ex Bunge growing in an arid environment and heavy metal contaminated sites
MWP	2011	M. Govender (PhD)	P.J. Dye, E.T.F. Witkowski / I.M. Weiersbye, F. Ahmed	Assessing water access by trees growing above contaminated groundwater plumes originating from gold tailings storage facilities
MWP	2012	K. Stausebach (BSc Hons)	P.J. Dye, I.M. Weiersbye	Estimating water-use by *Tamarix usneoides* grown on acid mine drainage in a semi-arid environment: the Mine Woodlands Project
MWP	2013	Z. Ndlovu (BSc Hons)	E.N. Bakatula, I.M. Weiersbye / H.	The influence of three tree species on cyanide degradation and

			Tutu	evolution in a gold tailings storage facility (TSF)
MWP	2013	A. Mader (BSc Hons)	D. Mycock, I.M. Weiersbye	The impact of pH on growth and gold allocation in *Tamarix usneoides* E. Mey ex Bunge *in vitro*
MWP	2014	S. Arendse (MSc)	H. Tutu / I.M. Weiersbye	The effect of trees on physical and chemical properties of substrata contaminated by gold mine waste disposal
MWP	2014	M. Molepo (BSc Hons)	I. Weiersbye, D. Mycock	Hyperaccumulation of sulfur by the halophyte, *Tamarix usneoides* E. Mey ex. Bunge *in vitro*
MWP	2014	S. Grindley (MSc)	P.J. Dye, C. Curtis / I.M. Weiersbye	Modelling the effects of trees on a contaminated groundwater plume from a gold tailings storage facility in the Orkney district
MWP	2015	H. Wilson (MSc - Upgraded)	D. Mycock / I.M. Weiersbye	Elemental accumulation and excretion processes in the tissues of *Tamarix usneoides* (Tamaricaceae) and their relation to gold phytoextraction
BCP / MWP	2015	S.G. Mayonde (MSc)	M. Byrne, G. Cron / J. Gaskin, J. Botha, I.M. Weiersbye	Genotypic and phylogeographic investigation of indigenous and alien *Tamarix* species in southern Africa
MWP	2016	M. Magayuranga (MSc)	E.T.F. Witkowski / I.M. Weiersbye	Changes in floristic and functional composition on a complex of gold tailings dams near Carletonville, North West Province from 1996 to 2014
MWP	2018	A. Mader (MSc - Upgraded)	D. Mycock / I.M. Weiersbye	Tolerance, uptake and translocation of Ni, Co and Pt in *Tamarix usneoides* E.Mey ex Bunge
MWP	2018	D.P. de Villiers (MSc)	S. Webb / M. Jones, I.M. Weiersbye	Mapping acidic mine water using geophysics – impact of trees
MWP	2018	K. Lucioli (BSc Hons)	I.M. Weiersbye	Testing *Tamarix usneoides* E. Mey ex Bunge for tolerance to copper and zinc, and allocation pattern
BCP	2018	S. Allem (MSc)	S. Newete, M. Byrne	Salt excretion in alien and native *Tamarix* species in South Africa and the effects of salt on the *Tamarix* leafhopper (*Opsius stactogalus*)
BCP / MWP	2019	S.G. Mayonde (PhD)	M. Byrne, G. Cron / K. Glennon	Genetic diversity of *Tamarix* lineages in South Africa and heavy metal tolerance of the indigenous species
BCP	2019	E. Smit (MSc)	D. Marlin, M. Byrne	Host specificity testing of *Diorhabda carinulata* (Coleoptera: Chrysomelidae) as a biological control agent of *Tamarix* spp. (Tamaricaceae)
MWP	2020	H. Wilson (PhD - upgrade)	D. Mycock / I.M. Weiersbye	Elemental accumulation and excretion processes in the tissues of *Tamarix usneoides* (Tamaricaceae) and their relation to gold phytoextraction

MWP	2020	N.R. Ndou (MSc)	S.O. Bada, R. Falcon, I.M. Weiersbye	Combustion and co-combustion characteristics of *Searsia lancea* and *Tamarix usneoides* with coal
MWP	2020	R.L. Setsepu (MSc)	S.O. Bada, I.M. Weiersbye	The utilisation of *Searsia lancea* and *Tamarix usneoides* as feedstock for biochar production through hydrothermal carbonisation
BCP	2020	L. Drude (MSc)	D. Marlin, M. Byrne	Increased soil salinity has no effect on the host preference and suitability of the *Tamarix* biological control agent, *Diorhabda carinulata*
MWP	2022	A. Mader (PhD - upgrade)	D. Mycock / I.M. Weiersbye	Tolerance, uptake and translocation of Ni, Co, and Pt in *Tamarix usneoides* E. Mey ex Bunge
MWP	In abeyance	C. Sililo (MSc)	C. Curtis, J. Fitchett / I.M. Weiersbye	Modelling hydrological control and decontamination of smelter run-off and seepage effluents by natural vegetation and planted shrubs

Supplementary Table 1.3. Phytoremediation studies investigating the ability of different plant species to hyperaccumulate Pt, Ni, and Co under varying experimental conditions. Heavy metal hyperaccumulators were classified according to studies and the Global Hyperaccumulator Database (http://hyperaccumulators.smi.uq.edu.au/collection/) (Reeves *et al.*, 2018). Metal hyperaccumulation threshold was identified according to (Van der Ent *et al.*, 2013). No study has been conducted to investigate the ability of a halophyte to remediate land contaminated by Pt. Moreover, studies investigating Pt uptake by plants are generally amid the potential pollution of roadsides via the abrasion of catalytic converters and thus, the release of PGEs into the environment. Therefore, to the knowledge of the authors, no study investigating the ability of plants to take up Pt for its recovery/ remediation has been conducted. ***Note: Nature of plant species: H = halophyte; G = glycophyte; MH = metal hyperaccumulator; MA = metal accumulator; NMA = non-metal accumulator.**

Metal	Plant species	Nature of plant species*	Study conditions	Study Results and Outcome	Reference
Platinum (Pt)	*Sinapis alba* *Lepidium sativum*	G/MA G/MA	Plants were established in distilled water. *L. sativum* was transferred into growth media containing different Pt (as nanoparticles) concentrations (0, 1, 10, or 100 mg/L. *S. alba* plants were transferred to growth medium containing Pt (as nanoparticles) at 0, 1, 10, or 50 mg/L. Growth medium was supplemented with Pt nanoparticles or a salt [$Pt(NH_3)_4](NO_3)_2$] at 1 mg/L.	Pt was concentrated, by both plant species, in roots compared with shoots. Pt (as Pt-NPs) accumulation in shoots ranged from 3.1 - 563 mg/kg and 3.5 - 54 mg/kg whereas roots accumulated 148 – 7460 mg/kg and 190 – 8752 mg/kg in *L. sativum* and *S. alba*, respectively. When plants were exposed to the Pt-salt, Pt accumulation was approximately 220, 30, and 3 times higher in leaves, stems, and roots, compared with accumulation levels when plants were exposed to Pt-NPs. Moreover, TF was 45 % higher when plants were exposed to Pt-salts compared with Pt-N Ps. Study concluded that the presence of Pt-salt increased the bioavailability of Pt resulting in elevated accumulation by both plant species.	(Asztemborska *et al.*, 2015)
Pt	Grass spp (species not mentioned)		Plants were collected from four sampling sites along roadsides at 0, 1, 2, and 5 m sampling interval distances away from the road.	Pt accumulation in soils decreased as distance from road increased, where Pt accumulation was directly proportional to concentration of Pt in soil. Plants accumulated 12.04 ± 8.26, 8.27 ± 6.92, 5.52 ± 3.15, and 6.60 ± 6.69 µg/kg at 0 m (Pt_{soil} = 15.94 ± 7.54 µg/kg), 1m (Pt_{soil} = 9.18 ± 2.89 µg/kg), 2 m (Pt_{soil} = 4.13 ± 1.74 µg/kg), and 5m (Pt_{soil} = 2.04 ± 1.35 µg/kg) sampling distances. Influence of salinity was not investigated or discussed.	(Hooda *et al.*, 2008)
Pt	*Taraxacum officinale* (Dandelion) *Plantago lanceolata*	G/MA(Pb/Zn), G/MH,	Plants were grown in two types of soil, collected from agricultural sites, supplemented with Pt (as a salt, hexachloroplatinum, $[PtCl_6]^{2-}$) at 1000	Glycophytes [*T. officinale* (5.4 ± 0.4 ng/g), *P. lanceolate* (3.6 ± 0.5 ng/g), *V. pratense* (5.9 ± 0.6 ng/g), and *R. squarrosus* (30 ± 2 ng/g)] accumulated low levels of Pt where TF < 1 (0.004 and 0.008, for the two tested soil types). The accumulation of Pb by plants was	(Djingova *et al.*, 2003)

	(Plantain) *Lolium multiflorum* (Annual ryegrass) *Rhytidiadelphus squarrosus* (Moss)	G/NMA	µg/L.	positively concentrated to accumulated Pt concentrations.	
Pt	*Sinapis alba* (Mustard) *Zea mays* (Maize)	G/MA G/ NMA	Pot trials – plants were irrigated with nutrient solutions under greenhouse conditions. Nutrient solutions were supplemented with 500 mg/L Pt-salt [$Pt(NH_3)_4(NO_3)_2$].	Pt was mainly accumulated in roots (444 and 239 mg/kg) compared with shoots (~ 44 and 12.2 mg/kg) of *S. alba* and *z. mays*, respectively. Pt TF < 1 whereas BCF for roots > shoots for both plant species. Influence of salinity was not investigated or discussed.	(Kowalska *et al.*, 2004)
Pt	*Lolium multiflorum*	G/NMA	Plants were grown hydroponically, where growth medium was supplemented with medium and excess concentrations of Pt (3.6 mg/L and 38.7 mg/L, respectively), Pd (4.4 mg/L and 21.7 mg/L, respectively) and Rh (0.5 mg/L and 7.1 mg/L, respectively) at pH5.3. PGEs were supplemented as salts, namely $Pt(NH_3)_4(NO_3)_2$, $Pd(NH_3)_4(NO_3)_2$ and $Rh(NO_3)_3.2H_2O$	Pt (as well as Pd and Rh) accumulation in roots and leaves in a time-dependent response where PGE accumulation increased as exposure time increased. Elevated Pt concentrations were accumulated in roots (1 910 and 6230 mg/kg, respectively) compared with shoots (16.60 and 70.60 mg/kg, respectively) after 8-day treatment period at medium or elevated PGE treatment concentrations, respectively. Elevated Pt was accumulated, compared with Pd and Rh, which was attributed to the elevated translocation ability of Pt. Although the influence of salinity was not investigated, the metal-salt species may have increased the bioavailability of Pt and Pd, compared with Rh.	(Leśniewska *et al.*, 2004)
Pt	*Scutellaria baicalensis*	G/ MA	Plant samples were collected from natural habitat, horticultural areas, and in polluted areas surrounding ore deposits.	Elevated levels of Pt were generally concentrated in shoots (leaves and stems) compared with roots (i.e. TF > 1). For example, Pt_{soil} = 0.001 – 0.426 g/t; Pt_{roots} = 0.001 - 0.33 g/t Pt_{stem}= 0.040 - 0.43 g/t; Pt_{leaves}= 0.001 - 0.3 g/t; $Pt_{flowers}$ = 0.082 - 0.24 g/t; and Pt_{seeds}= 0.007 - 0.028 g/t. Influence of salinity was not investigated or discussed.	(Pshenichkina and Pshenichkin, 2018)

Pt	*Zea mays* (Maize) *Pisum sativum L.* (Pea)	G/MA G/ NMA	Seeds and seedlings were exposed to Pt (as a salt – $PtCl_4$)	Pt was accumulated in a dose- and time-dependent response, where Pt accumulation in *P. sativum* and *Z. mays* increased as Pt treatment concentration increased.	(Mikulaskova *et al.*, 2013)
Pt	*Elodea canadensis* *Peltandra virginica*	G/ Cr-MA G/NMA	Plants were grown hydroponically for 2 weeks in nutrient solutions, supplemented with Pt^{4+} at concentrations between 0.05 and 5 mg/L, or a 0.1 mg/L PGE (P^{t4+}, Rh^{3+}, Pd^{2+}) mixture. Plants were grown under controlled conditions. PGEs were added to nutrient solutions as salt complexes - Pt(IV) as hexachloroplatinic acid ($H_2[PtCl_6].6H_2O$), Pd(II) as $PdCl_2$ and Rh(III) as $RhCl_3$.	PGEs were accumulated in a dose-dependent response (R^2 = 0.9958) where *in planta* Pt accumulation by *E. canadensis* were 0.05 mg Pt/kg (6.4 ± 0.9 mg/kg; BF = 127.4), 0.1 mg Pt/kg (8.8 ± 1.1 mg/kg; BF = 87.7); 1.0 mg Pt/kg (216 ± 7.3 mg/kg; BF = 216), and 5.0 mg Pt/kg (1628.4 ± 95.7 mg/kg; BF = 325.7). PGE uptake did not interfere with Ca, Fe, or Cu uptake. Although the influence of salinity was not investigated, the type of PGE-salt constituent may have increased the bioavailability of Pt compared with Pd and Rh.	(Diehl and Gagnon, 2007)
Pt	*Sinapis alba*	G/MA	Plants were cultivated hydroponically for three weeks followed by a two-week exposure period of PGEs (as metal-chloride complexes), namely Pt, Pd, or Rh at 0.5 mg/L or 1.0 mg/L.	Pt was accumulated in a dose-dependent response. Pt was mainly concentrated in plant roots compared with shoots (TF < 1). At lower Pt treatment (0.5 mg/L), roots (762 ± 20 mg/kg) > stems (14.9 ± 0.4 mg/kg) > leaves (1.06 ± 0.02 mg/kg) whereas at elevated Pt treatment concentration, roots (5973 ± 140 µg/g) > aboveground plant parts (95.8 ± 1.2 mg/kg). Elevated concentrations of Pt were accumulated by plant roots compared with Pd or Rh. Exposure to PGE induced phytochelatin (PC) synthesis. Presence of PGEs as chloride complexes highlights the elevated bioavailability of Pt compared with Pd and Rh. Influence of salinity was not investigated.	(Kińska and Kowalska, 2019)
Pt	*Sarcocornia fruticosa*	H/MA	Plants samples (roots, stems, and leaves) were collected from two different salt marshes (where sites differed in traffic density) over two seasons (spring and summer).	Low traffic density site had an elevated salinity which increased Pt bioavailability and contributed to Pt uptake by *S. fruticosa*. Pt accumulation in plant roots increased as salinity in interstitial water increased. Pt was retained in roots where Pt concentrations in roots were 30 times higher compared with aboveground biomass (stems	(Almécija *et al.*, 2017)

