ECOPHYSIOLOGY AND PHYTOREMEDIATION POTENTIAL OF HEAVY METAL(LOID) ACCUMULATING PLANTS

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B.Hort.Sc.(Hons)

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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— MMVIII —

— CERTIFICATE OF ORIGINALITY —

I hereby declare that the text of this thesis is my own work, and that, to the best of my knowledge and belief, it contains no material that has been previously published or written by another person, nor any material that has been accepted as part of the requirements for any other degree or diploma in any university or other institute of higher learning, unless due acknowledgement has been made.

I also declare that the intellectual content of this thesis is original and the result of my own research and to the best of my knowledge and belief, any assistance I received in the experimentation presented, and all sources of information cited have been duly acknowledged.

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Anthony George Kachenko

Declared at ....
this ....day of
....2008

TO
MY PARENTS

— ABSTRACT —

Soil contamination with heavy metal(loid)s is a major environmental problem that requires effective and affordable remediation technologies. The utilisation of plants to remediate heavy metal(loid)s contaminated soils has attracted considerable interest as a low cost *green* remediation technology. The process is referred to as *phytoremediation*, and this versatile technology utilises plants to *phytostabilise* and/or *phytoextract* heavy metal(loid)s from contaminated soils, thereby effectively minimising their threat to ecosystem, human and animal health. Plants that can accumulate exceptionally high concentrations of heavy metal(loid)s into above-ground biomass are referred to as *hyperaccumulators*, and may be exploited in phytoremediation, geobotanical prospecting and/or phytomining of low-grade ore bodies. Despite the apparent tangible benefits of utilising phytoremediation techniques, a greater understanding is required to comprehend the ecophysiological aspects of species suitable for phytoremediation purposes.

A screening study was instigated to assess phytoremediation potential of several fern species for soils contaminated with cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb) and zinc (Zn). Hyperaccumulation was not observed in any of the studied species, and in general, species excluded heavy metal uptake by restricting their translocation into aboveground biomass. *Nephrolepis cordifolia* and *Hypolepis muelleri* were identified as possible candidates in phytostabilisation of Cu-, Pb-, Ni- or Zn-contaminated soils and *Dennstaedtia davallioides* appeared favourable for use in phytostabilisation of Cu- and Zn-contaminated soils. Conversely, *Blechnum nudum*, *B. cartilagineum, Doodia aspera* and *Calochlaena dubia* were least tolerant to most heavy metals and were classified as being least suitable for phytoremediation purposes

Ensuing studies addressed the physiology of arsenic (As) hyperaccumulation in a lesser known hyperaccumulator, *Pityrogramma calomelanos* var. *austroamericana*. The phytoremediation potential of this species was compared with that of the well known As hyperaccumulator *Pteris vittata*. Arsenic concentration of 3,008 mg kg^{-1} dry weight (DW) occurred in *P. calomelanos* var. *austroamericana* fronds when exposed to 50 mg kg^{-1} As without visual symptoms of phytotoxicities. Conversely, *P. vittata* was able to hyperaccumulate 10,753 mg As kg^{-1} DW when exposed to 100 mg kg^{-1} As without the onset

of phytotoxicities. In *P. calomelanos* var. *austroamericana*, As was readily translocated to fronds with concentrations 75 times greater in fronds than in roots. This species has the potential for use in phytoremediation of soils with As levels up to 50 mg kg^{-1}.

Localisation and spatial distribution of As in *P. calomelanos* var. *austroamericana* pinnule and stipe tissues was investigated using micro-proton induced X-ray emission spectrometry (μ-PIXE). Freeze-drying and freeze-substitution protocols (using tetrahydrofuran [THF] as a freeze-substitution medium) were compared to ascertain their usefulness in tissue preservation. Micro-PIXE results indicated that pinnule sections prepared by freeze-drying adequately preserved the spatial elemental distribution and tissue structure of pinnule samples. In pinnules, μ-PIXE results indicated higher As concentration than in stipe tissues, with concentrations of 3,700 and 1,600 mg As kg^{-1} DW, respectively. In pinnules, a clear pattern of cellular localisation was not resolved whereas vascular bundles in stipe tissues contained the highest As concentration (2,000 mg As kg^{-1} DW). Building on these μ-PIXE results, the chemical speciation of As in *P. calomelanos* var. *austroamericana* was determined using micro-focused X-ray fluorescence (μ-XRF) spectroscopy in conjunction with micro-focused X-ray absorption near edge structure (μ-XANES) spectroscopy. The results suggested that arsenate (As^{V}) absorbed by roots was reduced to arsenite (As^{III}) in roots prior to transport through vascular tissues as As^{V} and As^{III}. In pinnules, As^{III} was the predominant species, presumably as aqueous-oxygen coordinated compounds. Linear least-squares combination fits of μ-XANES spectra showed As^{III} as the predominant component in all tissues sampled. The results also revealed that sulphur containing thiolates may, in part sequester accumulated As.

