Bioaccumulation and physiological responses of Coontail, *Ceratophyllum demersum* exposed to copper, zinc and their combination

KOSAL HAK

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Master of Science in Earth System Science

Prince of Songkla University

2019

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Ceratophyllum demersum is a submerged aquatic angiosperm which is fast growing in contaminated water. This plant has no roots and so takes up nutrients slowly from the water column. This study aimed to compare the bioaccumulative capacities of Cu, Zn and their combination by *C. demersum* and physiological responses (growth, chlorophyll content, and photosynthetic rate) of *C. demersum* to Cu and Zn. Additionally, pulse amplitude modulation (PAM) technology was applied to detect copper and zinc toxicity effects on the light reactions of photosynthesis. Cu and Zn are essential trace elements for plant growth, development, and generally higher plants take up Cu in the form Cu^{2+} to around 10 µg/g in dry plant tissue. Increases of Cu in aquatic ecosystems is a consequence of various anthropogenic sources such as Cu mine drainage, Cu -based pesticides, industrial and domestic wastes and antifouling paints. It is commonly found in treated wastewaters. Toxicity of Cu causes oxidative stress, and the ions themselves could directly initiate oxidative breakdown of polyunsaturated lipids by two readily interconvertible oxidation states and it can catalyze the formation of free radicals through the Haber-Wiss reaction. Zinc is an essential element for the normal growth and metabolism which playsan important role in enzyme activation and is also involved in the biosynthesis of some enzymes and growth hormones of plants. Excess Zn has clearly visible effects (causes chlorosis) with inhibition of growth and decrease in biomass production; severe toxicity can also be fatal. Zinc toxicity is involved with metabolism through competitive inhibition of other essential ions, inactivation of enzymes, and displacement of essentialcofactor elements from functional sites of enzymes. Like Cu it is commonly found in domestic wastewaters.

There are many techniques used to remove contaminants from contaminated water. However, phytoremidiation is the best solution to remove the low contaminants from waste water. *C. demersum* is an aquatic plant that could be a good accumulator for Cu and Zn while they were together in solution. Additionally, RGR (relative growth rate), chlorophyll content of *C. demersum*show that acute toxic effects when exposed to Cu or Zn increased overtime. It was found that Cu and Zn effects manifested themselves more slowly than expected: at least 5 to 10 d were needed for noticeable effects both macroscopically (physical appearance), microscopically (Chloroplast morphology and numbers) and from measurements of photosynthesis using Pulse Amplitude Modulation (PAM) fluorometry. Moreover, the combination of Cu and Zn in the highest concentration was a higher toxic effect more than Cu or Zn in all parameters measured in this study.

Keywords: Phytoremediation, Cu, Zn, PAM, RGR, chlorophyll, contaminant, time course of toxicity.

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CHAPTER 1
INTRODUCTION

1.1 Introduction

Heavy metal environmental pollution is a serious problem that is widely distributed all over the world mainly through mobilization of heavy metals from ores (Ali *et al*., 2113). Increasing industrialization and disturbance of the natural biogeochemical cycle makes the problem of heavy metals pollution increasingly serious. Unlike organic substances, heavy metals cannot be degraded but can accumulate in the environment in toxic form. Heavy metal contamination of soil and water poses serious threat to aquatic life, human, food chain, and environment (Ali *et al*., 2113). Heavy metals have adverse effects on human health and contaminate both human food chains and food chains in the environment (Wuana and Okieimen, 2011; Modaihsh *et al*., 2004; Chehregani and Malayeri, 2007; Fulekar *et al*., 2009; Sabiha-Javied *et al*., 2009; Ali *et al*., 2013).

Cu and Zn are essential elements for plant growth, development. Generally higher plants require an uptake of Cu in the form Cu^{2+} of around 10 µg/g on a dry plant tissue basis (Baker and Brooks, 1989; Welch *et al*., 1993; Greger, 1999; Kanoun-Boulé *et al*., 2009). Increases of Cu in aquatic ecosystems is a consequence of various anthropogenic sources such as Cu mine drainage, Cu -based pesticides, domestic wastewater, industrial and domestic wastes and antifouling paints (Ma *et al*., 2003; Andrade *et al*., 2004). It is usually present in the effluent of sewage treatment plants. Toxicity of Cu causes oxidative stress of cellular metabolism, and the Cu^{2+} ions themselves and directly initiate oxidative breakdown of polyunsaturated lipids by two readily interconvertible oxidation states (Kanoun-Boulé *et al*., 2009). Another reason for Cu toxicity is that it can catalyze the formation of free radicals (Haber-Wiss reaction) (De Vos *et al*., 1993).

Many techniques have been developed to remove pollutants from soil including technical, physical, chemical, and combined methods (Ilyin, 1991; Osipov and Alekseev, 1996; Popesko and Solov'yev, 1996; Galiulin and Galiulina, 2003; Luo *et al*., 2006). These methods all have consequential effects on the environment, are costly and cannot remove pollutants 100% from soil or surface water. That is why the researchers have to focus on low cost techniques for saving soil and surface water, hence phytoremediation has been favoured as a cost-effective solution. Phytoremediation is a technique that uses plants for clean-up the pollutants from land and surface water (Pilon-Smits and Freeman, 2006).

There are several technologies of phytoremediation based on different uptake mechanisms such as phytodegradation, phytovolatilization, phytostimulation or rhizodegradation, phytoextraction, phytostabilizaltion, phytofiltration and phytodesalination. Suitable plants for phytoremediation of soils should have high biomass production, deep roots, be fast growing and be able to tolerate and accumulate high amounts of potentially toxic trace elements such as arsenic (As) (Alvarado *et al*.,2008), silver (Pinto *et al*., 1987), cadmium (Cd) (Agunbiade *et al*., 2009), chromium (Cr) (Mishra and Tripath, 2009), copper (Cu) (So *et al*., 2003), iron (Fe) (Jayaweera *et al*., 2008), nickel (Ni) (Hadad et al., 2011), lead (Pd) (Smolyakov *et al*., 2012), and zinc (Zn) (Lu *et al*., 2004). Plants also accumulate organics such as naphthalene (Nesterenko-Malkovskaya *et al*., 2012), ethion (Xia and Ma, 2006), paper mill waste containing tannins (Das & Mazumdar, 2016) and phenol (Nora and Jesus, 1997) in their shoots, fruits and other harvestable parts such as tubers. The aim of such processes is to lock away toxic trace elements in a biologically inert, usually insoluble form.

The phytoremediation requirements for aquatic plants may be slightly different to those of vascular terrestrial plants. *Ceratophyllum demersum* or coontail, is a dicotyledonous plant of the family Ceratophyllaceae. It is a widespread submerged aquatic plant, a rootless floating macrophyte (Mishra*et al*., 2006; Chen*et al*., 2015). Coontail has very small flowers located on the leaf below the water and plants have an average length of 50 cm to 110 cm due to ready fragmentation (Haberland, 2014; Umebese and Motajo,

2008). It grows rapidly in muddy, shallow water even under low illumination or high nutrient (Umebese, and Motajo, 2008). Coontail has a strong ability to accumulate many heavy metals such as Pb, Cu, Zn, Cr, Hg and Cd (Rai *et al*., 1995; Tripathi *et al*., 2007; Devi and Prasad, 1998; Aravind and Prasad, 2003; Chen*et al*., 2015). Coontail does not have roots and so removes metals from the water column and does not have the potential to remobilize metals already locked up in the sediment *Vallisneria natans* (Zhu *et al*., 2016).

This study compared the bioaccumulative capacities of Cu, Zn and their combination by *C. demersum* and physiological responses (growth, chlorophyll content, photosynthetic rate) of *C. demersum* to Cu and Zn. Additionally, pulse amplitude modulation (PAM) technology wasused to detect copper and zinc toxicity effects on the photosynthetic rate (light reactions of photosynthesis).

1.2 Objectives

1. To compare the bioaccumulative capacities of copper, zinc and their combination by *C. demersum.*
2. To study physiological responses (growth, chlorophyll content, photosynthetic rate) of *C. demersum* exposed to Cu and Zn.
3. To study the combination effects of Zn and Cu toxicity in *C. demersum* because Cu and Zn contamination often occurs in combination.
4. Particular attention was paid to the time course of development of toxicity symptoms.

1.3 Scope

This project was on the bioaccumulative capacities of Cu, Zn and their combination by *C. demersum.* Physiological responses including growth, cell and chloroplast characteristics, chlorophyll content, and photosynthetic rate wereinvestigated.

1.4 Expected outcomes

The understanding of phytoremediation techniques can help us to remove pollutants from contaminated sites successfully. Coontail (*C. demersum*) is one of the potential plants that has been used in phytoremediation technique. This study showed the capacity of this plant on removing Cu and Zn in aquatic environment. Moreover, the physiological responses of *C. demersum* exposed to Cu, Zn and Cu+Zn incombination were studied.

CHAPTER 2
LITERATURE REVIEW

2.1 Remediation technology

Remediation technology is the clean-up (or remediation) technology available for reducing the harmful effects at organic and inorganic-contaminated sites. There are many methods for reducing the bioavailability of heavy metals from contaminated soil: physical removal (soil replacement and thermal desorption), chemical inactivation (chemical leaching, chemical fixation into insoluble forms, electrokinetic conversion into insoluble forms, vitrification (glassification)) and biological conversion into unavailable insoluble forms (phytoremediation, bioremediation, animal remediation) (Yao *et al*., 2012). Soil replacementis replacing contaminated soil by using clean soil to cover it over or adding fresh soil to dilute pollutants (Yao *et al*., 2012) (Table 2.1). pH is an important consideration: low pH mobilizes Cu and Zn whereas under neutral and alkaline conditions insoluble oxides generally form which effectively locks Cu and Zn out of the ecosystem. Changes in soil redox/pH conditions can convert dangerous mobile forms of heavy metals into inert insoluble forms.

Table 2.1 Summary of remediation technologies of soil contaminated by heavy metals

Type of remediation	Pollutants	Advantage	Disadvantage
Soil replacement method	Heavy metals	To decrease the pollutant concentration in short term (6-9 months)	Mainly suitable for large scale projects. It can be very costly but can be adapted for small areas of severe soil pollution.
Thermal desorption	Hg, As	Simple process but very energy expensive, devices are usually mobile and the remediated soil can be reused (6-9 months).	Very expensive but especiall effective for mercury contamination. Energy costs are high.
Chemical leaching	As	Chemical leaching removes acid soluble and redox mobilisable metals (6-9 months).	What to do with the leachate can be a problem. Essentially eliminates microbial soil flora which would need to be replaced probably using soil conditioners.
Chemical fixation	Heavy metals	Conversion of soluble into inert insoluble forms by changing pH and Redox conditions without collecting leachate (6-9 months).	Adjust pH conditions using lime and limestone. The use of soil conditioning agents to change redox conditions may change the soil structure and have effects on the microbes in soil.
Electrokinetic remediation	Organic and inorganic	Electrokinetic remediation has the advantages of easy installation and operation, low cost and does not physically disturb the soil which might initiate undesirable redox reactions mobilizing metals (6-9 months).	Electrokinetic remediation needs pH control using lime or limestone to avoid acidification. Sometimes treatment efficiency was low.

Table 2.2 Summary of remediation technologies for heavy metal contaminated by soils (Continued)

Type of remediation	Pollutants	Advantage	Disadvantage
Vitrification technology	Very Dangerous Metals	Vitrification technology can release organic matters volatilized or decomposed (6-9 months)	Complex and very expensive basically locks up metals in inert glass. Needs lots of energy in the melting, which makes it very costly. Appropriate only for the most dangerous heavy metals. Volatilisation can negate effectivess.
Phytoremediation	Organic and inorganic	Particularly suitable for low concentrations of contaminants covering a large area; accumulates high concentrations of the contaminants. Well chosen plants can accumulate many different kinds of heavy metals. Removes biologically active metals and not heavy metals in soils that are already in biologically unavailable forms. Generally low cost.	Takes long time (18-60 months)
Biological remediation using microbes	Heavy metals	Microbes can perform many types of redox reactions that plants do not.	Take long time (18-60 months)
Animal remediation	Heavy metals	Soil fauna, particularly aerthworms, can contribute to immobilization of heavy metals by adsorbing heavy metals, degrading into insoluble forms in their faeces and allowing migration of the heavy metals into the subsoil.	Take long time (18-60 months)

2.2 Phytoremediation (adapted from UNEP 2019 with relevant references added)

Phytoremediation is a natural technique that uses plants and their associated microbes for environmental cleanup (Ojecgba and Fasidi, 2007; Mahar *et al*., 2016). This technique uses natural processes where plants and their microbial rhizosphere bacterial and fungal flora modify or extract hazardous substances from the environment and turn them into biologically more inertmetabolites (Pilon-Smits, 2006; Odjecgda and Fasidi, 2007; Mahar *et al*., 2016). Phytoremediation is an effective and inexpensive technology that can clean up hazardous waste sites contaminated with both inorganic and organic contaminants (Xia *et al*., 2006; Pilon-Smits &Freeman, 2006; Odjegba and Fasidi, 2007) (Fig. 2.1 and Table 2.2). There are many modern techniques of phytoremediation, based on different uptake mechanisms such as phytodegradation, phytovolatilization, phytostimulation or rhizodegradation, phytoextraction, phytofiltration, phytostabilizaltion, and phytodesalination.

2.2.1 Phytodegradation

Phytodegradation, also called phyto-transformation, reduces or breaks down contaminants through metabolic process in the plant (Pilon-Smits &Freeman, 2006). Enzymes produced by the plant breakdown contaminants surrounding the plant (Pilon-Smits &Freeman, 2006). Plants have abilities to degrade complex organic and inorganic pollutants into simpler molecules and incorporate them into their tissues to help them grow faster (Table 2.1) (Pilon-Smits & Freeman, 2006) or convert them into a biologically inert form. Enzymes in plants can catalyze and accelerate chemical reactions. Al-Baldawi *et al*. (2015) studied on phytodegradation of total petroleum hydrocarbon (TPH) in diesel-contaminated water using *Scirpus grossus* and showed that hydrocarbon (223.56 mg/kg) accumulated in stem and leaf samples 93.72%. The hydrocarbons accumulated were n-alkanes (C20–C34) (Table 2.3).

2.2.2 Phytovolatilization

Phytovolatilization is the uptake and transpiration of contaminants from one segment of the environment (soil or water) to another (atmosphere) through the plant by

converting liquid to gas (Pilon-Smits &Freeman, 2006; Boule *et al*., 2009). Some plants allow some contaminants (chlorinated solvents, some inorganic compounds) to pass through their body to leaves and evaporate or volatilize into the atmosphere (Pilon-Smits &Freeman, 2006). Moreno *et al*. (2008) investigated the removal of Hg from solutions by Indian mustard (*Brassica juncea*) grown in hydroponic conditions with solutions containing Hg concentrations from 0 to 10 mg/L (Table 2.3). Moreno *et al*. (2008) also showed thatphytofiltration effectively removed up to 95% of Hg by *B. juncea* from the contaminated solutions by both volatilization and plant accumulation.

2.2.3 Phytostimulation

Phytostimulation, also called rhizodegradation or plant-assisted bioremediation, is breakdown of contaminants in the soil by microorganisms in the rhizosphere (Boule *et al*., 2009; Jia *et al*., 2016). Organic substances are consumed and digested by microorganisms (yeast, fungi, and bacteria) for nutrition and energy. In biodegradation microorganisms digest organic substances (fuels or solvents which affect human health) and decrease their toxicities or remove them from the immediate environment by volatilisation. The plant roots can release natural organic compounds (sugar, alcohols, and acids) which contain nutrients and provide an energy source to enhance microbial activities in the soil to break down contaminating compounds in the environment and transpire water (Boule *et al*., 2009; Jia *et al*., 2016). Ramamurthy and Memarian, (2012) reported that *B. juncea* could accumulate Cd (II) (189.1 mg/g -327.3 mg/g) and $PbCl_2$ (5.4 mg/g) in shoots (Table 2.3).

2.2.4 Phytoextraction

Phytoextraction also called phytoaccumulation, refers to uptake, translocation and accumulation of organic and inorganic pollutants from soil by the plant roots to aboveground biomass (Mahar *et al*., 2016). Certain plants, called hyperaccumulators, absorb large amounts of toxicant in their bodies in comparison to other plants. Toxicant translocation from root to shoot is an essential biochemical process and may be a desirable and effective phytoextraction to remove contaminants in soils (Ali *et al*., 2013). Phytoextraction is the best technique of phytoremediation to remove organic

and inorganic pollutants (Table 2.2) because there are approximately 400 known plants that accumulate these heavy metals (Pilon-Smits &Freeman, 2006). For example, Ubugunovet *et al*. (2014) studied on Cd extraction potential of *Thlaspi caerulescens* and found that at maximum Cd dose 500 mg/kg, shoots accumulated 259 mg/kg in dry mass and the root system up to 609 kg/kg. Moreover, the removal of soil Cd by *T. caerulescens* per square unit varied from 0.13 to 12.92 mg/m^2 depending upon Cd dose in soil and its incubation period. Another study showed that plants (sunflower, Ricinus, and mustard) could extract Cd and Pb from soil with different abilities of phytoextraction and translocation of heavy metals (Lu and He, 2005; Niuet *et al*., 2007). *Helianthus annuus* could accumulate Cd (327.34 mg/kg) in 20 mg/L Cd solution. *H. annuus* could accumulate Pb (917.82 mg/kg) when Pb (200 mg/L) was added in solution. *B. juncea* could accumulate Pb (835.54 mg/kg) at the concentration of Pb (200 mg/L). Lu and He (2005) who studied on bioaccumulation of Cd by *Ricinus communis* and showed that Cd (4460.3 mg/kg) was accumulated from solution containing 360 mg/L Cd (Table 2.3). Harvesting of the plants that had accumulated the contaminants in effect removes the contaminant from the area. Sometimes remobilization of heavy metals from sediment is not desirable, for example remobilization of Cu by rooted aquatic plants such as *Vallisneria natans* (Zhu *et al*., 2016) and it may be better for Cd and Pb to be left in insoluble form in soils (Lu and He, 2005; Niu *et al*., 2007) if the pH and redox conditions in the soils do not favour remobilisation.

2.2.5 Phytofiltration

Phytofiltration is the absorption or precipitation of contaminants (heavy metals, organic compounds) from contaminated surface water or wastewater into the roots. Phytofiltration is used to cleanup contaminants in groundwater rather than soil (Pilon-Smits &Freeman, 2006; Boule *et al*., 2009). Das *et al*. (2016) studied on phytoremediation potential of a novel fern, Salvinia cucullata which could accumulate Cr (1-10 mg/kg), Cu (20-100 mg/kg), Ni (20-246 mg/kg), Pb (27 mg/kg), Fe (1000 mg/kg), Zn (100-500 mg/kg), Mn (400 mg/kg), P (no limited) in leaves and root (Table 2.3).

2.2.6 Phytostabilizaltion

Phytostabilizaltion or phytoimmobilization refers to the use of plants for stabilization of contaminants (in insoluble form) from soil and groundwater (Pilon-Smits &Freeman, 2006; Ali *et al*., 2013). This technique is used to re-establish the mobility and bioavailability of pollutants (heavy metals and chlorinated solvents) in soil and groundwater (Table 2.2). Plants can immobilize heavy metals through sorption by the roots. Chunkao *et al*. (2012) studied phytostabilizaltion of *Eichhornia crassipes* and showed effective removal of pollutants such as Cr (130.3 ppm), Pb (102.6 ppm), Cd (0.44 ppm), Hg (0.3 ppm) from aqueous solution (Table 2.3). pH and redox conditions can be manipulated to ensure that heavy metals remaining in soils in insoluble form.

2.2.7 Phytodesalination

Phytodesalination is the use of halophytic plants for removal of salts from salt-affected soils in order to enable them for supporting normal plant growth (Ali *et al*., 2013). Phytodesalination may also remove chlorinated solvents and inorganic compounds (Table 2.2) (Laghlimi *et al*., 2015). The cultivation of the halophyte on the salinized soil (phytodesalination culture) showed that *Sesuvium portulacastrum* has the ability to absorb Na^+ ions by roots and accumulate the Na^+ in above-ground biomass up to 872 mg/plantand 4.36 g/pot (about 1t/ha) (Table 2.3) (Rabhi *et al*., 2010). However, plants have different abilities to accumulate organic and inorganic pollutants. Another study reported on comparison of *Thellungiella salsuginea* to its glycophyte relative Arabidopsis thaliana and showed that *T. salsuginea* was more tolerant to phenanthrene stress as compared *to A. thaliana* (Shiri *et al*., 2016). The two halophytes, *Suaeda maritime* and *Sesuvium portulacastrum* could remove 504 kg and 474 kg NaCl from 1 ha of saline soil in a period of 4 months (Table 2.3) (Ali *et al*., 2013).

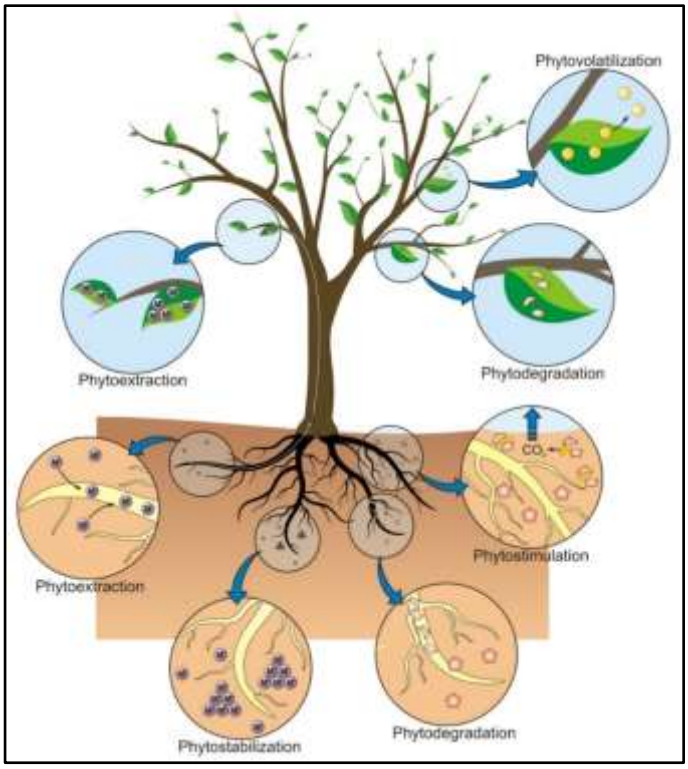


Figure 2.1 Diagram showing the phytoremediation techniques (source: http://systemsbiology.usm.edu/BrachyWRKY/WRKY/IMG/Phytoremediation-01.jpg)

Table 2.3 Summary of different techniques of phytoremediation

Techniques	Description	Process goals	Contaminants
Phytodegradation	Intracellular degradation of organic xenobiotics in plant tissues.	Contaminant extraction and degradation	Toxic metals and organic pollutants
Rhizodegradation	Degradation of organic xenobiotics by microbes living around roots.	Contaminant extraction and degradation	Toxic metals and organic pollutants
Phytoextraction	Pollutant accumulation in plant biomass not used as food i.e., shoots, floriculture, fibre etc	Physical removal from the area.	Toxic metals and Organic pollutants
Phytostabilization	Roots secrete organic compounds which immobilse harmfull metals.	Locking up contaminants in biologically unavailable form.	Heavy metals, chlorinated solvents
Phytofiltration	Mainly applies to aquatic plants taking up pollutants from the water column and from sediment in the case of rooted aquatics.	Capture in plant biomass for removal	Heavy metals, organic compounds
Phytodesalination	Removal of excess salts from saline soils by harvesting halophytes.	Removal of salt by salt accumulating plants	NaCl

Table 2.4 Examples of phytoremediation techniques that can be used to uptake heavy metals in some vascular plants.

Techniques	Plant species	Metal concentration	References
Phytodegradation	*Scirpus grossus*	Total petroleum hydrocarbon (223.56 mg/kg) in stem and leaf samples with 93.72% n-alkanes C20–C34	Al-Baldawi*et al*., 2015
Phytovolatilization	*Brassica juncea*	Hg emission from planted vessels (0.625 µg -314 µg) at 0-10 ml/l Hg.	Moreno *et al*., 2008
Phytostimulation	*Brassica juncea*	$CdCl_2$ (189.1 mg/g -327mg/g) in shoots$PbCl_2$ (5.4 mg/g)) in shoots	Ramamurthy,and Memarian, 2012
Phytoextraction	*Thlaspicaerulescens*	Cd (500 mg/kg) shoots, 259 mg/kg in dry mass and up to 609 kg/kg (the root system); Cd (12.92 mg/m^2)	Ubugunov, *et al*., 2014
	Helianthus annuus	Cd (327.34 mg/kg)	Niu, *et al*., 2007
	Brassica juncea	Pb (917.82 mg/kg)	Niu, *et al*., 2007
	Ricinus communis	Pb (835.54 mg/kg)	Niu, *et al*., 2007
	Typha angustifolia	Cd (4460.3 mg/kg)160.7 ng/g over a period of 342 days; ibuprofen carboxylic acid(1374.9 ng/g); 2- hydroxy ibuprofen (235.6 ng/g); and 1-hydroxy ibuprofen (301.5 ng/g) in the sheath	Lu and He, 2005 Li *et al*., 2016

Table 2.5 Examples of phytoremediation techniques that can be used to uptake heavy metals in some plants (Continued).

Techniques	Plant species	Metal concentration	References
Phytofiltration	*Salvinia cucullata*	Cr (1-10 mg/kg in leaf and root) Cu (20-100 mg/kg in leaf and root) Ni (20-246 mg/kg in leaf and root) Pb (27 $mg.kg^{-1}$ in leaf and root) Fe (1000 mg/kg in leaf and root) Zn (100-500 mg/kg in leaf and root) Mn (no limited)Mn (400 mg/kg in leaf and root)	Das *etal*., 2016
		P (not limited)	
Phytostabilization	*Eichhornia crassipes*	Cr (130.3 ppm) Pb (102.6 ppm) Cd (0.44 ppm) Hg (0.3 ppm)	Chunkao *et al*., 2012
Phytodesalination	*Suaeda maritime*	NaCl (504 kg/ha period of 4 months)	Ali *et al*., 2013
	Sesuvium portulacastrum	NaCl (474 kg/ha period of 4 months) accumulate in above-ground biomass up to 872 mg/plant and 4.36g/pot (about 1t/ha)	Ali *et al*., 2013, Rabhi *et al*., 2010

2.3 Heavy metals

Heavy metals are natural constituent elements and are present in varying concentrations in all ecosystem but sometimes they are present as inert insoluble forma of little environmental concern (Ilyin *et al*., 2003; Carranza *et al*., 2016). Some heavy metals

are found in volatile forms and adsorb onto fine particles that could be widely transported as dust in wind erosion (Ilyin *et al*., 2003; Carranza *et al*., 2016).

Heavy metals often have insidious effects, on human health and contaminate the food chain in the environment for both animals and humans (Wuana and Okieimen, 2011; Modaihsh *et al*., 2004; Chehregani and Malayeri, 2007; Fulekar *et al*., 2009; Sabiha-Javied *et al*., 2009; Ali *et al*., 2013). The high level of heavy metals present in the environment have become a serious threat to aquatic life and human health (Table 2.4). Table 2.6 is a summary chronic and acute heavy metal toxicity on human health. Clemens (2006) points out that toxic effects of heavy metals (Cadmium, Cd) can occur in humans at levels that are asymptomatic in plants.

Most heavy metals have relatively harmless insoluble oxides that can remain in soils and sediments with few adverse effects: problems arise if they are mobilised into soluble forms. The sources of various toxic metals are summarized in Table 2.7. The mobilization of heavy metals releases elements into the environment by many ways such as natural sources (volcanic, erosional, mineral) and anthropogenic sources (industrial, urban, agricultural, waste disposal) (Ali *et al*., 2013; Mahar *et al*., 2016). Atmospheric dispersal is an important component of lead pollution (Table 2.5) because of volatile Pb compounds (Thangavel and Subbhuraam, 2004; Wuana and Okieimen, 2011).

2.4 Toxicity of heavy metals in plants

Heavy metals such as Cu, Ni and Zn are essential trace micronutrients for plants, but in excess these metals can harmful to humans, animals and plants and can contaminate the food chain in the environment. Another class of heavy metals (for example, Pb, Cd and Hg) are those that are not trace elements and have no known positive biological function (Reeves, 2000; Hall, 2002). Cadmium is a toxic non-essential metal, which targets different components of the photosynthetic apparatus and can decrease electron transport efficiency, inhibit chlorophyll biosynthesis and reduce photosynthetic carbon assimilation. These effects are a consequence of Cd acting as a toxic analogue of

essential trace metals (Zn/Cd) (Waisberg *et al*., 2003; Maksymiec *et al*., 2007). On the other hand, Zn is an important component of a large number of enzymes in plants and animals but can be toxic at high concentrations (Hall, 2002; Cherif *et al*., 2011). Notably heavy metals such as Zn and Cu interfere with electron transport processes, the cytochromes of mitochondria and chloroplasts (Raven *et al*., 1999). Wang *et al*. (2009) showed that excessive accumulation of Zn accumulation delays or diminishes growth and root development and causes leaf chlorosis due to interference with chlorophyll synthesis (Hall, 2002). Excess Zn can cause the formation of ROS (Reactive Oxygen Species) in plant cell because of the redox properties of Zn ions. This results in general cellular oxidative damage and photooxidative problems in chloroplasts and membrane lipid peroxidation (Clemens, 2006; Jin *et al*., 2008).

Cadmium is a toxic heavy metal that unfortunately is recognized as an analogue for Zn. It seems to mainly act as a competitive inhibitor of Zn uptake, transport and metabolism. When both Cd, Zn were combined together, the toxic effect of Cd could be prevented by Zn in a competitive inhibitor effect (Clemens 2006; Cherif *et al*., 2011). Hassan *et al*. (2005b) showed that Cd poisoning could be partially releaved by adding high levels of Zn in culture media indicating a reduction in the Cd uptake and accumulation in roots without transport into the xylem stream in to the rest of the plant in rice cultivars. This is a consequence of a higher degree of discrimination at the endodermis for Zn over Cd. Hart *et al*. (2002) showed that in both durum and bread wheat, increased Zn decreased Cd accumulation in roots, possibly due to a competition between Zn and Cd uptake. Aravind and Prasad (2003) showed in *Ceratophyllum demersum* that Zn increased provided some protection of anti-oxidant enzymes from Cd-toxicity even at [Zn] as low as 200 μmol/l. Cherif *et al*. (2011) showed the Zn concentration at low level, strongly protected *Solanum lycopersicum* from Cd toxicity (through reducing Cd uptake, chlorophyll breakdown and lipid peroxidation and improving the ROS scavenging antioxidant enzymes activities). Interestingly, when Zn was increased in the medium in combination with Cd, there was a detectable increases in oxidative stress in the cytochromes in mitochondria and

chloroplasts, which was higher than that for Cd or excess Zn respectively indicating a significant effect of the ratio of Cd to Zn (Raven *et al*., 1999).

2.4.1 Plant growth parameters

Das*et al.* (2016) reported that *Eichhornia crassipes* without Cd treatment (control) showed increases in root, shoot, and leaf biomass increase, whereas Cd treatment group showed decrease in growth with increase in Cd concentration. The results (Table 2.6) showed that *E. crassipes* could tolerate high concentration of Cd in 21 days. However, *E. crassipes* showed toxicity symptoms (chlorosis, necrosis, wilting of old leaves) at high concentration of Cd (Figure 2.2; Table 2.5). These are conventional, well documented effects of Cd toxicity in plants (Clemens, 2006).

Table 2.6 Summary of harmful effects of specific heavy metals on human health.

Heavy metal	**Cause effect**	**References**
As	As (as arsenate) is an analogue of phosphate and thus interferes with essential cellular processes such as oxidative phosphorylation and ATP synthesis.	Tripathi *et al*. (2007)
Cd	Carcinogenic, mutagenic, and teratogenic; endocrine disruptor; interferes with calcium regulation in biological systems; causes renal failure and chronic anemia	Degraeve (1981); Salem *et al*. (2000); Awofolu (2005); Clemens (2006).
Cr	hair loss	Salem *et al*. (2000)
Cu	Elevated levels have been found to cause brain and kidney damage, liver cirrhosis and chronic anemia, stomach and intestinal irritation	Salem *et al*. (2000); Wuana and Okieimen (2011)
Hg	Anxiety, autoimmune diseases, depression, difficulty with balance, drowsiness, fatigue, hair loss, insomnia, irritability, memory loss, recurrent infections, restlessness, vision disturbances, tremors, temper outbursts, ulcers and damage to brain, kidney and lungs	Neustadt and Pieczenik (2007); Ainza *et al*. (2010), and Gulati *et al*. (2010)

Table 2.6 Summary of harmful effects of specific heavy metals on human health (Continued)

Heavy metal	Cause effect	References
Ni	Allergic dermatitis known as nickel itch; inhalation can cause cancer of the lungs, nose, and sinuses; cancers of the throat and stomach have also been attributed to its inhalation; hematotoxic, immunotoxic, neurotoxic, genotoxic, reproductive toxic, pulmonary toxic, nephrotoxic, and hepatotoxic; causes hair loss.	Tariq *et al*. (2006)
Pb	Aerial emission from combustion of leaded petrol, battery manufacture, herbicides and insecticides.	Salem *et al*. (2000); Padmavathiamma and Li(2007); Wuana and Okieimen (2011); Iqbal (2012)
Zn	Over dosage can cause dizziness and fatigue. Over-dosage of Zn could cause dizziness and fatigue in humans.	Hess and Schmid (2002)

Table 2.7 Anthropogenic sources of specific heavy metals in the environment.