				and leaves). Authors concluded that the active biogeochemical cycle of Pt occurs in belowground biomass. Pt concentrations ranged from roots (0.027 – 1.6 ng/g) > leaves (0.039 – 0.13 ng/g) > stem (0.012 – 0.086 ng/g (over two sampling seasons and two locations).	
Pt	*Arabidopsis thaliana*	G/NMA	Plants were grown hydroponically under controlled conditions. Plant growth media was supplemented with Pt^{2+} as a salt $[Pt(NH_3)_4](NO_3)_2]$ at various concentrations at pH 6.2.	Pt accumulation in plant roots and rosette increased in a dose-dependent response, where Pt accumulation significantly differed between plant parts. Pt was retained in roots (where TF < 1). Pt accumulation ranged from 100 ± 2.26 – 425 ± 5.86 ng/g in roots and 17.9 ± 0.41 – 210 ± 4.40 ng/g in the rosette at the Pt treatment range. Increasing Pt concentrations induced a hermetic response where growth was stimulated at lower Pt concentrations and impeded at elevated Pt concentrations. Exposure to Pt also induced GSH synthesis. The influence of salinity was not investigated or discussed.	(Gawrońska *et al.*, 2018)
Nickel (Ni)	*Alyssum bertolonii* *Nicotiana tabacum* (Tobacco)	Ni MH	Plant roots were cultured *in vitro* under controlled conditions (plant tissue culture)	Nickel accumulation by *N. tabacum* decreased as metal exposure time increased. After 30-day metal exposure period, *A. bertolonii* explants accumulated a maximum of 900 mg/kg whereas *N. tabacum* accumulated 400 mg/kg. Physiological growth of *A. bertolonii* roots were not affected by elevated Ni treatment, thus, *A. bertolonii* tolerated elevated Ni concentrations. Ni induced oxidative stress in both plant species.	(Boominathan and Doran, 2002)
Ni	*Mesembryanthemum crystallinum* *Brassica juncea*	H G	Pot trials – plants were grown under ambient environmental conditions where growing medium (soil) was supplemented with Ni as a salt ($NiCl_2$). For Ni treatments, the soil was artificially contaminated with 25, 50 and 100 mg Ni/kg.	Increasing addition of Ni-salt ($NiCl_2$) increased Ni^{2+} accumulation in roots and translocation (and subsequent accumulation) in shoots) of *M. crystallinum*. Elevated concentrations of Ni were accumulated in roots of both species whereas the *M. crystallinum* accumulated significantly higher Ni in shoots and roots compared with *B. juncea*. For example, 78 mg/kg and 57 mg/kg at 100 mg/kg $NiCl_2$ respectively; and roots: 371 and 152 mg/kg at 100 mg/kg $NiCl_2$, respectively. The effective salt tolerance mechanisms may have increased Ni uptake due to its presence as a salt in the medium. It must be noted that the uptake of Ni by *B. juncea* also increased in a dose-dependent response however, to a lower degree. Thus, Ni TF for *M. crystallinum* > *B. juncea*.	(Amari *et al.*, 2017)

Ni	*Sesuvium portulacastrum*	H	Plants were propagated via cuttings and grown under greenhouse conditions. Once established, plants were transferred to a hydroponic solution (Hoagland's complete nutrient solution) which was supplemented with only Ni or in combination with Cd.	Ni was mainly accumulated in roots compared with shoots. Presence of Cd reduced Ni accumulation in the root (potential competitive ion adsorption interaction). Ni concentration in xylem sap reached 1 450 µg/L – demonstrating the ability of the halophyte to effectively absorb and translocate metals.	(Mnasri *et al.*, 2015)
Ni	*Spirodela polyrrhiza*	G/ Cd-MH	Plants were grown hydroponically in Hoagland nutrient medium. Growth media was supplemented with doses of NaCl along with Ni as a salt [$Ni(NO_3)_2.6H_2O$] at 20 mg/L and Cd as a salt ($CdCl_2$) at 2 mg/L. Growth media pH = 7.	Heavy metal (Ni and Cd) accumulation significantly decreased as salinity level increased. Relative growth rate (RGR) of the Cd-hyperaccumulator decreased as salinity level increased. Increase in salinity (with presence of heavy metals) decreased photosynthetic pigment (chlorophyll a and b, and carotenoid) content of plants, resulting in chlorosis.	(Leblebici *et al.*, 2011)
Ni	*Brassica juncea* (Indian mustard)	G/NMA	Pot trials – seeds and seedlings (established in soil supplemented with $NiCl_2$ at 100 mg/kg) were grown in soil containing NaCl for 15-days followed by a spraying with salicylic acid. The influence of salinity on Ni accumulation was not investigated or discussed.	Plant exposure to Ni and/or NaCl negatively impacted the growth and physiology of *B. juncea*, namely leaf water potential, photosynthetic pigments, and overall growth. Spraying plants with salicylic acid reduced the negative impacts associated with Ni and/or NaCl stress by the glycophyte. The accumulation of Ni, as well as the influence of NaCl on Ni accumulation, was not investigated in this study.	(Yusuf *et al.*, 2011)
Ni	*Triticum aestivum* (Wheat)	G/NMA	Pot trials – growth medium was supplemented with Ni [as a salt, $Ni(NO_3)_2.6H_2O$] under two different salinity levels (0 or 10 dS/m).	Elevated soil salinity decreased Ni uptake and translocation across Ni treatments. Ni accumulation (concentrated in roots > shoots) was negatively impacted by increasing Ni treatments where plants had a hermetic response (i.e. plant growth was stimulated 20 mg Ni/kg but decreased at 40 mg Ni/kg). Potassium accumulation increased as Ni treatment concentration increased whereas Na^+ concentrations decreased (suggesting a potential Na - Ni antagonistic interaction in glycophytes).	(Ain *et al.*, 2016)

Ni	*Sesuvium portulacastrum* *Cakile maritima*	H H	Plants were grown hydroponically and supplemented with Ni (as a salt – $NiCl_2$) for 21 days.	Nickel was concentrated in roots by both halophytes, where Ni accumulation increased in a dose-dependent response. Elevated Ni was accumulated by *S. portulacastrum*, compared with *C. maritime*, across all Ni treatments. A maximum of 1050 and 550 mg Ni/g was accumulated in the shoots of *S. portulacastrum* and *C. maritima*, respectively. However, *C. maritima* accumulated elevated Ni in roots compared with *S. portulacastrum*. Authors concluded that *S. portulacastrum* had an elevated degree of Ni tolerance, compared with *C. maritima*, which may be attributed to the halophyte's elevated ability to sequester Ni in cell walls (restricting the accumulation of the soluble fraction of Ni).	(Fourati *et al.*, 2016)
Ni	*Atriplex hortensis* var. *purpurea* *Atriplex hortensis* var. *rubra* *Atriplex rosea*	H H H	Pot trials – plants grown in soil supplemented with differing metal (Ni, Cu, Pb, and Zn) concentrations (0, 25, 50, and 100 % strength) under greenhouse conditions.	Metal (Ni, Cu, Pb, and Zn) accumulation in roots > shoots increased in a metal dose-dependent response. Metals were generally concentrated in roots (TF < 1) suggesting a metal-exclusion tolerance mechanism at the root level. Accumulation of metals in shoots significantly differed between *Atriplex* spp. *Atriplex* spp did not hyperaccumulate Ni (> 1000 mg/kg) however, *A. hortensis* var. *rubra* accumulated elevated Ni in shoots (61 mg/kg) and roots (1593 mg/kg), compared with other species/ varieties. BCF for *A. hortensis* var *purpurea* (ideal candidate for phytostabilization), *A. hortensis* var. *rubra*, and *A. rosea* ranged 0.95 - 1.13, 0.95 - 1.37, and 0.36 - 0.55, respectively.	(Kachout *et al.*, 2012)
Ni	*Populus alba* *Morus alba*	G G	Pot trials – plants grown in soil supplemented with Ni (120, 240, and 480 mg/kg), Cd (40, 80, and 160 mg/kg), and Cr (60, 120, and 240 mg/kg), under ambient environmental conditions. EC = 1.2 mS/m.	Ni BCF increased in a dose-dependent response. BCF for *M. alba* increased but decreased for the 480 mg Ni/kg treatment for shoots and roots. Although TF decreased as Ni treatment increased, *P. alba* had TF > 1 across Ni treatments. Elevated Ni concentrations were accumulated in green leaves > fallen Leaves > roots > stems for *P. alba* whereas Ni accumulation was generally in the order; fallen leaves > green leaves > root > stem for *M. alba*. Due to the elevated TF and BCF in aboveground biomass, authors concluded that *P. alba* and *M. alba* are ideal candidates for phytoextraction of Ni (as well as Cd and Cr) and not phytostabilization. The influence of salinity on metal accumulation was not investigated or discussed.	(Rafati *et al.*, 2011)

Ni and Cobalt (Co)	*Alyssum murale* *Thlaspi apterum* *Alyssum murale* ssp. *picleri* *Alyssum heldreichii* *Alyssum markgrafii* *Thlaspi ochroleucum* *Thlaspi tymphaeum* *Thlaspi praecox*	Ni MH/G Ni MH/ G Ni MH/G Ni MH/ G Ni MH/ G Ni MH/ G Ni MH/ G Cd-Ni MH/ G	Plant and soil samples were collected from 18 serpentine sites ranging from natural conditions to anthropogenically impacted (i.e. historic chrome mining activity).	Ni_{soil} and Co_{soil} concentrations ranged from 1070 - 3280 mg/kg and 93 - 298 mg/kg. Elevated Ni (to the degree of hyperaccumulation) was accumulated in the leaves of investigated plant species, compared with Co. *A. murale* (Ni: 4700 - 20 100 mg/kg/ Co: 8.8 – 90 mg/kg) *A. murale* ssp. *pichleri* (Ni: 4 100 mg/kg / Co: 26 mg/kg) *A. heldreichii* (Ni: 5 400-11 800 mg/kg / Co: 16 mg/kg) *A. markgrafii* (Ni: 10 800 – 19 100 mg/kg / Co:12-25 mg/kg) *Th . ochroleucum* (Ni: 1 100-3 400 mg/kg / Co:4 - 16 mg/kg) *Th . tymphaeum* (Ni: 6 400 - 7000 / Co: 5.7 - 6.4 mg/kg) *Th . apterum* (Ni: 14 100 - 21 500 / Co: 22 - 173 mg/kg) *Th . praecox* (Ni: 1 100 – 13 600 / Co: 15 – 20 mg/kg). Authors concluded that *A. murale* and *A. markgrafii* are viable candidates for Ni phytoextraction and phytomining. Influence of salinity was not investigated or discussed.	(Bani *et al.*, 2010)
Ni and Co	*Glochidion* cf. *sericeum*	Ni and Co MH	Analysed frozen, hydrated plant parts, tissues, and vascular bundle (xylem and phloem).	Sulphur was accumulated in the series; roots (1954 mg/kg) > leaves (1453 mg/kg) > fruit (960 mg/kg) > twigs (679 mg/kg) > wood (291 mg/kg) and in the phloem (931mg/kg). Accumulation of Ca followed a similar accumulation pattern: roots (3180 mg/kg) > leaves (2725 mg/kg) > twigs (665 mg/kg) > fruit (658 mg/kg) > wood (405 mg/kg) and in the phloem (3414 mg/kg). Elevated concentrations of Ni (831 – 2447 mg/kg) and Co (159 - 1311 mg/kg) were accumulated in leaves compared to other plant parts. Co was accumulated in the order twigs (217 mg/kg) > wood (88 mg/kg) > roots = fruit (73 mg/kg) whereas Ni was in the order: twigs (282 mg/kg) > roots (271 mg/kg) > wood (235 mg/kg) > fruit (174 mg/kg). Phloem, collected during sample collection contained elevated Ni (1059 mg/kg) and Co (1354 mg/kg) concentrations. Both Ni and Co were associated with carboxylic acids (namely citrate for Ni and tartrate or malate for Co). Co oxidation to Co^{3+}	(van der Ent *et al.*, 2018)

				was observed which was attributed to Co secreted on the surface of leaves.	
Ni and Co	*Berkheya coddii*	Ni MH/ G	Plants were grown in artificial Co and Ni metalliferous soil under greenhouse conditions.	Co was taken up by *B. coddii* regardless of Ni presence whereas Ni uptake was impeded by presence of combination of Ni and Co at the same concentrations (attributed to competition between Ni and Co for binding sites – competitive ion adsorption). Co phytotoxicity threshold for *B. coddii* was 20 mg/kg. This suggests limitations for future phytoremediation trials aimed at remediating Ni/ Co in the presence of Co/ Ni. Ni and Co were hyperaccumulated when plants were grown on single-element artificial substrate. Effect of salinity was not investigated/ discussed.	(Keeling *et al.*, 2003)
Ni and Co	*Festuca rubra L.* *Juncus spp*	G/NMA NMA/H	Plant and soil samples were collected from serpentine quarry soils (four sampling sites) with elevated Co (ranged from 76 - 147 mg/kg) and Ni (1 342 – 20 139 mg/kg) concentrations.	Elevated Co and Ni were accumulated in roots compared with shoots. Lower concentrations of Co and Ni were accumulated from sites with higher pH levels attributed to retention of metals in the soil). Co and Ni TF < 1. *F. rubra* and *Juncus* spp are viable candidates for phytostabilization of Co and Ni (effective root accumulators where BCF > 1). Influence of salinity was not investigated or discussed.	(Lago-Vila *et al.*, 2015)
Ni and Co	*Berkheya coddii*	Ni MH/ G	Pot trials – plants were grown in Ni-rich ultramafic soil. Salts ($MgCO_3$, $CaCO_3$), sulphur, chelating agents (NTA, DTPA, EDTA) and acid mine tailings (AMT) were added to investigate effects of salts, AMD, and chelating compounds on Ni and Co uptake.	The addition of salts increased Ni uptake by *B. coddii* whereas the addition of S and AMT (which had low pH) significantly increased Ni and Co uptake.	(Robinson *et al.*, 2003)
Ni and Co	*Noccaea caerulescens*	Cd/Zn MH	Plants were grown in nutrient solutions supplemented with varying concentrations of metals (Ni, Co, Fe, and Zn) at pH 5.8, under controlled conditions.	Co inhibited Ni and Zn uptake by *N. caerulescens* (i.e., competitive ion adsorption) whereas Co inhibited Zn uptake and accumulation. Presence of Ni and Co increased Fe uptake by the plant whereas Zn reduced Ni uptake. Influence of salinity was not investigated or discussed.	(Deng *et al.*, 2021)

