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

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

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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

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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*.

CHAPTER 1

Stress response of seagrasses to trace metal toxicants

Abstract

Seagrasses (marine angiosperms) play an important role as the main food source and protective habitat for a diverse range of fishes and invertebrates; yet, they are under constant threat from the effects of trace metal pollution arising from industry and domestic waste as well as agriculture run-off in coastal areas. While the harmful effects of trace metals on seagrasses are well known at the physiological level, there is no data available examining how these physiological variations translate at the molecular level. The knowledge gaps include, but are not limited to, antioxidant enzyme response, the specificity of metal transporters and detoxification mechanisms.

This chapter brings together the current knowledge of seagrass responses to trace metal toxicity with special attention to copper (Cu). We identified the knowledge gaps in understanding how accumulation, tolerance and adaptation to trace metals occur in seagrasses relative to what is already known in terrestrial plants.

1. Seagrass: the submerge pasture

Marine angiosperms or seagrasses are a small group in the order of *Alismatales* comprising around 60 recognized species to date (Andrews & Abel 1979; Wissler et al. 2011). All species are distributed within four monocotyledonous families: *Posidoniaceae*, *Zosteraceae*, *Hydrocharitaceae* and *Cymodoceaceae* (Kenworthy et al. 2006; Wissler et al. 2011). A combination of characteristics derived from their ancestor saltmarsh plants as well as new adaptations have enabled seagrasses to successfully acclimatise to survive under constantly challenging shallow water environments (den Hartog 1970; Larkum & Den Hartog 1989; Den Hartog & Kuo 2006; Kuo & Den Hartog 2006).

1.1. Characteristics of the seagrass, *Zostera muelleri*

Zostera muelleri (Table 1-1 and Figure 1-1) is one of the fourteen species in the family of *Zosteraceae* with strap-shaped rounded tip leaves and a dark brown rhizome with < 3 mm in diameter (Waycott, Lavery & McMahon 2014; Lee et al. 2016). It is usually found in coastal waters of Australia, Papua New Guinea and New Zealand (Davey 2017; The IUCN Red List of Threatened Species 2018). As a result of their shallow growth habitat, the seagrass meadows are continuously threatened by local coastal development (Dennison et al. 1993; The IUCN Red List of Threatened Species 2018).

Figure 1-1. The morphology of *Z. muelleri* with long strap-shaped leaves, thin roots and rhizome. Image source: http://www.seagrasswatch.org/ id_seagrass.html.

Although Z. *muelleri* is categorized as "least concern" in the red list category of International Union for Conservation of Nature (IUCN 2018) (Table 1-1), the disturbance of their meadows by coastal development was repeatedly reported (The IUCN Red List of Threatened Species 2018).

Table 1-1. Identified species in the family of *Zosteraceace*, their current location and their status in the red list category of IUCN (data extracted from The IUCN Red List of Threatened Species (2018)).

***Zostera* species**	**Location**	**Red list category**
Zostera asiatica	Russian Far East, Japan, Korea, NE China	Near Threatened

Zostera caespitose	Russian Far East, Japan, Korea, NE China	Vulnerable
Zostera capensis	Madagascar; Kenya to Cape Province	Vulnerable
Zostera capricorni	New Guinea, Australia, New Zealand	Least Concern
Zostera caulescens	Russian Far East, Japan, Korea, NE China	Near Threatened
Zostera chilensis	Chile	Endangered
Zoastera japonica	Russian Far East, Japan, Korea, China, Vietnam	Least Concern
Zostera marina	Shores of North Pacific, North Atlantic, British isles Mediterranean, Black Sea	Least Concern
Zostera mucronata	Australia	Least Concern
Zostera muelleri	**Australia, Papua New Guinea, New Zealand**	**Least Concern**
Zostera nigricaulis	Australia	Least Concern
Zostera notii	Shores of North-eastern Atlantic, Mediterranean, Black Sea, Caspian Sea	Least Concern
Zostera polychlamys	Australia	Least Concern
Zostera tasmanica	Australia	Least Concern

1.2. Ecological importance of the seagrasses

Seagrass meadows are the third most valuable ecosystem globally with their value estimated at US \$28,916 ha^{-1} yr^{-1} worldwide for their ecological services (Costanza et al. 2014; Lefcheck et al. 2017). They provide fundamental services to marine ecosystems (Short et al. 2007; D`Esposito 2013) such as:

- Providing a protective habitat for a diverse range of fish eggs (Borum et al. 2004; Kenworthy et al. 2006; Short et al. 2007; Cox et al. 2015; Smith 2017).
- Serving as a main food source (leaves and/or epiphytes) for a number of herbivores such as dugongs, turtles, fishes, limpets and urchins (Boström & Mattila 1999; Dawes et al. 2004; Orth et al. 1984; Melville-Smith et al. 2017; Stoner et al. 1991; Tomas et al. 2006).
- Oxygenating the seawater as a by-product of photosynthesis (Antoine & Morel 1996; Borum et al. 2004; Maxwell et al. 2017).

- Improving water quality by concentrating trace metals in their leaves and root systems (Dawes et al. 2004).
- Protecting the shoreline against strong tidal waves and enhancing sediment accretion by their root system (Dawes et al. 2004; Short et al. 2007; Jiménez et al. 2017).
- Providing a major contribution to blue carbon storage, which is a term used for the carbon that has been captured in the ocean (Mcleod et al. 2011; Pendleton et al. 2012; Lavery et al. 2013; Trevathan-Tackett et al. 2018).

However, seagrasses are declining globally at an alarming rate of $> 7\%\ yr^{-1}$ due to anthropogenic perturbations (Waycott et al. 2009; Evans et al. 2018). In Australia only, 246 hectares of seagrasses have been lost in Western Australia between 1965 and 1995 (Lord et al. 1998).

2. Trace metal toxicity in marine environments

Coastal environments are continuously subjected to contamination by a wide range of trace metals as a consequence of both natural and anthropogenic activities (Cambridge & McComb 1984; Waycott et al. 2009; Nielsen et al. 2017; Birch, Cox & Besley 2018b). The earliest study on trace metal toxicity to higher plants was performed around 45 years ago with *Arabidopsis thaliana* (Antonovics et al. 1971). Since then, numerous studies have examined trace metal accumulation and tolerance in terrestrial plants (see review by Nagajyoti, Lee & Sreekanth (2010)). Natural sources of trace metals derived from volcanic emission and soil erosion comprises a small percentage of environmental pollution (Seaward & Richardson 1989; Nagajyoti, Lee & Sreekanth 2010). However, the majority of trace metal pollution is derived from anthropogenic activities (Figure 1-2) and are found in agricultural fertilizers, sewage, pesticides, and in waste run-off from mining operations, chemical and pharmaceutical manufacturing, petroleum refining and industrial printing (Silberstein, Chiffings & McComb 1986; Ross 1994; Board 2000; Yanqun et al. 2005; Halpern et al. 2008; Diamond 2012).

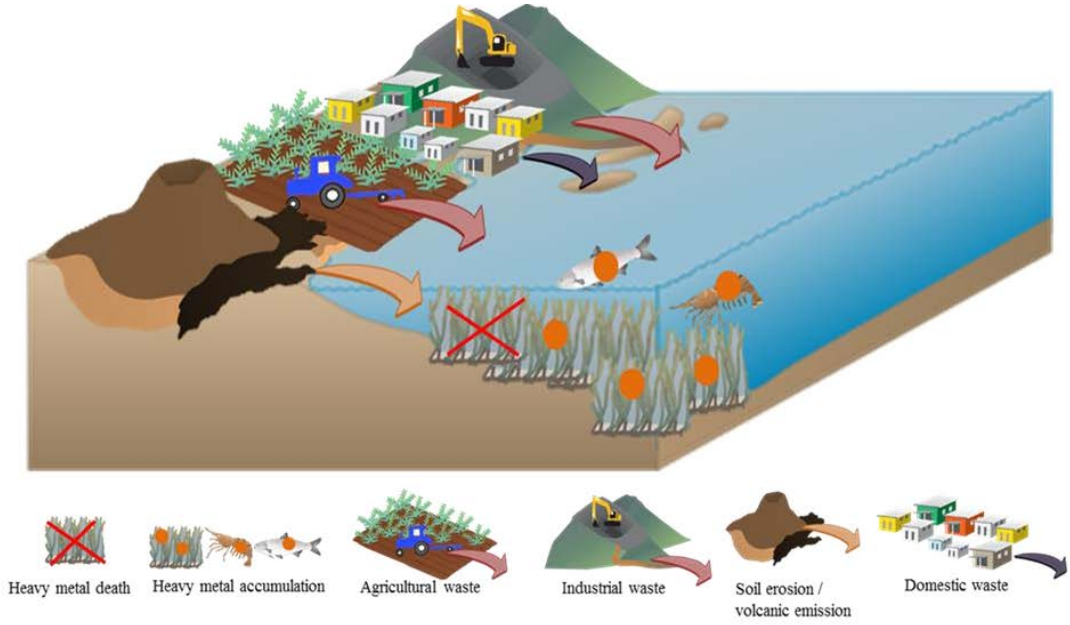

Figure 1-2. Main sources of trace metal pollution (acid mine drainage, domestic / industrial waste, storm drains, soil erosion and pesticide and algaecide run-off). Objects imported form ian.umces.edu/symbols.

At low concentrations, trace metals are essential for physiological and biochemical processes in plants (Niess et al. 1999; Gaur & Adholeya 2004; Schneider et al. 2018). For example, some trace metals that are essential for activation of plant enzymes include: Cu, Zn, Mn (superoxide dismutase); Cu (amine oxidases and ammonia monoxidase); Zn (RNA polymerase, carbonic anhydrase, alcohol dehydrogenase, mallic dehydrogenase and oxalosuccinic decarboxylase); Mn (PSII water splitting system); Co (B12 vitamin) and Fe (Cytochrome) (Nagajyoti, Lee & Sreekanth 2010). An excess level of any of these trace metals, however, can adversely affect plant health (DalCorso et al. 2013).

There is no clear explanation for the reasons why plants or seagrasses continuously absorb trace metals beyond their toxic levels (Pollard & Baker 1997; Boyd et al. 2002; Hanson et al. 2003). However, for some trace metals such as Se, Ni and Zn, it has been suggested to be a deterrent or a defense mechanism against pathogens, fungal infection and herbivores (Pollard & Baker 1997; Boyd et al. 2002; Hanson et al. 2003; Quinn et al. 2008; Quinn et al. 2010). For example, excess level of Se absorption by Indian mustard (*Brassica juncea*) has been shown to deter the root / stem pathogen, *Fusarium*, as well as the leaf pathogen, *Alternaria brassicicola* (Hanson et al. 2003). Furthermore, hyper-accumulation of Ni in leaves of *Senecio coronatus*, reduces the production of secondary

metabolites, such as alkaloids. Alkaloid metabolites are usually produced by plants as a defense mechanism, but as a result of the protection from Ni, the need for metabolic defense mechanism was reduced (Boyd et al. 2002).

Additionally, in most cases, a combination of toxicants occurs, which can enhance the sensitivity of plants to stress (Babu et al. 2001). For example, a study on the physiological responses of the seagrass, *Z. capricorni*, under combined Cu and herbicide stress, showed a greater negative impact on the physiology of the seagrass compared to individual stress responses (Macinnis-Ng & Ralph 2004a). Similar results have been reported for the combination of Cu and Polycyclic Aromatic Hydrocarbons (PAH) on the physiological state of duckweed (*Lemna gibba*) (Babu et al. 2001).

2.1. Trace metal-contaminated seagrass tissues as a threat to marine food web

When seagrasses take up trace metals, their contaminated tissues provide a trophic pathway for the distribution of trace metals throughout the marine food web (Ward 1987; Kalay, Ay & Canli 1999; McGeer et al. 2004; Navratil & Minarik 2005; Smith 2018). This situation is detrimental to the health of marine organisms, to human health if contaminated seafood is consumed, and to coastal fisheries, which risk closure (Basha & Rani 2003; Canli & Atli 2003; Wang et al. 2005; Baki et al. 2018). Trace metal pollution threats are not limited to shallow waters. In fact, trace metals have been found in organisms at 17 meters of water depth (Zhang et al. 2007) which illustrates the wide range of marine organisms that can be affected by excess levels of trace metals (Roach 2005; Van Dam et al. 2008; Lewis & Devereux 2009; Lin et al. 2016).

2.2. Factors influencing trace metal absorption in seagrasses

When seagrasses are in a healthy condition, trace metals can be absorbed from their shoots and roots as well as their root-associated microorganisms (bacteria and fungi), which play a critical role in the bio-availability of trace metals for seagrasses (Howley et al. 2006). It has been previously shown that Cu can alter the chemical property of the sediment and as a result, alter microbial activity (Eggleton & Thomas 2004). For example, oxidation of sediment can lead to an increase in the activity of sulfur bacteria such as *Thiobacteria*. Oxidation of sulfide (reactions in the next page) decreases the sediment pH and consequently changes the bio-availability of trace metals for plants and

their associated micro-organisms (Calmano, Hong & Förstner 1993; Eggleton & Thomas 2004; Lenntech 2004).

$H_2S + 2O_2 = SO_4^{-2} + 2H^+$

or

$FeS + 9/4\ O_2 + 3/2\ H_2O = FeOOH + SO_4^{-2} + 2H^+$

In this thesis, we focused on trace metal uptake via shoots to investigate the photosynthesis response of *Z. muelleri* to elevated Cu concentration along with other molecular stress responses that might occur.

Another factor influencing the absorption of trace metals is the sediment type. For example, Cu and Cd have binding sites for inorganic matter (Lopez-Sanchez et al. 1996; Shul'kin & Bogdanova 1998), whereas Cr, Cd and Mn have binding sites for oxide sediments (Lopez-Sanchez et al. 1996; Dong et al. 2000; Hursthouse 2001; Fan et al. 2002).

Water salinity is an additional factor that influences the bio-availability of trace metals in seagrasses. The uptake of Zn, Pb, Ag, Cr, Sb, and Cd from sediment is shown to linearly decrease with water salinity (Brinkhuis et al., 1980; Williams et al., 1994), whereas the opposite is true for Cu uptake (Förstner 2006).

3. Cu: a mixed-blessing for seagrass health

According to World Health Organization (WHO) list (1996), Cu is one of the essential trace metals for a great number of key enzymes including superoxide dismutase and key photosynthesis subunits such as plastocyanin (Katoh 1977; Barón, Arellano & Gorgé 1995; World Health Organization 1996; Kaufman Katz et al. 2003; World Health Organization 2011). However, elevated concentrations of Cu can cause physiological and molecular alterations in plant genes and deleterious effects on photosynthetic efficiency at different levels (Barón, Arellano & Gorgé 1995; Yruela 2005). More specifically, the alteration of the function of transporters and ion channels as a result of excess level of Cu can cause intra-cellular redox imbalances (Cambrollé et al. 2013; Tiecher et al. 2017) as well as cellular damage via the over-production of reactive oxygen species (Girotto et al. 2013; Tiecher et al. 2017).

3.1. Major source of Cu contamination

The level of Cu in coastal waters is mainly attributed to anthropogenic activities with major sources of contamination such as acid mine drainage, agricultural fungicides and pesticides discharging to the oceans (Nor 1987; Nriagu 1989, 1990; Barón, Arellano & Gorgé 1995; Richir & Gobert 2016). Given that trace metals including Cu are non-degradable and can accumulate over time, improper discharge has been a serious pollution concern over the last decades for the health of marine organisms by entry of Cu into the food chain (Ouzounidou 1993; Pandey & Sharma 2002).

3.2. Level of Cu contamination in Australian waters

Australian coasts have some of the most diverse marine habitats (Hamilton & Gehrke 2005; Carter et al. 2017). Unfortunately, the continuous growth in industrial, domestic and agricultural activities have jeopardized the well-being of many marine organisms in the last decades (Roach 2005; Van Dam et al. 2008). According to the Australian trace metal field measurement study in South coast of New South Wales (2018), Cu is measured within the range of 0.01 − 0.08 µg/L in water and between 0.01 − 605 µg/g in sediment (McVay et al. 2018). However, the range of Cu concentration seems to be region-specific. For example, the mean Cu concentration in detritus and in the tissue of *Z. capricorni* in Lake Macquarie were shown to be around 11 − 13 µg/g dry mass (Schneider et al. 2018). Whereas the investigation from Sydney estuaries by Birch, Cox & Besley (2018a) in 2013 − 2015 showed that the Cu level in the total sediment adjacent to *Halophila ovalis* meadows to be between 75 − 138 µg/g dry mass. The Cu concentration increased to 103 − 492 µg/g dry weight in the tissue of *H.ovalis*.

The Australian government has established an Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZECC / ARMCANZ 2000) to protect seagrasses (Van Dam et al. 2008). The Department of Primary Industries also regularly monitors seagrass health and has authorized research centers to perform limited sample collection of seagrasses.

4. Current knowledge of the mechanism of trace metal toxicity response in plants

The level of trace metal toxicity is directly associated with the type of plant species, the amount of absorbing surface and the concentration / period of trace metal exposure (Stobart et al. 1985; Küpper Hendrik, Küpper Frithjof & Martin 1996; Nguyen,

Amyot & Labrecque 2017). In general, studies have reported considerable trace metal-induced physiological modifications and immediate stress response in some seagrasses such as *Halophila sp., Z. caprocorni* and *Cymodocea serrulata* (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002a, 2004a). However, it is shown that the level of trace metal accumulation and tolerance are species-specific and toxicant-specific (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2004b). As for Cu, the range is shown to be within the range of 0.1 – 10 mg L^{-1} for Cu Clijsters & Assche 1985; Macinnis et al. 2002; Prange & Dennison 2000; Ralph & Burchett 1998b).

At the molecular level, trace metal toxicity is mainly studied in terrestrial plants and shown to adversely affect the maintenance of inter-cellular ion concentration (homeostasis) and initiate cell death (DalCorso et al. 2013). For example, Fe, Cr and Cu toxicity can be recognized by the overproduction of antioxidant enzymes and degradation of DNA and proteins (Nagajyoti, Lee & Sreekanth 2010). Similar toxicity responses have also been reported in seagrasses as mentioned subsequently in section 4.3 and 4.4. However, there is no clear information available for the molecular mechanism of trace metal toxicity in seagrasses to date.

4.1. The mechanism of trace metal absorption

Trace metal absorption can be regulated or non-regulated. Non-regulated absorption of trace metals can occur via passive uptake (Rathore et al. 1970; Lindsay 1972; Skipnes, Roald & Haug 1975) or diffusion through the cell wall (Moustakas et al. 1994). As an example, Blaudez, Botton & Chalot (2000) reported the absorption of Cd by its binding to the cell wall of *Paxillus involutus*. However, the exact mechanism is yet to be confirmed for most of terrestrial and submerged plants.

Trace metals transporters in terrestrial plants are recognized as a part of the regulatory maintenance of intra-cellular ion homeostasis. They operate at three stages; uptake, chelation and sequestration (Clemens 2001; Hall 2002). Trace metal transporters that have been extensively studied in higher plants are shown in Figure 1-3 and include:

- Natural resistance-associated macrophage proteins (NRAMPs), which are integrated membrane proteins for uptake of divalent ions such as Cd, Fe, Mn and Zn (Clemens 2001; Ishimaru, et al. 2011; Lin et al. 2012; Takahashi, Sasaki et al. 2012; Williams, Pittman & Hall 2000).

- Cu transporters (COPT/CTR), Cu chaperon for cytochrome c oxidase (COX), vacuolar iron transporter (VIT), permease in chloroplast (PIC) and mitochondrial iron transporter (MIT), which are trace metal chelators (Andrés-Colás et al. 2018; Clemens 2001; Eisses & Kaplan 2005; Logeman et al. 2017; Pilon 2011; Sancenón et al. 2004; Schulten & Krämer 2017).
- Zinc regulated transporters (ZRT) / Iron regulated transporters (IRT) or ZIP proteins, which are integrated membrane transporters involved in Fe and Zn uptake (Clemens 2001; Vert et al. 2002).
- Metal tolerance proteins (MTP), which are cytoplasmic proteins, involved in the influx of trace metal cations such as Zn into vacuoles (Clemens 2001; Blaudez et al. 2003; Krämer 2005; Krämer, Talke & Hanikenne 2007).
- Cation exchange proteins (CAX), which are cation / H^+ antiporters that mediate sequestration of trace metals into vacuoles (Clemens 2001; Mei et al. 2009).
- Pleiotropic drug resistance (PDR) proteins, which are members of ATP Binding Cassette (ABC) transporters involved in sequestration of Cd into vacuoles (Clemens 2001). Another member of the family of ABC transporters involved in trace metal transportation is lysosomal cysteine transporter (LCT), which was found to transport calcium along with Cd into the cells but its localization is yet to be determined (Williams, Pittman & Hall 2000; Rawat et al. 2018).
- Phytochelatine (PC) and Metallothionin (MT) proteins, which are two groups of cysteine-rich chelating proteins for metal detoxification especially for Cu and Cd (Clemens 2001; Clemens & Ma 2016; Chaudhary, Agarwal & Khan 2018).
- P-type trace metal ATPases (PAA and HMA proteins) which are cation / H^+ antiporters that use ATP to uptake trace metals from the cell membrane, mitochondria and chloroplast membrane (Verret et al. 2004; Kim et al. 2009; Morel et al. 2009; Ueno et al. 2010; Li et al. 2017; Chaudhary, Agarwal & Khan 2018).
- Yellow strip (YS) transporters, which are Fe chelator proteins (Clemens 2001; Schaaf et al. 2004; Banakar et al. 2017).

Trace metal transporters play an important role in detoxification by regulating the trace metal influx / efflux and sequestration (Hall 2002). For example, as mentioned above, P-type ATPase transporters and cation exchange proteins (CAX) are cation / H^+ antiporters which also play a role in sequestration of trace metals into vacuoles (Williams,

Pittman & Hall 2000; Clemens 2001; Morel et al. 2009). Furthermore, some trace metals were found to disrupt the absorption of other trace metals. For example, an excess level of Zn can induce Fe and Cu deficiency in plant roots (Marschner & Rimmington 1988) or Cd uptake is shown to be enhanced with iron deficiency (Cohen et al. 1998).

The production of microbial metabolites such as siderophores can also regulate the bio-availability of some trace metals such as Fe, Zn, Pb, Al and Cd uptake from the root system (Braud et al. 2009; Braud et al. 2010; Schalk, Hannauer & Braud 2011). Additionally in bacteria, another genetically regulatory mechanism for trace metal detoxification, called riboswitches, has been recently proposed. Riboswitches are RNAs that are shown to specifically bind to trace metal transporters such as Mg transport proteins in *Bacillus subtilis* (Ramesh & Winkler 2010) and Co transport proteins in *Clostridium scindens* (Furukawa et al. 2015). They control trace metal transportation by altering the transporter binding sites. Further investigations are required to test (i) whether the same regulatory mechanisms exist in plants and (ii) if this mechanism can be used in plants and algae for genetic modification and subsequent biotechnological purposes (Bocobza & Aharoni 2014).

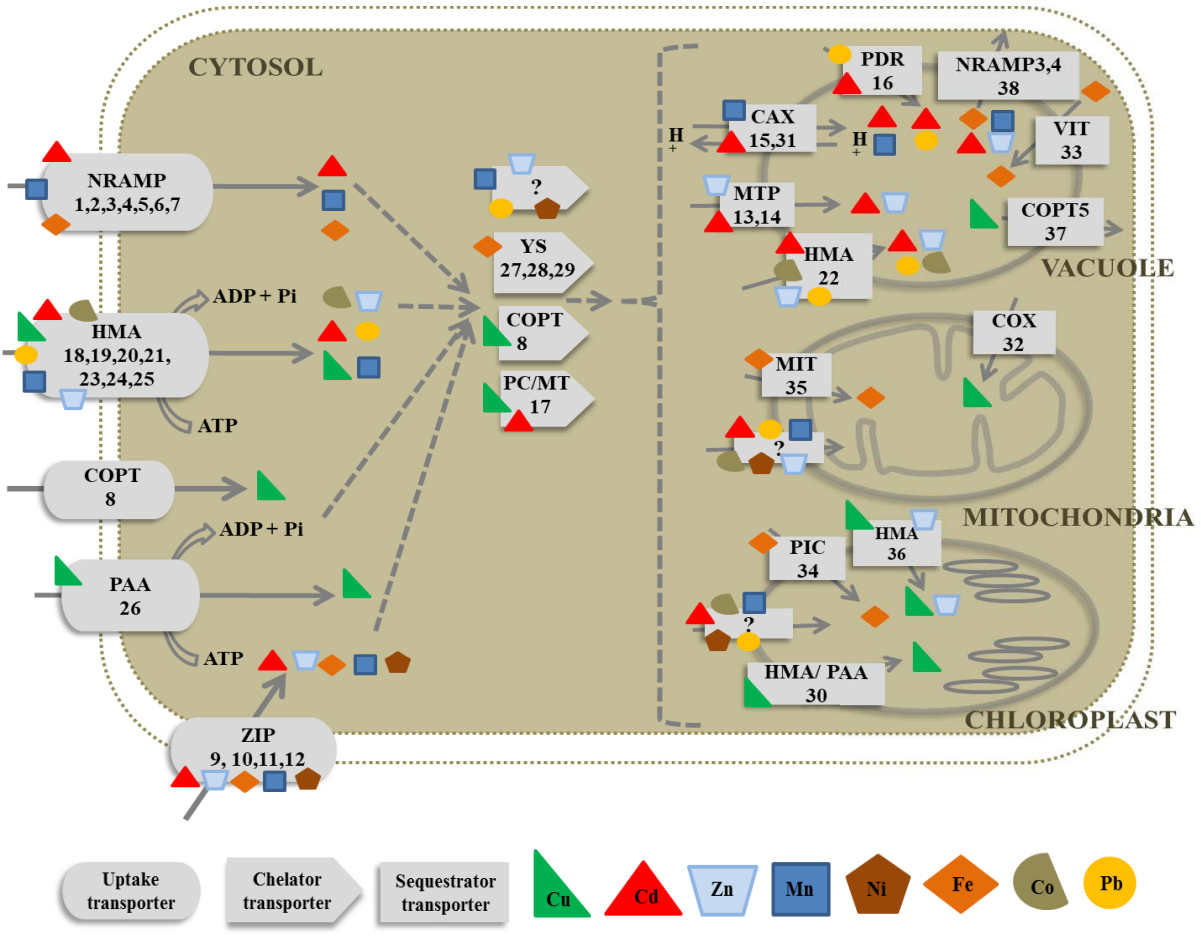

Figure 1-3. Scheme of possible trace metal transporters in cytosol, vacuole, chloroplast and mitochondria of the leaves of terrestrial plants. Transporters with question marks are unknown. NRAMP: Natural Resistance-associated Macrophage Protein; HMA and PAA: P-type trace metal ATPases protein; YS: Yellow strip protein; PC: Phytochelatine; MT: Metallothionin; COPT: Cu transporter protein; ZIP: ZRT/ IRT related protein; MTP: Metal Tolerance Protein; CAX: Cation exchange protein; PDR: Pleiotropic drug resistance protein; COX: Cu chaperon for cytochrome c oxidase; VIT: Vacuolar Iron Transporter; PIC: Permease in Chloroplast protein; MIT: Mitochondrial Iron Transporter. **1**: Narayanan, Vasconcelos & Grusak (2006); **2**: Sasaki et al. (2012); **3**: Takahashi, Ishimaru, et al. (2011); **4**: Curie et al. (2000); Lanquar et al. (2005); (Thomine et al. 2003) **5**: Cailliatte et al. (2010); **6**: Kaiser et al. (2003); **7**: Bereczky et al. (2003); **8**: Eisses & Kaplan (2005); Sancenón et al. (2004); Yruela (2009); **9**: Bughio et al. (2002); **10**: Nishida et al. (2011); **11**: Grotz & Guerinot (2006); Vert et al. (2002); **12**: Rogers, Eide & Guerinot (2000); **13**: Blaudez et al. (2003); Kobae et al. (2004); Krämer (2010); van der Zaal et al. (1999); **14**: Desbrosses-Fonrouge et al. (2005); **15**: Guerinot (2000); (Mei et al. 2009); **16**: Kim, Bovet, et al. (2006); Kim et al. (2007); **17**: Cobbett & Goldsbrough (2002); Murphy & Taiz (1995); Zhou & Goldsbrough (1994); **18**: Andrés-Colás et al. (2006); Hirayama et al. (1999); Puig et al. (2007); **19**: Becher et al. (2004); Hussain et al. (2004); **20**: Mills et al. (2003); **21**: Verret et al. (2004); **22**: Morel et al. (2009); **23**: Ueno et al. (2010); **24**: Lee et al. (2007); **25**: Roberts et al. (2004); Yamaji et al. (2013); **26**: Abdel-Ghany et al. (2005); **27**: Curie et al. (2001); Roberts et al. (2004); Schaaf et al. (2004); **28**: Jean et al. (2005); **29**: Koike et al. (2004); **30**: Grotz & Guerinot (2006); Shikanai et al. (2003); Yruela (2009); **31**: Abdel-Ghany et al. (2005); Edmond et al. (2009); **32**: Balandin & Castresana (2002); **33**: Kim, Punshon, et al. (2006); **34**: Duy et al. (2007); **35**: Bashir et al. (2011); **36**: Kim et al. (2009); Morel et al. (2009); Yruela (2013); **37**:Pilon 2011; **38**: Puig et al. 2009

Although the physiological responses to trace metal stress by seagrasses have been studied previously (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002a, 2004a, 2004b; Li et al. 2012), to our knowledge, very few studies have examined the molecular interaction of seagrasses with trace metals (Bond et al., 1988; Brinkhuis et al., 1980). The physiological responses to Cu stress across various seagrasses are shown to be species-specific and also, concentration and time-dependent

(Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2004b, Buapet et al. 2019).

It is currently unknown whether the same transporters in higher plants are active in seagrasses. For example, NRAMP transporters have been previously reported as a stress-response candidate in the seagrass *Posidonia oceanica,* but their function has not been confirmed yet (Lauritano et al. 2015). Furthermore, the biological function and specificity of metal transporters are still not completely understood even though several studies have been reported in higher plants (Cho-Ruk et al., 2006; Clemens and Ma, 2016; Kupper and Kroneck, 2005; Li et al., 2009; Perriguey et al., 2008; Sunitha et al., 2013; Jalmi et al., 2018; Zhang et al., 2018; Chaudhary et al., 2018; Shi et al., 2018).

4.2. Photosynthetic response to Cu toxicity

Photosystems of higher plants and seagrasses are shown to be sensitive to elevated levels of Cu, Zn, Fe, Mn and Cd at a wide range of concentration (0.1 – 10 mg L^{-1} for Cu) and therefore, it can be an effective and sensitive tool for the early detection of trace metal toxicity (Clijsters & Assche 1985; Ralph & Burchett 1998b; Prange & Dennison 2000; Wang et al. 2007; Kaur et al. 2012; Reale et al. 2016; Alirzayeva et al. 2017; Bayçu et al. 2017; Iori et al. 2017; Sebastian, Nangia & Prasad 2018). More specifically, the ratio of maximum quantum yield (F_v / F_m) is a reliable indicator of the efficiency of photosystem II (PSII) since damage to the PSII reaction centre leads to less electron transport and consequently, a decrease in F_v / F_m ratio (Ouzounidou 1993; Demmig-Adams et al. 2014, Buapet et al. 2019).

The prominent impact on chloroplasts by trace metals in terrestrial plants include: inhibition of nitrogen fixation and disruption in plasma membrane permeability by Cd (Costa & Morel 1994; Balestrasse et al. 2003); induction of stomata closure by Cd (Perfus-Barbeoch et al. 2002); decline in water content of the shoot by Ni (Gajewska et al. 2006) and inhibition of chlorophyll synthesis by Mn (Clairmont, Hagar & Davis 1986). Some toxic trace metals can be transported to chloroplast and competed with co-factors of the corresponding subunits of enzymes. For instance, previous studies have reported displacement of Mg from chlorophyll with $Cr_{(III)}$ and $Cr_{(VI)}$ in wheat (Sharma & Sharma 1996), with Hg in spinach (Šeršeň, Král'ová & Bumbalova 1998) and with Zn in bean (Van Assche & Clijsters 1986). Quinine A (Q_A) activity has also been shown to be drastically reduced by the displacement of its $Fe_{(II)}$ with $Cu_{(II)}$ (Jegerschoeld et al. 1995).

Furthermore, ATP biosynthesis can be affected by trace metal toxicants via AsO_4 displacement with phosphate in ATP biosynthesis pathway (Meharg & Hartley-Whitaker 2002). In terrestrial plants (Figure 1-4), the data suggests that PSII is more sensitive to trace metal exposure than PSI. There are few studies that have shown changes in chlorophyll content (Purnama et al. 2015) and chlorophyll fluorescence (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002a, 2004b; Moustakas et al. 2017; Zheng et al. 2018) in seagrasses as a response to Pb, Cu, Cd and Zn exposure, which also indicate the sensitivity of PSII towards trace metal stress. The precise trace metals binding sites in photosystems of seagrasses, nonetheless, are still unknown.

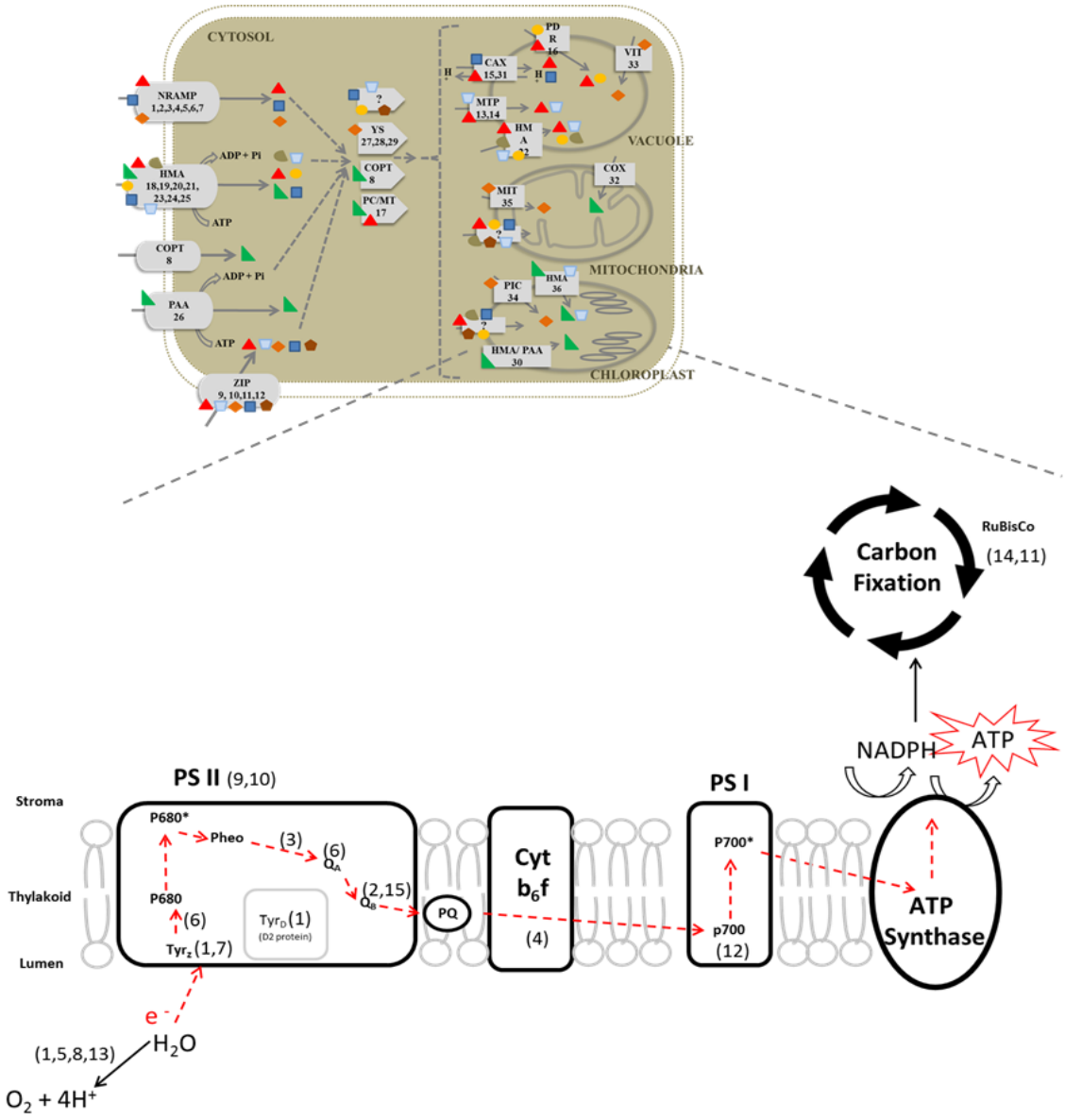

Figure 1-4. Proposed trace metal binding sites in plant photosystem where they might negatively impact the electron transfer chain (dash line) and photosystem efficiency. Reference numbers 1 – 7, 9 – 11 and 15 are related to Cu stress.
1:Šeršeň et al. (1997) **2**:Mohanty, Vass & Demeter (1989) **3**:Yruela et al. (1993) **4**:Babu et al. (2001) **5**:Vierke & Struckmeier (1977) **6**: Jegerschoeld et al. (1995) **7**:Schröder et al. (1994) **8**: Van Assche & Clijsters (1986); **9**: Cedeno-Maldonado, Swader & Heath (1972); **10**: Arellano et al. (1995); **11**: Siedlecka et al. (1997); **12**: Millaleo et al. (2013); **13**: Tamura & Cheniae (1987); **14**: (Van Assche & Clijsters 1986); **15**: (Geiken et al. 1998); **16**: Sigfridsson et al. (2004).

Apart from Cu, Zn and Mn, which are essential trace metals and expected to have binding sites in the photosystem of plants, Cd also seems to be able to bind to PSII subunits and negatively affect the functionality of the photosystem (Geiken et al. 1998). In terrestrial plants, the mechanism of Cd damage is thought to be via stomata (i) by entering the cells through calcium channels (Perfus-Barbeoch et al. 2002) and (ii) by inducing stomata closure in plant cells. However, seagrasses have no stomata and the mechanism of Cd damage has not been described to date. There are few studies that specifically focused on the physiological impact of Cu stress on seagrasses and there are summarized in

Table **1-2.** Current knowledge of physiological responses of seagrasses to Cu stress.

Species	**Experimental design**	**Result**	**Reference**
Halophila sp. *Z. caprocorni* *C. serrulata*	Concentration : 1 mg / L (+EDTA) Duration : 12 days +5 days recovery Location: Australia	Decline in photosynthesis efficiency	Prange & Dennison (2000)

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Halophila sp. *Z. caprocorni* *C. serrulata*	Concentration : 1 mg / L (+EDTA) Duration : 12 days +5 days recovery Location: Australia	Decline in photosynthesis efficiency	Prange & Dennison (2000)

H. ovalis	Concentration: 5 – 10 mg / L Duration: 4 days Location: Australia	Decline in chlorophyll a and pigment responses	Ralph & Burchett (1998)
H. ovalis	Concentration: 0.5 – 4 mg / L Duration : 50 days Location: Australia	Reduce growth rate	Ambo-Rappe, Lajus & Schreider (2011)
Z. capricorni	0.1 – 1 mg / L injection Duration :10 hr, 3 days recovery Location: Paradise Beach (Australia)	Decrease photosynthesis efficiency	Macinnis-Ng & Ralph (2002b)
Z. capricorni	0.1 – 1 mg / L injection Duration :10 hr, 3d recovery Location: Pittwater, Sydney Harbour and Botany Bay (Australia)	Fluorescence quenching	Macinnis-Ng & Ralph (2004a)
Z. muelleri	0.250 and 0.500 mg / L Duration : 7 days Location : Pittwatter, Sydney	Decrease photosynthesis efficiency	Buapet et al. 2019

4.3. Enzymatic defense mechanism to Cu toxicity in plants and seagrasses

Reactive oxygen species (ROS) are regularly produced in the cytosol, chloroplast, mitochondria and peroxisomes of plants during cellular activities (Keunen et al. 2011; Bhat et al. 2015). Environmental stress factors including trace metals, leads to over production of ROS; followed by the oxidation of macromolecules and initiation of apoptosis (Panda, Baluška & Matsumoto 2009; Iwase et al. 2014; Bhat et al. 2015; Moustakas et al. 2017, Buapet et al. 2019). In terrestrial plants, over production of ROS is reported in several cases as a response to excess level of Cd and Hg (Cho & Park 2000; Maksymiec & Krupa 2006; Hsu & Kao 2007; Rodríguez-Serrano et al. 2009; Hernández et al. 2012; Zhao et al. 2012; Sawaki et al. 2016). The first signaling response to trace metal stress exposure is the activation of antioxidant enzymes including superoxide dismutase (SOD); catalase (CAT); peroxidase (POD); glutathione peroxidase (GPX); and ascorbate peroxidase (APX) (Tan et al. 2002; Sureda et al. 2006; Prasad et al. 2016). Current knowledge of antioxidant enzymes involved in trace metal detoxification in terrestrial plants and seagrasses are summarized in Figure 1-5.

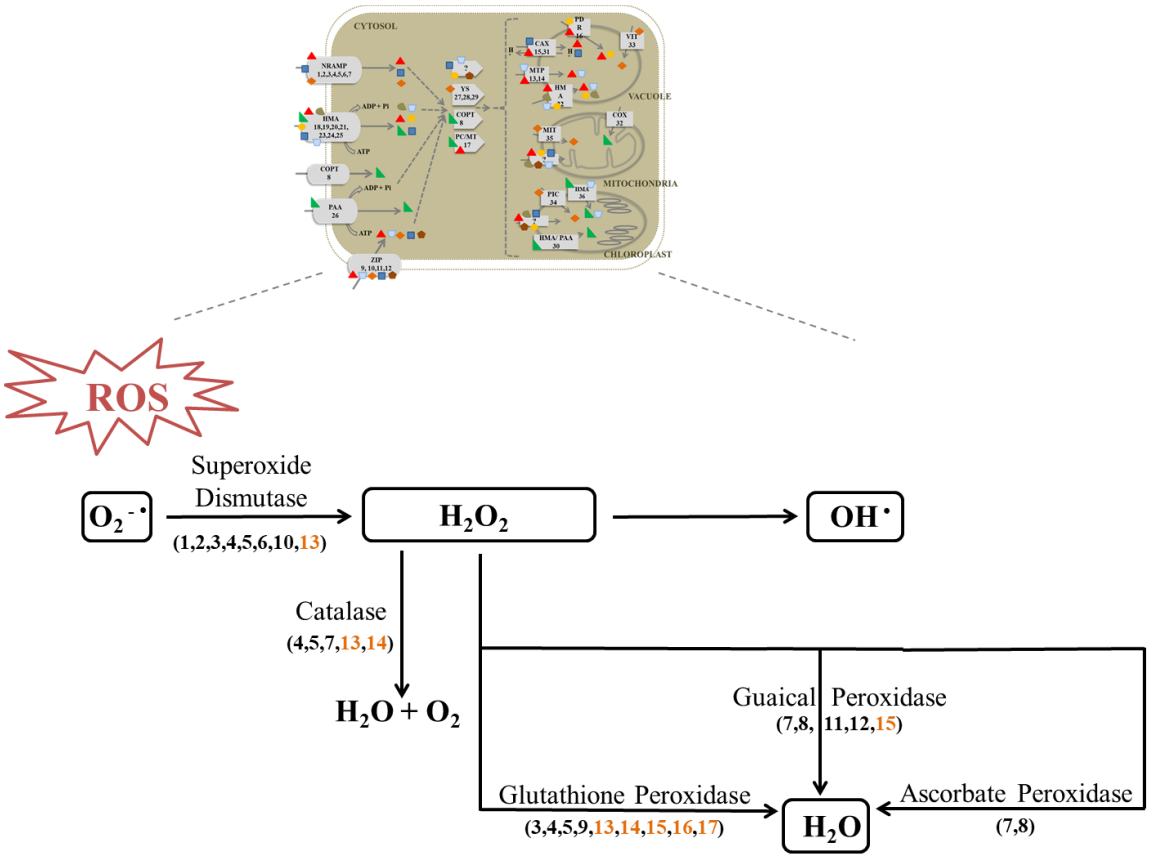

Figure 1-5. Antioxidant responses to trace metals. The corresponding numbers are illustrating the antioxidant enzymes as a response to trace metals in plants in black and seagrasses in orange. Reference numbers 1, 6, 13, 18–20 are related to Cu stress.
1: Chongpraditnun, Mori & Chino (1992); **2**: Sandalio et al. (2001); **3**: Kaur et al. (2012);**4**: Rodrigues-Serrano et al. (2006); **5**: Cho & Park (2000); **6**: Cuypers et al. (2011); **7**: Shaw (1995); **8**: (Chaoui et al. 1997); **9**: Schickler & Caspi (1999); **10**: Baccouch, Chaoui & El Ferjani (2001); **11**: Gajewska et al. (2006); **12**: Šimonovičová et al. (2004); **13** and **14**: Lin et al. (2016); **15**: Ferrat, Bingert, et al. (2002); Ferrat, Romeo, et al. (2002); **16**: Hamoutene et al. (1996);**17**: Bucalossi et al. (2006). **18**: Pim 2018; **19**: Zheng et al. (2018); **20**: Nanda & Agrawal (2016); **21**: Anjum et al. (2016); **22**: Rui et al. (2016).

4.4. Chemical defense mechanism to Cu toxicity in plants and seagrasses

Aside from direct toxicity response of plants to trace metal stress (enzymatic response), it is previously shown that plants also have a regulated signaling pathway to respond to environmental stress factors using hormones (Jonak, Nakagami & Hirt 2004; Opdenakker et al. 2012; Dattolo et al. 2013; Kong et al. 2014; Masood et al. 2016; Bücker-Neto et al. 2017; Jalmi et al. 2018). The most studied phytohormones linked to trace metal detoxification include:

- Abscisic acid (ABA), which accumulates in cell as a protective mechanism against trace metal stress in plants and seagrasses (Bartels & Sunkar 2005; Danquah et al. 2014; Davey et al. 2017). Specifically, it is found as a response to Hg, Cd and Cu (Munzuroğlu, Zengin & Yahyagil 2008; Stroiński et al. 2010; Kim et al. 2014), Zn (Wang, Wang, et al. 2014) and Pb (Atici, Ağar & Battal 2005).
- Ethylene, which is shown to have role in detoxification of abiotic stress factors in higher plants (Potuschak et al. 2003; Raab et al. 2009). Cu and Cd have been shown to induce the expression of precursor genes of ethylene production in plants (Schlagnhaufer, Arteca & Pell 1997; Schellingen et al. 2014). However, the ethylene biosynthesis and signalling seemed to be lost in the family of *Zosteraceae* (Golicz et al. 2015). It is not clearly known how the gene loss has occurred and whether an alternative pathway is replaced by ethylene signalling in this family of seagrasses.
- Auxin, which is increased as a response to trace metals in plants (Wang et al. 2015). Auxin metabolism can also be indirectly induced by ethylene in some cases (Růžička

et al. 2007). On the other hand, Yuan & Huang (2016) reported the suppression of auxin production under nitric acid and Cd treatment.

- Plant steroids (brassinosteroids), that regulates senescence in plants (Mandava 1988) and have a physiological protective role against abiotic stress factors (Abbas, Latif & Elsherbiny 2013; Mahesh et al. 2013; Wang, Shu, et al. 2014; Olsen et al. 2016). In trace metal stress, it is shown that brassinosteroids also elevates the activity of antioxidant enzymes (Fariduddin et al. 2009; Dhriti et al. 2014).
- Proline and choline, which mainly protect cellular components of plants and seagrasses during stress by accumulation in mitochondria and chloroplast, respectively (Rhodes & Hanson 1993; Prange & Dennison 2000; Ashraf & Foolad 2007).
- Cytochrome P450, that is shown to have a role in detoxification of Pb and Cd in plants and fungi as suggested by Zhang et al. (2015).
- Senescence regulator, with a role in Cu-specific detoxification in plants (Breeze et al. 2008).
- Multicopper oxidase, which converts the toxic form of Cu (Cu^{+1}) to less toxic ionic form (Cu^{+2}) in cytosol as a detoxification mechanism in plants (Teitzel et al. 2006).

5. Identification of biomarkers for early assessments of trace metal toxicity

The use of molecular tools for early detection of environmental stress in seagrasses has been suggested as an effective way to improve conservation management of seagrass meadows (Macreadie et al. 2014; Schliep et al. 2015; Pernice et al. 2016). Therefore, cellular, molecular and biochemical changes related to environmental pollution (in this case, trace metal pollution) known as biomarkers, have the potential to be a useful tool for the early detection of eco-toxicological stress in plants as well as in seagrasses (Depledge, Aagaard & Györkös 1995; Ferrat, Pergent-Martini & Roméo 2003; Rainbow 2006).

Photosystem subunits of higher plants and seagrasses are sensitive to elevated levels of Cu, Zn, Fe, Mn and Cd and therefore, could be considered as effective biomarkers for the early detection of trace metal toxicity (Clijsters & Assche 1985; Ralph & Burchett 1998b; Prange & Dennison 2000; Wang et al. 2007; Kaur et al. 2012; Reale et al. 2016). Antioxidant enzymes are also shown to be early stress response detectors against abiotic stress factors including trace metal stress (Keunen et al. 2011; Bhat et al.

2015). Thus, differential expression level of antioxidant enzymes can be used as biomarkers to evaluate changes in the physiological conditions of plants (Ferrat et al. 2003; Dazy, Masfaraud & Férard 2009). However, no biomarkers have been suggested for *Z. muelleri* under trace metal stress to date.

6. Hyper-accumulation of trace metals in seagrasses: a combined role in adaptation and phytoremediation

Trace metals are considered as hazardous contaminants since they are not biodegradable (Gaur & Adholeya 2004). According to the National Environment Protection Council, there are more than 80,000 trace metal contaminated sites in Australia, which will cost billions of dollars to decontaminate (Salt et al. 1995; Sinha et al. 2010). With continuous coastal development, there is a need for efficient and cost-effective decontamination methods, such as the use of plants in phytoremediation (Baker et al. 1994; Hinchman, Negri & Gatliff 1996; Clemens, Palmgren & Krämer 2002; Cho-Ruk et al. 2006). Understanding the tissue preference and tolerable toxicity level of trace metals in seagrasses is useful not only to better comprehend their adaptation to trace metals, but also for identifying their potential phytoremediation capacity (Lyngby & Brix 1984; Ward 1987; Catsiki & Panayotidis 1993; Benemann et al. 1994; Campanella et al. 2001; DalCorso et al. 2013).