Heavy metal	Source	References
As	Pesticides and wood preservatives, contaminant of phosphate fertilizers.	Thangavel and Subbhuraam (2004)
Cd	Cadmiumis a commonly used industrial catalyst. Incidentally present inpaints and pigments, plastic stabilizers, electroplating, incineration of Cd-containing plastics, natural contaminant of phosphate fertilizers	Salem *et al*. (2000); Pulford and Watson (2003)
Cr	Tanneries, steel industries, fly ash	Khan *et al.* (2007)
Cu	Pesticides, fertilizers, waste domestic water	Khan *et al.* (2007)
Hg	Release from Au–Ag mining and coal combustion, medical waste	Memon *et al*. (2001); Wuana and Okieimen (2011); Rodrigues *et al*. (2012)

Table 2.7 Anthropogenic sources of specific heavy metals in the environment (Continued).

Heavy metal	Source	References
Ni	Industrial effluents, kitchen appliances, surgical instruments, steel alloys, automobilebatteries	Tariq *et al*. (2006)
Pb	Aerial emission from combustion of leaded petrol, battery manufacture, herbicides andinsecticides.	Thangavel and Subbhuraam (2004); Wuana and Okieimen (2011)
Zn	Emission from traffic, agricultural and industrial products.	Aksu (2015)

2.4.2 Chlorophyll and carotenoid contents

Water hyacinth was affected on the chlorosis and wilting of leaves with higher concentrations of Cd. Concomitant with these symptoms, the chlorophyll and carotenoid contents were also reduced (Table 2.7). The total chlorophyll contents were 6.15 mg/g FW in control plants and they were significantly reduced 5.38, 3.04, and 1.68 mg/g at 10, 15, and 20 mg/l Cd, respectively (Clemens, 2006; Das *et al*., 2016).

Table 2.8 Dry biomass (g/plant) of different plant tissues along with root length (cm) and total leaf area (cm^2) of *E. crassipes* grown in Cd solution (Das *et al*., 2016).

$CdCl_2$ (mg/l)	**Day(d)**	**Root**	**Shoot**	**Leaf**	**Root length (cm)**	**Total leaf area (cm^2)**
Control	0 d 21d	0.44 ± 0.002 1.58 ± 0.36	0.51 ± 0.003 2.13 ± 0.19	0.62 ± 0.009 2.35 ± 0.22	9.9 ± 0.264 20.3 ± 0.45	165.0 ± 8.88 311.4 ± 4.20
5	0 d 21d	0.44 ± 0.002 0.86 ± 0.02* (-45.56%)	0.51 ± 0.003 1.25 ± 0.25* (-41.31)	0.62 ± 0.003 1.22 ± 0.19* (-48%)	9.9 ± 0.173 18.2 ± 0.50* (-10.48%)	165.6 ± 1.52 276.5 ± 7.31* (-11.21%)
10	0d 21d	0.44 ± 0.003 0.67 ± 0.01* (-57.34%)	0.51 ± 0.003 0.76 ± 0.02* (-64.08%)	0.62 ± 0.003 0.83 ±0.008* (-64.46%)	9.9±0.2017. 2 ±0.37* (-15.27%)	165.6 ± 3.21 254.7 ± 10.14 (-18.21%)

Table 2.8 Dry biomass (g/plant) of different plant tissues along with root length (cm) and total leaf area (cm^2) of *E. crassipes* grown in Cd solution (Das *et al*., 2016) (Continued)

$CdCl_2$ (mg/l)	**Day(d)**	**Root**	**Shoot**	**Leaf**	**Root length (cm)**	**Total leaf area (cm^2)**
15	0d 21d	0.44 ± 0.003 0.55 ± 0.01 (-64.6%)	0.50 ± 0.002 0.61 ± 0.01* (-71.12%)	0.62 ± 0.006 0.72 ±0.008* (-69.19%)	9.96 ± 0.251 15.4 ± 0.40* (-24.13%)	165.3 ± 3.20 225.9 ±12.15* (-27.45%)
20	0d 21d	0.44 ± 0.001 0.46 ± 0.01* (-70.75%)	0.50 ± 0.003 0.53 ± 0.01* (-75.16%)	0.62 ± 0009 0.65 ± 0.01* (-72.17%)	9.9 ± 0.0057 14.5 ± 0.20* (-28.57)	164.66 ± 4.5 205.8 ± 4.32* (-33.91%)

Table 2.8 is an example of heavy metal accumulation in an aquatic vascular plant over time, compared to the controls (no Cd and t = 0). Cd had a consistent inhibitory effect on the biomass of the roots, shoots and leaves compared to the control after 21d and also inhibited root length and leaf surface area. All concentrations of Cd were inhibitory but inhibition was highest at the highest concentration used. Although *E. crassipes* is a rooted aquatic plant the roots were extracting the Cd from the water column, not from soil.

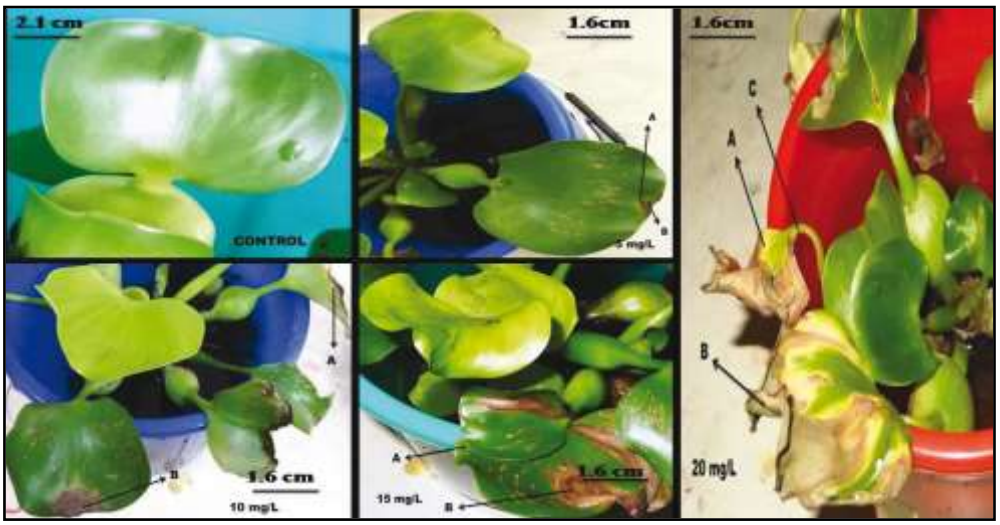


Figure 2.2 *E. crassipes* treated with different concentrations of Cd after 21 days. A – yellowing, B – necrosis, and C – wilting (Das *et al*., 2016). Cd caused significant necrosis of leaves.

Table 2.9 Effect of Cd treatments on leaf pigment contents of *E. crassipes* after 21 days. Redrawn from Das *et al*. (2016).

$CdCl_2$ (mg/L)	**Chlorophyll (mg/gfresh weight)**			**Carotenoid**
	Chl_a	**Chl_b**	**Chl_{a+b}**	**$Carot_{x+c}$**
0	6.15 ± 0.081	1.67 ± 0.225	7.83 ± 0.225	2.09 ± 0.035
5	5.69 ± 0.09*	1.86 ± 0.072ns	7.55 ± 0.159ns	1.8 ± 0.047*
10	4.07 ± 0.042*	1.30 ± 0.132*	5.38 ± 0.174*	1.49 ± 0.022*
15	2.27 ± 2.18*	0.767 ± 0.1*	3.04 ± 0.122*	1.03 ± 0.087*
20	1.48 ± 0.117*	0.202± 0.096*	1.68 ± 0138	0.687 ± 0.042*

Cd effects were significantly different from control at $P < 0.05$; values are mean ± SD of 3 replicates; percent decrease in mean values as compared to the corresponding control values is shown the parentheses. Chl*a* = Chlorophyll *a*, Chl *b* =Chlorophyll *b*, Chl_{a+b}= total chlorophyll; $Carot_{x+c}$ = carotenoid. ; * = significantly different and ns = not significantly different at $P < 0.05$ at various doses of Cd for a particular plant pigment as compared to control values.

2.5 Bioaccumulation of heavy metals in plants

A bioaccumulation factor (BCF>1000) indicates a favourable accumulative ability of a plant. Such a plant may have potemtial in bioindication and phytoremediation projects (Rajfur *et al*., 2010). Begum and HariKrishna (2010) studied on bioaccumulation of trace metals by aquatic plants to evaluate the usefulness of different macrophytic species in reducing the nutrient contents of the water to reduce the pollution level of water. In their research, *Hydrilla vercillata*, Elodea (*Elodea canadensis*), *Salvinia sp*., were tested for removal of three important heavy metals: Fe, Cu and Ni from metal solution. These plants removed up to 98% of Fe, 95% of Cu and 90 % of Ni after 10 days. Results indicated that Fe over the period of 10 days was reduced to found harmless levels. The plants did not show any toxicity symptoms of Fe toxicity. However, all the plants showed some morphological toxicity symptoms caused by Cu and Ni after 5 days. *Salvinia sp.* improved water quality to the maximum extent by reducing all metal concentrations. Phukan *et al*., (2015) studied *Hydrilla verticillata* treated with 15 mg/l Cr and Cd in solution for 11 days. Plantsaccumulated Cr in leaves from Cr solution, 20 mg/l highest in roots and Cd accumulation was maximum in 3 mg/l in both roots and shoots. This study showed that bioaccumulation of both Cr and Cd in root was significantly lower than that in leaf in all concentrations.

2.5.1 Metal uptake capacity

Das*et al*. (2016) showed that *E. crassipes* accumulated high concentrations Cd to up to 15 mg/l. However, at 20 mg/l of $CdCl_2$ the accumulation started to decrease (Tables 2.9 and 2.10 below). This suggests an upper (but very high) tolerance limit for Cd in *E. crassipes*.

Table 2.10 Cadmium accumulation in different plant parts (roots, shoots, and leaves) of *E. crassipes* after 21 days (Das *et al*., 2016).

$CdCl_2$ (mg/L)	Cd concentration (µg/gdry wt) in plant parts			
	Root	**Shoot**	**Leaf**	**Whole plant**
5	846. 6 ± 43.22	937.9 ± 61.84	850.2 ± 52.57	878.3 ± 51.68
10	956.0 ± 43.44	986.0 ± 76.39	958.8 ± 68.24	966.9 ± 61.16
15	1908.6 ± 18.88*	1966.1 ± 28.58*	1908.6 ± 5.72*	1927.8 ± 17.03*
20	921.97 ± 38.13	1966.33 ± 21.79	848.22 ± 76.77	912.5 ± 40.46

Mean ± SD (n = 3); = * indicate significance at $P < 0.05$ at different does for a particular plant tissue.

2.5.2 Bioconcentration factor

Table 2.11 Bioconcentration factor (BCF) of Cd in different parts of *E. crassipes* (Das *et al*., 2016).

$CdCl_2$(Mg/L)	BCF_{root}	BCF_{shoot}	BCF_{leaf}	$BCF_{whole\ plant}$
5	169.3 ± 8.64	187.5 ± 12.3	170 ± 10.49	526 ± 31.0
10	95.6 ± 4.45	98.6 ± 7.63	95.8 ± 6.8	290 ± 18.35
15	127.2 ± 1.25	131.07 ± 1.9	127.2 ± 0.38	385 ± 3.40
20	46.09 ± 1.90	48.36 ± 1.08	42.41 ± 3.83	121 ± 33.76

Bioconcentration factor (BCF) is the ability of plants to bioaccumulate a particular metal in its tissue taking into account the concentration of that element in the substrate (Umebese and Motajo, 2008; Zayed *et al*., 1998). The bioconcentration factor is the ratios between concentration of heavy metal in plant tissue and initial concentration of metal in external solution. After 21 days of growth, the bioconcentration factor decreased with increasing Cd concentrations (Das *et al*., 2016) (Tables 2.9, 2.10 and 2.11). Cadmium is an insidious poison because it acts as an analogue in Zn and Cu transport mechanisms and so is inadvertently accumulated by the mechanisms plants use to obtain trace metals (Clemens, 2006).

2.6 Copper and its toxicity to plants

Copper an essential micronutrients in plants (Sheldon *et al*., 2004; Yruela, 2005; Burkhead *et al*., 2009). Its particularly critical role is a cofactor in enzymes that are involved in electron transfer reactions (Yruela, 2005; Burkhead *et al*., 2009). Mining and smelting, urban, industrial and agricultural wastes, and the use of agrochemicals are the main human activities which mobilize Cu into the environment (Sheldon *et al*., 2004; Khan *et al*., 2007; Ali *et al*., 2013).

Copper also acts as a structural element in regulatory proteins and ezymes. Copper is an essential functional component in photosynthetic electron transport, oxidative stress response, cell wall metabolism and hormone signaling (Marschner, 1995; Mocquot *et al*., 1996; Raven *et al*., 1999). Undertypical environmental conditions, Cu is present in many forms in soils with free Cu^{2+} and Cu^{+} activities considered the best indicator of bioavailability but much Cu in soils is not readily available to plants and plants mobilise it using chelation compounds such as citrate (Sauve *et al*., 1996; Yruela, 2005).

Both, Cu deficiency and excess Cu can be toxic to plants (Hall, 2002; Sheldon *et al*., 2004; Yruela, 2005; Burkhead *et al*., 2009). Mocquot *et al*. (1996) reported that Cu caused decreases in root and leaf biomass. High levels of Cu cause characteristic symptoms: chlorosis, abnormal colouration and necrosis of leaves and at the chloroplast level chlorophyll deficiencyiy and alterations of chloroplast structure and thylakoid membrane composition and at the whole-plant leaf stunting of growth and inhibition of root growth (Baszynski *et al*., 1988; Van Assche & Clijsters, 1990ab; Lidon and Henriques, 1993; Marschner, 1995; Ciscato *et al*., 1997; Pätsikkä*et al*., 1998; Quartacci *et al*., 2000; Yruela, 2005). Hu *et al*. (2007) showed that high concentrations (5 mg/l and 10 mg/l) of Cucould reduce chlorophyll pigment concentration of *E. crassipes.* In contrast, malondiadehyde (MDA) increased when the *E. crassipes* wasexposed to high Cuconcentrations. However, protein content increased at Cu < 0.5 mg/l but then decreased with exposure to Cu ≈ 1 mg/l. Devos *et al*. (1993) showed that cell membrane properties were affected when Cu was increased by oxidation of membrane lipids. Hu *et al*. (2007)

reported that Chl *a* decreased rapidly after exposure to 5 mg/l Cu or higher concentrations to below 50% of the controls after 3 days followed by a slight rise of relative ratio to < 60% after 3 days to 10 days. Chl *b* also decreased rapidly to < 40% from 1 to 5 days at Cuconcentration of 5 mg/lor higher, followed by a rise of the relative ratio to nearly to 70% at 10 days. Carotenoid content decreased rapidly to 50% at 3 days followed by a slight increase to < 60% at 5 to 10 days at 5 mg/l Cu or higher concentrations. The soluble protein content decreased significantly to more than 80% of control after 6 days and then it still decreased to 50% and 40% for 14 days, respectively. Malondiadehyde increased significantly to three fold higher than control at 5 mg/l - 10 mg/l Cu. *S. sagittifolia* and *P. crispus* are aquatic plants which great accumulation of toxic metals such as Fe, Pb, Ni, Cd, and Cu (Ali*et al*., 2005).

2.7 Zinc and its toxicity to plants

Zinc is essential micronutrient for protein synthesis, development and is the structural component of ribosome but is toxic at higher concentration levels in plants (Hall, 2002; Radic *et al*., 2010; Mousvi *et al*., 2012). Zinc also acts as a constituent or cofactor for several enzymes and not only involved in protein sytnthesis but also in carbohydrate, nucleic acid, lipid metabolism (Radic *et al*., 2010; Mousvi *et al*., 2012). Zn also has an important role in interaction with other eseential elements such as Cu. Excess Zn is toxic to plants as amino acids accumulated in plant tissues and protein synthesis both decline (Marschner, 1995; Outten *et al*., 2001; Pandey *et al*., 2006; Mousvi *et al*., 2012). Excess Zn interferes with redox reactions in plants (Hall, 2002) in particular it interferes with the function of cytochromes in mitochondria and chloroplasts (Raven *et al*., 1999). At a whole plant level, high concentration of Zn can causestunting of shoots, curling and rolling of young leaves (growth abnormalities), death of leaf tips, inhibition of root growth and chlorosis and meristematic damage (Rout *et al*., 2003). Ye *et al*. (1997) reported that Zn ~ 80 μM led to chlorosis in *Typha latifolia* seedlings. Shen *et al*. (1997) found that 1 mM Zn after 4 days of culture was toxic in *Thlaspi ochroleucum* seedlings. Aquatic macrophytes

can reduce metal concentrations and serve as indicators of metal contamination which usually show metals significantly higher than in the environment (Miretzky *et al*., 2004; Thiébaut *et al*., 2010; Martinez and Shu-Nyamboli, 2011; Bácsi *et al*., 2015). Ceglowska *et al*. (2016) studied Cu and Zn toxicity in *Elodea canadensis* from rivers with various pollution levels. *E. canadensis* is now a cosmopolitan submerged macrophyte rooted in the sediment which plays an important role in the ecology of many littoral zones of lakes and rivers. *E. Canadensis* accumulates metals and certain organic compounds and has been used for bioremediation (Maleva *et al*., 2004; Malec *et al*., 2009; Thiébaut *et al*., 2010; Dosnon-Olette *et al*., 2011; Hansen*et al*., 2011; Martinez and Shu-Nyamboli, 2011). *E. canadensis* growing in Cu-polluted water had decreased accumulation of other heavy metals metals (Cd, Co, Cr, Mn, Ni, and Zn) (Samecka-Cymerman and Kempers, 2003). Xue *et al*. (2010) found similar results with copper toxicity in *Hydrilla verticillat*a which is very similar to *Elodea canadiensis*. Ceglowska *et al*. (2016) concluded that, *E. canadensis* seems to be a better indicator of environmental pollution because it could accumulate and survive at the relevant heavy metal levels, and indicated a great potential for the accumulation of heavy metals. Ceglowska *et al*. (2016) monitored toxic effects based on growth criteria and cell morphology. The combination of Zn and Cu affected *E. canadensis* younger leaves more strongly than in older leaves (Ceglowska *et al*., 2016). The negative effects of Zn and Cu on the old leaves of *E. Canadensis* were oncell structure, although the influence of Cu was greaterthan Zn. The cell disintegration effect of Cu + Zn mixture on *E. canadensis* was less pronounced than when Cu was added separately (Ceglowska *et al*., 2016). *Ipomoea aquatic*can be employed in biomonitoring of Zn polluted aquatic ecosystems using root browning, root and shoot growth inhibition as simple criteria, and chlorophyll and total carotenecontents as more sensitive biomarkers (Jayasri & Suthindhiran, 2017).

2.8 Pulse amplitude modulation fluorometry

Pulse amplitude modulation (PAM) fluorometer technique investigates direct information on the photosynthetic light reactions of plants (Silva *et al*., 2004; Ritchie 2012) (Table 2.12). In this study rapid light curves were used to measure photosynthetic performance of the plants based on the methods of Ralph & Gademann (2005) developed by Ritchie (2012) and Ritchie and Mekjinda (2016). Beer *et al*. (2000) used PAM fluorometer technology to measure photosynthetic ratesin marine macroalgae. Beer *et al*. (2000) attempted to compare the effective electron transfer quantum yield of photosystem II measured for *Ulva lactuca* and *Ulva fasciata* at various irradiances and inorganic carbon (Ci) concentrations. Silva *et al*. (2004) determined the relationship between the oxygen evolution and the electron transport rate (light reactions) for the seagrass *Zostera noltii* by using PAM fluorometry. Beer *et al*. (1998) studied on photosynthetic activity of symbiotic zooxanthellae in corals under natural growth conditions of *Platygyra lamellina* and *Favia favus* measurements by using diving-PAM for photosynthetic rate. Both species showed a reduction in photosystem II (Y) with increasing irradiances. Ritchie (2012) used PAM fluorometer to measure photosynthesis as the electron transport rate (ETR) through PSII photosynthesis in floating leaves of *Nymphaea caerulea* and systematically determined whether they expressed SAM/CAM physiology but not heavy metal issues. Beer *et al.* (2000) compared photosynthetic ETRs based on fluorescence parameters with rates of photosynthetic O_2 evolutionof *Halodule stipulacea*; *Halophila wrightii* and *Halophila ovalis* by using PAM fluorometry. PAM has been used for toxicity testing. For example, Ritchie and Mekjinda (2016) studied arsenic toxicity effects in *Wolffia arrhiza*where photosynthesis was measured using PAM technology and investigated the most useful PAM parameters to be used as arsenic toxicity criteria in an aquatic vascular plant. They found that Yield (Y) and Electron Transport Rate (ETR) and photosynthetic efficiency measurements gave the most readily interpretable results. In this study it was found that

Non-Photochemical quenching measurements were not very consistent and were difficult to interpret (cf.Brestic & Zivcak 2013).

Table 2.12 Using of pulse amplitude modulation (PAM) fluorometry techniques on plants.

Plants	Purpose of using PAM	Parameters	References
Ulva lactuca *Ulva fasciata*	PAM fluorometry can be used as a practical tool for quantifying photosynthetic rates	Electron transport rates (ETR); photosynthetic O_2 evaluation; yield	Beer *et al*., 2000
Zostera noltii	Determine the relationship between theoxygen evolution and the electron transport rate for the seagrass *Zostera noltii*	O_2 production and Electron transport rates (ETR)	Silva *et al*., 2004
Platygyra lamelina *Favia favus*	Using the diving-PAM for in situ measurements of photosynthetic responses to light.	Electron transport rates (ETR); Yield	Beer *et al*., 1998
Nymphaea caerulea	Using PAM techniques to investigate photosynthesis in floating leaves of *Nymphaea* and systematically determine whether they expressed SAM/CAM physiology.	Electron transport rates (ETR), Yield and non-Photochemical quenching.	Ritchie, 2012

Table 2.13 Using of pulse amplitude modulation (PAM) fluorometry techniques on plants. (Continued)

Plants	Purpose of using PAM	Parameters	References
Halodulestip ulacea Halophila wrightii Halophila ovalis	Compare calculating photosynthetic electron transport rates (ETRs) based on fluorescence parameters with rates of photosynthetic O_2 evaluation	Electron transport rates (ETR); photosynthetic O_2 evaluation	Beer *et al*., 2000
Wolffia arrhiza	Arsenic toxicity effects in *Wolffia arrhiza* was detected by PAM technology and investigates the most useful PAM parameters to be used as toxicity criteria in a vascular plant	Electron transport rates (ETR), Yield and non-Photochemical quenching.	Ritchie and Mekjinda,2016

2.9 *Ceratophyllum demersum*

Coontail *(C. demersum)* is a perennial and widespread submerged aquatic plant. It will grow under low illumination in dark coloured on muddy water. It can be live well in either oligotrophic or eutrophicwaters (Chen*et et al*., 2015). *C. demersum* although originally of North American origin it is a now a cosmopolitan species in tropical, temperate and cold regions (Fassett, 1953; Les, 1989; Winton *et al*., 2012). Some studies have shown that non-rooted *C. demersum* (stems, leaves and epidermis) can assimilate nutrients (Paterniti and Mantai, 1986). Although a common species it is usually not considered a major pest species like Water Hyacinth. This makes it a plausible candidate for bioremediation studies where the use of Water Hyacinth would be considered inappropriate.

Figure 2.3 Coontail, *Ceratophyllum demersum* L. This plant is about 250 cm long. Coontail has no roots and generally increases by fragmentation. Flowering was observed during the project but not seeding.

Coontail is a flowering plant with very small red flowers which are uncommonly seen. The reproduction of coontail is both sexual (seed) and asexual (vegetative) fragmentation growth of plant fragments (Haberland, 2014). Coontail's flowers are located on the leaf base below the water. Importantly, Coontail is rootless and so nutrients are taken up by the stems and leaves. Coontail is capable of removing nutrients and biosorbent for Pb, Cu and Zn from the water column effectively (Keskinkan *et al*., 2004; Krems *et al*., 2013). This growth habit has the important implication that it does not mobilise metals bound up in insoluble form in sediments. Nevertheless, Coontail decomposes much more easily than other aquatic plants after death. The decomposition rate (0.0568/d) of coontail was higher than those of other macrophytes (Chimney and Pietro, 2006). Rai *et al*. (1995) indicated that *C. demersum* could remove Pb more than 70% in a sample of pond water in 15 days. El-Khatib *et al*. (2014) also reported *C. demersum* had strong abilities to accumulate and tolerate Pb and Cd after 7 days of exposure (Mishra *et al*., 2006; Duman and Koca, 2014; El-Khatib *et al*., 2014).

The classification of *C. demersum* is the followings:

Kingdom Plantae

Phylum Tracheophyta

Class Magnoliopsida

Order Nymphaeales

Family Ceratophyllaceae

Genus *Ceratophyllum*

Species *Ceratophyllum demersum* (Fassett, 1953)*

*An alternative name for the organism is Coontail (*Cerstophyllum demersum* (Haberland, 2014). For systematic and taxonomic difficulties with the species see Les (1989).

CHAPTER 3
MATERIALS AND METHODS

3.1 Plant material

Ceratophyllum demersum were obtained from a farm in Samutprakarn, Thailand. The plants (10 cm, top portion) (Figure 3.1) were acclimatized under laboratory conditions (180 µmol /m^2/ s light with 12-h photoperiod at room temperature in a culture solution which is similar in composition to the artificial freshwater. They were grownat least for one week before the experiments.

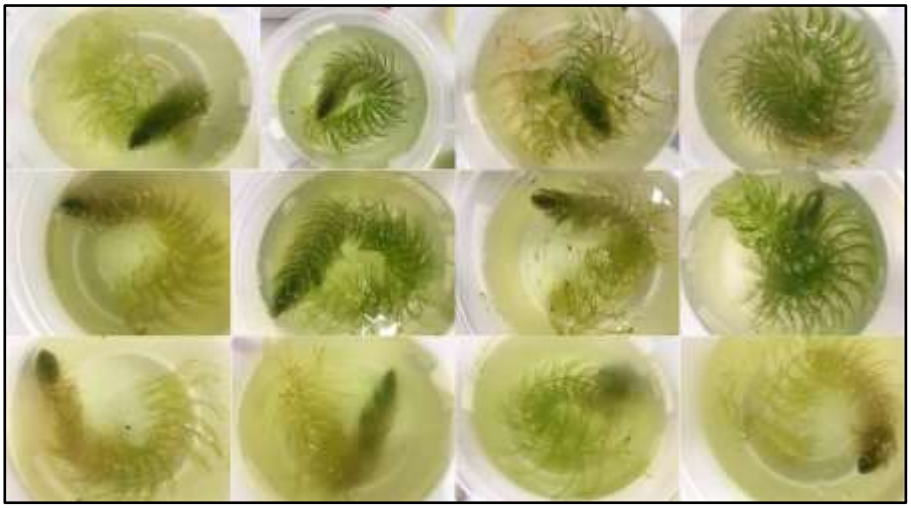


Figure 3.1 Coontail grown in plastic culture dishes (800 ml).

Plants that were uniformly green were selected for experiments. When Coontail was not growing satisfactorily the older parts of the plant became yellowed but the tip of the plant usually remained green. In the selection of 12 plants shown only about 6 plants would have been selected for an experiment.

3.2 ReagentsPreparation

3.2.1 Artificial Freshwater

Reagents used for preparingexperimental water are listed below. This is a low dissolved solids medium consistent with the waters in which Coontail is found.

This medium is based on BG-11 medium that is used for algal culture *(*Allen and Stanier, 1968). The original BG-11 medium was found to be unsatisfactory for growing Coontail because its mineral and nutrient content was too high. A soft water artificial freshwater medium was developed based on Smith *et al*. (2002). It has a low level of dissolved salts. The trace element mix for BG-11 medium was used but at 1/10 dilution (0.1 ml/l rather than 1 ml/l).

Table 3.1 Reagent used in the preparing Artificial Freshwater for *C. demersum.*

Additive	**MW**	**Stk [mM]**	**[mM]**	**volume/l**	**g/l**
NaCl	58.5	1M	1.0	1.0	0.0585
$NaHCO_3$	84.01	0.5 M	0.5	1.0	0.04201
KCl	74.6	0.1 M	0.1	1.0	0.0074
$MgCl_2.6H_2O$	203.3	0.197 M	0.197	1.0	0.040
$CaCl_2.2H_2O$	47.0	0.147 M	0.147	1.0	0.022

BG-11 Trace element mix 0.1 ml/l

Fe-EDTA (50 mM) 0.1 ml/l

$NaNO_3$ (1M stock) 0.1 ml/l (100 µM)

Na_2HPO_4 (100 mM Stk) 0.1 ml/l (10 µM)

3.2.2 BG-11 low nutrient media

Table 3.2 Chemical composition of BG-11 traceelement mix 1 ml/l for normal BG-11, 0.1 ml/l for low nutrient media.

Salt	**MW**	**g/l**	**Stock [mM]**	**Final 1 ml/l [µM]**
$(NH_4)_6Mo_7O_{24}$	1235.9	0.088	0.071	0.071
$Co(NO_3)_2.6H_2O$	291.0	0.0494	0.170	0.170
$Cu(SO_4).5H_2O$	249.7	0.079	0.316	0.316
Ferric Citrate	244.94	6	24.5	24.5
Na_2EDTA	292.24	1	3.42	3.42
H_3BO_3	61.8	0.6	9.71	9.71
$MnSO_4.5H_2O$	241.1	2.41	10.0	10.0
Na_2SeO_4	263	0.003	0.0114	0.0114
$NiSO_4.6H_2O$	262.9	0.132	0.502	0.502
$ZnSO_4.7H_2O$	287.6	0.287	1.00	1.00

Trace element mix was based on Allen and Stanier (1968).

3.2.3 Cu solution

$CuSO_4$ was used as the source of copper stock solution. All the required solutions were prepared with analytical reagent and distilled water. To prepare 0.1 g/l of Cu, 0.393 g/mol of $CuSO_4$ was dissolved in 1000 ml distilled water in 1 L volumetric flask. 0.05 mg/l Cu were taken 0.5 ml from stock solution to put in 1000 ml of distilled water. Similarly solutions with different metal concentrations (0.1, 0.15 and 0.2 mg) were prepared.

3.2.4 Zn solution

$ZnSO_4$ was used as the source of copper stock solution. 1 Mole of $ZnSO_4$ in 1000 ml contain 65.39 g of Zn. One mililiter of stock solution contain 0.0065 mg of Zn. 15.38 ml of stock solution contain 1.0 mg of Zn put in 1000 ml of distilled water. Similarly solutions with different metal concentrations (5.0, 10.0 and 15.0 mg) were prepared.

3.2.5 Cu + Zn solution

0.05 mg/l Cu were taken 0.5 ml from stock solution and 15.38 ml of stock solution contain 1.0 mg of Zn to put in 1000 ml of distilled water. Similarly solutions with different metal concentrations (0.05 + 5.0, 0.1 + 1.0 and 0.1 + 5.0) were prepared.

3.3 Metals accumulation study

3.3.1 Procedures

Table 3.3 Treatment of heavy metals

Concentrations	Treatment
Control	No heavy metals
Cu treatment	0.05 mg/l 0.1 mg/l 0.15 mg/l 0.20 mg/l
Zn treatment	1.0 mg/l 5.0 mg/l 10.0 mg/l 15.0 mg/l
Cu + Zn treatment	0.05 mg/l + 0.1 mg/l 0.05 mg/l + 5.0 mg/l 0.1 mg/l + 0.1 mg/l 0.1 mg/l + 5.0 mg/l

3.3.2 Relative growth rate (RGR)

Plants were harvested at day 5, 10, and 15 of experiment to determine the growth rates. Plants were rinsed by tap water. Plants were absorbed the water by using tissue paper to avoid the error weight. Plantsamples were weighed and measured the length before and after cultivation (Figure 3.2).

The relative growth of submerged plant was calculated using the standard relative growth equation (Hoffmann & Poorter, 2002):

$$\text{RGR} = \frac{ln(W_2) - ln\,(W_1)}{t_2 - t_1}$$

Where:

RGR: relative growth rate,

W_1& W_2 (g): weight of the plant sample at the beginning and in the end of the time,

t_1& t_2 (d): time of the beginning and at the end of the experiment.

Figure 3.2 The measurement of the length and weight of the plants were standard for the calculation of relative growth rates.