Ni and Co	*Noccaea kovatsii* *Noccaea praecox*	Ni MH Ni MH	Plant (two plant species) and soil samples were collected from sixteen (16) sites covering both ultramafic and non-ultramafic soil types.	Both *N. kovatsii* and *N. praecox*, collected from the ultramafic soils type, hyperaccumulated Ni (> 1000 mg/kg), where average Ni accumulation was elevated in shoots, compared with roots. For example, *Noccaea* spp accumulated elevated Ni in shoots (2060 ± 3120 mg/kg) compared with roots (231 ± 355 mg/kg) whereas Co accumulation were similar for roots (6.82 ± 5.08 mg/kg) and shoots (6.08 ± 4.61 mg/kg) across both ultramafic and non-ultramafic soils. Zn was not hyperaccumulated (i.e., 3000 mg/kg hyperaccumulation threshold) however Zn was accumulated to elevated levels (595 mg/kg) and hyperaccumulated in one case (4 921 mg/kg).	(Mišljenović *et al.*, 2020)
Co	*Alyssum murale* *Alyssum corsicum* *Nyssa sylvatica* *Brassica juncea*	Ni-MH/ G Ni-MH/ G Co-MA/ G NMA/ G	Pot trials – Plants grown on (i) sandy loam soil [amended with Co (58.9 mg/kg) as $CoSO_4.7H_2O$] and (ii) two Ni-smelter soils [where concentrations ranged from 24 – 37 mg Co/kg and 1720 – 2570 mg Ni/kg] under greenhouse conditions.	*Alyssum murale* (1320 mg/kg), *A. coriscum* (> 1 000 mg/kg), and *N.sylvatica* (800 mg/kg) hyperaccumulated Co (> 300 mg/kg). Although the influence of Co-salt constituents (namely SO_4^{2-}) were not investigated, the presence of Co as a salt ($CoSO_4.7H_2O$) may have contributed to elevated Co accumulation by *Alyssum* spp and *N. sylvatica*.	(Malik *et al.*, 2000)
Co	*Panicum antidotal* (Panikum) *Pennisetum purpureum* (Napier grass) *Cucurbita pepo* (Squash) *Gossypium hirsutum* (Cotton) *Helianthus annuus* (Sunflower)	G G G MH/ G	Pot trials – Plants were grown in two different soil types (S1 and S2) which were previously irrigated with contaminated water. EC_1 = 8.43dS/m (Co = 146 mg/kg where bioavailable fraction was 0.9 mg/kg); EC_2 = 1.23 dS/m (Co = 83 mg/kg where bioavailable fraction was 1.4 mg/kg). Main contributor to salinity was SO_4^{2-}.	Co removal efficiency ranged from 13.8 - 43.7 % where elevated Co was retained in roots (~ 56.9 – 69.3 %) compared with shoots. Elevated levels of Co were accumulated by *Helianthus annuus* (57.7 mg/kg) where 24.8 mg/kg and 32.9 mg/kg were accumulated by roots and shoots, respectively (i.e., TF > 1).	(Lotfy and Mostafa, 2014)
Co	*Crotalaria cobaltica* (Devil-bean)	Co MH/ G	Cells of each plant species were cultured (hydroponically) *in vitro* under	Co accumulation increased as Co exposure period increased. *C. cobaltica* accumulated Co (1168 mg/kg) in cells after a seven-day	(Oven *et al.*, 2002)

	Rauvolfia serpentina	NMA/ G	controlled conditions (i.e., plant tissue culture).	exposure period. *C. cobaltica* tolerated Co concentrations as no phytotoxic symptoms were observed relative to measured physiological growth parameters. Co was mainly bound to cell walls of both plant species. Influence of salinity was not investigated or discussed.	
Co	*Lycopersicon esculentum* (Tomato) *Triticum aestivum* (Wheat)	G/NMA G/ NMA	Plants grown in hydroponic solutions, with varying Co treatments, under controlled conditions. Co was added as Co-salts [$Co(NO_3)_2$].	Elevated Co was accumulated in roots compared with shoots. Root concentrations for *L. esculentum* ranged from 38.2 – 4 423 mg/kg in roots and 0.6 – 492 mg/kg Co when exposed to different treatments, whereas *T. aestivum* ranged from 2.5 – 9 319 mg/kg in roots and 0.8 - 395 mg/kg Co. The influence of salinity was not considered or discussed.	(Bakkaus *et al.*, 2005)
Co	*Brassica napus* (Canola)	G	Pot trials - plants grown in soil amended with Co-salts ($CoSO_4$) at 100 mg/kg where bioavailable fraction of Co was 0.08 mg/kg (which increased to 2.25 mg/kg after 30 days) (pH = 6.81). EC = 128.3 dS/m.	Plants accumulated 1.95 mg/kg in roots and 2.18 mg/kg in shoots (translocation factor > 1). Accumulation of Co increased as EDTA concentration increased. Effect of salinity was not investigated or discussed.	(Adiloğlu, 2016)
Co	*Alectra sessiliflora* *Haumaniastrum katangense* *Anisopappus chinensis (L.)* *Buchnera henriquesii* *Celosia trigyna L.*, *Hibiscus rhodanthus* *Crepidorhopalon perennis* *Crepidorhopalon tenuis*	Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G Cu-Co MH/ G	Plant samples were collected from a Cu/Co mine located in different locations across Katanga, Democratic Republic of Congo (DRC).	Uptake and translocation of Co to shoots Plant species, including *A. chinesis* (933 - 1948 mg/kg), *C. perennis* (433 – 1156 mg/kg), and *T. welwitschii* (123 - 1544 mg/kg), accumulated elevated Co concentrations across study sites. Influence of salinity was not investigated or discussed.	(Faucon *et al.*, 2007)

	Triumfetta welwitschii,	Cu-Co MH/ G			
	Vernonia petersii	Cu-Co MH/ G			
	Vigna dolomitica	Cu-Co MH/ G			
	Acalypha cupricola	Cu-Co MH/ G			
Co	*Brassica oleracea* (Cauliflower)	G/ MA	Pot trials - plants were grown in refined sand under greenhouse conditions. Plants were exposed to excess concentrations of Co (as Co-salt, namely $CoSO_4^{2-}$), Cr, and Cu.	Co was mainly accumulated in roots (1274 mg/kg) > leaves (507 mg/kg) > stem (273 mg/kg). Co impeded the translocation of P, S, Mn, Zn, and Cu. Co accumulation also may have displaced other metals (namely Fe) from physiologically important plant parts/ sites resulting in lower biomass production by *B. oleracea*. Influence of salinity was not investigated or discussed.	(Chatterjee and Chowdhury, 2003)
Co	*Crepidorhopalon tenuis*	Co-Cu MH	Plant samples were collected from four different sites within the Katanga region, DRC.	Although the degree of Co accumulation differed between sites/ plant populations, some *A. chinensis* and *C. tenuis* individuals hyperaccumulated (> 300 mg/kg) Co (range: 3 – 1334 mg/kg and 8 – 605 mg/kg, respectively). Free Co ions in soil was positively correlated to Co accumulation in shoots by plant species.	(Lange *et al.*, 2014)
	Anisopappus chinensis	Co-Cu MH			
Co	*Salicornia europaea*	H/MA,	Plants were collected from five locations (from coastal and inland saline areas) the field and separated into different plant parts.	Salinity influenced Co and Ni uptake by *H. portulacoides* and *S. maritima*, where Co and Ni uptake were positively correlated to salinity in roots, stems, and leaves of *H. portulacoides* (and in the roots of *S. maritima*). Co and Ni was mainly accumulated in roots > stems > leaves. Co was retained in roots (TF < 1) by all halophytes whereas *S. maritima* had a TF > 1 for Ni. Elevated metal concentrations were accumulated by plants sampled from coastal saline areas compared with inland areas.	(Milić *et al.*, 2012)
	Suaeda maritima	H/MA,			
	Salsola soda	H/MA			
	Halimione portulacoides	H/ MA			
Co	*Triticum aestivum* (Wheat)	G/NMA	*T. aestivum* seedlings were grown in field conditions (previously under saline irrigation) and exposed to Co (as a Co-salt, $CoSO_4$) at different concentrations (0, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0 mg/kg. EC = 26.6 dS/m.	Co was accumulated in a dose-dependent response. Plants had a hormetic response to Co treatments where Co concentrations below 15 mg/kg promoted growth and depressed growth after 15 mg/kg. Fe, Na, and Cl accumulation decreased in a Co-dose dependent response, whereas Co treatments increased the accumulation of other elements such as N, P, K, Ca, Mg, Mn, Zn, and Cu.	(Gad and Kandil, 2011)

Co	*Cucumis sativus L.* (Cucumber)	G/NMA	Seeds were sown in soils at three different salinities (3.35, 3.85 and 5.40 dS/m) and subsequently irrigated with Co at 0.0, 10.0, 12.5, 15.0, 17.5 and 20 mg/kg).	Uptake of Co, as well as Mn, Zn, Cu, and Fe, generally decreased as salinity level increased. Co accumulation increased in a dose-dependent response.	(Gad *et al.*, 2018)

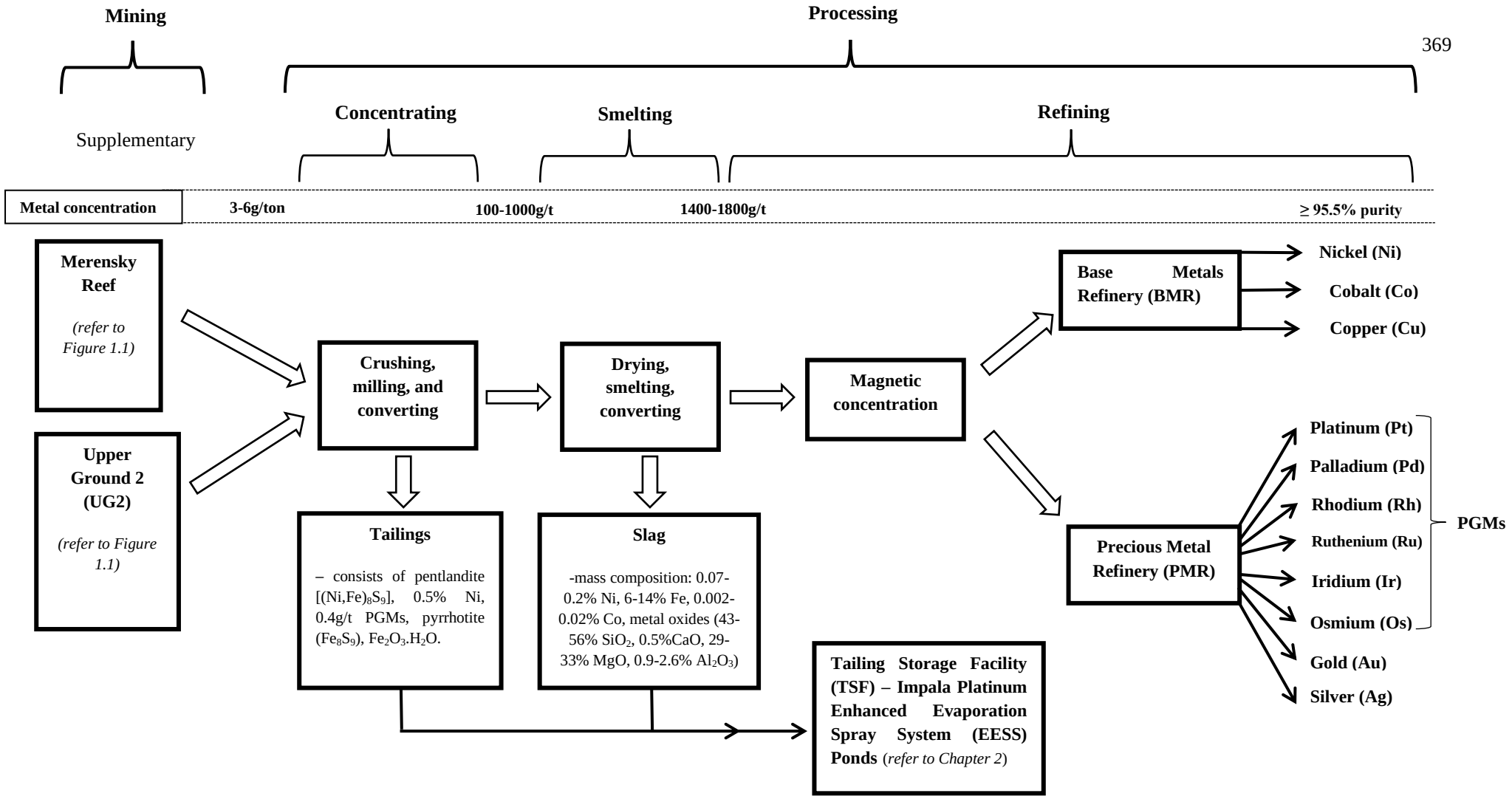


Figure 1.1. Mining and processing of platinum (Pt), nickel (Ni), and cobalt (Co). Ore is mined, concentrated, and smelted at the Bushveld Igneous Complex (BIC), Rustenburg, whereas refining processes are conducted at the Impala Platinum Refinery Services (IRS), located in Springs, Ekurhuleni, South Africa. Adapted from Crundwell *et al.*, (2011), De Clerk and De Vaal, (2012), and Warner *et al.*, (2007).