The ability of seagrasses to uptake trace metals from both roots and shoots makes them valuable candidates for phytoremediation (Boudouresque et al. 2000; Ferrat, Pergent-Martini & Roméo 2003; Malea, Adamakis & Kevrekidis 2013). Most candidate plant species are capable of accumulating high concentrations of trace metals in their leaves (Table 1-3). In terrestrial plants, some trace metals, such as Pb and Cr, seem to only accumulated in below-ground tissues (Dahmani-Muller et al. 2000; Bose & Bhattacharyya 2008; John et al. 2012), whereas in seagrasses, their accumulation has been reported in both below and above-ground tissues (Lyngby & Brix 1984; Catsiki & Panayotidis 1993; Warnau et al. 1995; Howley, Morrison & West 2006). Therefore, tissue preference in each candidate species is an important factor to consider in the application of using seagrasses in trace metal phytoremediation.

Another important factor for seagrass candidates in phytoremediation is seasonal variation, which is known to considerably affect Cd, Cu, Mn, Ni and Pb accumulation in the leaves of *P. australis* (Ward 1987); Cu, Cd, Zn accumulation in *P. oceanica* (Malea

& Haritonidis 1989; Warnau, Fowler & Teyssié 1996); and Al, Fe, Cd, Pb, Cu accumulation in *C. nodosa* (Malea 1993; Malea & Haritonidis 1995).

Although seagrasses have these valuable capabilities for hyper-accumulation of trace metals, their use for phytoremediation remains challenging: firstly some seagrass species such as *P. australis*, *Z. caespitosa* and *Z. caulescense* are near threatened according to the International Union for the Conservation of Nature (IUCN) and should not be considered for this application. Secondly, most of *Posidonia* species are slow growing with low rate of recovery so they cannot be considered financially (Larkum 1976). Lastly, harvesting seagrasses that grow in deep water would be challenging and not cost effective. Therefore, algal species (macro and micro), which are more abundant and fast growing, would be a more convenient option for phytoremediation purposes than seagrasses (Chekroun & Baghour 2013; Labbé et al. 2017; Riaz et al. 2017).

Table 1-3. Trace metals translocation and tissue preference in seagrasses.

Plant species	**Toxicant**	**Tissue preference**	**References**
Croton bonplandium	Fe, Zn, Ni, Al, Si	Shoot	Kumari, Lal & Rai (2016)
Giant reed *(Arundo donax L.)*	Se	Shoot	El-Ramady et al. (2015)
Cyperus malaccensis	Zn	Shoot	Chayapan et al. (2015)
Psoralea pinnata	Fe, Cr	Seed	Ochonogor & Atagana (2014)
Cupressus lindleyi and *Juniperus deppeana*	Zn, Mn	Shoot	Morton-Bermea et al. (2014)
Salix atrocinerea	Zn	Shoot	Monterroso et al. (2014)
Brassica juncea	Cd, Pb	Root	John et al. (2012)
Oryza japonica	Cd	Shoot	Kashiwagi et al. (2009)
Oryza sativa		Shoot (grain)	Rodda, Li & Reid (2011)
		Shoot	Uraguchi et al. (2009)
Oryza sativa			
Triticum aestivum	Ni, Fe Cd, Cr	Shoot Root	Bose & Bhattacharyya (2008)

Cardaminopsis halleri *Armeria maritima ssp. halleri*	Zn, Cd Pb, Cu	Shoot Root	Dahmani-Muller et al. (2000)
Seagrasses			
Z. marina	Cd	Root	Faraday & Churchill (1979)
	Cd	Shoot and Root	Brinkhuis, Penello & Churchill (1980)
	Mn	Shoot	
	Cd, Zn	Shoot	Lyngby & Brix (1984)
	Cu, Hg, Pb	Root	
Z. muelleri	Cu	Shoot	Carter & Eriksen (1992); Catsiki & Panayotidis (1993)
Z. capricorni	Cu, Mn, Pb, Cd, Ni, Zn	Shoot	Howley, Morrison & West (2006)
	As	Root	
Thallasia testudinum	Mn, Zn	Shoot and root	Schroeder & Thorhaug (1980)
P. oceanica	Hg	Shoot	Maserti, Ferrara & Paterno (1988)
	Zn, Cu	Shoot	Warnau et al. (1995)
	Cd	Shoot and Root	
	Pb, Fe	Root	
	Cr	Rhizome	
	Cd, Zn	Shoot	Warnau, Fowler & Teyssié (1996)
	Hg	Rhizome	Pergent-Martini (1998)
H. sipulacea	Cd	Root	Malea (1994)
H. stipulacea	Zn	Shoot	Malea & Haritonidis (1995)
H. minor	As	Root	

	Cu	Rhizome	Ahmad, Azman & Said (2015)
H. ovalis	Cd	Shoot	
C. nodosa	Cr	Shoot	Catsiki & Panayotidis (1993)
Tape seagrass (Enhalus acoroides)	Zn, Cu, Ni, Pb, Cd	Shoot, root	Sidi et al. (2018)
Thalassia hemrichii	Pb, Cd	Shoot, root	Tupan & Uneputty (2017)

7. Conclusion

Major knowledge gaps still remain in understanding trace metal toxicity in seagrasses, including the identification of: (i) transporters for uptake, chelation and sequestration of trace metals by seagrasses; (ii) the environmental factors affecting bio-availability; and (iii) the precise binding sites within the photosystems of seagrasses. Knowledge of tissue-specific accumulation of trace metals in seagrasses and the antioxidant response to trace metals exposure are also limited. Further studies are therefore, required to address how seagrasses will respond to on-going anthropogenic disturbance and how seagrasses species could be used for phytoremediation purposes.

With recent advances in molecular techniques, some of the current knowledge gaps in understanding seagrass responses to abiotic stresses, such as light and heat and trace metal stress, have been answered at both transcriptomic and proteomic levels (Serra et al. 2012; Dattolo et al. 2014; Franssen et al. 2014; Kong et al. 2014; Pernice et al. 2015; Schliep et al. 2015; Kumar, Padula, et al. 2016; Pernice et al. 2016; Lin et al. 2018, Buapet et al. 2019). Therefore, the same techniques hold great potential to study the molecular mechanisms of Cu stress in the seagrass, *Z. muelleri*.

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CHAPTER 2

Excess Cu promotes photoinhibition and modulates the expression of antioxidant-related genes in *Zostera muelleri.*

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Abstract

Copper (Cu) is an essential micronutrient for plants and as such is vital to many metabolic processes. Nevertheless, when present at elevated concentrations, Cu can exert toxic effects on plants by disrupting protein functions and promoting oxidative stress. Due to their proximity to the urbanised estuaries, seagrasses are vulnerable to chemical contamination via industrial runoff, waste discharges and leachates. *Zostera muelleri* is a common seagrass species that forms habitats in the intertidal areas along the temperate coast of Australia. Previous studies have shown the detrimental effects of Cu exposure on photosynthetic efficiency of *Z. muelleri*. The present study focuses on the impacts of sublethal Cu exposure on the physiological and molecular responses. By means of a single addition, plants were exposed to 250 and 500 μg Cu L^{-1} (corresponding to 3.9 and 7.8 μM, respectively) as well as uncontaminated artificial seawater (control) for 7 days. Chlorophyll fluorescence parameters, measured as the effective quantum yield (ϕPSII), the maximum quantum yield (F_v/F_m) and non-photochemical quenching (NPQ) were assessed daily, while Cu accumulation in leaf tissue, total reactive oxygen species (ROS) and the expressions of genes involved in antioxidant activities and trace metal binding were determined after 1, 3 and 7 days of exposure. *Z. muelleri* accumulated Cu in the leaf tissue in a concentration-dependent manner and the bioaccumulation was saturated by day 3. Cu exposure resulted in an acute suppression of ϕPSII and F_v/F_m. These two parameters also showed a concentration-and time-dependent decline. NPQ increased sharply during the first few days before subsequently decreasing towards the end of the experiment. Cu accumulation induced oxidative stress in *Z. muelleri* as an elevated level of ROS was detected on day 7. Lower Cu concentration promoted an up-regulation of genes encoding Cu/Zn-superoxide dismutase (*Cu/Zn-sod*), ascorbate peroxidase (*apx*), catalase (*cat*) and glutathione peroxidase (*gpx*), whereas no significant change was detected with higher Cu concentration. Exposure to Cu at any concentration failed to induce regulation in the expression level of gene encoding metallothionein type 2 (*mt2*), metallothionein type 3 (*mt3*) and cytochrome c oxidase Cu chaperone (*cox17*). It is concluded that chlorophyll fluorescence parameters provide timely probe of the status of photosynthetic machinery under Cu stress. In addition, when exposed to a moderate level of Cu, *Z. muelleri* mitigates any induced oxidative stress by up-regulating transcripts coding for antioxidant enzymes.

1. Introduction

Seagrasses are one of the most productive marine plant communities on Earth. They colonize in shallow coastal water, serving as habitat for many ecologically and economically important marine organisms. Extensive root systems of seagrass act as natural stabilizer of marine sediments, protecting the coasts from wave action and erosion (Nordlund et al., 2016). An increasing body of evidence supports the high carbon production and sequestration capacity of seagrass meadows which suggests they have an additional role in mitigating the effects of global climate change (Duarte, 2017). Regardless of their valuable ecosystem services, habitat destruction and human disturbances have continued causing a decline in seagrass meadows worldwide (Waycott et al. 2009; Unsworth & Cullen 2010; Short et al. 2011).

Among many anthropogenic pressures in the coastal ecosystems, trace metal contamination is a major threat to the health of marine organisms (Gledhill et al. 1997; Correa et al. 1999; Livingstone 2001). Copper (Cu) is a widespread trace metal contaminant (Barón et al., 2018). It is introduced into the coastal environment via various pathways, including waste discharges and leachates from industries, as well as runoff from mines and agricultural areas in which Cu is used in fertilizers, fungicides and pesticides (Gledhill et al. 1997; O'Gara et al. 2004; Bonnard, Romeo & Amiard-Triquet 2009). Although Cu is an essential micronutrient for plants, it can become very toxic at higher concentrations. Common toxic effects of Cu include slow growth rates, chlorosis, senescence and eventual death (Singh et al., 2016). At the physiological level, Cu excess disrupts photosynthetic activity by inhibiting both the donor and acceptor sides of photosystem II (Dewez et al. 2005; Barón et al. 2018) as well as substituting magnesium in chlorophyll molecule (Küpper, Küpper & Spiller 1996). In addition, Cu can induce oxidative stress by catalyzing the generation of reactive oxygen species (ROS) via Haber-Weiss and Fenton reactions (Halliwell, 2006; Palmer and Guerinot, 2009), leading to a loss in integrity of biological membranes, lipid peroxidation, protein and DNA damage (Körpe and Aras, 2011; Thounaojam et al., 2012). In higher plants, ROS level is determined by a balance between its production and scavenging (Mittler et al., 2004). When facing an overproduction of ROS, plant cells increase their scavenging activity by utilising antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX) (Foyer, Lelandais & Kunert 1994; Asada 2006) and a non-enzymatic antioxidant such as ascorbate and glutathione

(Noctor & Foyer 1998). These antioxidants work in concert to transform reactive oxygen species to less toxic products. Superoxide dismutase catalyses the dismutation of superoxide radical (O_2^-) into hydrogen peroxide (H_2O_2). H_2O_2 is then detoxified either via the activity of APX or CAT or GPX (Singh et al., 2016). The protective role of antioxidants against harmful oxidative damage induced by trace metal stress has been highlighted in many plant species (Shahid et al., 2014; Singh et al., 2016). Exposure to Cu resulted in an increase in the activity of SOD, APX, CAT and GPX (Thounaojam et al. 2012; Goswami & Das 2016), as well as in the transcription level of the genes coding for these antioxidant enzymes (Xiang & Oliver 1998; Fidalgo et al. 2013; Da Costa & Sharma 2016). This suggests that cooperative actions of antioxidant systems at both transcription and activity levels play essential roles in mitigating Cu stress.

In addition to defence against ROS, tolerance of plants to trace metals depends on their capacity to maintain homoeostasis of trace metals once they have entered the cells. Intracellular Cu must be tightly controlled to avoid oxidative toxicity brought about by the free Cu ion (Ravet and Pillon, 2013). Various metals including the free Cu ion can be bound by a group of Cys-rich proteins, metallothioneins (MTs), to their thiol groups of the Cys residue (Huang & Wang 2010; Liu et al. 2015), making Cu less harmful. At the same time, Cu which is required for assembly of many proteins and enzymes is chelated by specific Cu chaperones and delivered to where it is needed. Cytochrome c oxidase (COX) which resides in a plants' mitochondria is one of the target proteins that uses Cu as a metal cofactor (Garcia et al., 2014). The cytochrome c oxidase Cu chaperone (COX17) has been proposed to play a role in shuttling Cu from the cytosol to the mitochondria. It has been shown that expression of *mt* and homologues of *cox17* found in *Arabidopsis thaliana* (*atcox17*) are up-regulated by Cu exposure (Zhou & Goldsbrough 1994; Attallah, Welchen & Gonzalez 2007; Attallah et al. 2007; Liu et al. 2015) and other oxidative stress inducers (Attallah et al., 2007; Liu et al., 2015), suggesting a role of these Cu-binding proteins in maintaining Cu homoeostasis and oxidative balance.

Zostera muelleri (syn. *Z. capricorni*) is a common seagrass found in intertidal areas along the temperate coastlines of Australia (Davey et al., 2018). It has been reported that accumulation of different metals including Cu in the biomass of *Z. muelleri* corresponds with ambient concentrations, suggesting the possibility to use this seagrass as a bioindicator for trace metal pollution (Carter & Eriksen 1992; Howley, Morrison &

West 2006; Birch, Cox & Besley 2018b). Previous experimental studies have provided insights into the capacity of *Z. muelleri* to act as a biomonitor of Cu contamination, through the use of chlorophyll fluorescence-based ecotoxicological bioassays (Macinnis-Ng & Ralph 2002a, 2004a, 2004b; Ralph et al. 2007). In these works, Cu was shown to be highly toxic within the previously reported concentration range of 0.1 − 1 mg Cu L^{-1} (Macinnis et al. 2002) and it exerted rapid and irreversibly adverse damage, as seen by chlorophyll fluorescence parameters. Investigations of Cu toxicity effects in seagrasses have increased in the recent years, covering species from various bioregions (Lin et al. 2016; Llagostera et al. 2016; Zhao et al. 2016; Gamain et al. 2017; Moustakas et al. 2017; Nielsen et al. 2017; Zheng et al. 2018). It has been shown that Cu affects physiological processes of seagrasses and alters defence mechanisms. Cu-treated seagrasses exhibited a decrease in photosynthesis, chlorophyll content and growth rates (Llagostera et al. 2016; Zhao et al. 2016; Moustakas et al. 2017; Nielsen et al. 2017; Zheng et al. 2018). An increase in SOD, CAT and GPX activity in response to Cu exposure was observed in *Zostera japonica* (Lin et al., 2016), while *Thalassia hemprichii* displayed an increase in peroxidase (POD) activity concomitant with a decrease in the activity of SOD and CAT (Zheng et al., 2018). Although these vital physiological mechanisms are primarily controlled at the transcription levels, the molecular responses have, to our knowledge, been probed only in the seagrasses, *Z. noltei* and *Posidonia oceanica* (Giordani et al. 2000; Gamain et al. 2017). Genes associated with photosynthesis, antioxidants and mitochondrial metabolism in *Z. noltei* were investigated and all genes studied were found to be inhibited by Cu except for *gpx1* at 20 ºC whereas the genes encoding metallothionein type 2 in *P. oceanica* was found to be induced by Cu exposure (Giordani et al., 2000).

The main objective of our study was to characterize the physiological and molecular processes of *Z. muelleri* in response to sub-lethal exposure to Cu. We applied chlorophyll fluorescence to detect the toxicity effects on photosystem II (PSII) efficiency and quantitative real-time PCR to detect differential expressions of genes associated with antioxidative enzymes and metal-binding. Cu effects were subsequently linked to Cu bioaccumulation and ROS production. The results obtained in this study will facilitate a better understanding of the effects of Cu on the health of seagrass meadows, as well as provide insight into the use of biomarkers for early and timely detection of Cu exposure and for quantifying the potential impact of trace metal contamination on this critical marine habitat that is at risk.

2. Materials and Methods

2.1. Seagrass samplings, aquaria setup and experimental design

Sods of *Zostera muelleri* with intact sediment were collected from Pittwater, New South Wales, Australia (33°38′45.6″S, 151°17′12.8″E). Additional sediment from the same site was also collected for aquaria setup. The plant samples were covered with wet paper towels to prevent water loss and within 2 hr transferred to seagrass aquarium facility at the University of Technology Sydney.

At the aquarium facility, epiphytes were removed from seagrass leaves by washing with artificial seawater (salinity of 30). The horizontal rhizomes were separated, and individual ramets/shoots from different sods were combined into a tub. The sediment used for planting was prepared by mixing approximately 50% of washed sand and 50% of natural sediment. Sixty individual ramets/shoots were planted in each aquarium (50 L), and in total six aquaria were filled with artificial seawater (salinity of 30). Stirring and aeration were provided using a submersible pump and diffusive airstone. Irradiance of approximately 200 μmol photons m^{-2} s^{-1} (minimal saturating irradiance derived from the rapid light curves) with a light-dark cycle (12:12 h) provided by RGBW LEDs (Cidley 250W). The temperature of the aquarium facility was kept constant at 22 ºC. The plants were allowed to acclimate to the aquaria conditions for 2 weeks.

The experiment consisted in three treatments: (1) artificial seawater with no addition of Cu, serving as control (2) artificial seawater with 250 μg Cu L^{-1} (using a stock solution of $CuCl_2$ with concentration of 1000 μg Cu L^{-1}), corresponding to final concentration of 3.9 μM), and (3) artificial seawater with 500 μg Cu L^{-1} (corresponding to 7.8 μM $CuCl_2$). Cu exposure was introduced by means of a single dose addition at the beginning of the experiment in the middle of the photoperiod.

Photosynthetic efficiency of photosystem II was assessed daily using chlorophyll fluorescence technique. After 1, 3 and 7 days of Cu exposure, whole plant leaves were collected in three biological replicates from all treatments and frozen in liquid N_2 before being stored at −80°C for further analysis of tissue Cu, total reactive oxygen species (ROS) and RNA extraction for quantification of transcript levels.

2.2. Detection of chlorophyll fluorescence

The impact of Cu exposure on photosynthetic efficiency of *Z. muelleri* was assessed using a Diving-PAM (Walz GmbH, Effeltrich, Germany). Leaf clips were attached to the middle areas of the second leaves on a ramet to ensure that the measurements on different days were done on the same area of the same leaf from the same distance and angle. Each day, after the overnight recovery in the dark and before the LEDs were turned on, the maximum quantum yield of PSII, i.e. $F_v/F_m = (F_m-F_0) / F_m$ where F_0 and F_m are, respectively, minimum and maximum fluorescence of dark-adapted sample was measured. Six hours later in the middle of the photoperiod, the effective quantum yield of PSII, i.e. $\phi PSII = (F_{m'}- F) / F_{m'}$ where F and $F_{m'}$ are, respectively, minimum and maximum fluorescence of light-adapted sample, was determined. Non photochemical quenching (NPQ) was calculated as $(F_m-F_{m'}) / F_{m'}$.

2.3. Detection of Cu level

Approximately 200 mg of frozen plant materials was acid-digested with a mixture containing 2.5 mL of 65% HNO_3, 0.25 mL of concentrated HCl and 0.5 mL of H_2O_2 (all reagents used were high purity grade, Baseline, Choice Analytical Australia). Clear solution obtained after digestion and dilution with 2% nitric acid were analysed for Cu concentrations using a microwave plasma-atomic emission spectrometer (Agilent MP 4100, USA), equipped with a concentric nebulizer and a double pass cyclonic spray chamber (Karlsson et al., 2015).

2.4. Detection of ROS

Total reactive oxygen species (ROS) concentration in the leaves was quantified using 2′,7′–dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich) based on a protocol adapted from Buapet et al. (2017). Extraction was done by homogenizing 200 mg of leaf tissue in 1 mL of 10 mM Tris–HCl (pH 7.2). After centrifugation at 12,000×g for 20 min at 4 °C, the supernatant was collected and adjusted to a volume of 3 mL with 10 mM Tris–HCl. The reaction was initiated by adding 40 µL of 1 mM H_2DCFDA to the diluted samples. H_2DCFDA, when oxidized by ROS to dichlorofluorescein (DCF); this generates a fluorescence signal, which was subsequently measured after 10 min of dark incubation (excitation at 490 nm, emission at 530 nm, Gary 50 Bio spectrophotometer, Agilent Technologies).

2.5. Quantification of transcripts encoding antioxidant enzymes and Cu-binding proteins

2.5.1. Primer design

The present study conforms to the Minimum Information for Publication of Quantitative Real-Time PCR guidelines (Bustin et al., 2009). In-depth analysis of *Zostera muelleri* transcriptome (Davey et al., 2016), revealed transcripts encoding proteins with high similarities to the domains of: Cu/Zn-superoxide dismutase (Cu/Zn-SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX), metallothionein type 2 (MT2), metallothionein type 3 (MT3) and cytochrome c oxidase Cu chaperone (COX17) proteins. The functional domains of these sequences were used as a template to design sequence-specific primers for RT-qPCR using Primer3 0.4.0 (Koressaar and Remm, 2007) using default settings. The specificity of each selected primer pair was observed prior to RT-qPCR by PCR amplification as single band of the expected size resolved via agarose gel electrophoresis. Primers for reference genes used in this study included some designed previously such as those for genes encoding calmodulin (*calmo*), actin (*actin*) and polyadenylate RNA polymerase (*poly A*) (Schliep et al., 2015) and some designed specifically for this study: for the genes encoding glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), RuBisCo large subunit (*rbcl*), chloroplast ribosomal protein S2 (*rps 2*), and chloroplast ribosomal protein S11 (*rps 11*) (Table 2-1). The quality of the amplification products was checked for each selected primer pair (i) by PCR amplification as single bands at the expected size resolved via agarose gel electrophoresis and (ii) by a melting curve analysis during the RT-qPCR analysis. Two genes (*rbcl* and *rbcl*) were selected as quality control for qPCR plates (Appendix 1). We used Normfinder to measure stability value for each reference gene and select for the best combination of reference genes for the experimental conditions as previously described by Andersen et al. (2004). We also ran complementary analysis using a second software, GeNorm (Vandesompele et al., 2002), which led to similar results as for NormFinder. The best combination of two reference genes according to NormFinder: *rps 11* and *poly* A were then used to detect constitute expression and to normalize target gene expression profile in *Z. muelleri* under Cu exposure.

Table 2-1. Reference genes and target genes investigated in *Zostera muelleri* by using RT-qPCR. Accession numbers, primers sequences, amplicon length, melting temperature and RT-qPCR efficiency are indicated. *Cu/Zn-sod*: Cu/Zn-superoxide dismutase; *apx*: ascorbate peroxidase; *cat*: catalase; *gpx*: glutathione peroxidase; *mt2*: metallothionein type 2; *mt3*: metallothionein type 3; *cox17*: cytochrome c oxidase Cu chaperone; *calmo*: calmodulin; *actin*: actin; *rbcl*: RuBisCo large subunit; *gapdh*: glyceraldehyde-3-phosphate dehydrogenase; *rps2*: chloroplast ribosomal protein S2; *rps11*: chloroplast ribosomal protein S11; *poly A*, poly (A) RNA polymerase.

Gene abbreviation	Accession number	Forward primer	Reverse primer	Amplicon length (°C)	Melting Temperature (°C)	RT-qPCR efficiency (%)
Cu/Zn-sod	ZOSMA_5G01030	AACGGCTCGACCAATGATAG	GCTGGTGATCTGGGAAATGT	112	59.4	109
apx	ZOSMA_46G00350	ACGTGGTCCGAAACCTACAG	CCCAAGAACCTGATCCTGAA	102	60.2	105
cat	ZOSMA_228G00030	ACTTCAAGCAGCCAGGAGAA	CGGCCTGAGACCAGTAAGAG	105	60.5	99
gpx	ZOSMA_41G01010	TGTGGACTCACCAACTCCAA	CCTGCCCTCCAAACTGATTA	108	59.7	105
mt2	ZOSMA_154G00400	TGCGGAAGAAACATGGAGAT	CCTTCATCAACGGACTTGGT	115	59.5	106.2

mt3	ZOSMA_148G00060	CCTTCCATGACCTCAGCAGT	CTGTGCCGACAAGACCAAC	98	60.3	92
cox17	ZOSMA_273G00140	CGAAGTTGTTGCCACTGAAA	TATCTGGACAGGCACAGCAG	118	60.5	92
calmo	Zoma_B_i07192	ATCCATCCTGGTCTTTGTCG	CACTGTGATCCACTCGTTGG	197	60.1	96.2
actin	Zoma_ZMF02257	TAAGGTCGTTGCTCCTCCTG	ACTCTGCCTTTGCAATCCAC	104	60.4	102.4
rbcl	ZM194765	CAGGGGGTATTCATGTTTGG	ATCACGCCCTTCATTACCGA	172	59.8	105.5
gapdh	Zoma_C_c6252	TTGAGGGTTTGATGCCACA	GAATCCTGCAGCTCTTCCAC	101	60.5	93.7
rps2	ZM209552	GCAGGAGTTCATTTTGGCA	GTTTTCCTCCACTTGCTGCA	163	60.3	100
rps11	ZM209799	TGGTTTCTTGGTCCTCTGCA	GCATCTCTTCCTAGACCCGG	163	60.9	103.2
polyA	Zoma_C_c36619	GCTGGTCGTTCAAATTCCTC	ATGACCGCATTTAATCTGC	112	59.9	106.5

2.5.2. RNA extraction and synthesis of DNA

Total RNA was extracted from 80 mg of leaf tissue using the PureLink™ RNA Mini Kit (Life Technologies) according to the manufacturer's protocol. During the extraction, on-column DNase digestion was performed using PureLink™ DNase Set (Invitrogen). A spectrophotometer (NanoDrop 2000, NanoDrop Technologies) was used to analyze RNA quantity and purity. The cDNA synthesis was carried out using the iScript™ cDNA Synthesis Kit (Biorad) with a total of 500 ng of RNA as template for each sample.

2.5.3. Differential gene expression analysis

RT-qPCR assays were set up using an Eppendorf epMotion 5075 Robotics System (Eppendorf) in 384-well plate in technical triplicate with a total volume of 10 µL containing 4.8 µL SYBR Green PCR Master Mix (Applied Biosystems), 4 µL 1:15 diluted cDNA, 0.2 µL H_2O and a final concentration of 100 nM of each specific primers. Controls with no template and controls with no reverse transcription were included for each primer set to ensure that the PCR reactions were free of DNA contamination. Amplification was conducted with the following conditions: 10 min at 95 °C followed by 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 68 °C for 30 s. Real-time PCR efficiency for each gene and each treatment were determined from a cDNA dilution gradient of 243, 81, 27, 9 and 3 ng and a linear regression model previously described by Pfaffl (2001). The corresponding real-time PCR efficiencies were calculated according to the equation described by Radonić et al. (2004). All RT-qPCRs displayed efficiencies between 92% and 106% (r^2 of calibration curve: 0.98).

Data from RT-qPCR was analyzed using the Bio-rad CFX manager software. Gene expression was determined based on the cycle threshold set at 0.03 for all genes and normalized to the best combination of reference genes according to Normfinder (Andersen et al., 2004).

2.5.4. Statistical analyses

All the statistical tests were performed on Statistica Academic (StatSoft, Tulsa, USA). The effects of Cu exposure on F_v/F_m, ϕPSII and NPQ were tested using repeated-measures ANOVA (days after treatment as the within-group factor and Cu level as the categorical factor). Functional relationships between exposure duration and the chlorophyll fluorescence parameters were tested using an analysis of

covariance (ANOVA). The differences in tissue Cu and total ROS among the treatments were analyzed using two-way ANOVA. Gene regulations were determined from the difference between control and treated samples using one-Way ANOVA. Where significant factors' effects were detected, Fisher's least significant difference (LSD) test was used to compare the physiological and qPCR data among Cu levels and days after treatment. A p-value < 0.05 was considered to be significantly different. Cochran's test was used to test the assumption of homogeneity of variances before ANOVA was performed.

3. Results

3.1. Content of Cu in leaves

Cu content in controls remained similar throughout the experiment, ranging from 1.15 ± 0.19 to 1.66 ± 0.62 µg Cu mg^{-1} FW. Cu content in leaves of treated-plants increased in a time and concentartion-dependent manner (Figure 2-1,Table 2-2). The difference among treatments was observed on day 3, after which the level of Cu in leaves reached a plateau (Figure 2-1).

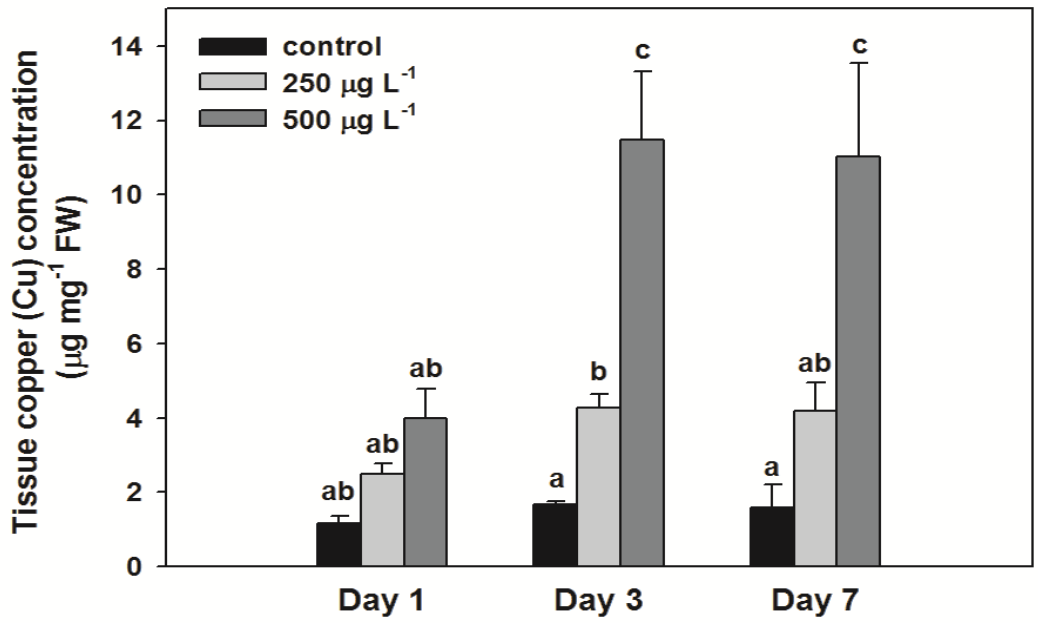

Figure 2-1. The accumulation of Cu in the leaves of *Zostera muelleri* exposed to control (black bars), 250 µg Cu L^{-1} (light grey bars) and 500 µg Cu L^{-1} (dark grey bars) on day 1, 3 and 7. Error bars show standard error, n = 3. Bars without shared letters are statistically different (Fisher's LSD post-hoc test, $p < 0.05$).

Table 2-2. Results of the repeated-measures ANOVA for effective quantum yield of PSII (ϕPSII), 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 *Zostera mulerii* after exposure to 0, 250 and 500 µg Cu L^{-1}. (Df: degrees of freedom, Sum Sq: sum of squares, Mean Sq: mean squares).

Source of Variation	Df	Sum Sq	Mean Sq	*F*-value	*p*-value
ϕPSII					
Treatment (Cu levels)	2	3.15744	128.504	70.066	< 0.001
Day after treatment	7	0.98531	30.007	6.258	< 0.05
Treatment x Day after treatment	14	0.59011	12.988	2.709	0.081
Fv/Fm					
Treatment (Cu levels)	2	1.04695	1.579	18.644	< 0.001
Day after Treatment	7	0.98872	0.141	15.389	< 0.001
Treatment x Day after treatment	14	0.69462	0.042	4.608	< 0.001
NPQ					
Treatment (Cu levels)	2	13.28569	6.643	13.587	<0.001
Day after Treatment	7	10.30256	1.472	5.359	<0.001
Treatment x Day after treatment	14	10.28406	0.735	2.675	<0.01
Cu content					
Treatment (Cu levels)	2	257.0074	128.504	33.748	<0.001
Day after Treatment	2	60.0131	30.007	7.881	<0.01
Treatment x Day after treatment	4	51.9539	12.989	3.411	<0.05
Total ROS					
Treatment (Cu levels)	2	32.1007	16.050	14.989	<0.001
Day after Treatment	2	56.3122	28.156	26.294	<0.001
Treatment x Day after treatment	4	37.0367	9.259	8.647	<0.001

3.2. Changes in photosynthetic parameters

Both Cu concentrations and time of exposure were found to affect the photosynthetic efficiency of *Z. muelleri* (Figure 2-2 and Table 2-2). The effective quantum yield of PSII (ϕPSII) of controls showed slight variations among days ranging from 0.70 to 0.74, but there was no statistical difference. Cu exposure resulted in a significant decrease in the ϕPSII and the maximum quantum yield of PSII (F_v/F_m). A more rapid decline in ϕPSII was observed in the 500 µg Cu L^{-1} than in 250 µg Cu L^{-1} treatment (ANCOVA, $p < 0.05$). ϕPSII in the 500 µg Cu L^{-1} treatment started to show a statistically significant reduction from controls on day 1 (Fisher's LSD post-hoc test, $p < 0.05$), whereas ϕPSII in 250 µg Cu L^{-1} started to show a statistically significant reduction from controls on day 2 (Fisher's LSD post-hoc test, $p < 0.05$). At the end of the experiment, the lowest ϕPSII was observed in 500 µg Cu L^{-1} (0.15 ± 0.11) followed by 250 µg Cu L^{-1} (0.42 ± 0.11) and controls (0.71 ± 0.03). Maximum quantum yield (F_v/F_m) exhibited similar trend to ϕPSII. F_v/F_m of controls remained constant throughout the experiment, ranging from 0.75 to 0.77. F_v/F_m exhibited a statistically significant decrease on day 3 in 500 µg Cu L^{-1} treatment and day 5 in 250 µg Cu L^{-1} (Fisher's LSD post-hoc test, $p < 0.05$). Similar to ϕPSII, a more rapid decline in F_v/F_m was observed in 500 µg Cu L^{-1} treatment than in 250 µg Cu L^{-1} (ANCOVA, $p < 0.05$). At the end of the experiment, the F_v/F_m in 500 µg Cu L^{-1} treatment was the lowest (0.22 ± 0.11), followed by the F_v/F_m measured in 250 µg Cu L^{-1} (0.58 ± 0.10) and controls (0.77 ± 0.02). NPQ of controls remained constant throughout the experiment. Cu enrichment promoted an increase in non-photochemical quenching (NPQ) on day 1 and 2 in both Cu levels (Fisher's LSD post-hoc test, $p < 0.05$). On day 3 and 4, NPQ in 250 µg Cu L^{-1} remained higher than controls (Fisher's LSD post-hoc test, $p < 0.05$), whereas NPQ in 500 µg Cu L^{-1} decreased to reach similar values to controls. On day 5 to 7, no difference between treatments was observed.

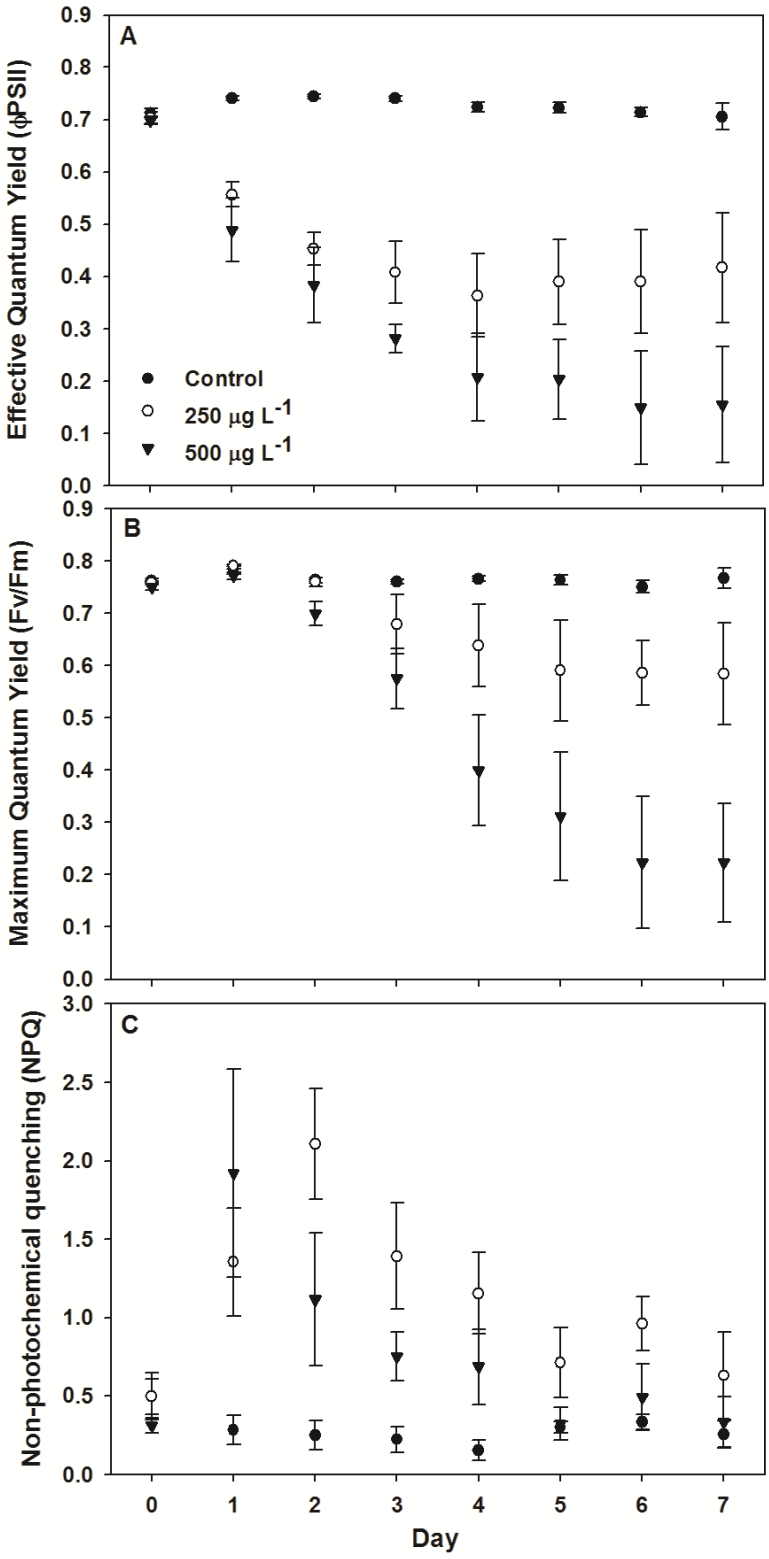

Figure 2-2. Time course of effective quantum yield (ϕPSII) (A) maximum quantum yield (Fv/Fm) (B) and non-photochemical quenching (NPQ) (C) of *Zostera muelleri* up to 7 days exposure to various Cu concentrations: control (closed circles), 250 µg L^{-1} (open circles) and 500 µg L^{-1} (triangles).

3.3. ROS accumulation

Total ROS in control plants remained constant throughout the time course of the experiment. Cu exposure increased ROS production in *Z. muelleri*. An increase in total ROS accumulation induced by Cu enrichment was found on day 7 (Figure 2-3). The leaves of *Z. muelleri* exposed to both 250 and 500 µg Cu L^{-1} showed a statistically significant increase in ROS compared to control on day 3 and day 7 (Fisher's LSD post-hoc test, $p < 0.05$). No difference was observed between the two Cu treatments on day 3 but higher ROS in 500 µg Cu L^{-1} compared to 250 µg Cu L^{-1} was detected on day 7 (Fisher's LSD post-hoc test, $p < 0.05$).

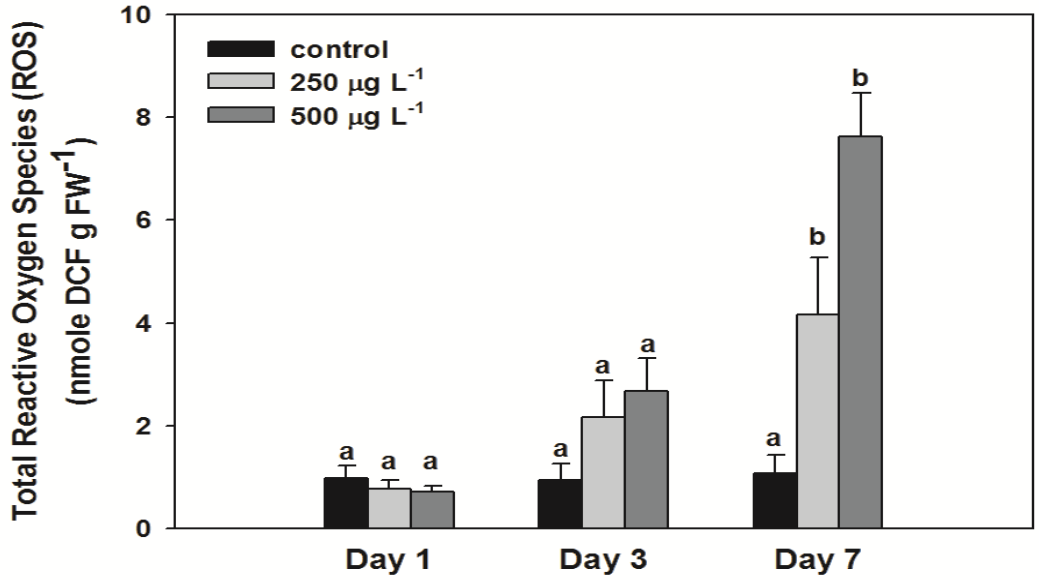

Figure 2-3. Total Reactive Oxygen Species (ROS) in the leaves of *Zostera muelleri* exposed to controls (black bars), 250 (light grey bars) and 500 µg Cu L^{-1} (dark grey bars) on day 1, 3 and 7. Error bars show standard error, n = 3. Bars without shared letters are statistically different (LSD test, $p < 0.05$).

3.4. Changes in gene expression

The level of transcripts encoding Cu/Zn-SOD, APX, CAT, GPX, MT2, MT3 and COX17 in *Z. muelleri* exhibited differences depending on the concentration of Cu and time of exposure (Figure 2-4 and Figure 2-5).

A concentration-dependent response was observed in the expression of *Cu/Zn-sod*, *apx*, *cat* and *gpx* (A to D). Overall, 250 µg Cu L^{-1} elicited a statistically significant

up-regulation in the expression of these genes, whereas 500 µg Cu L^{-1} induced varying response, but this was not statistically significant. An up-regulation of *Cu/Zn-sod*, *cat* and *gpx* in 250 µg Cu L^{-1} was highly responsive as it was detected after only 1 day of exposure. All the antioxidant genes (except for *apx* on day 1 and *cat* on day 3), were up-regulated throughout the time course of the experiment. Among target genes in the present study, *gpx* expression was significantly up-regulated (up to 6.0 fold) followed by *Cu/Zn-sod*, *apx* and *cat*.

Transcripts of genes encoding Cu-binding proteins *mt2, mt3* and *cox17* were not affected by 250 µg Cu L^{-1} but down-regulated following exposure to 500 µg Cu L^{-1} on day 3 and day 7, respectively (Figure 2-5 A to C, Fisher's LSD post-hoc test, $p < 0.05$).

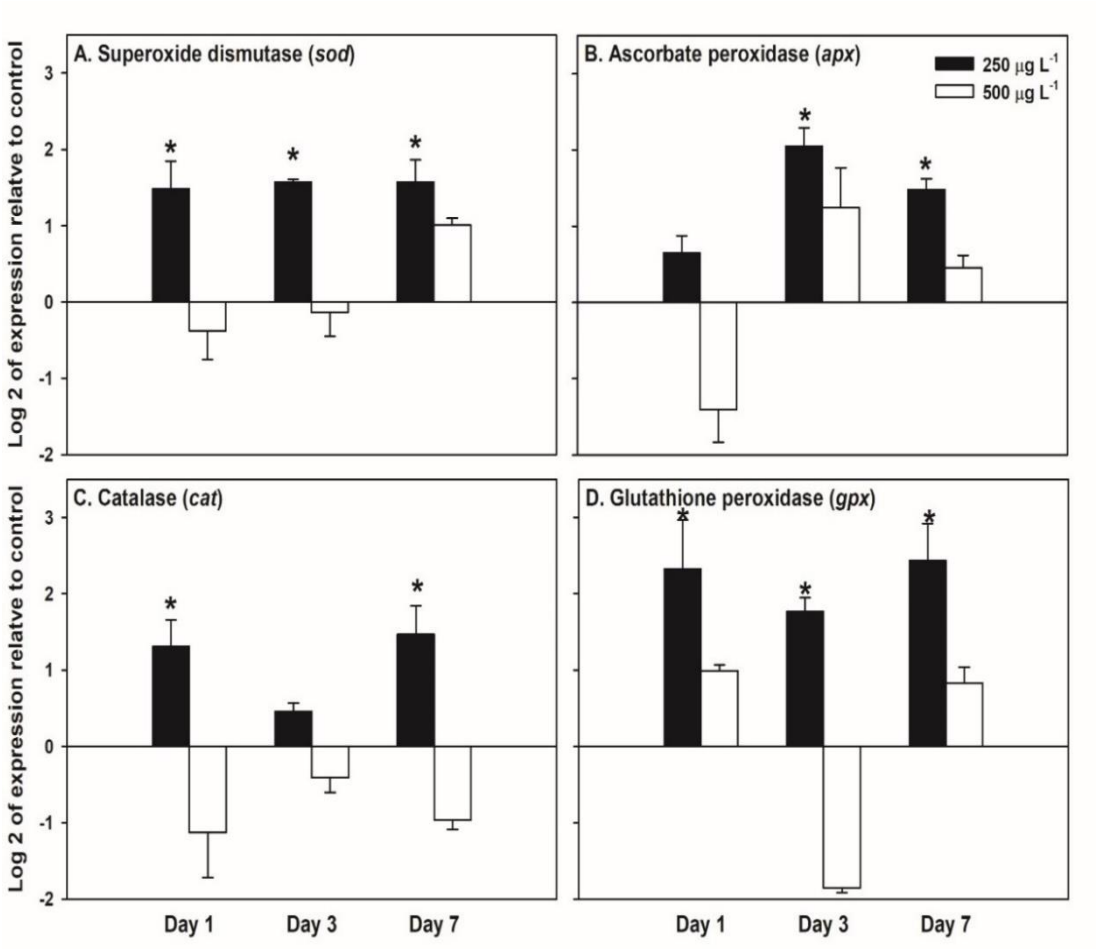

Figure 2-4. Quantitative PCR (qPCR) analyses for expression of *sod* (A), *apx* (B), *cat* (C) and *gpx* (D). Data were expressed relative to expression of control. Asterisk indicates significant difference from control (One-Way ANOVA, $p < 0.05$). Error bars show standard error, n = 3. For each replicate, 3 seagrass shoots were sampled.

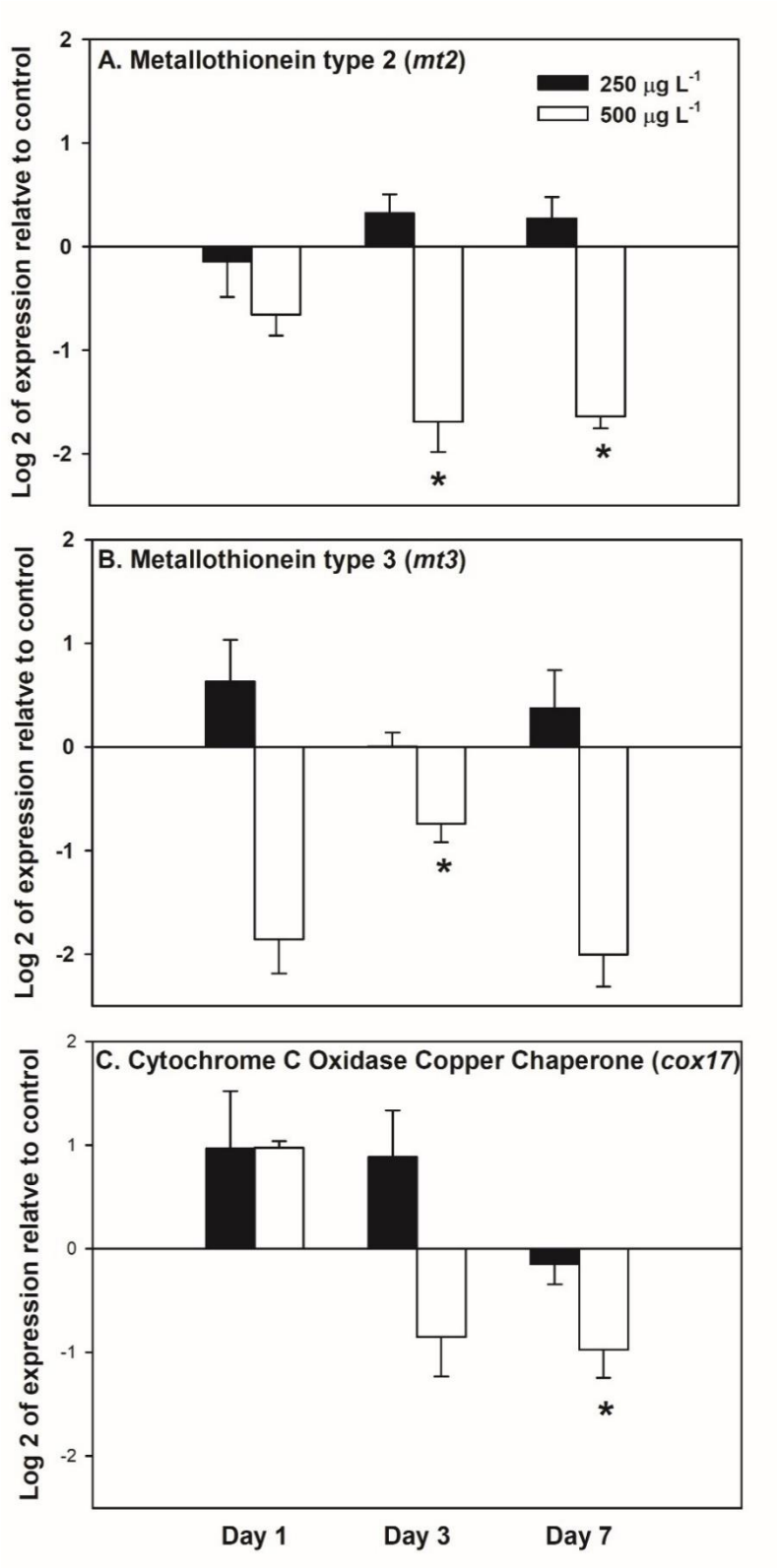

Figure 2-5. Quantitative PCR (qPCR) analyses for expression of *mt2* (A), *mt3* (B) and *cox17* (C). Data were expressed relative to expression of control. Asterisk indicates significant difference from control (One-Way ANOVA, $p < 0.05$). Error bars show standard error, n = 3. For each replicate, 3 seagrass shoots were sample.

