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)

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

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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.

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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 Isoelecric 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_6f	Cytochrome b_6f
^oC	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 Onthology
GPX	glutathione peroxidase

h	hour
HCl	Hydrochloric acid
Hg	Mercury
H_2O_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
Tm	Melting temperature
W	watt
Zn	Zinc

Table of Contents Pg.

List of Figures Pg.

List of Tables Pg.

List of Appendices

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 µg Cu L^{-1} and 500 µg 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 µg Cu L^{-1} and 500 µg 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 µg 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*.