

Molecular and physiological investigation of trace metal stress in seagrass, *Zostera muelleri*.

Thesis submitted to the University of Technology Sydney for the degree
of DOCTOR OF PHILOSOPHY (PhD)

Submitted July, 2019

Nasim Shah Mohammadi, BSc. MSc.

Supervisors: Professor Peter Ralph, Dr Mathieu Pernice and
Dr Manoj Kumar

The Thesis presented meets the standards and requirements set out by the University
of Technology Sydney.

Certificate of original ownership

I hereby declare that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as part of the doctoral degree and/or fully acknowledged within the text. I also certify that this submission is my own work (Nasim Shah Mohammadi). Any help that I have received in my research work and the preparation of this thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in this thesis. This research is supported by an Australian Government Research Training Program.

Production Note:

Signature removed prior to publication.

01/07/2019

Acknowledgments

I would like to thank all who made the completion of this thesis possible. First and foremost, I wish to express my immense gratitude to my supervisor, Professor Peter Ralph for giving me the exciting opportunity to work within the Climate Change Cluster (C3) at the University of Technology Sydney and for his guidance throughout my PhD both scientifically and personally.

I would like to extend my appreciation to my co-supervisors, Dr Mathieu Pernice and Dr Manoj Kumar for their advice and support. I would also like to express my profound gratitude to Dr Mathew Padula for all the generous support and mentoring that helped me grow into my potential.

Many people helped me along the way and I would like to thank them for all their support. I would like to thank the C3 technical staff; Paul Brooks, Gemma Armstrong and Kun Xie for providing first-class support in the laboratories. In relation to fieldwork, Scott Allchin, Graeme Poleweski and Mikael Kim should be thanked. Also, I received generous supports from Dr Tim Kahlke, Dr Bernhard Tschitschko and Dr Raffaella Abbriano Burke in bioinformatics analysis.

Finally, I wish to express my gratitude for the love and support that my family and friends gave me during this journey. I cannot thank my parents enough specially my mother, Nahid, who I am forever indebted to for her continuous support of my goals and dreams. I express my special gratitude to my sister, Tina; my partner, Ben and my dear friends Dr Farzad Noorian, Ipek Karacan, Navpreet Ahluwalia Mangat, Angus Rawl, Shawn Price and Liis Vahtra. I thank them for their continuous support and encouragement. Most specifically, I would like to thank Dr Parisa Noorian for all her help and support and being by my side during all my highs and lows. I am so grateful for love, care and true friendship of all these people in my life.

Preface

The Chapters within this PhD thesis have been written with the intention of submission to scientific journals. The chapters are therefore presented in a journal format, ready for submission. Chapter 2 has been recently published in *Aquatic Toxicology*. Chapter 3 is under review to be published in *Marine Pollution Bulletin*. Chapter 4 – 6 will be submitted in the near future to scientific journals as original research articles. Given that this thesis is presented as a series of ready to submit manuscripts, there is an element of repetition in the introduction of some of the chapters.

General Abbreviations

2D-IEF	Two Dimensional Isoelectric focusing
Ag	Gold
Al	Aluminium
ANOVA	Analysis of variance
APX	ascorbate peroxidase
ASC	Ascorbate
ATP	Adenosine 5- triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
C3	Climate Change Cluster
Ca ²⁺	Calcium
CAT	catalase
Cd	Cadmium
cDNA	Complementary Deoxyribonucleic Acid
Co	Cobalt
COX	Cytochrome c oxidase
COX17	cytochrome c oxidase Cu chaperone
Cr	Chromium
Cu	Copper
Cys	Cystein
Cyt.b ₆ f	Cytochrome b ₆ f
°C	Celsius
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
Fe	Iron
F _v /F _m	maximum quantum yield of photosystem II
FW	Fresh weight
g	Relative centrifugal force
GO	Gene Ontology
GPX	glutathione peroxidase

h	hour
HCl	Hydrochloric acid
Hg	Mercury
H ₂ O ₂	Hydrogen peroxide
InterPro	a database of protein families
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
LED	light-emitting diodes
MAPK	Mitogen-activated protein kinases
Mg	Magnesium
mM	milimolar
Mn	Manganese
MQ	MiliQ
MT2	metallothionein type 2
MT3	metallothionein type 3
MTs	metallothioneins
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH/ NADP(H)	Reduced nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information
nM	nanomolar
NPQ	non-photochemical quenching
NSW	New South Wales
ORF	Open reading frame
PAM	Pulse-Amplitude-Modulation
Pb	Lead
PC	Plastocyanin
PCR	Polymerase Chain Reaction
PQ	Plastoquinone
PSI	Photosystem I
PSII	Photosystem II
psu	Practical salinity units
ΦPSII	effective quantum yield of PSII