3.4 Appropriate Cu and Zn treatment

Range – finding test

Typically toxicity studies are done in two stages. Firstly a range finding test is used to find the appropriate range of concentrations of a toxin that give a measureable effect and are not an overdose. The aim is to find a range of concentrations giving a range of effects small to large effects so that a dose response curve can be developed. Range-finding test was used to find the concentrations of Cu and Zn that cause measureable effects (0 – 100%). Firstly, the plants were cut at 10 cm from the top and weighted. The plant material was transferred to a 1000 ml plastic bowl with 800 ml soft water culture solution (Umebese and Motajo, 2008; Ceglowska *et al*., 2016). The Coontail samples were exposed to Cu as $CuSO_4$ at 4 concentrations including 0, 0.1 mg/l, 0.20 mg/l, and 0.30 mg/. The concentrations of Zn as $ZnSO_4$, at 4 concentrations including 0, 1.0 mg/L, 10.0 mg/l, 20.0 mg/l were used.

Definitive test

From the range-finding test, the concentrations that have a measureable effect but did not kill the plants outright were chosen for application in the definitive experiments. For copper treatment 0, 0.05 mg/l, 0.10 mg/l, 0.15 mg/l, 0.20 mg/l were used to do the experiment. For zinc treatment 0, 1.0 mg/l, 5.0 mg/l, 10 mg/l, 15.0 mg/l were found to be appropriate.

Table 3.4 *C. demersum* (Alive/Dead): Cu treatment, Cu as $CuSO_4$

Concentration (mg/l)	**Day 0**	**Day 5**	**Day 10**	**Day 15**
Control	5/0	5/0	5/0	5/0
Cu = 0.05	5/0	5/0	5/0	5/0
Cu = 0.1	5/0	5/0	5/0	5/0
Cu = 0.15	5/0	5/0	5/0	5/0
Cu = 0.20	5/0	5/0	5/0	5/1

Table 3.5 *C. demersum* (Alive/Dead): Zn treatment, Zn as $ZnSO_4$

Concentration (mg/l)	**Day 0**	**Day 5**	**Day 10**	**Day 15**
Control	5/0	5/0	5/0	5/0
Zn = 1.0	5/0	5/0	5/0	5/0
Zn = 5.0	5/0	5/0	5/0	5/0
Zn = 10.0	5/0	5/0	5/0	5/1
Zn = 15.0	5/0	5/0	5/0	5/5

Table 3.6 *C. demersum* (Alive/Dead): Cu + Zn treatment

Concentration (mg/l)	Day 0	Day 5	Day 10	Day 15
Control	5/0	5/0	5/0	5/0
Cu = 0.05 + Zn = 1.0	5/0	5/0	5/0	5/0
Cu = 0.05 + Zn = 5.0	5/0	5/0	5/0	5/0
Cu = 0.1 + Zn = 1.0	5/0	5/0	5/0	5/2
Cu = 0.1 + Zn = 5.0	5/0	5/0	5/0	5/0

All of the experiments were conducted with 5 replications. The solution was replaced after harvesting. *C. demersum* plants were rinsed by using tap water and the growth of plant was measured on day 0, 5, 10 and 15. The pH in culture solutions was around 7.0 and did not greatly change over the course of the experiments.

At the beginning of the test (day 0: control) and atday 5, 10 and 15, plants were collected for growth measurement, metal accumulation, leaf cell, pigment content and photosynthesis by PAM fluorometry measurement.

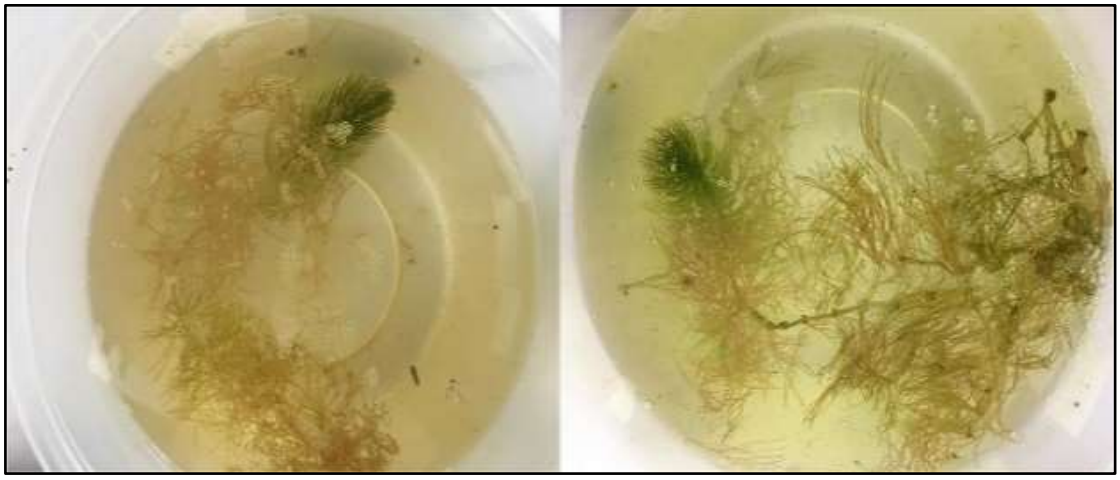


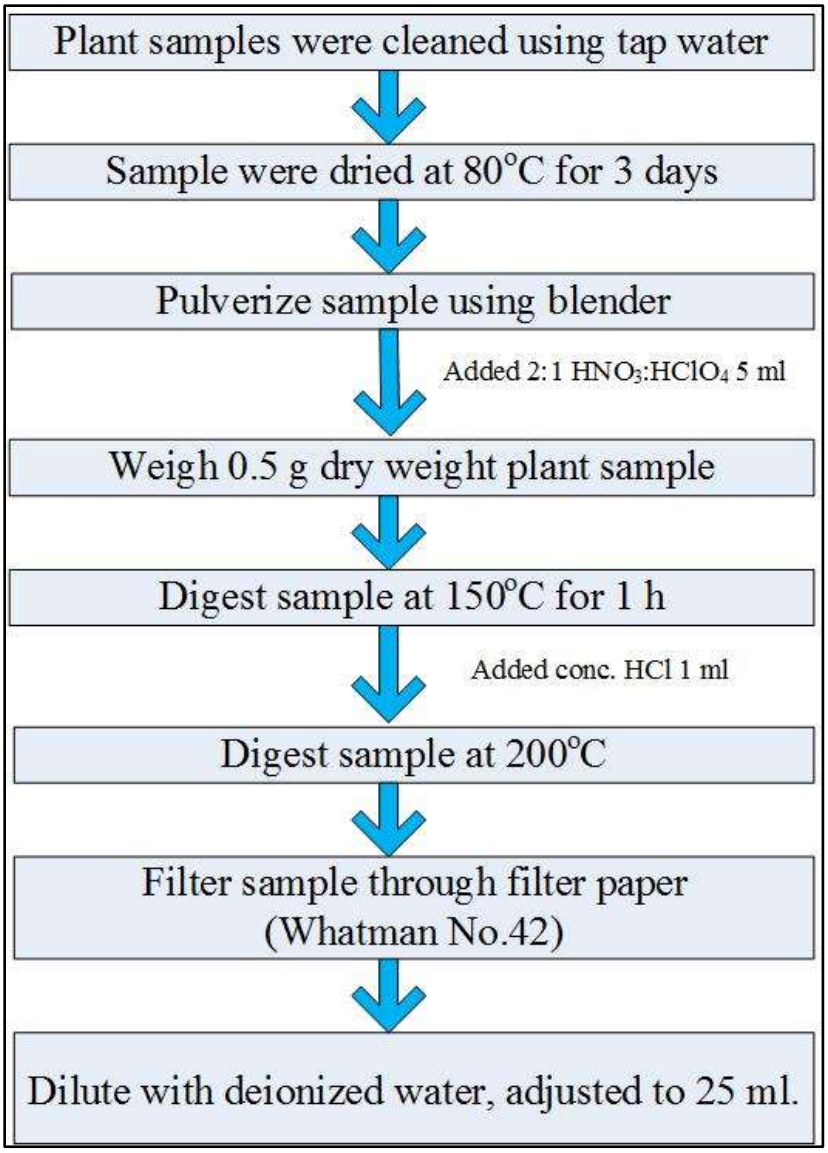
Figure 3.3 Physical observation of *C. demersum* (Unhealthy).

Figure 3.4 Physical observation of *C. demersum* (Healthy).

A characteristic symptom of toxicity in *C. demersum* was a dying-off of the older parts of the plant. The young tip was the most resistant. See also Fig. 3.1. Healthy plants were green from the tip to the end of the stem.

3.5 Cu and Zn accumulation

Plant samples were collected, washed and dried at 80^{o}C for 3 days and then pulverized. Dry samples (0.5 g) were weighed, transferred to a test tube and digested with nitric acid: concentrated until digestion was completed. Test tubes were cooled at room temperature. Samples were filtered (Whatman No.42 filter paper), diluted and adjusted to 25 ml volume using deionized water. The full procedural flow chart is shown in Figure 3.5(Dummee *et al*., 2012). Prepared extracts were then sent to the Central Lab Faculty of Science, Prince of Songkla University, Hat Yai Campus where Cu and Zn concentrations were determined using an inductively coupled plasma optical emission spectrometry (ICP-OES).



Analysis of Cu and Zn using ICP-OES.

Figure 3.5 Diagram showing processes of heavy metal analysis in the plant based on Dummee *et al*. (2012).

3.5.1 Bioconcentration factor

A bioconcentration factor (BCF) can be used to express Copper and Zn uptake in terms of it ability to accumulate metal with respect to its concentration in the soil

substrate (Zayed *et al*., 1998). In the case of Coontail, which has no roots (unlike many other freshwater aquatics), the plant obtains all its nutrients from the water in which it is grown. For a plant taking up metals only from the water column the appropriate form of the BCF equation is (Zayed *et al*., 1998).

$$\boldsymbol{BCF} = \frac{\textbf{Metal concentration in plant tissue (mg/kg)}}{\textbf{Initial concentration of metal in solution (mg/l)}}$$

3.6 Photosynthetic pigment estimation

Vascular plants have Chlorophyll *a* as their primary photosynthetic pigment and use chlorophyll *b* as an accessory pigment. The chlorophylls are located in chlorophyll-protein complexes and the ratio of Chl *b* to Chl *a* varies with the quality of light. Fresh materials (100mg) were ground in 7:2 acetone and ethanol for 24 h at 4^{o}C and in darkness. Chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) contents were determined using spectrophotometric methods and the equations described by Ritchie (2018). Pure ethanol was used as the extractant medium in some Chorophyll estimations where the equations of Ritchie (2006) were used. The 7:2 acetone/ethanol mixture was found to be a much more effective solvent extractant. The algorthim formaluae for acetone/ethanol solvent (Ritchie 2018) are:

Chl *a* ($\mu g\ mL^{-1}$) = $-2.34435 \times (A_{648}-A_{850}) + 12.4552 \times (A_{665}-A_{850})$

Relative error ± 0.396%

Chl *b* ($\mu g\ mL^{-1}$) = $2629242 \times (A_{648}-A_{850}) - 9.00689 \times (A_{665}-A_{850})$

Relative Error ±0.799%

Where, A_{648}, A_{665}& A_{850} are the absorbance of the solvent extract at 648, 665 and 850 nm respectively.

These formulae use 850 nm as a blank rather than the more usual 750 nm (Ritchie 2006, 2008 & 2018). This is to avoid interference from any bacteriochlorophyll that might be present in environmental samples. Ratios of Chl *a* & Chl *b* are a reflection of chromatic adaptation of green algae and vascular plants to light conditions. Plants grown in low light and in the filtered light of understories where plants are growing underneath other plants usually have higher relative amounts of Chl *b* than plants grown in full sunlight. In the present study, Chl *b*/Chl *a* ratios were calculated.

To extract Chl *a* and *b* from Coontail, the top of plants were cut and the fresh weight (0.1–0.15 g) of each sample was recorded. Plants were cut into small pieces with scissors acetone/ethanol mixture (5 ml) then frozen and stored at 4°C in 24h in a freezer. Provided the sediment was not disturbed, clearing by centrifugation was not normally needed. Then 3 ml samples were transferred into a spectrophotometric cuvette to put into the Spectrophotophotometer Shimadzu UV-1601 UV–visible double beam spectrophotometer (Ritchie, 2006) using a using standard scanning settings (1 nm bandwidth and 1 nm sampling interval). The wavelengths used were 648 & 665 nm with an 850 nm blank for the actone/ethanol based equations (Ritchie, 2018) and 649 & 665 nm with an 850 nm blank for when ethanol solvent was used (Ritchie, 2006).

Chlorophyll is used as a standard on which to calculate plant biomass and the amounts of Chl *a*, *b* and the Chl *b/a* ratio provide information on chromatic adaptation and the relative amounts of Chl *a* (the primary photosynthetic pigment) to Chl *b* which acts as an accessory photosynthetic pigment in higher plants. Chl *a* is also used as a standard for the calculation of photosynthetic rates.

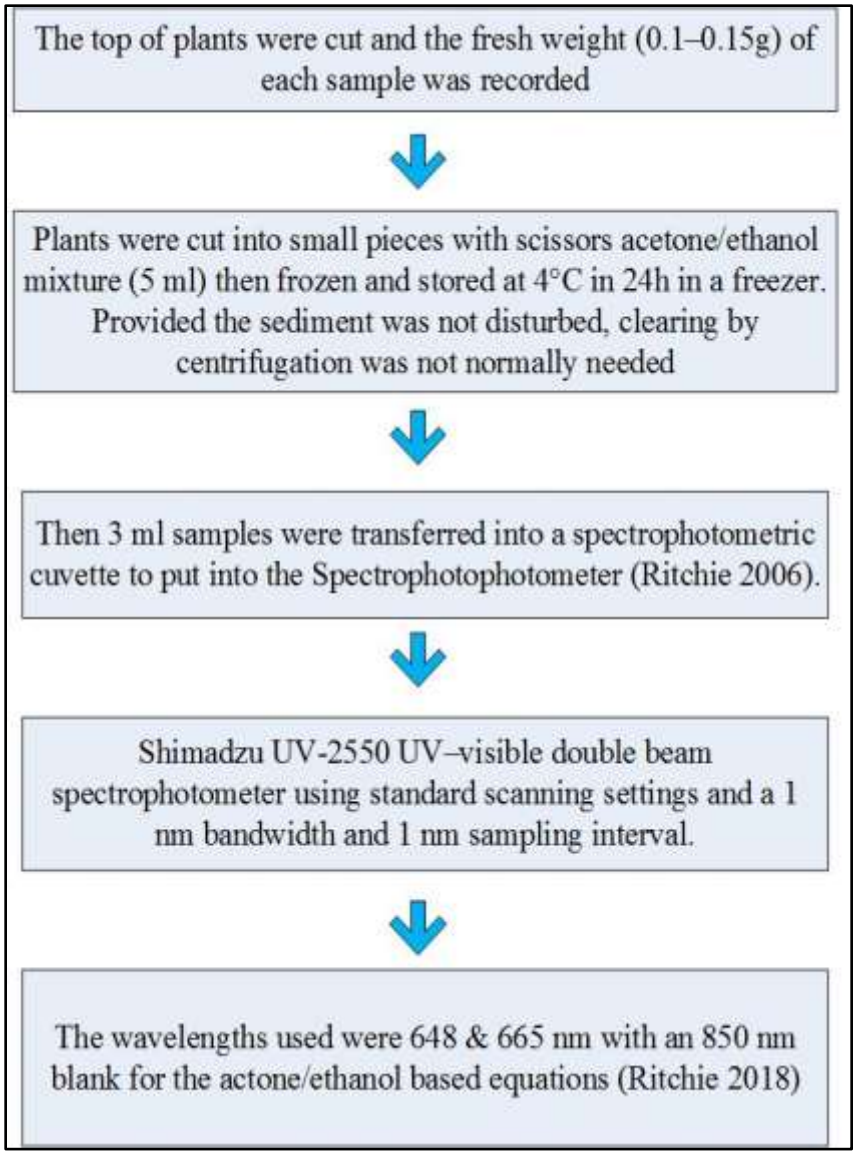


Figure 3.6 Diagram showing processes of chlorophyll pigment extraction

3.7 Leaf cell observations

The wet mount technique were used. The fresh leaf were observed and captured with Human microscope with a P/N: TP 605 100A digital camera (Figure 3.7). Notes were made of the colour, number of chloroplasts in the epidermal cells, and cytoplasmic streaming activity were investigated and compared with those of the control.

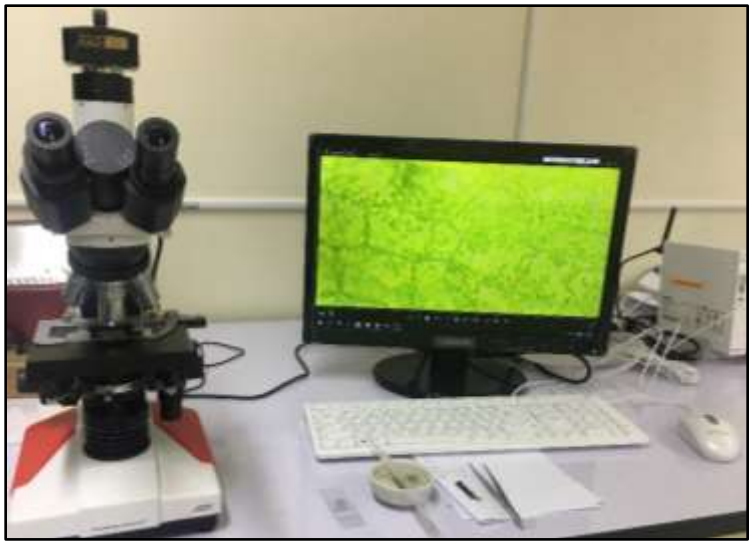


Figure 3.7 Standard Clinical microscope with P/N: TP 605 100A

3.8 PAM machine

Theory of PAM fluorometry (summarised from Ritchie 2008, Ritchie and Mekjinda, 2016; Apichatmeta *et al*., 2017; Quinnell *et al*., 2017)

A Junior PAM portable chlorophyll fluorometer (Gademann Instruments, Würzburg, Germany). This simple PAM device has a 1.5 mm-diameter optic fibre and a blue diode light source (465 ± 40 nm) (see Diagramatic Figure 3.8). The Walz-type PAM was fitted with a simple highpass filter (>695 nm) to protect the detector diode from transmitted blue light and could measure fluorescence from Chl*a* (Ritchie 2008; Ritchie and Larkum 2013; Ritchie and Mekjinda, 2016; Apichatmetaet *et al*., 2017; Quinnell *et al*., 2017). PAM parameters (Y, rETR, qN, NPQ) were automatically calculated using the WINCONTROL software (v2.08 & v2.13; Heinz WalzGmbh, Effeltrich, Germany) using the standard default settings for rapid light curves (default absorptance factor, AbtF = 0.84, PSI/PSII allocation factor = 0.5) to calculate the relative electron transport rate (rETR) (Ralph & Gademann, 2005; Brestic & Zivcak, 2013; Ritchie and Mekjinda, 2016).

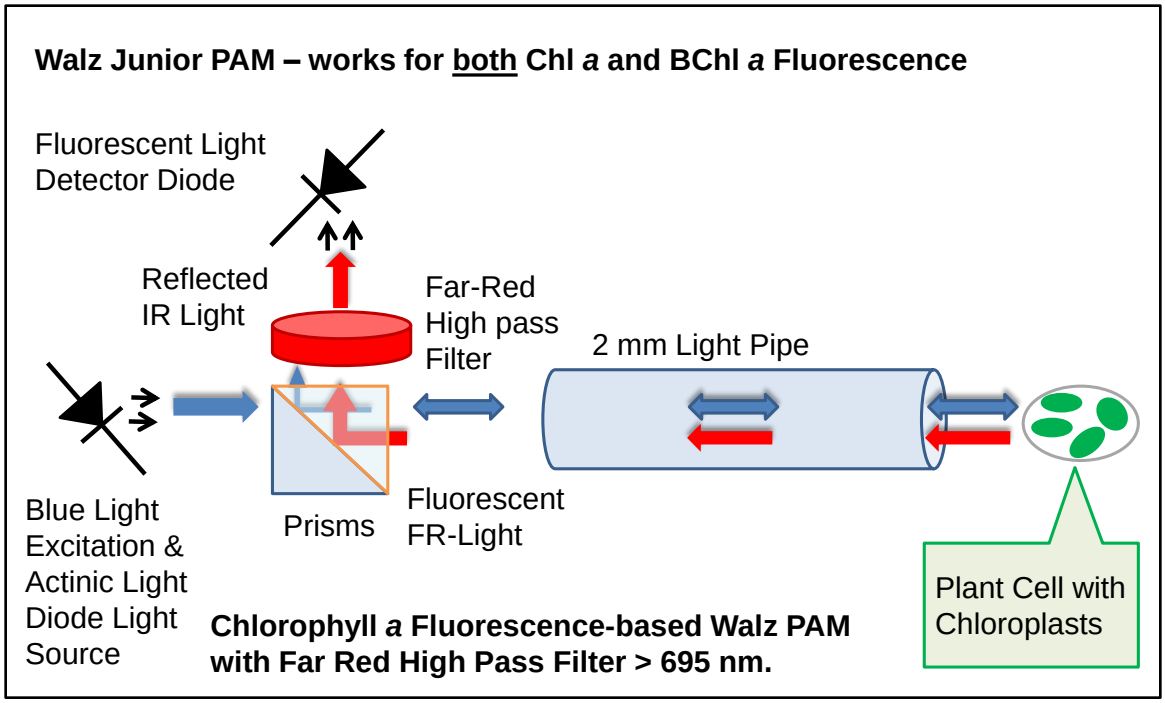


Figure 3.8 Arrangement of actinic light source and detector diode of Walz-type Junior PAM used in this study.

The detector diode is protected by a high pass filter such that only chlorophyll fluorescence reaches the detector diode. Source: Modified after, Ritchie, Chandaravithoon and Runcie (2018).

Yield was calculated by the walz PAM machine software as:

$Y = 1 – Fo/Fm'$

where, Fo is the fluorescence in the modulated measuring light and Fm' is the fluorescence in the light acclimated state after a flash of actinic light (Brestic and Zivcak 2013). If Y is plotted against irradiance (*E*), it follows a simple exponential decay function of the form $y = e^{-kx}$ (Ritchie 2008; Ritchie and Larkum 2013; Ritchie and Mekjinda, 2016; Apichatmeta *et al*., 2017, Quinnell *et al*., 2017).

Photosynthetic electron transport rate (ETR) is proportional to the product of the yield (Y) × Irradiance (E). The Walz software uses a default absorptance of 0.84

and so calculates relative ETR (rETR): if absorptance (Abt) is measured experimentally the actual ETR can be calculated. Experimental measurements of the Absorptance of Coontail apices (youngest shoots) were made at 465 nm ($Abt_{465\ nm}$) using a Reflectance-Absorptance-Transmission (RAT) machine (Ritchie and Runcie 2014).The allocation factor was taken as 0.5 as the default by the Walz software (PSI/PSII ratio) (Ritchie 2008; Ritchie and Larkum 2013; Ritchie and Mekjinda, 2016; Apichatmetaet *et al*., 2017; Quinnell *et al*., 2017).

rETR

$rETR = Y \times E \times (PSII/PSI = 0.5) \times (Abt_F = 0.84)$

Oxygenic Photosynthesis

$ETR = Y \times E \times 0.5 \times Abt_{465nm}/0.84$

$ETR = rETR \times Abt_{465nm}/0.84$

The lectron source in oxygenic photosynthesis is water: $2H_2O \rightarrow 4H^+ + 4e^- + O_2$ and hence 1 μmol O_2 g^{-1}Chl *a* $s^{-1} \equiv 4$ μmol e^- g^{-1}Chl *a* s^{-1}) The ETR is an estimates of the Photosynthetic Oxygen Evolution Rate (POER) from the light reactions of photosynthesis. ETR does not take photorespiration into account (Ritchie and Larkum, 2012; Ritchie and Mekjinda, 2016; Apichatmeta*et al*., 2017, Quinnell *et al*., 2017).

The Waiting-in-Line equation is a good model for rapid light curves of ETR vs Irradiance (Ritchie and Larkum, 2012; Ritchie and Mekjinda, 2016; Apichatmeta *et al*., 2017, Quinnell *et al*., 2017),

$$\mathrm{ETR} = \frac{\mathrm{ETR}_{\max} \times E}{E_{\mathrm{opt}}} \times \mathrm{e}^{1-E/E_{\mathrm{opt}}}$$

Where, ETR is electron transport rate as a measure of the photosynthetic electron transport rate (μmol e^- m^{-2} s^{-1}), *E* is the irradiance (μmol photon m^{-2} s^{-1} 400–700 nm PPFD), E_{opt} is the optimum irradiance, ETR_{max} is the maximum photosynthetic electron transport rate.

The maximum photosynthetic efficiency (α_0) is the initial slope of the curve at $E = 0$ ($\alpha = ETR_{max} \times e/E_{opt}$) (Ritchie and Larkum, 2012; Ritchie and Mekjinda, 2016; Apichatmeta *et al*., 2017, Quinnell *et al*., 2017).

PAM machines calculate ETR on a surface area basis. If estimates of Chl *a* per unit surface area are available it is possible to express ETR as mol e^{-} g^{-1} Chl *a* s^{-1}.

3.9 Statistical analysis

Mean and standard errors of the metal concentrations, RGR and chlorophyll *a* and *b* in Coontail were calculated using reoutine calculations written for Microsoft Excel (2013) and SPSS was used for routine statistics. For the PAM data non-linear least squares fitting programs were used to fit Yield, ETR and chemical and non-chemical quenching data to models described by, Ritchie and Larkum (2013), Ritchie and Mekjinda (2016), Apichatmetaet *et al*. (2017) and Quinnell *et al*. (2017). Errors of fitted parameters were calculated as described by Ritchie (2008). One way ANOVA followed by LSD (SPSS version 17.0) was used to analyze the difference of metal concentrations, RGR and chlorophyll *a* and *b* in the plant at the different treatment.

3.10 Waste management

The solid waste, e.g contaminated samples, plastic and paper were collected and sent to Phuket incinerator. The liquid waste were collected in plastic containers. The alkaline chemical, hydrated potassium aluminium sulfate (potassium alum), were added to make the metals precipitate. The clear solution were released into wastewater treatment plant system. The sediment were dried and sent to the Phuket incinerator as dry waste.

CHAPTER 4
RESULTS

4.1 Effects of copper, zinc, and their combination on growth rate

In all these experiments on the effects of added Cu and Zn *C. demersum* was grown on soft water media with very low dissolved salts in media described above in the Materials and methods.

4.1.1 Copper

4.1.1.1 Shoot length

Figure 4.1 shows the relative growth rate (RGR) of shoot length of *C. demersum* exposed to copper. The results showed that there was no significant difference in every concentration ($P < 0.05$).

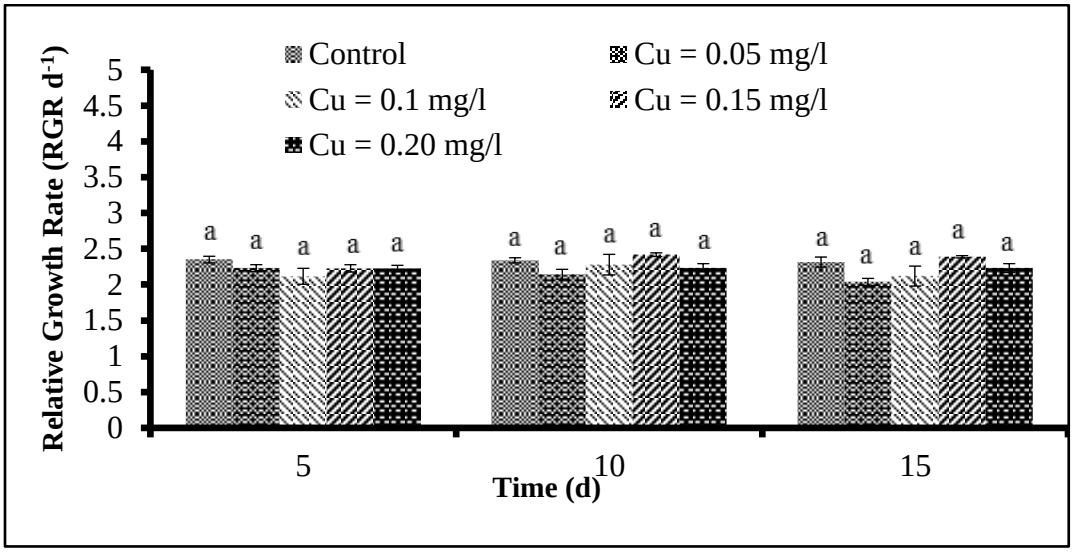


Figure 4.1 The RGR of copper on shoot length. The data presented are mean ± SE of five replicates. Values with different letters are significantly different at $P < 0.05$.

4.1.1.2. Weight

Figure 4.2 shows the relative growth rate (RGR) of wet weight of the plants exposed to copper. The results indicated that RGRs were significantly decreased ($P < 0.05$) when copper was added to 0.05, 0.1, 0.15 and 0.2 mg/l (Figure 4.2). At day 5 of the experiment, RGRs of Cu 0.10 mg/l, Cu 0.15 mg/l and Cu 0.20 mg/l treatment were lower than those of control and Cu 0.05 mg/l ($P < 0.05$). At day 10, all signifcantly RGRs were significantly lower than that of control. In addition, the RGR of Cu 0.05 mg/l (0.46 ± 0.05 per day) was the lowest on day 10. At day 5, the RGR of control was 2.35 ± 0.05 per day which was the highest.

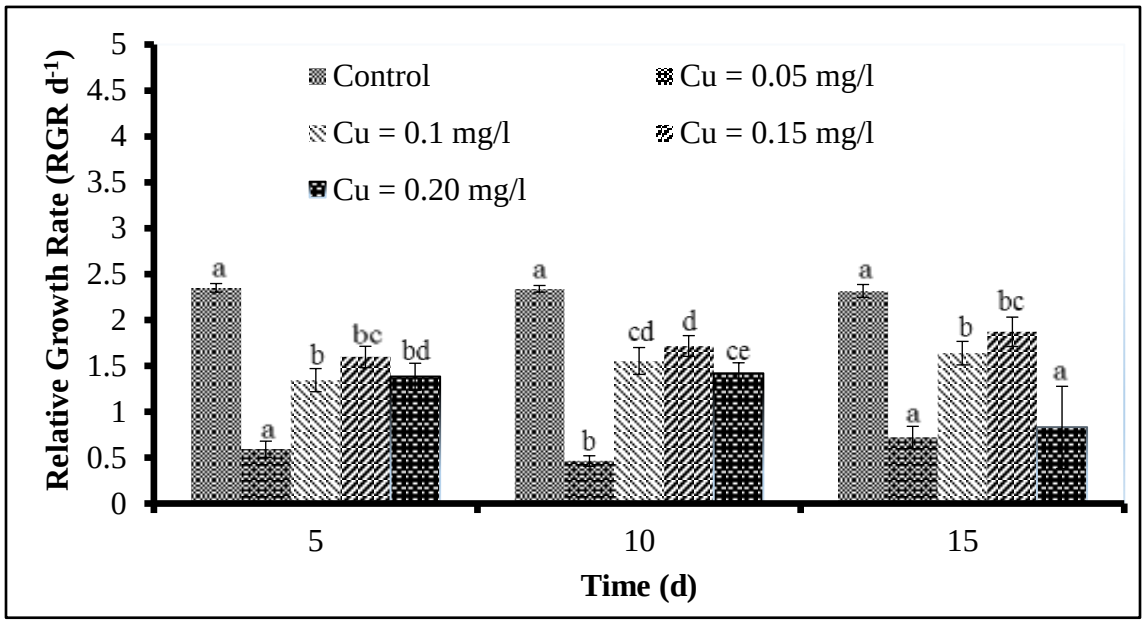


Figure 4.2 The RGR of Coontail, *C. demersum*, wet weight exposed to Cu. The data presented are mean ± SE of five replicates. Values with different letters are significantly different at $P < 0.05$.

4.1.2 Zinc

4.1.2.1 Shoot length

Figure 4.3 shows the relative growth rate (RGR) of the shoot length of Coontail exposed to zinc. The results showed that there was no significant difference in every concentration (P< 0.05) at day 10 and day 15 of the experiment. Moreover, at day 5the RGRs of Zn 5.0 mg/l and Zn 10 mg/l were significantly lower than those of other treatments (P<0.05). The highest RGR was found in Zn 1.0 mg/l (2.41± 0.04 per day) at day 15 and control (2.35 ± 0.05 per day) at day 5.

The lowest RGR was found in Zn 5.0 mg/l (2.04 ± 0.10 per day) at day 5, and Zn 5.0 mg/l (2.06±0.11 per day) at day 10.