4. Discussion

Cu content in leaf tissue of *Z. muelleri* observed in this study increased linearly as levels of Cu exposure increased, indicating that this seagrass can accumulate Cu from their surrounding environment. This is in line with observations for the same species (*Z. capricorni* syn. *Z. muelleri*) (Prange & Dennison 2000; Macinnis-Ng & Ralph 2004a; Howley, Morrison & West 2006; Birch, Cox & Besley 2018b) and other seagrass species such as *Z. marina* (Lyngby and Brix, 1982; Nielsen et al., 2017), *Z. japonica* (Lin et al., 2016), *Z. noltei* (Gamain et al., 2017), *H. ovalis*, *H. spinulosa*, *Halodule uninervis*, *C. serrulata* (Prange and Dennison, 2000), *C. nodasa* (Moustakas et al., 2017), *Thalassia testudinum* (Govers et al., 2014) and *P. oceanica* (El Zrelli et al., 2017). In these studies, content of Cu in leaves of the seagrasses investigated appeared to accumulate at higher Cu concentration. Since Cu is an essential micronutrients, it can be taken up via various transporters such as COPT and ZIP transporters protein families (Yruela 2009; Pilon 2011). Seagrasses are able to acquire nutrients from both the water and sediment through the cells of the root/rhizome complex and the surrounding water column through the leaf cells (Vonk, Christianen & Stapel 2008; Alexandre et al. 2011; Pernice et al. 2016). Since Cu was supplied in the water column in our experiment, it is, thus, assumed that Cu accumulation in the leaves was primarily a result of leaf uptake rather than translocation from below-ground parts. Higher concentrations of Cu in above-ground compared to below-ground tissue have been reported in various seagrass species (*Z. marina*, Lyngby and Brix, 1982; *Z. capricorni*, Howley et al., 2004; *Z. japonica*, Lin et al., 2016 and *P. oceanica*, El Zrelli et al., 2017). Leaf Cu content could, therefore, be used as an indicator of Cu contamination. However, time of exposure should be carefully considered as an increase in leaf Cu in enriched treatments was observed after 3 days of exposure. This is relatively slow compared to the kinetics of Cu bioaccumulation in the leaves of *C. nodosa* using CuO as the source of Cu (Moustakas et al., 2017). In this work, the bioaccumulation was rapid during the first few hours of exposure and reached a steady state between $10 - 27$ hours (Moustakas et al., 2017).

However, Cu exposure in our experiment was by means of a single dose addition, which might be the reason for a plateau observed in Cu bioaccumulation after 3 day. The results may be different with multiple-pulse exposure which provide constant level of Cu available for leaves for the duration of the experiment which could

be a future direction for this project to be tested. Cu exposure resulted in a marked reduction in photosynthetic efficiency (Figure 2-2). A rapid decrease in ϕPSII was observed after 1 day of Cu exposure followed by a decrease in F_v/F_m. ϕPSII and F_v/F_m dropped at a greater rate in 500 µg Cu L^{-1} than in 250 µg Cu L^{-1}, indicating that in addition to being fast, the response was also concentration-dependent. Photosystem II is the most sensitive site to Cu toxicity (Barón, Arellano & Gorgé 1995; Dewez et al. 2005). It has been reported that Cu inhibits water-splitting systems (Rijstenbil et al., 1994), as well as electron transports from photosystem II by interacting with quinone and pheophytin (Mohanty, Vass & Demeter 1989; Yruela et al. 1993; Yruela et al. 1996), resulting in a reduction in quantum efficiency of PSII. Nevertheless, a down-regulation of quantum efficiency in the light may be one of the photoprotective mechanisms which helps reducing absorbed light energy, minimizing ROS production and thus preventing photodamage (Osmond & Grace 1995; Kitao et al. 2000; Takahashi, Kopriva, et al. 2011; Burdett et al. 2014; Buapet 2017; Phandee & Buapet 2019). This is supported by a simultaneous increase in NPQ upon Cu addition. Elevated NPQ indicates excess light energy could be dissipated from light harvesting antenna (Demmig-Adams and Adams, 1992; Muller, 2001; Ort, 2001). Enhanced NPQ during trace metal stress has previously been reported in other plants (Maksymiec et al., 2007; Vernay et al., 2007). Nevertheless, a rise in NPQ was only observed during the first few days of exposure, after which decreasing NPQ was accompanied by a reduction in F_v/F_m. Photodamage (observed as F_v/F_m inhibition) may be partly due to a loss of the capacity of excess excitation energy dissipation. During the first few days, excess excitation energy could be efficiently dissipated, but this photoprotective mechanism did not work well after Cu started accumulating in the plant tissue. Impaired NPQ due to trace metal toxicity was reported in an aquatic plant, *Microsorium pteropus* exposed to mercury. Deng et al. (2014) shown that a high mercury exposure, results in transthylakoid ΔpH and the formation of zeaxanthin were diminished. This actual mechanism of toxicity remains to be investigated in Cu using chlorophyll fluorescence coupled with metabolites profiling.

Cu exposure induces production of reactive oxygen species (ROS) directly via Haber-Weiss and Fenton reactions (Halliwell, 2006; Palmer and Guerinot, 2009). It seems that ROS level in *Z. muelleri* was effectively scavenged as an increase in ROS was only observed on day 7 in our experiment. ROS is regulated by maintaining the

redox balance of the electron transport chain, thus suppressing its production and by ROS-scavenging systems (Moustakas et al., 2017). Excess energy dissipation by NPQ may help to limit ROS generation in the chloroplast, but in the present study, NPQ appeared to be impaired as a result of a longer exposure to Cu. Our results suggest a significant role of antioxidant enzymes in ROS regulation in *Z. muelleri*. Under 250 µg Cu L^{-1}, an up-regulation of *Cu/Zn-sod, sod*, *cat* and *gpx* was observed on day 1, and genes encoding all the antioxidant enzymes (*Cu/Zn-sod, sod*, *apx, cat* and *gpx*) remained up-regulated throughout the experiment. These enzymes play a dominant role in scavenging ROS in different plant organelles (Mittler et al. 2004; Das 2018). However, such up-regulation of these antioxidant genes failed to prevent photodamage and an eventual increase in ROS level on day 7. Our results suggested that Cu is highly toxic and its mode of action involves induction of oxidative stress. It has been reported that Cu can also accumulate in mitochondria, another important site of ROS production in a cell, and disturb its metabolisms (Tan et al. 2010; Dai et al. 2018). An over-generation of ROS observed on day 7 may be associated with dysfunction of mitochondria (Dai et al. 2018) or an increase in the activity of mitochondrial electron transport chain due to high energy demand under Cu stress (Sako et al. 2016). ROS generated as a result of Cu exposure may have led to a breakdown of antioxidant enzymes by protein oxidation or by enhanced proteolytic activity, leading to a failure to eliminate ROS (Salo et al. 1990; Pena et al. 2012). Also, some trace metals including Cu are able to non-specifically bind to the sulfhydryl groups of proteins and consequently alter the structure and inhibit the activity of enzymes (Nagalakshmi and Prasad, 2001; Yruela, 2009). Such post-translational modification of antioxidant enzymes can lead to discrepancies between gene expressions and enzymatic activities observed under trace metal excess in various organisms (Regoli, Benedetti & Giuliani 2011; Regoli & Giuliani 2014; Hattab et al. 2016). This may partially explain an increase in ROS observed on day 7, despite high expression levels of antioxidant genes. Nevertheless, no change in the expressions of antoxidant genes was observed in 500 µg Cu L^{-1} treatment. The lack of response suggests that 500 µg Cu L^{-1} was too high. Such high Cu concentrations may lead to severe cellular damage, impairing metabolic processes involved in transcription. Strong toxicity of 500 µg Cu L^{-1} was also demonstrated by a greater accumulation of ROS on day 7 compared to 250 µg Cu L^{-1}, indicating higher level of oxidative stress. This may be a result of a collapse of antioxidant defence systems as observed at the transcriptional level.

Photosynthetic parameters and antioxidant genes expression were very responsive to Cu addition; a modulation in these parameters was observed before a difference in leaf Cu content between treatments was detected. Excess trace metal in the surrounding environment may trigger signalling cascades that activate early physiological responses to maintain cellular redox homeostasis. It has been shown that trace metals, as well as ROS, generated from exposure to trace metals including Cu can induce MAPK, oxylipin, Ca^{2+} and phytohormones signalling pathways (Jonak, Nakagami & Hirt 2004; Opdenakker et al. 2012; Jalmi et al. 2018). The detailed mechanisms involved in trace metal sensing and signalling in submerged plants like seagrasses may be different from what has been reported in land plants and remain to be elucidated.

Exposure to Cu at any concentration failed to induce the expressions of *mt2, mt3* and *cox17* (Figure 2-5). The high binding capacity of metallothionein (MTs) to Cu suggests that it plays a significant role in detoxification and homeostasis of trace metal in the cytosol (Cobbett and Goldsbrough, 2002). Previous studies showed different effects of Cu on *mt* expression levels, which could be up-regulated (Zhou & Goldsbrough 1994; Giordani et al. 2000; Guo, Meetam & Goldsbrough 2008; Contreras-Porcia et al. 2011; Laporte et al. 2016), down-regulated (Castiglione et al., 2007; Chang et al., 2004) or unaffected (Kohler et al. 2004; Gonzalez-Mendoza, Moreno & Zapata-Perez 2007; Usha, Venkataraman & Parida 2009), depending on plant species, concentration and time of exposure and MT type. Our results suggest that *mt2* and *mt3* may not play a major role in detoxification of Cu in *Z. muelleri*. There are four subfamilies of MTs (Cobbett and Goldsbrough, 2002) as well as various *mt* genes identified in plants (such as six *mt* genes in *Arabidopsis*, Guo et al., 2008) and they showed varying responses to trace metal stress (Zhou & Goldsbrough 1994; Cobbett & Goldsbrough 2002; Kohler et al. 2004; Laporte et al. 2016). Thus, it is possible that Cu excess may be chelated and detoxified by other MTs not investigated in the present study. Additionally, other sequestration mechanisms, such as phytochelatins and ABC transporters, may play a more dominant role in detoxification and compartmentalization of trace metals in seagrasses (Gonzalez-Mendoza, Moreno & Zapata-Perez 2007; Guo, Meetam & Goldsbrough 2008; Hassinen et al. 2011; Sharma, Dietz & Mimura 2016). A down-regulation of *mt2* and *mt3* in 500 μg Cu L^{-1} suggests that this Cu level may have caused excessive cell damage, leading to

disturbed transcription processes and reduced mRNA levels. It has been shown in previous studies that *mt* expression in plants is stimulated in a concentration-dependent manner at a lower range of Cu, but is inhibited by higher levels of Cu (Choi et al., 1996; Huang and Wang, 2010). A study by Giordani et al. (2000) shows an accumulation of *mt* transcript in the seagrass, *P. oceanica* (*Pomt2b*) in response to Cu exposure at a concentration of 10 µM (equal to 635 µg Cu L^{-1}). This suggests that *Z. muelleri* may be more sensitive to Cu than *P. oceanica*. Some authors have suggested that mitochondrial cytochrome *c* oxidase (*cox*) and its metallochaperones are involved in Cu homeostasis and signal transduction (Garcia et al., 2014). The lack of response of *cox17* and its down-regulation on day 7 in 500 µg Cu L^{-1} indicates that *cox17* may not contribute in Cu homeostasis under excessive Cu in *Z. muelleri*. The impairment of mitochondria metabolism is further supported by a recent study which suggests that Cu excess may impair mitochondrial metabolism as demonstrated by a down-regulation of subunit 1 of cytochrome c oxidase (*cox1*) in *Z. noltei* (Gamain et al. 2018).

5. Conclusion

Rapid loss of seagrass habitat highlights the urgency of understanding the mechanisms of stresses that threaten seagrass health, as well as developing preventive measures for further seagrass decline such as an effective suite of biomarkers. Based on our physiological and molecular investigation, *Z. muelleri* is highly susceptible to Cu and exhibiting dramatic responses at sub-lethal concentrations (summarized by the conceptual illustration shown in Figure 2-6). The mechanisms of Cu toxicity involve induction of photoinhibition and oxidative stress, whereas defence mechanisms of *Z. muelleri* adjust excess light energy dissipation from light harvesting antenna and activate antioxidant genes. Disruption of redox homeostasis under Cu stress could render the seagrass more sensitive to other stresses which tend to occur simultaneously in the natural settings. Further investigations into combined stresses are needed to determine how Cu may impact *Z. muelleri* populations in the field and differential vulnerability among populations. Although further studies using non-targeted approaches (e.g. whole transcriptome and proteome sequencing), are critically needed to identify other candidate genes which are specific to trace metal contamination, we suggest that the photosynthetic efficiency and antioxidant enzymes genes expression should be integrated as a biomarker suite for the assessment of Cu exposure in *Z.*

muelleri. Due to their responsiveness, they can provide early warnings which allow immediate remedial measures to take place.

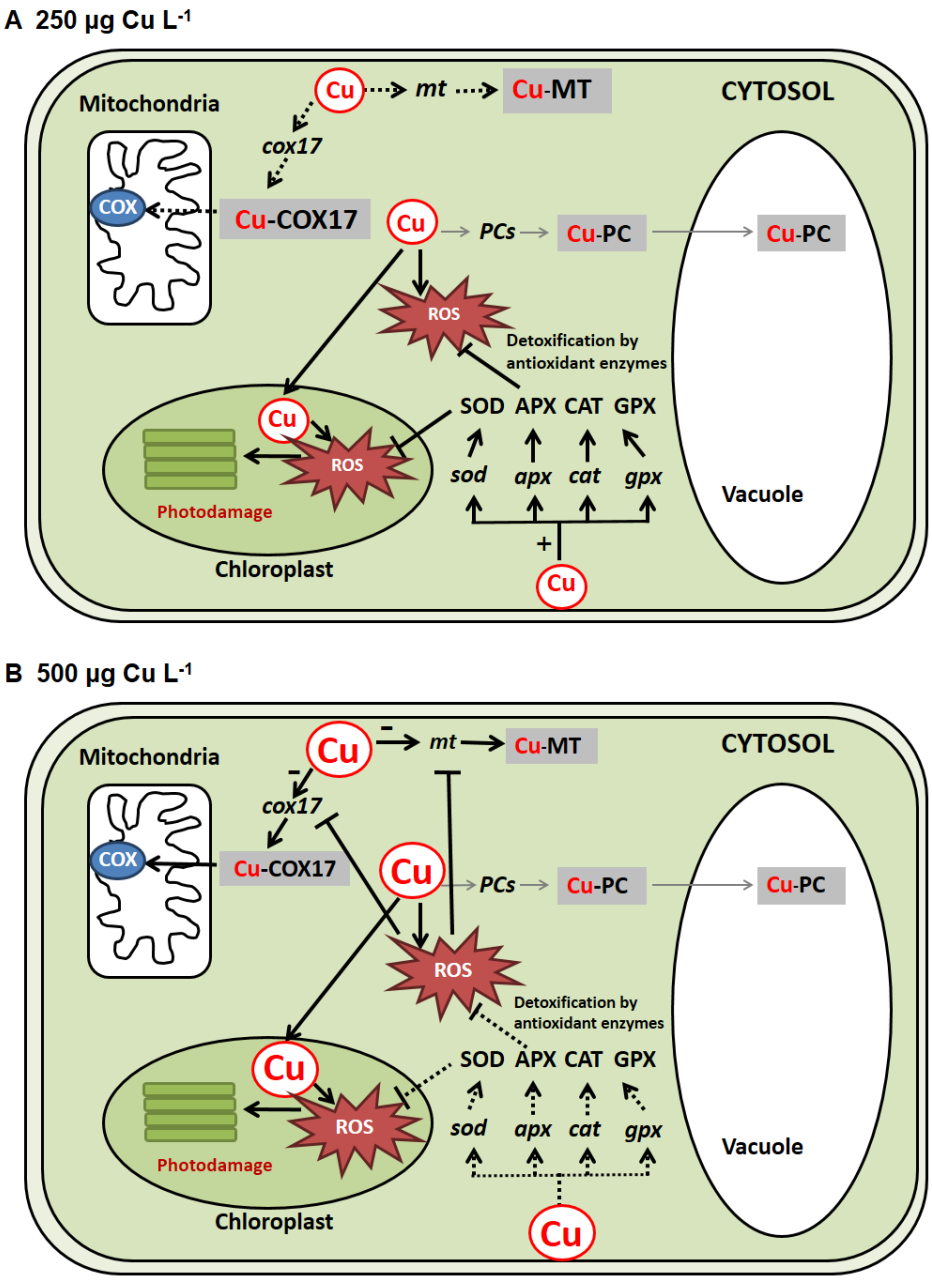

Figure 2-6. Conceptual illustration showing differential expression of genes and physiological responses in plants exposed to 250 µg Cu L^{-1} (A) and 500 µg Cu L^{-1} (B). Solid lines represent gene expressions and physiological responses observed in this study; gene up-regulation is depicted by + whereas gene down-regulation is depicted by - . Dotted lines indicate no change. Grey lines show related pathway not investigated in this study. *sod* = superoxide dismutase; *apx* = ascorbate peroxidase;

cat = catalase; *gpx* = glutathione peroxidase; *mt* = metallothionein; *cox17* = cytochrome c oxidase Cu chaperone; *PCs* = phytochelatin synthase; PC = phytochelatin; ROS = reactive oxygen species.

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CHAPTER 3

Transcriptome profiling analysis of the seagrass, *Zostera muelleri* under Cu stress.

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Abstract

Copper (Cu) in an essential trace metal but it can also contaminate coastal waters at high concentrations mainly from agricultural run-off and mining activities which are detrimental to marine organisms including seagrasses. The molecular mechanisms driving Cu toxicity in seagrasses are not clearly understood yet. Here we investigate the molecular responses of the Australian seagrass, *Z. muelleri* at the whole transcriptomic level after 7 days of exposure to 250 μg Cu L^{-1} and 500 μg Cu L^{-1}. The leaf-specific whole transcriptome results showed a concentration-dependent response in photosynthesis, carbon fixation, glycolysis, enzymatic defense mechanism and chemical defense mechanism.

This study provided new insights into the responses of seagrasses to trace metal stress and reported possible candidate genes which can be considered as biomarkers to improve conservation and management of seagrass meadows.

1. Introduction

Globally, coastal habitats are increasingly affected by a wide range of anthropogenic discharges which often contain high levels of trace metals (Cambridge & McComb 1984; Waycott et al. 2009; Leng et al. 2015). Once introduced to the marine environment, they are taken up by seagrasses via roots and shoots (Howley et al. 2006). Consequently, as a food source for many marine organisms, seagrasses represent a trophic pathway for the distribution of trace metals into the marine food chain (Ward 1987; Kalay, Ay & Canli 1999; Campanella et al. 2001; McGeer et al. 2004; Navratil & Minarik 2005). Trace metals are not only detrimental to the health of fishes, dugongs, turtles and marine invertebrates that feed on contaminated seagrass tissues, but also to human health as the trace metals subsequently find their way into our foods through contaminated seafood (Basha & Rani 2003; Canli & Atli 2003; Wang et al. 2005).

In particular, agricultural run-off and acid mine drainage often contain high concentrations of Cu (Tokar et al. 2013). Under normal conditions, Cu is vital for the function of many key enzymes and proteins including superoxide dismutase and plastocyanin (Katoh 1977; Barón, Arellano & Gorgé 1995; World Health Organization 1996; Kaufman Katz et al. 2003; Nagajyoti, Lee & Sreekanth 2010; World Health Organization 2011). However, high concentrations of Cu in the environment can cause deleterious effects to living organisms including seagrasses at

both physiological and molecular levels (Barón, Arellano & Gorgé 1995; Gupta & Gupta 1998; Theophanides & Anastassopoulou 2002; Yruela 2005; Stern 2010). More specifically, the alteration of the function of transporters and ion channels as a result of excess level of Cu can cause intra-cellular redox imbalances (Cambrollé et al. 2013; Tiecher et al. 2017) as well as cellular damage via the over-production of reactive oxygen species (Girotto et al. 2013; Tiecher et al. 2017).

Zostera muelleri is a fast growing species of seagrasses in the family of *Zosteraceae* found predominantly in coastal regions of Eastern and Southern Australia (den Hartog 1970; Kenworthy et al. 2006; Wissler et al. 2011; Davey 2017). The destructive effects of hyper-accumulation of Cu in the family of *Zosteraceae* have been reported previously as irreversible suppression in photosynthetic efficiency (Prange & Dennison 2000; Macinnis-Ng & Ralph 2002, 2004b, Buapet et al. 2019) and over production of reactive oxygen species (ROS) (Greco et al. 2019; Buapet et al. 2019). In higher plants, regulatory scavenging mechanisms for the detoxification of Cu include chelation (Sancenón et al. 2003; Yruela 2009), alteration to less toxic ionic form (Gill & Tuteja 2010; Thounaojam et al. 2012) and sequestration into vacuoles (Himelblau & Amasino 2000). However, less in known about Cu-related toxicity responses in seagrasses.

Transcriptomic profiling can be useful to better understand the toxicology response of seagrasses to a range of environmental stress factors. Recent reports of complete genomes of *Z. muelleri* and *Z. marina* (Lee et al. 2016; Olsen et al. 2016) have opened new avenues to deepen our understanding about the molecular basis of stress responses in the family of *Zosteraceae* (Franssen et al. 2011 and 2014; Kong et al. 2014; Kumar, Padula, et al. 2016; Lin et al. 2018; Martin-Guirao et al. 2017; Pernice et al. 2015; Pernice et al. 2016; Ruocco et al. 2017; Schliep et al. 2015). However, with few exceptions (Davey et al. 2017; Procaccini et al. 2017; Lin et al. 2018; Ruocco et al. 2017), most of the differential gene expression studies so far are limited to few targeted genes and do not investigate the effect of certain stress factors at the whole transcriptome level. In addition, none of these studies have investigated the molecular responses of the family of *Zosteraceae* to Cu stress.

As previously mentioned in Chapter 1 (page 12), the family of *Zosteraceae*, is sensitive to high level of Cu concentration within the range of $0.1 - 10$ mg L^{-1} (Clijsters & Assche 1985; Macinnis et al. 2002; Prange & Dennison 2000; Ralph & Burchett

1998b). Additionally, the physiological response of *Z. muelleri* under 250 µg Cu L^{-1} and 500 µg Cu L^{-1} (corresponding to 3.9 and 7.8 µM, respectively) has been recently studied after 1, 3 and 7 days in chapter 2 (page 60) showing a concentration and time-dependent decline in effective quantum yield (ϕPSII) and maximum quatum yield (F_v/F_m) parameters. Additionally, in the same chapter, a RT-PCR investigation illustrated an elevation in ROS production, as well as up-regulation of the transcript expression of antioxidant enzymes including glutathione peroxidase (*gpx)*, catalase (*cat* – only for 250 µg Cu L^{-1}), superoxide dismutase (*Cu/Zn sod*) and ascorbate peroxidase (*apx*) after day 7.

Therefore, in this study we continued our investigation using whole transcriptomic analysis to further investigate how transcriptome of *Z. muelleri* altered under 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days of Cu exposure and identify which genes were specifically expressed in response to Cu stress.

2. Materials and Methods

2.1. Sample collection and aquaria setup

Samples were collected at Pittwater, New South Wales, Australia (33°38′45.6′′S. 151°17′12.8′′E) in July 2016 at the approximate depth of 1 meter. Whole vertical plants of *Z. muelleri* were collected and transferred to the aquarium facility at the University of Technology Sydney (UTS) within 2 – 3 hours of collection and in dark containers to avoid additional stress as previously described by Davey et al. (2017).

Aquaria were prepared according to Chapter 2 (page 51). Briefly, six aquaria (two tank replicates for control and two for each treatments) were established with conditions mimicking the natural environment at the sampling time, i.e. salinity of 30 psu and temperature of 21°C and diel cycle of 12 hour light: 12 hour dark with maximum light intensity of 200 µmol photons m^{-2} s^{-1} at midday. One LED aquarium light (Cidley 250W), one submerged pump (Elite mini, Hagen, Canada) and one air stone were also equipped for each tank. The sediment for planting seagrasses was as a mixture of 50% washed sand and 50% natural sediment (4 – 5 cm for each tank). Individual plants (30 – 40) were rinsed with artificial seawater (30 psu) to remove epiphytes and transplanted randomly into each aquaria.

2.2. Experimental design

Plants were kept in 6 allocated aquaria to acclimatise to the closed-system conditions for 14 days with daily monitoring of photosynthetic efficiency using Diving-PAM (Walz GmbH, Effeltrich, Germany) until stable effective quantum yield (ϕPSII) was measured (data not shown) as explained in Chapter 2 (page 52). Aftrewards, a stock solution of $CuCl_2$ (concentration of 1000 µg Cu L^{-1}) was prepared to make the final concentration of 250 µg Cu L^{-1} and 500 µg Cu L^{-1} and added to treatment tanks as a single dose at midday on day 0. Leaves of three biological replicates were harvested randomly from allocated tanks for each treatments on day 7 for RNA extraction. Collected leaves were rinsed with saline water, tap dried and were frozen in liquid N_2 prior for storage at – 80 °C for further analysis.

2.3. RNA extraction

Frozen leaf tissue (80 – 100 mg) from each treatments (control, 250 and 500 µg Cu L^{-1}) were ground into a fine powder using a pre-chilled pestle and mortar. RNA extraction was performed using an Ambion PureLink RNA Mini kit (Fisher Scientific) according to the manufacturer`s instructions. On-column DNA digestion was performed during RNA extraction using Ambion PureLink DNase set (Thermo Fisher Scientific).

2.4. Library preparation and RNA sequencing

Nine RNA samples (three for control, three for each treatments) were sent to the Ramacciotti Center for Genomics (University of New South Wales, Australia) for quality control and sequencing. Quality and quantity measurement were conducted using a 2100 Bioanalyzer (Agilent Technology) with quality cutoff for RNA Integrity (RIN) numbers of > 6. Library preparation was performed using TruSeq mRNA standard total library preparation kit (Illumina) for sequencing of 380 million base-pair reads (42 million pair-end reads per sample) using a HiSeq2500 system from Illumina.

2.5. Genome-guided transcriptome assembly and annotation

The quality of raw reads were checked using FastQC software (version 0.11.05) (Andrews 2017; Davey et al. 2017). Adaptors and low quality reads were trimmed using Trimmomatic (version 0.2.35) with the following settings: ILLUMINACLIP: TruSeq3-PE2.fa: 2:30:10; LEADING:14; TRAILING:14;

SLIDING-WINDOW: 4:10; MINLEN: 90 (Bolger, Lohse & Usadel 2014; Davey et al. 2017). Trimmed reads were aligned to the exons of reference genome using the STAR RNA aligner (version 2.5.2b) (Dobin et al. 2013). Functional annotations of transcripts were taken from the *Z. muelleri* genome annotation file (Lee et al. 2016). Read counts were created using HTSeq (version 0.6.1) (Love, Anders & Huber 2014) and the exon counts were imported into the R package DESeq2 (version 3.7) for differential gene expression analysis (Anders, Pyl & Huber 2015; Love et al. 2015). Conditions of 250 and 500 µg Cu L^{-1} were compared to the control and genes with a p-value of < 0.05 were reported.

The genome of *Z. muelleri* is not completely annotated yet. Therefore, the FASTA file of significantly expressed genes in both 500 µg Cu L^{-1} and 250 µg Cu L^{-1} were submitted in Blast2Go (version 5.0.5) for gene identification based on the best hit in NCBI database search (cutoff e-value of 1×10^{-3}) as well as for sister species, *Z. marina*. GO distribution of expressed genes for both Cu treatments were exported for most induced biological process, molecular function and cellular process using Blast2Go data analysis.

Lastly, the functional classification of correspondent proteins were reported using BlastKOALA (version 2.1- https://www.kegg.jp/blastkoala/).

3. Results

3.1. Transcriptome assembly and functional annotation

Extracted RNA from control and treated samples generated an average of 22 million Illumina pair reads per sample. The quality check of raw reads (using FastQC package) gave an average quality score of 30 (1:1000 probability of incorrect base), which was sufficient for the further quantitative analysis of the transcriptome. After trimming low quality reads and adaptors using Trimmomatic (Love, Anders & Huber 2014; Davey 2017), an average of 44 million reads (97.8%) per sample were used for downstream analysis. HTSeq (Love, Anders & Huber 2014) analysis revealed an average of 17 million reads (42%) that mapped back to the reference genome unambiguously and were used for differential expression analysis with DESeq2 (Love, Anders & Huber 2014). DESeq2 results revealed significant changes in the transcriptome expression of both 500 µg Cu L^{-1} and 250 µg Cu L^{-1} samples in comparison to the control (Figure 3-1).

Among the total of 651 expressed exons in 250 µg Cu L^{-1} and 500 µg Cu L^{-1}, 141 exons (84 genes) were in common to both treatments. Additionally, 22 exons were not identified in BLAST searches for both treatments. For the remaining exons, a total of 247 genes were up-regulated and 112 genes were down-regulated in our Cu treatments (Table 3-1). The list of significantly expressed genes in both 500 µg Cu L^{-1} and 250 µg Cu L^{-1} with their correspondent GO, KEGG and InterPro identifications can be found in Supplementary 1-A and 1-B.The list of up and down-regulated genes in each Cu treatments with their correspondent log2 fold change can be found in Supplementary 2 and 3.

Table 3-1. Total number of expressed genes under 500 µg Cu L^{-1} and 250 µg Cu L^{-1}.

	Up-regulated (genes)	**Down-regulated (genes)**
250 µg Cu L^{-1}	66	41
Uncharacterised	15	4
500 µg Cu L^{-1}	208	90
Uncharacterised	25	1
Total	**314**	**136**
Common	**67**	**24**
Total (unique)	**247**	**112**

The list of expressed genes in both Cu treatments were also investigated for GO analysis (Figure 3-2 to 3-4). The most highly induced biological processes were metabolic and cellular processes. At the molecular function level, catalytic activity

and binding were the most affected. The most affected cellular component were membrane and cell.

Lastly, the functional classification of correspondent proteins were investigated for both treatments using BlastKOALA (Figure 3-5). Energy metabolism followed by carbohydrate metabolism were the most affected pathways.

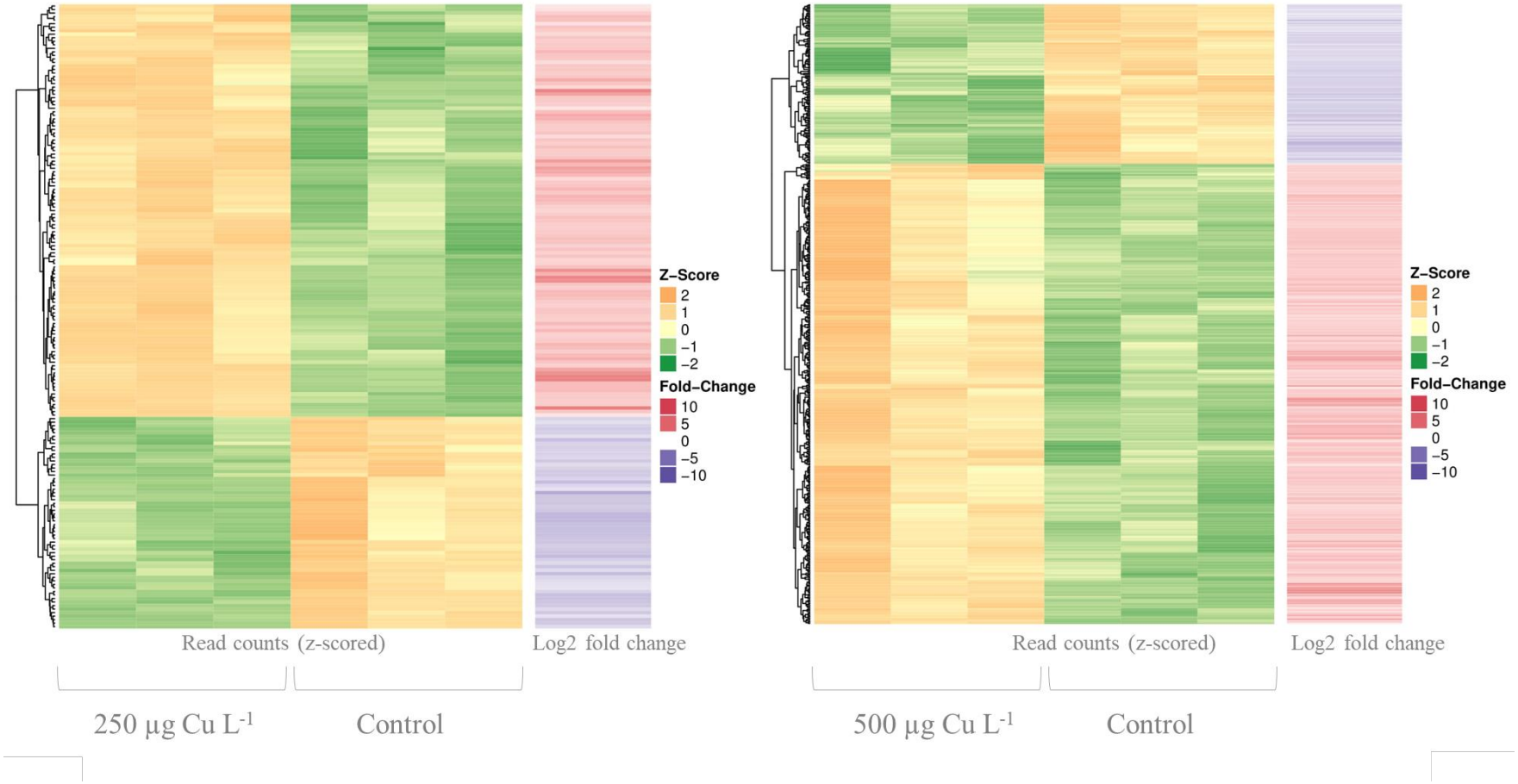

Figure 3-1. Heatmaps of the log 2 fold change and z-score of normalized read counts for significantly differentially expressed genes in response to 250 µg Cu L^{-1} (left) and 500 µg Cu L^{-1} (right) after 7 days.

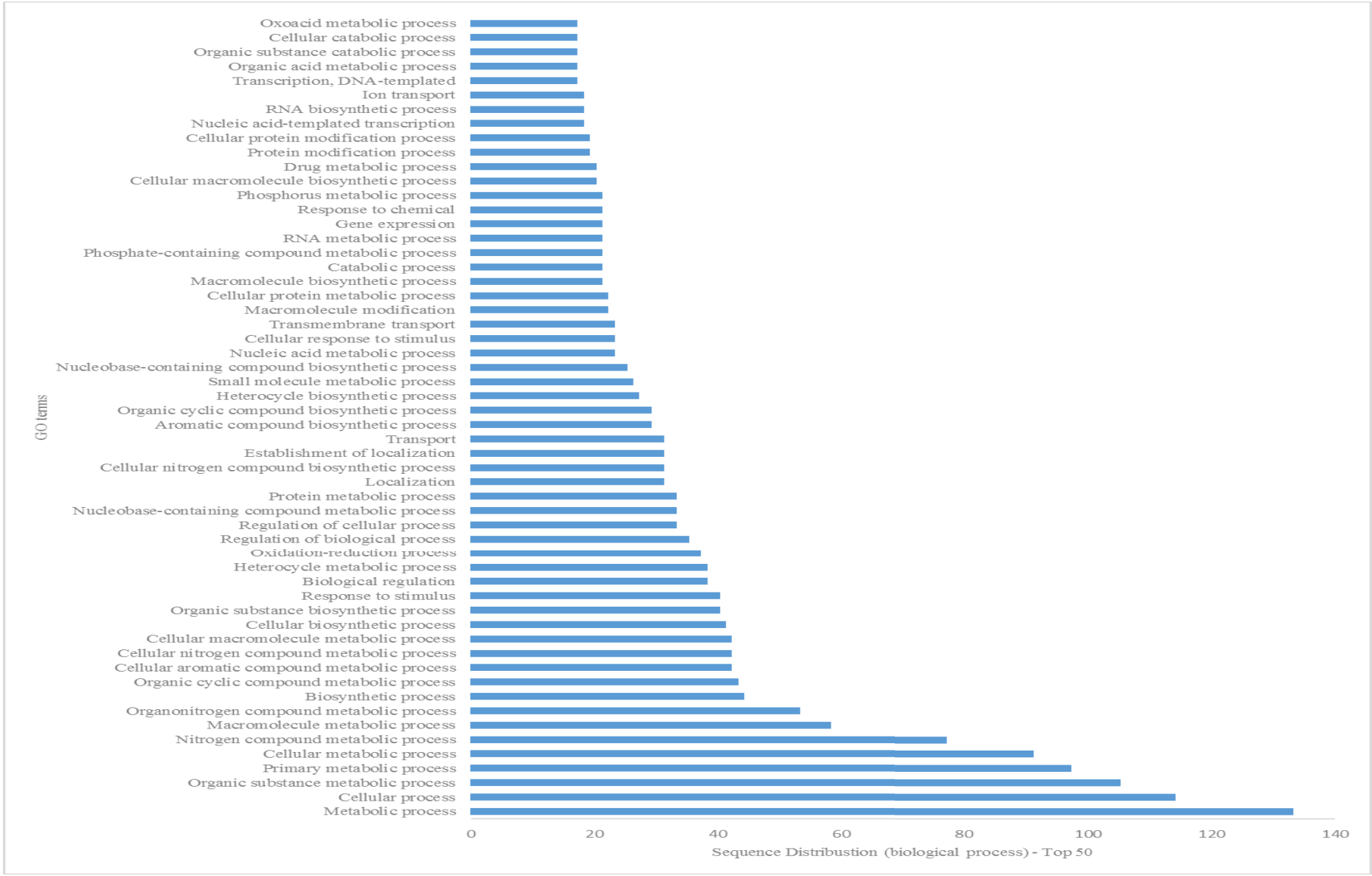
Oxoacid metabolic process
Cellular catabolic process
Organic substance catabolic process
Organic acid metabolic process
Transcription, DNA-templated
Ion transport
RNA biosynthetic process
Nucleic acid-templated transcription
Cellular protein modification process
Protein modification process
Drug metabolic process
Cellular macromolecule biosynthetic process
Phosphorus metabolic process
Response to chemical
Gene expression
RNA metabolic process
Phosphate-containing compound metabolic process
Catabolic process
Macromolecule biosynthetic process
Cellular protein metabolic process
Macromolecule modification
Transmembrane transport
Cellular response to stimulus
Nucleic acid metabolic process
Nucleobase-containing compound biosynthetic process
Small molecule metabolic process
Heterocycle biosynthetic process
Organic cyclic compound biosynthetic process
Aromatic compound biosynthetic process
Transport
Establishment of localization
Cellular nitrogen compound biosynthetic process
Localization
Protein metabolic process
Nucleobase-containing compound metabolic process
Regulation of cellular process
Regulation of biological process
Oxidation-reduction process
Heterocycle metabolic process
Biological regulation
Response to stimulus
Organic substance biosynthetic process
Cellular biosynthetic process
Cellular macromolecule metabolic process
Cellular nitrogen compound metabolic process
Cellular aromatic compound metabolic process
Organic cyclic compound metabolic process
Biosynthetic process
Organonitrogen compound metabolic process
Macromolecule metabolic process
Nitrogen compound metabolic process
Cellular metabolic process
Primary metabolic process
Organic substance metabolic process
Cellular process
Metabolic process
GO terms
0
20
40
60
80
100
120
140
Sequence Distribustion (biological process) - Top 50

Figure 3-2. Sequence distribution (biological process) of top 50 genes expressed in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

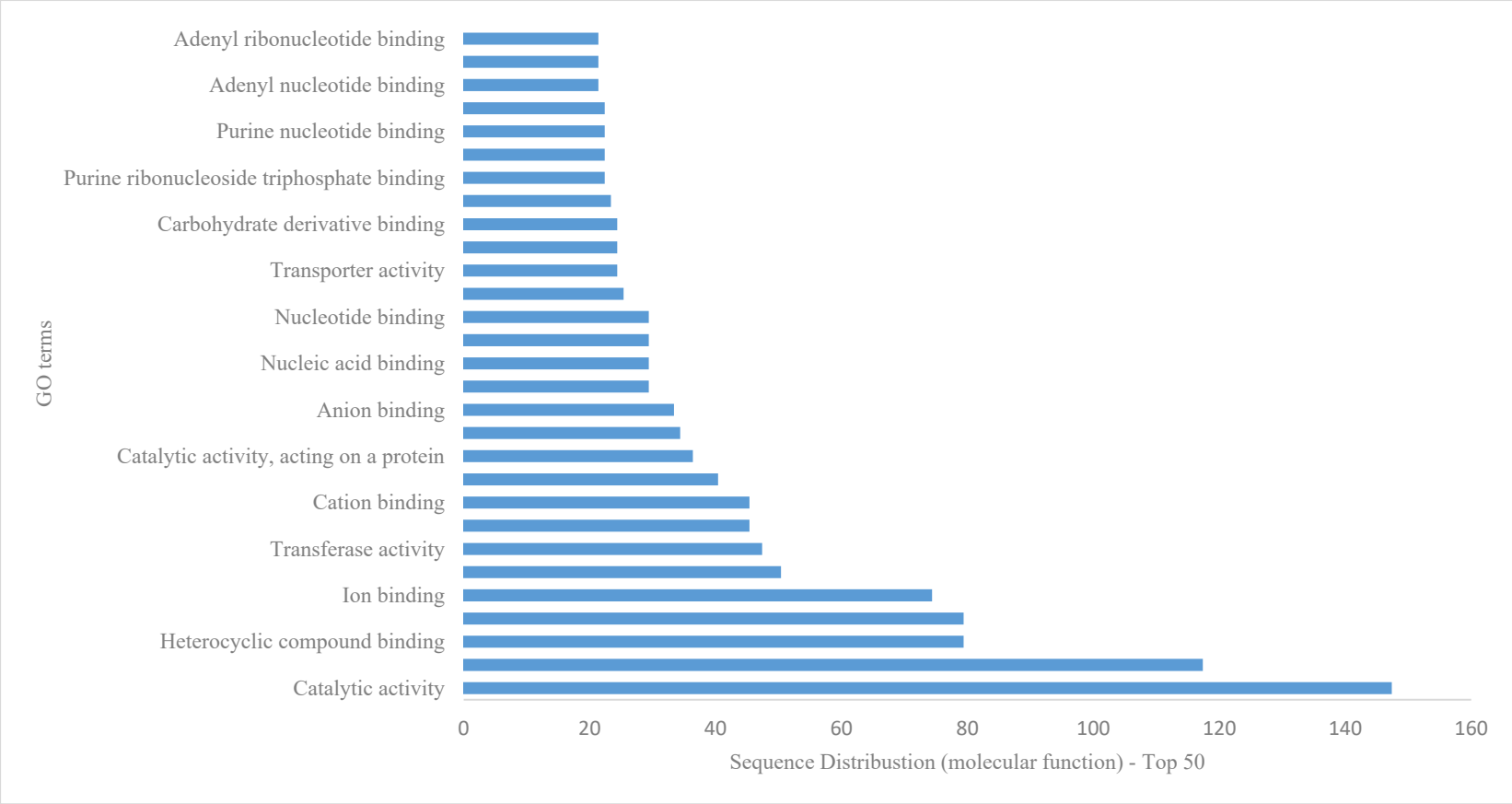

Figure 3-3. Sequence distribution (molecular function) of top 50 genes expressed in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

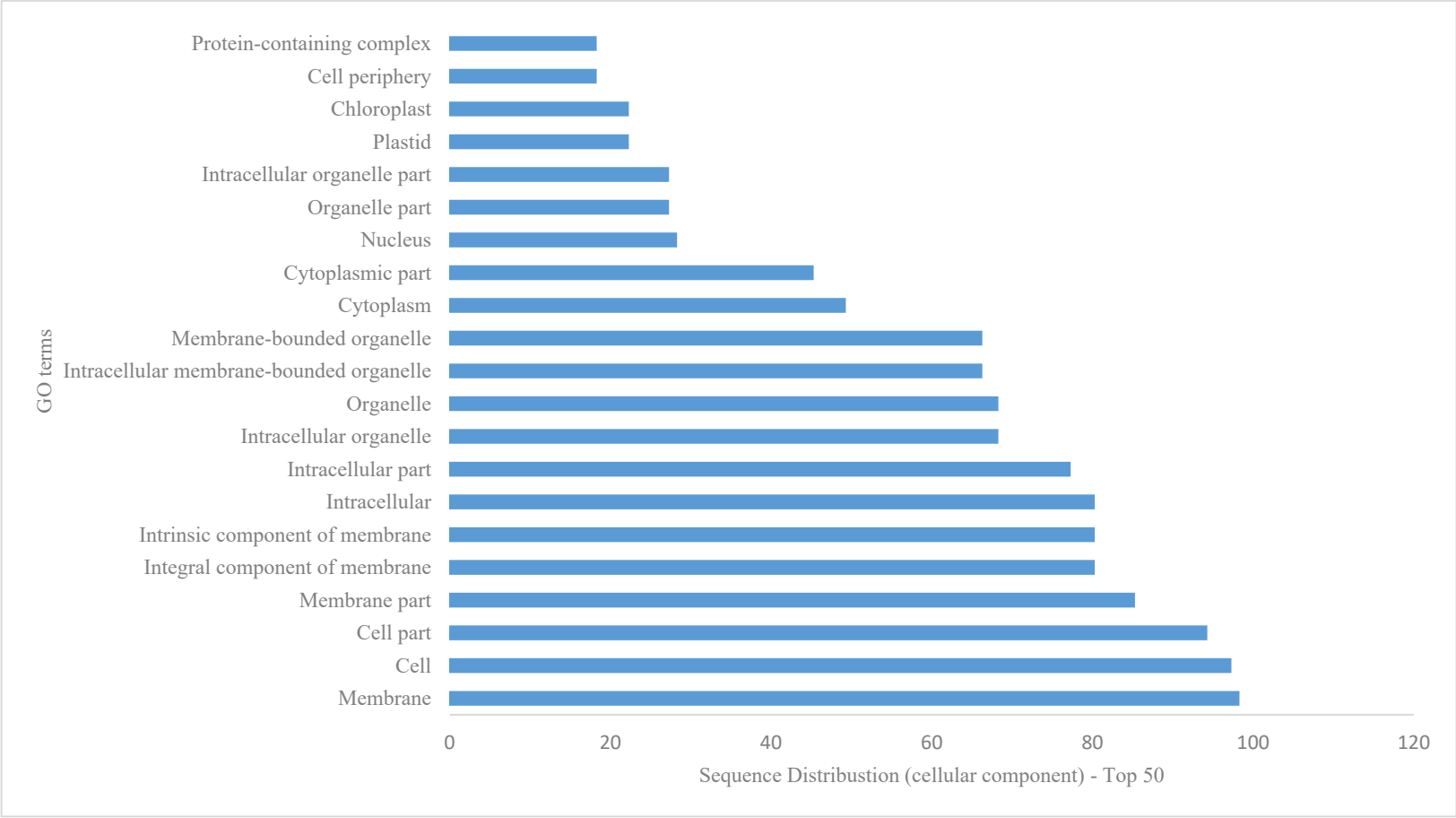

Figure 3-4. Sequence distribution (cellular component) of top 50 genes expressed in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

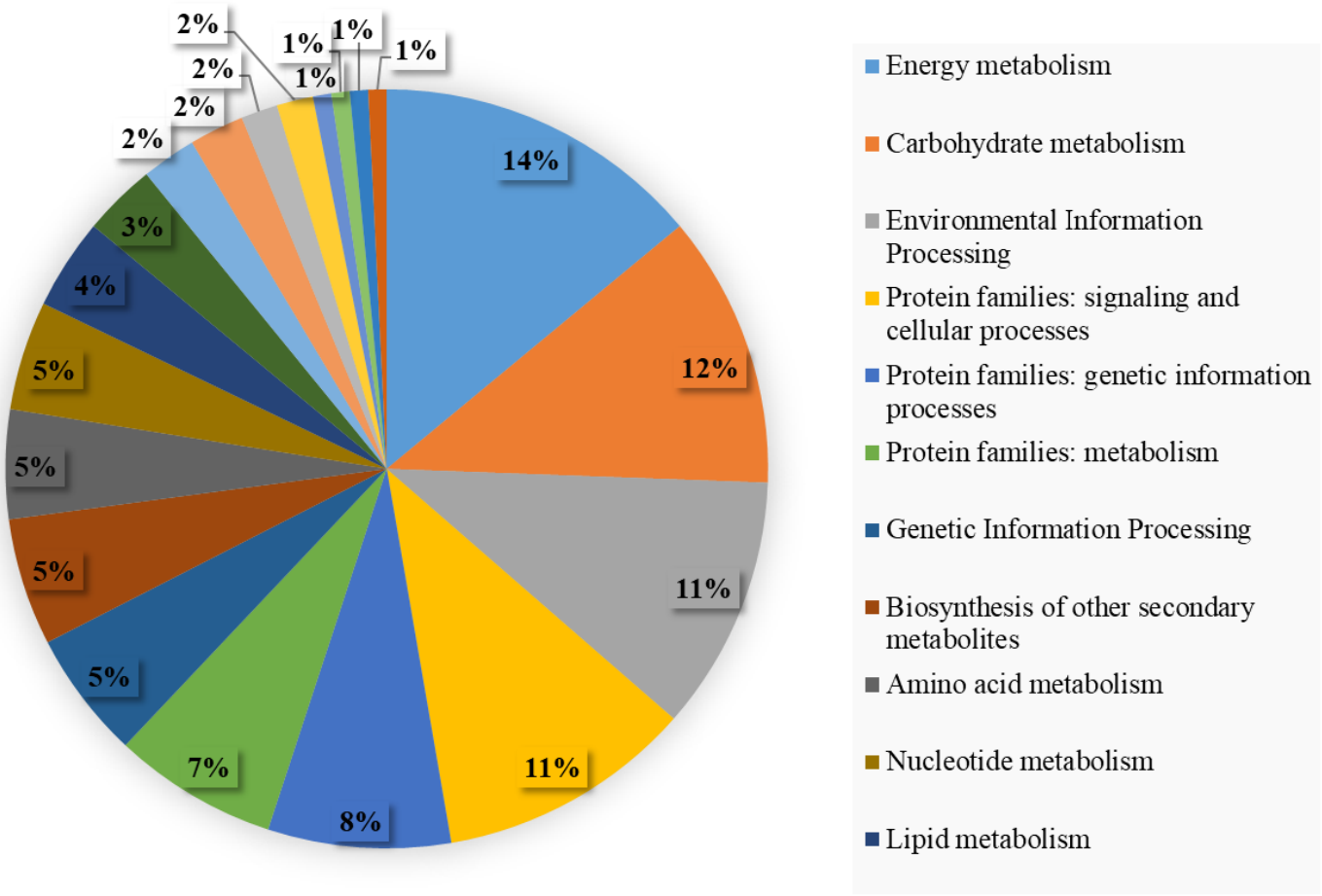

Figure 3-5. Functional classification of proteins in 250 µg Cu L^{-1} and 500 µg Cu L^{-1} after 7 days.