qPCR	Quantitative Polymerase Chain Reaction
RGBW LEDs	red, green, blue and white light-emitting diodes
RNA	ribonucleic acid.
RNA-Seq	RNA-Sequencing
ROS	Reactive Oxygen Species
RT-qPCR	Real time quantitative polymerase chain reaction
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
Sb	antimony
SOD	superoxide dismutase
Se	Selenium
STAR	Spliced Transcripts Alignment to a Reference
T _m	Melting temperature
W	watt
Zn	Zinc

Table of Contents	Pg.
Certificate of original ownership	II
Acknowledgments.....	III
Preface.....	IV
General Abbreviations	V
List of Figures	XIV
List of Tables	XVII
List of Appendices	XVIII
Thesis summary	1
PhD aims and objectives	3
CHAPTER 1	4
Abstract	5
1. Seagrass: the submerge pasture	5
1.1. Characteristics of the seagrass, <i>Zostera muelleri</i>	5
1.2. Ecological importance of the seagrasses	7
2. Trace metal toxicity in marine environments	8
2.1. Trace metal-contaminated seagrass tissues as a threat to marine food web	10
2.2. Factors influencing trace metal absorption in seagrasses.....	10
3. Cu : a mixed-blessing for seagrass health	11
3.1. Major source of Cu contamination.....	12
3.2. Level of Cu contamination in Australian waters.....	12
4. Current knowledge of the mechanism of trace metal toxicity response in plants	12
4.1. The mechanism of trace metal absorption.....	13
4.2. Photosynthetic response to Cu toxicity	17
4.3. Enzymatic defense mechanism to Cu toxicity in plants and seagrasses ..	22

4.4. Chemical defense mechanism to Cu toxicity in plants and seagrasses ...	23
5. Identification of biomarkers for early assessments of trace metal toxicity	24
6. Hyper-accumulation of trace metals in seagrasses: a combined role in adaptation and phytoremediation.....	25
7. Conclusion	28
References	28
CHAPTER 2	47
Abstract	48
1. Introduction	49
2. Materials and Methods	52
2.1. Seagrass samplings, aquaria setup and experimental design	52
2.2. Detection of chlorophyll fluorescence	53
2.3. Detection of Cu level	53
2.4. Detection of ROS	53
2.5. Quantification of transcripts encoding antioxidant enzymes and Cu-binding proteins.....	54
2.5.1. Primer design	54
2.5.2. RNA extraction and synthesis of DNA	57
2.5.3. Differential gene expression analysis.....	57
2.5.4. Statistical analyses	57
3. Results	58
3.1. Content of Cu in leaves	58
3.2. Changes in photosynthetic parameters.....	59
3.3. ROS accumulation	62
3.4. Changes in gene expression	62
4. Discussion.....	65
5. Conclusion	69
Acknowledgements	71

References	71
CHAPTER 3	83
Abstract	84
1. Introduction	84
2. Materials and Methods	86
2.1. Sample collection and aquaria setup	86
2.2. Experimental design.....	87
2.3. RNA extraction	87
2.4. Library preparation and RNA sequencing	87
2.5. Genome-guided transcriptome assembly and annotation.....	87
3. Results	88
3.1. Transcriptome assembly and functional annotation.....	88
3.2. Changes in the expression of genes in <i>Z. muelleri</i> in response to Cu stress	96
4. Discussion.....	107
4.1. Elevated Cu concentration impacts plant photosynthesis	107
4.2. Activation of inorganic carbon fixation in response to Cu stress in <i>Z. muelleri</i>	108
4.3. Activation of energy production as a possible defense mechanism in Cu-stressed leaves of <i>Z. muelleri</i>	109
4.4. Induced enzymatic and chemical defense mechanism in response to Cu stress	109
5. Conclusion	111
Acknowledgement	111
References	111
CHAPTER 4	122
Abstract	123
1. Introduction	123