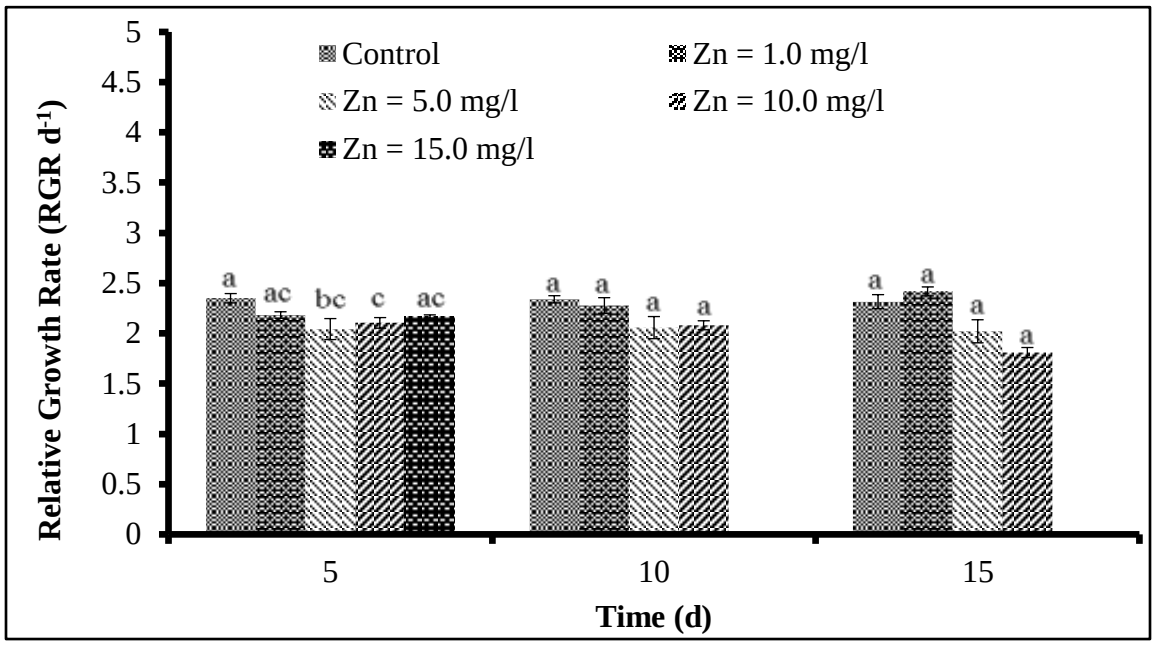


Figure 4.3 The RGR of Coontail, *C. demersum*, shoot length exposed to Zn. The data presented are mean ± SE of five replicates.Values with different letters are significantly different at P < 0.05.

4.1.2.2Weight

Figure 4.4 shows the relative growth rate (RGR) of the wet weight of *C. demersum* exposed to zinc. The results showed that there was no significant difference

among concentrations (P< 0.05) on day 15. The highest RGR was found in Zn 1.0 mg/l (1.70 ± 0.15 per day) of day 15 and zinc 1.0 mg/l (1.61 ± 0.13 per day) at day 10.

The lowest RGR was found in Zn 10.0 mg/l (0.13 ± 0.11 per day) at day 15 and Zn 15.0 mg/l (0.54 ± 0.02 per day) at day 5. In addition, plants died on day 10 when Zn was increased to 15.0 mg/l. However, there was a significant difference at between RGRs at day 5 and day 10.

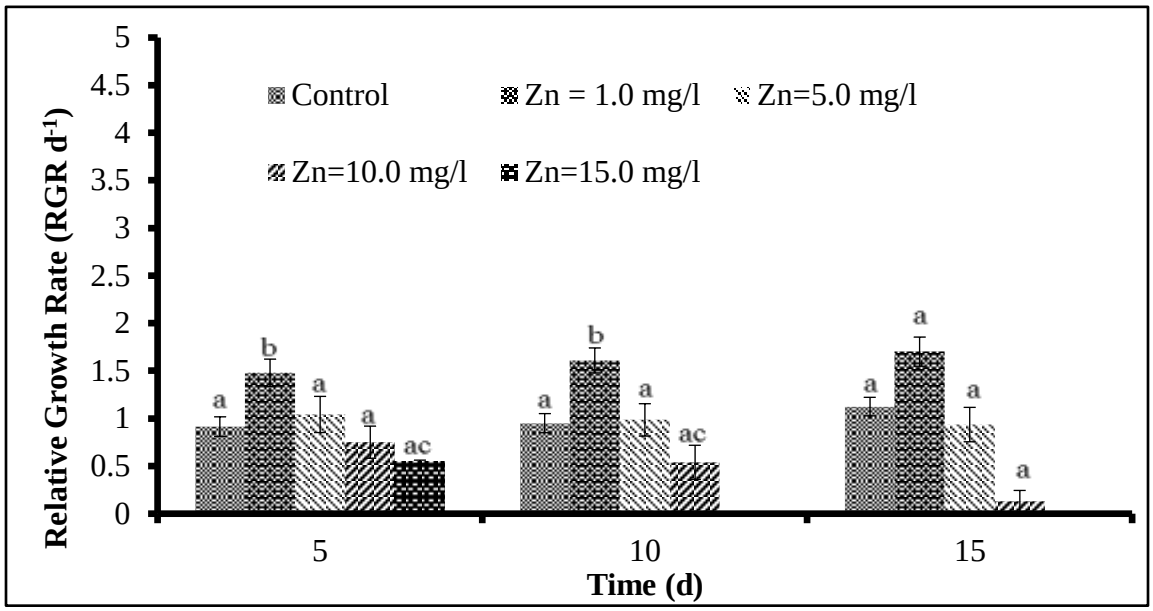


Figure 4.4 The RGR of zinc on shoot weight (biomass). The data presented are mean ± SE of five replicates. Values with different letters are significantly different at P<0.05.

4.1.3 Copper plus zinc

4.1.3.1 Shoot length

Figure 4.5 shows the relative growth rate (RGR) of shoot length of Coontail exposed to copper plus zinc. The results showed that there were significant difference among concentrations (P< 0.05) at day 5, day 10, and day15 of the experiment. The highest RGR was found in Cu 0.05 mg/l + Zn 1.0 mg/l (2.60 ± 0.02 per day) at day 10 and Cu 0.05 mg/l + Zn 1.0 mg/l (2.58 ± 0.02 per day) at day 5.

The lowest RGR was found in Cu 0.1 mg/l + Zn 1.0 mg/l (1.61 ± 0.19 per day)at day 10 and Cu 0.1 mg/l + Zn 1.0 mg/l (1.68 ± 0.2 per day) at day 5.

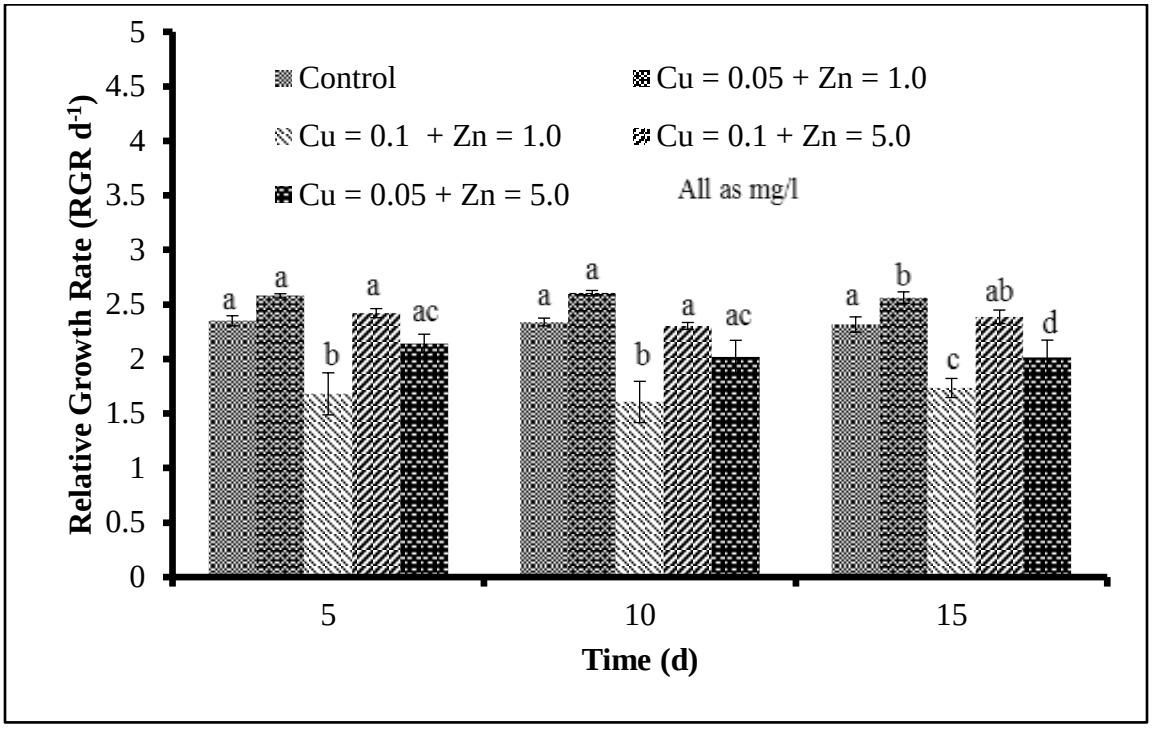


Figure 4.5 The RGR of Coontail, *C. demersum*, shoot length exposed to Cu + Zn.Values with different letters are significantly different at P<0.05.

4.1.3.2Weight

Figure 4.6 shows the relative growth rate (RGR) of the wet weight of *C. demersum* exposed to copper plus zinc. The results showed that there was no significant difference in every concentrations (P< 0.05) on day 5 and day 10 of the experiment. In addition, the highest RGR was found in control (2.35 ± 0.05 per day) at day 5 and control (2.34 ± 0.04 per day) at day 10.

The lowest RGR was found inCu0.1 mg/l + Zn 1.0 mg/l (-0.10 ± 0.4 per day) at day 10, and in Cu 0.1 mg/l + Zn 5.0 mg/l

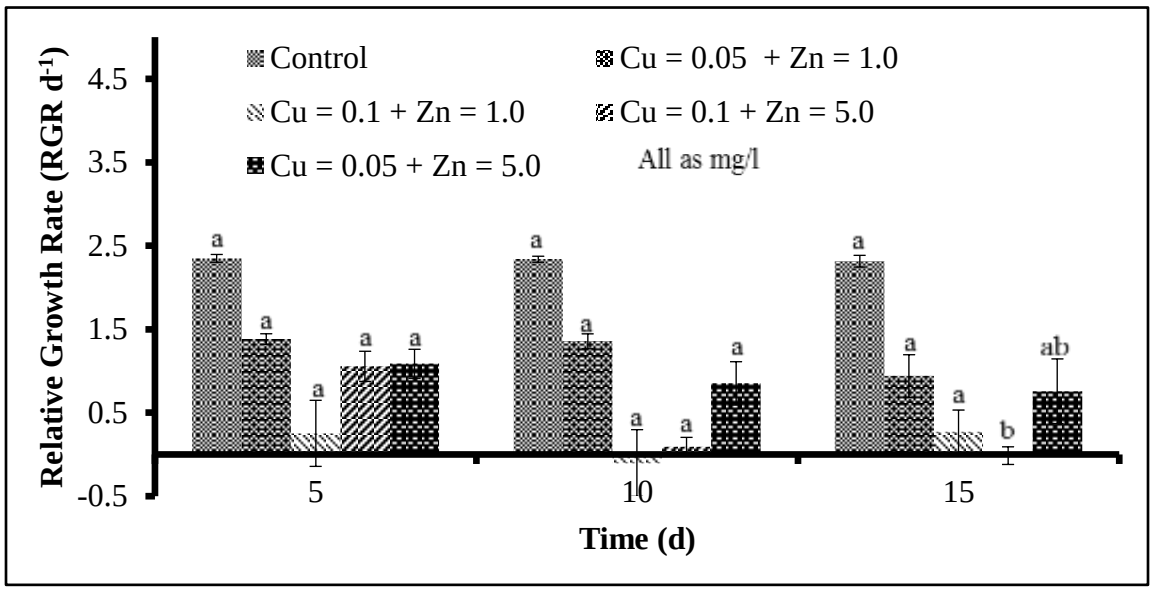


Figure 4.6 The RGR of Coontail, *C. demersum*, wet weight exposed to Cu + Zn. The data is presented are mean ± SE of five replicates. Values with different letters are significantly different at P<0.05.

4.2 Effects of copper, zinc, and their combination on chlorophyll content

4.2.1 Copper

4.2.1.1 Chlorophyll *a*

Figure 4.7 shows the chlorophyll *a* content of *Ceratophyllum demersum* exposed to copperat various concentration. The results showed that there was no significant difference among concentrations (P< 0.05) on day 10 of the experiment. The highest chlorophyll *a* content was found in Cu 0.05 mg/l (694.37 ± 57.27 µg/g) of fresh weight at day 15, and Cu 0.1 mg/l (625.85 ± 20.58 µg/g) at day 10.

The lowest chlorophyll *a* content was found in Cu 0.05 mg/l 122.92 ± 20.76 µg/g of fresh weight of day 5, Cu 0.15 mg/l 138.65 ± 53.33 µg/g of fresh weight of day 10, respectively.

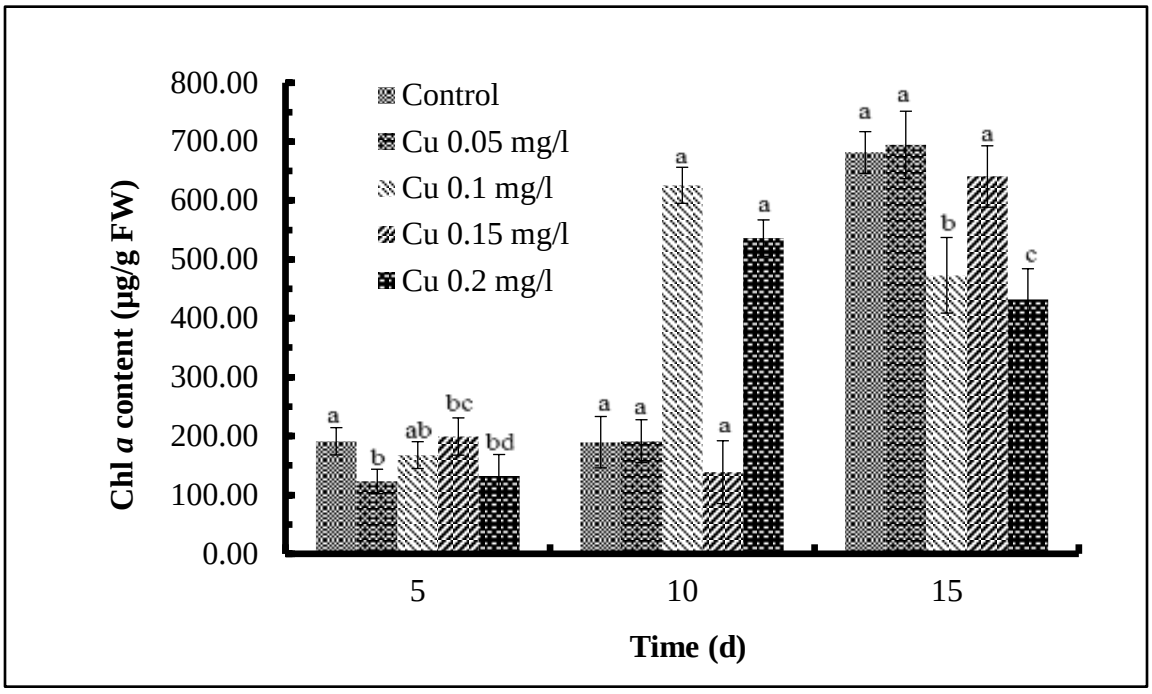


Figure 4.7 Chlorophyll *a* content of Coontail, *C. demersum* exposed to varied concentrations of Cu. Values with different letters are significantly different at P<0.05.

4.2.1.2 Chlorophyll *b*

Figure 4.8 shows the chlorophyll *b* content of Coontail exposed to copper. The results showed that there was significant difference among concentrations (P< 0.05) on day 10 of the experiment. The highest chlorophyll *b* content was found in Cu 0.20 mg/l (178.68 ± 10.18 µg/g) at day 10, and Cu 0.15 mg/l (154.76 ± 16.91µg/g) at day 15.

The lowest chlorophyll *b* content was found in Cu 0.05 mg/l (22.54 ± 4.32 µg/g) at day 5, and control (37.88 ± 9.40µg/g) at day 10.

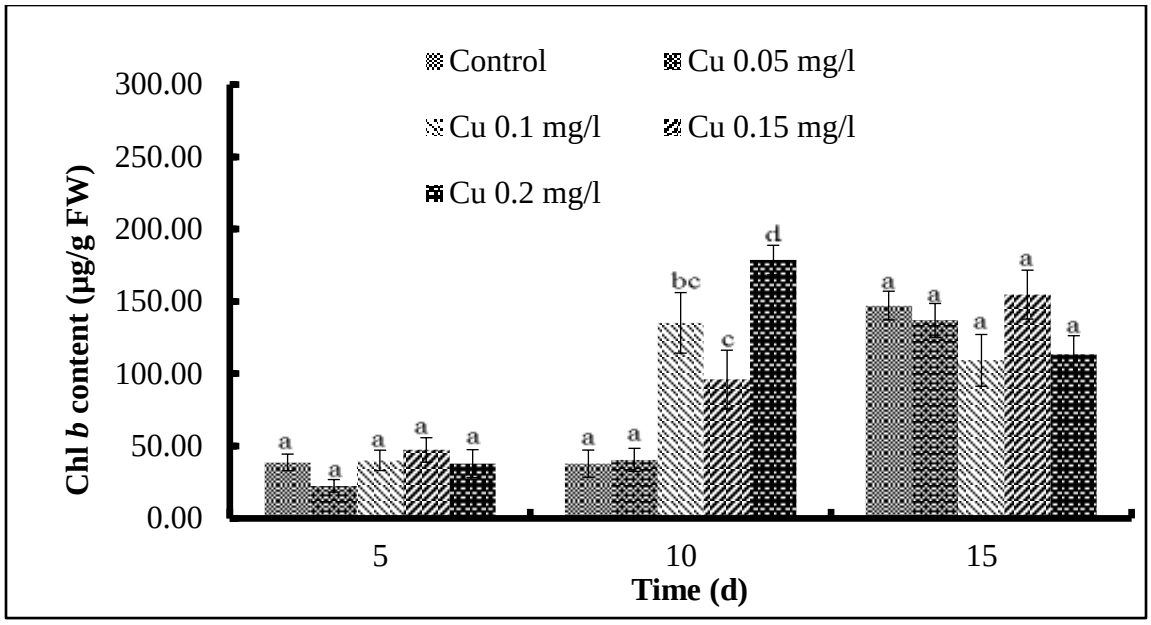


Figure 4.8 Chlorophyll *b* content of Coontail, *C. demersum* exposed to varied concentration of Cu. Values with different letters are significantly different at P<0.05.

4.2.1.3 Chlorophyll *b/a*

Figure 4.9 shows the content of ratio chlorophyll of Coontail exposed to copper. The results showed that there were significant difference in every concentration (P< 0.05) on day 5, day 10 and day 15 of the experiment. The highest content of ratio chlorophyll was found in Cu 0.15 mg/l (0.78 ± 0.11 µg/g) at day 10,and Cu 0.20 mg/l (0.32 ± 0.06 µg/g) at day 5.

The lowest content of ratio chlorophyll was found in Cu 0.05 mg/l (0.18 ± 0.01 µg/g) at day 5, and control (0.20 µg/g) at day 10. Chl *b* is an accessory photosynthetic pigment compared to Chl *a* which is the primary photosynthetic pigment. The Chl *b/a* ratio tended to increase over time in plants exposed to high Cu.

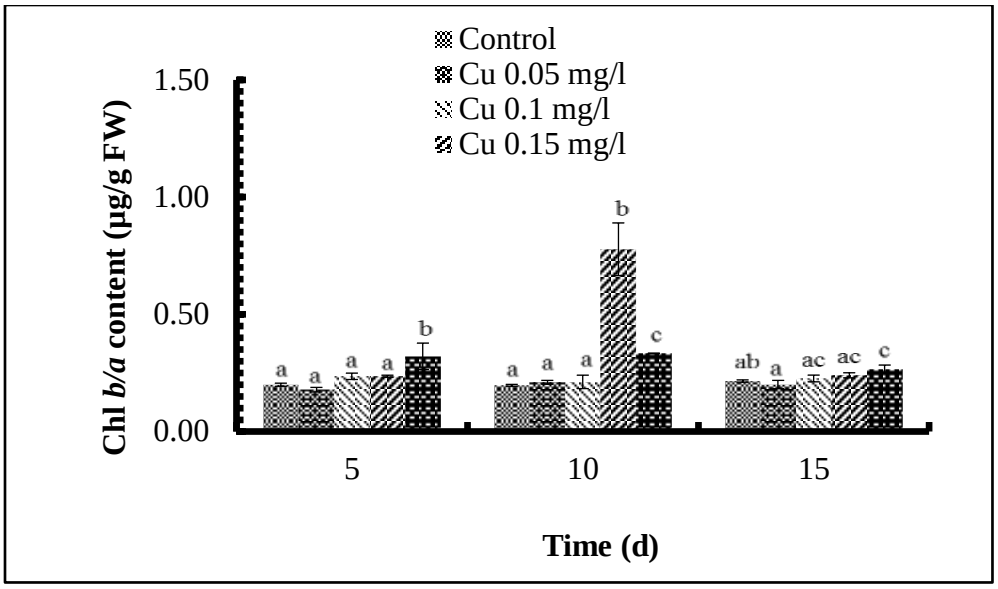


Figure 4.9 Chlorophyll *b/a* content of Coontail, *C. demersum* exposed to varied concentration of Cu. Values with different letters are significantly different at P<0.05.

4.2.2 Zinc

4.2.2.1 Chlorophyll *a*

Figure 4.10 shows the chlorophyll *a* content of Coontail exposed to zinc. There were lethal and sublethal effects. The results showed that there was no significant difference in every concentration (P< 0.05) on day 10 and day 15 of the experiment. The highest chlorophyll *a* content was found in Zn 10.0 mg/l (707.93 ± 52.82 µg/g) at day 15 andZn 5.0 mg/l (462.18 ± 46.59 µg/g) at day 10. However, 15 mg/l of Zn was toxic after 10 and 15 days. Sublethal concentrations of Zn (5 and 10 mg/l) led to an increase in Chl *a* over time.

The lowest chlorophyll *a* content was found incontrol (189.56 ± 43.63 µg/g) of fresh weight of day 10 and control (191.06 ± 23.21 µg/g) of fresh weight of day 5, respectively.

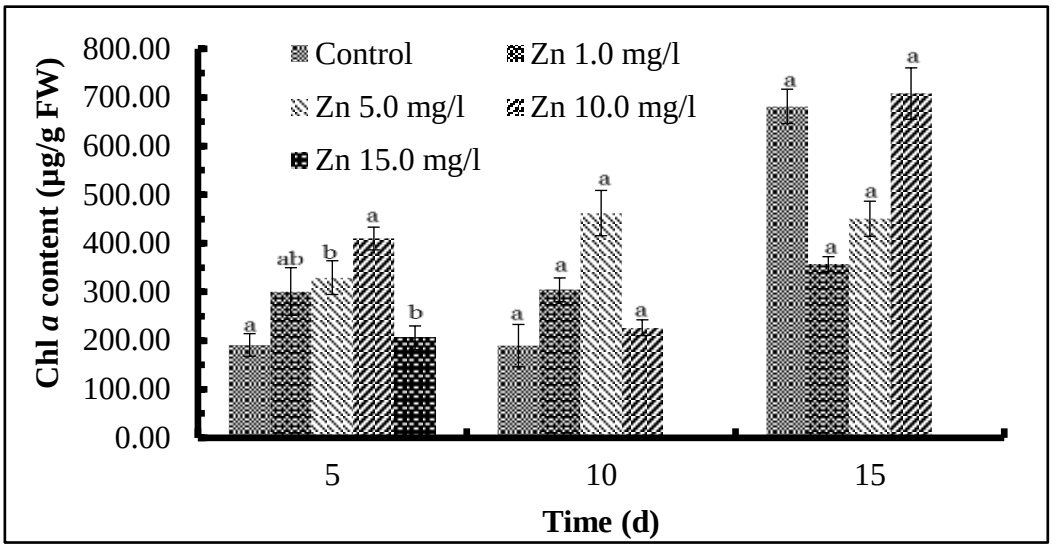


Figure 4.10 Chlorophyll *a* content of Coontail, *C. demersum* exposed to varied concentration of Zn. Values with different letters are significantly different at P<0.05. 15 mg/l Zn was lethal at 10 & 15 days.

4.2.2.2 Chlorophyll *b*

Figure 4.11 shows the chlorophyll *b* content of Coontail exposed to zinc. As for Chl *a* (Fig 4.11) there were lethal effects for 15 mg/l Zn after 10 and 15 days. The results showed that there were no significant difference in every concentration (P< 0.05) on day 10 and day 15 of the experiment for 5 and 10 mg/l Zn. The highest chlorophyll *b* content was found in Zn 10.0 mg/l (226.34 ± 16.31 µg/g) at day 15 and Zn 5.0 mg/l (121.02 ± 12.84 µg/g) at day 10.

The lowest chlorophyll *b* content was found incontrol mg/l (37.88 ± 9.40µg/g) day 10 and control (38.73 ± 5.77µg/g) at day 5. As for Chl *a* (Fig 4.10), Zn at sublethal concentrations increased Chl *b* over time.

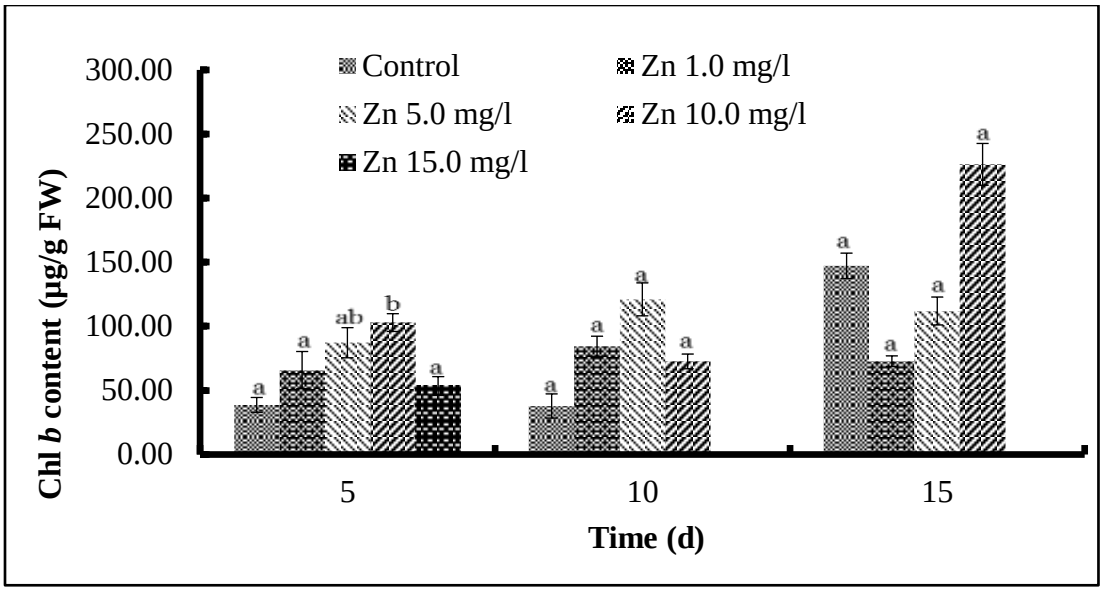


Figure 4.11 Chlorophyll *b* content of Coontail, *C. demersum* exposed to varied concentration of Zn. Values with different letters are significantly different at P<0.05.

4.2.2.3 Chlorophyll *b/a* content of plants exposed to zinc

Figure 4.12 shows the content of ratio chlorophyll of Coontail exposed to zinc. The results showed that there was no significant difference in every concentration (P< 0.05) on day 10 and day 15 of the experiment. The highest content of ratio chlorophyll was found in Zn 10.0 mg/l (0.32 ± 0.01 µg/g) at day 10. As for the Chl *a* and Chl *b* results above, there is no Chl *b/a* ratio data for plants exposed to 15 mg/l Zn after 10 and 15 days because the plants had died.

The lowest content of ratio chlorophyll was found in control (0.20 ± 0.01 µg/g) at day 5, Fig 4.12 shows that there were no large changes in the Chl *b/a* ratio over time at 5 or 10 mg/l Zn compared to the controls.

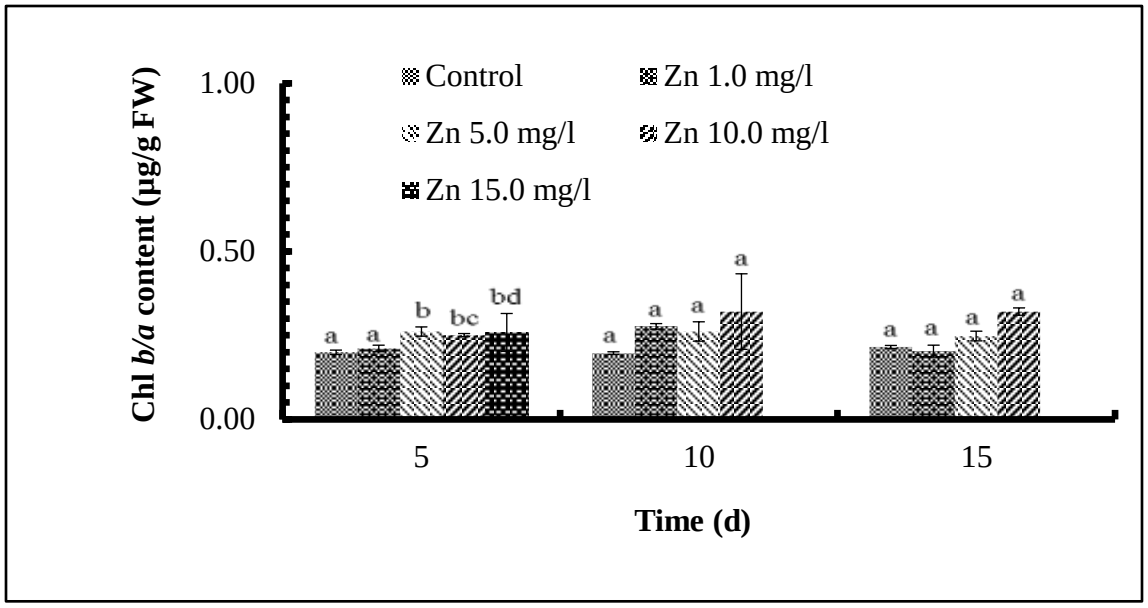


Figure 4.12 Chlorophyll *b/a* content of Coontail, *C. demersum* exposed to varied concentration of Zn. Values with different letters are significantly different at P<0.05.

4.2.3 Copper plus zinc

4.2.3.1 Chlorophyll *a*

Figure 4.13 shows the chlorophyll *a* content of Coontail exposed to copper plus zinc. The results shows that there was no significant difference in every concentration (P< 0.05) on day 5 and day 10 of the experiment. The control had much higher Chl *a* than any other treatments at day 15 and much high than the controls at day 5 and 10. The combination of high Cu + Zn was strongly inhibitory after 15 days.

The highest chlorophyll *a* content was found in control (681.55 ± 35.41 µg/g) day 15 and Cu 0.1 mg/l + Zn 1.0 mg/l (332.86 ± 20.64 µg/g) day 10.

The lowest chlorophyll *a* content was found inCu 0.1 mg/l + Zn 5.0 mg/l (163.99 ± 21.23 µg/g) at day 15 andcontrol (189.56 ± 43.63µg/g) at day 10.

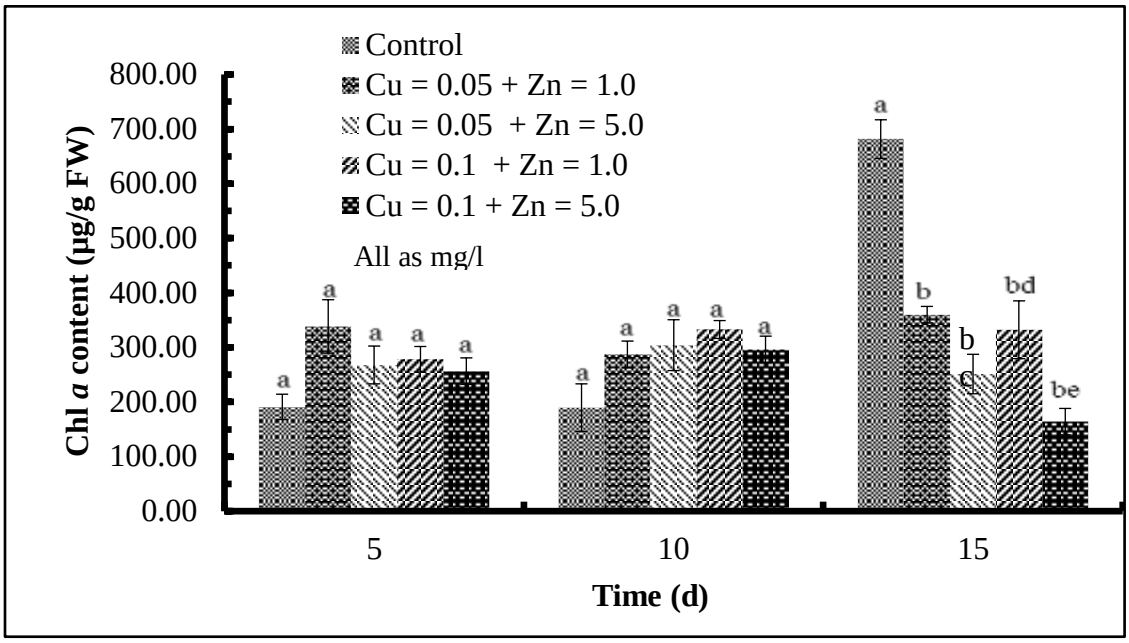


Figure 4.13 Chlorophyll *a* content of Coontail, *C. demersum* exposed to varied concentration of Cu + Zn. Values with different letters are significantly different at P<0.05.