Chapter 2

Supplementary Table 2.1. Level of agreement, LoA (%) between certified values and analysed values for the certified reference materials (CRMs) analysed using energy dispersive X-Ray Fluorescence (ED-XRF). "Norm" represents normalised data; agreement between ED-XRF measurements normalized using Equation 2.3. “Re-run” measurements: samples reanalysed using a different ED-XRF calibration method.

	CRM	**Stream sediment-GSD-12**			**Spinach_1570a**		**Orchard_1571**			**Maize_IPE200**			**Dandelion_IPE140**		
Element	**Symbol**	**Initial**	**Re-run**	**Norm.**	**Initial**	**Norm.**	**Initial**	**Re-run**	**Norm**	**Initial**	**Re-run**	**Norm**	**Initial**	**Re-run**	**Norm**
Sodium	**Na**	77.25	40.73	97.93			86.85	49.86	93.35						
Magnesium	**Mg**	50.61	51.82	98.72			38.92	64.56	85.98	39.44	52.79	94.28	37.15	42.05	81.68
Aluminium	**Al**	80.18	80.54	99.37	34.94	98.46				35.17	17.11	82.96	19.29	22.58	76.02
Silicon	**Si**	91.12	93.73	99.36											
Phosphorous	**P**	61.51	61.93	98.75			66.89	87.07	89.63	51.16	56.94	95.18	69.37	63.05	88.18
Sulphur	**S**	95.06	95.31	98.92			57.35	67.05	88.67	44.62	44.16	89.82	58.22	71.01	92.64
Chlorine	**Cl**	90.87	86.60	97.05			50.75	39.13	73.42	60.98	64.23	92.77	68.54	23.16	88.38
Potassium	**K**	91.50	91.56	99.42						48.76	46.44	82.46	65.72	59.79	96.01
Calcium	**Ca**	91.43	90.93	99.28						45.39	44.53	94.75	58.13	54.13	94.60
Vanadium	**V**	85.79	88.07	94.43	33.96	56.50							36.44	39.84	50.58
Chromium	**Cr**	67.77	70.60	97.44			20.52	23.59	92.20	2.90	36.57	92.47			
Manganese	**Mn**	88.97	88.23	99.35	63.47	97.22	57.81	75.28	87.45	33.62	33.63	93.88			
Iron	**Fe**	97.89	96.42	97.98			72.21	96.60	89.79	57.24	52.70	93.22	63.06	53.20	91.20
Cobalt	**Co**	34.09	11.36	100.00	6.75	72.30	6.67	17.30	92.35	5.74	59.86	80.65	58.47	50.44	78.17
Nickel	**Ni**	54.69	50.26	96.87	25.17	91.28	20.95	15.88	96.42	22.83	25.33	86.59	71.61	82.85	92.21
Copper	**Cu**	97.63	96.86	99.52	64.68	95.18	59.36	73.86	97.48	32.88	37.00	91.17	46.57	49.22	92.72
Zinc	**Zn**	91.41	90.67	99.52	86.13	97.71	77.70	75.87	79.36	77.57	79.90	89.88	71.10	62.56	92.18
Gallium	**Ga**	83.33	78.37	87.09											
Germanium	**Ge**	26.74	8.91	97.85											

Arsenic	**As**	91.25	97.04	98.89	13.60	95.05	56.11	1.67	91.83	65.26	86.42	80.77	71.96	39.66	66.71
Selenium	**Se**	50.00	66.67	95.65	23.04	98.22	16.00	48.00	95.65	N/A	N/A	N/A	40.20	82.92	92.44
Rubidium	**Rb**	95.63	94.36	96.89											
Strontium	**Sr**	97.63	95.53	97.69	76.41	98.47	95.45	81.22	76.10	63.92	68.85	92.53	96.51	93.08	95.88
Molybdenum	**Mo**	N/A	N/A	N/A											
Yttrium	**Y**	90.60	95.48	94.12											
Zirconium	**Zr**	93.92	87.21	97.37											
Niobium	**Nb**	93.64	90.32	93.44											
Silver	**Ag**	57.55	54.35	98.90											
Cadmium	**Cd**	60.97	51.25	72.99	39.64	75.67	5.50	52.44	82.56	20.31	58.20	30.07	53.86	55.26	29.21
Tin	**Sn**	79.16	74.88	94.97											
Antimony	**Sb**	67.31	72.24	91.76											
Tellurium	**Te**	1.53	1.42	86.70			2.58	5.69	73.02	3.44	4.21	80.64			
Iodine	**I**	58.89	55.56	84.47											
Caesium	**Cs**	49.09	16.88	91.39											
Barium	**Ba**	80.43	75.24	92.32											
Lanthanum	**La**	16.88	90.83	55.77											
Cerium	**Ce**	34.17	20.90	26.80											
Praseodymium	**Pr**	31.37	15.89	77.52											
Hafnium	**Hf**	12.05	4.02	75.61											
Tantalum	**Ta**	9.32	8.75	89.17											
Tungsten	**W**	86.76	88.02	96.67											
Mercury	**Hg**	59.53	16.80	98.44	6.49	39.90	15.50	46.50	98.58	N/A	N/A	N/A	2.38	7.14	96.55
Thallium	**Tl**	60.20	51.14	79.81											
Lead	**Pb**	92.32	89.95	99.53			18.58	22.59	68.20	11.89	15.62	70.19	2.11	8.49	75.24

Bismuth	**Bi**	53.89	53.44	89.26					
Thorium	**Th**	88.50	85.81	97.13	5.49	60.29	6.40	23.11	59.36
Uranium	**U**	69.57	65.38	84.13					

Supplementary Table 2.2. Soil chemical properties, namely pH, EC (µS/cm), and Eh (mV), of seven soil pits sampled from the study site soil. Two opposing soil profile faces were sampled (n = 2) at each depth interval down the soil profile. Note, only one soil profile face was sampled for each unplanted control pit (Control 1, 2, and 3). Control pits were void of any *T. usneoides* trees within 10 m radius

Tree	**AM8**			**AM4**			**AM2**			**AM10**			**Control 1**			**Control 2**			**Control 3**		
Soil Depth	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**	**pH**	**EC**	**Eh**
0-2	6.68	4100	279	6.49	1552	386	7.08	8575	302	5.27	2172	344	6.45	663	234	8.02	9950	253	5.74	3320	289
2-5	7.89	2040	279	6.08	4979	380	6.09	9280	292	5.46	1810	357	7.38	159	267	7.26	15850	270	9.99	24500	177
5-10	8.17	2385	247	6.55	472	372	5.40	3865	316	5.94	1745	364	7.60	182	281	5.70	1382	280	10.00	19400	149
10-15	8.08	3255	254	7.29	305	333	5.40	3770	350	5.78	1479	306	8.77	622	320	7.40	1186	247	9.90	18730	145
15-20	7.75	3995	254	7.51	572	330	5.40	3640	349	5.80	1595	347	9.15	703	249	9.01	5840	213	9.71	13590	163
20-30	7.16	5165	281	7.67	1058	346	5.65	3875	281	6.34	167	393	9.45	985	224	9.20	4150	229	9.23	9310	168
30-40	6.71	5670	292	7.25	1969	348	5.96	3450	290	5.53	2340	324	9.74	2960	188	9.30	4600	220	8.79	11470	167
40-50	6.73	6660	286	6.88	3039	363	6.24	7000	296	5.70	3480	400	9.76	3470	176	6.20	7680	224	8.85	12820	157
50-60	6.83	7430	283	6.55	3130	375	6.18	4170	297	6.37	3190	361	9.47	4230	187	5.85	9610	239	8.83	13170	155
60-70	6.61	8425	275	6.03	4145	381	5.97	4935	310	5.90	4175	374	9.87	4420	247	6.00	11440	244	8.61	14100	158
70-80	6.40	7360	281	5.62	4290	385	5.82	9380	307	5.85	3720	360	8.86	4970	196	6.17	10460	248	8.45	16470	156
90-100	5.38	7835	290	5.93	3585	377	5.38	6020	313	6.27	3025	326	7.56	6430	222	5.97	11800	255	8.16	19670	161
120-130	4.82	6990	295	5.28	3205	383	5.24	5090	310	6.07	3495	362	5.98	7630	237	5.57	14890	257	8.10	24400	158
145-155	4.77	6915	301	5.18	2765	385	5.15	1311	316	5.80	3755	341	5.82	8240	244	5.22	16250	262	8.36	23100	155
170-180	4.77	6375	303	5.39	2965	379	5.24	676	336	6.03	3210	265	5.74	8490	248	4.46	9740	295	8.47	14800	150
190-210	4.78	5820	307	5.50	2243	379	5.37	423	336	6.02	3095	287	5.76	8060	250	5.20	15630	268	8.53	26300	158
240-260	5.17	3695	320	5.68	1210	382	5.57	365	343	5.91	3100	302	5.74	7340	254	5.49	9180	280	8.29	2300	152
280-300	5.95	1571	301	5.72	870	395	5.76	354	320	5.85	1816	306	5.72	6610	259	5.52	7200	283	7.99	18540	167
320-340	5.81	2244	317	5.81	488	403	5.70	348	351	5.65	1243	318	5.60	5430	269	5.65	5640	289	NA	NA	NA

Supplementary Table 2.3. Density (fresh mass) of tree trunk sections taken at 0.3 m (stem basal diameter - SBD) and 1.2m (stem diameter at breast height - DBH) above ground from four *Tamarix usneoides* trees harvested from the study site. Density was calculated as follows; Density (p) = mass/volume.

Tree	Tree Size/Class	Sample	Density (kg/m^3)
AM4	Very Small	SBC_ 0.3 m	9.41
		SD_ 1.2 m	6.93
AM8	Small	SBC_ 0.3 m	11.21
		SD_ 1.2 m	7.01
AM2	Medium	SBC_ 0.3 m	21.72
		SD_ 1.2 m	24.75
AM10	Large	SBC_ 0.3 m	37.05
		SD_ 1.2 m	32.89
Average		SBC_ 0.3 m	79.39
		SD_ 1.2 m	71.58

Supplementary Table 2.4. Amount of platinum (Pt) (µg), nickel (Ni) (mg), cobalt (Co) (mg), and sulphur (S) (g) allocated in plant organs of four *T. usneoides* trees harvested from the study site.

Tissue type	Tree	Pt amount (µg)	Ni amount (mg)	Co amount (mg)	S amount (g)
Leaves	Large	311	429	16.3	294
	Medium	525	525	20.7	284
	Small	326	355	11.8	313
	Very Small	444	274	8.88	290
Twigs	AM10	313	350	12.8	181
	AM2	340	442	17.5	176
	AM4	289	414	14.6	201
	AM8	165	286	9.13	185
Wood	AM10	358	686	13.6	209
	AM2	200	503	12.8	194
	AM4	86	143	4.69	124
	AM8	212	168	5.92	154
Coarse Roots	AM10	335	411	20.4	240
	AM2	432	572	15.2	247
	AM4	451	577	47.4	202
	AM8	644	257	27.2	228
Flowers	AM10	511	962	37.7	172
	AM2	740	1702	68.1	158

Chapter 3

Supplementary Table 3.1. Constituents of Murashige and Skoog (MS) standard plant growth medium (Murashige and Skoog, 1962).

Compound	Compound formula	Concentration (mg/L)	Molar mass (g/mol)
Major salts			
Ammonium nitrate	NH_4NO_3	1650	80.05
Calcium chloride	$CaCl_2.2H_2O$	440	36.03
Magnesium sulphate	$MgSO_4.7H_2O$	370	126.11
Potassium phosphate	KH_2PO_4	170	136.09
Potassium nitrate	KNO_3	1900	101.10
Minor salts			
Boric acid	H_3BO_3	6.20	61.83
Cobalt chloride	$CoCl_2.5H_2O$	0.025	90.08
Cupric sulphate	$CuSO_4.4H_2O$	0.025	90.08
Ferrous sulphate	$FeSO_4.7H_2O$	27.80	126.11
Manganese sulphate	$MnSO_4.4H_2O$	22.30	72.06
Potassium iodide	KI	0.83	166.00
Sodium molybdate	$NaMoO_4.2H_2O$	0.25	36.03
Zinc sulphate	$ZnSO_4.7H_2O$	8.60	126.11
Na_2EDTA	$C_{10}H_{14}N_2Na_2O_8$	37.20	45.98
Vitamins and organics			
i-inositol	$C_6H_{12}O_6$	100	180.16
Niacin	$C_6H_5NO_2$	0.50	123.11
Pyridoxine.HCl	$C_8H_{11}NO_3$	0.50	205.64
Thiamine.HCl	$C_{12}H_{17}ClN_4OS.HCl$	0.10	337.27
IAA	$C_{10}H_9NO_2$	1.00– 30	175.18
Kinetin	$C_{10}H_9N_5O$	0.04 - 10.00	215.21
Glycine	$C_2H_5NO_2$	2.00	75.07
Ethylenediamine	$C_2H_4(NH_2)_2$	1000	60.10

Supplementary Table 3.2. Studies conducted on a range of Indole-3- butyric acid (IBA) concentrations, and period of auxin treatment, which was used to induce rooting in various plant species *in vitro*.