3.2. Changes in the expression of genes in *Z. muelleri* in response to Cu stress

Based on the results of the most functional classification of related proteins to our expressed genes, it is shown that energy metabolism and carbohydrate metabolism were the most induced pathways in response to 250 µg Cu L^{-1} and 500 µg Cu L^{-1} and selected for further investigation. Additionally, according to the results in Chapter 2, photosynthesis and defense mechanisms (enzymatic and chemical) were induced in response to Cu stress and therefore, genes linked to these pathways were also selected for further investigation in this study. As a result, a total of 24 significantly expressed genes (44 ORFs) were selected for the study of toxicity response of *Z. muelleri* under elevated levels of Cu (Table 3-2).

Out of the 24 differentially expressed genes of interest, 6 were related to photosynthesis (Figure 3-6 and Table 3-2) including three subunits of the transmembrane F_1F_0-ATPase (beta, epsilon and CF0 subunit III), cytochrome b_6f and two membrane protein subunits of Photosystem I (psaA and psaB) which were all down-regulated at 500 µg Cu L^{-1} only. The level of expression of PSII subunits did not show any significant change related to Cu stress on day 7.

Glycolysis was up-regulated at both 500 µg Cu L^{-1} and 250 µg Cu L^{-1}. One fold of up-regulation in phosphofructokinase, that is a key regulatory enzyme in glycolysis and gluconeogenesis pathways, was recorded at 500 µg Cu L^{-1} only. A second glycolysis enzyme (fructose bisphosphate aldolase- class I) was also shown to be up-regulated with 1.8 fold change for 250 µg Cu L^{-1} and 1.6 fold change for 500 µg Cu L^{-1} compared to control. In contrary, in the mitochondria, ATPase subunit 1 (mitochondrion) was down-regulated at 1.3 fold change.

From the carbon fixation pathway, two genes were recorded with significant alteration in their gene expression at 500 µg Cu L^{-1}. Ribulose bisphosphate carboxylase (RuBisCo) large subunit was shown to be down-regulated whereas an induction of inorganic carbon fixation, was recorded by up-regulation of carbonic anhydrase. The Cu concentration of 250 µg Cu L^{-1} did not elicit any statistically significant change in the regulation of genes related to photosynthesis and carbon fixation (Table 3-2). The expression level for all the genes in energy metabolism

category were approximately 1.5 fold change, except for two ORFs of NADH-plastoquinon oxidureductase subunit 7 with -2 and -0.86 log 2 fold change.

Enzymatic defense mechanism (oxidative response) was strongly active for two of the antioxidant enzymes after 7 days of Cu exposure. Two peroxidase enzymes were recorded in our results: peroxidase P-like were up-regulated with 1.73 fold change in 250 µg Cu L^{-1} and peroxidase 5 were up-regulated with 2.07 fold change in 500 µg Cu L^{-1}. Glutathione s transferase (F3 and T1) genes were up-regulated at 500 µg Cu L^{-1} only, with an average of 1.6 fold change.

Additionally, three genes encoding enzymes involved in chemical defense mechanisms were found to be significantly expressed in both Cu concentrations. Cytochrome P450 (94B3 and A2-like) for example, was strongly up-regulated in both treatments with fold change between 1.3 and 2. However, Cytochrome P450 (89A1-like) were expressed in 500 µg Cu L^{-1} only and was down-regulated.

Lastly, the senescence regulator was up-regulated at 2.3 and 2.6 fold change at 250 µg Cu L^{-1} and 500 µg Cu L^{-1}, respectively. Proline dehydrogenase 2, on the other hand, was down-regulated in both treatments.

Table 3-2. List of 25 differentially expressed genes selected to investigate in this study.

	Gene ID	Description	*Z. marina* accession ID	Log 2 fold change	
				250 µg/L	500 µg/L
	Photosynthesis				
1	1:snap_masked-2238_86636--0.21-mRNA-1:exon:10998	ATP synthase beta subunit (chloroplast)	MYP_009433313.1		-1.572
3	0:maker-12051_14742--0.6-mRNA-1:exon:30225	Photosystem I chlorophyll a apoprotein A1	MYP_009433313.1		-1.877
4	0:maker-20826_7649--0.2-mRNA-1:exon:33678	Photosystem I P700 apoprotein A1 (chloroplast)			-1.600
5	0:maker-20826_7649--0.2-mRNA-1:exon:33679				-1.724
6	0:snap_masked-48_303880--0.7-mRNA-1:exon:418	Photosystem I P700 apoprotein A1 (chloroplast)			-1.593
7	0:maker-18706_10964--0.4-mRNA-1:exon:32487	Photosystem I P700 apoprotein A2 (chloroplast)	YP_009433312.1		-1.597

8	0:maker-18706_10964--0.4-mRNA-1:exon:32488			-1.678
9	0:maker-18706_10964--0.4-mRNA-1:exon:32489			-1.791
10	0:maker-18706_10964--0.4-mRNA-1:exon:32491			-1.452
11	0:augustus_masked-17538_37452_1_6784--0.1-mRNA-1:exon:32258	ATP synthase CF0 subunit III (chloroplast)	YP_009433302.1	-1.447
12	0:augustus_masked-15085_10303--0.0-mRNA-1:exon:32519	ATP synthase CF1 epsilon subunit (plastid)	YP_009433321.1	-1.513
13	0:augustus_masked-19104_15484_1_10973--0.1-mRNA-1:exon:32520	Cytochrome f (chloroplast)	YP_009433326.1	-1.437
	Energy metabolism			

14	0:maker-9308_28514--0.21-mRNA-1:exon:27364	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (chloroplast)	YP_009433319.1	-1.440
15	1:augustus_masked-634_55655--0.4-mRNA-1:exon:4186	Carbonic anhydrase, chloroplastic-like	KMZ73035.1	1.662
16	0:augustus_masked-12871_24276--0.0-mRNA-1:exon:30707	NADH-plastoquinone oxidoreductase subunit 7 (chloroplast)	YP_009433365.1	-2.000
17	1:snap_masked-1780_39850--0.13-mRNA-1:exon:9412		N/A	-0.869
18	0:snap_masked-17538_37452_24635_37452--0.1-mRNA-1:exon:32661	NADH-plastoquinone oxidoreductase subunit 2 (chloroplast)	YP_009433355.1	-1.555
19	0:maker-4413_38735--0.10-mRNA-1:exon:17560	ATP-dependent 6-phosphofructokinase 3-like	KMZ63705.1	1.095
20	0:maker-4413_38735--0.10-mRNA-1:exon:17561			1.130

21	1:maker-4123_63504--0.9-mRNA-1:exon:17147	Fructose-bisphosphate aldolase 6, cytosolic	KMZ58915.1	1.854	1.607
	Enzymatic defence mechanism				
22	0:augustus_masked-15899_5858--0.1-mRNA-1:exon:31786	Peroxidase P7-like	KMZ56929.1	1.735	
23	0:snap_masked-790_133743--0.25-mRNA-1:exon:5065	Peroxidase 5	KMZ75156.1		2.076
24	0:maker-1390_135030_66551_135030--0.20-mRNA-1:exon:7737	Glutathione s-transferase F13	KMZ67334.1		1.728
25	0:maker-1390_135030_66551_135030--0.20-mRNA-1:exon:7739				1.628
26	0:maker-1173_166881_1_148987--0.46-mRNA-1:exon:7200	Glutathione s-transferase T1	KMZ72094.1		1.730
27	0:maker-1173_166881_1_148987--0.46-mRNA-1:exon:7207				1.588

	Chemical Defence mechanism				
28	0:maker-1210_109775--0.31-mRNA-1:exon:6906	Proline dehydrogenase 2, mitochondrial-like	KMZ67632.1	-1.730	-1.421
29	0:maker-1210_109775--0.31-mRNA-1:exon:6904			-2.051	
30	0:augustus_masked-4411_36512--0.2-mRNA-1:exon:17387	Cytochrome P450 84A1-like	N/A		-1.353
31	1:augustus_masked-5439_21644--0.1-mRNA-1:exon:20377	Cytochrome P450 94B3	N/A	2.004	2.573
32	1:augustus_masked-5439_21644--0.1-mRNA-1:exon:20378				1.845
33	1:snap_masked-2454_54672_1_25020--0.9-mRNA-1:exon:11239			1.849	
34	0:maker-8118_116443--0.34-mRNA-1:exon:25615	Cytochrome P450 89A2-like	N/A	1.426	

35	0:maker-8118_116443--0.34-mRNA-1:exon:25616				1.350	
36	0:maker-14927_11295--0.5-mRNA-1:exon:31410	Cytochrome P450 89A2-like	N/A		2.586	2.457
37	0:maker-14927_11295--0.5-mRNA-1:exon:31411				2.079	1.825
38	0:augustus_masked-4883_85142_1_69478--0.1-mRNA-1:exon:18884	Cytochrome P450 94B3-like	N/A			1.675
39	0:maker-8118_116443--0.34-mRNA-1:exon:25615	Cytochrome P450 89A2-like		N/A		1.392
40	0:maker-8118_116443--0.34-mRNA-1:exon:25616					1.337
41	0:maker-14927_11295--0.5-mRNA-1:exon:31410	Cytochrome P450 89A2-like		N/A	2.586	
42	0:maker-14927_11295--0.5-mRNA-1:exon:31411				2.079	2.685

43	1:augustus_masked-2632_68683_1_61125--0.2-mRNA-1:exon:12148	Senescence regulator	N/A	2.324	
44	0:snap_masked-894_68702_4923_68702--0.5-mRNA-1:exon:5381	Putative senescence regulator S40	N/A		1.517

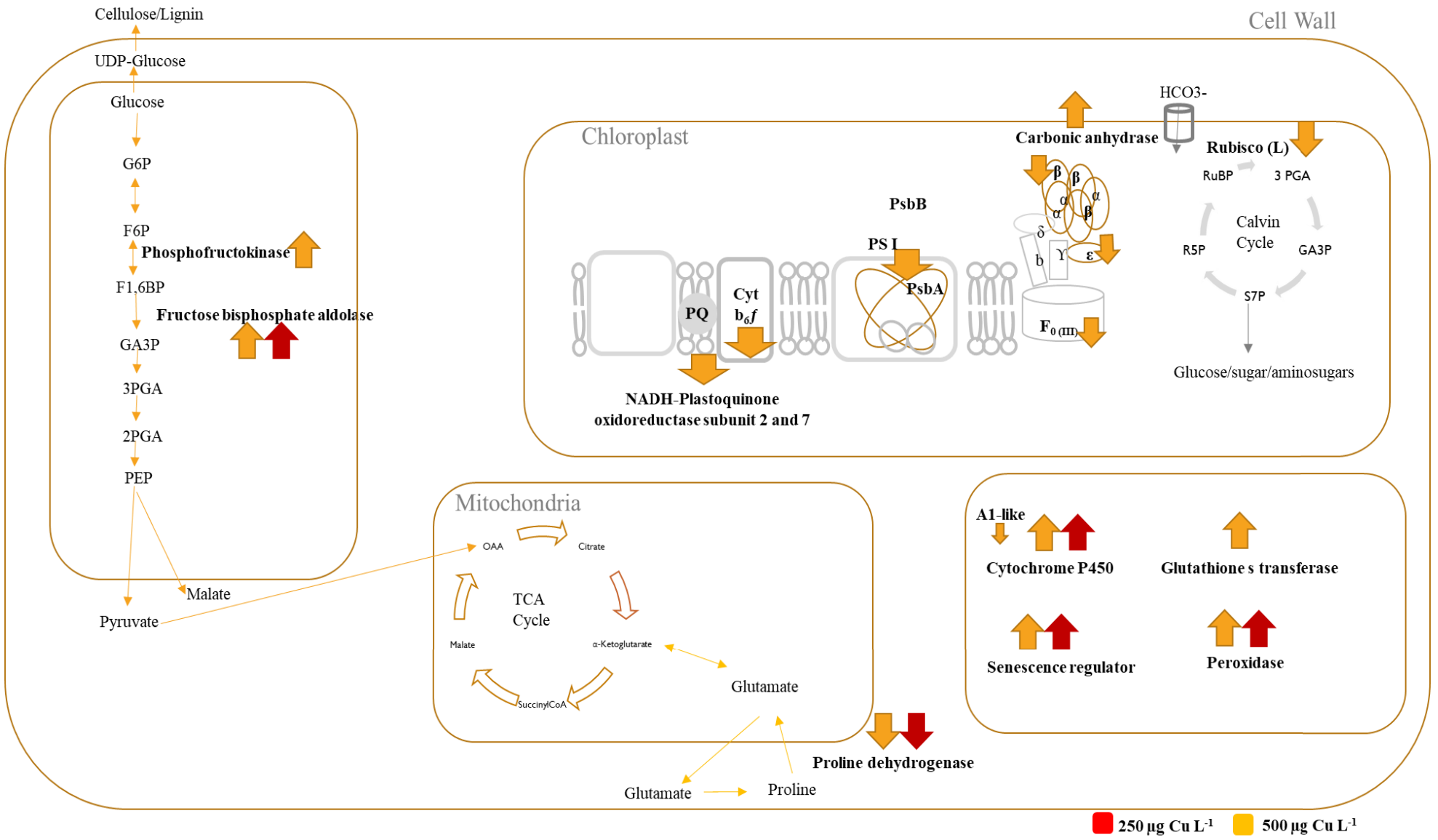
Cellulose/Lignin
UDP-Glucose
Glucose
G6P
F6P
Phosphofructokinase
F1,6BP
Fructose bisphosphate aldolase
GA3P
3PGA
2PGA
PEP
Malate
Pyruvate
Cell Wall
Chloroplast
HCO3-
Carbonic anhydrase
Rubisco (L)
RuBP
3 PGA
Calvin Cycle
R5P
GA3P
S7P
Glucose/sugar/aminosugars
PsbB
PS I
PsbA
PQ
Cyt b6f
F0 (III)
NADH-Plastoquinone oxidoreductase subunit 2 and 7
Mitochondria
OAA
Citrate
TCA Cycle
Malate
α-Ketoglutarate
SuccinylCoA
Glutamate
Proline dehydrogenase
Glutamate
Proline
A1-like
Cytochrome P450
Glutathione s transferase
Senescence regulator
Peroxidase
250 µg Cu L-1
500 µg Cu L-1

Figure 3-6. Differentially expressed genes related to photosynthesis, carbon fixation, energy metabolism, enzymatic and chemical defense mechanism under 250 µg Cu L^{-1} and 500 µg Cu L^{-1}. **Chloroplast:** PSI: photosystem I; RuBP: Ribulose 1,5-bisphosphate; 3 PGA: 3-phosphoglycerate; GA3P: Glyceraldehyde 3-phosphate; S7P: Sedoheptulose 7-phosphate; R5P: Ribulose 5-phosphate; PQ: plastoquinone; $Cytb_6f$: cytochrome b_6f. **Cytosol:** G6P: Glucose-6-phosphate; F6P: Fructose-6-phosohate; F1,6BP: Fructose 1,6 bis-phosphate, GA3P: Glyceraldehyde-3-phosohate; 3 PGA: 3-phospho glycerate; 2 PGA: 2-phospho glycerate;PEP: phosphoenolpyruvate. **Mitochondria:** TCA cycle: citric acid cycle; OAA: oxaloacetate.

4. Discussion

Investigation of differential gene expression of *Z. muelleri* in response to increased levels of Cu illustrated high sensitivity of this seagrass species to Cu as previously reported in the family of *Zosteraceae* (Lee et al. 2004; Lin et al. 2018, Buapet et al. 2019). As a result, four Cu-specific responses were that observed in this study:

4.1. Elevated Cu concentration impacts plant photosynthesis

Significant down-regulation of chloroplast-related genes were recorded at 500 µg Cu L^{-1} only. ATP synthase (subunit beta, epsilon and CF0 subunit III), cytochrome b_6f and PSI (subunit psaA and psaB) were found to be significantly expressed. Although cytochrome b_6f and photosystem subunits were also reported by Leng et al. (2015) in grape leaves under Cu stress, the subunits, were slightly different. The function of the photosystem is thought to be different in seagrasses compared to the higher plants given their adaptation to shallow coastal marine habitat and more especially the absence of stomata (Kumar & Ralph 2017). This could be an explanation for different photosynthesis subunits involved in Cu stress in seagrasses compared to higher plants. Additionally, NADH-plastoquinone oxidoreductase (subunit 2 and 7), was down-regulated at 500 µg Cu L^{-1}. Zaripova et al. (2011) reported up-regulation of NADH-plastoquinone oxidoreductase (subunit 1) under Cd, Cu and Ni but at much higher concentrations of trace metals. Therefore, it seemed like the response of NADH-plastoquinone oxidoreductase might be concentration-dependent.

Surprisingly, we did not find any statistical difference in the expression level of PSII subunits in our results. Even though there are previous reports showing that PSII is more sensitive to Cu stress than PSI in higher plants and seagrasses (Cedeno-Maldonado, Swader & Heath 1972; Mohanty, Vass & Demeter 1989; Arellano et al. 1995; Jegerschoeld et al. 1995; Ralph & Burchett 1998; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002a, 2004a; Dattolo et al. 2014; Lin et al. 2018). Additionally, a decline in photosynthetic efficiency was recorded from our previous experiments which shows the negative effect of Cu on PSII (Buapet et al. 2019). Cu toxicity is reported to change the conformation and function of the photosystem over time (Yruela 2005). This could be an explanation why Cu did not show any significant impact on the regulation of PSII at the transcriptomic level. We also investigated whether the PSII subunits were damaged by searching for any possible active repairing

mechanism in our list of expressed genes. Previous studies have shown that phytohormones can have a direct or indirect role in regulating and repairing PSII under abiotic stress factors (Gururani, Venkatesh & Tran 2015). For example, in higher plants, auxin is shown to increase the ability of energy trapping by PSII reaction centres under Cu stress (Ouzounidou & Ilias 2005). However, we observed down-regulation of auxin-responsive factor 15 in both treatments and factor 17 in 500 µg Cu L^{-1} in our results. On the other hand, jasmonic acid which is another suggested phytohormone with repairing activity for PSII under Cu stress in higher plants (Maksymiec, Wojcik & Krupa 2007) was up-regulated in our results for both Cu treatments (Appendix 3 - entry 18 and 57, Appendix 4- entry 68 and 193) indicating a possible initiation of the repair of PSII.

4.2. Activation of inorganic carbon fixation in response to Cu stress in *Z. muelleri*

As for CO_2 fixation, one major difference between seagrasses and higher plants is their ability to uptake bicarbonate from water via carbonic anhydrase as an alternative source of inorganic carbon (Tiwari et al. 2005). Previous studies reported a decrease in the activity of carbonic anhydrase as a response to Cu stress (Fariduddin et al. 2009). However, our result showed the opposite response with a 1.5 fold up-regulation of carbonic anhydrase in 500 µg Cu L^{-1}. One possible interpretation is that carbonic anhydrase has previously been shown to have a role in the regulation of pH within the chloroplast (Bhat, Ganai & Uqab 2017) which might indicate a defense mechanism role for carbonic anhydrase in response to damage of carbon fixation process as a result of Cu stress.

Increased levels of Cu has been shown to negatively affect the RuBisCo activity and carbon fixation in higher plants (Gonzalez-Mendoza et al. 2013). Our results showed that the large subunit of RuBisCo was down-regulated at 500 µg Cu L^{-1} only. The down-regulation of RuBisCo large subunit was congruent with previous studies of Cu stress in higher plants (Rakwal, Agrawal & Yonekura 1999; Hajduch et al. 2001; Leng et al. 2015; Roy et al. 2016). The oxidation of a cysteine residue in RuBisCo as a result of Cu stress was hypothesized to make conformation changes to RuBisCo in higher plant (Stiborova, Ditrichova & Brezinova 1988; Hajduch et al. 2001). A similar process may be responsible for the observed down-regulation of RuBisCo in our study for *Z. muelleri*. Additionally, given that the large subunit of

RuBisCo is encoded in the chloroplast genome, increased levels of Cu may also affect the chloroplast DNA, as suggested by Shinozaki et al. (1986).

4.3. Activation of energy production as a possible defense mechanism in Cu-stressed leaves of *Z. muelleri*

Previous studies have reported a decrease in glucose production and in turn down-regulation of glycolysis pathway under Cu stress (Leng et al. 2015; Roy et al. 2016). However, our results indicate the opposite result with up-regulation of the two key enzymes of glycolysis; phosphofructokinase and fructose bisphosphate aldolase (class I). Both enzymes were significantly up-regulated at 500 µg Cu L^{-1}. The latter was also shown to be up-regulated at 250 µg Cu L^{-1}. One hypothesis for this observation is that deterioration of photosynthesis under Cu stress may have induced the over-reduction of electron transfer chain leading to ROS production (Mittler et al. 2004). As a result, the plant was forced to use storage glucose sources (such as starch) due to reduction in glucose production to keep the glycolysis pathway active (Geiger, Servaites & Fuchs 2000; Hego et al. 2016).

4.4. Induced enzymatic and chemical defense mechanism in response to Cu stress

ROS production via Haber-Weiss and Fenton reaction is the first stress response towards most of the common stresses in plants (Hall 2002; Halliwell 2006). Activation of enzymatic defense mechanism to quench over-expression of ROS remained active for two antioxidant enzymes after 7 days of Cu exposure. Peroxidase (POX) and glutathione s transferase (GST) have been shown to be up-regulated in our experiment in a concentration-dependent manner. Peroxidase, a key enzyme in terrestrial plants for scavenging over-produced ROS (Hiraga et al. 2001), was up-regulated in both treatments. Glutathione s transferase, which also has a protective function and is a carrier for photochemicals (Edwards, Dixon & Walbot 2000) was up-regulated at 500 µg Cu L^{-1} only. Cu exposure was previously hypothesized to inhibit some antioxidant enzymes activity by binding to their sulfhydryl groups of proteins and alter their structure (Yruela 2009; Pena et al. 2012, Buapet et al. 2019). However, peroxidase and glutathione s transferase seemed to be unaffected by Cu in *Z. muelleri*.

Secondary stress responses also play a significant role against the stress factors at later stages of toxicity (Jonak, Nakagami & Hirt 2004; Opdenakker et al. 2012; Jalmi et al. 2018). Our results showed an up-regulation in the expression of

cytochrome P450 (CytP450) and senescence regulator at both 500 µg Cu L^{-1} and 250 µg Cu L^{-1}. CytP450 is shown to have a role in detoxification of Pb and Cd in plant and fungi as suggested by Zhang et al. (2015). Our results indicated that CytP450 may also play a significant role in Cu detoxification in *Z. muelleri*. Additionally, senescence regulator has previously been reported to have a role in Cu-specific detoxification (Breeze et al. 2008). The up-regulation of the gene coding for senescence regulator in our study supports its role in Cu-specific detoxification mechanism in *Z. muelleri* as well.

Proline, which is involved in secondary defense mechanisms and protecting cellular components during stress (Rhodes & Hanson 1993; Ashraf & Foolad 2007), was found to be inactive after 7 days of Cu exposure in our results. Proline dehydrogenase, which is involved in the transportation of proline to the mitochondria was down-regulated at both Cu concentrations in our results. Proline accumulation was previously reported in detoxification of salt and drought stress by stabilizing sub-cellular structures and scavenging free radicals (Öztürk & Demir 2002; Hsu, Hsu & Kao 2003; Kishor et al. 2005). The mechanism is thought to be via breaking-down of proline inside mitochondria to induce oxidative phosphorylation and ATP production to recover the damage. In our results, proline dehydrogenase that catalyzes proline catabolism was down-regulated at both 250 µg Cu L^{-1} and 500 µg Cu L^{-1}. Therefore, proline seemed not to be able to recover any mitochondrial damage under treatments used in this study.

Lastly, we found some inconsistency in the final results using different statistical approaches. We reported the result of a set of 7 genes in Chapter 2 and conducting RT-PCR method. Some of the genes reported as not significant in that study on day 7 which are superoxide dismutase, catalase, ascorbate peroxidase and glutathione peroxidase (at 500 µg Cu L^{-1}), metallothionein type 3 (at 250 and 500 µg Cu L^{-1}), metallothionein type 2 and cytochrome c oxidase (at 250 µg Cu L^{-1}) are also not reported in the results of this chapter as significant and could be considered as validation of our study. However, the reported significant genes are not in line with our results which could be due to using different statistical methods (ANOVA vs DESeq 2) and possible method limitations. Validation of all the Cu-induced genes reported in this chapter is suggested as a future direction of this project.

5. Conclusion

To summarize, our results showed that the concentration of the exposure seemed to play a critical role in Cu toxicity responses of *Z. muelleri*. Photosynthesis (PSI and ATP synthase), carbon fixation (RuBisCo large subunit), enzymatic defense mechanism (peroxidase and glutathione s transferase), chemical defense mechanisms (CytP450 and senescence regulator) and glycolysis (phosphofructokinase and fructose bisphosphate aldolase) were heavily regulated in Cu stress. Our study provides a knowledge base for the development of specific biomarkers for trace metals and Cu toxicity in the seagrass *Z. muelleri*. Further studies can enrich this dataset by analyzing Cu-contaminated seagrasses ideally from contaminated sites as biologically relevant samples to compare with our close-system results.

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CHAPTER 4

Protein-centric and peptide-centric proteome analysis of the Australian seagrass, *Zostera muelleri.*

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Abstract

A comprehensive proteomic study was carried out on the seagrass *Zostera muelleri* using protein-centric and peptide-centric approaches. A protein-centric (two dimensional electrophoresis 2-DE) and four peptide-centric approaches including in-solution digest (ISD), one dimensional electrophoresis (1-DE), peptide isoelectric focusing (IPE) and mixed mode (SDB-RPS based StageTips columns) were conducted followed by LC-MS/MS analysis in order to 1) identify the total proteins expressed in *Z. muelleri* leaves from each method and 2) determine which approach is best suited to yield an extensive protein reference map of the seagrass. A total number of identified proteins was significantly increased using peptide-centric approaches (5,189 proteins from combined peptide-centric methods versus 350 proteins using 2-DE method). 1-DE followed by SDB-RPS based StageTip desalting method provided the greatest proteome coverage (1,155 and 894 uniquely identified proteins, respectively) among the peptide-centric approaches evaluated in this study.

This comprehensive analysis reinforced the fact that characterisation of the entire proteome of a cell is beyond the capability of any single proteomic method. Thus, the combination of multiple methods is required to provide the most comprehensive proteome coverage. This work represents the most extensive proteomic identification of *Z. muelleri* leaves to date and can serve as a reference map for future comparative proteomic and functional genomic studies of *Z. muelleri* to biotic and abiotic stress.

1. Introduction

Seagrasses are ecological engineers of coastal marine habitats and are rated as the third most valuable ecosystem globally with an estimated value of US $28,916 ha^{-1} yr^{-1} for their ecological services (Costanza et al. 2014). However, seagrasses are declining globally at an alarming rate of > 7% yr^{-1} due to both natural and anthropogenic disturbances (Waycott et al. 2009). Therefore, understanding the acclimation and / or tolerance responses of seagrasses to external perturbations is crucial for developing strategies to prevent their ongoing decline as well as assist with conservation and management (Davey et al. 2016; Kumar, Kuzhiumaparambil, et al. 2016).‘Omics’ technologies aim to investigate the structure and function of an organism through its biological molecules and can be applied to identify molecular markers that can help to determine early warning signals to prevent the demise of

meadows before they pass a point of no return (Macreadie et al. 2014; Kumar, Kuzhiumaparambil, et al. 2016; Jiang et al. 2017).

Proteomic is the comprehensive study to identify and quantify changes in protein and proteoform (single protein isoform) abundance in an organism, cell, tissue or organelle at a specific time under specific conditions (Pandey & Mann 2000; Graves & Haystead 2002). Therefore, study of the abundance of proteoforms can provide an insight into the regulation of metabolic pathways and their interactions with other biochemical pathways (Tan, Lim & Lau 2017). Additionally, phenotypic variability can be examined using proteomic, to identify possible biomarkers to monitor eco-physiological responses and adaptations (Slattery et al. 2012; Vanderschuren et al. 2013; Tomanek 2014).

Proteomic studies are technically complex, especially for plant materials (Carpentier et al. 2008). Extracting sufficient proteins from plant tissues is difficult due to the rigid cell wall and the presence of proteases and secondary metabolites (Carpentier et al. 2008; Wang, Tai & Chen 2008). Regardless, progress has been achieved in understanding stress response processes in higher plants since the first plant proteomic study in maize was reported back in the 1980s (Tan, Lim & Lau 2017).

Proteomic in seagrasses, on the other hand, remains in its infancy for two primary reasons: 1) the lack of efficient and optimised protein extraction methods, and 2) the limited availability of properly annotated genomic sequence information. The recent development of an efficient protein extraction method for *Z. muelleri* (Jiang et al. 2017), has allowed the proteome profiling of this seagrass species. Moreover, completion of the genome sequencing of *Z. marina* (Olsen et al. 2016) and *Z. muelleri* (Lee et al. 2016) have enabled seagrass researchers to start undertaking system-wide approaches (integrated omics) with seagrasses. The available genomic information assists the understanding of the molecular responses across the family of *Zostraceae* to environmental processes. However, the functional annotation of the large number of predicted Open Reading Frames (ORFs), which are potentially translated eukaryotic internal exons with stop codons (Sieber, Platzer & Schuster 2018) and their corresponding protein products are far from complete (see the NCBI database for *Z. marina* and http://appliedbioinformatics.com.au for *Z. muelleri*). Proteomic approaches have been shown to be a more effective solution to elucidate biological

function than transcriptomic approaches, because the abundance of mRNA does not necessarily correlate with protein abundance or indicate that the protein is active in the cell (Libault et al. 2017). Furthermore, post-translational modifications and translational rate can only be investigated by proteomic approaches (Aebersold & Mann 2003; Mata, Marguerat & Bähler 2005; Bucalossi et al. 2006; Higashi et al. 2006; Altelaar, Munoz & Heck 2013). In comparison with transcriptomic analysis, proteomic is able to examine the broader effects of adaptive / tolerance strategies of seagrasses to environmental stress factors.

Characterization and quantification of proteins can be achieved through protein-centric as well as peptide-centric approaches (Klont et al. 2018). A protein-centric or top-down proteomic approach allows the fractionation of proteoforms, using one- or two-dimensional gel electrophoresis (Padula et al. 2017). In 2-DE, protein spots that change in abundance between treatments are then enzymatically digested to peptides using trypsin followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis (Kumar, et al. 2016). Two dimensional gel electrophoresis (2-DE) based proteomic has three main advantages: 1) quantification is performed on intact proteoforms; 2) protein-derived peptide connectivity is retained and 3) peptides from a single protein are present in the MS data file, often increasing protein sequence coverage (Kumar, Padula, et al. 2016; Padula et al. 2017). Although it should be noted that multiple proteins can be present in the same spot. Additionally, issues such as limited number of spots, gel-to-gel variations, insensitivity towards low abundant proteins, protein co-migration, selected range of isoelectric point (pI) and difficulty in the separation of hydrophobic proteins are still major challenges for 2-DE approaches (Carpentier et al. 2008; Rabilloud 2014; Padula et al. 2017).

On the other hand, peptide-centric (bottom-up) approaches for whole proteome analysis performed without any prior separation are referred to as ‘shotgun proteomic’ or gel-free techniques. The identification and quantification of the products of ORFs (proteins) in peptide-centric techniques are achieved by matching the fragmentation spectra of the ionised peptides with the sequences derived from a database most often generated from genome sequencing data (Jiang et al. 2017; Tan, Lim & Lau 2017; Klont et al. 2018). Peptide-centric approaches provide three main advantages over protein-centric methods: 1) unsupervised automated throughput, 2) enhanced detection of lower abundance proteins, and 3) broader proteome coverage (Tan, Lim

& Lau 2017). However, peptides are disconnected from their parent proteoform and can often be shared by multiple proteins, also known as non-unique or degenerate peptides (Neilson et al. 2011). Furthermore, a protein is often inferred by a small number (>2) of detected peptides which can only be assigned to a protein if the sequence of that protein is known and present in the database (Lehmann 2017) and identification of a specific proteoform is often not possible.

It is apparent that no single proteomic method can cover all the subsets of the entire expressed proteome of a cell (Klont et al. 2018). Each technique can provide new insight into characterization, identification and quantification of proteins (Carpentier et al. 2008). Recently, proteome profiling of *Z. muelleri*, *Z. marina* and *P. oceanica* have been carried out using 2-DE to study their stress response to altered light and salinity (Spadafora et al. 2008; Mazzuca et al. 2009; Serra, Mazzuca & Pirog 2011; Dattolo et al. 2013; Jiang et al. 2017; Kumar et al. 2017). However, to our knowledge, peptide-centric proteomic analysis for *Z. muelleri*, has not been investigated yet.

Here, we provide a baseline information for a comprehensive proteome reference map of Australia's dominant and model seagrass, *Z. muelleri,* using both protein-centric and peptide-centric approaches. We have undertaken proteomic analysis using our recently developed protein extraction method and LC-MS/MS analysis to ensure wide proteome coverage (Jiang et al. 2017). In addition to 2-DE, we investigated the number of identified proteins using the in-solution digestion method alone and in combination with 1D-PAGE and peptide IEF (isoelectric focusing). Additionally, SPE (solid phase extraction**)** and SDB-RPS (styrenedivinylbenzene reverse phase sulfonate) based desalting and fractionation analysis were assessed to obtain a comprehensive proteome profile of *Z. muelleri*. Our results will assess the relative capacity of several proteomic methods for their efficiency to identify the highest number of peptides in *Z. muelleri.*

2. Materials and Methods

2.1. Plant material

Samples of *Z. muelleri* were harvested from Pittwater (33°38′45.6′′S. 151°17′12.8′′E), Sydney, NSW, Australia. Turfs of seagrass with 10 – 15 cm of intact sediment were carefully removed from the meadow using a hand spade and placed in

plastic tubs. Wet paper towels were placed over the plants to prevent desiccation during transport. Plants were cleaned of epiphytes and grazers once transported back to the aquaria facility at the University of Technology Sydney. Any intact sediments were washed from roots and rhizome using seawater with salinity of 32 psu. Leaves were then separated at the horizontal creeping rhizome, and frozen at −80°C for protein extraction. All the reagents used in this study (otherwise stated) were purchased from Sigma-Aldrich.

2.2. Protein extraction, purification, alkylation and reduction

Proteins were extracted from whole leaves of *Z. muelleri* following the optimized protocol for seagrass protein extraction according to Kumar et al. (2016). In brief, the frozen leaf tissues were pulverized using a cryomill (Retsch-MM200) and 1 cm stainless steel ball pre-cooled with liquid nitrogen. The ground tissue was re-suspended in 8 mL of ice-cold extraction buffer (100 mM Tris (pH 8.0), 100 mM EDTA-Na_2, 50 mM borax, 0.5% SDS (w/v), 50 mM ascorbic acid, 2% PVPP (w/v), 1.5% Triton X-100 (v/v), 0.5% β-mercaptoethanol (v/v), 30% sucrose (w/v)) and a protease inhibitor cocktail (Roche, Germany) prior to protein extraction from this solution using 2 volumes of Tris-saturated Phenol (pH 8.0). After another wash with equal volume of BPP buffer, the proteins were precipitated using 0.1 M ammonium acetate in methanol at −20°C overnight and washed three times in 80% ice cold acetone. After removing the supernatant of the third wash, the pellet was air-dried and solubilised in rehydration buffer containing UTC7 (7 M urea, 2 M thiourea and 0.5% C7BzO) for 2-DE analysis only. For the peptide-centric methods, the proteins were solubilised in 8 M urea in 100 mM triethylammonium bicarbonate (TEAB) which was a more compatible buffer for LC-MS/MS applications (Annesley 2003; Chen et al. 2015; Peláez-García et al. 2015; Shi et al. 2017). The cysteine of solubilized proteins were reduced and alkylated using tributylphosphine (reducing agent, 5 mM) and acrylamide monomers (alkylating agent, 20 mM). The reaction was quenched using dithiothreitol (DTT, 20 mM).

Assessment of protein concentration was carried out by SDS-PAGE using bovine serum albumin as a standard (Gallagher 2012). Briefly, protein samples were electrophoresed into an SDS-PAGE gel for ~3 minutes before fixation using 40% methanol and 10% acetic acid and stained with Flamingo fluorescent stain (Bio-Rad). The gel was scanned using a laser scanner (Typhoon 9500-GE Healthcare) at an

excitation wavelength of 512 nm and emission wavelength of 535 nm for densitometry of the protein bands compared to the standards.

The protein sample from the whole leaves of *Z. muelleri* were subsequently used for protein-centric (2-DE) as well as the following peptide-centric methods; Method A: In-solution digestion (ISD), Method B: SPE-based desalting and HILIC fractionation, Method C: SDB-RPS-based desalting and fractionation, Method D: 1D-PAGE with SDB-RPS-based desalting and Method E: Peptide IEF with SDB-RPS-based desalting.

2.3. Protein-centric analysis (2-DE)

Protein sample (300 µg) were desalted using MicroBioSpin columns (Bio-Rad) equilibrated with UTC7 according to the manufacturer's instructions and then separated by isoelectric focusing (IEF) using a cup-loading method according to Jiang et al. (2017). Briefly, Immobilized pH gradient (IPG) strips (Bio-Rad, pH = 5 − 8, 11 cm) were passively rehydrated in UTC7 rehydration solution for a minimum of 6 h at room temperature. Isoelectric focusing was conducted in four steps on a Protean IEF device (Bio-Rad): 250 V rapid ramp for 15 min, 4000 V slow ramp for 8 h, 10,000 V linear ramp for 5 h and then constant 10,000 V for >10 h (total ∼100 kV h) with a current of 50 mA per IPG strip (Jiang et al. 2017). The focused strips were then equilibrated and directly applied onto a precast 4 – 20% polyacrylamide gel (Criterion™ Precast Gels, Bio-Rad) for the second dimension electrophoresis at constant voltage of 300 V, for 25 min. Gels were then fixed with 40% methanol, and 10% acetic acid for 30 minutes before staining with Coomassie stain G-250 and scanned with a laser scanner (Typhoon 9500 GE Healthcare). Stained gels were analysed using PD Quest 2-D analysis software, version 8.0 (Bio-Rad, USA). Molecular masses were estimated using a broad-range standard (Precision Plus, Bio-Rad). Protein spots after staining were excised, trypsin digested and analyzed by LC-MS/MS.

2.4. Peptide-centric analysis

2.4.1. In-solution digestion (ISD)

Reduced and alkylated proteins were diluted to 8 M urea using 100 mM TEAB buffer and digested with trypsin (TrypsinGOLD, Promega; 1 µg trypsin for 30 µg

protein) overnight at 37 °C. The digested peptide solution (1 µg / 5 µL) was analysed by LC-MS/MS. This method will be referred to as Method A in this work.

2.4.2. ISD, solid phase extraction (SPE)-based desalting and HILIC fractionation:

Tryptic peptides were introduced in the reverse phase column (Discovery ® DSC-18 SPE column-Sigma) to remove contaminants (Poole 2002). The column was conditioned with 100% acetonitrile (ACN) and MS loading solvent (2% ACN and 0.2% trifluoracetic acid). Peptides were eluted with 50% ACN and 0.1% trifluoracetic acid (TFA).

Eluted peptides were subsequently introduced to Hydrophilic Interaction LIquid Chromatography (HILIC) column (PolyHYDROXYETHYL resin, PolyLC) for fractionation since SPE column is not an efficient technique for removing hydrophobic contaminants (Li et al. 2011). The HILIC column was conditioned with 80% ACN and 15 mM ammonium formate (pH = 2.7). Peptides were eluted using eight sequential steps of increasing water percentage (McCalley 2010; Di Palma et al. 2011). This method will be referred to as Method B in this work.

2.4.3. ISD, SDB-RPS-based desalting and fractionation:

Stop-and-go-extraction tip (StageTip) column was made by placing a small portion of Empore SDB-RPS (styrenedivinylbenzene reverse phase sulfonate) in an ordinary pipette tip according to Rappsilber, Mann & Ishihama (2007). These SDB-RPS based tips were used for both desalting and fractionation of peptides. The contaminants will attach to the benzene side whereas the peptides will attach to the sulfonate side. The mechanism of desalting is to firstly, acidify the peptides to become positively charged and attach to the resin (by their amine side) and secondly, to neutralise their charge by increasing the pH of the solution for eluting (Rappsilber et al. 2007).

StageTip columns were initially activated with sequential washing with 100% ACN and MS loading solvent. The trypsin digested peptides were then pipetted into the top of StageTip columns. Columns were centrifuged at 3,000 xg until the sample completely passed through the column. Peptides were eluted using 5% ammonium hydroxide and 80% ACN, dried in a SpeedVac centrifuge (Savant DNA 120-Thermo

Scientific) and re-dissolved in MS loading solvent to be analysed by LC-MS/MS system at the concentration of 1 µg / 5 µL.

The same column preparation and activation protocols were used for peptide fractionation. Peptides at concentration of 20 − 25 µg were fractionated into a total of six fractions; the first four elution solutions containing 100 mM ammonium formate, 0.5% formic acid and 10, 20, 30 and 40% of ACN, respectively. The fifth elution solution had slightly higher concentration of ammonium formate (150 mM) to increase pH and ACN (60%). The last fraction contained 5 % ammonium hydroxide and 80% ACN. All fractions were dried in a SpeedVac centrifuge, re-dissolved in MS loading solvent and analysed by a LC-MS/MS system at the concentration of 1 µg / 5 µL. This method will be referred to as Method C in this work.

2.4.4. One-dimensional electrophoresis (1-DE) and SDB-RPS-based desalting:

According to Shevchenko et al. (2006), proteins in concentration of 100 µg was separated on a precast 4 − 20% polyacrylamide gel, fixed and stained as described in 2-DE method. The gel was sliced into 11 pieces (1 cm each), and each slice was chopped out into several small pieces of 1 − 2 mm in dimension with a clean scalpel. Gel pieces of each slice were washed using 100 µL of 100 mM ammonium bicarbonate (pH = 7.8) and 50% ACN (50:50), followed by 100 µL of 100% ACN. Proteins were then in-gel digested using trypsin (12.5 ng/µL trypsin in 10 mM ammonium bicarbonate). Peptides from each gel slice (total of 11 slices) were desalted using SDB-RPS based StageTip columns (see section peptide desalting in Method C) and analysed by LC-MS/MS at the concentration of 1 µg / 5 µL. This method will be referred to as Method D in this work.

2.4.5. Peptide isoelectric focusing (PEP-IEF) and SDB-RPS-based desalting:

Peptide IEF was carried out according to the protocols developed by Zaman, Urlaub & Abbasi (2018) and Jiang et al. (2017) with slight modification. Peptides (100 µg) were applied to an 11 cm, pH = 3 −10 IPG strip (BioRad) that was passively rehydrated in MQ water for 6 h at room temperature. Isoelectric focusing was conducted on a Protean IEF device (Bio-Rad) using the following program: 150 − 3000 V over 3 hours, 3000−10,000 V over 5 hours, 10,000 V for at least 5 − 6 hours until 100,000 V was achieved. Each peptide was migrated in IPG strip until it reached a pH of zero net charge (Padula et al. 2017). The IPG strip was then cut to approximately 1

cm length and peptides were extracted by sonicating each gel slices (total of ten slices) for 15 min each in 50% ACN and 0.1% trifluroacetic acid, twice. Extracted peptides were lyophilized and desalted using SDB-RPS based StageTip columns (see section peptide desalting in Method C) and analysed by LC-MS/MS at the concentration of 1 µg / 5 µL. This method will be referred to as Method E in this work.

2.5. LC-MS analysis of peptides

Using an Acquity M-class chromatography system (Waters, USA), 5 µL of each sample was loaded at 15 µl / min onto a nanoEase C18 trapping column (Waters, USA) with 2% ACN and 0.1% formic acid for 3 minutes. The peptides were eluted from the trapping column and onto a PicoFrit column (75 µm ID x 250 mm; New Objective, Woburn, MA) packed with magic C18AQ resin (3 µm; Michrom Bioresources, Auburn, CA). Peptides were eluted from the column and into the source of a Q Exactive Plus mass spectrometer (Thermo Scientific) using the following program: 5 − 30% MS buffer B (98% Acetonitrile + 0.2% Formic Acid) over 90 minutes, 30 − 80% MS buffer B over 3 minutes, 80% MS buffer B for 2 minutes, 80 − 5% for 3 min. The eluting peptides were ionised at 2000 V. A data dependant MS/MS (dd-MS2) experiment was performed, with a survey scan of 350 − 1500 Da performed at 70,000 resolution for peptides of charge state 2^+ or higher with an Automatic Gain Control (AGC) target of $3e^6$ and maximum injection time of 50 ms. The top 12 peptides were fragmented in the HCD cell using an isolation window of 1.4 m/z and an AGC target of $1e^5$ and maximum injection time of 100 ms. Fragments were scanned in the Orbitrap analyser at 17,500 resolution and the product ion fragment masses measured over a mass range of 100 − 2000 Da.

The resulting raw MS files from each analysis were searched against the Uniprot database of *Z. marina* and the customized proteome database of *Z. muelleri*, using PEAKS Studio v8.5 (Bioinformatics Solutions, Waterloo, ON) for protein identification. Variable modifications were set to cysteine propionamide, oxidised methionine and deamidated asparagine while enzyme specificity was set to semi-trypsin. Three missed cleavage sites were allowed, with a parent and fragment mass error tolerance of 20 ppm and 0.1 Da, respectively. The threshold selection for random protein sequences was a PEAKS probability-based ions score greater than 15 and false-discovery rate of 0.1% which represented the p-value of 0.03 (Zhang et al. 2012).

Proteins identified by at least one peptide in PEAKS were identified as expressed proteins. In this work, the term 'protein' refers to an Open Reading Frame (ORF) in the database rather than a mature proteoform.

2.6. Bioinformatics analysis

Gene ontology (GO) terms for *Z. muelleri* transcripts were determined using the InterPro annotation which was combined with the GO terms of related *Z. marina* genes. The PEAKS results from individual peptide-centric methods were initially exported in a mzIdentML format and searched in Scaffold (version 4.8.5) using a merged database of *Z. muelleri* and *Z. marina*. Scaffold used Bayesian statistical analysis to combine peptides assigned to each protein (Searle 2010). GO terms of all hits of a cluster determined by Scaffold (for peptide-centric methods) were combined and added to all *Z. muelleri* transcripts found in the same cluster using custom Linux scripts. For the 2-DE method, the FASTA protein sequence from individual PEAKS results for each spot was BLAST searched against *Z. marina* database and GO terms of the best hits (e-value of $< e^{-5}$) were assigned to the query sequence. Gene ontology terms were summarized using the GOSUM package (https://github.com/timkahlke/GOSUM), which estimates the number of child terms (is _a relationship) in a list of GO terms from all terms of the depth of level 1 where depth is the level that GOSUM sums the GO terms at.

For the assessment of the hydropathy of the identified proteins, the grand average of hydropathy (GRAVY) index was calculated using the calculator tool (http://www.bioinformatics.org). The relative (pI)/ (MW) of individual peptides were calculated using Expasy compute Pi/Mw tool (https://web.expasy.org/compute_pi/).

3. Results

3.1. Proteome analysis using protein-centric approach

According to the basic 2-D separation method for resolving proteome information, it was appear that most of the membrane proteins are neglected (Luche, Santoni & Rabilloud 2003; Gilmore & Washburn 2010). In addition, one has to consider the tradeoff between resolving power and the width of the gel pH gradient. Our recent work (Kumar et al, 2016, Ziang et al, 2017) suggested that more than 80% of Z. *muelleri* proteome was resolved within pI range 5 − 8. Therefore, this pH range

was chosen to be the standard analytical window in the present study of Z. *muelleri* using 2-D gel electrophoresis.

We performed a 2-DE separation of 500 µg of leaf proteins. A large number of protein spots (690 ± 58) were detected on Coomassie stained gels, of which 412 spots that were clearly visible were excised (Appendix 5), digested with trypsin, and analysed by LC-MS/MS.

Protein identification using the Uniprot *Z. marina* protein database of the *Z. muelleri* protein database (http://appliedbioinformatics.com.au/index.php/ Seagrass Zmu_Genome) yielded significant protein identifications ($p < 0.05$) for 325 spots using PEAKS Studio v8.5, some of which gave two different identifications in the same spot. A schematic workflow for protein identification using PEAKS for gel based proteomic is presented in Figure 4-1.Overall, 350 protein identifications were obtained, corresponding to 230 different accession numbers. Altogether, 325 spots yielded a positive identification (Appendix 6).