The final aspect of this thesis examined several ecophysiological strategies of Ni hyperaccumulation in *Hybanthus floribundus* subsp. *floribundus*, a native Australian perennial shrub species and promising candidate in phytoremediation of Ni-contaminated soils. Micro-PIXE analysis revealed that cellular structure in leaf tissues prepared by freeze-drying was adequately preserved as compared to THF freeze-substituted tissues. Elemental distribution maps of leaves showed that Ni was preferentially localised in the adaxial epidermal tissues and leaf margin, with concentration of 10,000 kg^{-1} DW in both regions. Nickel concentrations in stem tissues obtained by μ-PIXE analysis were lower than in the leaf tissues (1,800 mg kg^{-1} vs. 7,800 mg kg^{-1} DW, respectively), and there was no clear pattern of compartmentalisation across different anatomical regions. It is possible that storage of accumulated Ni in epidermal tissues may provide Ni tolerance to this species, and may further act as a deterrent against herbivory and pathogenic attack. In *H. floribundus* subsp. *floribundus* seeds, μ-PIXE analysis did not resolve a clear pattern of Ni compartmentalisation and suggests that Ni was able to move apoplastically within the seed tissues.

The role of organic acids and free amino acids (low molecular weight ligands [LMW]) in Ni detoxification in *H. floribundus* subsp. *floribundus* were quantified using high performance liquid chromatography (HPLC) and ultra performance liquid chromatography (UPLC). Nickel accumulation stimulated a significant increase in citric acid concentration in leaf extracts, and based on the molar ratios of Ni to citric acid (1.3:1–1.7:1), citric acid was sufficient to account for approximately 50% of the accumulated Ni. Glutamine, alanine and aspartic acid concentrations were also stimulated in response to Ni hyperaccumulation and accounted for up to 75% of the total free amino acid concentration in leaf extracts. Together, these LMW ligands may complex with accumulated Ni and contribute to its detoxification and storage in this hyperaccumulator species.

Lastly, the hypothesis that hyperaccumulation of Ni in certain plants may act as an osmoticum under water stress (drought) was tested in context of *H. floribundus* subsp. *floribundus*. A 38% decline in water potential and a 68% decline in osmotic potential occurred between water stressed and unstressed plants, however, this was not matched by an increase in accumulated Ni. The results suggested that Ni was unlikely to play a role in osmotic adjustment in this species. Drought stressed plants exhibited a low water use efficiency which might be a conservative ecophysiological strategy enabling survival of this species in competitive water-limited environments.

* * *

— ACKNOWLEDGEMENTS —

"A journey of a thousand miles begins with a single step"

Lao-tzu, Chinese Philosopher (604 BC –531 BC).

It must be said that when I first commenced my postgraduate studies, I was somewhat overwhelmed with the thought of several years of *original* research, extensive field studies, hours of laboratory analyses, conference presentations, manuscript submissions – the litany could go on for ever. However, with careful planning, sound advice and support from several remarkable people, it was possible to negotiate hurdles, maintain momentum and focus on the onerous tasks at hand. During my final year as an undergraduate student, I can clearly recall the Dean of the Faculty of Agriculture, Food and Natural Resources (FAFNR), Professor Les Copeland, encouraging students to apply for postgraduate research scholarships. In his address, he proceeded to say that from his own experience, postgraduate study was one of the best, yet challenging times in your life. To Professor Les Copeland, I would have to say that to this day, I wholeheartedly agree with you.