4.2.3.2 Chlorophyll *b*

Figure 4.14 shows the chlorophyll *b* content of Coontail exposed to copper plus zinc. The results showed that there was significant difference in every concentration (P< 0.05) on day 5, day 10, and day 15 of the experiment. The highest chlorophyll *b* content was found in control (147.19 ± 9.93 µg/g) at day 15 and Cu 0.05 mg/l + Zn 1.0 mg/l (105.12 ± 12.18.74 µg/g) at day 5. As in the case of Chl *a* (Fig. 4.14) the highest Cu + Zn inhibited Chl *b* content but this was most apparent at day 15.

The lowest chlorophyll *b* content was found incontrol (37.88 ± 49.40µg/g) at day 10 and control (38.73 ± 5.77µg/g) day 5.

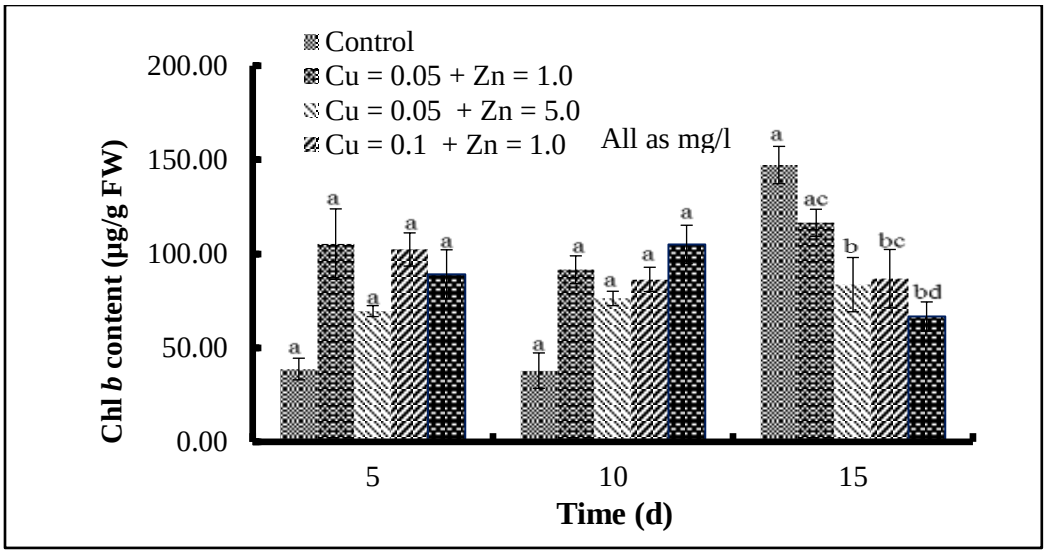


Figure 4.14 Chlorophyll *b* content of Coontail, *C. demersum* exposed to varied concentration of Cu + Zn. Values with different letters are significantly different at P<0.05.

4.2.3.3 Chlorophyll *b/a*

Figure 4.15 shows the content of ratio chlorophyll of Coontail exposed to zinc. The results showed that there was significant difference in every concentration (P< 0.05) at day 5, day 10 and day 15 of the experiment. The highest content of ratio chlorophyll was found inCu 0.1 mg/l + Zn 5.0 mg/l (0.41 ± 0.01 µg/g) at day 15 and Cu 0.1 mg/l + 1.0 mg/l (0.37 ± 0.01 µg/g) at day 5. High Cu + Zn tended to consistently increase the Chl *b/a* ratio at day 5, 10 and 15. The Chl *b/a* ratio of the controls was very consistent.

The lowest content of ratio chlorophyll was found in control (0.20 ± 0.01 µg/g) of fresh weight of day 5, control mg/l (0.20 µg/g) at day 10.

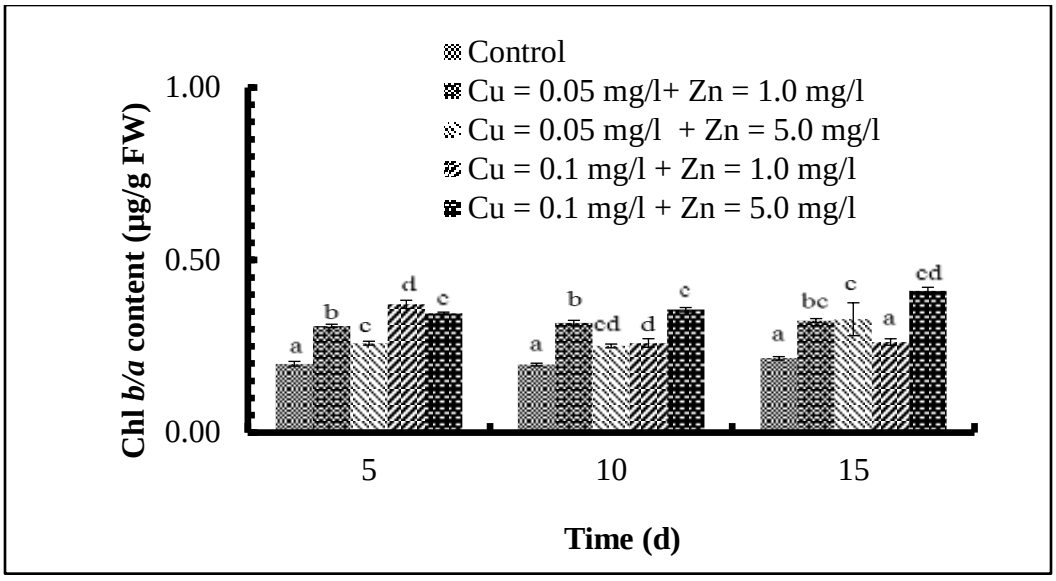


Figure 4.15 Chlorophyll *b/a* content of Coontail, *C. demersum* exposed to varied concentration of Cu + Zn. Values with different letters are significantly different at $P<0.05$.

4.3 Leaf cell observation

4.3.1 Copper

Figure 4.16 shows the leaf cell of *C. demersum* in the control and various concentration of Cu (0.05, 0.1, 0.15 and 0.20 mg/l) on day 5 of the experiment. The cells were retangular in shape. The cloroplasts were green and oval in shape. In addition, the vacuole of the treatments were bigger than control. For these experiments *C. demersum* was grown on soft water media with very low dissolved salts.

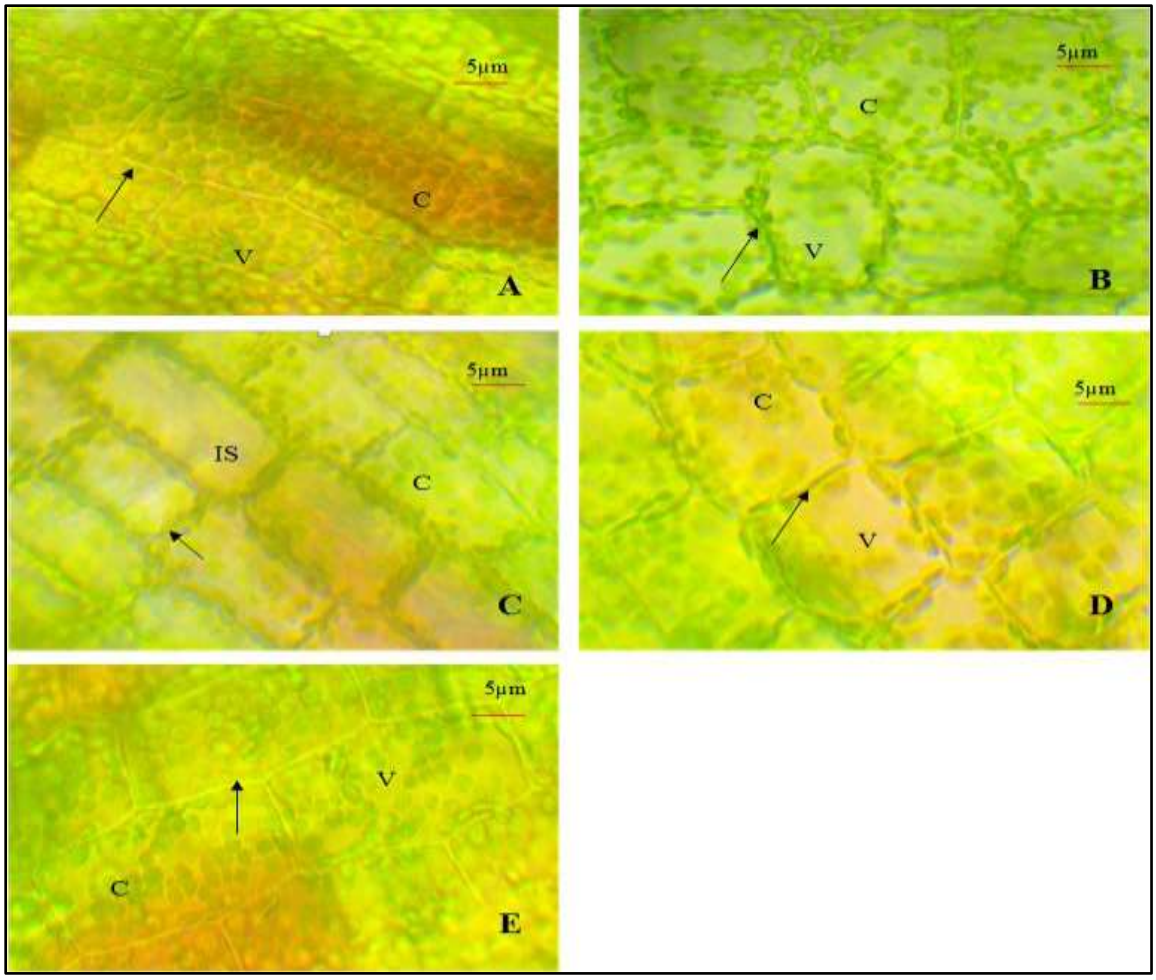


Figure 4.16 Leaf cell of *C. demersum* in control (A) and varied concentration of Cu onday 5, B: 0.05 mg/l, C: 0.10 mg/l, D: 0.15 mg/l and E: 0.20 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

Figure 4.17 shows the leaf cell of *C. demersum* in the control and various concentrations of Cu (0.05, 0.1, 0.15 and 0.20 mg/l) on day 10 of the experiment.The significant changes were found. The intercellular space of the treatment cells were wider when compared with that of control. At high concentrations of Cu (0.15 and 0.20 mg/l) the cloroplasts were broken, swollen and deformed (Figure 4.17 E). Moreover, plasmolysis of cells was found (Figure 4.17 D)

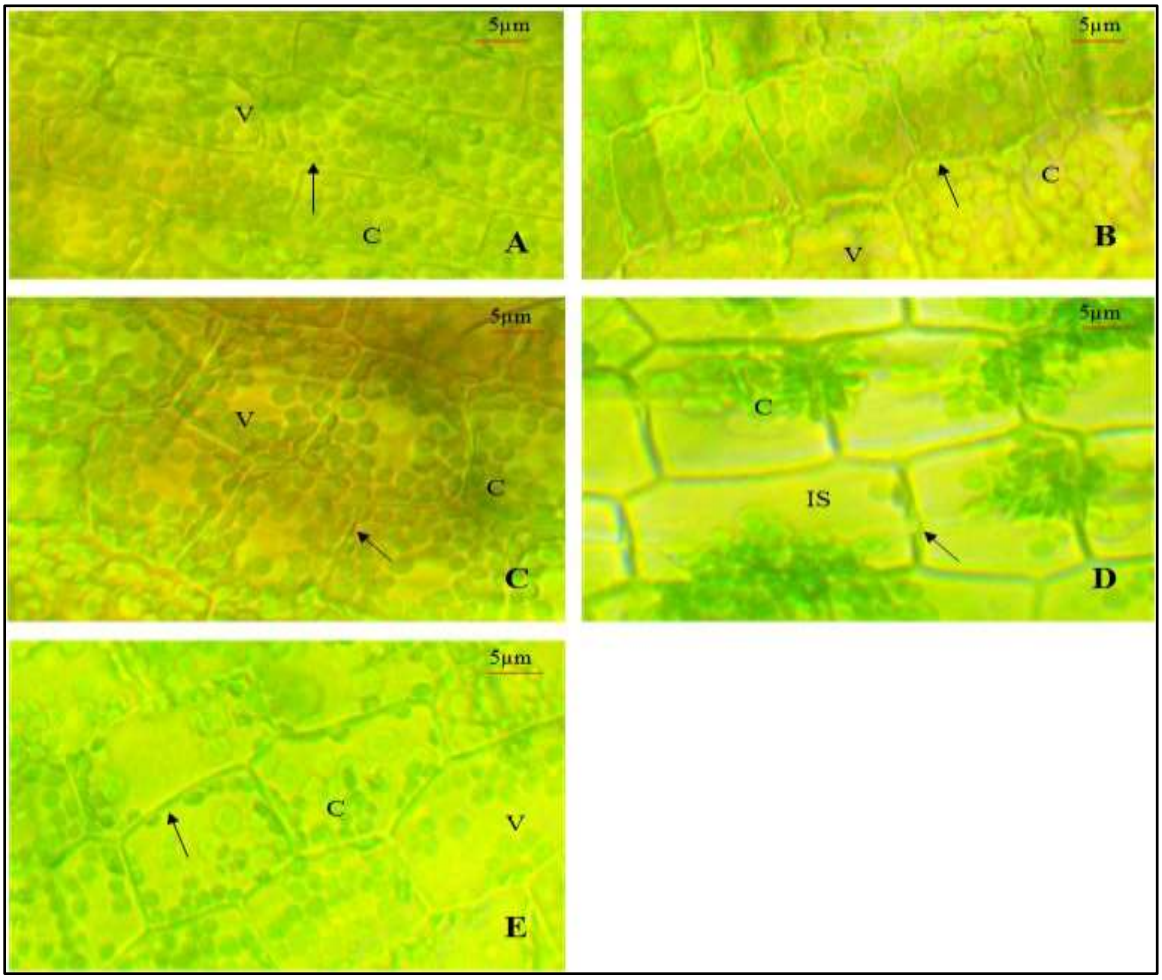


Figure 4.17 Leaf cell of *C. demersum* in control (A) and varied concentrationson of Cuon day 10, B: 0.05 mg/l, C: 0.10 mg/l, D: 0.15 mg/l and E: 0.20 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow). Fig. 4.17D shows severe plasmolysis of the cells.

Figure 4.18 showsthe leaf cell of *C. demersum* in the control and various concentration of Cu (0.05, 0.1, 0.15 and 0.20 mg/l) on day 15 of the experiment. Broken shoots and leaves were found at 0 .15 and 0.20 mg/l. The vacuoles of the tratments were as big as the cell size. The cloroplasts were foundand the edge of the cell (Figure 4.18 B and Figure 4.18 C). The swollen and deformed chloroplasts were found in all treatments except for Cu 0.05 mg/l. In addition, broken chloroplasts were also found at Cu 0.20 mg/l, resulting in chloroplast fragments being dispersed throughout the cell.

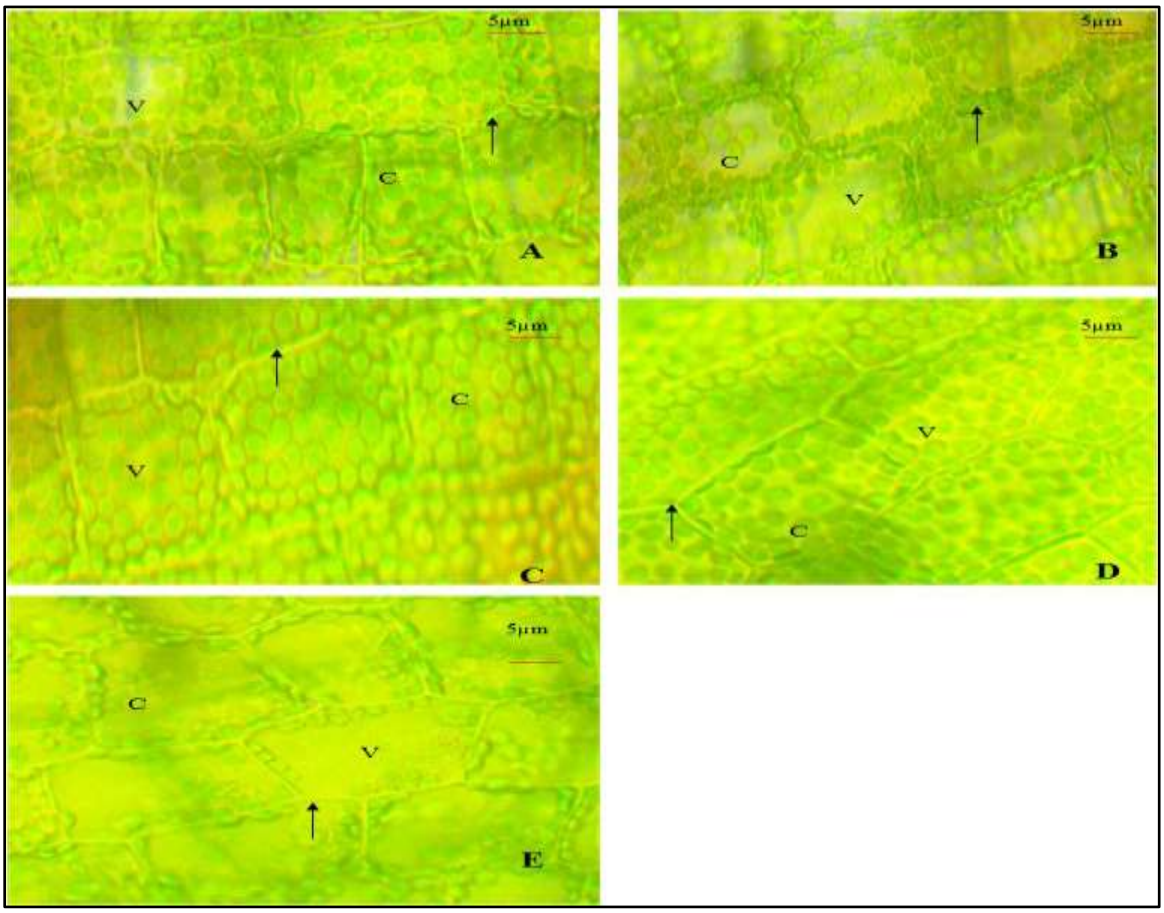


Figure 4.18 Leaf cell of *C. demersum* in control (A) and varied concentration of Cu onday 15, B: 0.05 mg/l, C: 0.10 mg/l, D: 0.15 mg/l and E: 0.20 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

4.3.2Zinc

Figure 4.19 shows the leaf cell of *C. demersum* in the control and various concentrations of Zn (1.0, 5.0, 10.0 and 15.0 mg/l Zn on day 10 of the experiment. The significant change was found in 5.0 mg/l, 10.0 mg/l and 15.0 mg/l Zn. Broken, swollen and deformed chloroplasts were found in Zn 10.0 mg/l. Additionally, the chloroplast fragments were spread throughout the cell and in the intercellular space as well (Figure 4.19 C, D and E).

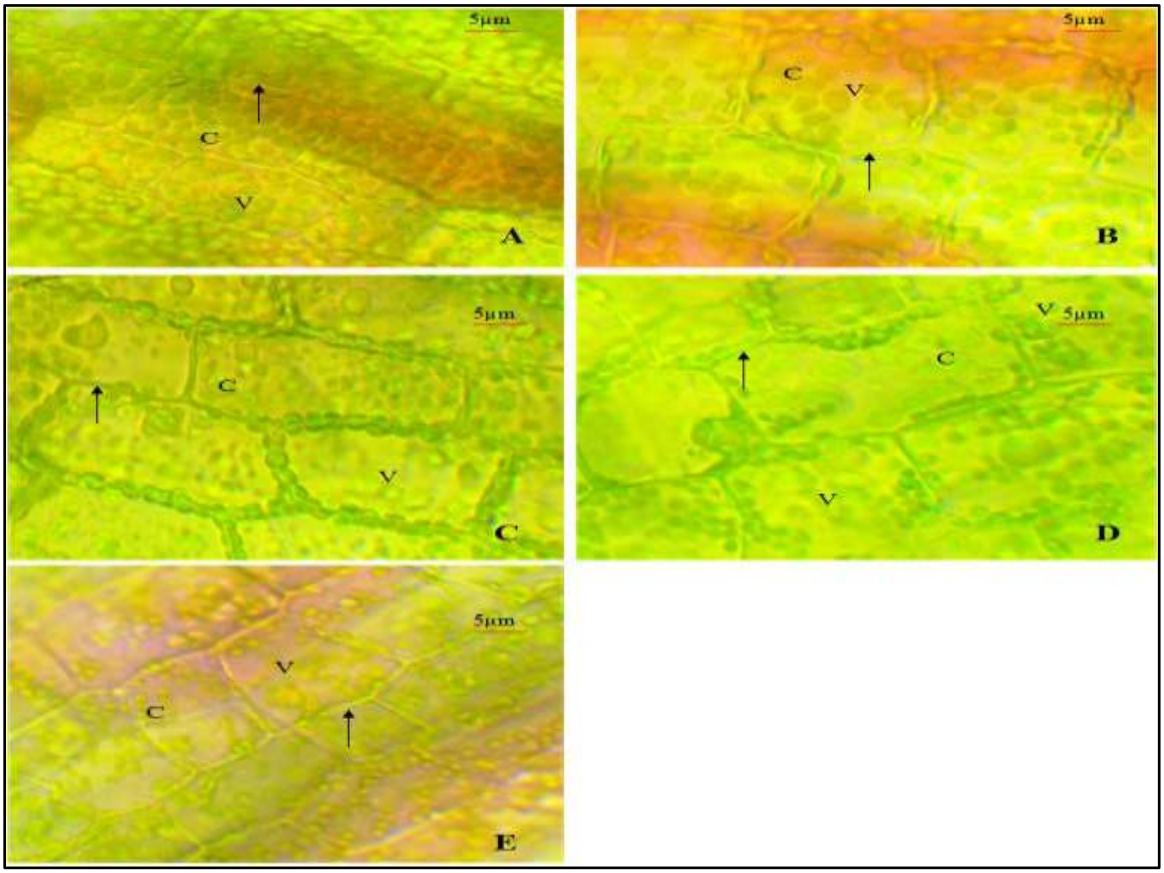


Figure 4.19 Leaf cell of *C. demersum* in control (A) and varied concentration of Zn on day 5, B: 1.0 mg/l, C: 5.0 mg/l, D: 10.0 mg/l and E: 15.0 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

Figure 4.20 shows the leaf cells of *C. demersum* in the control and various concentrations of Zn (1.0, 5.0, 10.0 and 15.0 mg/l Zn) on day 10 of the experiment.Significant changes were found in Zn 10.0 mg/l with broken, swollen and deformed chloroplasts. Additionally, thechloroplast fragments were found spread throughout the cell and in the intercellular spaces (Figure 4.20 D). Moreover, at concentration of Zn 15.0 mg/l, plants died on day 12.

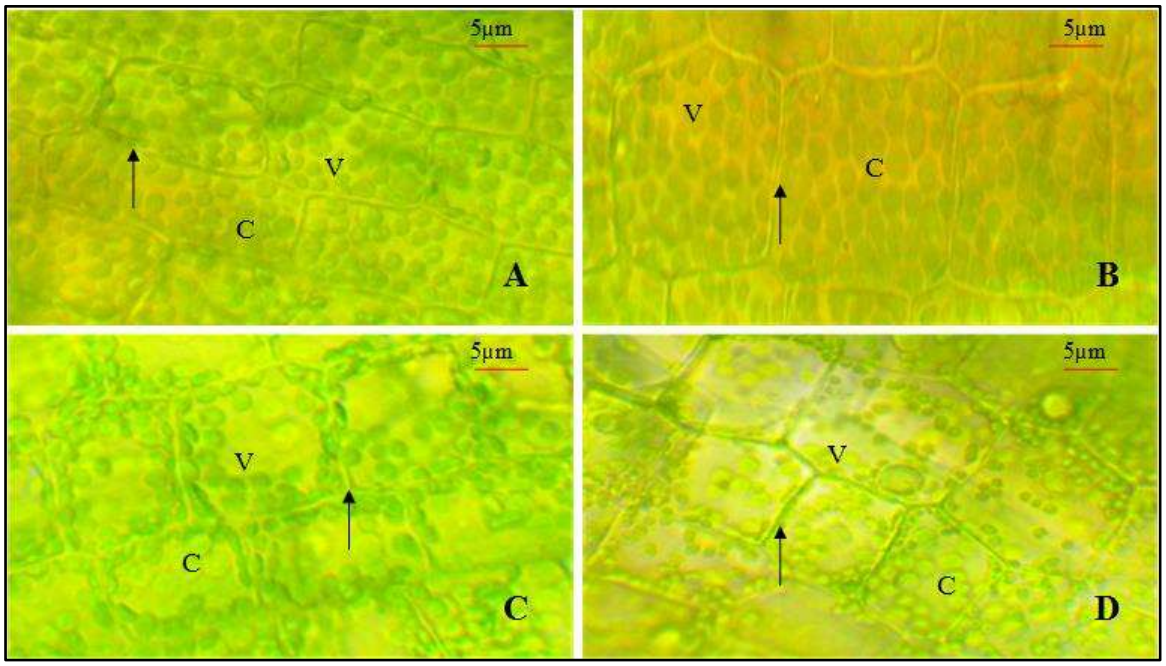


Figure 4.20 Leaf cell of *C. demersum* in control (A) and varied concentrations of Zn as $ZnSO_4$ in day 10, B: 1.0 mg/l, C: 5.0 mg/l, D: 10.0 mg/l and E: 15.0 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

Figure 4.21 shows the leaf cells of *C. demersum* in the control and various concentrations of Zn (1.0, 5.0 and 10.0 mg/l) on day 15 of the experiment. The broken, swollen and deformed chloroplasts were found in every treatment especially at Zn 10.0 mg/l. At Zn 10.0 mg/l the chloroplasts were smaller and wereflattened and scaly in shape. In addition, the chlorophyll was found in intercellular spaces and throughout the cell in all treatment.

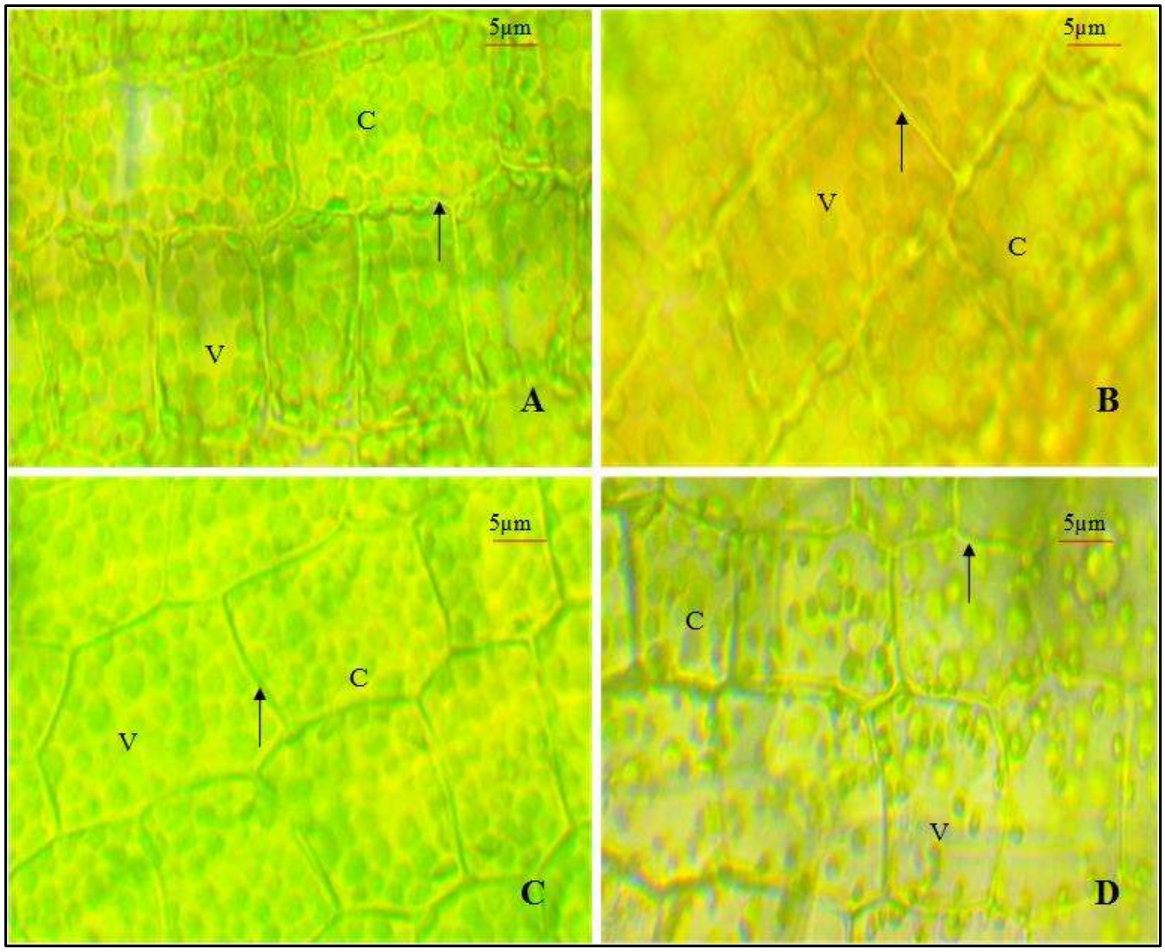


Figure 4.21 Leaf cell of *C. demersum* in control (A) and varied concentration of Zn on day 15, B: 1.0 mg/l, C: 5.0 mg/l, D: 10.0 mg/l and E: 15.0 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

4.3.3Copper + Zinc

Figure 4.22 shows the leaf cells of *C. demersum* in the control and various concentrations of Cu + Zn (0.1 mg/l + 1.0 mg/l, 0.1 mg/l + 5.0 mg/l, 0.05 mg/l + 5.0 mg/l and 0.05 mg/l + 1.0 mg/l) on day 5 of the experiment. The significant changes were found in every treatment. The vacuoles of the treatments were as big as the cell size. The broken, swollen and deformed choroplast, chloroplast fragments appeared in the intercellular speces. In addition, the exposure of 0.1 mg/l + 5.0 mg/l Cu + Zn, showed the most severe symptoms. In this treatment, the number of chloroplast was lesser than the others and they became small fragment or flattened and scale-like in shape.

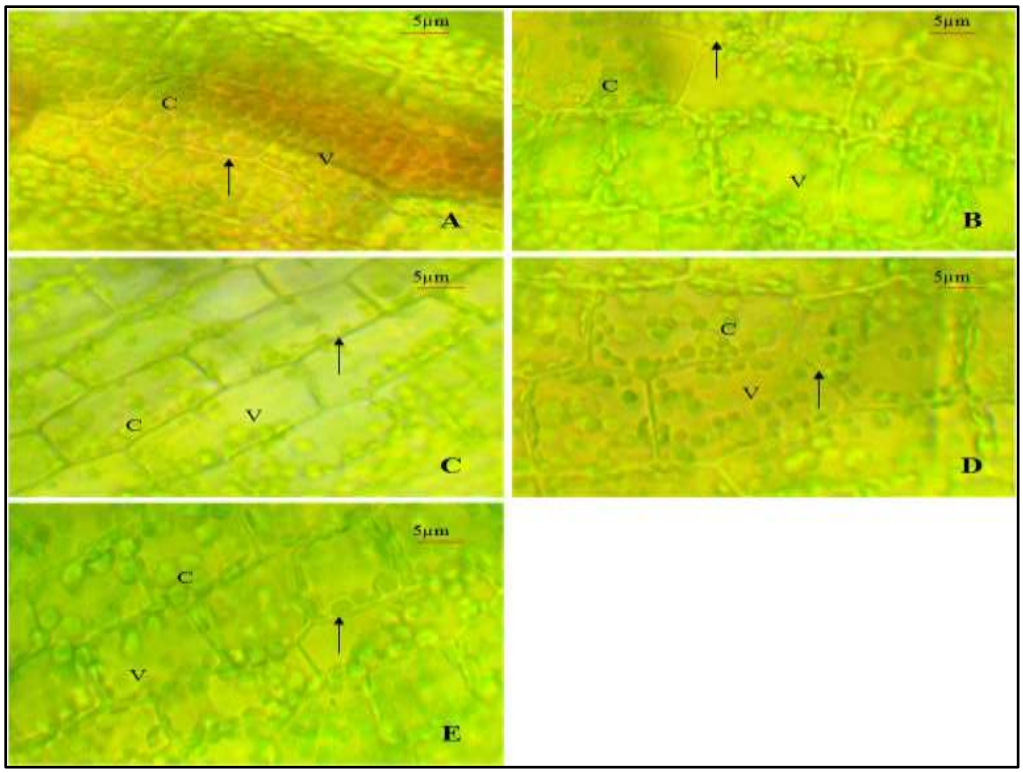


Figure 4.22 Leaf cell of *C. demersum* in control (A) and varied concentration of Cu as $CuSO_4$ and Zn on day 5, B: 0.1 mg/l + 1.0 mg/l, C: 0.1 mg/l + 5.0 mg/l, D: 0.05 mg/l + 5.0 mg/l and E: 0.05 mg/l + 1.0 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

Figure 4.23 shows the leaf cell of *C. demersum* in the control and various concentrations of Cu + Zn (0.1 mg/l + 1.0 mg/l, 0.1 mg/l + 5.0 mg/l, 0.05 mg/l + 5.0 mg/l and 0.05 mg/l + 1.0 mg/l) on day10 of the experiment. Fragmented chloroplasts were found in all treatments (Figure 4.23 B-E). The chloroplast fragmentswere spreading throughout the cell and in the intercellular spaces. Broken chloroplasts were found in every treatment except in 0.05 mg/l + 1.0 mg/l Cu + Zn treatment.