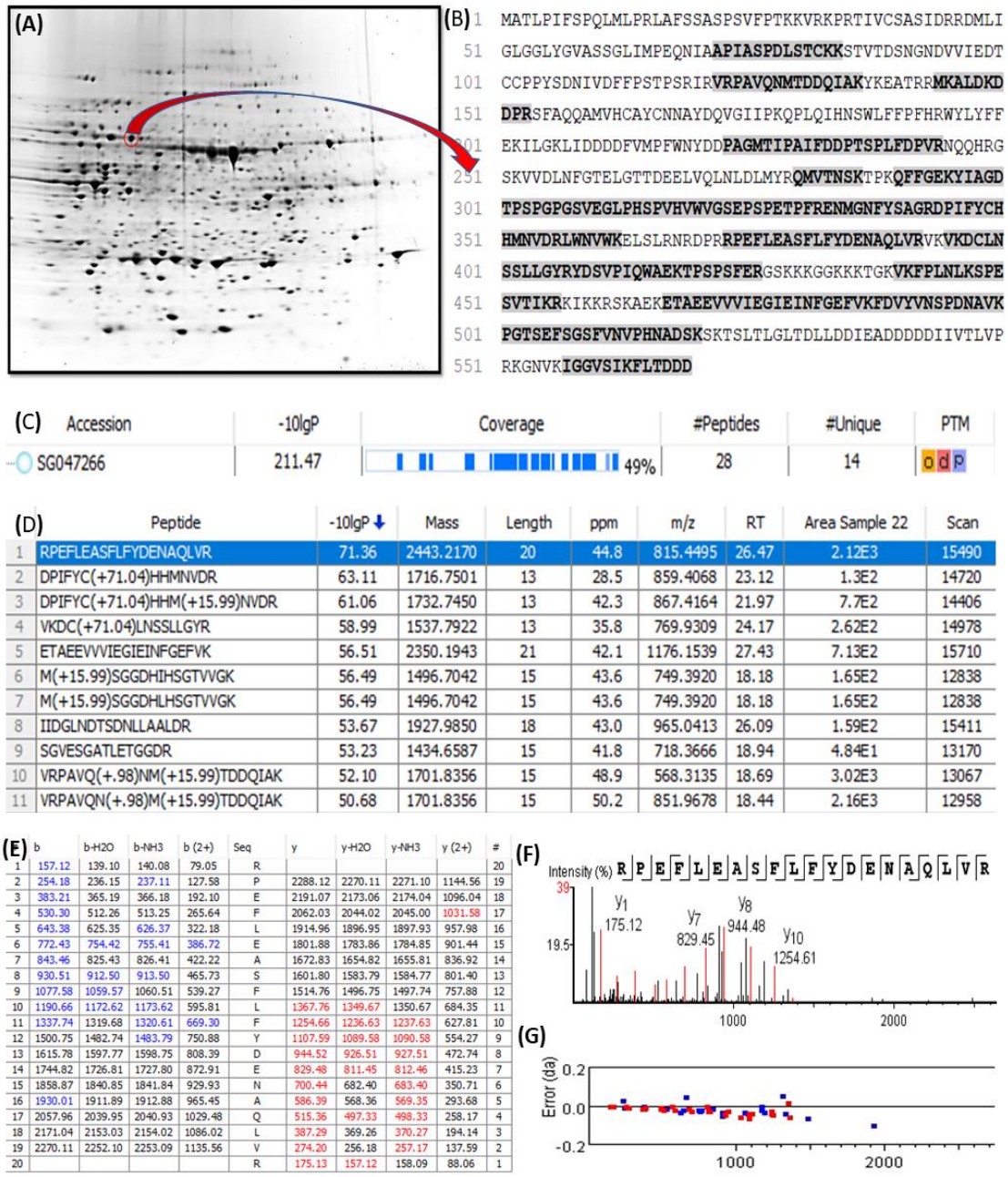


Figure 4-1. Demonstrative example for the identification of proteins extracted from leaves of seagrass *Zostera muelleri* .**A)** A protein spot was excised, trypsin digested and analysed using LC-MS/MS; **B)** the spectra of fragmented peptides were then matched to peptides present in protein sequences in a database; **C)** matched protein accession showing -10logP protein score value, protein sequence coverage unique and exclusive identified peptides and types and numbers of modfications present in the peptide shown in color-coded icons; **D)** top 10 annotated peptides with -10logP score and related peptide statistics; **E)** ion match summary of identified peptide; **F)** annotated peptide mass spectrum; **G)** peptide error. The shaded peptide sequence

marked with dark letters in Figure B represents the matched peptides. The blue and red marked values in Figure E represent the identified and matched amino acids from N- and C-terminal of a peptide sequence.

Among the identified 350 proteins, > 90% were identified with ≥ 2 unique peptides, of which a large proportion (67%) showed a high number of unique peptides ranging from 615 and log score value of 50 − 150 (Figure 4-2A and B) with protein sequence coverage > 30%. The detailed statistical parameters for protein and peptide identification including their score (-10lgP), exclusive peptide, unique peptide, sequence coverage, identified peptide with their ions score and PTMs are listed in Appendix 6. The sequences of identified proteins obtained from a customised seagrass proteome database (including *Z. marina*, *Z. muelleri* and other closely related seagrass species) were also BLAST-searched against a *Z. muelleri* proteome database to retrieve their protein sequences, more accurate descriptions and their theoretical pI and Mw values (Appendix 7).

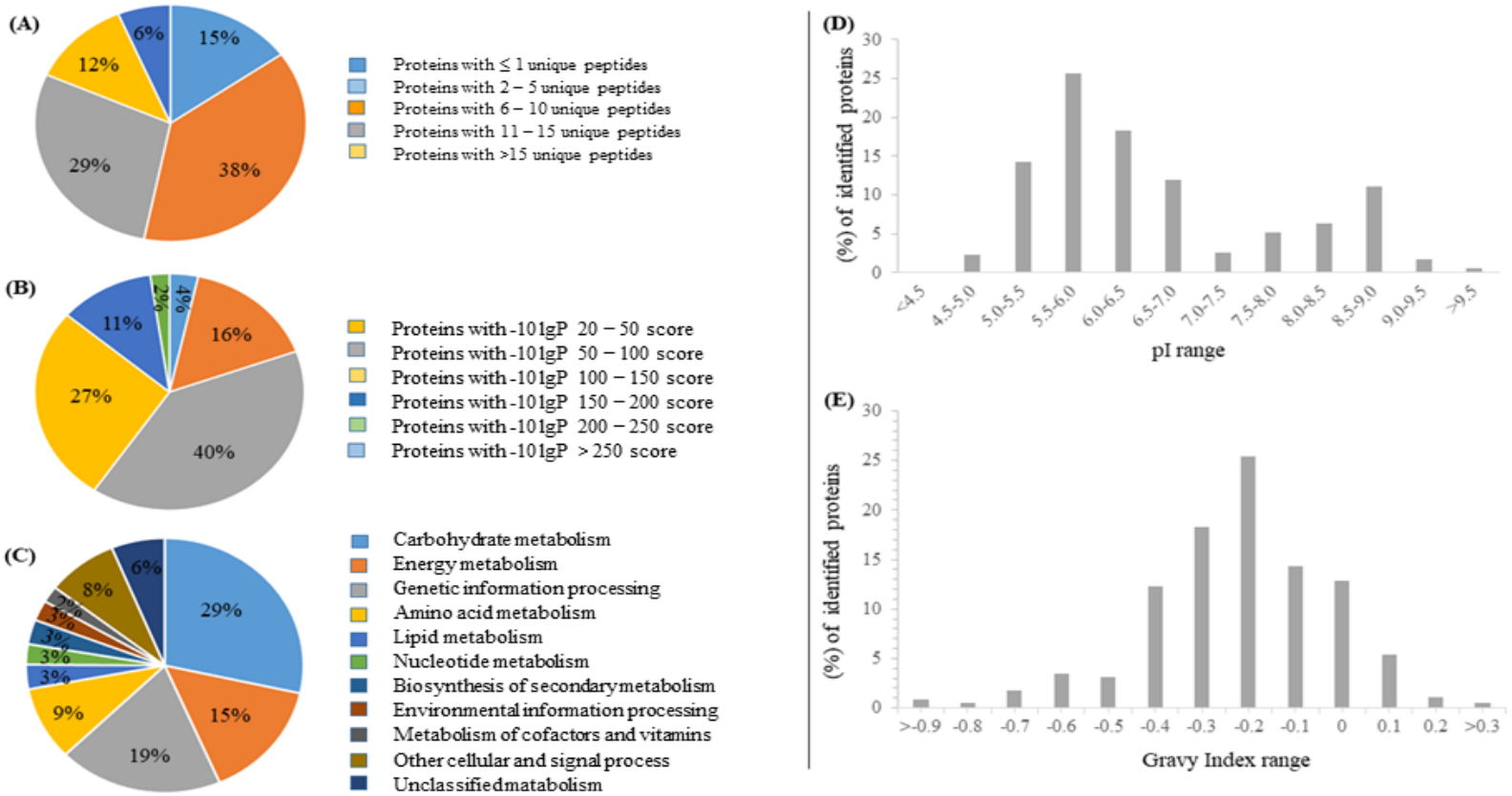


Figure 4-2. Protein percentage identified with unique peptides coverage (A), and protein score (-10lgP) (B), functional classification of identified proteins (%) belonging to diverse cellular metabolism (C), with corresponding Pi value (D) and GRAVY scores (E) for the 2D-IEF based leaf proteome analysis of *Z. muelleri.*

Isoelectric point and GRAVY index:

The theoretical pI values of the 350 identified proteins ranged from 4.5 – 9.6 (Figure 2 D). This theoretical pI value distribution showed a bi-modal pattern with the majority of the leaf proteins (246 proteins) in the 5 – 7 range representing > 72% of total proteins identified. A second peak was observed in the alkaline pI range of 7.5 – 9.6 with 87 proteins (correspond to 25% of total proteins identified). However, the observed pI value of the 350 identified proteins on 2D gels ranged from 5.0 – 7.8 (see protein spots distribution in Appendix 7), with > 85% of identified proteins in the range of pI 5.3 – 7.2. This clearly showed a significant difference between the theoretical and observed pI values (particularly the proteins with theoretical alkali pI values). Similarly, the theoretical molecular weight (Mw) of the identified proteins ranged from 12 – 118 kDa, which varied moderately ($R^2 = 0.83$) from the observed Mw values which ranged from 15 – 106 kDa. Some of the spots (spot 76, 91, 95, 96, 98, 139, 204, 205, 213, 221, 249 and 266) showed a significant variation in their theoretical and observed Mw values (Appendix 7).

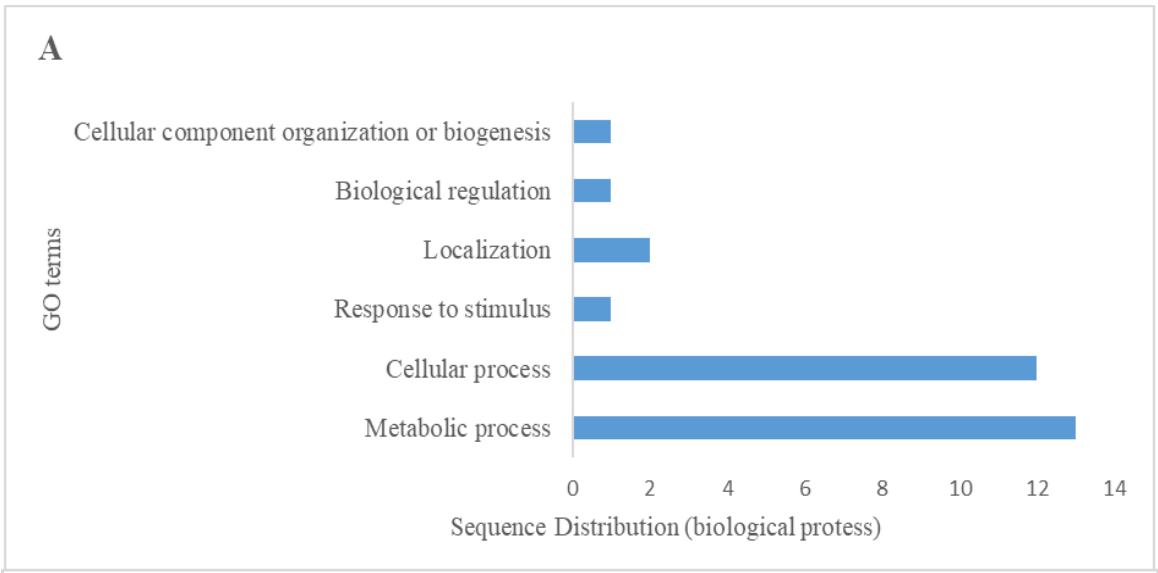
The GRAVY scores (also called the Kyte and Doolittle grand average hydropathy score), which is a measure of hydrophobicity, were utilized to categorize hydrophilic and hydrophobic peptides (Figure 4-2 E). Proteins with negative GRAVY scores are hydrophilic and those with positive values are hydrophobic. The majority of proteins (77%) had a GRAVY score ranging between − 0.6 and 0, indicating that most of them are hydrophilic.

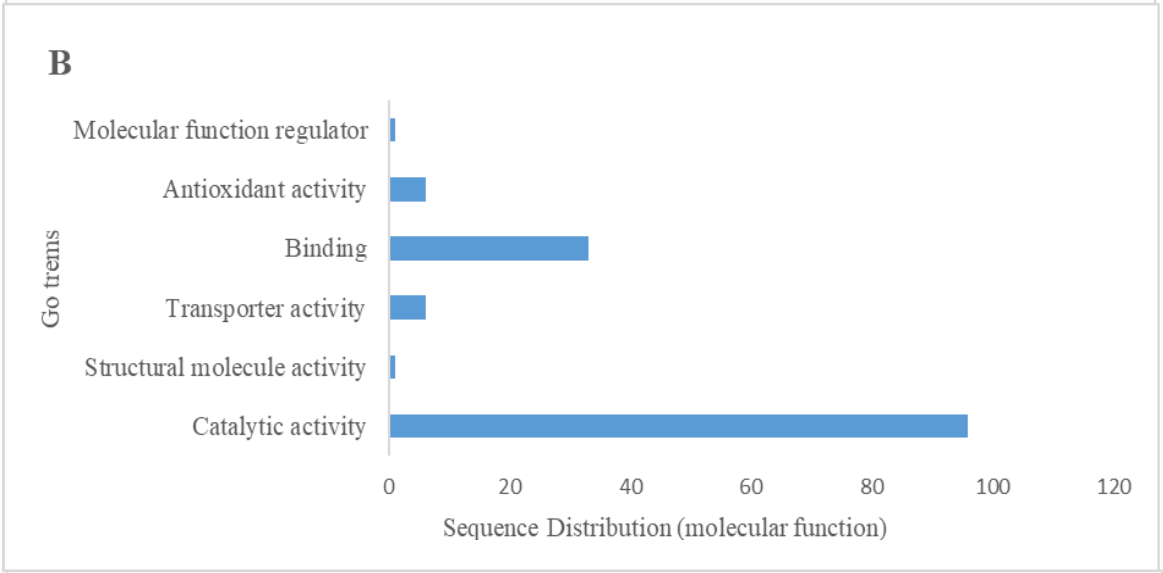
Gene Ontology (GO) and functional classification:

The complete set of proteins identified was subjected to classification according to their Gene Ontology. The proteins were catalogued based on their association with different biological processes, molecular functions and sub-cellular locations. Homology-based searches were performed against the NCBI database, using the identified protein query sequence, followed by mapping of the GO terms associated with the hits obtained. Generation of combined graphs showed the distribution of identified proteins according to their cellular components, biological process and molecular function (Figure 4-3). The majority of the identified proteins in a biological process category were involved in cellular and metabolic processes. According to molecular functions, the proteins were classified into six functional groups that include transporters, antioxidant activity, structural molecules, molecular

function regulators, binding and catalytic regulators with the majority of proteins belonged to the last two categories (Figure 4-3). Among the cellular components, the majority of the proteins belonged to the categories of cell, membrane or organelle components as well as protein containing complexes.

The identified proteins were further functionally classified into 11 categories according to KEGG functional annotations (Figure 4-2 C). Among the identified proteins, a high number of proteins were putatively involved in carbohydrate and energy metabolism (44%), genetic information processing (19%), amino acid metabolism (9%) and defence response (6%) were identified. We also identified several proteins including catechol oxidase (spots 47, 91, 95 and 98); cinnamoyl CoA reductase-like protein (spots 74 and 86); dihydroflavonol-4-reductase (spot 225); dihydrolipoyl dehydrogenase (spot 239); and chorismate synthase (spot 296), that are involved in secondary metabolites synthesis (Appendix 6). The FASTA protein sequence of the spots and the LC-MS/MS results of expressed proteins can be found in Appendix 8 and 10.


A
Cellular component organization or biogenesis
Biological regulation
Localization
Response to stimulus
Cellular process
Metabolic process
GO terms
0
2
4
6
8
10
12
14
Sequence Distribution (biological protess)


B
Molecular function regulator
Antioxidant activity
Binding
Transporter activity
Structural molecule activity
Catalytic activity
Go trems
0
20
40
60
80
100
120
Sequence Distribution (molecular function)

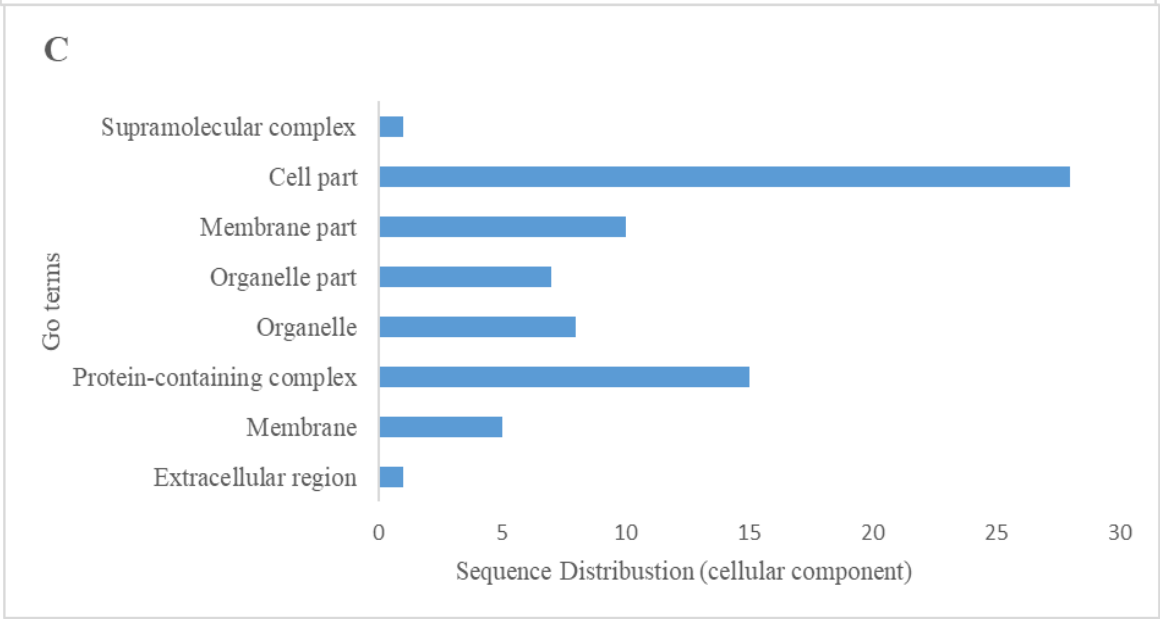

C
Supramolecular complex
Cell part
Membrane part
Organelle part
Organelle
Protein-containing complex
Membrane
Extracellular region
Go terms
0
5
10
15
20
25
30
Sequence Distribustion (cellular component)

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 *Z. muelleri*.

3.2. Proteome analysis using peptide-centric approach

The raw peptide MS files obtained from each method A – D were initially searched against a combined proteome database of *Z. marina* and *Z. muelleri* using PEAKS Studio software for general evaluation of the efficiency of each methods. The total number of proteins identified for all methods were over 2,500, except for Method B (ISD and by SPE based column), which showed inconsistency among the technical replicates and therefore, this method was discontinued from further analysis (Appendix 10). Subsequently, for Method C – D, desalting and/or fractionation of peptides were performed using SDB-RPS based StageTip columns.

Later, for all tested methods, proteins were identified against the *Z. muelleri* proteome database to identify proteins in our species of interest using PEAKS Studio (Table 4-1). The proteome coverage among the tested methods were as follows: Method E (4,440 identified proteins using 1-DE and SDB-RPS-based desalting) > Method C (3,831 identified proteins using SDB-RPS-based desalting and fractionation) > Method D (3,018 identified proteins using Peptide-IEF and SDB-RPS-based desalting) > Method A (2,017 identified proteins using ISD alone).

Approximately, 50% of the identified proteins from Method E were identified with more > 2 matched peptides. This number decreased to 33% for Method C, 39% in Method D and 36% in Method A. The search result of the peptide MS files, as well as the FASTA protein sequences of each method against *Z. muelleri* database can be found in Appendix 11 – 18.

Table 4-1. Total number of proteins and matched peptides from each peptide-centric methods searched against *Z. muelleri* database.

Method	Peptide-centric method	Desalting and fractionation method	Total number of putative proteins	Matched peptides		
				=1	>2	=2
A	In solution digestion (ISD)	_	2,017	733	396	828
C	In solution digestion (ISD)	SDB-RPS	3,831	1,280	894	1511
D	Peptide IEF	SDB-RPS	3,018	1,206	620	1,063
E	1D-PAGE	SDB-RPS	4,440	2,243	863	1,201

*Method B (ISD with Reversed phase / HILIC-based SPE column) was discontinued from further analysis due to insufficient amount of identified proteins.

The protein identifications from individual methods searched against the *Z. muelleri* database were used to create a Venn diagram to identify method-exclusive number of proteins (Figure 4-4). Method E showed the highest percentage of uniquely identified proteins (1155) followed by Method C (894) > Method D (255) > Method A (122). A total of 1293 proteins were identified as common in all methods.

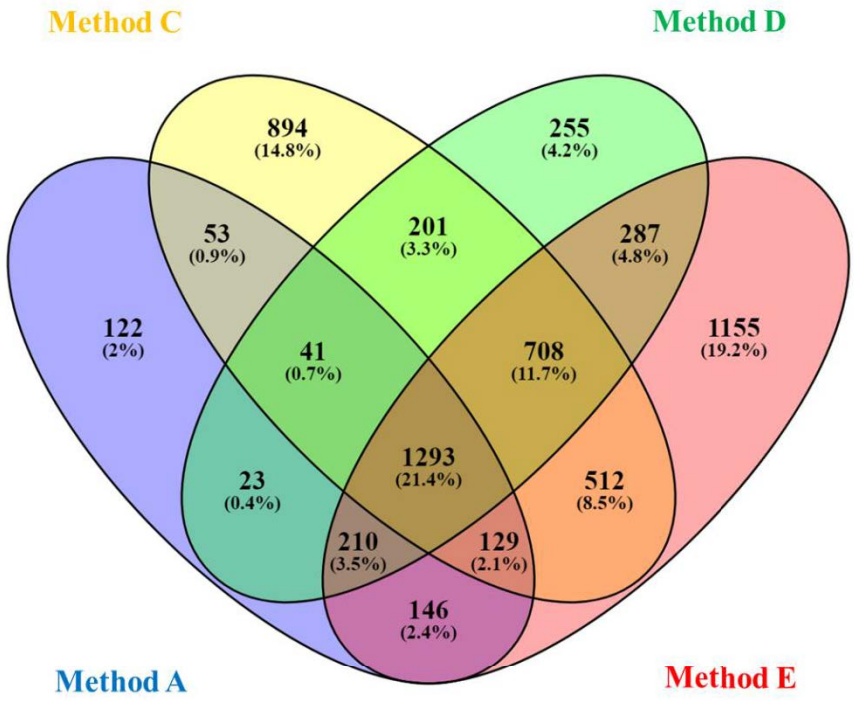


Figure 4-4. Venn diagram showing the total number of identified proteins as well as the number of proteins uniquely identified in each method and number of common proteins in all methods for *Z. muelleri* using Venny software.

Additionally, the number of unique peptides as well as their correspondent -10 lgP (protein score value) were investigated for each methods to evaluate the quality of proteome analysis. As it is shown in Figure 4-5 and Figure 4-6, we have a similar pattern for all peptide-centric methods which the majority of proteins were identified with ≥ 2 exclusively unique peptides and -10 lgP = 20 – 100. Methods E represented slightly better quality of proteome analysis with 52% of proteins identified based on ≥ 6 exclusively unique peptides. Method A and E were the only methods with score value (-10 lgP) ≥ 250.

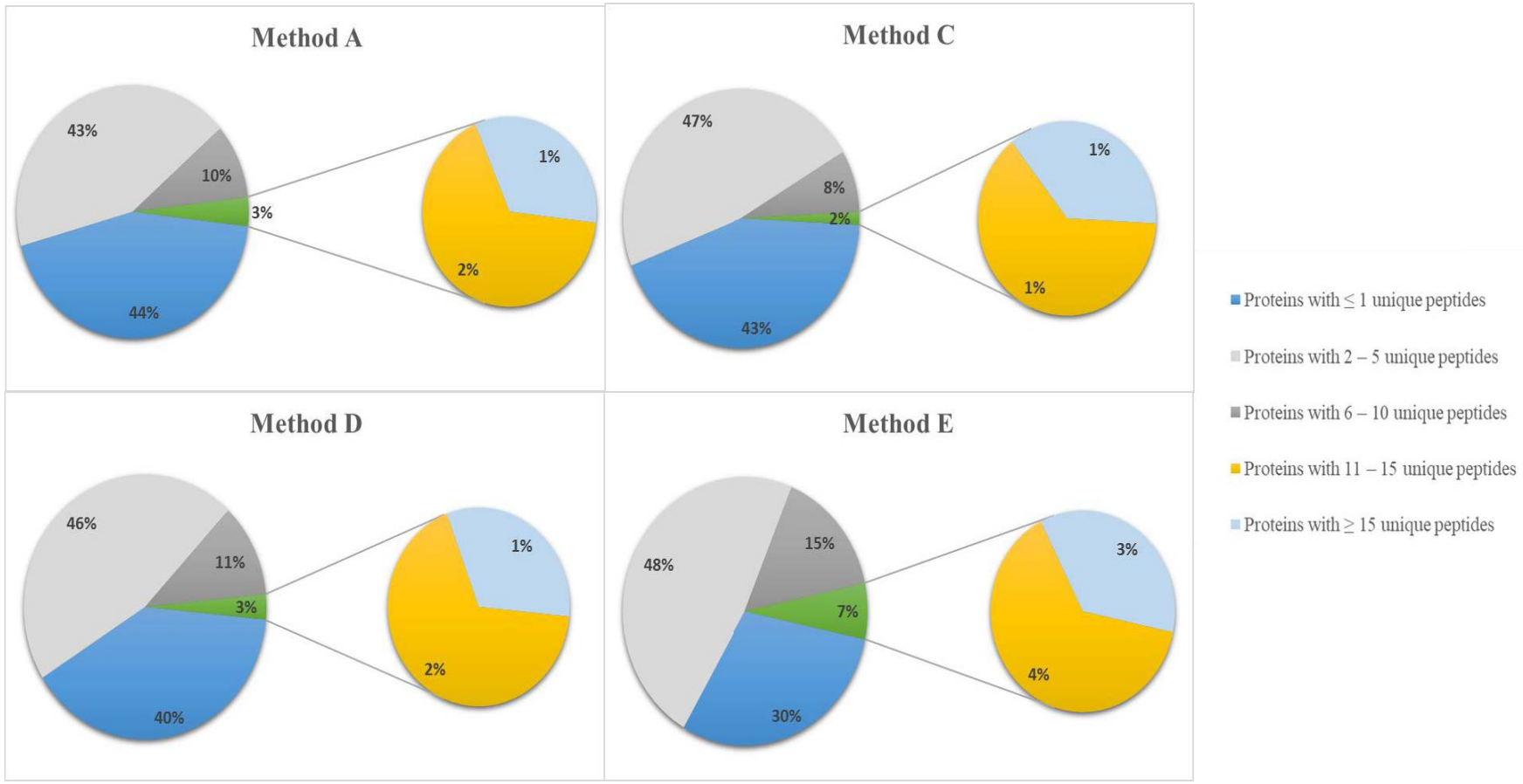


Figure 4-5. The percentage of unique identified peptides from each peptide-centric methods.

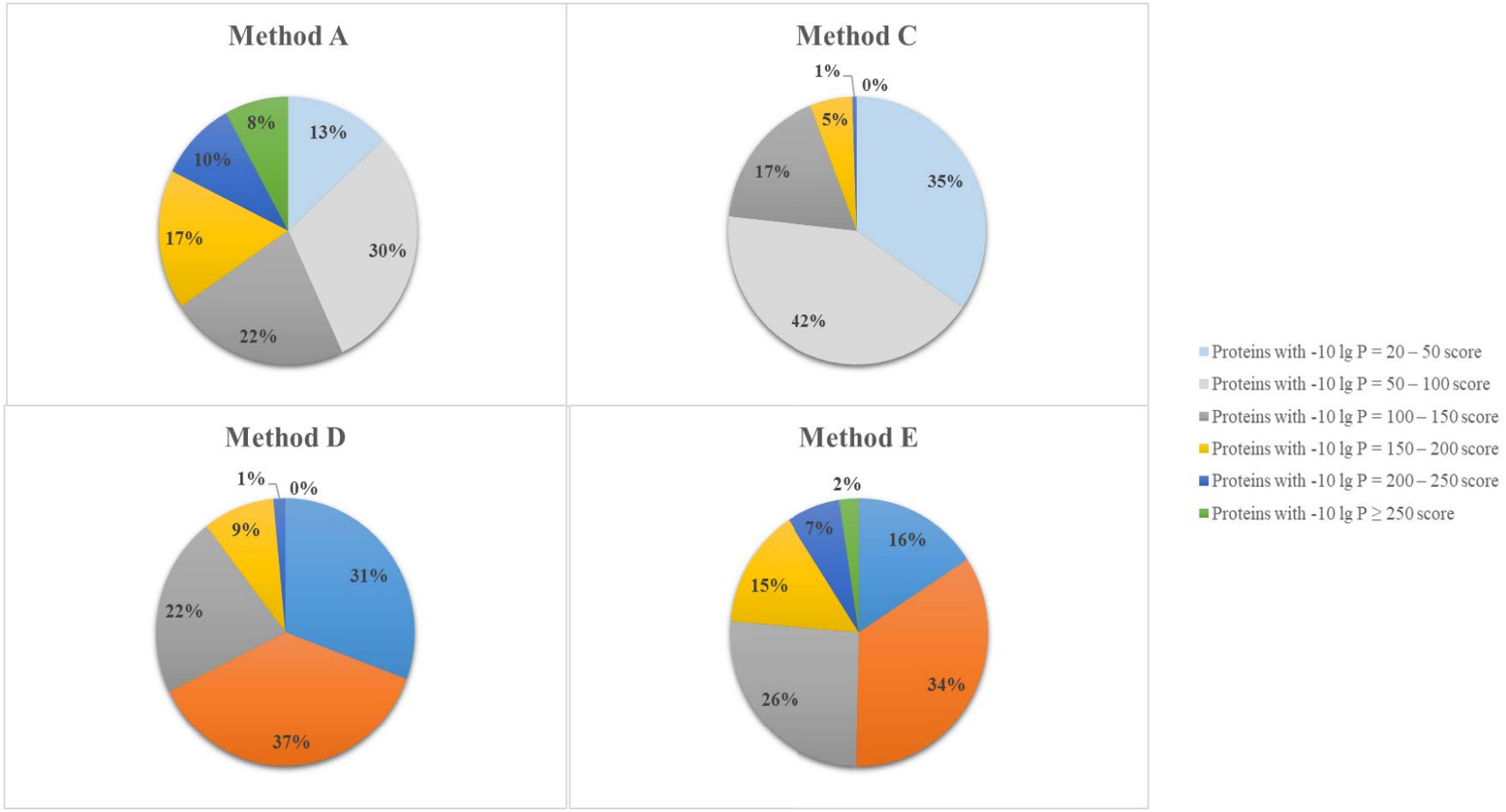


Figure 4-6. The percentage of the -10lgP value of unique peptides from peptide-centric methods.

3.2.1. Isoelectric point and GRAVY index:

The result of GRAVY scores showed the majority of proteins (average of 84.5%) to be hydrophilic with highest GRAVY index of -0.4 to 0.0 in all peptide-centric methods (Figure 4-7). Approximately 15 % of the proteins shown positive GRAVY index which is related to hydrophobic proteins such as membrane proteins. Furthermore, the pI value distribution showed a bi-modal pattern for the majority of the identified proteins from peptide-centric methods which showed where the isoelectric points were 5 – 6 or 8 – 9 (Figure 4-8).

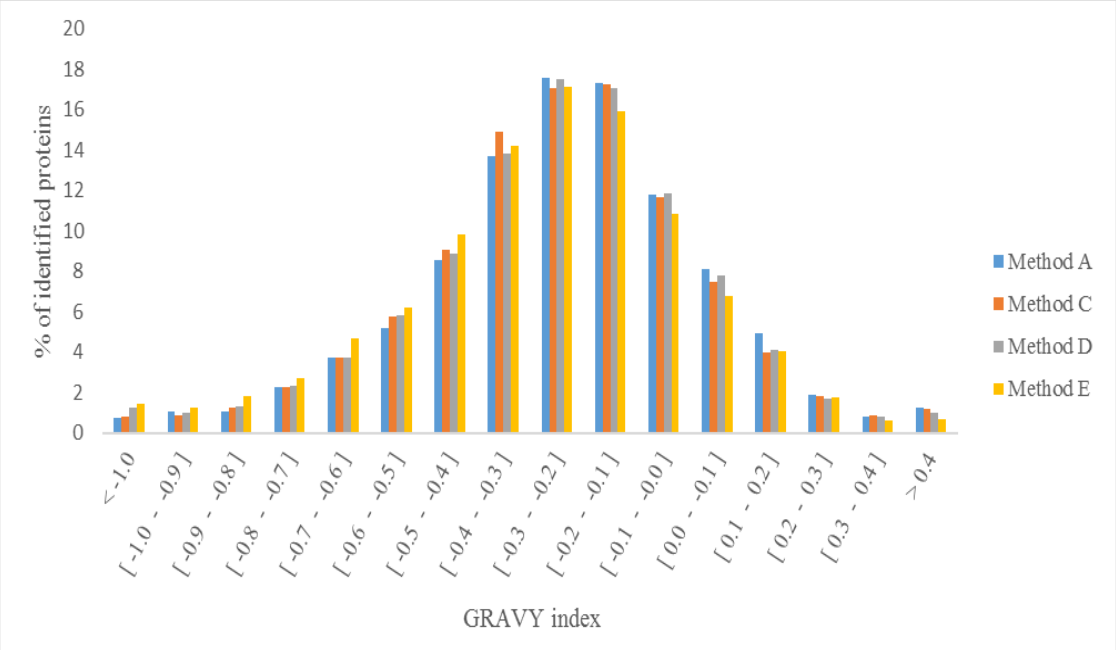


Figure 4-7. GRAVY score of the identified proteins from peptide-centric methods.

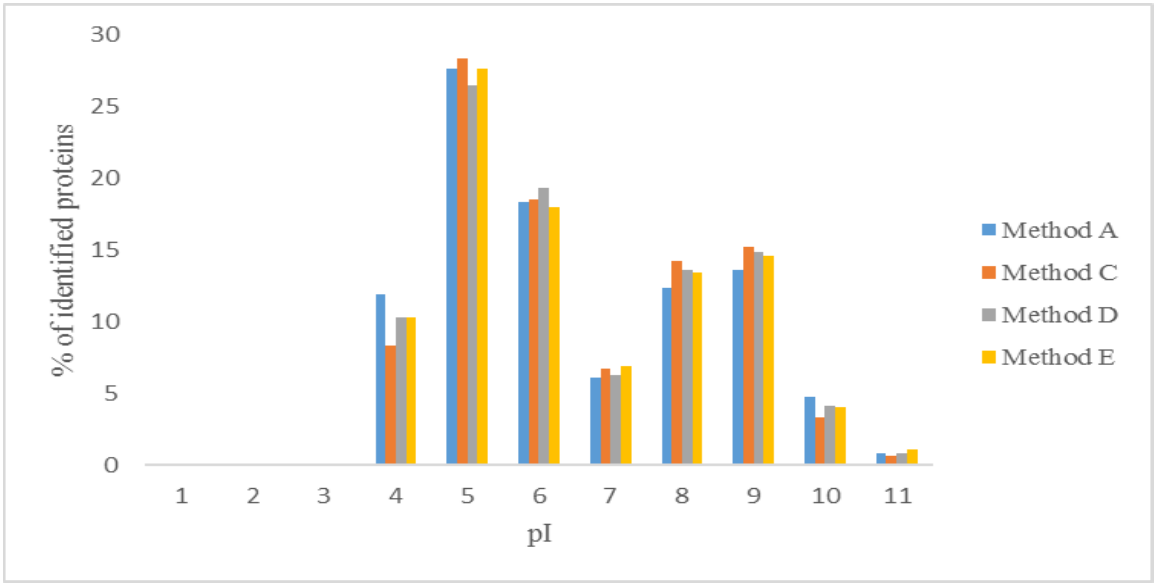
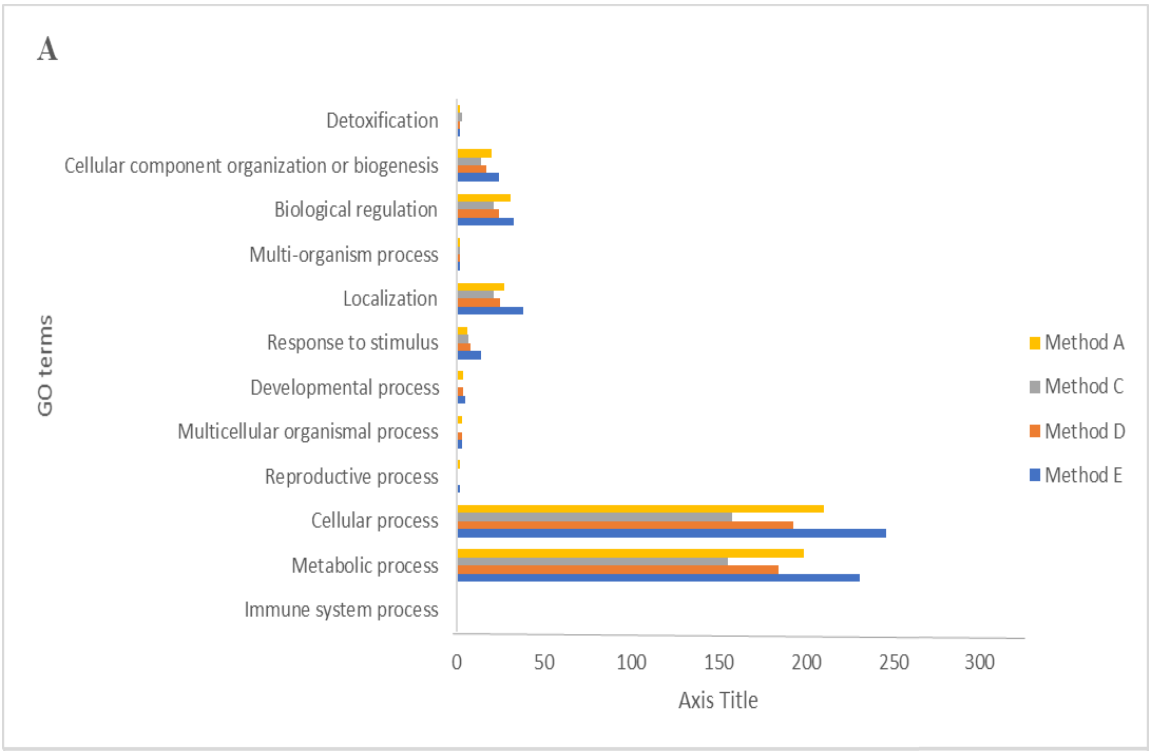
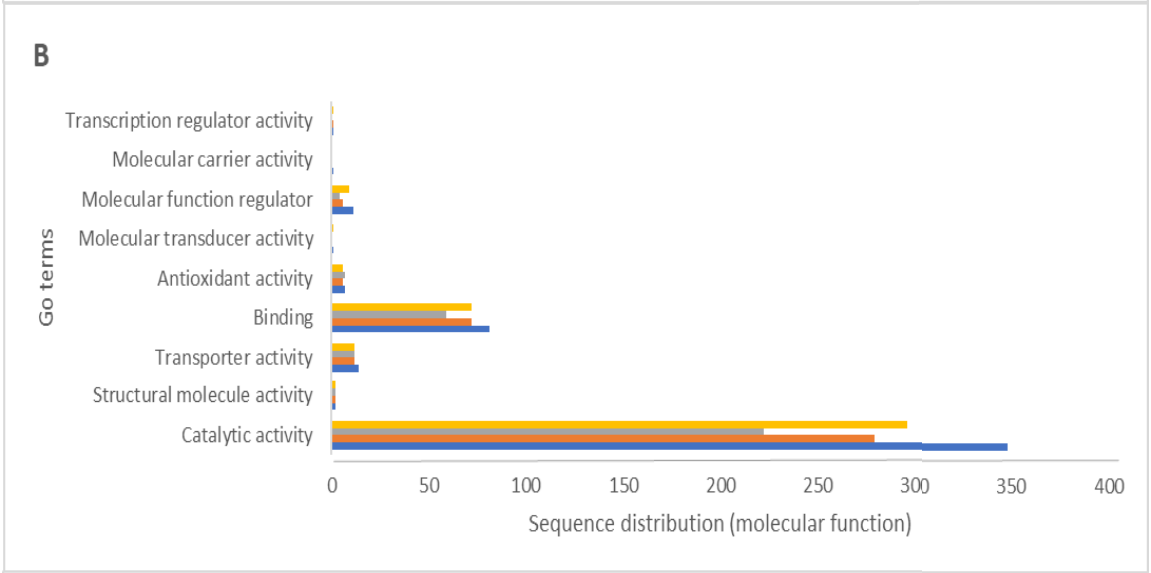


Figure 4-8. Distribution of isoelectric focusing of the identified proteins in peptide-centric methods.

3.2.2. Gene Ontology (GO) and Functional annotation:

The resulting PEAKS Studio files from each peptide-centric methods were exported in a mzIdentML format and analysis for their gene ontology in the three broad categories of biological process, molecular function and cellular component. The majority of the expressed proteins were shown to be involved in metabolic and cellular processes with catalytic (enzymatic) and binding activities, located in intra cellular and within a protein-macromolecule complexes (Figure 4-9).


A
Detoxification
Cellular component organization or biogenesis
Biological regulation
Multi-organism process
Localization
Response to stimulus
Developmental process
Multicellular organismal process
Reproductive process
Cellular process
Metabolic process
Immune system process
GO terms
Method A
Method C
Method D
Method E
0
50
100
150
200
250
300
Axis Title


B
Transcription regulator activity
Molecular carrier activity
Molecular function regulator
Molecular transducer activity
Antioxidant activity
Binding
Transporter activity
Structural molecule activity
Catalytic activity
Go terms
0
50
100
150
200
250
300
350
400
Sequence distribution (molecular function)

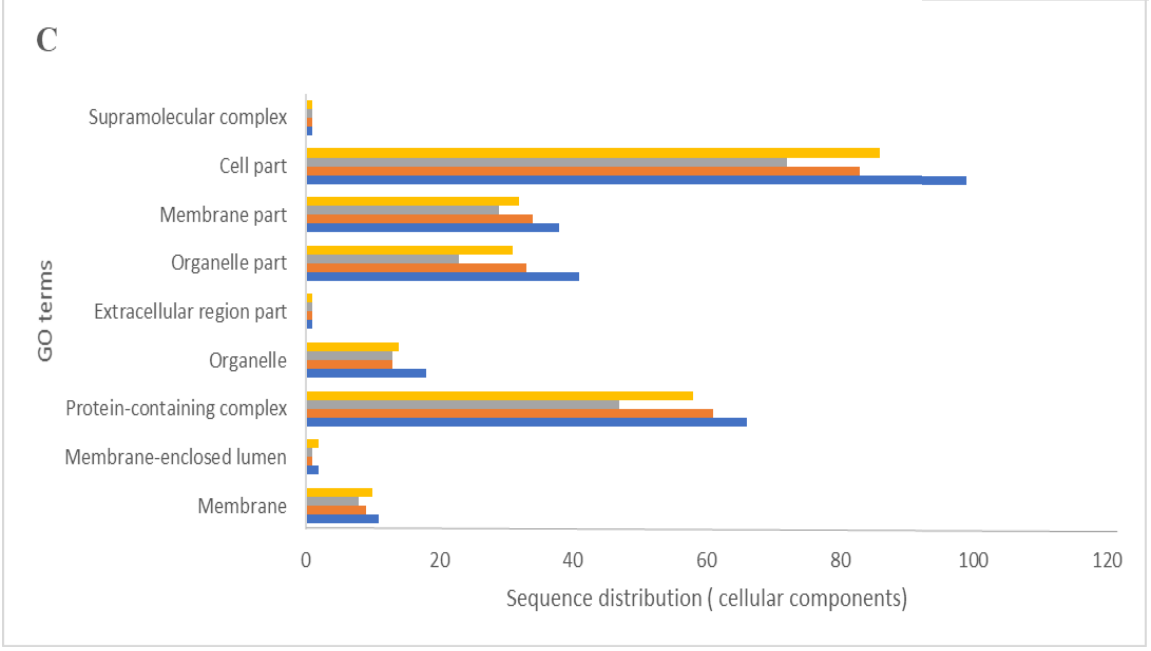

C
Supramolecular complex
Cell part
Membrane part
Organelle part
Extracellular region part
Organelle
Protein-containing complex
Membrane-enclosed lumen
Membrane
GO terms
0
20
40
60
80
100
120
Sequence distribution (cellular components)

Figure 4-9. Biological process (A), molecular function (B) and cellular component (C) of proteins uniquely identified in *Z. muelleri* using peptic-centric methods.

3.3. Identification of the total number of expressed proteins in *Z. muelleri* from peptide-centric methods

The resulting MS files of peptide-centric methods (combined) were searched against the *Z. muelleri* database using PEAKS Studio in order to identify the total number of expressed proteins from all peptide-centric methods performed. A total number of 5189 including 2,663 proteins with > 2 , 1,033 proteins = 2 and 1,304 = 1 matched peptide were identified. PEAKS results showed only the top hit for each identified protein and therefore, some duplication in the results might be expected. Thus, a Scaffold analysis for the combined methods was also conducted to identify the total number of uniquely identified proteins from *Z. muelleri* database. Parallel analysis with Scaffold using *Z. marina* and *Z. muelleri* databases showed a total number of 3,488 and 1,765 proteins were uniquely identified from Z. *muelleri* and *Z. marina,* respectively (with 247 proteins in common). The total number of identified proteins, against *Z. muelleri* was approximately 14.4% of the sequence database (5,189 out of 35,875 predicted genes the published annotation). The Scaffold file containing the list of identified proteins from Z. *muelleri* and *Z. marina* can be found in Appendix 19.

4. Discussion

In this work, our aim was to characterize the basal expressed proteome of the seagrass, Z. *muelleri* and provide the most comprehensive proteome dataset to date. Additionally, we determined the potential to quantify protein abundance changes using a protein-centric method (2-DE) compare to four peptide-centric methods. So far, few protein-centric proteomic studies have been published with seagrass species e.g. *Posidonia oceanica* (Spadafora et al. 2008; Mazzuca et al. 2009; Serra, Mazzuca & Pirog 2011; Dattolo et al. 2013), *P. australis* (Jiang et al. 2017) and *Z. muelleri* (Kumar, Padula, et al. 2016; Jiang et al. 2017). To our knowledge, there is no evaluation of protein-centric and gel-peptide-centric methods available for seagrasses to date.

4.1. 2-DE method provided functional classification for 350 expressed proteins in *Z. muelleri*

Despite the fact that 2-DE has the best resolving power and is a versatile and informative tool for comparative proteomic, it has several limitations as well. Issues reported include reproducibility (owing to the gel-to-gel and operator-to-operator variations), low sensitivity and narrow linear dynamic ranges in the detection methods, poor resolution to identify low abundant proteins, poor representation of highly acidic/basic proteins or proteins with extreme size or hydrophobicity (Carpentier et al. 2008; Abdallah et al. 2012; Padula et al. 2017). Further, the co-migration of multiple proteins in a single spot highlights possible inaccuracy for comparative quantification (Rabilloud et al. 2010; Abdallah et al. 2012). This study also faced some of these challenges, wherein among a total of 325 identified proteins most were populated primarily by acidic proteins on a pI range 5 – 8. This is in agreement with Candiano et al. (2002) who reported that current commercially available 4% total acrylamide IPG gel strips limit the high molecular weight proteins visualized with 2-DE.

A small number of protein spots yielded more than one protein identification; thus, a total of 350 proteins were identified in the 325 protein spots indicating co-migration of multiple proteins in the first and second dimensions. For example, spot 59 contained peptides that were confidently identified both a Aldehyde dehydrogenase and a Adenosylhomocysteinase. Both proteins have very close theoretical Mw (58.51 and 53.53 kDa) and pI (6 and 5. 89), and could quite possibly co-migrate due to differing post-translational processing events (Ly 2018). Similarly, spot 60 showed the presence of Proteasome subunit alpha type-3 and Catalase-peroxidase with theoretical Mw (27.62 and 28.14) and pI (5.85 and 5.99). The presence of co-migrating proteins could complicate quantification in future experiments and will necessitate alternative methods to confirm quantitative differences (Lei et al. 2005). Further, the identification of only 325 proteins in 350 spots, probably reflects multiple post-translational modification to create new proteoforms, which appeared in more than one spot. Similar findings were observed in the 2-DE based proteome reference mapping of the model higher plant *Arabidopsis* (Braun & Senkler 2012), chickpea (Barua et al. 2016), soybean (Komatsu et al. 2017) and Medicago (Lei et al. 2005).

Some of the protein spots differed in accession and descriptions but belonged to similar protein family in terms of their functionality when searched against *Z. marina* and *Z. muelleri* individually. Since the *Z. muelleri* genome has not been completely annotated, it is possible that some proteins that are slightly different in protein sequence, but belong to the same family, were identified with the different accession number corresponding to a protein from another species. In this case, it is expected that the identification relies on the peptides that are the most conserved and thus that are common between isoforms. Redundancy might also result from proteolytic cleavage that occurs *in vivo* or during homogenization (Lei et al. 2005; Rabilloud et al. 2010).