2. Materials and Methods	126
2.1. Plant material	126
2.2. Protein extraction, purification, alkylation and reduction.....	127
2.3. Protein-centric analysis (2-DE).....	128
2.4. Peptide-centric analysis.....	128
2.5. LC-MS analysis of peptides	131
2.6. Bioinformatics analysis	132
3. Results	132
3.1. Proteome analysis using protein-centric approach.....	132
3.2. Proteome analysis using peptide-centric approach	140
3.3. Identification of the total number of expressed proteins in <i>Z. muelleri</i> from peptide-centric methods	148
4. Discussion.....	148
4.1. 2-DE method provided functional classification for 350 expressed proteins in <i>Z. muelleri</i>	149
4.2. The combination of 1D-PAGE and SDB-RPS provided the best protein coverage for expressed proteins in <i>Z. muelleri</i> among four tested peptide-centric methods	151
5. Conclusion	154
Acknowledgement	155
References	155
CHAPTER 5	164
Abstract	165
1. Introduction.....	166
2. Materials and Methods.....	167
2.1. Sample collection and set-up for aquaria	167
2.2. Treatment of <i>Z. muelleri</i> with Cu	168
2.3. Protein extraction	168

2.4. In-solution digestion (ISD) of proteins	169
2.5. Peptide labelling by iTRAQ reagents	169
2.6. An optimized SDB-RPS-based desalting method.....	169
2.7. Bioinformatic analysis of peptides.....	170
2.7.1. iTRAQ analysis.....	170
2.7.2. Identification of differentially expressed proteins	170
3. Results.....	170
3.1. LC-MS/MS data analysis and functional annotation	170
3.2. Investigation of Cu-induced, differentially expressed proteins in <i>Z. muelleri</i> plants	177
4. Discussion	187
4.1. Excess Cu negatively affects photosynthesis and carbon fixation in <i>Z. muelleri</i>	187
4.2. Activation of oxidative stress in <i>Z. muelleri</i> under 500 µg Cu L ⁻¹	188
4.3. An enhanced energy production is required to respond to Cu stress in <i>Z. muelleri</i>	189
4.4. Excess Cu alters genetic information processing of <i>Z. muelleri</i>	191
5. Conclusion	191
Acknowledgement	192
References	192
CHAPTER 6.....	199
Abstract	201
1. Introduction	201
2. Materials and methods.....	204
2.1. Sample collection.....	204
2.2. Isolation of intact chloroplasts from <i>Z. muelleri</i>	204
2.3. Estimation of chlorophyll a/b ratio of intact chloroplasts	205

2.4. Estimation of the size of intact chloroplast using Differential interference contrast (DIC) microscopy	205
2.5. Protein extraction	205
2.6. Immunoblotting.....	206
2.7. In-solution digestion (ISD)	206
2.8. Proteomic data analysis	207
3. Results	207
3.1. Assessment of intact chloroplasts isolation.....	207
3.2. Assessment of intact chloroplast isolation	209
3.3. Isoelectric point and GRAVY index:	211
4. Discussion.....	216
5. Conclusion	219
Acknowledgement	219
References	219
Synthesis, outlook and conclusion	227
Overview	228
1. Photo-physiological damage occurs early in <i>Z. muelleri</i>	229
2. Only two antioxidant enzymes continued to scavenging ROS over-production in response to Cu stress in <i>Z. muelleri</i>	230
3. Possible defense mechanisms in <i>Z. muelleri</i> after 7 days exposure to 500 µg Cu L ⁻¹	232
4. Possible biomarkers for monitoring seagrass health were suggested.....	233
5. Evaluation of omics methods for future considerations	237
6. Optimised methods were introduced and future work were addressed in this PhD thesis.....	238
References	239
Appendices	246