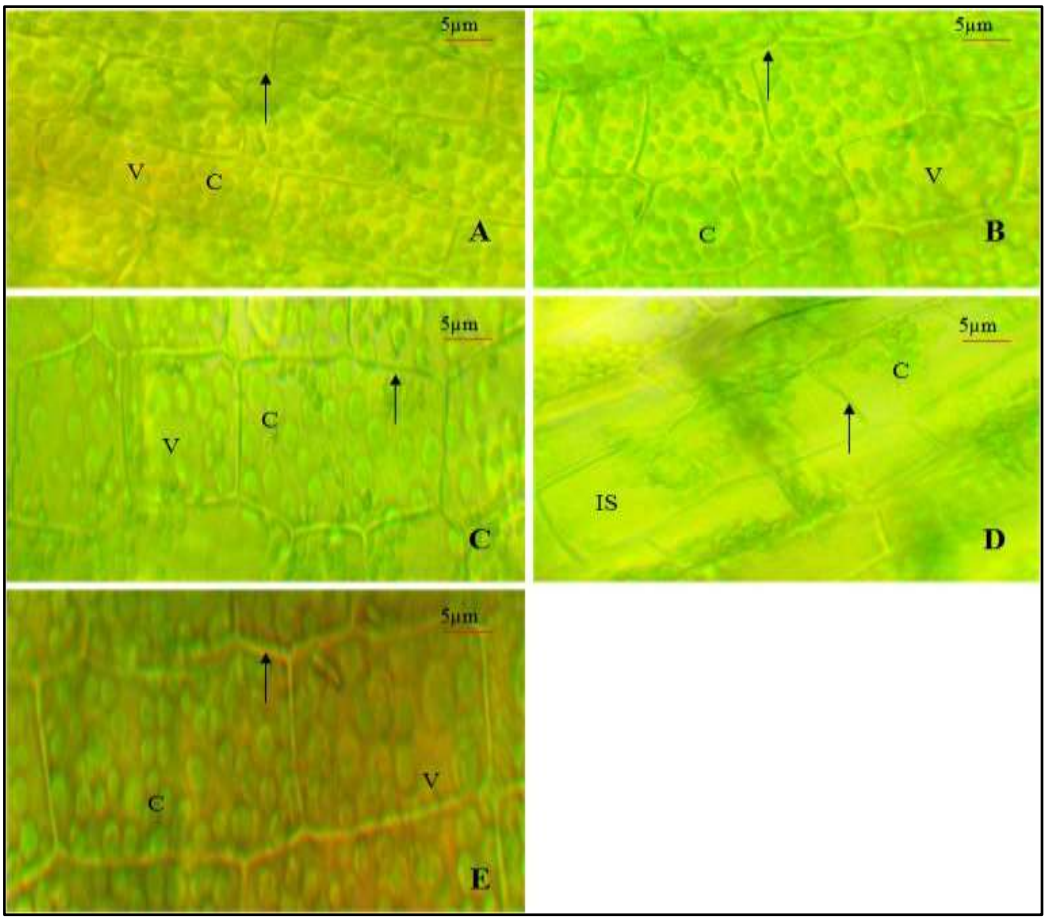


Figure 4.23 Leaf cell of *C. demersum* in control (A) and varied concentration of Cu and Zn on day 10, B: 0.1 mg/l + 1.0 mg/l, C: 0.1 mg/l + 5.0 mg/l, D: 0.05 mg/l + 5.0 mg/l and E: 0.05 mg/l + 1.0 mg/l showing the chloroplast (C), intercellular space (IS) and cell wall (arrow).

Figure 4.24 shows the leaf cells of *C. demersum* in the control and various concentrations of Cu + Zn (0.1 mg/l + 1.0 mg/l, 0.1 mg/l + 5.0 mg/l, 0.05 mg/l + 5.0 mg/l and 0.05 mg/l + 1.0 mg/l) on day 15 of the experiment. Broken, swollen and deformed choloroplasts were found (Figure 4.24 B-E). The fragmented or caly chloroplasts were found in 0.1 mg/l + 5.0 mg/l Cu + Zn (Figure 4.24 C). Additionally, the chloroplast fragments were found to be spread throughout the cell and in intercellular spaces (Figure 4.24 C-E).

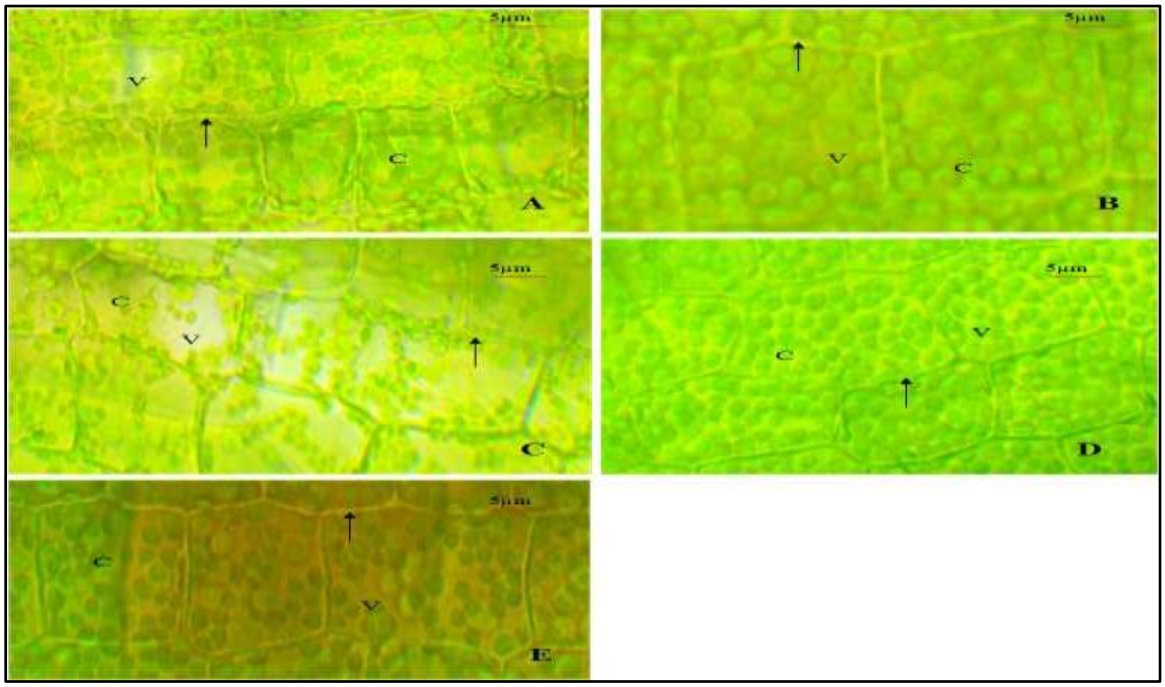


Figure 4.24 Leaf cell of *C. demersum* in control (A) and varied concentrations of Cu and Zn on day 15, B: 0.1 mg/l + 1.0 mg/l, C: 0.1 mg/l + 5.0 mg/l, D: 0.05 mg/l + 5.0 mg/l and E: 0.05 mg/l + 1.0 mg/l showing the chloroplast (C), vacuole (V) and cell wall (arrow).

4.4 The Bioconcentration factor (BCF)

4.4.1The BCF of Cu

Figure 4.25 shows Cu uptake by *C. demersum* after 15 days. The highest BCF was found in *C. demersum* (BCF = 3753), exsposed to 0.05 mg/l Cu on day 15 while the lowest was presented in 0.15 mg/l Cu at day 15 (Figure 4.25).

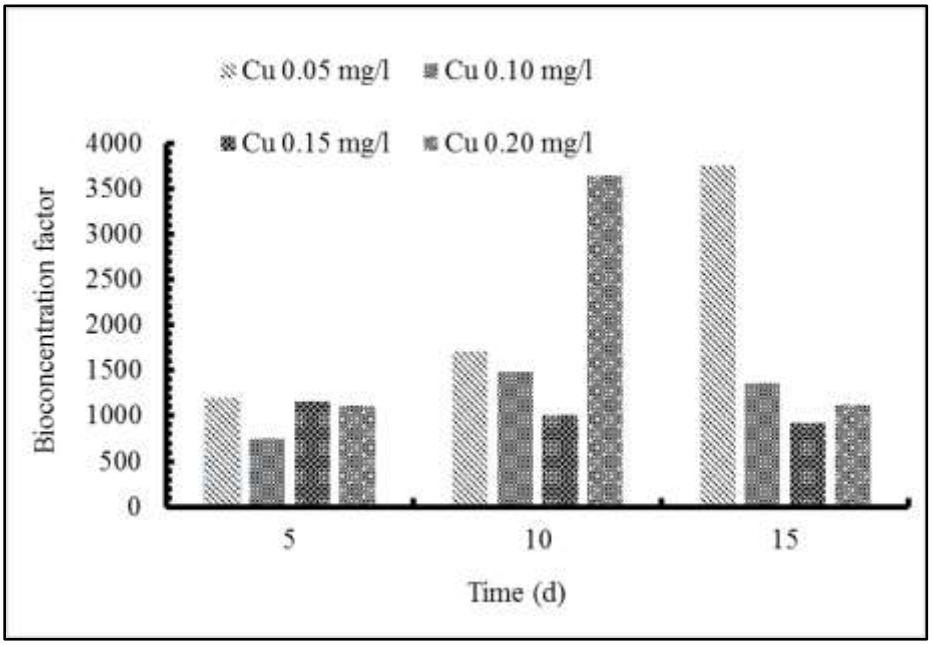


Figure 4.25 Bioconcentration of Cu in shoot of Coontail after up to 15 days.

4.4.2 The BCF of Zn

Figure 4.26 shows Zn uptake by Coontail after 15 days. After 15 days of the experiment, 10.0 mg/l Zn showed the highest (BCF = 16042) while the lowest was found in *C. demersum* (BCF = 2009) exsposed to 1.0 mg/l.

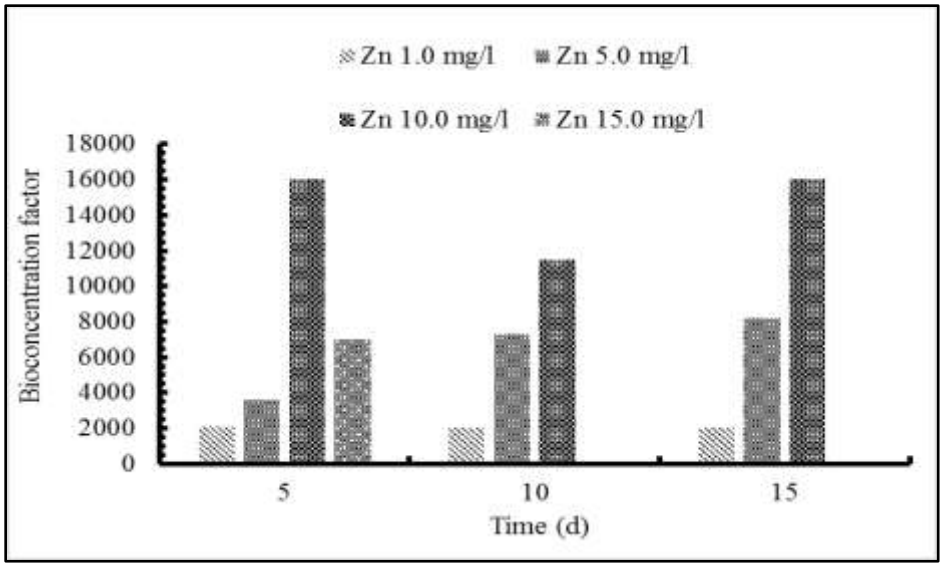


Figure 4.26 Bioconcentration of Zn in shoot of Coontail after 15 days. The data presented are mean of five replicates.

4.4.3 The BCF of Cu + Zn

Figure 4.27 shows Cu and Zn uptake by Coontail after 15 days.The highest BCF of Cu was found in *C. demersum* (BCF = 23039) in Cu 0.1 mg/l +Zn 5.0 mg/l on day 15.

The lowest BCF of Cu presented in Cu 0.05 mg/l + Zn 1.0 mg/l (BCF = 3604) on day 15. In addition, the highest BCF of Zn was found in *C. demersum* (6359) in 0.1 mg/l Cu + 5.0 mg/l Zn on day 15. However, the lowest BCF of Zn was found in Cu 0.05 mg/l +Zn 1.0 mg/l (BCF = 1060) on day 15.

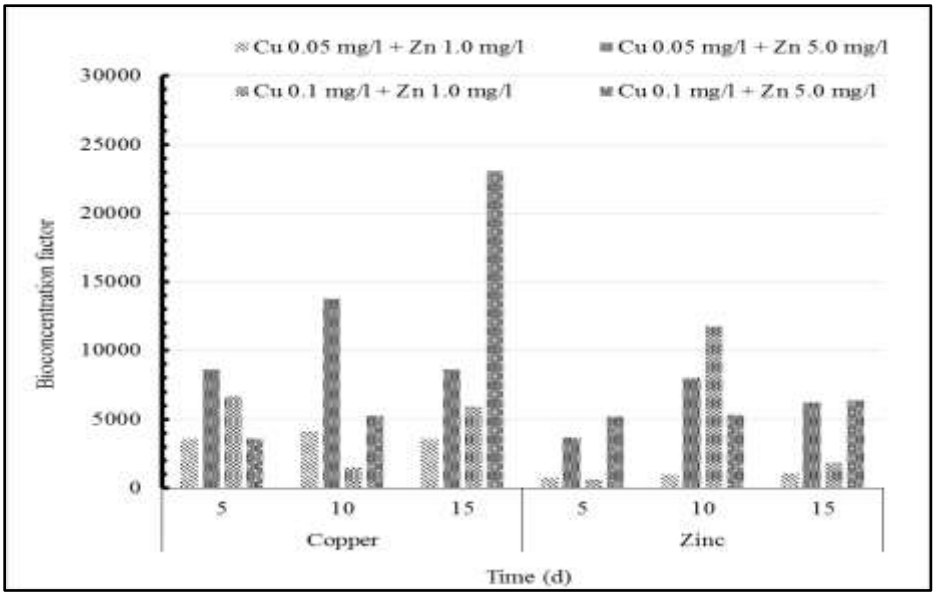


Figure 4.27 Bioconcentration factor of Cu + Zn of Coontail, *C. demersum*, after 15 days.

4.5 Photosynthesis of *C. demersum*inresponse to copper, zinc and their combination treatments.

Each PAM experiment to measure photosynthetic performance was done in 5 replicates. Experiments were run in a darkened laboratory with a 10 minute dark pretreatment following standard procedures (Ritchie, 2012; Ritchie &Mekjinda, 2016). Absorptance was measured using the RAT machine (Ritchie &Mekjinda, 2016) and so ETR quoted here is actual ETR not relative ETR (rETR).

In the interests of space all the PAM experiments are fully documented in the Appendix. For illustrative purposes selected PAM experiments are shown in this Chapter. All the calculated values are shown in Table 4.2: Maximum Yield(Y) (dimensionless), half saturation point for Yield ($Y_{1/2}$) (units: µmol photon m^{-2} s^{-1}), optimum irradiance (E_{opt}) (units: µmol photon m^{-2} s^{-1}, maximum photosynthetic electron transport rate (ETR_{max}) (units: µmol e^- m^{-2} s^{-1}). The maximum photochemical quenching (qP) is by definition unity, the half point of decay of qP ($k_{1/2}$qP**)** **(**units: µmol photon m^{-2} s^{-1}) and maximum non-Photochemical quenching (NPQ_{max}) is dimensionless and the half

dsaturation point for NPQ ($NPQ_{1/2}$) is in units of µmol photon m^{-2} s^{-1} (Brestic & Zivcak, 2013).

4.5.1 Control

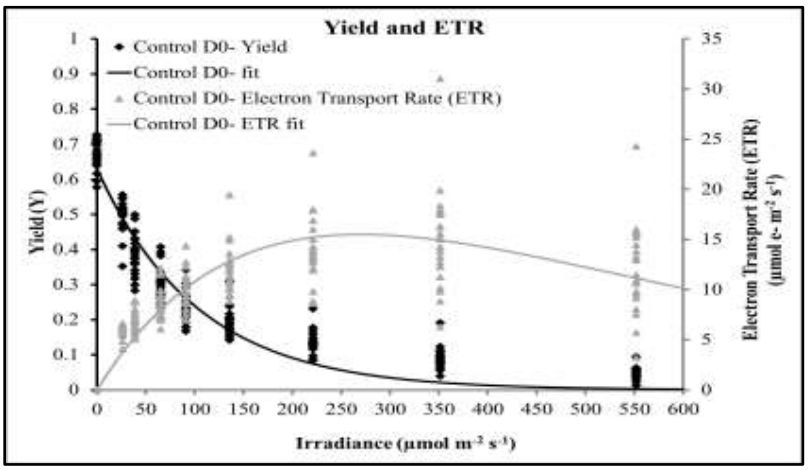


Figure 4.28 Yield (Y) and irradiance (E) and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 0.

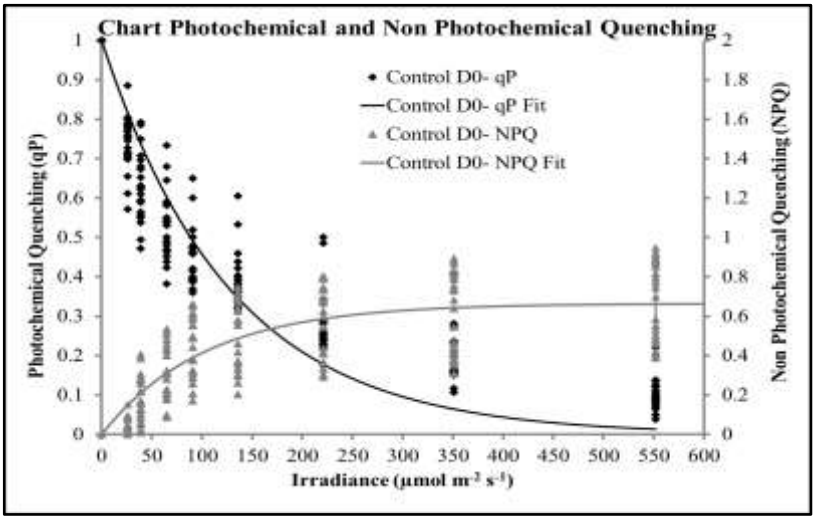


Figure 4.29 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 0.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and maximum electron transport rate (ETR_{max}) is 13.43 ± 0.52 µmol e^- m^{-2} s^{-1} on day 0 of the experiment (Figure 4.28).

Figure 4.29 shows that Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 0. Half point of Photochemical Quenching (qP) is 110.24 ± 8.62 µmol photon m^{-2} s^{-1} and the half-point of Non Photochemical Quenching (NPQ) is 66.86 ± 9.73µmol photon m^{-2} s^{-1} on day 0 (Figure 4.29).

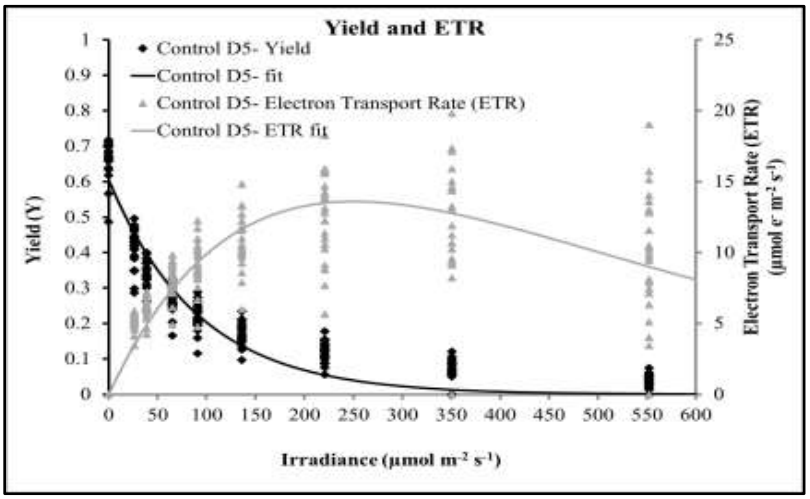


Figure 4.30 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 5.

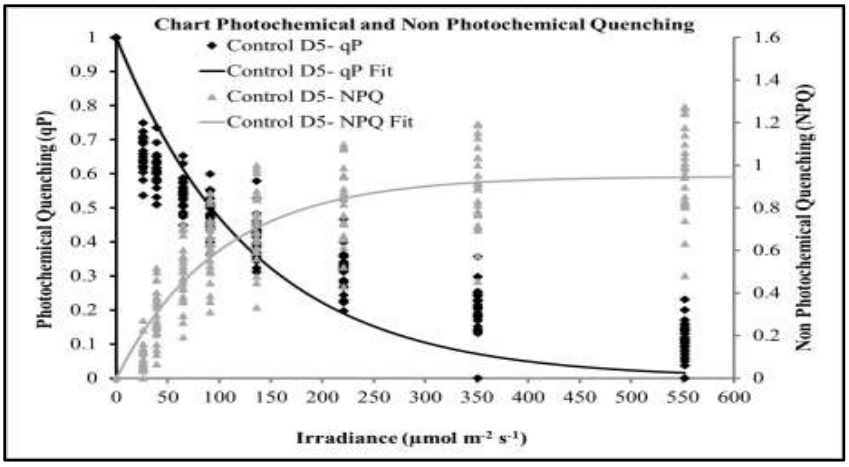


Figure 4.31 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 5.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.61 ± 0.02 and electron transport rate (ETR_{max}) is 13.61 ± 0.50 µmol e^- m^{-2} s^{-1} on day 5 (Figure 4.30).

Figure 4.31 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 5.The half point of Photochemical Quenching (qP) is 92.18 ± 5.62 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.15 ± 8.70 µmol photon m^{-2} s^{-1} on day 5 (Figure 4.31).

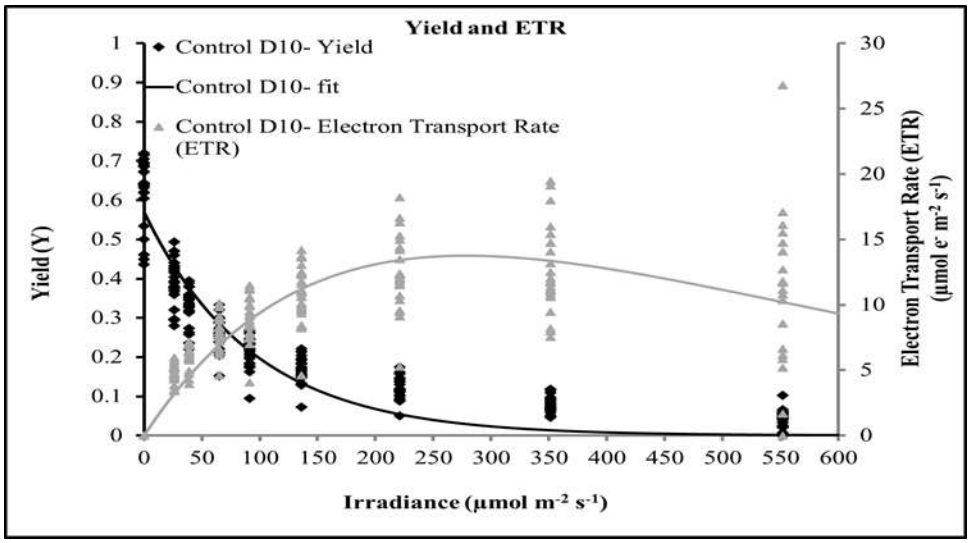


Figure 4.32 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 10.

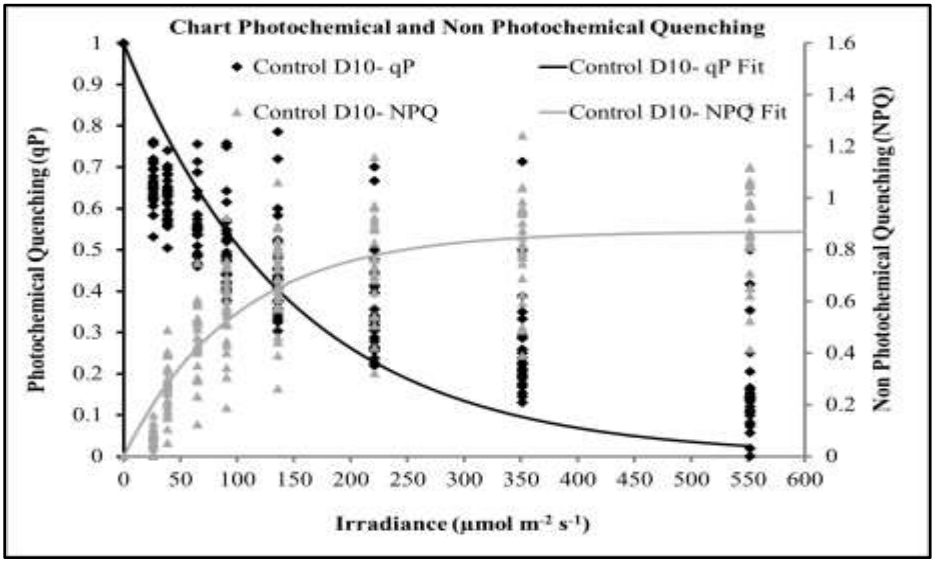


Figure 4.33 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 10.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and electron transport rate (ETR_{max}) is 13.76 ± 0.54 µmol e^- m^{-2} s^{-1} on day 10 (Figure 4.32).

Figure 4.33 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 10. The half pointof Photochemical Quenching (qP) is 103.57 ± 19.87µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 68.44 ± 9.88 µmol photon m^{-2} s^{-1} on day 10 (Figure 4.33).

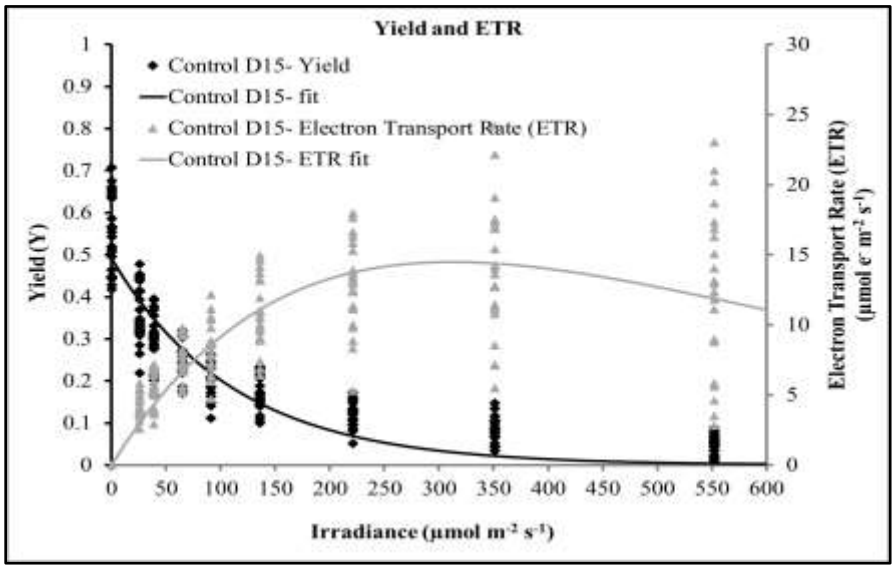


Figure 4.34 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 15.

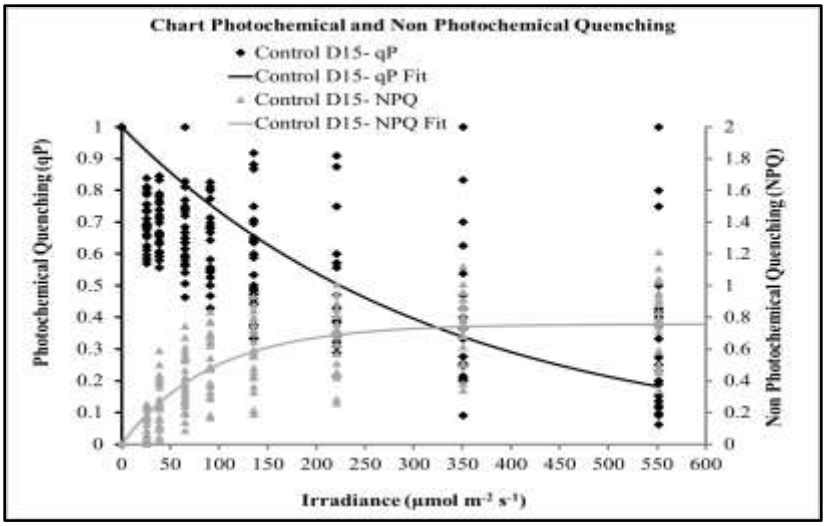


Figure 4.35 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 15.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.49 ± 0.02 and electron transport rate (ETR_{max}) is 14.49 ± 0.66 µmol e^- m^{-2} s^{-1} on day 15 (Figure 4.34).

Figure 4.35 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 15. The half point of Photochemical Quenching (qP) is 224.45 ± 50.32 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 63.49 ± 12.32 µmol photon m^{-2} s^{-1} on day 15 (Figure 4.35).

Figures 4.28 to 4.35 show that *C. demersum* plants kept in the laboratory over 15 days under control culture conditions did not show great changes in Yield, optimum irradiance (E_{opt}) or ETR and no great changes in photochemical and non photochemical quenching.

In experiments where the plant was exposed to Copper, Zinc and a combination of copper and zinc the general observation was that toxic effects only became apparent slowly (see Appendix). The lowest concentrations of Cu, Zn and Cu + Zn generally had little effect and toxic effects were only apparent at higher concentrations.

Toxic effects tended to increase with time and were most apparent after 15 days. For illustrative purposes the results of 15 day incubations at the highest metal concentrations are shown here but all results are shown in the Appendix.

4.5.2 Copper Effects at highest concentration.

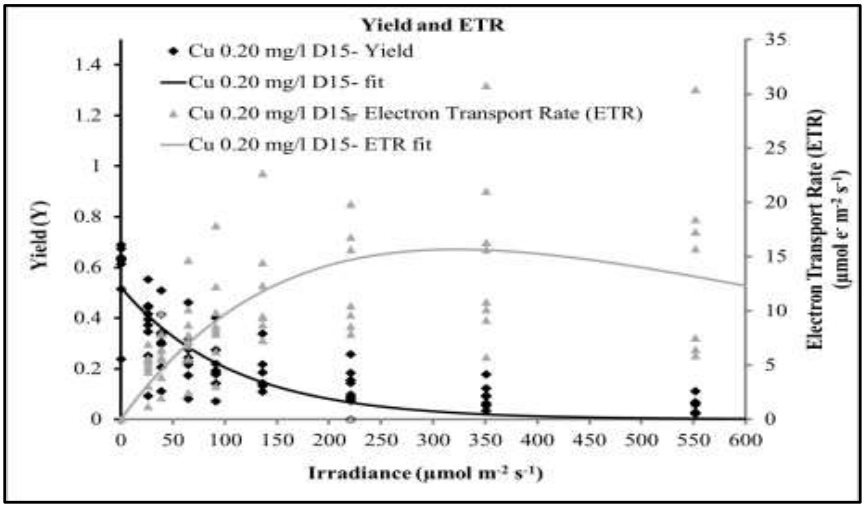


Figure 4.36 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.20 mg/l Cu on day 15.

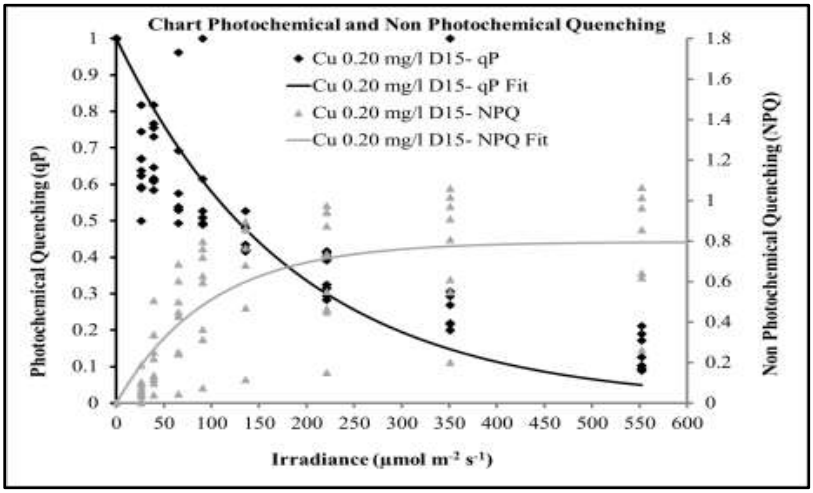


Figure 4.37 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu on day 15.