To Associate Professor Balwant Singh, you have always travelled above and beyond your duty as supervisor in all aspects of my candidature, and have at all times maintained committed and dedicated to my research endeavours. I sincerely thank you for initiating this project, advising and encouraging me, and most importantly, for maintaining a high level of contact with me throughout the years, particularly during your sabbatical in 2006. I would like to sincerely thank Dr. Naveen Bhatia who steered me towards researching the ecophysiology of hyperaccumulation and the fascinating world of histology and μ-PIXE spectroscopy. Naveen played an integral part in several aspects of this work, notably the choice of hyperaccumulating species to research. Naveen always provided enthusiasm, encouragement and tremendous support throughout all aspects of my research, particularly during times when I couldn't see the light.

"I am not discouraged, because every wrong attempt discarded is another step forward."

Thomas Alva Edison, American Inventor (1847 –1931)

My sincere thanks to Dr. Markus Gräfe (University of Sydney) and Dr. Rainer Siegele (Australian Nuclear Science and Technology Association: ANSTO) who were 'de facto' supervisors, and at all times provided invaluable support, criticisms and guidance when I needed it the most. Both Balwant and Markus introduced me to the intriguing world of synchrotron science and I feel honoured to have worked with such passionate scientists, and to have had the opportunity to conduct a beamline experiment and visit the Australian Synchrotron. I thank Dr. Rainer Siegele who bent over backwards to assist me with my μ-PIXE studies and made me feel welcome each and every visit to ANSTO. Rainer always had an answer to every question I threw at him, and at all times was enthusiastic and dedicated towards my studies. I would also like to thank Dr. David Cohen and Dr. Mihail Ionescu (ANSTO) who also made life a lot easier for me during my many ANSTO visits.

The bulk of this research would not have been possible without generous funding from several sources. I would like to acknowledge the support of the University of Sydney (USYD) for offering me a Commonwealth of Australia, Australian Postgraduate Award scholarship, and to the FAFNR for providing financial support. I also wish to acknowledge financial assistance from the Australian Institute of Nuclear Science and Engineering (AWARD No. AINGRA 05150 and 06160) and the Australian Institute of Agricultural Science & Technology Drought Research Fund. I wish to thank the Australian Synchrotron Research Program, for enabling me to travel to the Advanced Photon Source, Chicago, USA to conduct my beamline experiments. Lastly, I wish to acknowledge the U. S. Department of Energy, Office of Science, Office of Basic Energy Sciences, for funding my beam time.

I am indebted to the support of several people, who at some stage along the way provided much needed encouragement and/or support. To Mr. George and Mrs. Valerie Sonter from Sonters Fern Nurseries, thank you for introducing me to the world of fern propagation, showing me around your nursery, and supplying me with hundreds of sporelings. To Marilyn Sprague from Goldfields Revegetation Nursery, Mandurang, Victoria, thank you for your time in the field, providing me with your commentary on all things 'native' and supplying me with *Hybanthus* cuttings. To Associate Professor Nanjappa Ashwath from Central Queensland University, thank you for accompanying me to Mt. Morgan Gold Mine and assisting with field work. I wish to thank the Electron Microscope Unit for assistance with specimen preparation, particularly Ms. Emine Korkmaz, Dr. Louise Cole, Ms. Anne Simpson and Mr. Tony Romeo. I would also like to extend my gratitude to Mrs. Carol Campbell for assisting me with anatomical interpretations of plant material and Dr. Edith Lees for providing constructive comments on this thesis. Thanks also goes to Dr. Steve Heald and Dr. Matt Newville (Advanced Photon Source, Argonne National Laboratory, Illinois, United States of America) and Dr. Euan Smith (University of South Australia, Adelaide) for assisting with various aspects of my synchrotron experiments. Special thanks to Mr. Bernie McInerney and Mr. Leon McQuade from the Australian Proteome Analysis Facility, Macquarie University, and Mr Malcolm Nobel from the University of New South Wales for sample analyses.