List of Figures	Pg.
Figure 1-1. The morphology of <i>Z. muelleri</i>	6
Figure 1-2. Main sources of trace metal pollution	9
Figure 1-3. Scheme of possible trace metal transporters in cytosol, vacuole, chloroplast and mitochondria of the leaves of terrestrial plants	16
Figure 1-4. Proposed trace metal binding sites in plant photosystem.....	19
Figure 1-5. Antioxidant responses to trace metals	23
Figure 2-1. The accumulation of Cu in the leaves of <i>Zostera muelleri</i> exposed to control (black bars), 250 $\mu\text{g Cu L}^{-1}$ (light grey bars) and 500 $\mu\text{g Cu L}^{-1}$ (dark grey bars) on day 1, 3 and 7.	58
Figure 2-2. Time course of effective quantum yield (ϕPSII) (A) maximum quantum yield (F_v/F_m) (B) and non-photochemical quenching (NPQ) (C) of <i>Zostera muelleri</i> up to 7 days exposure to various Cu concentrations	61
Figure 2-3. Total Reactive Oxygen Species (ROS) in the leaves of <i>Zostera muelleri</i> exposed to controls (black bars), 250 (light grey bars) and 500 $\mu\text{g Cu L}^{-1}$ (dark grey bars) on day 1, 3 and 7.	62
Figure 2-4. Quantitative PCR (qPCR) analyses for expression of <i>sod</i> (A), <i>apx</i> (B), <i>cat</i> (C) and <i>gpx</i> (D). Data were expressed relative to expression of control. Asterisk indicates significant difference from control (One-Way ANOVA, $p < 0.05$). Error bars show standard error, $n = 3$. For each replicate, 3 seagrass shoots were sampled.	63
Figure 2-5. Quantitative PCR (qPCR) analyses for expression of <i>mt2</i> (A), <i>mt3</i> (B) and <i>cox17</i> (C).....	64
Figure 2-6. Conceptual illustration showing differential expression of genes and physiological responses in plants exposed to 250 $\mu\text{g Cu L}^{-1}$ (A) and 500 $\mu\text{g Cu L}^{-1}$ (B).	69
Figure 3-1. Heatmaps of the log 2 fold change and z-score of normalized read counts for significantly differentially expressed genes in response to 250 $\mu\text{g Cu L}^{-1}$ (left) and 500 $\mu\text{g Cu L}^{-1}$ (right) after 7 days.....	91
Figure 3-2. Sequence distribution (biological process) of top 50 genes expressed in 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ after 7 days.	93
Figure 3-3. Sequence distribution (molecular function) of top 50 genes expressed in 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ after 7 days.	93

Figure 3-4. Sequence distribution (cellular component) of top 50 genes expressed in 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ after 7 days.	94
Figure 3-5. Functional classification of proteins in 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ after 7 days.	95
Figure 3-6. Differentially expressed genes related to photosynthesis, carbon fixation, energy metabolism, enzymatic and chemical defense mechanism under 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$	106
Figure 4-1. Demonstrative example for the identification of proteins extracted from leaves of seagrass <i>Zostera muelleri</i>	134
Figure 4-2. Protein percentage identified with unique peptides coverage (A), and protein score (-10lgP) (B), functional classification of identified proteins (%) belonging to diverse cellular metabolism (C), with corresponding Pi value (D) and GRAVY scores (E) for the 2D-IEF based leaf proteome analysis of <i>Z. muelleri</i>	136
Figure 4-3. Biological process (A), molecular function (B) and cellular component (C) of proteins uniquely identified for the 2D-IEF based leaf proteome analysis of <i>Z. muelleri</i>	140
Figure 4-4. Venn diagram showing the total number of identified proteins as well as the number of proteins uniquely identified in each method and number of common proteins in all methods for <i>Z. muelleri</i> using Venny software.....	142
Figure 4-5. The percentage of unique identified peptides from each peptide-centric methods.	143
Figure 4-6. The percentage of the -10lgP value of unique peptides from peptide-centric methods.	144
Figure 4-7. GRAVY score of the identified proteins from peptide-centric methods.	145
Figure 4-8. Distribution of isoelectric focusing of the identified proteins in peptide-centric methods.	146
Figure 4-9. Biological process (A), molecular function (B) and cellular component (C) of proteins uniquely identified in <i>Z. muelleri</i> using peptic-centric methods.	148
Figure 5-1. Heatmap of 171 differentially expressed proteins isolated from <i>Z. muelleri</i> plants exposed to 500 $\mu\text{g Cu L}^{-1}$ for 7 days.....	172
Figure 5-2. Sequence distribution of biological process (A), molecular function (B) and cellular component (C) of the top 50 differentially expressed proteins exposed to 500 $\mu\text{g Cu L}^{-1}$	175

Figure 5-3. Functional annotation of proteins differentially expressed in <i>Z. muelleri</i> when exposed to 500 $\mu\text{g Cu L}^{-1}$.	176
Figure 5-4. Proteins related to photosynthesis, carbon fixation, energy metabolism, genetic information processing and defence mechanism which their abundance altered in response to 500 Cu L^{-1} .	180
Figure 6-1. Intact chloroplast isolates as a layer in method A and as a ring at the interface of 40/80% Percoll in method B.	208
Figure 6-2. Microscopic visualization of an intact chloroplast under DIC microscopy.	208
Figure 6-3. Immunoblotting of chloroplast (A –C) and mitochondrial (D –E) marker proteins.	209
Figure 6-4. The percentage of unique matched peptides for identified proteins from method B.	210
Figure 6-5. The percentage of -10lgP value of identified proteins from method B.	210
Figure 6-6. GRAVY score indicating the hydropathy of the identified proteins from method B.	211
Figure 6-7. Distribution of isoelectric focusing of the identified proteins in method B.	212
Figure 7-1: Thesis conclusion representing: 1) Cu-induced protein and enzymes linked to photosynthesis, carbon fixation, glycolysis and enzymatic defence mechanism and 2) Five possible defense mechanisms in <i>Z. muelleri</i> in response to 7 day exposure to 500 $\mu\text{g Cu L}^{-1}$.	236