Photosynthesis parameters of *Ceratophyllum demersum* under 0.20 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.52 ± 0.06 and electron transport rate (ETR_{max}) is 15.67 ± 1.99 µmol e^- m^{-2} s^{-1} on day 15 (Figure 4.36).

Figure 4.37 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *Ceratophyllum demersum* in the 0.20 mg/l Cu treatment on day 15. The half point of Photochemical Quenching (qP) is 127.09 ± 21.76 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 67.36 ± 25.16 µmol photon m^{-2} s^{-1} on day 15 (Figure 4.37).

According to this study, toxic effect of Cu as $CuSO_4$ to *C. demersum* was significant when exposed to increasing concentrations andtoxicity increased over time. All of the concentrationtreatments showed a similar impact to the plants within 5 days, however up to 10 days to 15 days of the experiment *C. demersum* seem to show more effects on photosynthesis. Overall, this study showed that Cu increased in toxicity to *C. demersum* overtime. Up to 15 d were needed to show significant toxity effects.

4.5.3 Zinceffects at maximum tolerable concentration 10 mg/l tolerable for 15 d.

Treatments at day 10 & day 15 died at the highest concentrations of Zn used (15 mg/l).

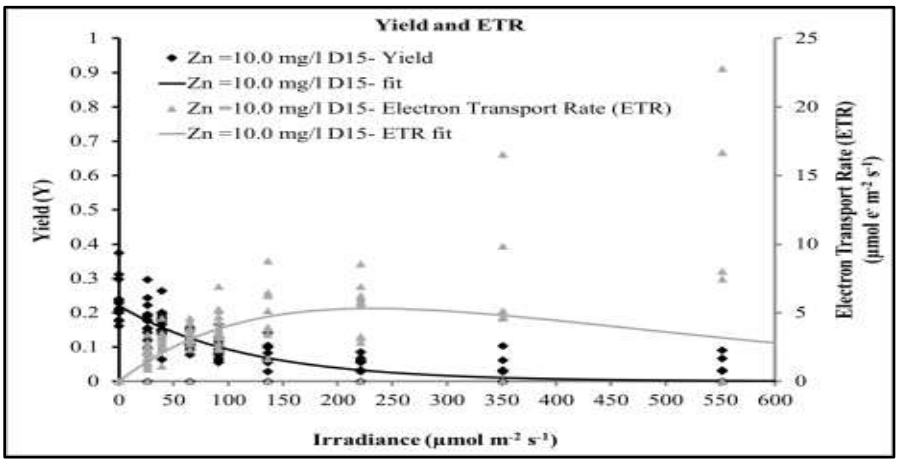


Figure 4.38 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 10.0 mg/l Zn on day 15.

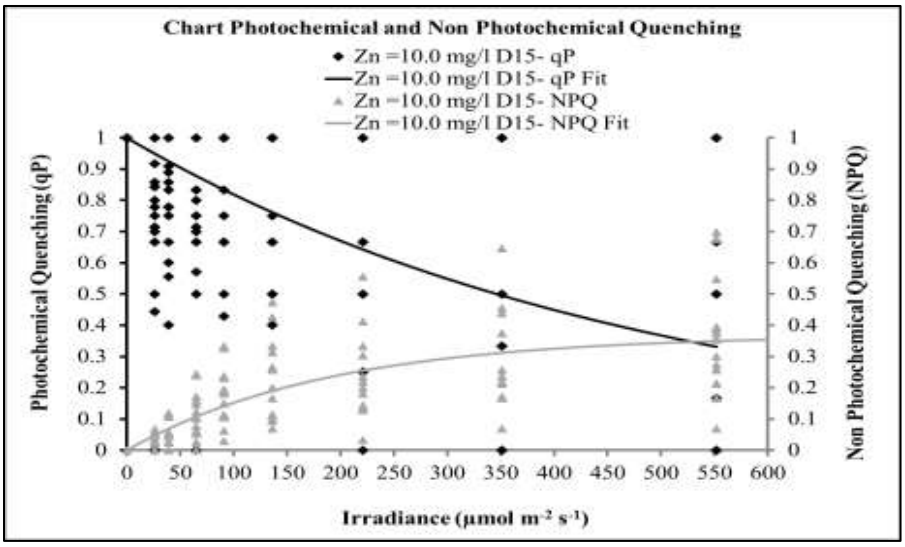


Figure 4.39 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn on day 15.

Photosynthesis parameters of *C. demersum* under 10.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.22 ± 0.02 and electron transport rate (ETR_{max}) is 5.31 ± 0.83 µmol e^- m^{-2} s^{-1} on day 15 (Figure 4.38).

Figure 4.39 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn treatment on day 15. The half point of Photochemical Quenching (qP) is 346.49 ± 93.41 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 130.76 ± 53.19 µmol photon m^{-2} s^{-1} on day 15 (Figure 4.39). These plants are very close to death as a result of the exposure to Zn and show abnormal qP & NPQ. Least square fitting of PAM curves on moribund plant material is difficult.

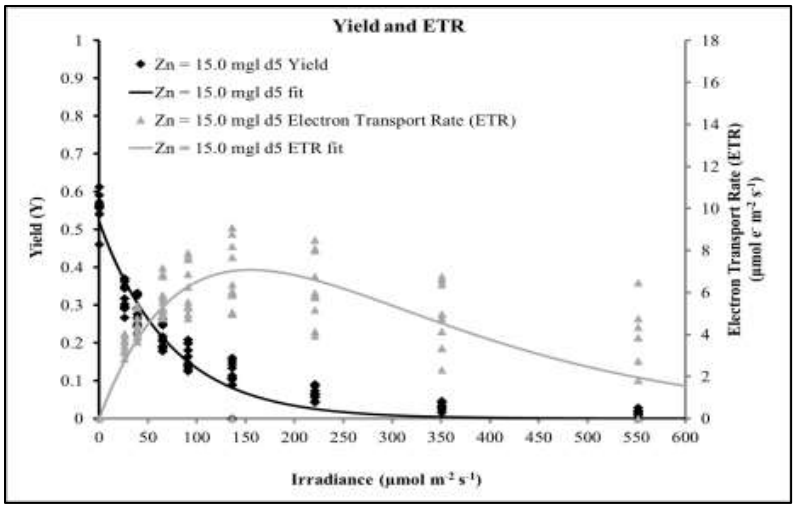


Figure 4.40 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 15.0 mg/l Zn on day 5.

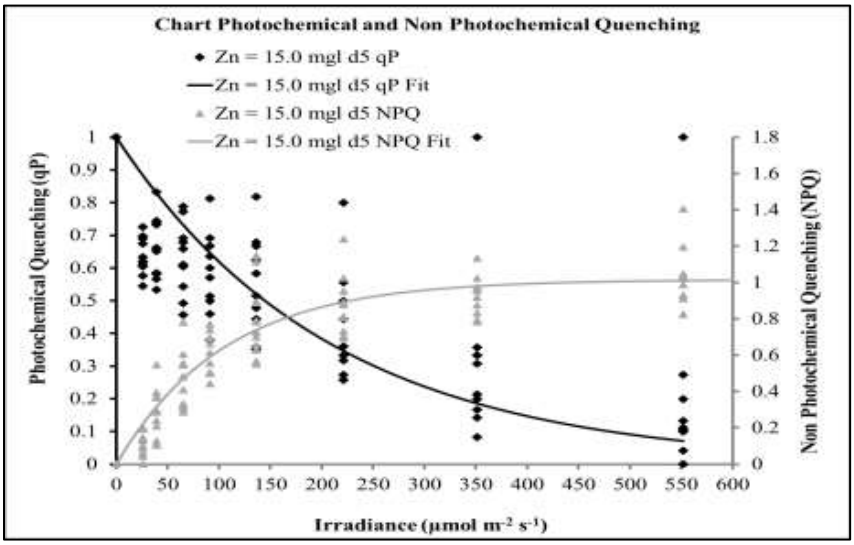


Figure 4.41 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 15.0 mg/l Zn on day 5.

Photosynthesis parameter of *Ceratophyllum demersum* under 15.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.52 ± 0.02 and electron transport rate (ETR_{max}) is 7.80 ± 0.44 µmol e^- m^{-2} s^{-1} on day 5 (Figure 4.40).

Figure 4.41 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 15.0 mg/l Zn treatment on day 5. The half point of Photochemical Quenching (qP) is 144.68 ± 24.12 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 72.50 ± 12.10 µmol photon m^{-2} s^{-1} on day 5 (Figure 4.41). As in the previous figure (4.39) these plants show signs of Zn stress (abnormal qP and NPQ) and low ETR.

The results showed that toxic effect of Zn as $ZnSO_4$ on *C. demersum* appeared to increase when Zn was increased to high concentrations (10.0 mg/l and 15.0 mg/l) after 10 days of the experiment. This study showed that *C. demersum* could tolerate Znmore than Cu after 15 days of the experiment and responses to Zn were slower than for Cu. The maximum concentration of Zn that *C. demersoum* could tolerate for the full course of the experiment was 10.0 mg/l Zn. 15 mg/l Zn was toxic after 5 days.

4.5.4 Copper plus Zinc Combinational Effects

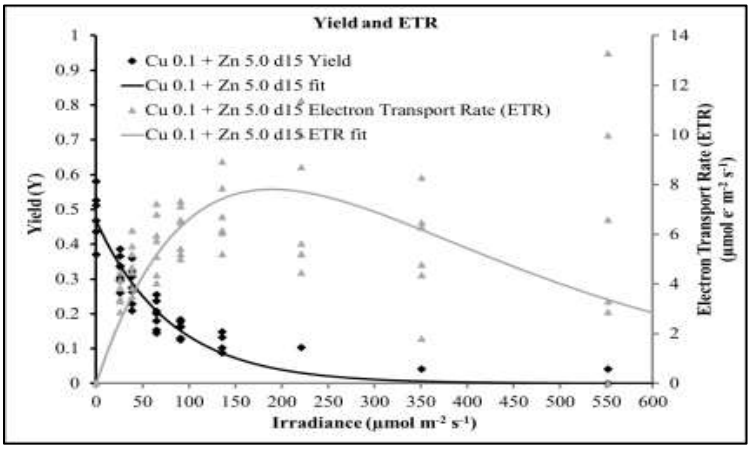


Figure 4.42 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate(ETR), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectivelyon day 15.

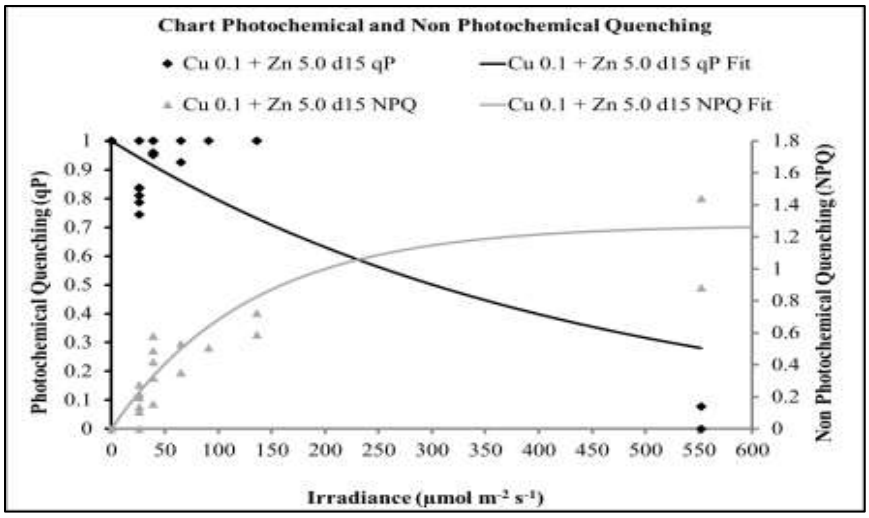


Figure 4.43 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 15.

Photosynthesis parameter of *C. demersum* under 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.47 ± 0.03 and electron transport rate (ETR_{max}) is 7.81 ± 0.84 µmol e^- m^{-2} s^{-1} on day 15 (Figure 4.42).

Figure 4.43 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 15. The half point of Photochemical Quenching (qP) is 301.19 ± 123.69 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 90.24 ± 25.66 µmol photon m^{-2} s^{-1} on day 15 (Figure 4.43). Acute metal stress is evident from the very poor qP results and NPQ. Only a few of the qP and NPQ calculations were valid calculations. A qP value greater than 1 is spurious and the Walz software flags spurious NPQ values. Plants that are nearly dead give very aberrant PAM result

Table 4.1 Summary of PAM results (Time = 0, 5, 10 and 15 days, including the Appendix data)

Concentration (mg/l)	Experiment (Day)	Y_{max}	$Y_{1/2}$	E_{opt}	ETR_{max}	$k_{1/2}qP$	NPQ_{max}	$NPQ_{1/2}$
Control	**0**	0.57±0.02	64.4±4.97	278 ± 19	13.4±0.5	110±9	0.88±0.05	66.9±9.7
	5	0.61±0.02	63.6±4.39	251 ±15	23.6±0.5	92±6	0.95±0.04	69.2±8.7
	10	0.57±0.02	65.0±5.12	278 ± 19	13.8±0.5	104±20	0.87±0.05	68.4±9.9
	15	0.49±0.02	78.0±7.98	312±26	14.5±0.7	252±50	0.76±0.05	63.5±12.3
Cu 0.05	**5**	0.60±0.01	62.3±3.48	238±10	12.7±0.3	90±5	0.89±0.03	68.9±6.5
	10	0.57±0.02	63.3±4.57	258±13	12.6±0.4	141±14	1.09±0.06	61.6±9.4
	15	0.28±0.03	109±31.8	422±107	12.8±1.7	438±121	0.47±0.08	49.8±21.1
Cu 0.1	**5**	0.62±0.02	67.0±3.99	249±13	14.6±0.5	102±7	0.96±0.04	68.0±7.9
	10	0.60±0.02	63.6±4.39	251±15	14.1±0.5	92±6	0.95±0.044	69.2±8.7
	15	0.57±0.02	51.1±3.98	230±18	8.9±0.4	104±10	1.04±0.06	66.5±9.7
Cu 0.15	**5**	0.58±0.03	75.3±7.64	257±22	14.5±0.7	142±16	0.90±0.08	78.9±18.0
	10	0.63±0.02	61.9±3.12	221±12	12.6±0.4	103±6	1.03±0.05	72.3±9.9
	15	0.55±0.02	66.9±4.28	241±14	12.2±04	118±8	0.87±0.0	67.6±11.4
Cu 0.20	**5**	0.57±0.03	65.7±6.67	228±25	12.1±0.6	92±6	0.94±0.4	69.2±8.7
	10	0.55±0.02	60.8±4.13	227±16	11.5±0.5	160±19	1.09±0.07	67.4±11.8
	15	0.52±0.06	74.4±18.90	322±80	15.7±2.0	127±22	0.79±0.11	67.4±25.2
Zn 1.0	**5**	0.61±0.01	77.5±4.64	280±14	15.4±0.4	98±6	0.79±0.04	88.6±12.7
	10	0.58±0.02	68.6±5.70	259±17	13.6±0.5	89±6	0.91±0.04	78.8±9.2
	15	0.59±0.02	62.3±4.30	242±13	13.0±0.4	87±6	0.88±0.05	77.7±11.9

Table 4.2 Summary of PAM results (Continued)

Concentration (mg/l)	Experiment (Day)	Y_{max}	$Y_{1/2}$	E_{opt}	ETR_{max}	$k_{1/2}qP$	NPQ_{max}	$NPQ_{1/2}$
Zn 5.0	**5**	0.60±0.02	51.2±4.0	223±17	10.46±0.50	83±10	0.85±0.08	68.3±16.8
	10	0.52±0.03	60.2±6.5	256±27	9.83±0.67	456±130	1.16±0.09	73.5±13.6
	15	0.52±0.02	60.2±6.4	240±26	10.18±0.70	455±130	1.16±0.08	73.7±13.8
Zn 10.0	**5**	0.50±0.02	64.2±6.6	221±26	9.40±0.68	135±12	0.82±0.06	84.3±15.8
	10	0.43±0.02	38.0±3.3	125±17	3.82±0.33	184±35	0.89±0.07	52.2±10.5
	15	0.22±0.02	80.4±14.9	5±1	5.31±0.3	347±93	0.37±0.07	130.0±53.2
Zn 15.0	**5**	0.52±0.02	50.8±4.3	155±14	7.08±0.44	145±24	1.02±0.06	72.5±12.1
Cu 0.1 + Zn 1.0	**5**	0.55±0.02	77.2±5.3	296±20	15.11±0.57	145±10	0.89±0.05	70.8±11
	10	0.53±0.03	61.2±7.4	210±21	10.56±0.69	152±19	0.98±0.08	69.4±14.5
	15	0.38±003	48.3±8.5	166±25	5.63±0.60	195±43	0.67±0.1	68.5±25.8
Cu 0.1 + Zn 5.0	**5**	0.41±0.02	54.9±6.4	219±26	8.00±0.59	309±51	0.77±0.07	58.8±16.5
	10	0.54±0.03	55.7±7.6	151±26	10.32±1.44	393±181	1.21±0.21	72.6±33.5
	15	0.47±0.03	56.0±8.7	176±34	8.22±1.02	301±124	1.19±0.16	68.1±19.1
Cu 0.05 + Zn 1.0	**5**	0.58±0.02	50.0±3.56	225±24	10.04±0.65	132±16	1.11±0.06	62.3±9.7
	10	0.52±0.02	52.2±5.0	209±22	10.02±0.66	260±44	0.96±0.04	68.0±7.9
	15	0.50±0.01	46.0±2.8	202±30	8.07±0.82	144±27	1.39±0.09	64.0±11.9
Cu 0.05 +Zn 5.0	**5**	0.53±0.01	59.1±3.5	205±11	10.12±0.32	113±7	0.77±0.04	56.5±8.5
	10	0.47±0.02	61.9±4.8	233±19	9.32±0.46	243±200	0.88±0.05	64.5±10.4
	15	0.38±0.03	48.1±8.7	165±25	6.02±0.65	195±43	0.67±0.1	70.9±27.3

Units of Fitted parameters:

Y_{max} - dimensionless, **$Y_{1/2}$** – µmol photon m^{-2} s^{-1}, **E_{opt} -** µmol photon m^{-2} s^{-1},

ETR_{max} - µmol e^{-} m^{-2} s^{-1}, **$k_{1/2}qP$ -** µmol photon m^{-2} s^{-1},

NPQ_{max} – dimensionless, **$NPQ_{1/2}$ -** µmol photon m^{-2} s^{-1}

CHAPTER 5
DISCUSSION

This chapter describes the effectiveness of *C. demersum* on Cu, Zn and Cu + Zn treatment. During the experiment, the pH of Cu, Zn and Cu + Zn treatment contaminated water was around 7.0. The temperature was between 23 - 25 ^{o}C.

5.1 Effects of heavy metals on growth

Zinc and copper are well known for their toxic effects on plant growth. *C. demersum* is fairly sensitive to Cu (0.05 and 0.20 mg/l). The relative growth rate (RGR) of shoot length of *C. demersum* exposed to copper showed that there was no significant difference in every concentration ($P < 0.05$). The relative growth rate (RGR) of wet weight of the plants exposed to copper indicated that RGRs were significantly decreased ($P < 0.05$) when copper was added to 0.05, 0.1, 0.15 and 0.2 mg/l (Figure 4.2). After 15 days of the experiment, RGRs of control (2.32 ± 0.07 per day) was higher than other concentrations ($P < 0.05$). At the end of the experiment, RGRs of wet weight were found 0.72 ± 0.12 per day. Shoot length hence was not a very good indicator of toxicity but biomass (wet weight) was sensive to Cu.

The relative growth rate (RGR) of the shoot length of *C. demersum* exposed to zinc showed that there was no significant difference in every concentration ($P < 0.05$) at day 10 and day 15 of the experiment. The highest RGR of the shoot length was found in Zn 1.0 mg/l (2.41± 0.04 per day) after 15 days. The lowest RGR of the shoot length of *C. demersum* was found (1.81 ± 0.05 per day) in Zn (10.0 mg/l) after 15 days.

The relative growth rate (RGR) of the wet weight of *C. demersum* exposed to zinc showed that there was no significant difference among concentrations ($P < 0.05$) on day 15. The highest RGR was found in Zn 1.0 mg/l (1.70 ± 0.15 per day) of day 15. The

lowest RGR was found in Zn 10.0 mg/l (0.13 ± 0.11 per day) at day. In addition, plants died after 7 days when Zn was increased to 15.0 mg/l. Neither shoot length nor wet weights were good indicators of Zn toxicity even though plants died as a result of Zn poisoning.

The relative growth rate (RGR) of shoot length of *C. demersum* exposed to Cu + Zn showed that there were significant difference among concentrations ($P < 0.05$) at day 5, day 10, and day 15 of the experiment. The highest RGR was found in Cu 0.05 mg/l + Zn 1.0 mg/l (2.56 ± 0.05 per day) after 15 days. The lowest RGR was found in Cu 0.1 mg/l + Zn 1.0 mg/l (1.73 ± 0.09 per day) after 15 days.

The relative growth rate (RGR) of the wet weight of *C. demersum* exposed to Cu + Zn showed that there was no significant difference in every concentration ($P < 0.05$) on day 5 and day 10 of the experiment. In addition, the highest RGR was found in control (2.32 ± 0.07 per day) after 15 days.

In Coontail, the lowest RGR was found in Cu 0.1 mg/l + Zn 1.0 mg/l (-0.01± 0.11 per day) after 15 days. An analysis of the underlying mechanism led to the conclusion that the growth of barley was controlled principally by the amount of plant-available zinc, which depended on the amounts of both added zinc and added copper (Luo & Rimmer, 1995). The effect of the added copper was to increase the toxicity of the added zinc (Luo & Rimmer, 1995). Excess Cu inhibited both frond growth and frond multiplication of the fern *L. paucicostata* (Nasu *et al*. 1984) and it decreased the content of chlorophyll *a* and photosynthetic CO_2 uptake in the freshwater aquatic *Lemna minor* (Filbin & Hough, 1979). Zn was is more toxic than Al to duckweed (*Lemna minor*) for the concentrations applied (Radić, Babić, Škobić, Roje, & Pevalek-Kozlina, 2010). Radić *et al*. (2010) showed that dry matter content significantly increased in response to Zn and Al higher metal concentrations, especially 0.3 mM Zn, probably reflecting high amounts of these metals in duckweed (*Lemna minor*) plants. Excess Cu inhibited both frond growth and leaf multiplication of L*emna paucicostata* (Nasu *et al*. 1984). Copper at 0.2 mg/l promoted the growth of *Lemna minor* leaves(Kanoun-Boulé *et al*., 2009; Khellaf & Zerdaoui, 2009).

5.2 Heavy metals accumulation

In this study, *Ceratophyllum demersum* could accumulate Cu (0.05 mg/l) after 15-day in Cu treatment. At high concentrations Zn (10.0 mg/l) in Zn treatment, *C. demersum* could accumulate a BCF of 16041 Zn (10.0 mg/l) after 15 – day. Cu and Zn uptake by *C. demersum* after 15 days in Cu + Zn treatment, Cu was found in *C. demersum* (BCF = 23039) in Cu 0.1 mg/l + Zn 5.0 mg/l. In addition, the highest BCF of Zn was found in *C. demersum* (BCF = 6359) in 0.1 mg/l Cu + 5.0 mg/l Zn on day 15. Mishra and Tripathi (2009) showed the BCF value of 300 was estimated in duckweed *Spirodela polyrrhiza* after 15-day of exposure to 5.0 mg/l Zn and so Coontail had a much higher BCF factor.

5.3 Toxicity of heavy metals in plants

Ivanova, Kholodova, & Kuznetsov (2010) found that copper was considerably more toxic than zinc in *Brassica napus.*Transfer of copper and zinc taken up by the roots was different. Zinc was strongly taken up by the roots but had little effect on transfer of copper to the rest of the plant (Ivanova *et al*., 2010). High zinc concentrations helped copper uptake by the roots but reduced its transfer to the aboveground organs of the plant (*Brassica napus*) (Ivanova *et al.*, 2010). $CdCl_2$ significantly reduced most of the studied growth parameters (shoot length, number of roots and leaves, and fresh weight) for both *S. nigrum* and *S. lycopersicum* (Al Khateeb & Al-Qwasemeh, 2014). *Solanum nigrum* exhibited higher tolerance than *Solanum lycopersicum* for all types of stress (Al Khateeb & Al-Qwasemeh, 2014). In addition, a higher accumulation rate of $CdCl_2$was observed in the cropwild relative (*Solanum nigrum*) than in cultivated *S. lycopersicum* (Al Khateeb & Al-Qwasemeh, 2014). These are all the typical, well documented effects of Cadmium on plants (Clemens, 2006). In the case of Coontail it has no roots and so it does not have the benefit of the discriminatory selective transport of metals from roots to shoots found in rooted plants. The rooted plants described above took up Zn by the roots but did not transfer it to the rest of the plant because of selective loading of metals into the xylem

stream at the rootss cortex. The cells of the Coontail take up Cu and Zn from the water column.

5.4 Effects of heavy metal on chlorophyll content and photosynthesis rate

Chlorophyll *a* content of *Ceratophyllum demersum* exposed to copper at various concentration showed that there was no significant difference among concentrations ($P < 0.05$) on day 10 of the experiment. The highest chlorophyll *a* content was found in Cu 0.05 mg/l (694.37 ± 57.27 µg/g) of fresh weight after 15 days. Chlorophyll *b* content of *C. demersum* exposed to copper showed that there was significant difference among concentrations ($P < 0.05$) on day 10 of the experiment. The highest chlorophyll *b* content was found in Cu 0.15 mg/l (154.76 ± 16.91 µg/g) after 15 days. The chlorophyll ratio of *C. demersum* exposed to copper showed that there were significant difference in every concentration ($P < 0.05$) on day 5, day 10 and day 15 of the experiment. The chlorophyll ratio found in Cu 0.20 mg/l was 0.27 ± 0.02 µg/g after 15 days.

No evidence of the formation of Cu-Chlorophylls were found spectrophotometrically in Coontail (De Philippis 1979; Küpper *et al*., 2000; Küpper *et al*., 2002; Hall 2002; Küpper *et al*., 2003; Kanoun-Boulé *et al*., 2009). Cu-chlorophylls have peaks at Cu-Chl *a* = 650 nm and Cu-Chl *b* = 628 nm in acetone and would also have been easily detected (Küpper*et al*., 2000, 2002, 2003). In the alga *Scenedesmus* severe copper toxicity did result in significant accumulation of CuChl *a* and CuChl *b* and spectrophotometric peaks due to these compounds. Cu chlorophylls were not obvious in scans of solvent extracts of Coontail (*Lemna minor:*Kanoun-Boulé *et al*., 2009).

There were lethal and sublethal effects of exposure to Zinc in *C. demersum* and effects on chlorophyll content. The results showed that there was no significant difference in every concentration ($P < 0.05$) on day 10 and day 15 of the experiment. Chlorophyll *a* content was found in (707.93 ± 52.82 µg/g) after 15 days. However, 15.0 mg/l of Zn was toxic after 10 and 15 days. Sublethal concentrations of Zn (5.0 and 10.0 mg/l) led to an increase in Chl *a* over time. The chlorophyll *b* content of *C. demersum*

exposed to zinc was examined. As for Chl *a* (Fig 4.11) there were lethal effects for 15.0 mg/l Zn after 10 and 15 days. The results showed that there were no significant difference in Chl *b* at every concentration ($P < 0.05$) on day 10 and day 15 of the experiment. The highest chlorophyll *b* content was found in Zn 10.0 mg/l (226.34 ± 16.31 µg/g) at day 15. The chlorophyll ratio of *C. demersum* exposed to zinc showed that there was no significant difference in every concentration ($P < 0.05$) on day 10 and day 15 of the experiment. The chlorophyll found in Zn 10.0 mg/l was 0.32 ± 0.01 µg/g) at day 15. As for the Chl *a* and Chl *b* results above, there is no Chl *b/a* ratio data for plants exposed to 15.0 mg/l Zn after 10 and 15 days because the plants had died. Cherif *et al.* (2011) working on tomato plants showed that high Zn decreased Chl *a* and Chl *b* but did not appreciably affect Chl *b/a* ratio.In their study of metal ore waste dumps (Trzcińska & Pawlik-Showrońska, 2008) found that high Zn inhibited Chlorophyll content of soil algae. These are similar results to those found in the present study.

In the present study, no evidence of the formation of Zn-chlorophylls were found spectrophotometrically in Coontail (De Philippis 1979; Hall 2002**;** Ikegami *et al*. 2005; Mikulic& Beardall, 2014). Zn-chlorophylls would have been easily detected because it has a peak at 654 nm (Ngo & Zhao, 2007). Ikegami *et al.* (2005) found that *Chlorella* did synthesise Zn-chlorophyllsand incorporate them into chlorophyll-protein complexes but only under heterotrophic conditions in the dark and at Zn concentrations that were lethal when the cells were grown phototrophically.

Chlorophyll *a* content of *C. demersum* exposed to Cu + Zn showed that there was no significant difference in every concentration ($P < 0.05$) on day 5 and day 10 of the experiment. If there had been substantial Zn or Cu substitution for Mg in the chlorophylls of Coontail large changes in Chl *a*&*b* and Chl *b/a* ration would have been found (De Philippis 1979, Hall 2002, Mikulic & Beardall 2014). No spectroscopic evidence was found for either Cu or Zn chalorophyll in the Cu + Zn treated plants (see above). The control had much higher Chl *a* than any other treatments at day 15 and much high than the controls at day 5 and 10. The combination of high Cu + Zn was strongly inhibitory after 15 days. Chlorophyll *b* content of *C. demersum* to copper plus zinc showed that there was

significant difference in every concentration ($P < 0.05$) on day 5, day 10, and day 15 of the experiment. The highest chlorophyll *b* content was found in control (147.19 ± 9.93 µg/g) after 15 days.

As in the case of Chl *a* (Fig. 4.14) the highest Cu + Zn inhibited Chl *b* content but this was most apparent at day 15. Content of ratio chlorophyll of *C. demersum* exposed to zinc showed that there was significant difference in every concentration ($P < 0.05$) at day 5, day 10 and day 15 of the experiment. The highest content of ratio chlorophyll was found in Cu 0.1 mg/l + Zn 5.0 mg/l (0.41 ± 0.01 µg/g) at day 15 and high Cu + Zn tended to consistently increased the Chl *b/a* ratio at day 5, 10 and 15. The Chl *b/a* ratio of the controls was very consistent. This indicates that Cu and Zn did not have large differential effects on Chl *a* vs. Chl *b* synthesis.

E. crassipes (Water Hyacinth) has been reported that has strong capacity to remove Cu^{2+} from Cu-contaminated water and also accumulates other metals through uptake from aquatic media (Chua, 1998; Soltan & Rashed, 2003; So *et al*., 2003). However, *E. crassipes* is a notorious aquatic weed pest which discourages it from being used for bioremediation. The results of this study illustrates that *C. demersum* accumulates copper as much as 270 mg/kg dry weight in plant tissues in copper treatment, and 285.16 mg/kg dry weight in plant tissues in copper + zinc treatment when it was treated with 0.20 mg/l and 0.1 mg/l, Cu + 5.0 mg/l, zincfor 15 days. The bioconcentration factors in water hyacinth exceed 2000 mg/kg for Cu, Pb, Zn and Cd (with the exception of Zn and Cd at pH 6)(Smolyakov, 2012). This study indicated that the water hyacinth can be successfully used for fast removal of metals in the initial stage of water body remediation (Smolyakov, 2012). However, water hyacinth is a notorious invasive aquatic weed which limits it attractively for bioremediation making Coontail a more attractive bioremediation proposition.

5.4.1 Chloroplast Microscopy

There were clear changes in the size of chloroplasts in the cell when *Ceratophyllum demersum*was treated with Cu, Zn or Cu + Zn in combination. Moreover, the size of vacuoles and intercellular spacewere shown clearly by this method and were

sensitive to exposure to heavy metal. Microscopy can be used to demonstrate clear toxicity effects and conformed the growth and PAM studies which both showed that responses to Ca and Zn in *Ceratophyllum demersum* took about 10 to 15 days to manifest themselves. Fragmentation of chloroplasts and flattening into disks was commonly observed in the present study as a consequence of metal toxicity but it was not obvious that Cu and Zn had different effects but it was apparent that Cu + Zn was more toxic than Cu and Zn separately. In the present study, simple light microscopy was used. A fluorescence microscope setup to more clearly visualize chloroplasts and measure chlorophyll fluorescence would have been an advantage.