As expected, the vast majority of proteins identified in this work were soluble proteins as 2-DE analysis of membrane proteins still remains a significant challenge for their hydrophobic properties (Lei et al. 2005; Rabilloud et al. 2010; Rawling 2016). The overall hydrophobicity of a protein is helpful in predicting its solubility in a buffer. In the present study, only a handful of membrane-associated proteins were identified (peptides with positive GRAVY score in Appendix 7).

In the present study, we were able to excise 412 spots that were visible to the naked eye after Coomassie blue (CB) staining. Since, the CB has low sensitivity (50 ng of detectable protein/spot) and narrow linear dynamic range of detection, a better stain compatible for mass spectrometry such as florescent stains including Sypro Ruby, Flamingo and Deep Purple which have sensitivities of 1 ng of detectable protein/spot could be used for future 2DE based studies in seagrasses; however an automated spot picker robot will be essential when working with such fluorescent dyes. Moreover, to overcome the protein ratio error due to low gel-to-gel reproducibility, the inclusion of 2D-DIGE (difference gel electrophoresis) should be tested, which enables protein detection at sub-picomolar levels and relies on pre-electrophoretic labelling of samples with fluorescent CyDyes (Cy2, Cy3, and Cy5) (Tannu & Hemby 2006). Despite several drawbacks linked with the gel based proteomic approach, 2D gels are still preferred because it exhibit highest resolution on protein level, displays hundreds to thousands of protein species, their isoforms and post-translational modifications at the same time (Westermeier 2016).

4.2. The combination of 1D-PAGE and SDB-RPS provided the best protein coverage for expressed proteins in *Z. muelleri* among four tested peptide-centric methods

We investigated four peptide-centric proteomic methods in this study as it is expected that these methods would increase proteome coverage, but possibly at the expense of individual proteoform sequence coverage (Zhang et al. 2013). The challenge for peptide-centric methods arises with protein digestion which separates peptides from their parent proteoform and complicates the reliability of matching peptides for accurate protein identification (McDonald & Yates 2002). Also, similar to the majority of peptide-centric proteomic studies across organisms, the presence of gene products translated to proteins are identified rather than mature functional proteoforms. In this study, identification was even more challenging since the *Z. muelleri* database is only partially annotated and therefore, the identification of expressed gene products was mainly based on the better-curated *Z. marina* database. Therefore, proper identification of expressed proteins would be challenging when applying peptide-centric methods in *Z. muelleri* samples. For these reasons, we only evaluated the peptide recovery from each peptide-centric methods in this study.

The most simplified peptide-centric method is in-solution digestion (ISD) or direct protein digestion (Klont et al. 2018). ISD (Method A) was our second least effective method with 2,017 identified proteins. A possible reason for low peptide coverage with this method might be due to the presence of surfactants from previous protein extraction steps that were not compatible with protein digestion, chromatography and MS (Aebersold & Mann 2003). Although the number of identified proteins was low in this method (Klont et al. 2018), a good precision of protein quantification was obtained since this method does not include any contaminant removal step where peptides could be lost. Our results also showed the highest percentage of significant proteins identification (with -10lgP > 200) in ISD method compared to other peptide-centric methods. Therefore, minimal protein loss could usually be achieved with careful reagents selection in order to avoid column contamination and poor resolution chromatography (Klont et al. 2018).

Removal of contaminants using desalting methods significantly increased the detection of peptides in LC-MS/MS (Camerini & Mauri 2015; Feist & Hummon 2015;

Hernandez-Valladares et al. 2016; Gao et al. 2017). Therefore, we tested the efficiency of two desalting methods here; HILIC-based and SDB-RPS based columns. We were not able to retain sufficient quantity of peptides with C18 SPE-based column (Method B) for reasons that were not clear. The type of resin that we used for HILIC (polyhydroxyethyl resins) was previously shown to be effective for separation of proteins, amino acids, carbohydrates and metabolites (Jandera 2011). One possible explanation for the results is that residual plant compounds such as lipids, flavonoids, pigments and polysaccharides attached to the resins and interrupted the protein elution (Tolstikov, Fiehn & Tanaka 2007) in spite of our efforts to remove them. Furthermore, the separation of compounds in the HILIC column was based on polarity, hence some protein loss may occur (Berthod et al. 1998; Wang, Jiang & Armstrong 2008). Another previously reported challenge with HILIC columns is that the pH of the mobile phase cannot be accurately measured, which can also affect the elution of the compounds (McCalley 2010). Additionally, adding electrolyte (triethylammonium), salt or changing the resin types (or using magnetic beads) are also suggested to optimise the retention of proteins using SPE-based columns (Jandera 2011; Stoychev et al. 2012).

Conversely, SDB-RPS column improved peptide retention in our study. We used SDB-RPS alone (Method C) and in combination with peptide-IEF (Method D) and 1D-PAGE (Method E). Successful application of the SDB-RPS method in the present study was consistent with previous plant-based experiments using the same desalting method (Muller et al. 2007; Escher et al. 2008; Svačinová et al. 2012). The combination of 1D-PAGE and SDB-RPS (Method E) provided the highest number of identified proteins (4,440) and highest percentage of method-exclusive proteins (1,155). SDB-RPS method alone (Method C) was the second most effective method with 3,831 proteins identified. The improvement in the number of identified proteins using the SDB-RPS column is likely to be due to the ability to remove non-protein contaminants from the gel pieces prior to trypsin digestion and peptide recovery. Recent improvements in the method to remove extremely hydrophobic contaminants, such as lipids and surfactants, may further improve peptide recovery and proteome coverage (Meier et al. 2018).

On the other hand, the combination of SDB-RPS with peptide IEF (Method D) seemed to slightly decrease the number of identified proteins (3,018 identified proteins as our third best method). Isoelectric focusing separates peptides based on their charge

at a particular pH and is a powerful technique to reduce the complexity of a sample as well as increasing the possibility of identification of low abundance proteins (Cargile et al. 2004; Issaq et al. 2005). However, the recovery from immobilized strips has been reported as challenging for some of the peptides (Hubner, Ren & Mann 2008). Jafari et al. (2012) reported a similar method comparative analysis between 1D-PAGE and IEF-IPG method and reported slightly lower depth of protein profiling in IEF-IPG method. According to their experiment, a significant loss of highly acidic and basic proteins, as well as low retention rate of low molecular weight peptides from gel matrix are expected using IPG strips (pH = 3 − 10). These results correlate with those observed in this work.

We investigated the physio-chemical characterization properties of identified proteins including hydropathy, molecular weight and isoelectric points (pI) for deeper method evaluation and proteome analysis. As expected, all peptide-centric methods shown similar patterns in all three characteristics. An average of 85% of the identified proteins from peptide-centric methods were shown to be hydrophilic with maximum GRAVY scores between -0.4 and 0.0; which is similar to higher plants such as barely (Mahalingam 2017), rice (Yang et al. 2013) and spinach (Sahay & Shakya 2010). The percentage of hydrophilic proteins were slightly lower in our peptide-centric results (77%), but the range was wider (− 0.6 and 0). This suggested the possibility that the majority of our identified proteins to be interactive in water (Sahay & Shakya 2010). Approximately 15% of the proteins were shown to be hydrophobic which can be interpreted as membrane proteins.

The pI value of identified proteins from peptide-centric methods showed a bi-modal pattern with the majority of the proteins to be in the range of 5 − 6 or 8 − 9. Similar patterns are reported by Mahalingam (2017) for barely with ISD method and by Salvato et al. (2013) for potato with 1D-PAGE method. The pI value in protein-centric approach was similar but with slightly narrower range (5 – 8). It should be noted that since a single protein extraction method is used for all peptide-centric methods in this work, any protein that is insoluble at the extraction level, will not be identified. Therefore, the physio-chemical characterization properties of identified proteins in peptide-centric methods in this work was mainly used for method evaluation rather than the property of the whole proteoform. In fact, our GO results showed that most of the identified proteins were annotated as intra-cellular proteins or

parts of protein-macromolecule complexes which also confirms that the extraction procedure is favouring soluble protein extraction rather than membrane proteins (Rowling 2016). In fact, there is a significant percentage of identified proteins with catalytic and binding activities as part of cellular and metabolic processes, which further supports our assumption of preferential extraction of soluble proteins. However, given the high number of genes without GO annotation this may also be an artifact of incomplete or insufficient genome annotation in *Z. muelleri* and *Z. marina*.

5. Conclusion

To summarize, we evaluated five proteomic methods to profile the extent of the expressed proteome of *Z. muelleri* using protein-centric and peptide-centric approaches that will further contribute to the accurate characterisation of seagrass responses to environmental stresses. Peptide-centric methods provided better protein coverage compared to the protein-centric method. The SDB-RPS fractionation method provided the greatest proteome coverage of all of the techniques evaluated. However, proper classification of expressed proteins was challenging due to separation of peptides from their original proteins as well as the partially annotated genome of *Z. muelleri*. Thus, we only assessed the efficiency of each method and reported the correspondent accession IDs. According to our results, only 21% of the identified proteins were common to all of the methods, which further reinforced the fact that characterisation of the entire proteome of a cell is beyond the capability of any single proteomic method (Ong et al. 2003). Therefore, the combination of multiple methods is required to provide a wide proteome coverage. This was the first attempt, to our knowledge, to evaluate the amount of peptides that can be mapped back to gene products in a database as well as indicating the total number of expressed proteins in the seagrass, *Z. muelleri* from five proteomic methods under specific time and environmental conditions. We acknowledge that the comprehensive profiling of *Z. muelleri* is still far from completion, but the list of 5,189 expressed proteins in this study can be a useful resource to further enrich the *Z. muelleri* protein database. Further investigations for identification of these expressed proteins is suggested for future work.

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CHAPTER 5

Analysis of the proteomic profile of the seagrass, *Zostera muelleri* under Cu stress.

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Abstract

Seagrasses are exposed to a wide range of environmental stresses, including pollution, as a consequence of population growth. Industrial waste as well as agricultural and domestic run-off are sources of pollutions and often rich in trace elements, which can be deleterious to the physiology of seagrass meadows. The molecular response to trace metal toxicity to seagrasses remains poorly understood compared to the response in terrestrial plants.

The emergence of omics technology (e.g. proteomic, transcriptomic and metabolomics) enables the investigation of the molecular mechanisms and pathways that drive stress responses in cells. These technologies, which are now emerging in seagrass research, hold great potential for the discovery of biomarkers that can enable the early detection of toxicity in seagrasses exposed to elevated levels of trace elements such as Cu.

We examined the transcriptome of the Australian seagrass, *Z. muelleri* that had been exposed to 250 µg Cu L^{-1} and 500 µg Cu L^{-1} for 7 days in Chapter 3. We observed a concentration-dependent regulation of the genes involved in photosynthesis, energy production and defence mechanisms. To better understand the molecular mechanisms involved in the Cu stress response, quantitative proteomic was performed in this chapter on *Z. muelleri* that had been exposed to 500 µg Cu L^{-1} for 7 days using iTRAQ (isobaric tags for relative and absolute quantification). A total of 171 differentially expressed proteins mostly related to carbohydrate metabolism and genetic information processing pathways were identified. Additionally, proteins related to photosynthesis, energy metabolism and enzymatic defence mechanisms were selected for further identification and comparison with transcriptomic data that was previously described in Chapter 3. From a total of 76 proteins analysed, the majority of the proteins shown an increase in their abundance as a result of exposure to Cu stress, except for proteins related to photosynthesis and carbon fixation.

This study provided new insights into the molecular processes of trace metal toxicity in seagrasses at the proteomic level as well as a protocol that was developed to assist with identification of the proteins whose abundance are altered by Cu stress in *Z. muelleri*. Since seagrasses are globally declining, the development of biomarkers is crucial for early detection of the type of stress level in seagrasses. Hence, proteins

correlated with Cu stress in this study have the potential to be used as potential biomarkers for implementing more effective conservation strategies with seagrass meadows.

1. Introduction

Industrialisation and anthropogenic pollution have been shown to have considerable impacts on the physiology and health of terrestrial and submerged plants, including seagrasses (Waycott et al. 2009; Miransari 2011; Singh et al. 2016). An imbalance between a plant's energy production and metabolism in combination with impairment of photosynthetic subunits as a result of environmental stress causes perturbations in the metabolic pathways (Kishor et al. 2005). However, the identification and characterization of stress-induced proteins in this process is still an open question for seagrasses.

Cu is an essential trace metal for many of the physiological and biological processes in plants and seagrasses including photosynthesis and enzymatic defence mechanisms (Katoh 1977; Barón, Arellano & Gorgé 1995; World Health Organization 1996; Kaufman Katz et al. 2003; World Health Organization 2011; Singh et al. 2016). However, the release of excessive levels of Cu from agriculture and mining sites into the environment can adversely affect seagrass health by inhibiting its growth and photosynthetic efficiency (Barón, Arellano & Gorgé 1995; Yruela 2005). Furthermore, Cu contaminated seagrass meadows can negatively affect the marine food chain and jeopardise the existence of the marine species that depend on seagrasses as a source of food and habitat (Ouzounidou 1993; Pandey & Sharma 2002; Short et al. 2007; Kouki et al. 2012; Grassi et al. 2015).

Zostera muelleri is one of the dominant seagrass species that inhabits the shallow waters along the coast of Australia, New Zealand and Papua New Guinea (Dennison et al. 1993; The IUCN Red List of Threatened Species 2018). Recent advances in transcriptomic and proteomic methods have identified the complex regulatory mechanisms that *Z. muelleri* employs in response to light stress (Davey et al. 2017; Kumar et al. 2017). The establishment and implementation of such omics tools provided useful information that was implemented in Chapter 2 and 3 in order to have a better insights into the mechanisms of Cu stress tolerance in seagrasses.

Transcriptomic analysis is an essential step to understand Cu toxicity mechanism in *Z. muelleri* by explaining the functionality of the genome and molecular basis of the cellular response (Wang, Gerstein & Snyder 2009). However, the elucidation of the toxicity response at phenotypic level can only be shown at the proteomic level (Gygi et al. 1999; Hossain & Komatsu 2013). Additionally, proteomic profiling is useful to identify the interaction between biological processes involved in trace metal toxicity responses (Ahsan, Renaut & Komatsu 2009). Nonetheless, the toxic effects of Cu stress have not been studied for *Z. muelleri* at the proteomic level to date.

Recent improvement in mass spectrometry-based proteomic techniques including iTRAQ has enabled the investigation of protein abundance of multiple samples at the same time which reduces the chance of technical error (McDonagh et al. 2012). Identification and quantification of Open Reading Frame (ORF) products from up to eight samples can be achieved simultaneously using isobaric tags that specifically label the primary amines of peptides (Ross et al. 2004; Evans et al. 2012). In this study, we used six iTRAQ labels to analyse the proteomic profile of *Z. muelleri* exposed to 500 µg Cu L^{-1} which was shown as the most sensitive Cu concentration in Chapter 3 (page 88). Additionally, in order to identify the ORF products whose abundance were altered by Cu in *Z. muelleri*.

2. Materials and Methods

2.1. Sample collection and set-up for aquaria

Whole plants of *Z. muelleri* were collected from Pittwater, New South Wales, Australia (33°38′45.6′′S. 151°17′12.8′′E) and transferred to the University of Technology Sydney (UTS) within 2 hours of collection under dark conditions as previously described in Chapter 2 (page 51). The plants were rinsed with artificial seawater (30 psu salinity) prior to transplantation into aquaria containing 4 cm of sand (50% washed sand and 50% natural sediment). The aquaria (2 control and 2 for Cu treatment) were set up as described in Chapter 2 (page 51) under the following conditions: 30 psu salinity, 21°C temperature and 200 µmol photons m^{-2} s^{-1} light intensity. Each aquarium contained one RGBW aquarium light (Cidley 250W), one submerged pump (Elite mini, Hagen, Canada) and one air stone.

2.2. Treatment of *Z. muelleri* with Cu

Z. muelleri plants were acclimatise for 14 days and photosynthetic efficiency was measured daily using a Diving-PAM (Walz GmbH, Effeltrich, Germany) until stable effective quantum yield (ϕPSII) was measured (data not shown) as explained in Chapter 2. $CuCl_2$ was added to treated aquaria as a single dose out of a stock solution (concentration of 1000 µg Cu L^{-1}) to give a final concentration of 500 µg Cu L^{-1} (7.8 µM) at midday. Leaf samples from control and Cu treatment aquaria were harvested on day 7 randomly as three biological triplicates from two replicate tanks per treatment (n = 6). Samples were further rinsed with natural seawater and snap frozen in liquid nitrogen prior to proteomic analysis.

2.3. Protein extraction

Proteins were extracted from the leaves of *Z. muelleri* plants following the protocol described in Chapter 4 (page 125) with one modification: Tris was replaced with triethylammonium bicarbonate (TEAB) in the protein extraction buffer as preliminary results indicated that presence of this reagent improved the quality and yield of extracted protein (protein assay result of 5.867 µg/ µL using TEAB versus 5.487 µg/ µL using Tris). Also, the reagents containing primary amines such as Tris buffer should be avoided due to their interference with the efficiency of iTRAQ labelling (Chiappetta et al. 2009). Briefly, the ground leaf tissue of *Z. muelleri* was re-suspended in extraction buffer, mixed with Tris-saturated phenol (pH 8.0) and precipitated in 0.1 M ammonium acetate in methanol. Similarly, precipitated proteins were re-suspended in 8 M urea in 100 mM TEAB, reduced and alkylated as described in Chapter 4 (page 125).

After fixing the electrophoresed SDS-PAGE gel containing protein samples with 40% methanol and 10% acetic acid, a protein assay was carried out to assess protein concentration as described in Chapter 4 (page 125) by staining the gel with Flamingo fluorescent stain (Bio-Rad). The gel was scanned in a Typhoon Laser Scanner 9500 (GE Healthcare) at an excitation wavelength of 512 nm and emission wavelength of 535 nm. Protein concentration determined by densitometry using bovine serum albumin as a standard as mentioned in Chapter 4 (page 125).

2.4. In-solution digestion (ISD) of proteins

Reduced and alkylated proteins isolated from Cu treated samples of *Z. muelleri* as well as control samples were digested using trypsin and analysed at the concentration of 1 µg / 5 µL by LC-MS/MS as described in Chapter 4 (page 126).

2.5. Peptide labelling by iTRAQ reagents

iTRAQ labels were used to quantify proteins by mass-spectrometry. Protein (30 µg) extracted from control and Cu treatment samples (6 samples in total) were labelled by mixing them individually with 8-plex iTRAQ labelling reagents (113, 114 and 116 for the control; 118, 119 and 121 for the Cu treated samples) that were pre-dissolved in isopropanol (50 µL). The pH of the samples were adjusted to approximately 8, using dissolution buffer (provided by manufacturer) prior to incubation at room temperature for 120 min.

2.6. An optimized SDB-RPS-based desalting method

Samples were pooled (60 µg) by combining 10 µg of each labelled sample and desalted using a SDB-RPS (styrenedivinylbenzene reverse phase solfonate) column. The tip containing the column was prepared as described in Chapter 4 (page 127) and Humphrey et al. (2018). Briefly, pooled labelled peptides were first acidified with 10% Trifluoroacetic acid (TFA) to a final concentration of 1% and then loaded onto a StageTip column. The tip was centrifuged at 3,000 xg until the sample completely passed through the column. The peptides trapped in the columns were washed twice (once with 90% isopropanol, 1% TFA and once with 1% TFA only), incubated for 10 min at RT with elution buffer (5% ammonium hydroxide and 80% ACN) and eluted by centrifugation. Eluted peptides were concentrated in a SpeedVac centrifuge (Savant DNA 120-Thermo Scientific), re-dissolved in MS loading solvent and analysed by LC-MS/MS system at the concentration of 1 µg / 5 µL using the same analysis setting as described in Chapter 3 (page 129).

Desalted pooled samples were also prepared for peptide fractionation. The steps were as described above except that at the elution step, the sample was fractionated using four elution buffers: 1) 100 mM ammonium formate, 0.5% formic acid and 20% ACN; 2) 100 mM ammonium formate, 0.5% formic acid and 40% ACN; 3) 150 mM ammonium formate (to increase pH) and 60% ACN; 4) 5% ammonium

hydroxide and 80% ACN. Peptides were concentrated and analysed in LC-MS/MS as mentioned above.

2.7. Bioinformatic analysis of peptides

2.7.1. iTRAQ analysis

Analysis of peptides were conducted as described in Chapter 4 (page 129) using PEAKS Studio v8.5 software (Bioinformatics Solutions, Waterloo, ON) with variable modifications set as cysteine propionamide, oxidised methionine, deaminated asparagine, as well as iTRAQ 8-plex. Parent mass error set at 30 ppm and fragment mass error tolerance selected to 0.1 Da. Reporter ion quantitation were performed for iTRAQ using ≥ 1.5 fold change, ≤ 1% false discovery rate (FDR) and ≥ 2 unique peptides. The search result of the peptide MS files, as well as the FASTA protein sequences of each method against *Z. muelleri* database can be found in Appendix 20 and 20. As stated in Chapter 4 (page 129), the term 'protein' in this work refers to an Open Reading Frame (ORF) in the database rather than a mature proteoform.

2.7.2. Identification of differentially expressed proteins

As described in Chapter 3, the FASTA file of differentially expressed proteins (171 proteins) was submitted to Blast2Go (version 5.05) for gene description, GO term, and InterPro IDs using best hits in the non-redundant database. Additionally, functional annotation of the expressed proteins was investigated for their correspondent biological process, molecular function and cellular component from Blast2Go results. The list of the proteins was also searched for *Z. marina* IDs and reported. The significance of expressed proteins was investigated using ANOVA (single factor) for p-value ≤ 0.05 and 0.01. Standard deviation of three biological replicates were calculated. Lastly, the functional classification of proteins was identified using BlastKOALA (v. 2.1).

3. Results

3.1. LC-MS/MS data analysis and functional annotation

We identified 171 differentially expressed proteins in *Z. muelleri* plants under Cu stress using iTRAQ (Figure 5-1). The FASTA file of differentially expressed proteins was submitted to Blast2Go software to obtain gene descriptions, GO terms and InterPro IDs. According to our results, the most widely induced biological processes were metabolic and cellular pathways (Figure 5-2). At the molecular level,

proteins with catalytic activity and binding properties were most affected by Cu stress. In the cellular components result, the categories of cell and cell part were shown to be mainly affected by Cu. The list of significantly expressed genes in both 500 µg Cu L^{-1} and 250 µg Cu L^{-1} with their correspondent GO, KEGG and InterPro identifications can be found in Appendix 22-A and B.

Statistical analysis of the data using ANOVA revealed 1) no statistical significance for differential expression for 3 proteins which were excluded from further analysis ; 2) a significant p-value ≤ 0.05 for 14 of proteins and 3) a significant p-value ≤ 0.01 for the remaining 154 proteins. The correspondent iTRAQ ratio, SD, p-value and significance results of all 171 proteins can be found in Appendix 23.

The functional annotation showed that the majority (33%) of Cu-induced proteins in *Z. muelleri* plants were linked to the carbohydrate metabolism (most specifically glycolysis) followed by genetic information processing (21%). As for the assessment of changes in the abundance of proteins, more than 80% (143 proteins out of 171 Cu-induced proteins) were increased in their abundance. The list of alterations in proteins abundance with their corresponding log2 fold change can be found in Table 5-1.

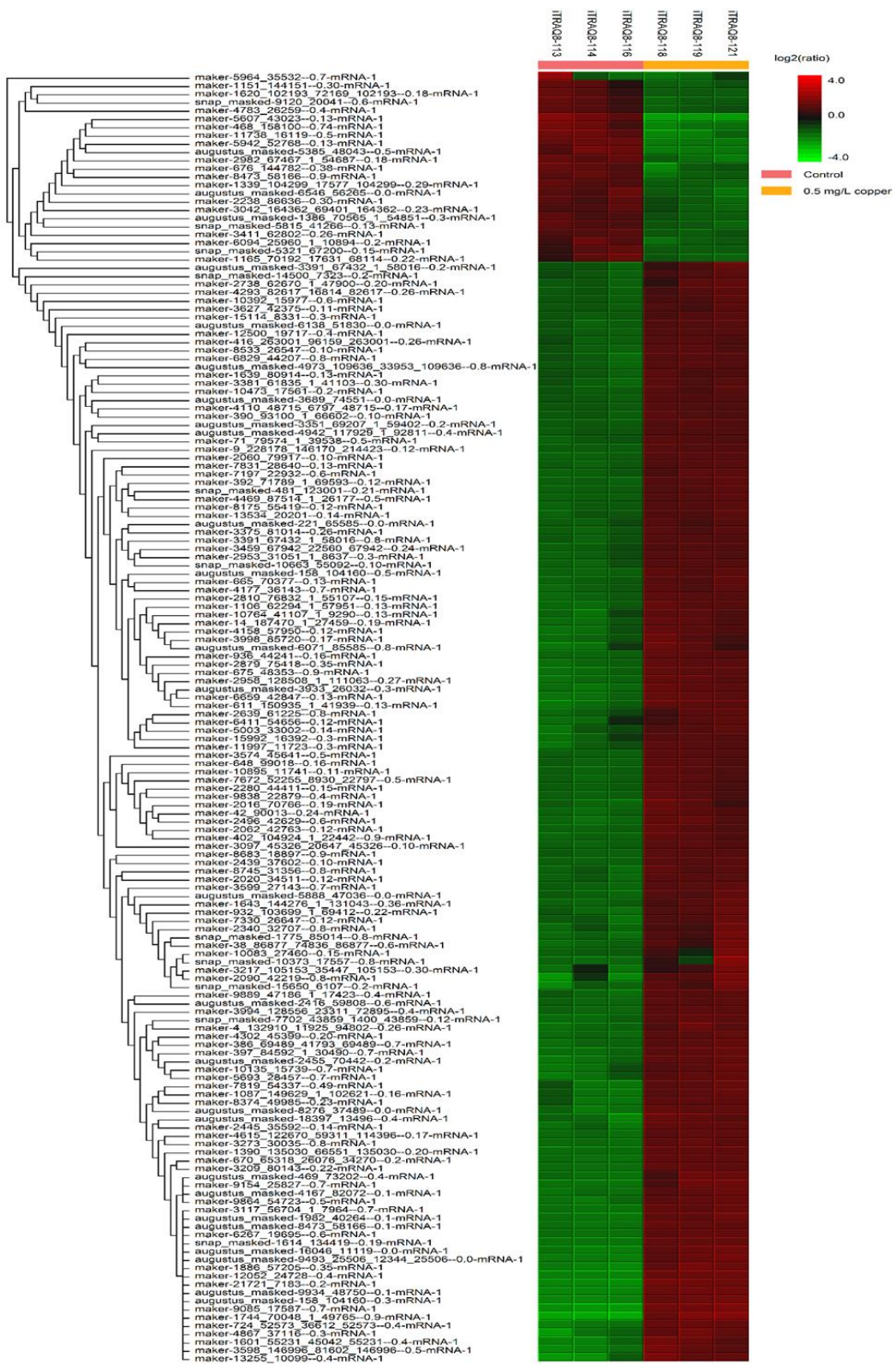
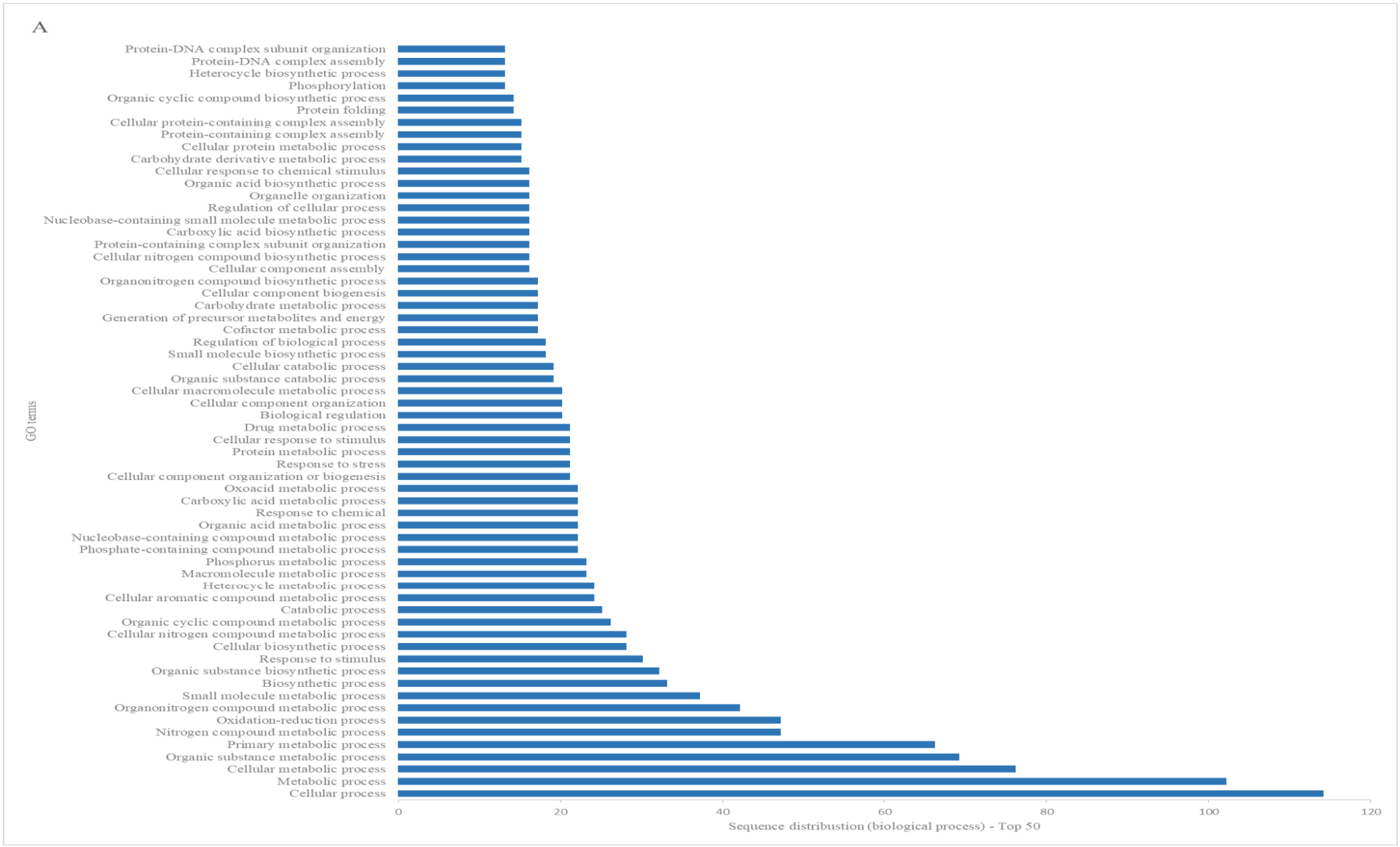
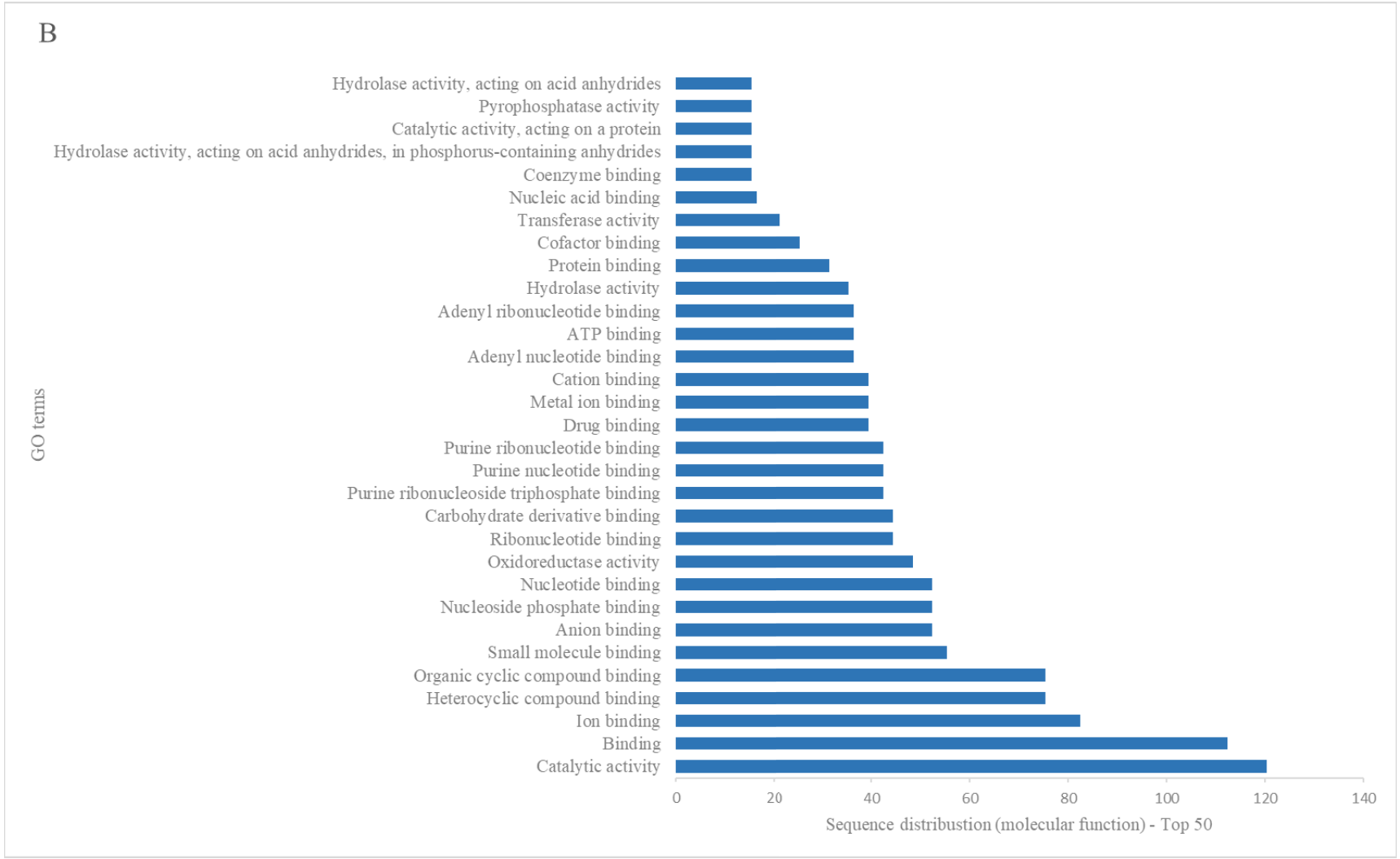


Figure 5-1. Heatmap of 171 differentially expressed proteins isolated from *Z. muelleri* plants exposed to 500 µg Cu L^{-1} for 7 days.


A
Protein-DNA complex subunit organization
Protein-DNA complex assembly
Heterocycle biosynthetic process
Phosphorylation
Organic cyclic compound biosynthetic process
Protein folding
Cellular protein-containing complex assembly
Protein-containing complex assembly
Cellular protein metabolic process
Carbohydrate derivative metabolic process
Cellular response to chemical stimulus
Organic acid biosynthetic process
Organelle organization
Regulation of cellular process
Nucleobase-containing small molecule metabolic process
Carboxylic acid biosynthetic process
Protein-containing complex subunit organization
Cellular nitrogen compound biosynthetic process
Cellular component assembly
Organonitrogen compound biosynthetic process
Cellular component biogenesis
Carbohydrate metabolic process
Generation of precursor metabolites and energy
Cofactor metabolic process
Regulation of biological process
Small molecule biosynthetic process
Cellular catabolic process
Organic substance catabolic process
Cellular macromolecule metabolic process
Cellular component organization
Biological regulation
Drug metabolic process
Cellular response to stimulus
Protein metabolic process
Response to stress
Cellular component organization or biogenesis
Oxoacid metabolic process
Carboxylic acid metabolic process
Response to chemical
Organic acid metabolic process
Nucleobase-containing compound metabolic process
Phosphate-containing compound metabolic process
Phosphorus metabolic process
Macromolecule metabolic process
Heterocycle metabolic process
Cellular aromatic compound metabolic process
Catabolic process
Organic cyclic compound metabolic process
Cellular nitrogen compound metabolic process
Cellular biosynthetic process
Response to stimulus
Organic substance biosynthetic process
Biosynthetic process
Small molecule metabolic process
Organonitrogen compound metabolic process
Oxidation-reduction process
Nitrogen compound metabolic process
Primary metabolic process
Organic substance metabolic process
Cellular metabolic process
Metabolic process
Cellular process
GO terms
0
20
40
60
80
100
120
Sequence distribustion (biological process) - Top 50


B
Hydrolase activity, acting on acid anhydrides
Pyrophosphatase activity
Catalytic activity, acting on a protein
Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides
Coenzyme binding
Nucleic acid binding
Transferase activity
Cofactor binding
Protein binding
Hydrolase activity
Adenyl ribonucleotide binding
ATP binding
Adenyl nucleotide binding
Cation binding
Metal ion binding
Drug binding
Purine ribonucleotide binding
Purine nucleotide binding
Purine ribonucleoside triphosphate binding
Carbohydrate derivative binding
Ribonucleotide binding
Oxidoreductase activity
Nucleotide binding
Nucleoside phosphate binding
Anion binding
Small molecule binding
Organic cyclic compound binding
Heterocyclic compound binding
Ion binding
Binding
Catalytic activity
GO terms
0
20
40
60
80
100
120
140
Sequence distribustion (molecular function) - Top 50

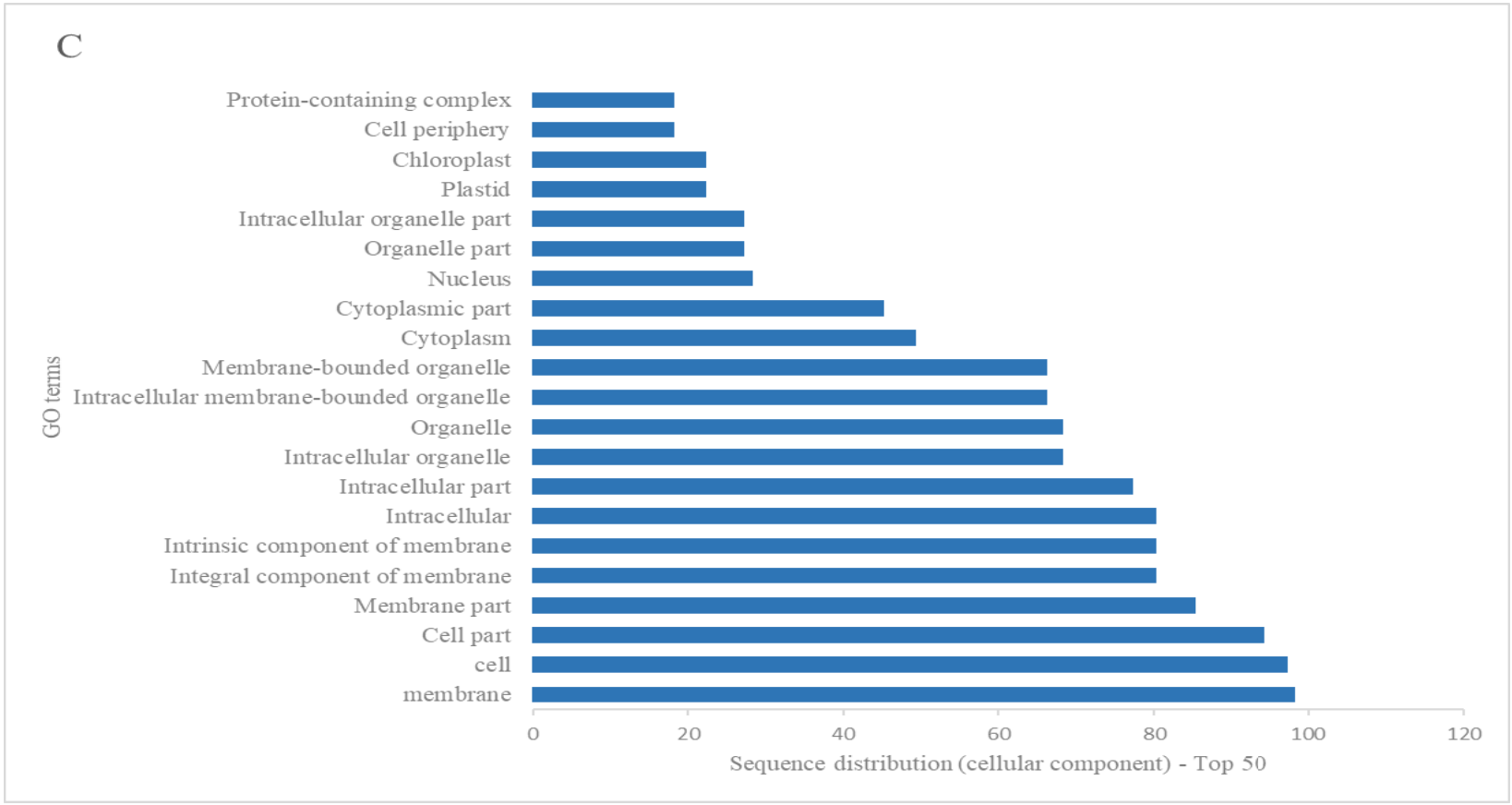


Figure 5-2. Sequence distribution of biological process (A), molecular function (B) and cellular component (C) of the top 50 differentially expressed proteins exposed to 500 µg Cu L^{-1}.

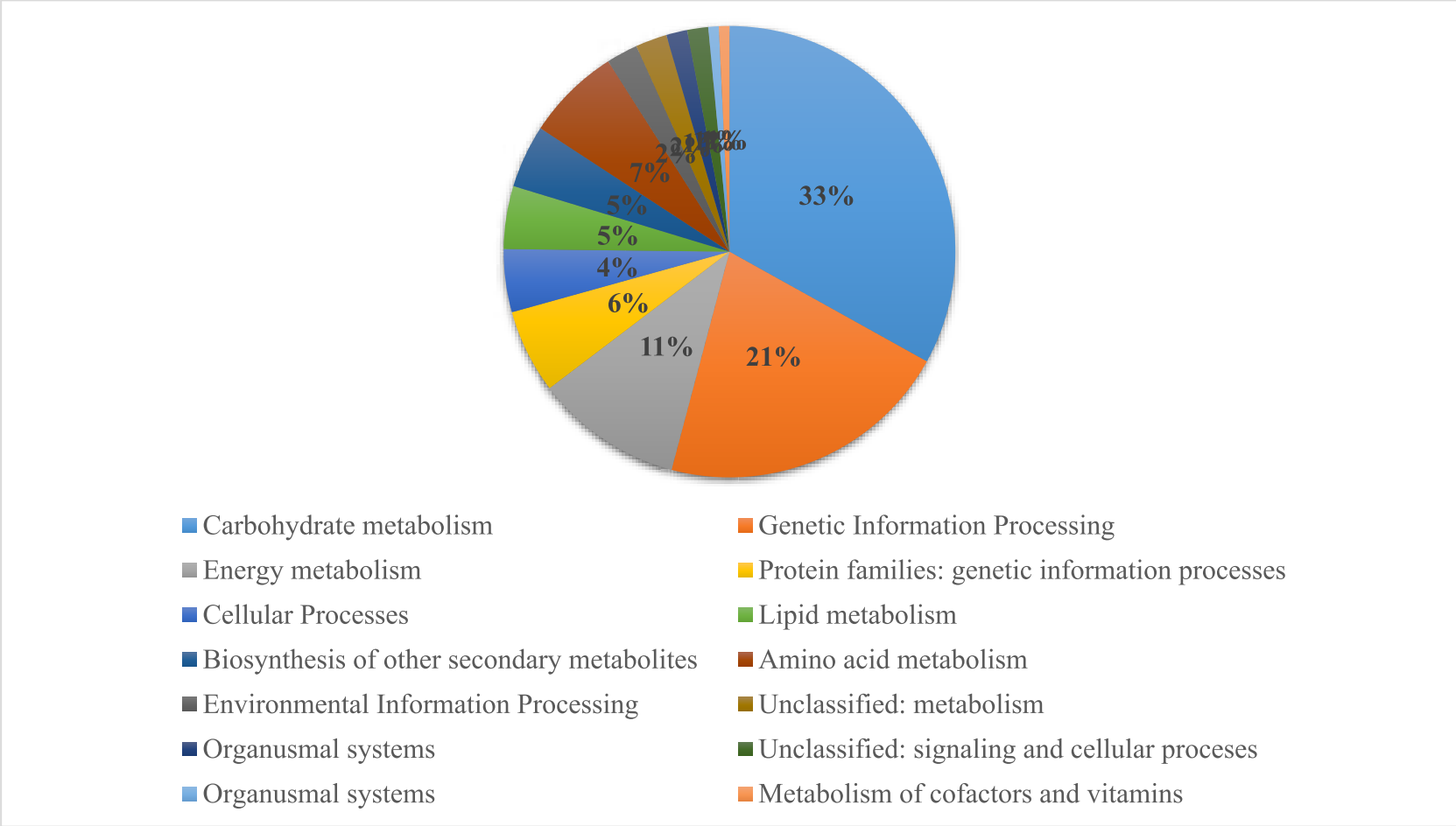


Figure 5-3. Functional annotation of proteins differentially expressed in *Z. muelleri* when exposed to 500 µg Cu L^{-1}.

3.2. Investigation of Cu-induced, differentially expressed proteins in *Z. muelleri* plants

Upon analysis of functional annotation of the expressed proteins, it was shown that carbohydrate metabolism and genetic information processing pathways comprised more than half of the expressed proteins that were significantly affected by Cu stress in *Z. muelleri* plants (Figure 5-3). At the transcriptomic level, as described in Chapter 3, transcriptional regulation of genes involved in photosynthesis, carbon fixation, energy metabolism and defence mechanisms were most affected by Cu stress. Therefore, to continue our investigation, 76 proteins expressed in this study that were linked to energy metabolism (in particular carbohydrate metabolism), genetic information processing, photosynthesis and enzymatic defence mechanisms were further investigated for their role and functionality in cellular pathways in order to better understand the mechanisms driving Cu toxicity in *Z. muelleri* plants (Figure 5-4 and Table 5-1).

Thirty proteins linked to energy metabolism were identified as Cu-induced in our study (Table 5-1). The abundance of the proteins involved with carbon fixation, including the RuBisCo large and small subunits and glyceraldehyde-3-phosphate dehydrogenase (cytosolic and chloroplastic) were decreased. On the contrary, proteins involved in glycolysis were all increased in abundance except for fructose bisphosphate aldolase (chloroplastic) which showed a decline in its abundance (-0.62 log 2 fold change). All proteins were significantly increased in abundance compared to the control at p-value ≤ 0.01 except for NADH dehydrogenase (mitochondrial), which was only significant at a p-value ≤ 0.05. One of the isomers of RuBisCo large subunits showed the highest level of log 2 fold change (1.47) and the RuBisCo small subunit showed the lowest (-0.97).

Twenty five identified proteins were annotated as being involved in genetic information processing pathways (Table 5-1), including cell division cycle protein 48 (CDC48), 60S acidic ribosomal protein, elongation factor (1 and Tu), proteasome subunit alpha (type 3,5 and 7), HSP (70, 80, 16.9 and 90.5), chaperonin 10kD and CPN60-2. All proteins showed positive change in their abundance and were significant for p-value ≤ 0.01, except one of the CDC48 proteins and the 10 kDa chaperonin which were only significant at p-value ≤ 0.05. Elongation factor Tu had the highest log 2 fold change of 1.02 and HSP70 had the lowest (0.59).

Six proteins associated with the photosynthesis process (Table 5-1) were reported in our study with significant decrease in their abundance (except PsbD2) at a p-value ≤ 0.01. Expression of chlorophyll a-b binding protein, were only significant at p-value ≤ 0.05. Cytochrome b_6f had the highest level of log 2 fold change (1.18) and PSII 10 kDa subunit had the lowest (-2.0).

Lastly, eleven antioxidant enzymes linked to the oxidative stress response (Table 5-1) were identified as Cu-induced in our results including glutathione s transferase (F13), peroxidase (2 and 5), glutathione peroxidase, copper/zinc superoxide dismutase 2, catalase (and catalase isozyme 3), ascorbate peroxidase, cytochrome c oxidase (subunit 6b), glutathione reductase and ascorbate peroxidase. All proteins had increased in their abundance and were considered significantly expressed at p-value ≤ 0.01. Peroxidase 5 had the highest log 2 fold change (2.27) and catalase isozyme 39 had the lowest (0.6).