IBA concentration (mg/L)	Time of treatment (hrs)	Plant species	Explant type	Summary	References
0.1 – 2.03	Continuous	*Crithmum maritimum*	Plantlet	Number of roots significantly increased at 1 and 2 mg/L IBA.	(Grigoriadou and Maloupa, 2008)
0.1-0.5	Continuous	*E. grandis*	Plantlet	0.1 mg/L resulted in 87, 45, and 41 % rooting establishment in different genotypes.	(Nakhooda *et al.*, 2012)
0.5 - 1	Continuous	*Azadirachta indica*	Nodes (3-4)	0.5 mg/L IBA resulted in 80 % rooting compared with 1.0 mg/L.	(Shivanna *et al.*, 2013)
0.5-3	Continuous	*Caralluma edulis*	Plantlet	1.0 – 3.0 mg/L resulted in an elevated number of roots and root length (1.5 mg/L IBA resulted in 90 % root induction).	(Patel *et al.*, 2014)
1	Continuous	*E. grandis x E. nitens*	Plantlet	Resulted in 75 % and 65 % rooting establishment for two *Eucalyptus* genotypes.	(Mokotedi *et al.*, 2000)
3	Continuous	*Salvadora persica* Linn	Plantlet	Highest rooting percentage (80 %) was induced during 3.0 mg/L treatment.	(Mathur *et al.*, 2002)
25	1 - 7 days	*Khaya senegalensis* (African mahogany)	Plantlet	IBA treatments at 0.5 and 28 for 1-7-day pulse treatments resulted in 71 (0.5 mg/L), 87 (1 day at 25 mg/L), and 96 % (7 days at 25 mg/L) rooting establishment, respectively.	(Danthu *et al.*, 2003)
20 - 250	1 – 72 hrs	*Santalum album*	Plantlet	24 hr and 48 hrs at 0.484 µg/L resulted in the highest root induction	(Sanjaya *et al.*, 2006)

Chapter 4

Supplementary Table 4.1. Plant tissue culture studies determining the phytoremediation potential of a particular plant species relative to heavy metal uptake, accumulation, translocation, and tolerance.

Metal	Symbol	Treatment Metal concentrations (mg/kg)	Plant species	Type of explant	Aim of study	Accumulation concentration (mg/kg)	Findings	Reference(s)
Aluminium	Al	Al: 0.54	*Nicotiana tabacum*	Cell culture	To determine Al uptake, localization, and tolerance.	Aluminium accumulation increased as treatment exposure time increased. Cells of *N. tabacum* accumulated 0.59 mg/kg Al per 10^6 cells.	Cells rapidly took up Al. Aluminium was accumulated in/near the cell wall of actively growing cells. The degree of Al uptake depended on the age of the cell culture. Cells tolerated Al treatments as relative growth rate was not affected by presence of Al. Aluminium complexed with cell walls. Increase in Ca resulted in lower *in planta* Al concentrations.	(Vitorello and Haug, 1996)
Arsenic **Cadmium** **Copper** **Zinc**	As Cd Cu Zn	As: 0, 0.93, 9.30, and 946.48 mg/kg Cd: 0, 1.04, 10. 42, and 52.12mg/kg Cu: 0, 0.90, 8.98, and 44.91 mg/kg Zn: 0, 40.37, 161.46, and 322.92 mg/kg	*Populus alba*	Shoots	To determine phytoremediation potential of *P. alba*.	Metal concentration accumulated by *P. alba* ranged from 150 - 1000 mg As/kg, 330-1020 mg Cd/kg, 15 - 350 mg Cu/kg, and 100 - 2400 mg Zn/kg.	Metal accumulation increased as treatment concentration increased. Metal concentrations in roots significantly differed from shoots.	(Di Lonardo *et al.*, 2011)
Arsenic	As	As: 0.75, 3.75, 7.49, 14.98	*Vetiveria zizanoides*	Plantlets	To determine the ability of *V. zizanoides* to accumulate, translocate, and tolerate As at varying concentration.	Arsenic concentrations were retained in roots resulting in a low TF. Maximum As accumulated by *V. zizanoides* was 169 mg/kg in roots compared with 83 mg/kg in shoots.	Maximum As accumulation occurred after 14 days. Roots accumulated elevated As concentrations compared with shoots. Arsenic accumulation increased as metal treatment concentration increased.	(Singh *et al.*, 2017)

Caesium **Strontium**	Cs Sr	Cs: 0.01, 0.13, 1.33, 13.29 Sr: 0.01, 0.09, 0.88, 8.76	*Tillandsia brachycaulos* and *Tillandsia stricta*	Shoots	To determine the ability of two *Tillandsia spp.* to accumulate Cs and Sr.	Caesium and Sr concentration increased as metal treatment concentration increased. *Tillandsia spp.*, accumulated concentrations of Sr ranging from; 0.14, 0.32, 1.03, and 12.11 mg Sr/kg and 0.14, 0.27, 2.64, and 26.47 mg Cs/kg.	Low concentrations of Cs and Sr stimulate metal uptake by plant species.	(Zheng *et al.*, 2017)
Cadmium	Cd	Cd: 2.81, 5.62, 11.24, 33.72, and 56.20	*Nicotiana tabacum*	Cell culture	To determine the molecular mechanisms involved in Cd response by *N. tabacum in vitro*.	Not analysed for *in planta* metal concentrations	Three-phase ROS generation resulted in cell mortality. Elevated H_2O_2 was produced by Cd-treated seeds *in vitro*.	(Garnier *et al.*, 2006); (Olmos *et al.*, 2003)
Cadmium	Cd	Cd: 1.12, 1.69, 5.62, 11.24, 16.86, and 22.48 mg/kg	*Adenophora lobophylla* and *A. potaninii*	Hairy roots	To determine plant species response to Cd exposure *in vitro*.	*A. lobophylla* accumulated elevated Cd compared with *A. potaninii*. Cadmium accumulation increased as metal treatment concentration increased. After seven days of Cd exposure *in vitro*, *A. lobophylla* accumulated 6, 16, 60, 100, and 190 mg Cd/kg whereas *A. potaninii* accumulated 5, 14, 40, 50, and 70 mg Cd/kg when root hairs were exposed to Cd at 1.12, 1.69, 5.62, 11.24, 16.86, and 22.48 mg/kg, respectively.	The endangered plant species, *A. lobophylla* accumulated elevated Cd levels compared to *A. potaninii*. Biochemical parameters (e.g. reduced glutathione) were elevated in *A. lobophylla* compared with *A. potaninii*.	(Wu *et al.*, 2001)
Cadmium	Cd	Cd: 1.00 - 15.00	*Dittrichia viscosa*	Shoot culture	To investigate the short-term response of *D. carota* plantlets to Cd exposure.	Plantlets accumulated 0.836 ± 0.24 – 1413 ± 2.40 mg Cd/kg where roots (813 mg Cd/kg) accumulated elevated levels compared with shoots (583 mg/kg). TF < 1 for all	Cadmium treatments reduced explant moisture content and shoot growth but did not significantly impact root elongation. Higher [Cd] were sequestered and stored in roots	(Fernández *et al.*, 2008)

						clones tested.	compared with shoots (heavy metal toxicity avoidance strategy).	
Cadmium	Cd	Cd: 11.24 - 112.40	*Daucus carota*	Root culture	To determine role of antioxidant mechanisms in heavy metal tolerance.	Not analysed for *in planta* metal concentrations	*D. carota* accumulated elevated [Cd] without any reduction in protein content and moisture content.	(Di Toppi *et al.*, 1999)
Cadmium	Cd	Cd: 20	*Thlaspi caerulescens* and *N. tabacum*	Root culture	Determine the degree of Cd-tolerance at molecular and physiological levels.	Cd accumulation by *T. caerulescens* decreased as exposure time increased whereas *N. tabacum* Cd accumulation increased as exposure time increased. *T. caerulescens* accumulated 1000 mg Cd/kg after 30 days (maximum of 2100 mg/kg after day 3) of exposure whereas *N. tabacum* accumulated 1750 mg Cd/kg after 30 days of exposure.	*T. caerulescens* tolerated [Cd] as no phytotoxicity symptoms were observed. Reactive oxygen species (ROS) levels were maintained at non-phytotoxic levels.	(Boominathan and Doran, 2003)
Cadmium **Zinc**	Cd Zn	Cd: 40 Zn: 150	*Linum usitatissimum*	Shoot culture Plantlet	To determine potential of linseed relative to Zn and Cd accumulation and tolerance.	*Linum usitatissimum* accumulated 675 mg Cd/kg and 1445 mg Zn/kg when exposed to 40 mg/kg $Cd(NO_3)_2$ and 150 mg/kg $Zn(NO_3)_2$, respectively.	[Zn] > 150 mg/kg and [Cd] > 40 mg/kg inhibited physiological growth and/or resulted in explant death. Degree of accumulation and tolerance to Zn and Cd *in vitro* was supported by field trials.	(Smykalova *et al.*, 2010)
Chromium **Cadmium**	Cr Cd	Cr: 0.00, 26.00, 52.00, 104.00, 176.80 Cd: 0.00, 33.72, 73.06, 146.12	*Prosopis laevigata*	Plantlets	To investigate the phytoremediation potential of *P. laevigata* relative to Cr and Cd at varying concentration.	Roots of *P. laevigata* accumulated 5035 and 8090 mg Cr/kg whilst shoots accumulated 2364 and 5461 mg Cr/kg when exposed to Cr at 104 and 177 mg/kg, respectively. Cadmium was accumulated at 9404 and 21 437 mg/kg in roots whereas shoots accumulated 3811 and 8176 mg Cr/kg when plantlets were treated with Cd at 0.30 and	Roots accumulated elevated levels of Cr and Cd compared with shoots however, elevated concentrations of metals in plantlet shoots suggests that *P. laevigata* efficiently translocates metals to aboveground tissue. This indicates that *P. laevigata* is a viable candidate plant species for phytoremediation	(Buendía-González *et al.*, 2010)

						0.65 mg/kg *in vitro*.	purposes.	
Cobalt	Co	Co: 130	*Crotalaria cobaltica* (Co-hyperaccumulator); *Rauvolfia serpentina*	Cell culture	To determine Co complexation in cells of a Co hyperaccumulator.	Co accumulation increased as exposure period increased. *C. cobaltica* accumulated 1168 mg/kg Co in cells after seven days.	Cobalt and Ni exposure resulted in increased [cysteine] which complexed metals. *C. cobaltica* tolerated elevated [Co] as no toxicity symptoms were observed relative to physiological growth parameters. Co ions bound to cell walls of both plant species.	(Oven *et al.*, 2002)
Copper	Cu	Cu: 0.03-63.55	*Populus x euramericana* (*P. deltoids x P. nigra*)	Plantlets	Investigate degree of Cu tolerance	Cu concentration in roots was elevated compared with shoots. An increase in Cu treatment resulted in increased Cu accumulation. Cu accumulation	[Cu] > 6.35 mg/kg reduced plant growth (impeded photosynthetic activity) whereas [Cu] ranging 0.03 – 1.27 mg/kg did not negatively impact physiological growth. Concluded that the plant species has the ability to tolerate high [Cu].	(Borghi *et al.*, 2007)
Copper **Zinc** **Manganese**	Cu Zn Mn	Cu: 0.01-12.71 Zn: 1.90-31.40 Mn: 5.49-87.90	*Ailanthus altissima*	Shoot culture	Determine impact of metal on *A. altissima* physiological growth.	Not analysed for *in planta* metal concentrations	All metals negatively impacted shoot growth. Demonstrated and concluded that tolerance levels *in vitro* were comparable to tolerance levels of plants which were being utilized for phytoremediation projects (in the field).	(Gatti, 2008)
Copper **Zinc**	Cu Zn	Cu: 0.32-31.78 Zn: 327 - 2616	*Eucalyptus globulus*	Plantlets	Determine impact of metals on physiological growth parameters (rooting percentage, root length, number of roots, and mean time taken to initiate rooting).	Not analysed for *in planta* metal concentrations	Cu treatment reduced ARF whereas Zn treatment induced dose-dependent phytotoxicity (as [Zn] increased, degree of phytotoxicity increased).	(Schwambach *et al.*, 2005)