List of Tables

Pg.

Table 1-1. Identified species in the family of <i>Zosteraceace</i> , their current location and their status in the red list category of IUCN	6
Table 1-2. Current knowledge of physiological responses of seagrasses to Cu stress.	20
Table 1-3. Trace metals translocation and tissue preference in seagrasses.	26
Table 2-1. Reference genes and target genes investigated in <i>Zostera muelleri</i> by using RT-qPCR.....	55
Table 2-2. Results of the repeated-measures ANOVA for effective quantum yield of PSII (Φ PSII), maximum quantum yield (Fv/Fm) and non-photochemical quenching (NPQ) and two-way ANOVA of leaf Cu content, and total reactive oxygen species (ROS) in the leaves of <i>Zostera mulerii</i> after exposure to 0, 250 and 500 $\mu\text{g Cu L}^{-1}$.59	
Table 3-1. Total number of expressed genes under 500 $\mu\text{g Cu L}^{-1}$ and 250 $\mu\text{g Cu L}^{-1}$	89
Table 3-2. List of 25 differentially expressed genes selected to investigate in this study.	98
Table 4-1. Total number of proteins and matched peptides from each peptide-centric methods searched against <i>Z. muelleri</i> database.....	140
Table 5-1. List of 76 proteins related to photosynthesis, energy metabolism, carbon fixation, defence mechanism and genetic information processing with their corresponding protein names that were selected to investigate in this study.....	180
Table 6-1. List of unique identified proteins from intact chloroplast isolates of <i>Z. muelleri</i> using Scaffold software	212
Table 7-1. Evaluation of four omics methods used in this PhD project based on the required time, the sensitivity of the technique, the cost and the coverage (range of identification).	238

List of Appendices

Appendix 1. Support documents for quality control genes used in qPCR.	
Appendix 2-A. Functional annotation of differentially expressed genes of <i>Z. muelleri</i> at 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ using Blast2Go (gene identification). B. Functional annotation of differentially expressed genes of <i>Z. muelleri</i> at 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ using Blast2Go (GO and InterPro IDs).	247
Appendix 3. Fold change of expressed genes exposed to 250 $\mu\text{g Cu L}^{-1}$	247
Appendix 4. Fold change of expressed genes exposed to 500 $\mu\text{g Cu L}^{-1}$	247
Appendix 5. Demonstrative example for the identification of proteins extracted from leaves of seagrass <i>Zostera muelleri</i> . Proteins were resolved on pI range 5–8 IPG strip followed by SDS-PAGE. In this example, protein spot 15 was a visible spot that was excised, trypsin digested and analysed using nanoLC-MS/MS.	247
Appendix 6. Functional annotation and proteomic information of expressed proteins using 2-DE gel.	247
Appendix 7. Physio-chemical characterisations of expressed proteins using 2-DE gel.	247
Appendix 8. Protein FASTA of expressed proteins using 2-DE gel.....	247
Appendix 9. LC-MS/MS results of expressed proteins using 2-DE gel.	247
Appendix 10. Total number of identified proteins from each peptide-centric methods	247
Appendix 11. LC-MS/MS results of expressed proteins from Method A.	247
Appendix 12. LC-MS/MS results of expressed proteins from Method C.....	247
Appendix 13. LC-MS/MS results of expressed proteins from Method D	248
Appendix 14. LC-MS/MS results of expressed proteins from Method E.....	248
Appendix 15. Protein FASTA of expressed proteins from Method A.....	248
Appendix 16. Protein FASTA of expressed proteins from Method C	248
Appendix 17. Protein FASTA of expressed proteins from Method D.....	248
Appendix 18. Protein FASTA of expressed proteins from Method E	248
Appendix 19. Representation of protein identification for expressed proteins from peptide-centric methods using Scaffold software.	248
Appendix 20. LC-MS/MS results of expressed proteins using 6 iTRAQ labels. ...	248
Appendix 21. Protein FASTA of expressed proteins from 6 iTRAQ labels.....	248