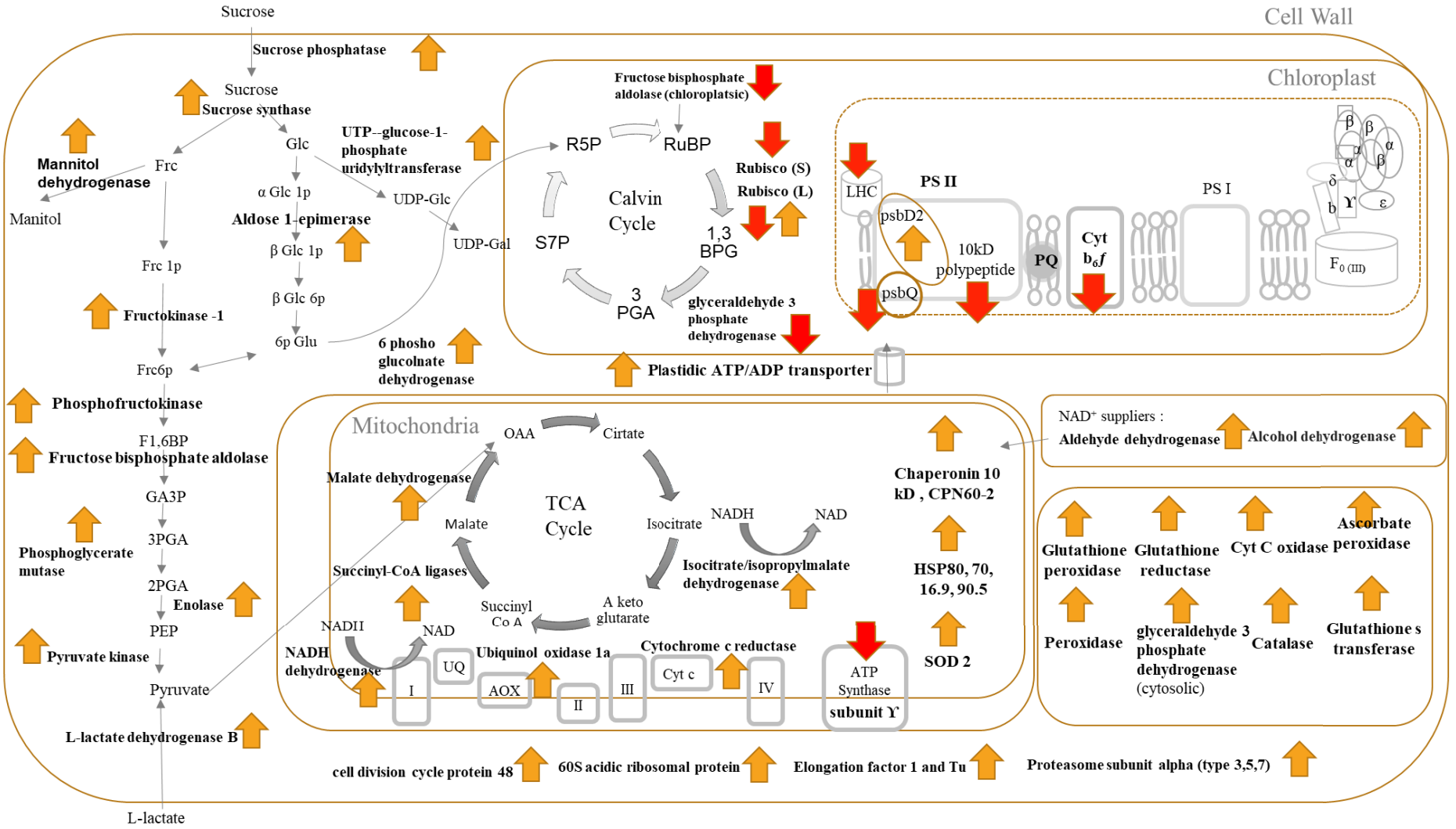

Cell Wall
Chloroplast
Sucrose
Sucrose phosphatase
Sucrose
Sucrose synthase
Glc
UTP--glucose-1-phosphate uridylyltransferase
Mannitol dehydrogenase
Frc
Manitol
α Glc 1p
UDP-Glc
Aldose 1-epimerase
β Glc 1p
UDP-Gal
Frc 1p
β Glc 6p
Fructokinase -1
6p Glu
6 phosho glucolnate dehydrogenase
Frc6p
Phosphofructokinase
F1,6BP
Fructose bisphosphate aldolase
GA3P
Phosphoglycerate mutase
3PGA
2PGA
Enolase
PEP
Pyruvate kinase
Pyruvate
L-lactate dehydrogenase B
L-lactate
Fructose bisphosphate aldolase (chloroplatsic)
R5P
RuBP
Rubisco (S)
Rubisco (L)
Calvin Cycle
S7P
1,3 BPG
3 PGA
glyceraldehyde 3 phosphate dehydrogenase
Plastidic ATP/ADP transporter
LHC
PS II
psbD2
10kD polypeptide
psbQ
PQ
Cyt b6f
PS I
F0 (III)
Mitochondria
OAA
Cirtate
TCA Cycle
Isocitrate
NADH
NAD
Isocitrate/isopropylmalate dehydrogenase
Malate dehydrogenase
Malate
Succinyl-CoA ligases
Succinyl Co A
A keto glutarate
NADH dehydrogenase
NADH
NAD
UQ
Ubiquinol oxidase 1a
AOX
Cytochrome c reductase
Cyt c
ATP Synthase subunit Υ
I
II
III
IV
NAD+ suppliers :
Aldehyde dehydrogenase
Alcohol dehydrogenase
Chaperonin 10 kD , CPN60-2
HSP80, 70, 16.9, 90.5
SOD 2
Glutathione peroxidase
Glutathione reductase
Cyt C oxidase
Ascorbate peroxidase
Peroxidase
glyceraldehyde 3 phosphate dehydrogenase (cytosolic)
Catalase
Glutathione s transferase
cell division cycle protein 48
60S acidic ribosomal protein
Elongation factor 1 and Tu
Proteasome subunit alpha (type 3,5,7)

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}. **Chloroplast:** PSII: photosystem II; RuBP: Ribulose 1,5-bisphosphate; 3 PGA: 3-phosphoglycerate; 1,3 BPG: 1,3 bis-phosphate glycerate; S7P: Sedoheptulose 7-phosphate; R5P: Ribulose 5-phosphate; PQ: plastoquinone; LHC: light harvesting complex; Cyt b_6f: cytochrome b_6f

Cytosol: Frc: Fructose;Glc: Glucose; UDP Glc: UDP Glucose, UDP Gal: UDP Galactose ; α/β Glc1P: α/β Glucose 1-phosphate; β Glc6P: α Glucose 6-phosphate; 6pGlu: 6 phosphor gluconate; Frc1p: Fructose 1 phosphate; Frc6p: fructose 6 phosphate; Frc 1,6 BP: Fructose 1,6 bis-phosphate; GA3P: Glyceraldehyde-3 phosohate; 3 PGA: 3-phospho glycerate; 2 PGA: 2-phospho glycerate; PEP: phosphoenolpyruvate;

Mitochondria: OAA: oxaloacetate; TCA cycle: citric acid cycle; I : Complex I; II: Complex II; III: Complex III; IV: Complex Iv; UQ : ubiquinone; Cyt c: cytochrome C; AOX : ubiquinol oxidase; SOD 2: superoxide dismutase 2.

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.

	Gene ID	**Description / protein name**	***Z.marina* accession ID**	**Log2 fold change**
	Photosynthesis			
1	maker-468_ 158100-0.74-mRNA-1	cytochrome b6-f complex iron-sulfur subunit, chloroplastic (*petC*)	KMZ63328.1	-1.18
2	maker-4783_26259-0.4-mRNA-1	oxygen-evolving enhancer protein 3-2, chloroplastic-like (*psbQ*)	KMZ70530.1	-0.59

3	maker-6094_25960_1_10894-0.2-mRNA-1	chlorophyll a-b binding protein CP29.1, chloroplastic-like (*LHC*)	KMZ74006.1	-0.76
4	maker-3391_67432_1_58016-0.8-mRNA-1	photosystem II protein D2, chloroplast (*psbD)*	YP_009433308.1	0.61
5	maker-5607_43023-0.13-mRNA-1	Photosystem II 10 kDa polypeptide, chloroplastic (*psbR*)	KMZ65203.1	-2
	Energy metabolism and carbon fixation			
6	maker-1165_70192_17631_68114-0.22-mRNA-1	ATP synthase subunit gamma, mitochondrial (*ATPC*)	KMZ62100.1	-0.68
7	augustus_masked-158_104160-0.3-mRNA-1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, chloroplast (*rbcL*)	BAD05100.1	1.47
8	maker-2238_86636-0.30-mRNA-1	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit, chloroplast (*rbcL*)	BAD05100.1	-0.62
9	maker-2982_67467_1_54687-0.18-mRNA-1	Chloroplast ribulose-1,5-bisphosphate carboxylase/oxygenase small subunit (*rbcS*)	KMZ72699.1	-0.97
10	maker-10083_27460-0.15-mRNA-1	Plastidic ATP/ADP-transporter	KMZ61857.1	0.67
11	augustus_masked-2455_70442--0.2-mRNA-1	Enolase (*eno*)	KMZ74878.1	1.02
12	maker-3042_164362_69401_164362-0.23-mRNA-1	Fructose-bisphosphate aldolase, chloroplastic (*FBA*)	KMZ64813.1	-0.62

13	maker-8175_55419-0.12-mRNA-1	Fructose-bisphosphate aldolase 6, cytosolic (*FBA*)	KMZ58915.1	0.66
14	maker-2445_35592-0.14-mRNA-1	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (*gpmI*)	KMZ75550.1	0.98
15	maker-2953_31051_1_8637-0.3-mRNA-1	6-phosphogluconate dehydrogenase, C-terminal domain (*PGD*)	KMZ60959.1	0.64
16	maker-10392_15977-0.6-mRNA-1	ATP-dependent 6-phosphofructokinase 3 (*PFK*)	KMZ71490.1	0.59
17	maker-4_132910_11925_94802-0.26-mRNA-1	probable fructokinase-1(*FRK-1*)	KMZ71113.1	0.88
18	maker-1106_62294_1_57951-0.13-mRNA-1	Pyruvate kinase 1, cytosolic (*PK1*)	KMZ68720.1	0.79
19	maker-8473_58166-0.9-mRNA-1	glyceraldehyde-3-phosphate dehydrogenase, cytosolic (*GADPH*)	KMZ64911.1	-0.81
20	snap_masked-5321_67200-0.15-mRNA-1	glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (*GADPH*)	KMZ61796.1	-0.66
21	augustus_masked-6546_56265-0.0-mRNA-1	Glyceraldehyde-3-phosphate dehydrogenase A, chloroplastic (*GADPH*)	KMZ61796.1	-0.81
22	maker-7831_28640-0.13-mRNA-1	Aldehyde dehydrogenase family 2 member B7, mitochondrial (*aldh*)	KMZ61155.1	0.68
23	augustus_masked-3689_74551-0.0-mRNA-1	aldehyde dehydrogenase family 7 member A1 (*aldh*)	KMZ69947.1	0.62

24	maker-3998_85720-0.17-mRNA-1	Malate dehydrogenase (*MDH*)	KMZ62786.1	0.73
25	augustus_masked-4973_109636_33953_109636-0.8-mRNA-1	L-lactate dehydrogenase B-like (*ldh*)	KMZ57501.1	0.69
26	snap_masked-1614_134419-0.19-mRNA-1	isocitrate dehydrogenase [NAD] catalytic subunit 5, mitochondrial (*Idh*)	KMZ65867.1	1.10
27	maker-12500_19717-0.4-mRNA-1	probable mannitol dehydrogenase (*MTD*)	KMZ69892.1	0.64
28	maker-8374_49985-0.23-mRNA-1	Aldose 1-epimerase	KMZ59492.1	0.91
29	maker-1886_57205-0.35-mRNA-1	Ubiquinol oxidase 1a, mitochondrial (*AOX1A*)	KMZ74933.1	1.41
30	maker-2090_42219-0.8-mRNA-1	NADH dehydrogenase [ubiquinone] iron-sulfur protein 1, mitochondrial (*NDUFS1*)	KMZ59646.1	0.96
31	snap_masked-10663_55092-0.10-mRNA-1	succinate--CoA ligase [ADP-forming] subunit beta, mitochondrial (*SUCLG2*)	KMZ74822.1	0.68
32	maker-11997_11723-0.3-mRNA-1	UTP--glucose-1-phosphate uridylyltransferase (*UGP1*)	KMZ76338.1	0.76
33	maker-2016_70766-0.19-mRNA-1	sucrose synthase 1 (*SUS1*)	KMZ65710.1	0.77
34	maker-3459_67942_22560_67942-0.24-mRNA-1	Alcohol dehydrogenase 1 (*Adh1*)	KMZ75204.1	0.58
35	maker-7330_26647-0.12-mRNA-1	sucrose-phosphatase 2-like (*SPP2*)	KMZ59293.1	0.74
36	maker-390_93100_1_66602-0.10-mRNA-1	cytochrome b-c1 complex subunit Rieske-4, mitochondrial-like	KMZ68400.1	0.61
	Enzymatic defence mechanism			

37	maker-1390_135030_66551_135030-0.20-mRNA-1	glutathione s-transferase F13 (*GSTF13*)	KMZ67334.1	0.94
38	maker-1744_70048_1_49765-0.9-mRNA-1	peroxidase 5 (*PER5*)	KMZ75156.1	2.27
39	augustus_masked-9934_48750-0.1-mRNA-1	Peroxidase 2 (*PER2*)	KMZ62607.1	1.50
40	maker-9085_17587-0.7-mRNA-1	Peroxidase 5 (*PER5*)	KMZ62607.1	1.76
41	maker-3599_27143-0.7-mRNA-1	ascorbate peroxidase (*APX*)	ALK24273.1	0.78
42	maker-4615_122670_59311_114396-0.17-mRNA-1	probable phospholipid hydroperoxide glutathione peroxidase (*GPX*)	KMZ63257.1	0.92
43	augustus_masked-16046_11119-0.0-mRNA-1	superoxide dismutase [Cu-Zn] 2 (*SOD2*)	KMZ72150.1	1.23
44	maker-9_228178_146170_214423-0.12-mRNA-1	Catalase (*CAT*)	AII23739.1	0.65
45	maker-10473_17561-0.2-mRNA-1	catalase isozyme 3 (*CAT3*)	AII23739.1	0.61
46	maker-3574_45641-0.5-mRNA-1	cytochrome c oxidase subunit 6b-1 (*COX6B-1*)	KMZ59275.1	0.72
47	maker-3598_146996_81602_146996--0.5-mRNA-1	glutathione reductase, cytosolic (*Gsr*)	KMZ69798.1	1.31
	Genetic information processing			
52	maker-611_150935_1_41939-0.13-mRNA-1	Heat shock cognate protein 80 (*HSP80*)	KMZ57743.1	0.93
53	maker-9864_54723-0.5-mRNA-1	heat shock cognate protein 80 (*HSP80*)	KMZ57743.1	1.04
54	maker-2958_128508_1_111063--0.27-mRNA-1	Heat shock protein 70, conserved site-containing protein (*HSP70*)	KMZ75128.1	0.89

55	augustus_masked-221_65585-0.0-mRNA-1	Heat shock protein 70, conserved site-containing protein (*HSP70*)	KMZ65999.1	0.59
56	augustus_masked-4167_82072-0.1-mRNA-1	70 kDa heat shock protein (*HSP70*)	KMZ75130.1	1.00
57	maker-6267_19695-0.6-mRNA-1	Heat shock 70 kDa protein, mitochondrial (*HSP70*)	KMZ59813.1	1.02
58	maker-397_84592_1_30490-0.7-mRNA-1	heat shock 70 kDa protein 15-like (*HSP70*)	KMZ59813.1	0.90
59	maker-2810_76832_1_55107-0.15-mRNA-1	heat shock 70 kDa protein 15-like (*HSP70*)	KMZ56466.1	0.77
60	augustus_masked-1982_40264-0.1-mRNA-1	heat shock protein 70, chloroplast (*HSP70*)	KMZ58558.1	1.10
61	maker-1087_149629_1_102621-0.16-mRNA-1	16.9 kDa class I heat shock protein 1-like (*HSP16.9*)	KMZ65668.1	0.91
62	maker-2639_61225-0.8-mRNA-1	heat shock protein 90-5, chloroplastic (*HSP90-5*)	KMZ74338.1	0.64
63	maker-4469_87514_1_26177-0.5-mRNA-1	10 kDa chaperonin-like	KMZ59992.1	0.71
64	maker-2738_62670_1_47900-0.20-mRNA-1	10 kDa chaperonin, mitochondrial-like	KMZ71583.1	0.64
65	maker-3994_128556_23311_72895-0.4-mRNA-1	Chaperonin CPN60-2, mitochondrial	KMZ67105.1	0.83
66	maker-2496_42629-0.6-mRNA-1	Cell division cycle protein 48 homolog	KMZ58253.1	0.91
67	maker-9889_47186_1_17423-0.4-mRNA-1	Cell division cycle protein 48 homolog	KMZ66154.1	0.79
68	maker-10895_11741-0.11-mRNA-1	60S acidic ribosomal protein P0	KMZ64797.1	0.70
69	maker-3375_81014-0.26-mRNA-1	Elongation factor 1-gamma 2	KMZ73245.1	0.67
70	augustus_masked-18397_13496--0.4-mRNA-1	Elongation factor Tu	KMZ57061.1	1.02

71	maker-3209_80143-0.22-mRNA-1	Proteasome subunit alpha type-3	KMZ65692.1	1
72	maker-3209_80143-0.19-mRNA-1	Proteasome subunit alpha type-3	KMZ65692.1	1
73	maker-3209_80143--0.19-mRNA-1	Proteasome subunit alpha type-3	KMZ65692.1	0.73
74	maker-665_70377--0.13-mRNA-1	proteasome subunit alpha type-5	KMZ65280.1	0.69
75	augustus_masked-6138_51830-0.0-mRNA-1	proteasome subunit beta type-5	KMZ65280.1	0.62
76	maker-7672_52255_8930_22797--0.5-mRNA-1	Proteasome subunit alpha type-7	KMZ65259.1	0.69

4. Discussion

The proteomic response of *Z. muelleri* to 500 µg Cu L^{-1} showed significant changes in the abundance of proteins involved in photosynthesis, carbon fixation, energy metabolism, enzymatic defence mechanisms and genetic information processing. Previous studies with terrestrial plants have shown that four regulatory mechanisms are involved in trace metal toxicity including: 1) competing for absorption; 2) deterioration of protein function as a result of alteration of their structure; 3) generation of ROS; and 4) modification of enzymatic activity by replacement of ions (Sharma & Dietz 2009; DalCorso et al. 2013). In our study, mechanisms 2, 3 and 4 were noted for Cu stress at the proteomic level.

4.1. Excess Cu negatively affects photosynthesis and carbon fixation in *Z. muelleri*

Exposure to 500 µg Cu L^{-1} showed that photosynthesis was significantly affected by Cu stress in *Z. muelleri*. We recorded a decrease in the abundance of PSII (subunits including psbQ, 10 kDa polypeptide) and light-harvesting complex (chlorophyll a-b binding protein CP29.1) in our study. The sensitivity of PSII to Cu stress was previously reported in higher plants at the physiological level (Cedeno-Maldonado, Swader & Heath 1972; Mohanty, Vass & Demeter 1989; Arellano et al. 1995; Jegerschoeld et al. 1995; Buapet et al. 2019). Additionally Hego et al. (2016) reported a decline in the abundance of PSII subunits and light-harvesting complex in *Agrostis capillaris* using 2-DE which is similar to our results with *Z. muelleri*. However, in contrast to the other PSII subunits, the abundance of protein psbD2 was shown to be increased as a result of exposure to 500 µg Cu L^{-1}. Komenda et al. (2004) reported that accumulation of the D2 protein is a required step for PSII assembly in *Synechocyctis sp* and therefore, the increased abundance of this protein might signify a repair mechanism induced in response to Cu-induced damage in PSII.

Carbon fixation was negatively affected by Cu stress. We identified a decline in the abundance of RuBisCo and glyceraldehyde 3 phosphate dehydrogenase (*GADPH*) in our study. Both small and large subunits of RuBisCo were negatively affected by Cu stress. Deterioration of RuBisCo as a result of Cu stress was in line with what was previously reported in higher plants at both physiological and molecular levels (Lidon & Henriques 1991; Schäfer, Simper & Hofmann 1992; Rakwal, Agrawal

& Yonekura 1999; Hajduch et al. 2001; Leng et al. 2015; Hego et al. 2016). It is thought that Cu is an inhibitor of RuBisCo for a wide range of species where it interacts with the cysteine residue of this enzyme (Stiborova, Ditrichova & Brezinova 1988; Hajduch et al. 2001). However, we noted that one of the RuBisCo large subunits showed the opposite result in our study with increase in their abundances which may refer to haplotype regions of the genome for RuBisCo where its confirmation was not affected by Cu stress (Andreakis et al. 2007; Galmés et al. 2014). The second carbon fixation protein that was affected by Cu was GADPH. In our study, the abundances of both isoenzymes of GADPH (cytosolic and chloroplastic) were decreased. Chloroplastic GADPH is thought to have a cyanobacterial origin whereas cytosolic GADPH reflects the evolution of the host and has more similarities to eukaryotes (Martin & Cerff 2017). While, chloroplastic GADPH only participates in glycolysis (Zaffagnini et al. 2013), cytosolic GDPH has an additional role to acts as a redox protein in response to abiotic stress (Yang & Zhai 2017). Similarly, inhibition of GADPH as a result of exposure to Cu and Cd was previously reported in cucumber (*Cucumis sativus)* (Burzynski & Zurek 2007). The mechanism of influencing the activity of GADPH is thought to be by binding the Cu to NAD which is a co-substrate of GADPH (Stanley et al. 2013).

4.2. Activation of oxidative stress in *Z. muelleri* under 500 µg Cu L^{-1}

Leakage of O_2^- linked to the impairment of the electron transfer chain and photosynthetic subunits could have initiated the oxidative stress response (Mittler et al. 2004). Similarly, we had increase in the abundance of glutathione s transferase, peroxidase, ascorbate peroxidase, glutathione peroxidase, superoxide dismutase 2 (mitochondrial), catalase, cyt c oxidase and glutathione reductase to scavenge extra ROS. It seems like the activation of antioxidant enzymes in response to Cu stress varies between species. For example, glutathione s transferase showed similar scavenging role in proteomic analysis of *Arabidiopsis* under Cu stress (Smith et al. 2004) whereas an increase in the abundance of superoxide dismutase was reported in *Oryza Sativa* exposed to elevated level of Cu (Hajduch et al. 2001). Additionally, proteome analysis of two Algae species (*Scytosiphon lomentaria* and *Scytosiphon gracilis*), showed activation of catalase, glutathione peroxidase, and ascorbate peroxidase similar to our results. Activation of ascorbate peroxidase was also reported

in the Cu toxicity response of German camomile (*Matricaria chamomilla) (*Kováčik et al., 2009).

4.3. An enhanced energy production is required to respond to Cu stress in *Z. muelleri*

A significant increase in protein abundances were recorded in the energy metabolism pathways, with 44 affected proteins. Activation of the pathways for catabolism for two particular carbohydrates, which are sources of energy storage were recorded: 1) sucrose, shown by an increase in the abundance of sucrose phosphatase (that transfers sucrose to cytosol) and sucrose synthase (that breaks down sucrose to fructose and glucose). 2) L-lactate shown by increase in the abundance of L-lactate dehydrogenase that converts lactate to pyruvate (Yamaguchi, Ikawa & Nisizawa 1966; Davies, Grego & Kenworthy 1974). On the other hand, we saw an increase in the abundance of enzymes corresponding to galactose and mannitol production were also recorded in our results which would infer that an activation of energy metabolism has also occurred. These precursors were udp-glucose 1 phospahte uridyltransferase and mannitol dehydrogenase in the galactose and mannitol pathways, respectively. Production of extra galactose and mannitol could also led to the production of another storage energy source which is glycogen and reported as a stress-induced metabolite as described by Doezema & Phillips Jr (1970).

Furthermore, we observed an increase in the abundance of energy suppliers such as ATP/ADP transporter protein (with one exception of ATP synthase subunit gamma) and NAD suppliers (aldehyde dehydrogenase 2 and 7 and alcohol dehydrogenase) in our study. Aldehyde dehydrogenase 7, in particular, was reported in previous studies as a conserved enzyme with a role in tolerance to abiotic stress in soybean and *Arabidopsis* (Kotchoni et al. 2006; Rodrigues et al. 2006) and might play the same role in Cu toxicity response of *Z. muelleri* as well.

Decrease in the abundance of protein subunits of PSII and light harvesting complex that was reported in section 4.1, was shown to initiate electron leakage and ROS production in chloroplast. Similarly in mitochondria of plants, an induction of ROS production was shown to taken place when exposing to a stress factor (Rhoads et al. 2006; Keunen et al. 2011). However, a Cu detoxification pathway seemed to be activated as a result of increase in the protein abundance of electron carriers in

mitochondria. Van Dongen et al. (2011) described an alternative pathway in his review that plants employ as a defence mechanism to scavenge ROS. The alternative bypasses ATP synthase in mitochondria to reduce O_2 production. In fact, we observed a similar result with the decline in the abundance of ATP synthase subunit gamma and increase in the abundance of NADH dehydrogenase, ubiquinol oxidase 1 and cytochrome c reductase. Induction of alternative respiratory pathway and an increase in the AOX protein content as a result of Cu stress were also reported in the higher plant, *A. pseudoplatanus* (Pádua et al. 1999). Therefore, this mitochondrial bypass pathway could be inferred as a defence mechanism against Cu stress in the mitochondria of *Z. muelleri*.

Correspondingly, glycolysis seemed to be highly active in our study. This included positive changes in the abundance of aldose 1 epimerase and 6 phospho gluconate dehydrogenase to provide glucose for the Calvin cycle, as well as for pyruvate production. Subsequently, the abundance of glycolysis enzymes such as fructokinase, phosphofructokinase, fructose bisphosphate aldolase, phosphoglycerate mutase, enolase and pyruvate kinase were also shown to be increased. The requirement for activation of carbohydrate catabolism to provide extra energy for detoxification of excess Cu was previously reported in higher plants at the molecular level which is in line with our results (Geiger, Servaites & Fuchs 2000; Lingua et al. 2012; Hego et al. 2016). Additionally, we found both isoenzymes of fructose bisphosphate aldolase (cytosolic and chloroplastic) in Cu-stressed leaves of *Z. muelleri* with different Cu responses; the abundance of cytosolic fructose bisphosphate aldolase which involves in glycogenesis (Rojas-González et al. 2015) was increased. In contrast, the abundance of chloroplastic isoenzyme which generates ribulose 1,5-bisphosphate (RuBP) and used in starch metabolism was decreased (Kroth et al. 2008; Rojas-González et al. 2015). These findings could suggest that a decrease in the abundance of the chloroplastic fructose bisphosphate aldolase resulted in further induction of the glycolysis pathway to provide extra energy required for Cu detoxification.

Likewise in mitochondria, the precursors of the TCA cycle showed increase in protein abundances in response to Cu stress in *Z. muelleri* similar to what previously reported in *Agrostis capillaris* (Hego et al. 2016). Pyruvate produced from glycolysis pathway is thought to be converted to oxaloacetic acid and carried on through the cycle

according to the positive proteins abundance of isocitrate dehydrogenase, succinyl-CoAa ligase and malate dehydrogenase that were found in our study.

4.4. Excess Cu alters genetic information processing of *Z. muelleri*

Exposure to 500 µg Cu L^{-1} significantly impacted the abundance of proteins annotated as being involved in genetic information processing in *Z. muelleri* as demonstrated by the increase in the abundance of the cell division cycle 48 (CDC48). It is an ubiquitin-dependent molecular chaperone, and suggested to mediate a variety of degradation and regulatory processes including maintenance of cellular homoeostasis (Latterich & Patel 1998). Also, activation of the 60S acidic ribosomal protein P0 and elongation factor 1 and Tu illustrated an induction in response to Cu stress at the nuclear level as a result of Cu exposure (Tchórzewski 2002; Sasikumar, Perez & Kinzy 2012). Additionally ubiquinone proteolysis, which was previously reported as a part of the plant stress response seemed to be active in our study as seen by the positive change in the abundance of the proteasome (subunit alpha) (Belknap & Garbarino 1996). Activation of a number of heat shock proteins (HSP70, HSP80, HSP 16.9, HSP 90.5) and chaperonins (CPN60-2 and 10 kD) confirmed that a strongly regulated induction occurs when plants were subjected to 500 µg Cu L^{-1}. HSP70 and CPN60-2 in particular, were also reported in *Agrostis capillaris* in response to Cu stress and therefore, it could be considered that these proteins are Cu- specific regulators (Hego et al. 2016).

5. Conclusion

In summary, 500 µg Cu L^{-1} induced an abundance of stress responses from the seagrass, *Z. muelleri* and therefore, the sensitivity of this seagrass species to elevated levels of Cu was confirmed. We identified 76 proteins with changes in their abundance as a direct response to Cu stress which are linked to energy metabolism (in particular carbohydrate metabolism), carbon fixation, photosynthesis, enzymatic defence mechanism and genetic information processing. Some of these identified proteins were also induced in respond to Cu stress at the transcriptomic level as mentioned in Chapter 3. It can be suggested that proteins with similar responses to Cu stress at both transcriptomic and proteomic level (RuBisCo, fructose bisphosphate aldolase, phosphofructokinase, glutathione transferase and peroxidase) can be further investigated as potential biomarkers of early stress in *Z. muelleri*. Also, we found six

proteins with similar behaviour to Cu stress in both *Agrostis capillaris* and *Z. muelleri* including light-harvesting complex, fructose bisphosphate aldolase, phospho glecerate mutase, isocistrate dehydrogenase, HSP70 and CPN60-2) which can be further studied for their potential as inter-species Cu-specific biomarkers.

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CHAPTER 6

Optimisation of an intact chloroplast extraction method for the Australian seagrass, *Zostera muelleri*.

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Abstract

Previous studies on seagrasses have illustrated the critical role of chloroplasts in the maintenance of the organisms' pathway as well as the sensitivity of chloroplast subunits towards environmental stress factors. Therefore, proteomic profiling of chloroplasts can provide fundamental information about the physiology of an organism at the time of exposure to the stress factor. However, isolation of intact chloroplasts is challenging and has not been reported for most of seagrass species. Here, a protocol for the isolation of intact chloroplast was developed and tested in the seagrass, *Z. muelleri* using a commercial chloroplast isolation kit (CPISO-Sigma). Both 40% Percoll layer (method A) and 40/80% Percoll gradient (method B) showed successful isolation of intact and functional chloroplasts evidenced by chlorophyll a/b ratio, microscopic observation and proteomic analysis. Furthermore, an immunoblot analysis of chloroplast and mitochondrial marker proteins suggested that the method B was more efficient not only in isolating the intact chloroplasts and its protein markers, but also in minimising contamination from mitochondrial proteins.

We also performed a peptide-centric proteomic analysis to investigate the expressed proteins extracted from intact chloroplasts using method B. A total of 94 proteins were identified with less than 2% contamination from non-chloroplastic proteins. The majority of identified proteins were associated with photosynthesis subunits (PSII and PSI), ATP synthase and chlorophyll a/b binding proteins.

This improved chloroplast isolation method for seagrass species will allow a better molecular understanding of their stress tolerance mechanism at the photosynthesis level.

1. Introduction

Seagrass ecosystems provide valuable services worldwide both ecologically and economically (Graham et al. 2018). Yet, their population is declining globally due to their exposure to a wide range of anthropogenic-driven pollutants (Cambridge & McComb 1984; Waycott et al. 2009). While seagrasses can endure short periods of environmental stress (Evans, Vergés & Poore 2017), extended exposure can severely damage their physiology and health (Ralph & Burchett 1998; Macinnis-Ng & Ralph 2002, 2004a; Purnama et al. 2015; Davey et al. 2017; Kim et al. 2017, Buapet et al. 2019).

Chloroplasts are one of the most fundamental organelles in plants and seagrasses, necessary to maintain physiology via photosynthesis and have been shown to be sensitive to environmental stress factors (Whatley 1978; Lopez-Juez & Pyke 2004). Successful isolation of intact chloroplasts from seagrass species would allow better understanding of environmental toxicity response of seagrasses at the photosynthesis-specific level. Apart from primary photosynthesis function, the chloroplast membranes also contains proteins and lipids, as well as a wide diversity of compounds deriving from the isoprenoid lipid biosynthetic pathway including carotenoids, chlorophylls, prenyl quinones, and a wide set of signalling molecules (carotenoid cleavage products, such as ABA; chlorophyll precursors derived compounds; gibberellins, etc.). This enormous array of chloroplast compounds perform vital functions within the plant cell for growth and development and environment stress response. The main stress effects include chlorosis, the decrease of photosynthetic pigments, chloroplast membrane damage, and impairment of the electron transport chain (Li et al. 2006; Ahsan et al. 2010; Singh, Parihar, et al. 2016).

Zostera muelleri is found in coastal waters of Australia, Papua New Guinea and New Zealand and their meadows are often exposed to anthropogenic-derived pollution (Dennison et al. 1993; The IUCN Red List of Threatened Species 2018). The photosynthetic efficiency across the family of *Zosteraceae* has been shown to rapidly decline under environmental stress factors such as low light, low oxygen and trace metal stress (Macinnis-Ng & Ralph 2002, 2004a; Kumar, Padula, et al. 2016; Davey et al. 2017; Kim et al. 2017, Buapet et al. 2019). However, the mechanism of photosystem damage are yet to be clearly identified (Ralph & Burchett 1998; Prange & Dennison 2000; Macinnis-Ng & Ralph 2004b; Campbell, McKenzie & Kerville 2006).

Traditional chloroplast isolation has been previously used for 2-DE proteomic analysis in *Arabidiopsis* (Schubert et al. 2002) and spinach (Ferro et al. 2002). A commercial chloroplast isolation kit (CPISO-Sigma) has previously been successfully used to isolate intact chloroplasts in terrestrial plants such as *Arabidiopsis thaliana* (Conte et al. 2009), *Lotus japonicas* (Sainz et al. 2010), *Jacobaea vulgaris* (Doorduin et al. 2011) and legumes (Dugas et al. 2015). However, limited progress has been achieved in studying chloroplast proteome across the wide diversity of plants due to some challenges. One is the abundance of the protein of interest. Chloroplasts are

shown to regulate their gene expression with signaling system which generally has low abundant proteins which makes the identification of these signaling factors very challenging specially in the presence of other highly abundant metabolic proteins (Baginsky & Gruissem 2004). Therefore, isolation of chloroplasts can resolve this issue by minimizing contamination by non-chloroplast proteins. Another challenge is the level of the solubility of the protein of interest. For example, unlike thylakoid lumen proteins which are soluble and therefore, easy to isolate, membrane bond proteins are hydrophobic and very hard to separate and identify (Baginsky & Gruissem 2004).

While recent studies have reported genome sequences of *Z. marina* and *Z. muelleri* (Lee et al. 2016; Olsen et al. 2016) and an optimized protein extraction method (Jiang et al. 2017) for *Z. muelleri*, chloroplast-specific proteomic approaches have only reported for *Posidonia oceanica* to date using different extraction method, different chloroplast isolation method and different proteomic approach (1-DE) than what we used (Piro et al. 2015). Therefore, using the chloroplast isolation kit is the first evaluation report for seagrass species.

It is shown in both of our transcriptomic (Chapter 3) and proteomic (Chapter 5) approaches that chloroplast was significantly affected by Cu stress. Therefore, it is imperative to study the chloroplast proteomic, while analysing the intact chloroplast to narrow down the investigation to sub-cellular level to acquire new insight into biochemical pathways in the chloroplast alone, including photosynthesis regulation and amino acid biosynthesis which are essential for retaining plant well-being under stress conditions (Lange & Ghassemian 2003). Therefore, in this study, we evaluated the efficiency of two intact chloroplast isolation methods suggested by the manufacturer (Sigma) for the Australian seagrass, *Z. muelleri*: 40% Percoll layer (method A) and 40/80% Percoll gradient (method B). Chlorophyll a/b ratio, percentage of isolated intact chloroplasts and observation of chloroplasts under microscope were used to evaluate the efficiency of each methods.

The ultimate goal of this study is therefore to evaluate the efficiency of two commercial kit from chloroplast isolation from seagrasses, as well as to investigate the quality of chloroplast isolates for proteomic analysis. Future studies on molecular investigation of environmental stress factors in seagrasses would significantly benefit

from this method development, allowing a detailed understanding of *Z. muelleri* biology at a much deeper molecular/biochemical level.

2. Materials and methods

2.1. Sample collection

Individual plants of *Z. muelleri* were collected from Pittwater (33°38′45.6′′S. 151°17′12.8′′E), New South Wales. Whole plants were rinsed to remove epiphytes, flash frozen in liquid nitrogen and transferred to the University of Technology Sydney (UTS) within 2 − 3 hours of collection as mentioned in previous Chapters. Frozen samples were stored at − 80°C for further analysis.

2.2. Isolation of intact chloroplasts from *Z. muelleri*

Intact chloroplasts were isolated using a commercial chloroplast isolation kit (Sigma-Aldrich) according to the manufacturer's instructions with some modifications: approximately 2 g of leaf tissue was used for method evaluation and 30 g for proteomic analysis. The leaf tissues were ground using a liquid nitrogen cooled bead beater (Retch MM 400) for 2 min (30 Hz) and the powder were mixed with chloroplast isolation buffer (1x CIB) containing 0.1% (w/v) bovine serum albumin (BSA). The solution was filtered and centrifuged at 400 x g for 3 min to remove cell debris. The supernatant was then centrifuged at 1200 x g for 7 min to collect the isolated intact chloroplasts.

Two methods were proposed by the manufacturer to isolate the intact chloroplasts: 40% Percoll layer (method A) and 40/80% Percoll gradient (method B). For method A, 2 mL of chloroplast suspension was mixed in a 1:2 ratio with 40% Percoll followed by centrifugation at 2000 x g for 10 min to collect intact chloroplasts as a pellet. For method B, an extra layer of 80% Percoll were added in a 1:1 ratio on top of the chloroplast suspension layer prior to addition of 40% Percoll. The suspension was centrifuged at 3000 x g for 25 minutes and the intact chloroplasts were collected as a layer between 40% and 80% Percoll. The chloroplasts were re-suspended in 3 volumes of 1x CIB without BSA and centrifuged at 2200 x g for 2 minutes to collect isolated chloroplasts. All the steps were carried out on ice and all centrifugations were performed at 4 °C.

The percentage of isolated intact chloroplasts was calculated for both methods according to the manufacturer`s protocol. Briefly, 50 µg of chloroplast isolates was

mixed with 1x CIB with BSA and 1.5 mM Ferricyanide to study the osmotic and non-osmotic shock. After illumination under a 40 W bulb, the reduction of Ferricyanide was measured every 2 min (for a total of 6 minutes) at the absorbance wavelength of 410 nm using UV visible spectrometer (Cary 50). The percentage of intact chloroplasts was calculated using the formula: (B – A / B) x100 where B and A are the absorbance of the osmotic and non-osmotic shock reactions, respectively.

2.3. Estimation of chlorophyll a/b ratio of intact chloroplasts

Estimation of the chlorophyll a/b ratio of the isolated chloroplasts of *Z. muelleri* from both methods was conducted to evaluate the physiological state of their photosystem I (PSI) according to Ritchie (2006). A dilution (1:50) of chloroplast isolates was prepared using 90% acetone. Chlorophyll a/b ratio was calculated using a UV visible spectrometer (Cary 50) at the absorbance wavelength of 664 nm (for chlorophyll a), 646 nm (for chlorophyll b) and 755 nm (for baseline).

2.4. Estimation of the size of intact chloroplast using Differential interference contrast (DIC) microscopy

Samples (2 µL) from both methods were observed under light microscope (Nikon Eclipse) with 100 x magnification using a DIC microscope technique to increase the contrast of the chloroplasts against the background (Shribak et al. 2008).

2.5. Protein extraction

Proteins were extracted following the optimized protein extraction protocol for *Z. muelleri* (Chapter 5-page 166) with slight modification. Intact chloroplast isolates were mixed with 1.2 mL ice-cold extraction buffer (100 mM TEAB, 100 mM EDTA-Na_2, 50 mM borax, 0.5% SDS w/v, 50 mM ascorbic acid, 1% PVPP w/v, 1.5% Triton X-100 (v/v), 0.5% β-mercaptoethanol (v/v), 30% sucrose (w/v)) and a protease inhibitor cocktail (Roche, Germany). The rest of the steps were exactly as mentioned in Chapter 4 (page 125). The protein pellets were solubilised in 8 M urea in 100 mM triethylammonium bicarbonate (TEAB) for immunoblotting and proteomic analysis. Assessment of protein concentration was carried out using bovine serum albumin as a standard as mentioned in Chapter 4 (page 125).

2.6. Immunoblotting

Immunoblotting was performed by running protein samples in 4 − 15% Mini-PROTEAN TGX Stain-free protein gels (Bio-Rad). The gel was then transferred onto a polyvinylidene fluoride (PVDF) membrane (Trans-Blot Turbo Transfer Packs PVDF MIDI, Bio-Rad) followed by 1 hour room temperature incubation in 5% milk solution. The membrane was individually incubated with five polyclonal antibodies with predicted activity in the family of *Zosteraceae* purchased from Agrisera (www.agrisera.com) for 1 hour at room temperature. Three chloroplastic marker proteins namely RuBisCo large subunit (AS03 037), ATP-dependent Clp protease proteolytic subunit 3 chloroplastic (AS16 4050) and Rieske iron-sulfur protein of Cyt *b6/f* complex (AS08 330) was selected for immunoblotting. RuBisCo (large subunit) is a major enzyme in carbon fixation in plants and seagrasses (Touchette & Burkholder 2000; Spreitzer & Salvucci 2002; Kim et al. 2018). The large subunit of RuBisCo is shown to be encoded in the chloroplast genome as suggested by Shinozaki et al. (1986). ATP-dependent Clp protease proteolytic subunit 3 (ClpP3) is a proteolytic enzyme with regulatory role in protein mis-folding (Porankiewicz, Wang & Clarke 1999). Rieske iron-sulfur protein of the Cyt b_6f complex (PetC) is a component of b_6f complex involved in the electron transfer chain (Kuras & Wollman 1994).

Additionally, two mitochondrial marker proteins namely malate dehydrogenase 2 (AS15 3064) and NAD-dependent malic enzyme (AS16 3932) were selected to test the percentage of mitochondrial contamination in immunoblotting. Malate dehydrogenase 2 (MDH2) is an enzyme that catalyses the oxidation of malate to oxaloacetate in metabolic and homeostasis pathways (Tomaz et al. 2010). NAD-dependent malic enzyme (NAD-ME) is a decarboxylation enzyme in bundle sheet of mitochondria (Gowik & Westhoff 2011).

Horseradish peroxidase was used as a secondary antibody conjugate (Bio-rad 1706515) before quantifying immune-stained signals using GE Amersham imager 600 (chemo-luminescence setting).

2.7. In-solution digestion (ISD)

Protein sample (100 µg) from method B was solubilized in 8 M urea and 100 mM TEAB followed by reduction, alkylation of cysteine residues and trypsin digestion as described in Chapter 4 (page 126). Digested peptides were analysed at a

concentration of 1 µg / 5 µL by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the same settings as mentioned in Chapter 4 (page 122).

2.8. Proteomic data analysis

The raw LC-MS/MS file from method B was analysed for the total number of identified proteins, the percentage of unique proteins and the significance of protein identification (-10 lgP) using PEAKS Studio software v8.5 (Bioinformatics Solutions, Waterloo, ON). Consistent with Chapter 4 and 5, the term 'protein' in this work, refers to an Open Reading Frame (ORF) in the database rather than a mature proteoform.

Additionally, the exported FASTA file from PEAKS Studio was submitted to the GRAVY calculator tool (http://www.bioinformatics.org) for the assessment of hydropathy of the proteins. The same FASTA file was used in Expasy compute pI/MW tool (https://web.expasy.org/compute_pi/) to identify the predicted isoelectric point (pI) and molecular weight of each identified proteins. Lastly, the list of identified proteins from PEAKS Studio were exported in a mzIdentML format and searched in Scaffold (version 4.8.5) software using both *Z. muelleri* and *Z. marina* proteome databases.

3. Results

3.1. Assessment of intact chloroplasts isolation

Intact chloroplasts were collected as a pellet at the bottom of falcon tube in method A and as a ring at the interface of 40/80% Percoll in method B (Figure 6-1). The estimated percentage of intact chloroplasts was determined to be approximately 60% (± 5) for method A and 71% (± 5) for method B. The chlorophyll a/b ratio, calculated according to Ritchie (2006), was 3.8 for both Method A and B. The size of intact chloroplasts using DIC microscopy was found to be 7 − 10 µm for both methods (Figure 6-2).

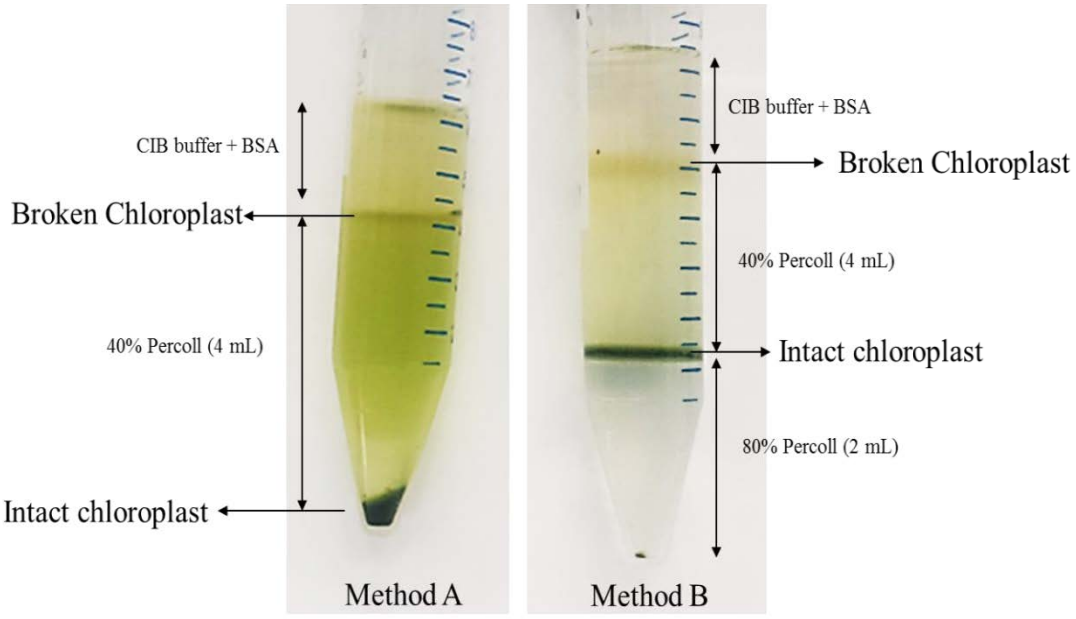

Figure 6-1. Intact chloroplast isolates as a layer in method A and as a ring at the interface of 40/80% Percoll in method B.

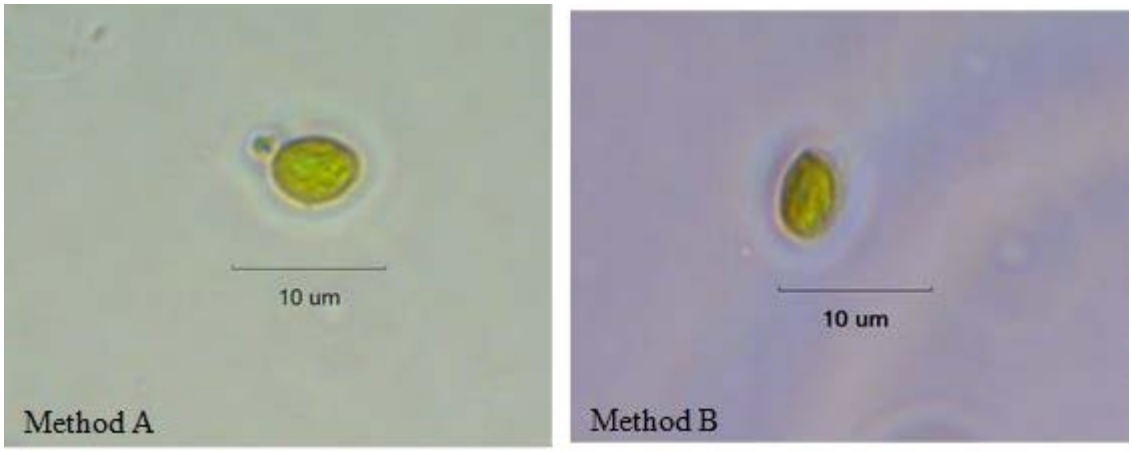

Figure 6-2. Microscopic visualization of an intact chloroplast under DIC microscopy.

Immunoblotting was performed using the whole leaf proteins extracted from isolated chloroplasts obtained in method A and B (Figure 6-3 and Appendix 24). Three selected chloroplast proteins (RuBisCo, Clpp3 and PetC) were positively detected in both method A and B, which confirmed the efficiency of both isolation methods. However, the presence of enzyme, NAD-ME, a mitochondrial contaminant, was also recorded for method A leaving method B as the preferred method for chloroplast isolation and further proteomic analysis in *Z. muelleri*.

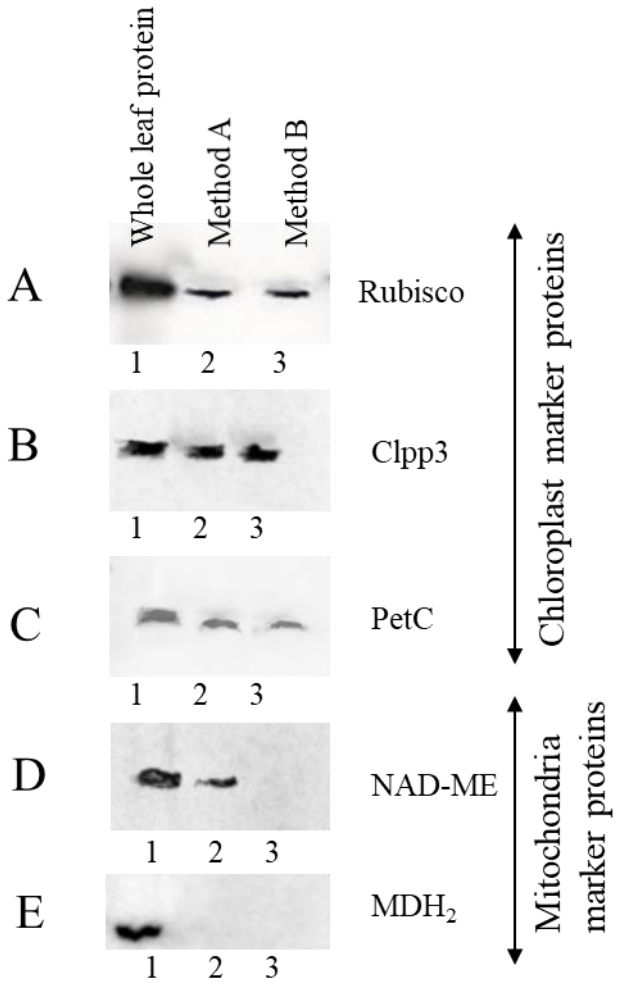

Figure 6-3. Immunoblotting of chloroplast (A −C) and mitochondrial (D −E) marker proteins.

A) RuBisCo large subunit (50 kD) 1:5000 dilution, **B)** Clpp3 antibody (28.5 kD) 1:2000 dilution , **C)** Pet C antibody (20 kD) 1:2500 dilution, **D)** NAD-ME antibody (65 kD) 1:1000 dilution, **E)** MDH2 antibody (35 kD) 1:1000 dilution.

1: whole protein; **2**: method A; **3**: method B.

3.2. Assessment of intact chloroplast isolation

The raw MS file from digested protein sample of intact chloroplasts obtained by using method B was searched against *Z. muelleri* database using PEAKS search using the same setting as mentioned in Chapter 4 and 5. A total number of 94 proteins were identified, where 19 proteins matched with > 2 peptides, 12 proteins matched with = 2 peptides and 59 proteins matched with 1 peptides. The resulting PEAKS and FASTA files from the *Z. muelleri* search can be found in Appendix 25 and 26.

The percentage of unique peptides and the significance of protein identification (-10lgP) were also evaluated for method B. Approximately (30%) of proteins had ≥ 2 matched unique peptides (Figure 6-4). For the significance of protein identification, 40% of the proteins were identified with -10 lgP of ≥ 50 (Figure 6-5).