Copper **Zinc**	Cu Zn	Cu: 131 Zn: 10 - 300	*Betula pendula*	Axillary bud culture	Determine the degree of Cu and Zn accumulation and tolerance *in vitro*.	*B. pendulina* accumulated an average of 0.06, 0.35, and 0.83 mg/kg of Cu when treated with 0.02, 0.51, and 1.91 mg/kg Cu *in vitro*. Elevated range of Zn was accumulated in roots (0.23 - 2.98 mg/kg) compared with leaves (0.20 - 0.43 mg/kg) when treated with Zn at 3.27 and 52.31 mg/kg whereas 262 mg/kg Zn treatments resulted in lower Zn accumulation.	*B. pendula* tolerated Cu and Zn, accumulated Zn in root tissue. Concluded that Zn-tolerant *B. pendula* clones cross-tolerated elevated [Cu].	(Utriainen *et al.*, 1997)
Lead **Zinc**	Pb Zn	Pb: 20.72-103.60 Zn: 6.54-32.69	*Populus tremula x tremuloides*	Plantlets	Determine phytoremediation potential of plant species *in vitro.*	The investigated *Populus* hybrid accumulated 1400 mg/kg and 3500 mg/kg Pb when treated with 20.72 and 103.60 mg Pb/kg solution *in vitro*.	Elevated [Zn] induced phytotoxicity, Pb accumulated by explants did not impact physiological growth parameters.	(Kališová-Špirochová *et al.*, 2003)
Nickel	Ni	Ni: 12 - 41	*Nicotiana tabacum* (Ni-tolerant cell line)	Cell culture	To investigate molecular and cellular mechanisms involved in Ni tolerance.	*N. tabacum* cells accumulated 117 mg/kg Ni when treated with Ni at 41 mg/kg.	Nickel was compartmentalized into vacuoles which conferred tolerance. Growth of non-tolerant cell line was inhibited when Ni > 18 mg/kg whereas 41 mg/kg Ni resulted in 99 % mortality.	(Saito *et al.*, 2005)
Nickel	Ni	Ni: 25	*Alyssum bertolonii; Nicotiana tabacum*	Root culture	To determine the role of antioxidant systems in conferring Ni-tolerance.	Nickel accumulation by *N. tabacum* decreased as metal exposure time increased. After 30-day metal exposure period, *A. bertolonii* explants accumulated a maximum of 900 mg/kg whereas *N. tabacum* accumulated 400 mg/kg.	Physiological growth of *A. bertolonii* roots were not affected by elevated [Ni], thus, *A. bertolonii* tolerated elevated [Ni]. Ni induced oxidative stress in both plant species.	(Boominathan and Doran, 2002)
Nickel	Ni	Ni: 20	*Alyssum bertolonii;*	Root culture	To determine phytoremediation	Hairy toots of *A. bertolonii* accumulated a maximum of 1500	Nickel, Cd, and Cu were hyperaccumulated *in vitro*. Plant	(Nedelkoska and Doran,

Cadmium **Copper**	Cd Cu	Cd: 20 Cu: 1000	*Thlaspi caerulescens; Hyptis capitata; Nicotiana tabacum*		potential of plant species relative to metal uptake and tolerance.	mg Ni/kg after 9 hrs of Ni exposure. *T. caerulescens* accumulated 1780 ± 100 mg Cd/kg whereas *N. tabacum* accumulated 1080 ± 52 mg Cd/kg.	species also tolerated range of metals as no significant reduction in physiological growth occurred.	2000)
Nickel	Ni	Ni: 29, 35, 41	*Nicotiana tabacum*	Cell culture	To determine biochemical involved in Ni tolerance.	Nickel was bioaccumulated (BCF > 1) by *N. tabacum* cells. Nickel was compartmentalized in cell vacuoles.	Ni tolerant cells elicited elevated tolerance to Co. Biochemical levels (phytochelatin synthesis, histidine, oxalate, and citrate-2-oxoglutarate) increased on exposure to Ni treatments *in vitro*.	(Nakazawa *et al.*, 2004)
Manganese **Zinc**	Mn Zn	Mn: 3.30 - 87.90 Zn: 3.92 - 31.39	*Brassica campestris* and *B. juncea*	Callus culture	Determine degree of metal tolerance relative to physiological growth parameters (moisture content, tolerance index, and metal uptake).	*B. campestris:* Mn (69 – 40 mg/kg) and Zn (74 - 32 mg/kg) decreased as metal treatment increased from 3.30 - 26 mg Mn/kg and 3.92 - 31 mg Zn/kg, respectively. *B. juncea*: Mn (62 - 37 mg/kg) and Zn (79 - 34 mg/kg) decreased as Zn treatment concentration increased.	*B. juncea* tolerated heavy metals at 44 mg Mn/kg and 15 mg Zn/kg. Metal accumulation decreased as metal treatment.	(Rout *et al.*, 1999)
Methyl mercury	Hg	Hg: 10 - 500	*Daucus carota*; *Lactuca sativa*	Cell culture	To determine the effect of light and plant growth regulator (2,4-D) on methyl-mercury phytotoxicity.	Not analysed for *in planta* metal concentrations.	Cell Hg retention decreased as [Hg] increased. *L. sativa* was more sensitive to Hg treatments compared with *D. carota.*	(Czuba, 1987)
Strontium	Sr	Sr: 25, 50, 100, and 200 mg/kg	*Solanum laciniatum*	Shoot	To determine phytoremediation potential of *S. laciniatum* as well as effect of Sr on explant growth parameters.	Explants accumulated 19, 40, 74, and 108 mg Sr/kg when exposed to 25, 50, 100, and 200 mg/kg Sr *in vitro*.	Elevated concentrations of Sr negatively impacted growth parameters as well as a decrease in Ca concentrations.	(Kartosentono *et al.*, 2001)

Uranium	U	U: 5.95 - 1190	*Brassica juncea; Chenopodium amaranticolor*	Root culture	To determine phytoremediation potential of plant species' roots to remove and tolerate uranium.	*Brassica juncea* accumulation decreased when treated with elevated U concentration (i.e. 238 - 1190 mg/kg), roots of *B. juncea* and *C.* amaranticolor accumulated lower concentration (230 and 130 mg U/kg, respectively) compared to accumulation (9000 and 8000 mg U/kg, respectively) when explants were treated with lower U concentration (5.95 - 1190 mg/kg).	Root growth was not affected by U treatments when U ≤ 119 mg/kg. Increased [U] treatment resulted in increased U uptake. Uranium accumulation by both plant species increased as metal treatment concentration increased.	(Eapen *et al.*, 2003)
Zinc	Zn	Zn: 130 - 262	*Populus alba*	Shoot culture	To determine plant's molecular and physiological response to Cu and Zn *in vitro*.	Zn concentration increased from 450 – 800 mg/kg as days increased (i.e., three-ten days, respectively) when grown on solid medium whereas after three days explants accumulated 910 mg Zn/kg when grown in a liquid medium.	Heavy metal treatments reduced adventitious root formation (ARF), increased leaf toxicity, induced elevated gene expression for polyamine biosynthesis, and induced ethylene production.	(Castiglione *et al.*, 2007)

Supplementary Table 4.2. Metal concentrations, bioconcentration factor (BCF), translocation factor (TF), and tolerance index (TI – calculated based on control samples) of five (5) *Tamarix usneoides* genotypes treated with 25% MS supplemented with Pt, Ni, or Co at 0 (control containing basal concentration of 15 µg Co/L), 25, 50, or 100 mg/L at pH 5.5 and 7.5 *in vitro* over a 14-day exposure period. Please note that metal concentration and BCF in roots and shoots, TF, and TI did not significantly differ (KW, $p > 0.05$) between pH levels and therefore, values (pH 5.5 and 7.5) were averaged accordingly. SE = standard error. Please note that averages were calculated where $n \geq 2$ and SE was calculated when $n \geq 3$. Low *in vitro* plantlet establishment percentage of LH31, LH40, and LH47 resulted in insufficient number of samples present for certain metal treatments and were therefore, "***not tested***". Basal concentrations of Co present in MS plant growth medium were 15 µg/L (control). ***All Pt concentrations in roots and shoots are expressed as µg/kg**.

Genotype	Metal	Metal dose (mg/L)	Number of plantlets (n=)	Metal concentration (± SE) (mg/kg)		BCF (± SE)		TF	TI
				Root	Shoot	Roots	Shoots		
LH31	Cobalt	0.0015	2	9	1	NA	NA	NA	NA
		25	2	1320	174	53	7	0.13	71
		50	2	1539	221	31	4	0.15	98
		100	*Not tested*						
	Nickel	0	2	4	1	N/A	N/A	N/A	N/A
		25	3	2265 ± 1038	71 ± 15	91 ± 42	3 ± 1	0.05 ± 0.02	86 ± 12
		50	2	1753	288	35	6	0.17	67
		100	1	753	334	8	3	0.44	54
	Platinum*	0	2	ND	ND	N/A	N/A	N/A	N/A
		25	1	1867.26	22.44	0.07	0.001	0.01	66
		50	2	990.07	762.15	0.02	0.02	11	103
		100	1	10 944.24	75.97	0.10	0.001	0.01	104
LH38	Cobalt	0.0015	2	4	0.34	N/A	N/A	N/A	N/A
		25	2	1088	38	43.50	2	0.04	91
		50	1	1857	69	37	1	0.04	99
		100	4	8 ± 4	0.77 ± 0.47	0.08 ± 0.04	0.01 ± 0.004	0.26 ± 0.16	122 ± 17
	Nickel	0	2	1	0.3	N/A	N/A	N/A	N/A
		25	1	652	53	26.09	2	0.08	104
		50	2	1588	195	31.75	4	0.14	103

		100	3	1897 ± 424	298 ± 88	18.97 ± 4.24	3 ± 0.88	0.18 ± 0.05	110 ± 38
	Platinum*	0	2	ND	ND	N/A	N/A	N/A	N/A
		25	3	1338 ± 223	40.27 ± 14.32	0.05 ± 0.01	0.002 ± 0.001	0.03 ± 0.01	263 ± 128
		50	2	3400.69	64.26	0.07	0.001	0.02	127
		100	3	6360 ± 5162	95.16 ± 41.67	0.06 ± 0.04	0.001 ± 0.000	0.04 ± 0.02	150 ± 35
LH40	Cobalt	0.0015	2	1	0.5	NA	NA	NA	NA
		25	*Not tested*						
		50	*Not tested*						
		100	2	1	0.1	0.01	0.001	0.1	77
	Nickel	0	2	4	2	N/A	N/A	N/A	N/A
		25	3	1521 ± 370	56 ± 4	61 ± 15	2 ± 0.17	0.04 ± 0.01	178 ± 80
		50	*Not tested*						
		100	1	7582	188	76	2	0.02	199
	Platinum*	0	2	ND	ND	N/A	N/A	N/A	N/A
		25	1	4192.93	21.86	0.17	0.001	0.01	112
		50	*Not tested*						
		100	*Not tested*						
LH47	Cobalt	0.0015	2	2	1	N/A	N/A	N/A	N/A
		25	2	716	79	29	3	0.11	59
		50	1	1580	214	32	4	0.14	27
		100	*Not tested*						
	Nickel	0	2	29	0.6	NA	NA	NA	NA
		25	*Not tested*						
		50	4	1489	151	30	3	0.11	60
		100	1	1637	310	16	3	0.19	117
	Platinum*	0	2	ND	ND	N/A	N/A	N/A	N/A
		25	*Not tested*						
		50	2	1896.42	32.11	0.04	0.001	0.02	108
		100	1	5997.92	40.92	0.06	0.001	0.01	108
LH48	Cobalt	0.0015	2	4	1	N/A	N/A	N/A	N/A

	25	5	719 ± 124	80 ± 21	29 ± 5	3.22 ± 0.83	0.14 ± 0.04	110 ± 24
	50	5	1440 ± 336	167 ± 54	29 ± 7	3 ± 1	0.15 ± 0.04	63 ± 13
	100	4	3 ± 0.39	1 ± 0.55	0.03 ± 0.01	0.01 ± 0.01	0.32 ± 0.18	124 ± 18
Nickel	0	2	436	35	N/A	N/A	N/A	N/A
	25	4	863 ± 147	78 ± 11	35 ± 6	3 ± 0.43	0.10 ± 0.02	50 ± 5
	50	2	1130	143	23	3	0.13	54
	100	4	6940 ± 3426	235 ± 80	69 ± 34	2 ± 1	0.07 ± 0.02	38 ± 13
Platinum*	0	2	ND	ND	N/A	N/A	N/A	N/A
	25	5	1512 ± 619	33.23 ± 20.07	0.06 ± 0.02	0.001 ± 0.001	0.03 ± 0.02	34 ± 8
	50	3	2165 ± 635	45.50 ± 14.27	0.04 ± 0.01	0.001 ± 0.001	0.02 ± 0.01	78 ± 7
	100	4	9294 ± 5439	210.56 ± 64.58	0.10 ± 0.05	0.002 ± 0.001	0.04 ± 0.01	57 ± 22

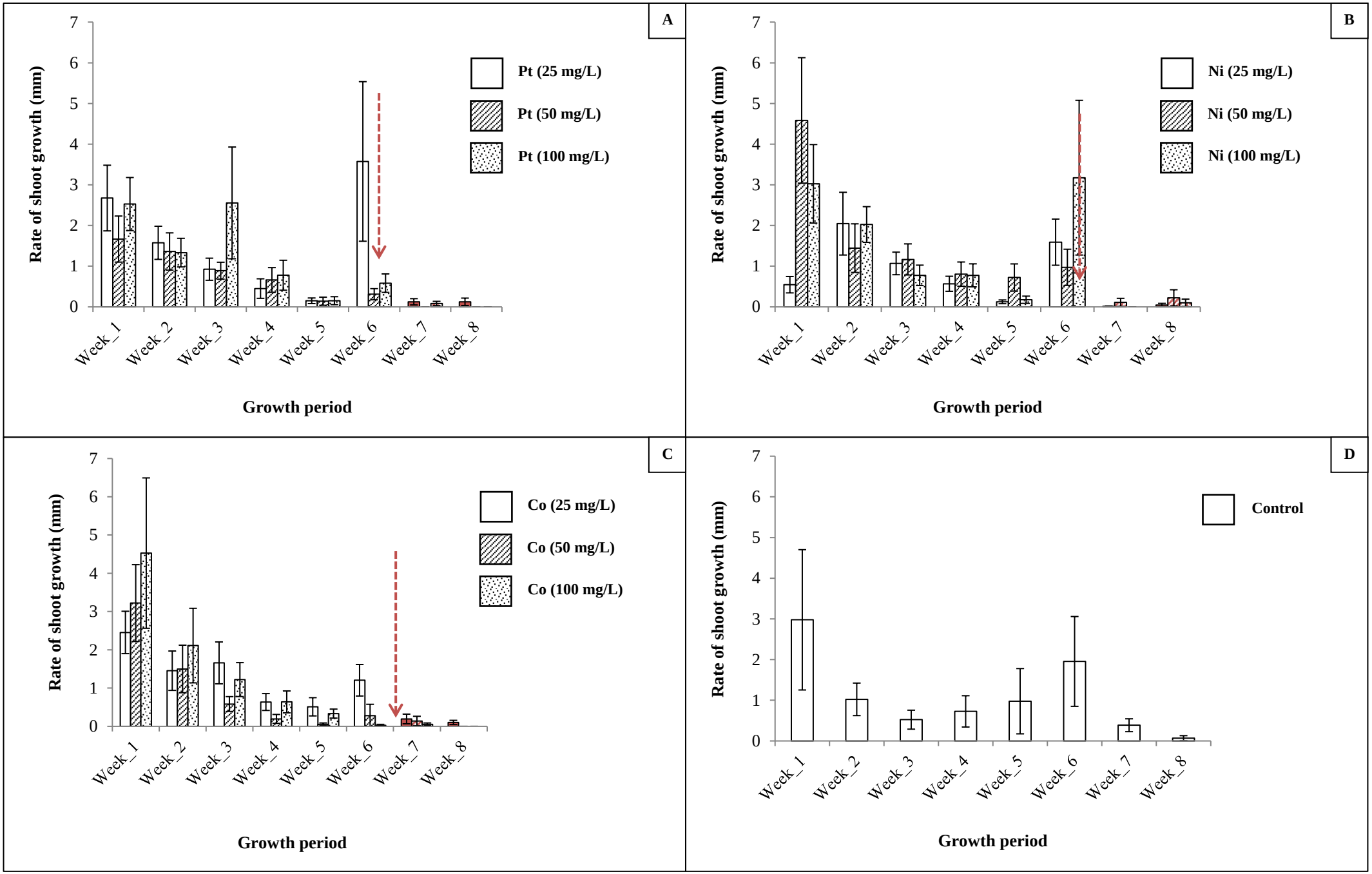
Supplementary Table 4.3. Average metal concentrations in roots and shoots of *T. usneoides* plantlets treated with 25% MS medium supplemented with Pt, Ni, or Co at 25, 50, 100 mg/kg at either pH 5.5 or 7.5, and associated, measured physiological growth parameters *in vitro*. C1 = LH31; C2 = LH38; C3 = LH40; C4 = LH47; C5 = LH48. ***All Pt concentrations in roots and shoots are expressed as µg/kg**.