To the dedicated technical staff who assisted me at some stage along the way, my warmest appreciation. You are the skeleton that supports research and without your support, research would falter. In particular, I wish to thank Ms Iona György (Aunty I), who "*at the drop of a hat*" kindly offered her expertise whenever it was 'demanded', and was a friend more than a colleague. Thank you to Mr. Colin Bailey, Mr. Kevin McLauchlan, Mrs. Jarka Geisler and Ms. Nazaneen Soran for your technical assistance and support. Thanks also go to Mr. Tom Savage for his invaluable assistance in ICP-AES analyses, and to Ms. Maryann Ebsworth and Dr. Mick O'Neill, who provided much needed statistical advice. A big thank you to the administration staff, Ms. Pamela Stern, Ms. Prue Winkler, Ms. Fortunée Cantrell, Mr. Kyle Kiefer, Ms. Nancy Cheng and Ms. Pamela Brass who like the technical staff, provided me with overwhelming levels of logistical support throughout the course of my study.

During my time at USYD, I was provided with ample opportunity to get involved in the postgraduate community (and university politics) at both faculty and university levels. Thank you to the university bureaucracy for providing me with this experience, and a thank you goes to all those intriguing people who shed light on the *inner mechanisms* of university politics. To my fellow comrades in arms, it has truly been an amazing experience and I am glad that I shared this chapter in my life with you – all the very best in your future endeavours. The lunches and coffees have been a pleasant distraction and a superb chance to escape Room 227 (the dungeon) of Ross Street. To Dr. Prashant Srivastava and Dr. Ghulam Mustafa who helped me stand on my own two feet and showed me the ropes from day one, thank you. To all of Balwants past and present students, who made lab work fun and office work enjoyable, and I sincerely thank you for your camaraderie over the years. To the Panebianco family from

Ralph's Café, Archie and Steve, my thanks for all the laughs and good times, for the mouth watering food and exceptional coffees – 'bit of this……bit of that'.

I wish to acknowledge the tremendous support form my family, particularly my sister Therese, who has always stood by me and given nothing but encouragement and praise. To all my friends, who have supported my studies over the years, thank you. I will no longer have to water plants on Saturday nights and on public holidays ever again (here's hoping). My passion for science, particularly biology began in high school, when Mrs. Margaret Shepherd captivated me by making the subject, interesting and thoroughly enjoyable. In particular, I wish to acknowledge Mr. Jim and Mrs. Jann Cloros (and George) and the EO crew, particularly Mr. Mike Mehigan and Ms. Susan Potthurst who have always offered support, friendship and shown interest in my affairs – thank you. In different ways, you have all taught me that, "When there is no vision, the people perish" (Proverbs 29:18).

And finally, I would like to convey my deepest love and heartfelt thanks to my parents who taught me that I can do all things through Christ, who has provided me with great strength and has sustained me throughout these challenging times. Mum and Dad, you have always told me to believe in myself and over the past 26 years and have bent over backward to provide me with a rich and fulfilling upbringing and a sound education. You instigated my passion for the great outdoors, the environment and all things horticultural. I fondly remember your encouraging words, in particular reaffirming that:

"Genius is one percent inspiration, ninety-nine percent perspiration"