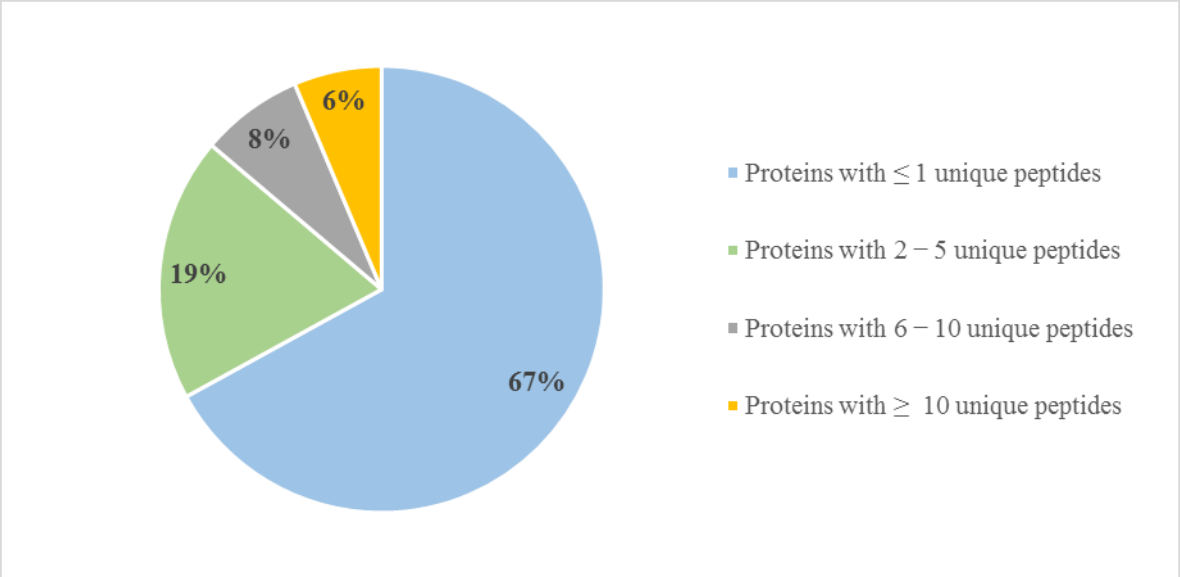

Figure 6-4. The percentage of unique matched peptides for identified proteins from method B.

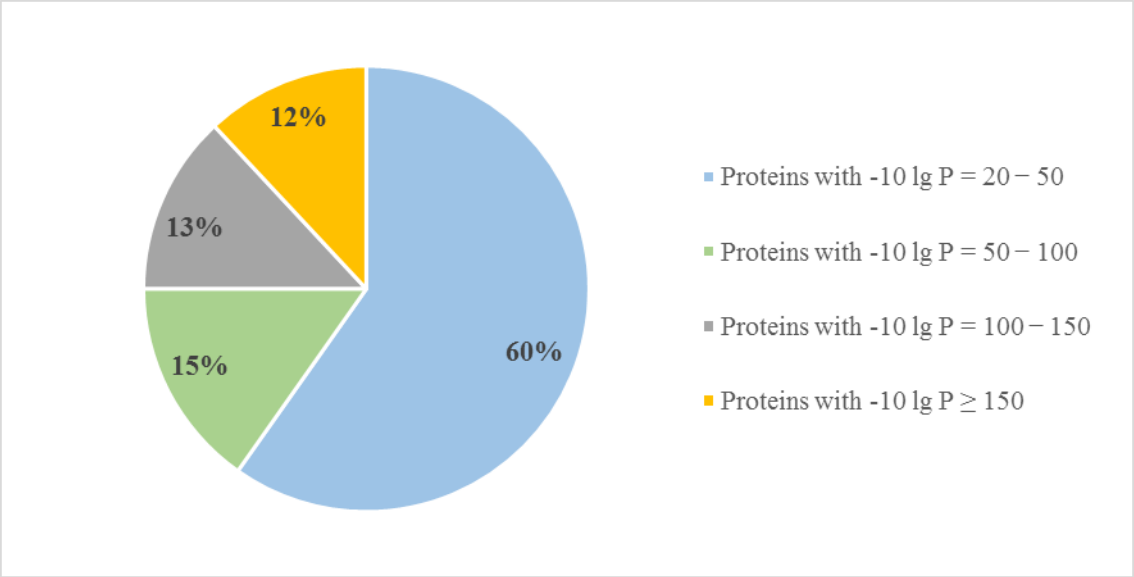

Figure 6-5. The percentage of -10lgP value of identified proteins from method B.

3.3. Isoelectric point and GRAVY index:

The hydropathy of the identified proteins in method B were assessed using the grand average of hydropathy (GRAVY) score. The results showed a majority of proteins (average of 76%) to be hydrophilic with the GRAVY score between −0.3 and 0.0 (Figure 6-6). An approximate of 20% of proteins showed positive GRAVY scores corresponding to hydrophobic proteins such as membrane proteins. Furthermore, the majority of the identified proteins were shown to have isoelectric points of either 5 or 9 (Figure 6-7).

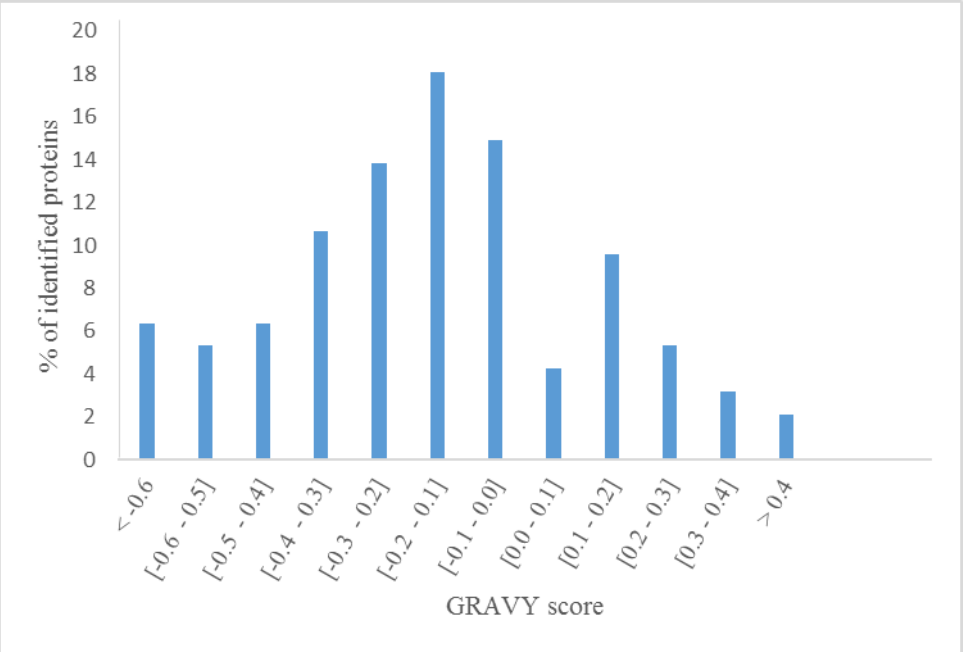

Figure 6-6. GRAVY score indicating the hydropathy of the identified proteins from method B.

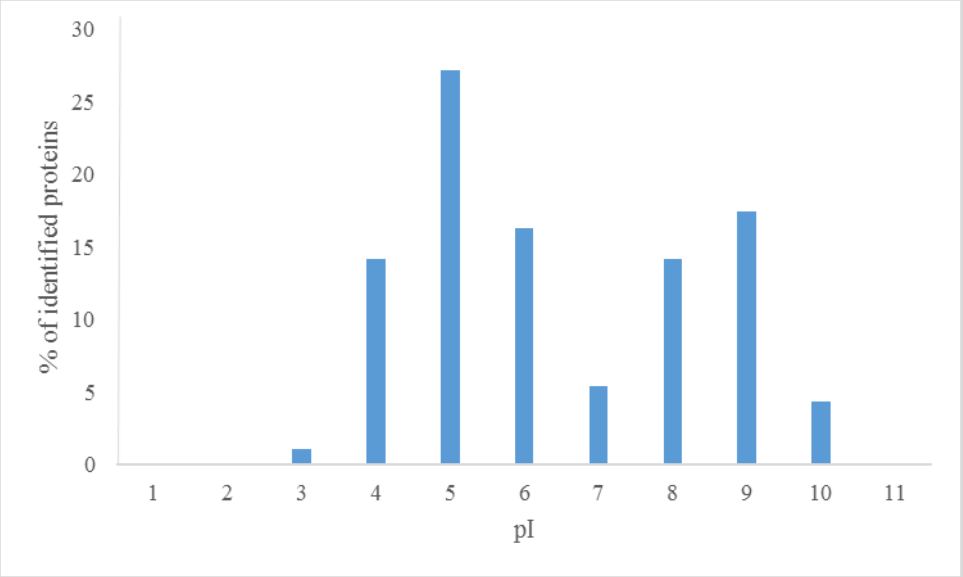

Figure 6-7. Distribution of isoelectric focusing of the identified proteins in method B.

The resulting PEAKS Studio file was exported as a mzIdentML format for a parallel database search in Scaffold (version 4) software. Scaffold search showed a total number of 43 uniquely identified proteins against *Z. marina* and Z. *muelleri* database (Table 6-1). All of the identified proteins except peptidyl-prolyl cis-trans isomerase and cytochrome c were identified as chloroplast related proteins. The exported Scaffold file with their correspondent molecular weight and protein identification probability can be found in Appendix 27.

Table 6-1. List of uniquely identified proteins from intact chloroplast isolates of *Z. muelleri* using Scaffold software

#	Identified Proteins	Accession Number	Alternate ID
PSII			
1	Photosystem II CP43 reaction center protein	A0A290Y2F4_ZOSMR	psbC
2	Photosystem II D2 protein	A0A290Y2E1_ZOSMR	psbD
3	Photosystem II protein D1	A0A290Y2E7_ZOSMR	psbA

4	Photosystem II CP47 reaction center protein	A0A290Y2J1_ZOSMR	psbB
4.1	Protein	augustus_masked-23760_5925--0.0-mRNA-1	
5	Cytochrome b559 subunit alpha	A0A290Y2H9_ZOSMR	psb E
6	Ycf48-like protein	maker-2783_154383_67051_154383--0.26-mRNA-1	
7	Repair protein PSB27-H1	snap_masked-6508_40704_1_22592--0.7-mRNA-1	
PSI			
8	Photosystem I iron-sulfur center	A0A290Y2L6_ZOSMR	psaC
9	Photosystem I P700 chlorophyll a apoprotein A2	A0A290Y2G9_ZOSMR	psaB
10	Photosystem I subunit N	maker-9498_13761--0.7-mRNA-1	Psa N
11	Photosystem I reaction center subunit II	A0A0K9P6C6_ZOSMR	ZOSMA_35G00140
12	Photosystem I reaction center subunit VI	A0A0K9NW28_ZOSMR	ZOSMA_5G00100
ATPase			
13	Cluster of ATP synthase subunit alpha, chloroplastic	A0A290Y2F1_ZOSMR	atpA
14	ATP synthase subunit alpha	A0A1Z1V5R5_ZOSMR	atp1
Light harvesting complex			
15	Chlorophyll a-b binding protein, chloroplastic	A0A0K9PJV3_ZOSMR	ZOSMA_217G00280
15.1	Protein	maker-11138_15675--0.12-mRNA-1	
15.2	Protein	augustus_masked-15490_11153--0.0-mRNA-1	

15.3	Protein	maker-395_77190--0.24-mRNA-1	
15.4	Protein	maker-718_54240_1_20820--0.7-mRNA-1	
16	Chlorophyll a-b binding protein, chloroplastic	A0A0K9PDW1_ZOSMR	ZOSMA_278G00220
16.1	Protein	augustus_masked-3379_66425--0.0-mRNA-1	
17	Chlorophyll a-b binding protein, chloroplastic	A0A0K9PYB0_ZOSMR	ZOSMA_137G00210
18	Chlorophyll a-b binding protein, chloroplastic	A0A0K9NKN1_ZOSMR	ZOSMA_89G01210
19	Chlorophyll a-b binding protein	maker-104_74335_26975_48195--0.9-mRNA-1	
20	Chlorophyll a-b binding protein	augustus_masked-6901_30015--0.0-mRNA-1	
ATP synthase			
21	ATP synthase subunit beta, chloroplastic	A0A290Y2H1_ZOSMR	atpB
21.1	ATP synthase subunit beta	A0A0K9NYL5_ZOSMR	ZOSMA_4G01180
21.2	Protein	snap_masked-158_104160--0.38-mRNA-1	
22	ATP synthase gamma chain	A0A0K9NRA9_ZOSMR	ZOSMA_69G00150
22.1	Protein	augustus_masked-8506_24755--0.3-mRNA-1	
23	ATP synthase CF0 subunit I	A0A290Y2F5_ZOSMR	atpF
Cytochrome			
24	Cytochrome f	A0A290Y2H0_ZOSMR	petA
25	Cytochrome c	maker-1491_92006_1_43032--0.14-mRNA-1	
Other			
26	H(+)-transporting two-sector ATPase	A0A0K9PHS8_ZOSMR	ZOSMA_238G00120

27	Ribulose bisphosphate carboxylase large chain (Fragment)	A0A1S0RZY9_ZOSMR rbcL
28	Ribulose bisphosphate carboxylase small chain	A0A0K9NJB1_ZOSMR ZOSMA_99G00830
29	GDP-mannose 3,5-epimerase 1	A0A0K9NZ95_ZOSMR ZOSMA_48G00360
30	Fructose-bisphosphate aldolase	A0A0K9P725_ZOSMR ZOSMA_34G00930
31	Putative 50S ribosomal protein L13	maker-1940_49596--0.18-mRNA-1
32	Glyceraldehyde-3-phosphate dehydrogenase (chloroplast)	augustus_masked-6546_56265--0.0-mRNA-1
33	Peptidyl-prolyl cis-trans isomerase	snap_masked-8961_14622--0.1-mRNA-1
Unknown		
34	Protein	maker-10130_14009--0.16-mRNA-1
35	Protein	maker-20826_7649--0.2-mRNA-1
36	Protein	snap_masked-2238_86636--0.21-mRNA-1
37	Uncharacterized protein	A0A0K9PHH8_ZOSMR
38	Protein	maker-468_158100--0.74-mRNA-1
39	Protein	augustus_masked-17535_10596_1858_10596--0.0-mRNA-1
40	Protein	augustus_masked-2796_55705--0.2-mRNA-1
41	Protein	maker-1749_90394--0.17-mRNA-1
42	Protein	augustus_masked-11227_31940_1_17964--0.1-mRNA-1
43	Protein	maker-15289_13413--0.9-mRNA-1

4. Discussion

We evaluated the efficiency of two commercial chloroplast isolation kit (A and B) for the Australian seagrass, *Zostera muelleri*. Chloroplast isolates were extracted successfully with both methods (as a pellet in method A and as a middle layer in method B) using 2 g of leaf tissue. The estimated chlorophyll a/b ratio in both methods were shown to be within an acceptable range (above 3) for healthy plants species (Ritchie 2006; Vitória et al. 2010; Meneguelli-Souza et al. 2016). Additionally, the size of the isolated chloroplasts in both methods also matched the expected size of 5 − 10 µm of chloroplasts in healthy condition (Tyagi 2009).

A difference between the two chloroplast isolation methods, however, was recorded after immunoblotting. As we expected, all three chloroplast antibodies showed positive protein expression in both methods. However, the NAD-ME enzyme also showed positive expression in method A suggesting the presence of mitochondrial contaminants. The most likely explanation for this difference between method A and B is that chloroplast isolates from method A were collected as a pellet, which increased the chance of presence of residue cell debris, whereas in method B, the intact chloroplast isolates were collected as a layer between two Percoll solutions. Therefore, method B was the preferred chloroplast isolation method for *Z. muelleri* method.

Proteomic analysis of method B purified chloroplasts revealed some challenges for further considerations. The first issue was the sample requirement. More leaf tissue (30 g) was needed in order to have sufficient biomass of protein for proteomic analysis (in our case, we obtained 32 µg of protein for method A and 16 µg for method B), which is a considerable limitation especially when working with endangered seagrass species with limited sampling due to collection permit. Additionally, if the seagrasses are stressed, obtaining healthy intact chloroplasts from damaged tissues could be even more challenging with reduced yield.

The second issue was the signal detection in LC-MS/MS. Ninety four proteins were identified from isolated chloroplasts from method B which is consistent with some previous chloroplast-specific proteomic studies in higher plants (Peltier et al. 2000; van Wijk 2000; Schubert et al. 2002) but drastically lower than other recent oreports (Zybailov et al.2008; Ferro et al. 2010; Beherns at al. 2013).

However, approximately 75% of identified proteins matched with ≤ 1 unique peptides. We were not be able to find a previous work using peptide-centric proteomic method for isolated chloroplast to investigate whether this issue arose from the extraction technique, the MS analysis or else, it is the expected results from low abundant chloroplast proteins. Therefore, we suggested 3 possible reasons that could explain our unusual result:

1) The low spectral quality in the presence of salt. Although the presence of NaCl has been reported to have no effect of protein ion intensity, it negatively affects the spectral quality of mass spectrometry (Metwally, McAllister & Konermann 2015). In our experiment, we rinsed the leaf tissues with miliQ water and tapped them dried prior to freezing in liquid nitrogen, but the presence of salt in leaf tissue was an inevitable factor. Furthermore, low quality of spectral can be an issue with really low abundant proteins such as our case.

2) Ion competition in electrospray ionization mass spectrometry. One of the challenges affecting bottom-up proteomic workflow is the relative intensity of analysed peptides. The intensity of identified peptides is greatly affected by the quantity of the ionised peptides at the electrospray ionization step (Xie et al. 2011; Beherns at al. 2013). Therefore, the presence of highly abundant proteins can negatively affect the intensity of co-ionized peptides (aka ion suppression) and as a result, decrease of the quality of the identification of the latter (Tang, Page & Smith 2004; Xie et al. 2011). In our case, the proteins with highest coverage that might cause ion suppression were chlorophyll a/b binding proteins and RuBisCo. These proteins were identified as two of the most abundant chloroplast proteins according to previous reports (Ellis 1979; Dolganov, Bhaya & Grossman 1995).

3) Interference of non-compatible reagents with MS analysis. We recorded the presence of a significant amount of non-specific contaminants after analysing the sample by LC-MS/MS. It seemed like one or some reagents used in the chloroplast isolation kit were not compatible with mass spectrometry (most probably contain surfactants or polypropylene glycol) (Ishihama, Katayama & Asakawa 2000). The contaminant reagent drastically decreased the sensitivity of the LC-MS/MS column. This result reinforce the fact that a method optimisation for sample preparation is requited in future work as also stated by Ferro et al. (2010). Our attempt to clean this

contaminant using a SDB-RPS-based desalting column with our improved method as mentioned in Chapter 5 (page 167) also failed (no binding of peptides to column resins). Therefore, we were not be able to proceed with more MS analysis of these samples.

We investigated the physio-chemical characterization properties of the identified proteins including hydropathy and isoelectric points (pI). A majority of the identified proteins were hydrophilic with maximum GRAVY scores between − 0.3 and 0.0 which is in consistent with previous results in higher plants (Sahay & Shakya 2010; Yang et al. 2013; Mahalingam 2017) and Chapter 4. Additionally, the pI value of the majority of the identified proteins were 5 or 9 similar to the bi-modal pattern that was reported in Chapter 4 for peptide-centric methods.

We identified 43 protein clusters mostly identified as chloroplast proteins (except two proteins) and mainly from photosynthesis subunits (PSII and PSI), ATP synthase, ATPase, Chlorophyll a/b binding proteins and cytochromes. Additionally, Ycf48-like protein, which has a role in binding chlorophyll to the membrane (Yu et al. 2018) and repair protein PSB27-H1 (Mabbitt, Wilbanks & Eaton-Rye 2014) were also recorded in our results. Additionally, our chloroplast isolates seemed to be healthy at the sampling time, since carbon fixation and glycolysis were active in our results. In carbon fixation, positive expression of RuBisCo (small and large subunits) was recorded whereas in glycolysis, fructose bis phosphate aldolase (chloroplastc) which generates ribulose 1,5-bisphosphate (RuBP) (Rojas-González et al. 2015) and glyceraldehyde-3-phosphate dehydrogenase (chloroplastic) which is an enzyme involve in glycolysis pathway (Holtgrefe et al. 2008) were also recorded. Furthermore, positive expression of Putative 50S ribosomal protein L13 (Phua, Srinivasa & Subramanian 1989) and GDP-mannose 3,5-epimerase (Wolucka & Van Montagu 2003) were considered as additional evidence of healthy conditions of chloroplast isolates.

Lastly, two non-chloroplast proteins were identified in our results: peptidyl-prolyl cis-trans isomerase and cytochrome c. Peptidyl-prolyl cis-trans isomerase is a cytosol enzymes which has a role in protein-folding (Kaur et al. 2015). Cytochrome c is a member of electron transfer chain in mitochondria (Yeagle 2016).

5. Conclusion

In summary, we successfully developed an intact chloroplasts isolation method from the leaves of *Z. muelleri* using optimized 40/80% Percoll method with low level of contamination. We also identified expressed proteins and reported some suggestions and considerations for LC-MS/MS-based proteomic analysis of seagrasses, if others use the same isolation kit. This proteomic study required further optimisation in terms of reagents and their compatibility with LC-MS/MS. The result of this study can be applied to future seagrass proteomic studies of *Z. muelleri* at photosynthesis-specific level.

Acknowledgement

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Synthesis, outlook and conclusion

Overview

Before the commencement of this PhD project, several reports of the mechanism of Cu toxicity in higher plants were published showing inter-cellular imbalances and physiological damages as a result of impaired photosystem and activation of reactive oxygen species (ROS) (Barón, Arellano & Gorgé 1995; Yruela 2005; Cambrollé et al. 2013; Girotto et al. 2013; Tiecher et al. 2017). Additionally, the omics techniques have been used and reported in higher plants since 1980s (Tan, Lim & Lau 2017). For seagrasses, however, there were few reports investigating the toxic effects of trace metals including Cu, but only at the physiological level (Ralph & Burchett 1998b; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002, 2004a, 2004b; Li et al. 2012).

At the commencement of this PhD thesis in August 2015, literature on transcriptomic in the family of *Zosteraceae* had started to be established with a focus on heat stress (Franssen et al. 2011; Franssen et al. 2014) and light stress (Kong et al. 2014; Davey 2017). Within one year, the genome of *Z. marina* (Olsen et al. 2016) and partially annotated genome of *Z. muelleri* (Lee et al. 2016) were published providing valuable information for omics studies in this family of seagrasses. With the exception of one report in 2016, showing the activation of ROS in response to Cu stress in *Z. japonica* (Lin et al. 2016), no omics investigations have been conducted as of 2016 on Cu toxicity response in the family of *Zosteracaeae*, leaving this niche field open for further work.

This PhD project aimed to study the acute response and acclimation of *Z. muelleri* to 250 and 500 µg Cu L^{-1} over a period of 7 days to better understand the toxicity response of this seagrass species at the molecular level. Starting with the physiological responses, Chapter 2 focused on the photosynthetic efficiency, Cu accumulation in the leaf tissue and total ROS production of *Z. muelleri* exposed to 250 and 500 µg Cu L^{-1} on days 1, 3 and 7. After the stress level was confirmed, targeted sequencing was also conducted as a part of this chapter with four antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, catalase and glutathione peroxidase) as well as metallothionein 2, 3 and cytochrome c oxidase. The Cu toxicity response was shown to be concentration and time-dependent. Therefore, in Chapter 3, we further investigated the Cu toxicity response of *Z. muelleri* exposed to 250 and 500 µg Cu L^{-1} on day 7 at the whole transcriptomic level. This continued into Chapter 4, where a

protein-centric (2-DE) and four peptide-centric proteomic methods were tested for their efficiency at identifying the highest number of expressed proteins in order to select the best possible proteomic method to be used in the Cu proteomic investigation in Chapter 5. The transcriptomic results from Chapter 3 showed concentration-dependent responses mainly at the photosynthesis, carbon fixation, glycolysis, enzymatic and chemical defence mechanism. Additionally, the most efficient proteomic analysis methods in *Z. muelleri* was shown to be SDB-RPS method (alone and combined with 1D-PAGE). For these reasons, we performed a SDB-RPS based proteomic study on Z. *muelleri* exposed to 500 µg Cu L^{-1} at day 7 in Chapter 5, with special attention to same induced pathways reported in Chapter 3. Lastly, based on the significant impact of Cu observed in photosystem of *Z. muelleri*, an attempt was made to isolate intact chloroplasts in Chapter 6 for future sub-cellular proteomic analysis.

More specifically, six main outcome and conclusions were achieved in this PhD thesis:

1. Photo-physiological damage occurs early in *Z. muelleri*

Upon analysis of the photosynthetic efficiency in Chapter 2, an acute suppression of ϕPSII was observed in both Cu treatments after 1 day of exposure. The decline in F_v/F_m in Cu treatments was slightly delayed (after 3 days) and was less intense than ϕPSII. The NPQ also rapidly increased in the first 2 days and gradually decreased afterwards. On examination of the available literature, the sensitivity of PSII to Cu toxicity was previously reported as a result of deterioration of electron transfer chain (Mohanty, Vass & Demeter 1989; Yruela et al. 1993; Barón, Arellano & Gorgé 1995; Yruela et al. 1996; Dewez et al. 2005). Additionally, enhanced NPQ in trace metal stress was also reported as a result of excess light energy dissipated from light harvesting antenna (Demmig-Adams and Adams, 1992; Muller, 2001; Ort, 2001). The results have also been shown to be concentration-dependent with greater negative impact at 500 µg Cu L^{-1} than in 250 µg Cu L^{-1}. These findings confirmed high sensitivity of *Z. muelleri* to Cu and also the toxicant level of 500 µg Cu L^{-1}, which is potentially a critical factor to the success of this seagrass species in colonising new habitat.

In Chapter 3, the decline in the expression of photosynthesis subunits were recorded at the transcriptional level but only at 500 µg Cu L^{-1}. ATP synthase (subunit

beta, epsilon and CF0 subunit III), cytochrome b_6f, PSI (subunit psaA and psaB) and NADH-plastoquinone oxidoreductase (subunit 2 and 7) were recorded as significantly expressed in our results (Figure 7-1). Although these findings confirmed the sensitivity of the photosystem of *Z. muelleri* to Cu stress, there were no records of significant differential gene expression for any of the PSII subunits as observed at the physiological level in Chapter 2 and also in previous results in higher plants and seagrasses (Cedeno-Maldonado, Swader & Heath 1972; Mohanty, Vass & Demeter 1989; Arellano et al. 1995; Jegerschoeld et al. 1995; Ralph & Burchett 1998; Prange & Dennison 2000; Macinnis-Ng & Ralph 2002, 2004a; Dattolo et al. 2014; Lin et al. 2018). There were previous reports about the alteration in the conformation of photosystem exposed to Cu (Yruela 2005). The decline in the photosynthesis efficiency that was observed in our results in Chapter 2, could similarly be implied as deterioration of PSII over time, potentially explaining why no significant gene expression was recorded in PSII subunits. The proteomic results in Chapter 5, however, showed decrease in the abundance of PSII (psbQ and psbR), cytochrome b_6f, and chlorophyll a-b binding protein and increase in the abundance of PSII (psbD2) (Figure 7-1). These results could be considered as extra line of evidence that PSII subunits were active at the starting of the Cu exposure (which represents as protein expression on day 7) and presumably gradually deteriorated by day 7 of Cu exposure (as transcriptomic results on day 7) as a result of alteration of functionality of photosystem subunits (as mentioned above) and ROS production (as discussed in next section).

An over-expression of jasmonic acid in our transcriptomic results, which has been shown to have a role in repairing PSII in higher plants (Maksymiec, Wojcik & Krupa 2007) could infer that the repairing mechanism of PSII was active on day 7 of exposure to 500 µg Cu L^{-1}.

2. Only two antioxidant enzymes continued to scavenging ROS over-production in response to Cu stress in *Z. muelleri*

Leakage of O_2^- as a result of deterioration of photosynthesis subunits was previously reported to initiate the oxidative stress response (Figure 7-1), which is the initial stress response towards most of the common stresses in plants (Hall 2002; Halliwell 2006; Mittler et al. 2004). In Chapter 2, a gradual increase in ROS

production was detected during the experiment in a concentration-dependent manner. Additionally, a fluctuation of up and down-regulations were recorded in our four selected antioxidant enzymes (superoxide dismutase, ascorbate peroxidase, catalase and glutathione peroxidase) at different days and different Cu concentrations. All four antioxidant enzymes were up-regulated on day 1, 3 and 7 and their gene expression were significant compared to the control at 250 µg Cu L^{-1}. However, we only had up-regulation of superoxide dismutase on day 7, ascorbate peroxidase on day 3 and 7 and glutathione peroxidase on day 3 and none of them were significant at 500 µg Cu L^{-1}. It seemed like Cu could alter the activity of antioxidant enzymes during the experiment and the damage was beyond the tolerable level at 500 µg Cu L^{-1} for *Z. muelleri*. The mechanism of inactivation of these enzymes seemed to be by non-specific binding of Cu to the sulfhydryl groups of proteins and altering their active sites as reported previously in higher plants (Nagalakshmi & Prasad 2001; Yruela 2009; Pena et al. 2012). However, it is likely that the inactivation of these enzymes were not permanent during the experiment.

Two antioxidant enzymes, however, seemed to be responsive to 500 µg Cu L^{-1} and unaffected by Cu. Transcriptomic results in Chapter 3 showed significant upregulation of peroxidase and glutathione s transferase transcripts in response to this Cu concentration (Figure 7-1). The same two antioxidant enzymes (along with six others as mentioned in Figure 7-1) also showed an increase in their abundance in our proteomic results in Chapter 5. These results could suggest that the activation of antioxidant enzymes in our results were initiated in response to Cu exposure most probably as a result of post-translational modifications to ensure a prompt response to stress as previously reported in higher plants (Mazzucotelli et al. 2008). However, their activity was blocked during the experiment except for peroxidase and glutathione s transferase. These two enzymes did not behave in a similar way in higher plants under Cu stress and were in fact, down-regulated in response in Cu exposure (Jouili & El Ferjani 2004; Ding et al. 2017). This could indicate that the resistance of these two enzymes to Cu is most probably species and concentration-specific.

3. Possible defense mechanisms in *Z. muelleri* after 7 days exposure to 500 µg Cu L^{-1}

Exposure to 500 µg Cu L^{-1} showed damaging effects in the quantum efficiency of PSII for *Z. muelleri* according to our results in Chapter 2. It has been previously reported in higher plants that Cu can inhibit PSII and reduce the quantum efficiency which is in line with our results as well (Mohanty, Vass & Demeter 1989; Yruela et al. 1993; Rijstenbil et al. 1994; Yruela et al. 1996). Therefore, the hypothesis that down-regulation of quantum efficiency (as a sign of unrepairable Cu exposure damage) could also be a defense mechanism to reduce ROS production and photo damage could be true in *Z. muelleri* as well (Osmond & Grace 1995; Kitao et al. 2000; Takahashi, Kopriva, et al. 2011; Burdett et al. 2014; Buapet 2017; Phandee & Buapet 2019).

The impairment of photosystem negatively affected carbon fixation process and Calvin cycle as shown by down-regulation of RuBisCo at both transcriptomic and proteomic levels. However, an upregulation in the expression of the gene encoding carbon fixation was recorded in our transcriptomic results (highlighted as number 1 in Figure 7-1). It is thought that the activation of inorganic carbon fixation was a defense mechanism to combat against photosystem and Calvin Cycle damage as a result of Cu stress. The role of carbonic anhydrase in regulation of the pH of the chloroplast during light stress has been previously reported in higher plants (Bhat, Ganai & Uqab 2017). It is thought that carbonic anhydrase could also have a similar role in protecting chloroplast stroma by regulating the pH during Cu stress.

On the contrary, we recorded over-activation of glycolysis and energy metabolism throughout the 7 days of Cu exposure and more specifically at 500 µg Cu L^{-1}. We had significant increase in the expression level of transcripts coding for two enzymes in glycolysis pathway: fructokinase and fructose bisphosphate aldolase (highlighted as number 2 in Figure 7-1). The same two enzymes showed an increase in their abundance in our proteomic investigation records along with phosphofructokinase, phosphoglycerate mutase, enolase and pyruvate kinase. It can be speculated that the activation of glycolysis pathways was a defense mechanism that *Z. muelleri* employed in response to Cu stress. The requirement for activation of carbohydrate catabolism to provide extra energy for conducting detoxification process

for excess Cu was also previously reported in higher plants at the molecular level which is in line with our results (Geiger, Servaites & Fuchs 2000; Lingua et al. 2012; Hego et al. 2016).

The enhanced provision of other energy sources that was recorded in our proteomic results was also thought to be a part of Cu toxicity response in *Z. muelleri*. Starting with catabolism of sucrose and L-lactate, which are energy storage sources, the plant started to add more glucose, fructose and pyruvate to their system, which is highlighted as number 3 and 4 in Figure 7-1. The extra pyruvate provided from glycolysis and L-lactate catabolism could keep the TCA cycle active. Pyruvate produced from the glycolysis pathway is thought to be converted to oxaloacetic acid and carried on through the cycle according to the positive proteins abundance of isocitrate dehydrogenase, succinyl-CoAa ligase and malate dehydrogenase that were recorded in our study.

Lastly, a possible bypassing pathway in mitochondria was recorded in our proteomic results by increasing the abundance of NADH dehydrogenase, ubiquinol oxidase 1 and cytochrome c reductase, but a decrease in the abundance of ATP synthase (subunit gamma), which could also be interpreted as a Cu toxicity response similar the hypothesis proposed by Van Dongen et al. 2011. It is thought that the bypassing pathway was triggered by the activation of oxidative phosphorylation in the mitochondria but avoiding the ATP synthase to reduce ROS (highlighted as number 5 in (Figure 7-1).

4. Possible biomarkers for monitoring seagrass health were suggested

According to our results in Chapter 2, alteration in the expression of the genes encoding photosynthetic efficiency and antioxidant enzymes could provide early warnings of stress in seagrass meadows and could be considered as possible biomarkers (highlighted in bold in Figure 7-1). More specifically, glutathione s transferase and peroxidase showed significant differential gene expression and alteration in protein abundance in our omics investigations. Additionally, RuBisCo, fructose bisphosphate aldolase and phosphofructokinase, have the potential to be considered as possible biomarkers for *Z. muelleri* meadows.

Furthermore, six proteins were reported in Chapter 5 with similar responses to Cu stress in both *Agrostis capillaris* and *Z. muelleri* including light-harvesting

complex, fructose bisphosphate aldolase, phosphoglecerate mutase, isocistrate dehydrogenase, HSP70 and CPN60-2. Since these proteins reacted similarly to Cu stress in both terrestrial and submerged plant species, it is likely that a better underestanding of the Cu toxicity in *Z.muelleri* could also be achieved by further investigating of the Cu-specific responses of these proteins in *Agrostis capillaris*.

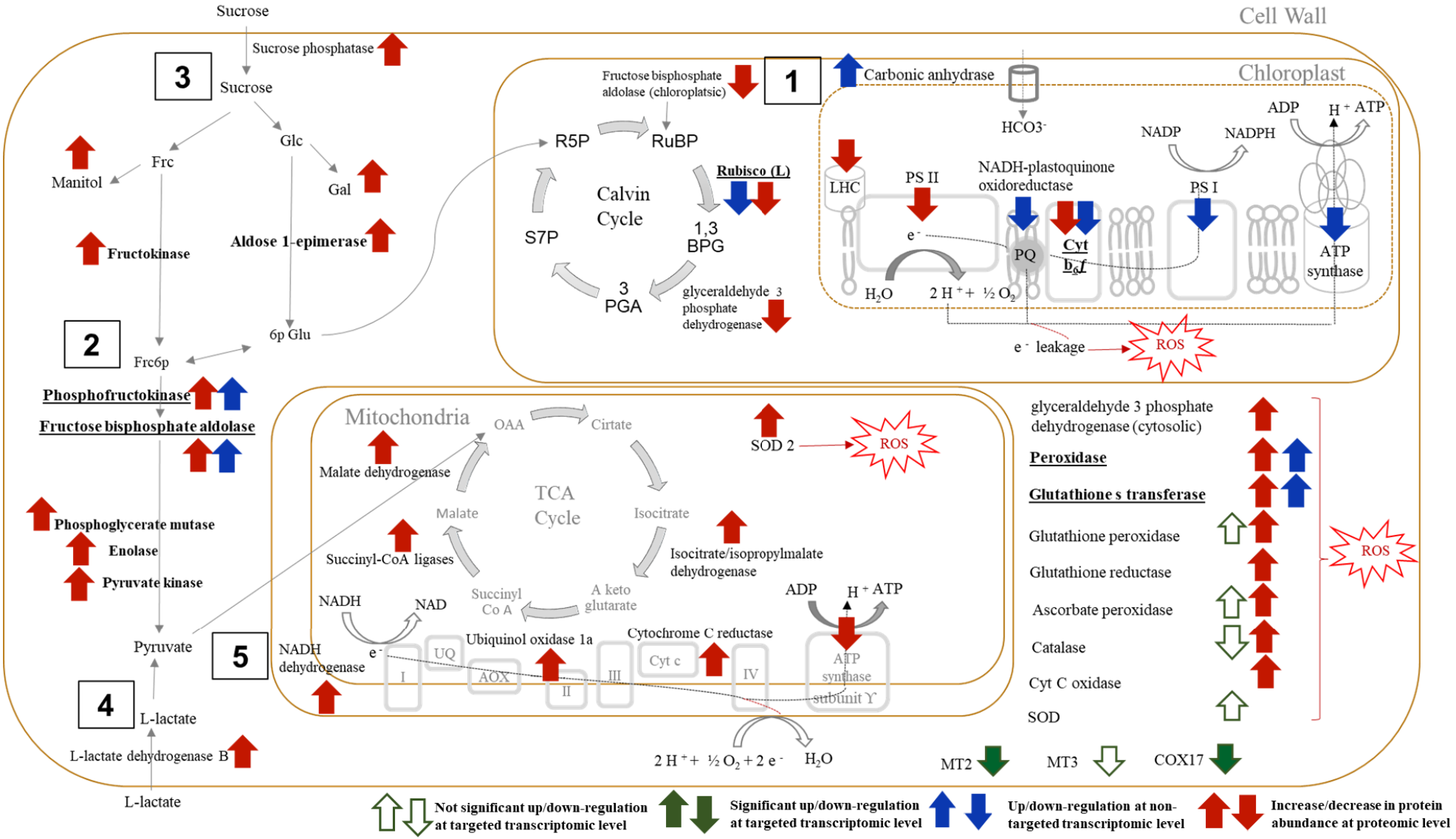
Cell Wall
Chloroplast
Sucrose
Sucrose phosphatase
3
Sucrose
Glc
Frc
Manitol
Gal
Fructokinase
Aldose 1-epimerase
2
6p Glu
Frc6p
Phosphofructokinase
Fructose bisphosphate aldolase
Phosphoglycerate mutase
Enolase
Pyruvate kinase
Pyruvate
5
4
L-lactate
L-lactate dehydrogenase B
L-lactate
Fructose bisphosphate aldolase (chloroplatsic)
1
Carbonic anhydrase
R5P
RuBP
Rubisco (L)
Calvin Cycle
1,3 BPG
S7P
3 PGA
glyceraldehyde 3 phosphate dehydrogenase
HCO3-
LHC
PS II
NADH-plastoquinone oxidoreductase
PS I
NADP
NADPH
ADP
H + ATP
ATP synthase
PQ
Cyt b6f
e -
H2O
2 H + + ½ O2
e - leakage
ROS
Mitochondria
OAA
Cirtate
Isocitrate
Isocitrate/isopropylmalate dehydrogenase
A keto glutarate
Succinyl Co A
Succinyl-CoA ligases
Malate
Malate dehydrogenase
TCA Cycle
SOD 2
ROS
NADH
NAD
NADH dehydrogenase
UQ
I
AOX
II
Ubiquinol oxidase 1a
III
Cytochrome C reductase
Cyt c
IV
ATP synthase subunit ϒ
2 H + + ½ O2 + 2 e -
H2O
glyceraldehyde 3 phosphate dehydrogenase (cytosolic)
Peroxidase
Glutathione s transferase
Glutathione peroxidase
Glutathione reductase
Ascorbate peroxidase
Catalase
Cyt C oxidase
SOD
ROS
MT2
MT3
COX17
Not significant up/down-regulation at targeted transcriptomic level
Significant up/down-regulation at targeted transcriptomic level
Up/down-regulation at non-targeted transcriptomic level
Increase/decrease in protein abundance at proteomic level

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 *Z. muelleri* in response to 7 day exposure to 500 µg Cu L^{-1}.

Blue arrows: up/down-regulation of an encoded gene recorded at transcriptomic level; Red arrows: increase/decrease in protein abundance at proteomic level. Bold highlight and underlined: possible biomarkers.

1) Activation of genes encoding carbonic anhydrase in response to deterioration of photosystem subunits and carbon fixation; **2)** activation of glycolysis most specifically by up-regulation of fructose bisphosphate aldolase and phosphofructokinase at both transcriptomic and proteomic level; **3)** Up-regulation of sucrose phosphatase at proteomic level ; **4)** Up-regulation of L-lactate dehydrogenase at proteomic level; **5)** Bypassing ATP synthase in oxidative phosphorylation process in mitochondria.

Chloroplast: PSII: photosystem II; PSI: photosystem II; RuBP: Ribulose 1,5-bisphosphate; 3 PGA: 3-phosphoglycerate; 1,3 BPG: 1,3 bis-phosphate glycerate; S7P: Sedoheptulose 7-phosphate; R5P: Ribulose 5-phosphate; PQ: plastoquinone. **Cytosol:** Frc: Fructose;Glc: Glucose; Gal: Galactose; Glc6P: α Glucose 6-phosphate; Frc6p: fructose 6 phosphate. **Mitochondria:** OAA: oxaloacetate; TCA cycle: citric acid cycle; I : Complex I; II: Complex II; III: Complex III; IV: Complex Iv; UQ : ubiquinone; Cyt c: Cytochrome C; AOX : ubiquinol oxidase; SOD: superoxide dismutase; SOD2: superoxide dismutase 2; MT2: metallothionein 2; MT3: metallothionein 3.

5. Evaluation of omics methods for future considerations

In this thesis four omics approaches were used which include: targeted transcriptomic (RT-PCR), non-targeted transcriptomic (sequencing), protein-centric proteomic and peptide-centric proteomic. There are some limitations for each methods according to our results in regards to the required processing time, sensitivity, cost and the coverage (range of identification) that we suggest should be taken into account for future work, as summarised in Table 7-2.

Starting with targeted transcriptomic approach, although it is a fast and relatively inexpensive procedure (Menzel et al. 2002), the technique is limited to one annealing temperature and restricted number of genes of interest (Gachon et al. 2004). Additionally, this method could not provide any information about the isoform of our encoded genes of interest. For example, two isomers of catalase showed an increase in the protein abundance in our results at the proteomic level (Table 5-1, page 177), whereas the result was the opposite in targeted transcriptomic approach (Figure 7-1). Even though this result was not significant in the targeted transcriptomic approach, we still were not able to report which isomer of catalase was amplifying in this method.

For non-targeted transcriptomic (sequencing) approach, it has the advantages of short sample preparation time and fully automated sample analysis with good sensitivity and good coverage (Ari & Arikan, 2016). However, the sequencing process is lengthy (3 months in our case), the post-sequencing analysis required bioinformatics experience and the sequencing cost is considerable (7,000 AUD for 9 samples).

As for the protein-centric method, since it is a non-automated technique, the cost is relatively cheap. Additionally, each spot in a 2-DE gel represents an intact proteoform which increases the reliability of the protein identification in MS analysis (Padula et al. 2017). However, gel-to-gel variations, difficulty in the separation of the mixed spots or identifying the spots for low abundant proteins are still very time consuming ($3-4$ days for each gel, in our case).

Lastly, for peptide-centric proteomic approach, similar to non-targeted transcriptomic approach, it is automated and provides high sensitivity and high coverage (Peng et al. 2003). However, the optimisation of the protein extraction as well as desalting and fractionation methods is usually needed for replacing reagents with more MS compatible one, while the total number of identified proteins should

ideally remain unchanged. Yet, it took 3-4 months in our case. Additionally, the cost is only substantial if a MS facility is not available for the research center.

In conclusion, a combination of non-targeted transcriptomic and peptide-centric proteomic approaches will be the best approach for a comprehensive and reliable toxicity study if the sequencer / MS facility is locally available or the cost is acceptable.

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).

Omics method	Time Personnel / Machine	Sensitivity	Cost	Coverage
Targeted transcriptomic		X		X
Non-targeted transcriptomic	X / X		X	
Protein-centric proteomic	X / -	X		
Peptide-centric proteomic	X / X			

6. Optimised methods were introduced opening new avenues for future work

I) In Chapter 3 and 5, we expanded the knowledge base for Cu-induced genes and correspondent proteins. We reported an improved protein extraction method in Chapter 5 by replacing Tris with TEAB resulting in better quality and yield of the proteins. However, we only focused on a few pathways namely photosynthesis, carbon fixation, energy metabolism, enzymatic and chemical defense mechanisms in this thesis due to their relevance to the physiological results that were recorded in Chapter 2. There are several other pathways including lipid and amino acid production, cellular processes and metabolism of secondary metabolites in our results with significant alteration in their gene / protein expression that were provided in the appendix 3, 4 and 22, which are suggested to be investigated in the future in order to enrich the knowledge about trace metal toxicity in seagrasses.

II) The possible biomarkers that we suggested in Chapter 3 and 5 require more investigation especially at Cu concentrations similar to field conditions in order to be employed for the assessment of the stress levels of seagrass meadows under trace metal stress. An example will be the Cu concentration that was reported from Lake to be around 11−13 µg / g dry mass (Schneider et al. 2018). Additionally, our results were based on a single-dose of Cu exposure to seagrasses. However, investigation of multiple Cu exposure in order to retain the specific Cu concentration throughout the experiment would also be informative to study.

III) In Chapter 4, a new desalting method was introduced and tested for the first time in seagrasses. We were able to improve the peptide retention in our study using SDB-RPS column to identify 3,831 proteins. The number of identified proteins increased to 4,440 by combining SDB-RPS column with 1D-PAGE. The SDB-RPS desalting method was further optimised in Chapter 5. However, since the genome of *Z. muelleri* is only partially annotated, the proper identification of these proteins required further intensive bioinformatics analysis. For these reasons, we only provided the list of the expressed proteins in this thesis. Therefore, identification of these proteins would be useful to enrich the current annotated database of *Z. muelleri*.

IV) In Chapter 6, we reported a chloroplast isolation method using 40/80% Percoll gradient method provided in Sigma chloroplast isolation kit with minimum contamination and functional isolated chloroplasts. However, further optimization should be conducted to improve the efficiency of isolated chloroplasts for future proteomic analysis. We provided 3 suggested ways to improve the efficiency of identified proteins based on our understandings that could be useful to resolve current issues.

In summary, in this PhD thesis new methods were developed and new knowledge was obtained to link the physiological response of Cu stress to the molecular basis that initiates these cellular alterations in *Z. muelleri* when exposed to elevated levels of Cu.

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Appendices

Due to the large size of most of the appendices files of the PhD thesis, each appendix file is provided individually as listed below in a folder namely "Nasim Shah Mohammadi thesis appendices" which can be accessed at: https://drive.google.com/drive/folders/1Mt2veF5oqylZkMvWIYewvi_Dr6lcUxvw?usp=sharing .

List of provided appendices:

Appendix1. Support documents for quality control genes used in qPCR.

Appendix 2-A. Functional annotation of differentially expressed genes of *Z. muelleri* at 250 µg Cu L^{-1} and 500 µg Cu L^{-1} using Blast2Go (gene identification). **B**. Functional annotation of differentially expressed genes of *Z. muelleri* at 250 µg Cu L^{-1} and 500 µg Cu L^{-1} using Blast2Go (GO and InterPro IDs).

Appendix 3. Fold change of expressed genes exposed to 250 µg Cu L^{-1}.

Appendix 4. Fold change of expressed genes exposed to 500 µg Cu L^{-1}.

Appendix 5. Demonstrative example for the identification of proteins extracted from leaves of seagrass *Zostera muelleri* . 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.

Appendix 6. Functional annotation and proteomic information of expressed proteins using 2-DE gel.

Appendix 7. Physio-chemical characterisations of expressed proteins using 2-DE gel.

Appendix 8. Protein FASTA of expressed proteins using 2-DE gel

Appendix 9. LC-MS/MS results of expressed proteins using 2-DE gel.

Appendix 10. Total number of identified proteins from each peptide-centric methods

Appendix 1. LC-MS/MS results of expressed proteins from Method A.

Appendix 2. LC-MS/MS results of expressed proteins from Method C

Appendix 3. LC-MS/MS results of expressed proteins from Method D

Appendix 4. LC-MS/MS results of expressed proteins from Method E.

Appendix 5. Protein FASTA of expressed proteins from Method A

Appendix 6. Protein FASTA of expressed proteins from Method C

Appendix 7. Protein FASTA of expressed proteins from Method D.

Appendix 8. Protein FASTA of expressed proteins from Method E

Appendix 9. Representation of protein identification for expressed proteins from peptide-centric methods using Scaffold software.

Appendix 10. LC-MS/MS results of expressed proteins using 6 iTRAQ labels.

Appendix 11. Protein FASTA of expressed proteins from 6 iTRAQ labels.

Appendix 12-A. Functional annotation of differentially expressed proteins of *Z. muelleri* at 500 µg Cu L^{-1} using Blast2Go (gene identification). **B**. Functional annotation of differentially expressed genes of *Z. muelleri* at and 500 µg Cu L^{-1} using Blast2Go (GO and InterPro IDs).

Appendix 13. Accession number, ratio of protein abundance, standard deviation (SD), p-value and statistical results of expressed proteins exposed to 500 µg Cu L^{-1}.

Appendix 14. Original images of immunoblotting of chloroplast and mitochondria marker antibodies.

Appendix 15. LC-MS/MS results of expressed intact chloroplast proteins.

Appendix 16. Protein FASTA of expressed intact chloroplast proteins

Appendix 17. Representation of protein identification probability of expressed proteins from intact chloroplasts using Scaffold software.