Metal	Treatment concentration (mg/L)	pH	Ontogeny	Genotype	Root metal concentration (mg/kg)*	Shoot metal concentration (mg/kg)*	Shoot length (mm)	Shoot branching	Root length (mm)	Number of roots	Degree of root branching	Degree of shoot branching
Pt*	25	5.5	Top	C5	436.97	4.16	52.25	19	57.00	2	3rd	3rd
			Middle	C2	939.31	11.77	37.00	26	54.00	4	2nd	2nd
			Middle	C2	1365.94	56.94	40.00	26	179.00	4	3rd	2nd
			Bottom	C5	1311.86	10.56	54.50	24	50.00	3	3rd	2nd
			Bottom	C5	869.50	9.30	42.75	16	30.00	7	2nd	2nd
		7.5	Top	C1	1867.27	22.44	42.00	21	37.00	7	3rd	2nd
			Top	C2	1708.57	52.09	52.75	18	40.00	6	2nd	2nd
			Top	C3	4192.92	21.86	43.00	22	48.00	1	2nd	2nd
			Top	C5	1018.09	111.43	60.00	15	18.00	1	3rd	0
			Bottom	C5	3921.28	30.66	50.75	22	22.00	9	3rd	2nd
	50	5.5	Top	C1	1913.26	70.43	45.00	27	57.00	2	3rd	2nd
			Top	C4	2398.72	49.52	42.00	26	56.50	5	3rd	0
			Middle	C5	3401.50	69.09	51.00	15	86.00	1	2nd	2nd
			Bottom	C2	3419.94	79.39	46.00	21	44.00	3	3rd	3rd
		7.5	Top	C1	1453.86	66.87	36.00	23	58.00	1	3rd	2nd
			Top	C4	1394.12	14.69	42.00	21	96.00	5	2nd	2nd
			Top	C5	1800.20	19.80	46.25	20	67.00	6	2nd	2nd
			Middle	C2	3381.43	49.13	41.25	24	44.00	4	2nd	2nd
			Bottom	C5	1292.72	47.61	55.25	25	89.00	5	3rd	2nd
	100	5.5	Top	C2	1226.62	17.69	44.00	16	32.00	3	3rd	2nd
			Middle	C5	2293.73	100.78	45.00	15	32.00	1	2nd	2nd
			Middle	C5	25 395.97	166.08	40.00	13	41.00	2	3rd	3rd
			Bottom	C2	1169.07	107.27	38.50	23	74.00	8	3rd	2nd

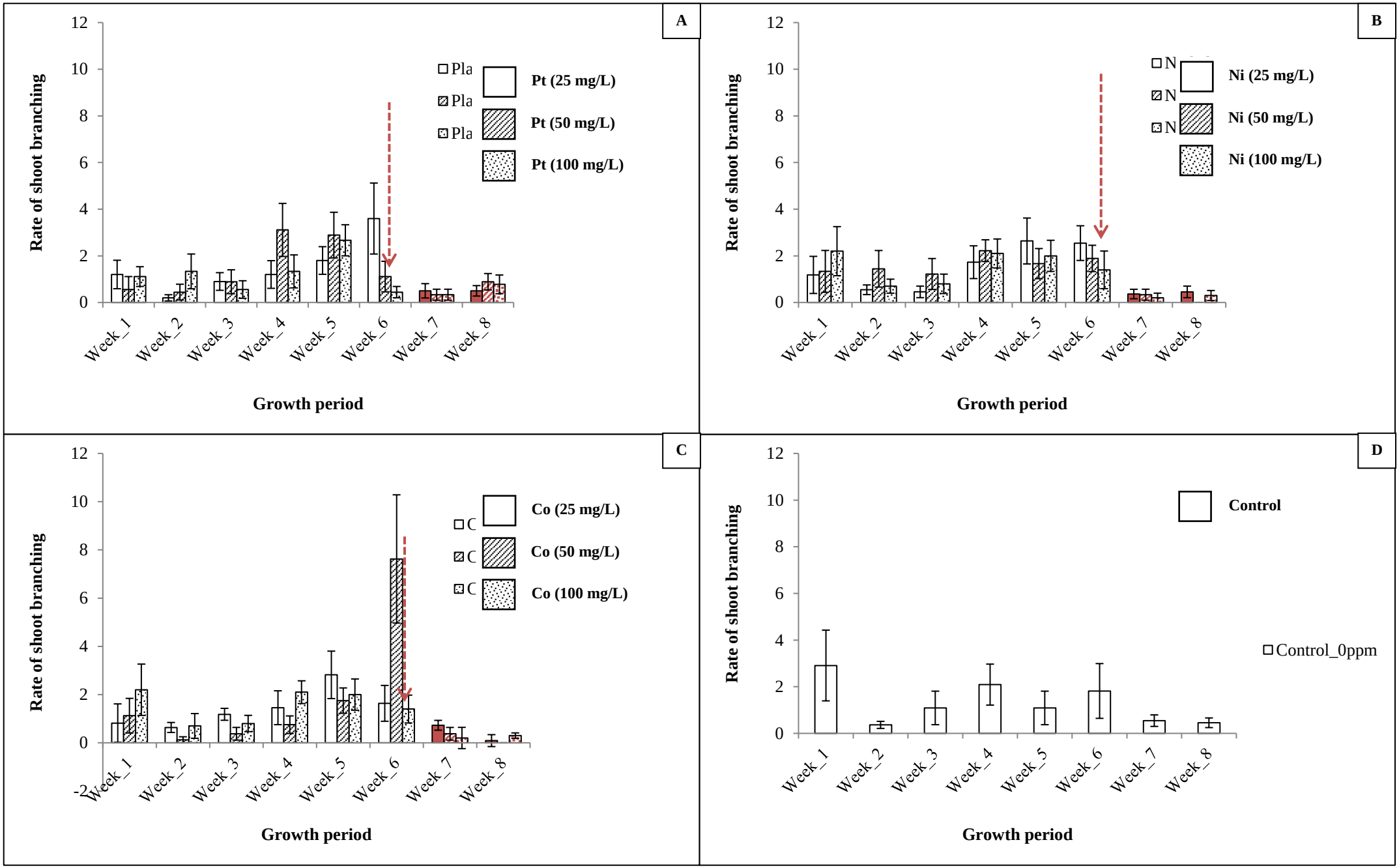
		7.5	Top	C4	6377.03	397.49	40.00	22	76.00	8	3rd	0
			Middle	C5	3110.80	177.87	62.00	15	103.00	2	2nd	2nd
			Bottom	C1	10 944.24	75.97	40.50	18	58.00	4	3rd	2nd
			Bottom	C2	16 683.9	160.50	42.00	26	50.00	4	3rd	2nd
	25	5.5	Top	C1	4795.73	60.48	39.00	16	38.00	2	2nd	0
			Top	C1	791.10	47.11	44.00	19	61.00	4	3rd	2nd
			Middle	C5	832.52	46.80	52.00	30	62.00	6	3rd	2nd
			Bottom	C3	2420.52	65.75	42.25	16	62.00	2	3rd	3rd
			Bottom	C3	972.03	56.06	49.00	26	142.00	5	2nd	2nd
		7.5	Top	C1	1208.15	106.29	38.25	23	45.00	5	3rd	2nd
			Top	C2	652.36	52.91	41.00	22	36.00	3	3rd	0
			Top	C5	1342.96	98.19	46.00	14	59.00	2	3rd	2nd
			Middle	C3	1169.07	47.46	44.50	21	26.00	4	2nd	0
			Bottom	C5	723.27	69.38	53.00	24	42.00	6	2nd	0
			Bottom	C5	553.87	97.48	62.00	24	44.00	6	3rd	2nd
Ni	50	5.5	Middle	C1	1807.80	177.44	41.25	18	31.00	3	3rd	2nd
			Middle	C4	1289.87	184.78	46.00	29	39.00	5	3rd	2nd
			Middle	C5	1136.17	85.11	62.00	33	45.00	4	3rd	0
			Bottom	C2	2041.87	178.04	43.00	25	34.00	2	3rd	2nd
		7.5	Top	C4	1688.25	116.58	42.50	22	46.00	9	2nd	2nd
			Top	C5	1124.17	200.78	48.00	12	66.00	3	3rd	2nd
			Middle	C1	1697.96	398.44	44.50	20	44.00	3	3rd	3rd
			Middle	C2	1133.35	212.44	43.50	22	37.00	3	3rd	2nd
	100	5.5	Top	C2	2506.25	164.47	39.75	24	21.00	3	3rd	2nd
			Middle	C3	7582.32	187.58	36.25	30	85.00	3	3rd	2nd
			Middle	C5	2581.49	92.69	67.00	27	42.00	6	3rd	0
			Bottom	C5	3829.06	487.47	65.25	6	1.50	2	0	0
		7.5	Top	C2	863.05	219.43	41.25	23	29.00	8	3rd	2nd
			Middle	C1	752.75	333.71	35.25	19	30.00	4	3rd	2nd

			Middle	C5	18 776.00	104.85	60.50	16	46.00	8	2nd	2nd
			Bottom	C2	2321.95	510.31	46.00	24	64.00	7	2nd	2nd
			Bottom	C4	1636.89	309.53	43.00	27	82.00	3	3rd	2nd
			Bottom	C5	2574.18	256.01	56.25	21	67.00	7	3rd	2nd
			Top	C1	967.32	133.49	37.00	15	40.00	5	2nd	0
			Top	C4	758.89	98.08	47.00	25	33.50	7	3rd	2nd
		5.5	Middle	C2	1319.79	39.64	46.00	23	35.00	7	3rd	2nd
			Middle	C5	842.17	46.12	52.00	26	191.00	4	2nd	2nd
			Bottom	C5	1147.79	69.43	58.25	25	102.00	3	2nd	2nd
	25		Top	C1	1672.16	215.47	43.25	23	40.00	8	3rd	2ND
			Top	C2	855.43	36.75	43.00	21	28.00	8	2nd	0
		7.5	Top	C4	673.12	59.34	42.00	22	49.00	5	3rd	2nd
			Top	C5	620.39	171.33	45.00	10	76.00	2	2nd	2nd
			Bottom	C5	681.48	47.48	50.50	16	142.00	3	3rd	3rd
			Bottom	C5	301.86	67.73	59.13	20	55.00	7	3rd	2nd
			Top	C5	1794.72	132.26	48.75	27	114.50	4	2nd	2nd
Co		5.5	Middle	C5	2173.70	291.98	60.00	32	40.00	4	3rd	0
			Middle	C5	1739.46	320.89	42.75	20	49.50	7	2nd	2nd
			Bottom	C1	1304.65	280.27	34.50	13	67.00	1	2nd	2nd
	50		Top	C4	1579.91	213.78	42.00	23	19.00	8	2nd	2nd
			Middle	C1	1772.56	162.58	43.00	14	43.00	1	2nd	2nd
		7.5	Middle	C2	1857.15	69.31	45.00	27	34.00	9	2nd	2nd
			Middle	C5	1490.42	89.94	44.25	21	51.00	4	3rd	2nd
			Bottom	C5	1.47	0.44	69.50	24	70.00	8	3rd	2nd
			Top	C2	6.23	0.46	46.00	22	32.00	6	2nd	2nd
		5.5	Middle	C3	1.43	0.09	39.50	14	33.00	1	3rd	2nd
	100		Middle	C5	2.25	0.20	58.75	24	151.00	9	3rd	2nd
			Bottom	C2	21.83	0.10	43.50	19	32.50	4	3rd	2nd
		7.5	Top	C2	0.91	0.16	48.00	21	49.00	8	3rd	2nd