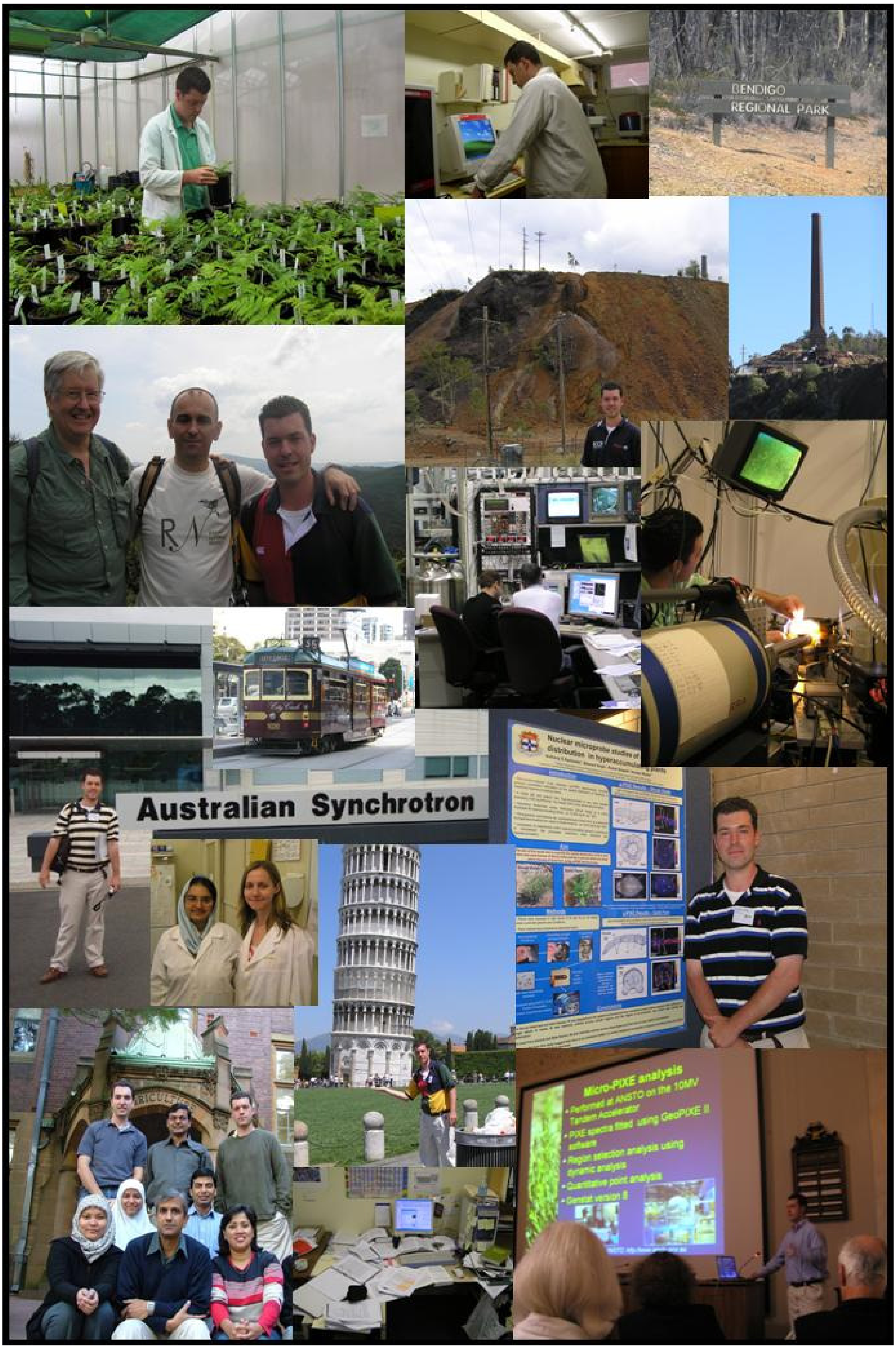
Thomas Alva Edison, American Inventor (1847–1931)

I thank you both for all you have sacrificed and for coping with my rollercoaster of ups and downs during the course of my studies. I can say that this experience had been a rich and rewarding one from which I have learnt that:

"The three great essentials to achieve anything worth while are, first, hard work; second, stick-to-itiveness; third, common sense."

Thomas Alva Edison, American Inventor (1847–1931)

— PICTORIAL TRAVELOGUE —



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2. Kachenko AG, Bhatia NP, Singh B, Siegele R (2007) Arsenic hyperaccumulation and localization in the pinnule and stipe tissues of the gold-dust fern (*Pityrogramma calomelanos* (L.) Link var. *austroamericana* (Domin) Farw.) using quantitative micro-PIXE spectroscopy. *Plant and Soil* **300,** 207–219.

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4. Siegele R, Kachenko AG, Bhatia NP, Wang YD, Ionescu M, Singh B, Baker AJM, Cohen DD (2008) Localisation of trace metals in metal-accumulating plants using μ-PIXE. *X-Ray Spectrometry* **37,** 133–136.

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2. Kachenko AG, Singh B, Siegele R and Bhatia N P (2007) Nuclear microprobe studies of metal(loid) distribution in hyperaccumulating plants. Proceedings of the 15th AINSE Conference on Nuclear and Complementary Techniques of Analysis & 10th Vacuum Society of Australia Congress. Melbourne; Australia; November 21–23.