Appendix 22-A. Functional annotation of differentially expressed proteins of <i>Z. muelleri</i> at 500 $\mu\text{g Cu L}^{-1}$ using Blast2Go (gene identification). B. Functional annotation of differentially expressed genes of <i>Z. muelleri</i> at and 500 $\mu\text{g Cu L}^{-1}$ using Blast2Go (GO and InterPro IDs).	248
Appendix 23. Accession number, ratio of protein abundance, standard deviation (SD), p-value and statistical results of expressed proteins exposed to 500 $\mu\text{g Cu L}^{-1}$	248
Appendix 24. Original images of immunoblotting of chloroplast and mitochondria marker antibodies.	248
Appendix 25. LC-MS/MS results of expressed intact chloroplast proteins.	248
Appendix 26. Protein FASTA of expressed intact chloroplast proteins	248
Appendix 27. Representation of protein identification probability of expressed proteins from intact chloroplasts using Scaffold software.	248

Thesis summary

Despite the vast research on the negative effects of anthropogenic pollution on marine organisms, little is known about the toxicity responses of seagrasses to such perturbations. Understanding seagrass responses at the molecular level will ensure adequate conservation strategies to mitigate the increasing decline rate of seagrasses as a result of climate change and anthropogenic driven disturbances. The meadows of the Southern hemisphere seagrass species, *Zostera muelleri*, encounter similar threats, which led to a significant loss along the Australia and New Zealand coasts. Trace metal pollution and most specifically copper (Cu), have been previously reported in industrial, agricultural and domestic run-off waste which often finds their way to the ocean and jeopardise the health of the seagrass meadows.

Although we have a firm undersetting of the deleterious effect of Cu stress at the physiological and ecological level, no current knowledge exists on how *Z. muelleri* responds to elevated levels of Cu at the molecular level. Upon our investigation of the physiological responses of *Z. muelleri* to 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ over a 7 day period of exposure, the Cu accumulation in the leaves, the continual production of ROS and the decline of photosynthetic efficiency were observed in *Z. muelleri* at both above mentioned Cu concentrations. However, the responses were concentration-dependent illustrating 250 $\mu\text{g Cu L}^{-1}$ and 500 $\mu\text{g Cu L}^{-1}$ as a tolerable and a toxic level for *Z. muelleri*, respectively.

The results of our molecular investigations indicated regulation shifts in the expression of genes and the abundance of proteins mainly at 500 $\mu\text{g Cu L}^{-1}$ were associated with energy metabolism, carbon fixation, photosynthesis and defence mechanism. While the expression of genes (and the abundance of proteins) involved in energy metabolism (mainly glycolysis) and defence mechanism have been shown to be mainly increased, the opposite was observed in the photosynthetic process and carbon fixation. As a result, whilst these results offers a new level of understanding into the seagrass toxicity responses at transcriptomic and proteomic levels, it also provides candidate molecular markers for future toxicology studies and seagrass monitoring.

This PhD thesis also evaluates a protein-centric and four peptide-centric proteomic methods and proposed an optimised peptide desalting protocol. Additionally, major alterations in photosynthesis process as a result of Cu stress has led us to report on an optimised intact chloroplast isolation method that can be used for future proteomic-based studies.

PhD aims and objectives

The overall aim of this thesis is to investigate how *Z. muelleri* responds to Cu stress using physiological and molecular approaches. By combining transcriptomic and proteomic techniques, we have obtained a deeper understanding of how this seagrass species responds to elevated levels of Cu exposure at a complete “omic” level.

Given the fact that seagrasses are declining globally by anthropogenic pollutions, this work can contribute to identify potential biomarkers for early detection of trace metal toxicity in seagrasses and assist with better restoration, conservation management of seagrass meadows.

As a result, the objectives of this PhD thesis include:

- To provide a critical literature review on the current understanding of trace metal toxicity responses in seagrass species and identifying knowledge gaps in previous studies.
- To address base knowledge associated with trace metals in higher plants and seagrasses with special attention to Cu.
- To complete characterisation of leaf-specific transcriptome and proteome of *Z. muelleri* under elevated Cu stress.
- To establish links between physiology, transcriptional regulation and protein expression as a result of Cu toxicity response of *Z. muelleri*.
- To investigate and report possible biomarkers for early detection of Cu stress signals in *Z. muelleri*.