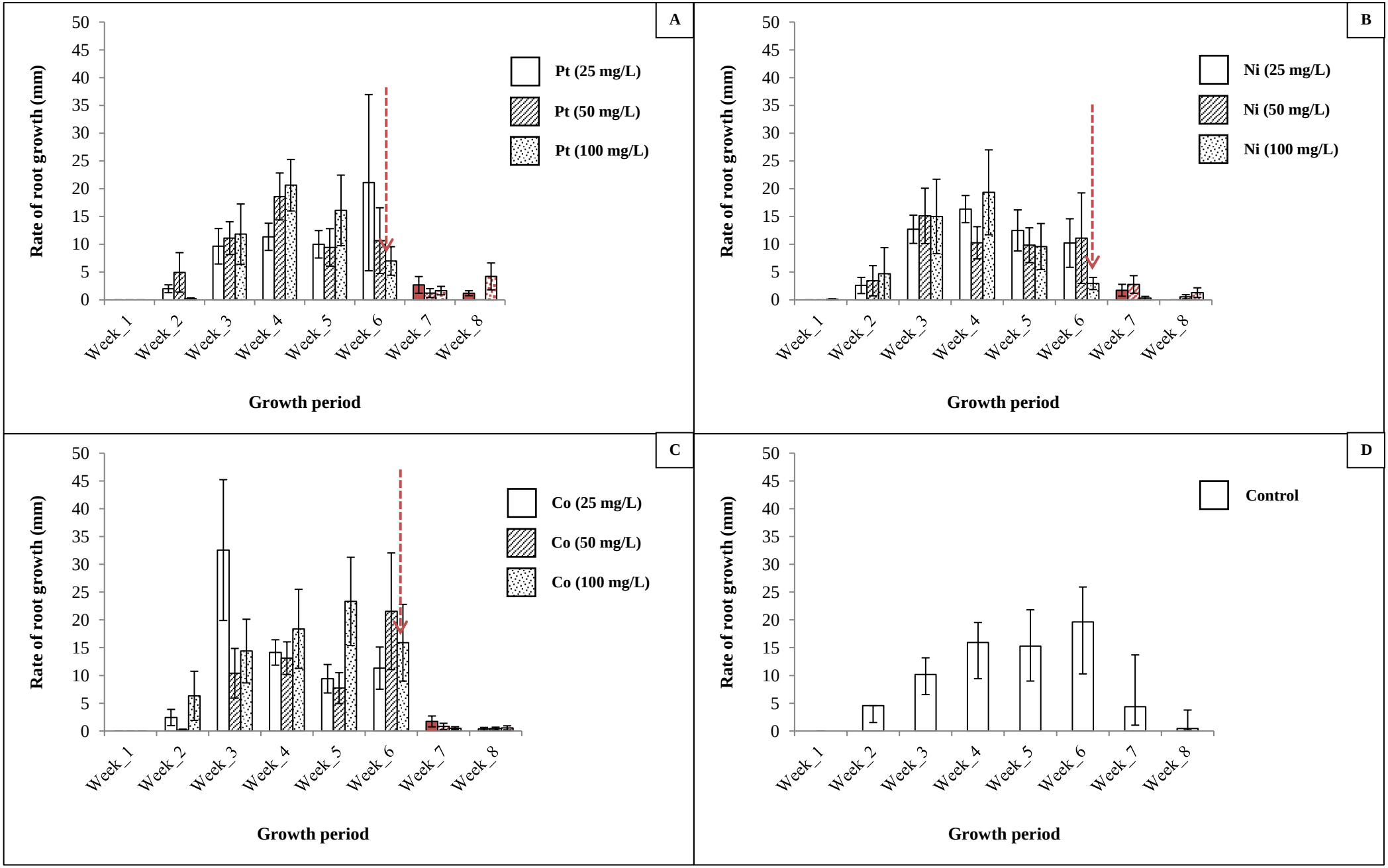
	Top	C5	3.01	2.83	44.25	20	167.00	2	2nd	2nd
	Middle	C5	2.44	0.11	47.00	20	89.00	4	3rd	2nd
	Bottom	C2	2.99	2.38	34.25	18	56.00	2	3rd	3rd
	Bottom	C5	4.24	0.83	48.75	21	105.00	3	3rd	2nd



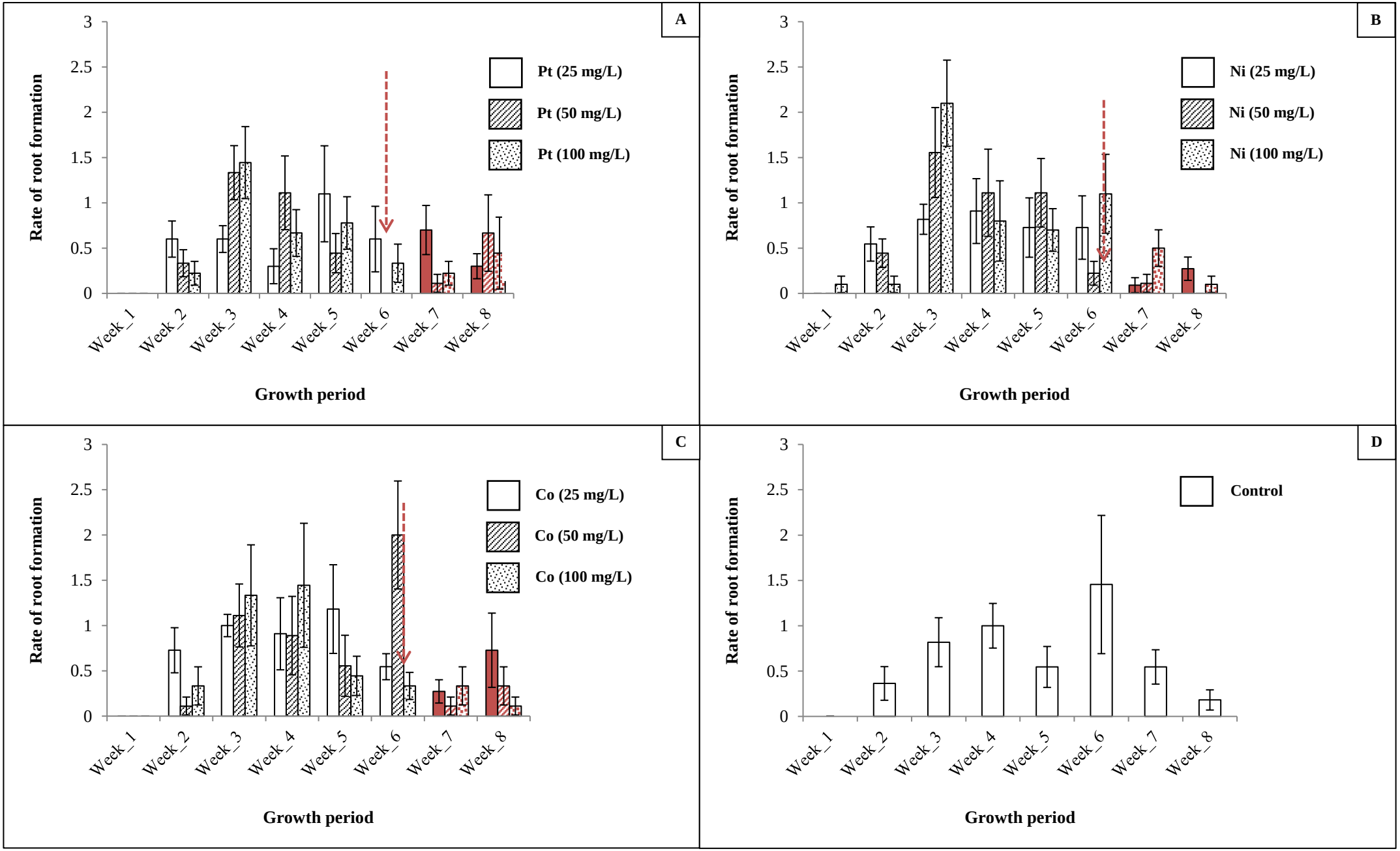
Supplementary Figure 4.1. Shoot growth (elongation) (mm) by *Tamarix usneoides* plantlets over a six-week growth period and two-week metal exposure period where plantlets were exposed to 25% MS medium supplemented with platinum (Pt), nickel (Ni), or cobalt (Co) at 0 (control), 25, 50, or 100 mg/L at pH 5.5 or 7.5. Plantlets were exposed to metal treatments (for two weeks) (represented by red dotted arrow and bars) after six weeks of culture. Note, basal concentrations of Co present in MS plant growth medium were 15 µg/L (control).



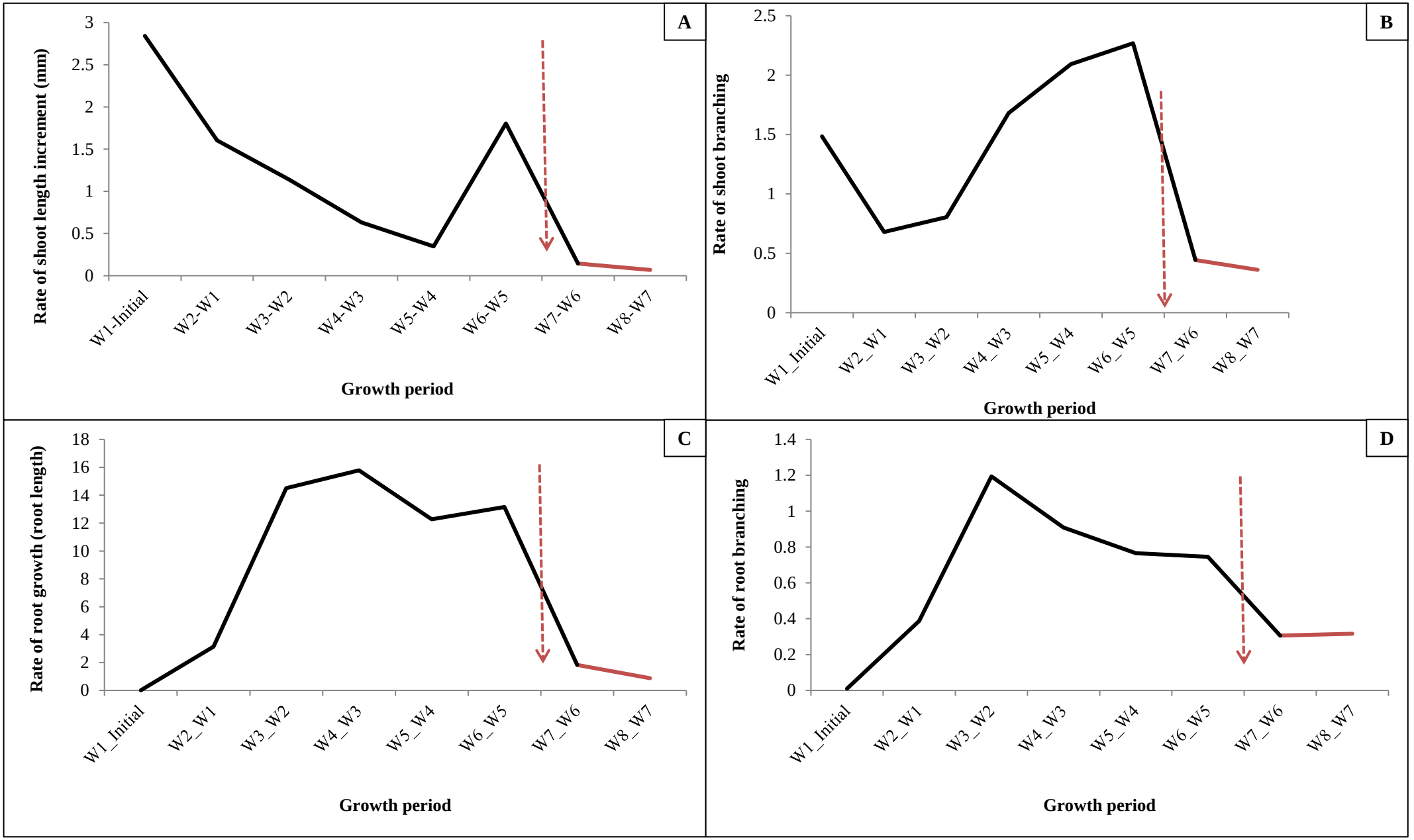
Supplementary Figure 4.2. Shoot branching (number of shoots developed) by *Tamarix usneoides* plantlets over a six-week growth period and two-week metal exposure period (represented by red dotted arrow and bars) where plantlets were exposed to 25% MS medium supplemented with platinum (Pt), nickel (Ni), or cobalt (Co) at 0 (control), 25, 50, or 100 mg/L at pH 5.5 or 7.5. Note, basal concentrations of Co present in MS plant growth medium were 15 µg/L (control).



Supplementary Figure 4.3. Root growth (elongation) (mm) by *Tamarix usneoides* plantlets over a six-week growth period and two-week metal exposure period (represented by red dotted arrow and bars) where plantlets were exposed to 25% MS medium supplemented with platinum (Pt), nickel (Ni), or cobalt (Co) at 0 (control), 25, 50, or 100 mg/L at pH 5.5 or 7.5. Note, basal concentrations of Co present in MS plant growth medium were 15 µg/L (control).



Supplementary Figure 4.4. Root formation by *Tamarix usneoides* plantlets over a six-week growth period and two-week metal exposure period (represented by red dotted arrow and bars) where plantlets were exposed to 25% MS medium supplemented with platinum (Pt), nickel (Ni), or cobalt (Co) at 0 (control), 25, 50, or 100 mg/L at pH 5.5 or 7.5. Note, basal concentrations of Co present in MS plant growth medium were 15 µg/L (control).



Supplementary Figure 4.5. Morphological growth, namely (A) shoot growth, (B) shoot branching, (C) root growth (elongation), and (D) root formation, by *Tamarix usneoides* plantlets over a six-week growth period and two-week metal exposure period where plantlets were exposed to 25% MS medium supplemented with platinum (Pt), nickel (Ni), or cobalt (Co) at 0 (control), 25, 50, or 100 mg/L at pH 5.5 or 7.5. Red arrow (dashed) represents addition of metal treatment whereas red line represents metal exposure period.

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