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1. Kachenko AG, Singh B, Bhatia NP, Siegele R (2006) Localisation & quantification of nickel in leaf and stem tissues of *Hybanthus floribundus* var. *floribundus* using micro-PIXE. Presented at the Fifth International Conference on Serpentine Ecology. Siena, Italy, May 9–13.

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3. Kachenko AG, Bhatia NP, Singh B, Siegele R (2006) Localisation & quantification of arsenic in *Pityrogramma calomelanos* (L.) Link var. *austroamericana*. Presented at the Faculty of Agriculture, Food and Natural Resources Symposium: New Horizons in Agricultural Research: Postgraduate Innovations. Sydney; Australia; November 24.

4. Kachenko AG, Gräfe M, Singh B and Heald SM (2007) Arsenic speciation in hyperaccumulating gold-dust fern using micro focussed X-ray absorption and X-ray fluorescence spectroscopies. ASRP/Australian Synchrotron Users Meeting. Melbourne; Australia; December 12–14.

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— TABLE OF CONTENTS —

CHAPTER 1: General introduction & background 1

CHAPTER 5: Quantitative nickel localisation in leaf, stem and seed tissues of *Hybanthus floribundus* subsp. *floribundus* 113

— LIST OF FIGURES —

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

— LIST OF TABLES —

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

— ABBREVIATIONS —

AAS	atomic absorption spectrometer
AFA	abstract factor analysis
AM	arbuscular mycorrhiza
ANOVA	analysis of variance
ANSTO	Australian Nuclear Science and Technology Organisation
ANU	Australian National University
APS	Advanced Photon Source
AR	analytical reagent
BDL	below detection limit
CDF	cation diffusion facilitator
cDNA	complimentary deoxyribonucleic acid
CMT	computed-microtomography
Cys	cysteine
DA	dynamic analysis
DMA	dimethylarsenic acid
DPX	distrene, plasticiser, xylene
DTPA	diethylenetriamine pentaacetic acid
DW	dry weight
EC	electrical conductivity
ECO	ectomycorrhiza
EDTA	ethylenediaminetetraacetic acid
EDXA	energy dispersive X-ray analysis
EELS	electron energy loss spectroscopy
EXAFS	extended X-ray absorption fine structure
FAFNR	Faculty of Agriculture, Food and Natural Resources
GC-MS	gas chromatography-mass spectrometry
GF-AAS	graphite furnace atomic absorption spectrometer
Glu	glutamate
Gly	glycine
GSH	glutathione
$HClO_4$	perchloric acid

HNO_3	nitric acid
HPLC	high precision liquid chromatography
HSP	heat shock protein
ICP-AES	inductively couples plasma-atomic emission spectrometry
IND	indicator value
IRT	iron-regulated transporter
LCF	linear least-squares combination fit
LMW	low molecular weight
LN_2	liquid nitrogen
LS	level of significance
LSD	least significant difference
MDL	minimum detection limit
μ-PIXE	micro-proton induced X-ray emission
μ-XANES	micro-X-ray absorption near-edge structures
μ-XRF	micro-X-ray fluorescence
μ-XRS	micro-X-ray spectroscopy
MDL	minimum detection limit
MMA	methylarsonic acid
MT	metallothioneins
nano-SIMS	nano-secondary ion mass spectrometry
NIST-SRM	National Institute of Standards and Technology- Standard Reference Materials
NRAMP	natural resistance-associated macrophage protein
NSS	normalised sum-square
PC	phytochelatins
PCA	principal component analysis
PNC/XOR	Pacific Northwest. Consortium/X-ray Operations Research
QPA	quantitative point analysis
REML	restricted maximum likelyhood
ROI	regions of interest
RSA	region selection analysis
RSD	relative standard deviation
RWC	relative water content
SD	standard deviation

SE	standard error
SS	sum-square
STEM	scanning transmission electron microscopy
TF	translocation factor
THF	tetrahydrofuran
TT	target transformation
UPLC	ultra performance liquid chromatography
USEPA	United States Environmental Protection Agency
VAM	vesicular arbuscular mychorrhiza
WUE	water use efficiency
XAS	X-ray absorption spectroscopy
ZIP	zipper interacting protein
ZRT	zinc regulated transporter