5.4.2 Photosynthesis of *Ceratophyllum demersum* response to copper, zinc and their combination treatment

The optimum irradiance for the Coontail plants was about 200 µmol photon m^{-2} s^{-1}, which is close to the light intensities under which the plants were grown in the laboratory (Table 4.2). This irradiance is very similar to the irradiance under which they were grown (see methods). The time required for significant effects to appear is important. Usually at least 10 days were needed for significant effects to be observable as effects on Yield, Optimum irradiance (E_{opt}) and ETR_{max}. Physiological stress was generally indicated by changes in E_{opt} and ETR_{max} (Figs 4.35 to 4.42, Appendix Figures and Table 4.2). Metal toxicity does not simply reduce the photosynthetic rate it affects the shape of rapid light curves of photosynthesis (ETR) (Ralph & Gademann, 2005; Brestic & Zivcak, 2013) as a function of irradiance. Both the ETR_{max} and the Optimum irradiance (E_{opt}) both decreased. Thus, poisoned plant have both a lower E_{opt} and ETR_{max}. This effect on the shape of rapid light curves is not obvious if only Yield and Fv/Fm parameters are measured in PAM studies (Beer *et al*., 1998, Beer & Bjork, 2000, Silva & Santos, 2004, Mikulic & Beardall, 2014). Coontail responded to toxic levels of Cu, Zn and Cu+Zn slower than was expected (Figs 4.35 to 4.42 & Table 2). The time lag phenomenon was also observed in morphological studies under the microscope. Signs of stress were low Yield (Y_{max}) but this was less obvious than the changes in the shape of the photosynthesis vs irradiance curves. Stress tended to concommittently affect ETR_{max} and E_{opt}: both E_{opt} and ETR_{max} were

lowered under conditions of stress. Effectively this meant that the photosynthetic efficiency (α_0) which is the product of $E_{opt}\times ETR_{max}$ is lowered in stressed plants. Although Cu was more toxic than Zn on a molar basis, Cu + Zn was conspicuously more toxic than Cu and Zn administered separately.

Photochemical and Non-photochemical quenching are often cited as good indicators of physiological stress (Ralph & Gademann, 2005; Brestic & Zivcak, 2013) but in this study it was found that effects were only apparent on moribund material, in other words, effects were observable in plants that were obviously dying but chronic stress could not be readily detected. The plants were generally nearly dead before the effects on photochmical and non photoschemical quenching became apparent (Figs 4.35 to 4.42; Table 2 and the Appendix rapid light curves). Photochemical quenching at zero irradiance is unity and decreases exponentially as irradiance increases with a decay constant comparable to the declined in Yield with increasing irradiance (Figs 4.35 to 4.42; Table 2). In stressed plants the decay constant decreased which means that the point at which the qP decays by half increases (see Figs 4.35 to 4.42, Table 2). Fig 4.40 is a good example of this effect. In moribund material the Walz software often cannot calculate a valid qP value, hence the missing data points in Fig. 4.42. In Coontail the maximum NPQ value (NPQ_{max}) was close to 1.0 under most experimental conditions and in the controls. Cu, Zn and Cu+Zn did not give very consistent effects on NPQ_{max} (Table 2). It might be supposed that physiological stress would consistently increase the ½ saturation point for NPQ but this is not apparent from the results of this study (see Figs 4.35 to 4.42, Table 2). Raven *et al*. (1999) pointed out that both Cu and Zn had conspicuous redox effects and so affect electron transport by cytochromes of mitochondria and chloroplasts. It would have been expected that Cu and Zn would have had apparent effects on Quenching parameters (qP & NPQ) but in the present study such effects were only apparent under severe metal poisoning. Effects on qP and NPQ are not as good indicators of physiological stress as generally thought (Ralph & Gademann, 2005; Brestic & Zivcak, 2013).

CHAPTER 6
CONCLUSIONS

The screening experiment with 0.05 mg/l, of Cu, shows that *Ceratophyllum demersum* has the ability to remove Cu from contaminated water. In view of relative growth rate (RGR) and biococentration factor (BCF), Cu 0.05 mg/l inform that *C. demersum* seem healthy and can be good accumulator. In addition, in Zn treatment, relative growth rate shown better in 1.0 mg/l Zn compared to bioconcentration. However, bioconcentration of Zn concentration was lower than Cu concentration. Hence, *C. demersum* can be a good accumulator for both Cu and Zn. On the other hand, biococentration factor (BCF) of the combination of Cu and Zn showed that *C. demersum* could accumulate Cu higher than in Cu treatment and Zn treatment separately. Also the accumulation of Zn in *C. demersum* in the combination treatment was higher than accumulation in Zn treatment separately. Relative growth rate (RGR) and chlorophyll content showed that *C. demersum* in Cu or Zn treatment separately were better than in combination treatment. This means that the combination treatment had a higher toxic effect than Cu or Zn separately.

This study shows that *C. demersum* show toxic effect of exposure to the high concentrations of Cu, Zn and Cu + Zn but responses are rather slow – generally about 5 to 10 days are needed for symptoms to appear. The PAM data and chlorophyll data show different result because of the differences in what they measure. Together they show that can be used together to observe the toxicity stress of aquatic plant by heavy metals. *C. demersum* can be a good accumulator of Cu and Zn from contaminated water because they usually co-occur. Cu and Zn and Cu + Zn toxicity can be detected using PAM fluorometry however, it was found that noticeable effects took about 10 to 15 days to become apparent. This was surprising because arsenic effects on the aquatic plant *Wolffia arrhizal* were apparent within an hour or two using PAM technology (Ritchie Mekjinda, 2016). Yield

was not a very good indicator of photosynthetic stress either as maximum yield (Y_{max}) or the ½ maximum of rapid light curves. Toxic effects were most apparent in the E_{opt} and ETR_{max} of rapid light curves (Ralph & Gademann, 2005). Metal stress decreased both E_{opt} and ETR_{max} more of less proportionally. Photochemical and Non-photochemical quenching are often cited as good indicators of physiological stress (Ralph & Gademann, 2005; Brestic & Zivcak, 2013) but in this study it was found that effects were only apparent on moribund material.

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Appendix 1: Complete Set of PAM fluorometry Experiments on the response of Photosynthesis of *Ceratophyllum demersum* to copper, zinc and their combination treatments. The data sets here include measurements made at times 0, 5, 10 and 15 days.

A.1 Control

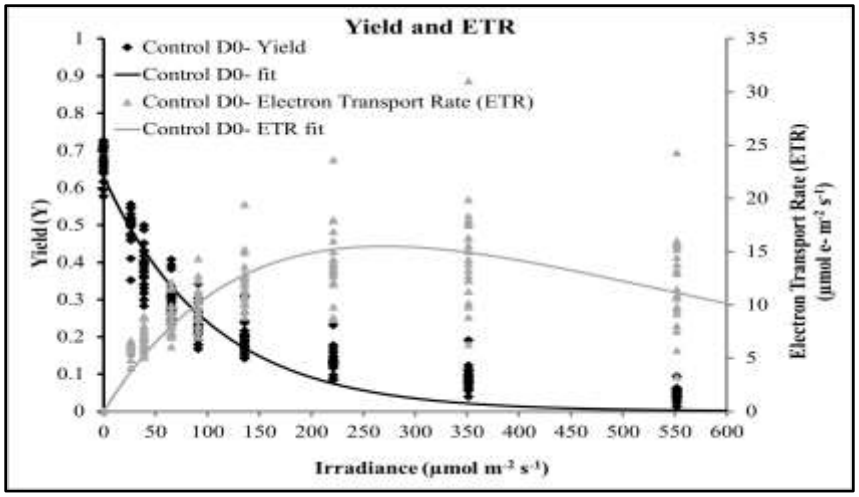

Figure A.1 Yield (Y) and irradiance (E) and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 0.

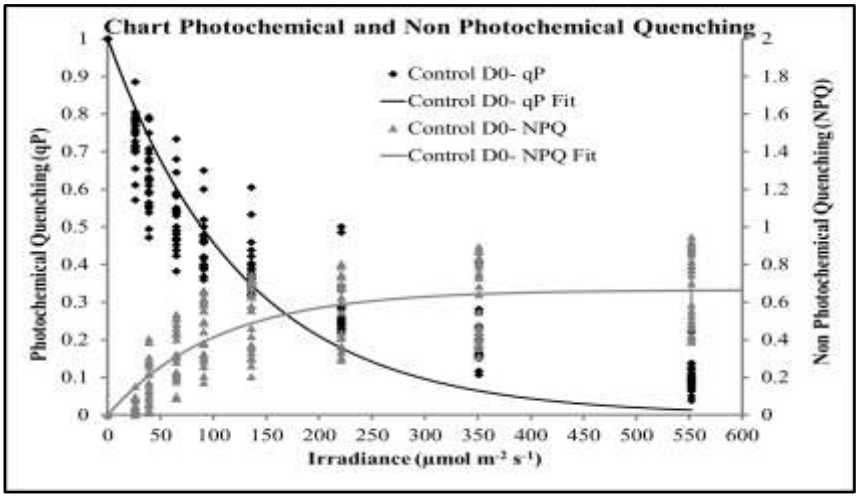

Figure A.2 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 0.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and maximum electron transport rate (ETR_{max}) is 13.43 ± 0.52 µmol e^- m^{-2} s^{-1} on day 0 of the experiment (Figure A.1).

Figure A.2Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 0. Half point of Photochemical Quenching (qP) is 110.24 ± 8.62 µmol photon m^{-2} s^{-1} and the half-point of Non Photochemical Quenching (NPQ) is 66.86 ± 9.73 µmol photon m^{-2} s^{-1} on day 0.

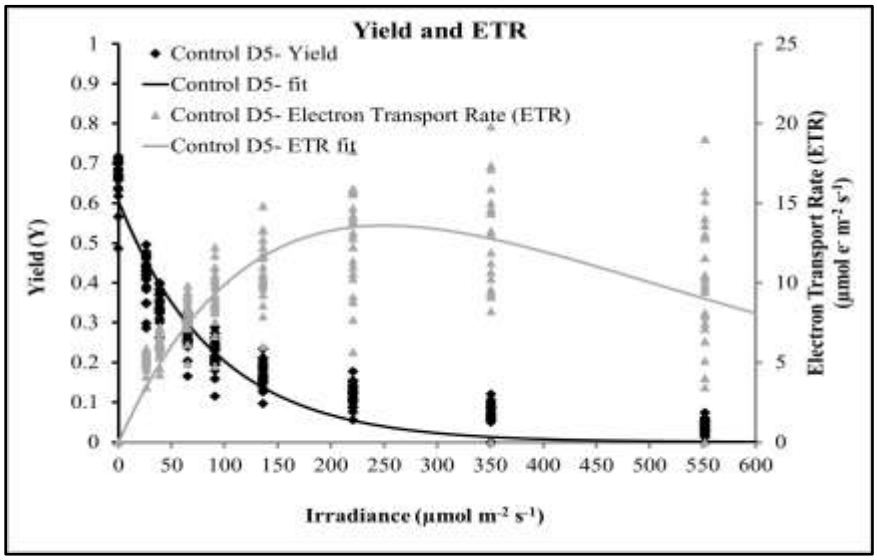

Figure A.3 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 5.

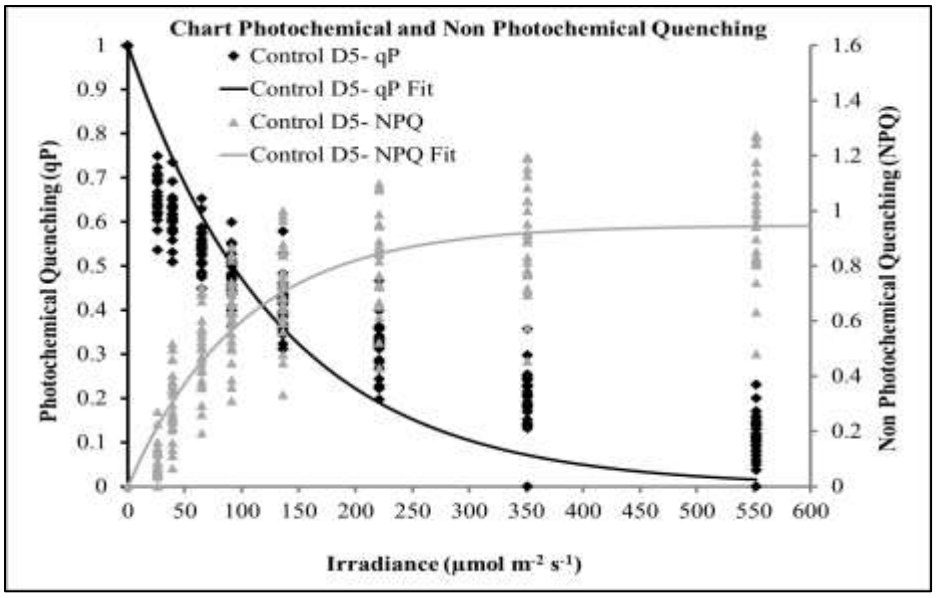

Figure A.4 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 5.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.61 ± 0.02 and electron transport rate (ETR_{max}) is 13.61 ± 0.50 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.3).

Figure A.4 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 5. The half point of Photochemical Quenching (qP) is 92.18 ± 5.62 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.15 ± 8.70 µmol photon m^{-2} s^{-1} on day 5.

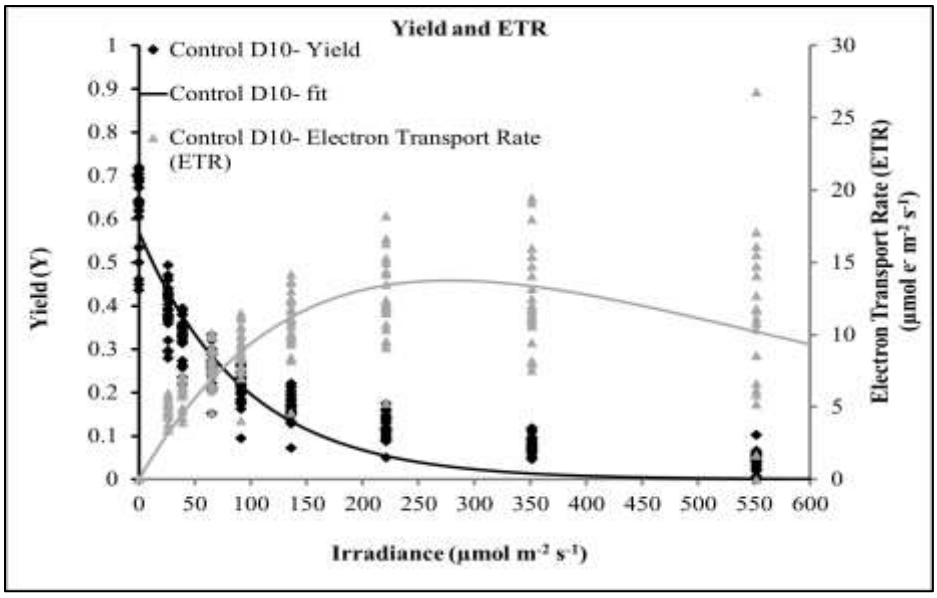

Figure A.5 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 10.

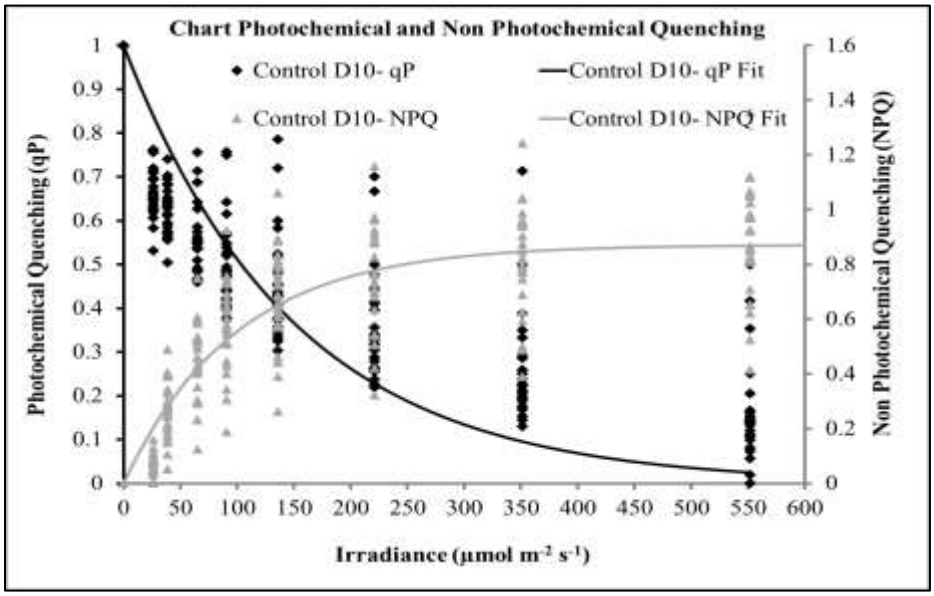

Figure A.6 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 10.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and electron transport rate (ETR_{max}) is 13.76 ± 0.54 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.5).

Figure A.6 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 10. The half pointof Photochemical Quenching (qP) is 103.57 ± 19.87µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 68.44 ± 9.88 µmol photon m^{-2} s^{-1} on day 10.

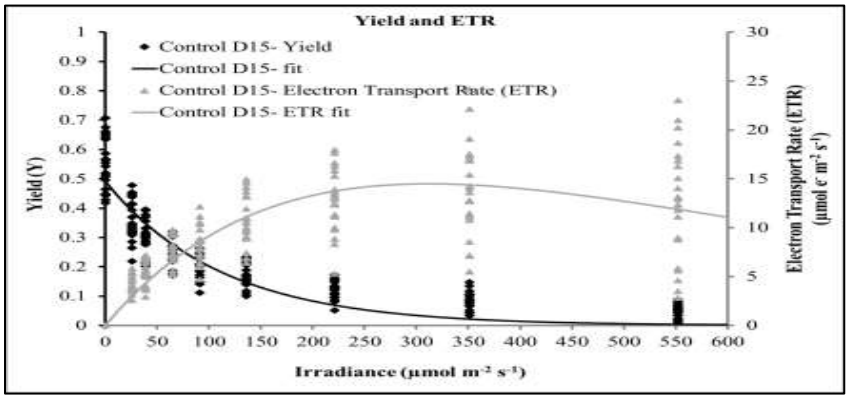

Figure A.7 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the control on day 15.

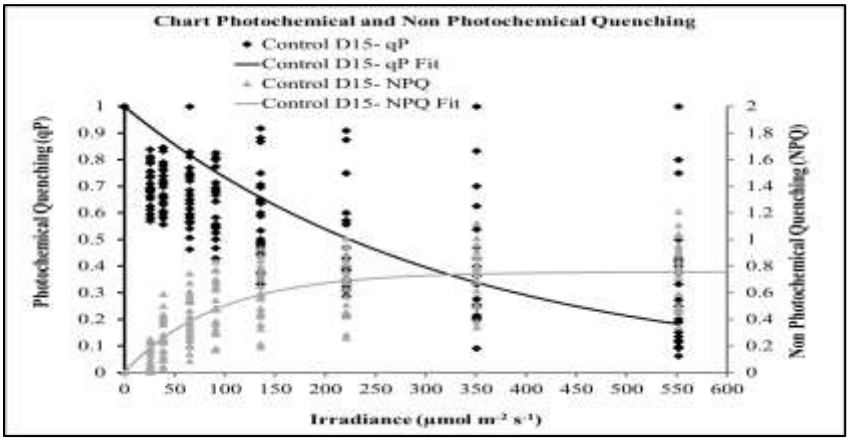

Figure A.8 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 15.

Photosynthesis parameters of *C. demersum* under control treatment including the maximum yield (Y_{max}) is 0.49 ± 0.02 and electron transport rate (ETR_{max}) is 14.49 ± 0.66 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.7).

Figure A.8 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the control on day 15. The half point of Photochemical Quenching (qP) is 224.45 ± 50.32 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 63.49 ± 12.32 µmol photon m^{-2} s^{-1} on day 15.

A.2 Copper Effects

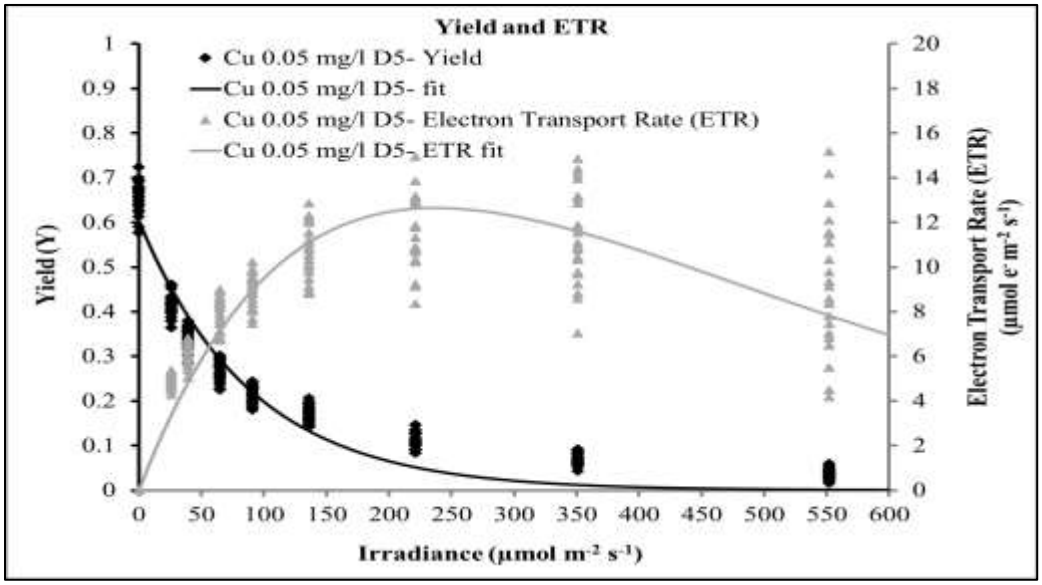

Figure A.9 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l Cu on day 5.

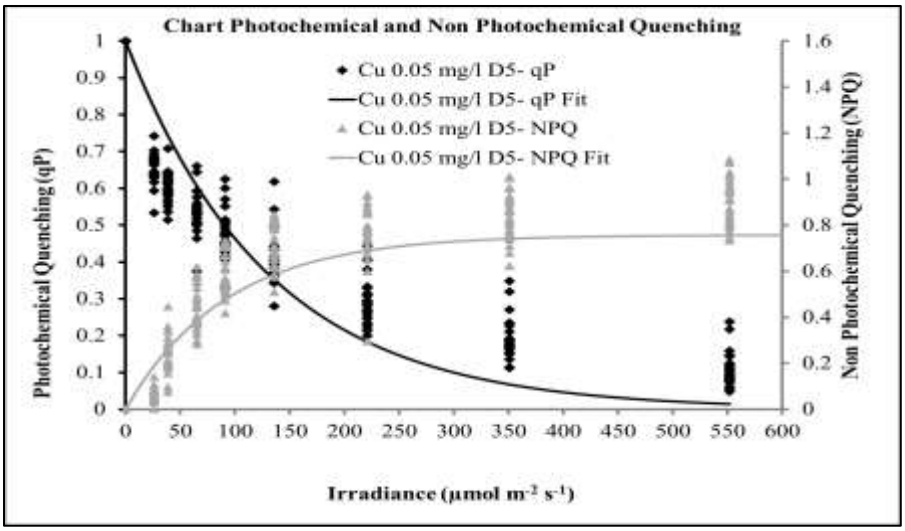

Figure A.10 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu on day 5.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.60 ± 0.01 and electron transport rate (ETR_{max}) is 12.65 ± 0.33 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.9).

Figure A.10 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu treatment on day 5. The half point of Photochemical Quenching (qP) is 90.25 ± 5.37 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 68.86 ± 6.51 µmol photon m^{-2} s^{-1} on day 5.

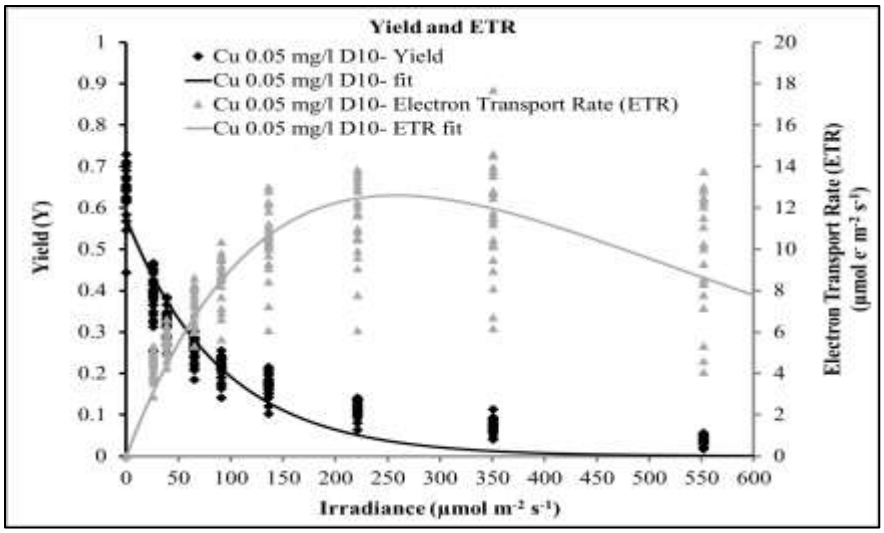

Figure A.11 Yield (Y), and irradiance (E) and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l Cu on day 10.

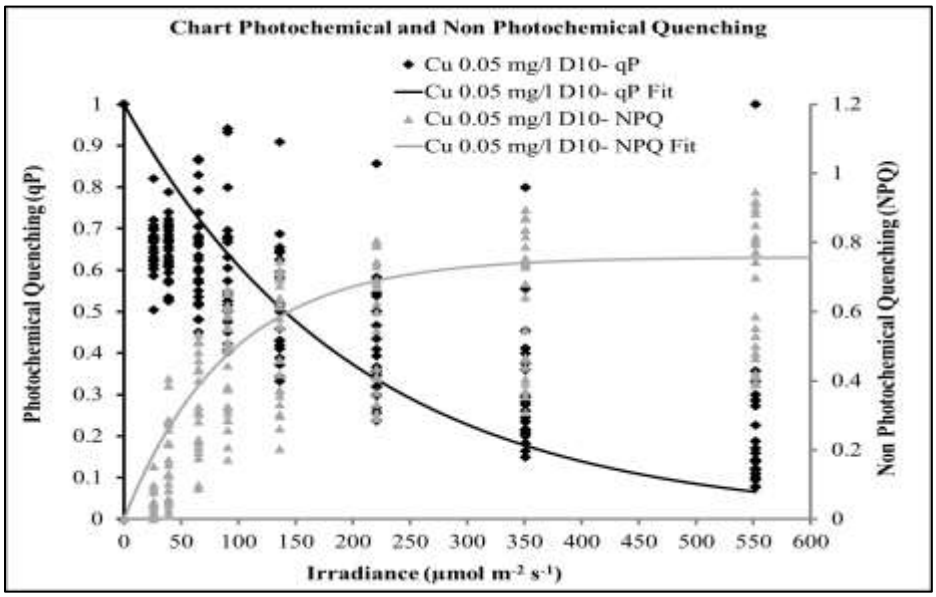

Figure A.12 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu on day 10.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.57 ± 0.01 and electron transport rate (ETR_{max}) is 12.61 ± 0.36 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.11).

Figure A.12 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu treatment on day 10. The half point of Photochemical Quenching (qP) is 140.67 ± 13.99 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 71.13 ± 14.31 µmol photon m^{-2} s^{-1} on day 10.

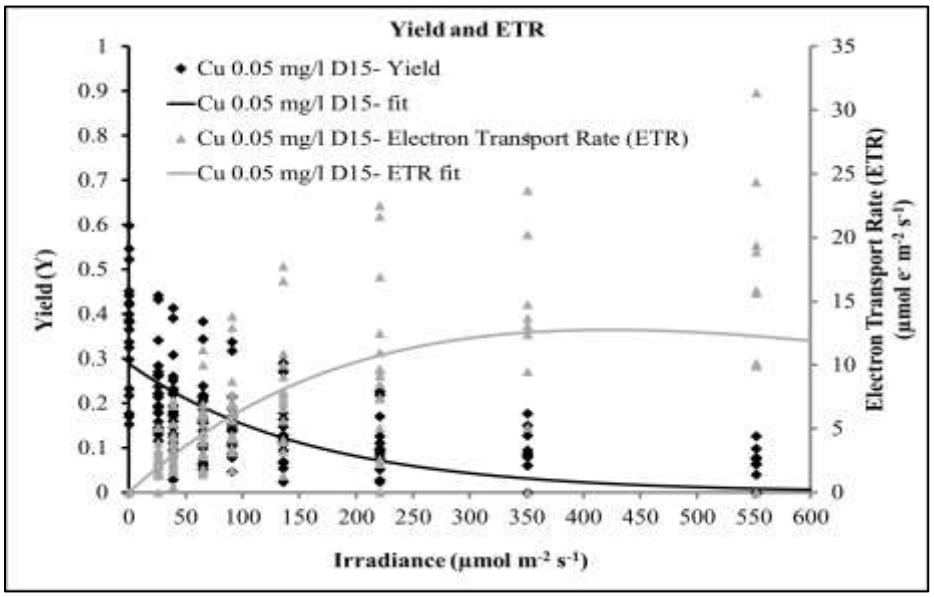

Figure A.13 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l Cu on day 15.

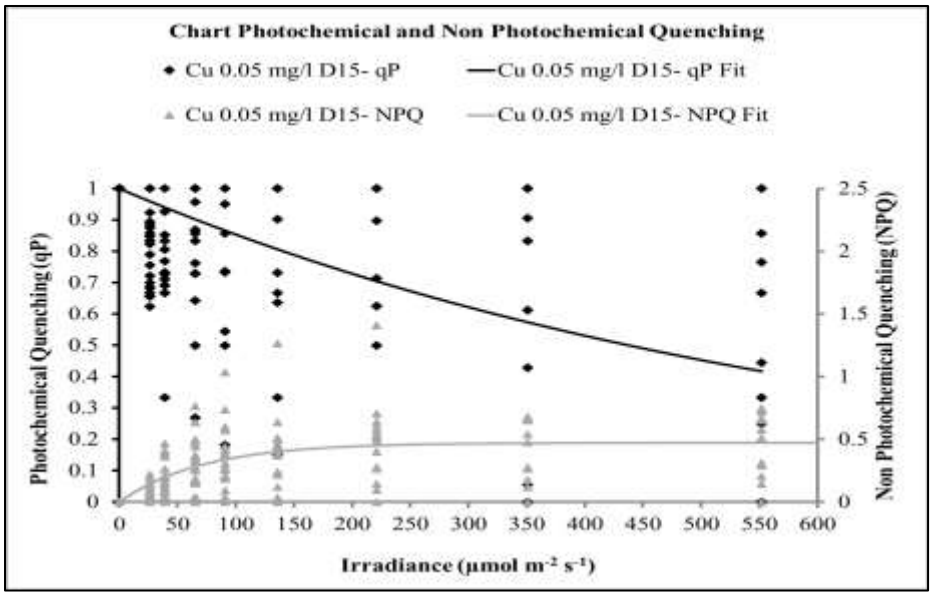

Figure A.14 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu on day 15.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.29 ± 0.03 and electron transport rate (ETR_{max}) is 12.77 ± 1.66 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.13).

Figure A.14 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l Cu treatment on day 15. The half point of Photochemical Quenching (qP) is 437.87 ± 121.42 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 49.77 ± 21.08 µmol photon m^{-2} s^{-1} on day 15. Yield, ETR, qP and NPQ show severe Cu toxicity effects after 15 d.

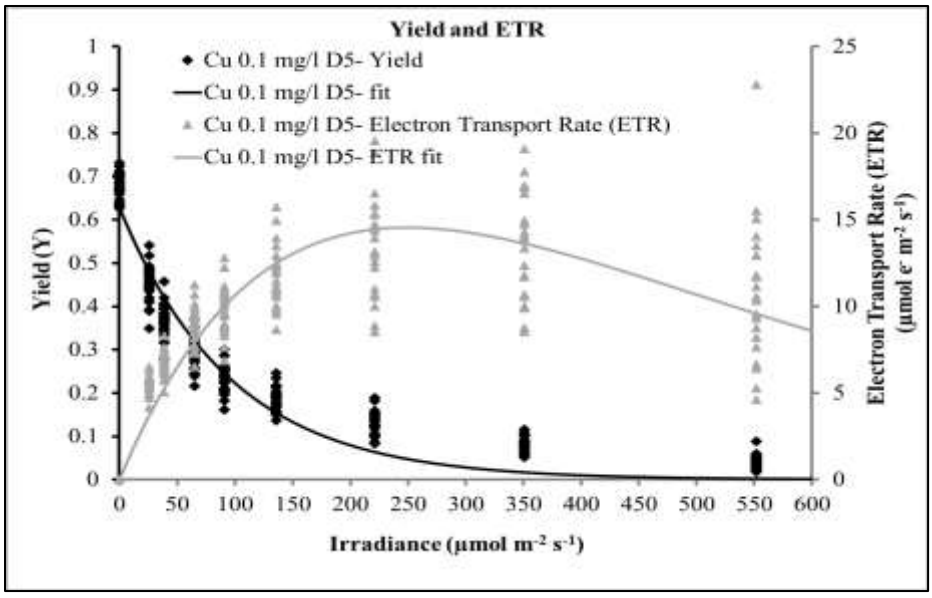

Figure A.15 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l Cu on day 5.

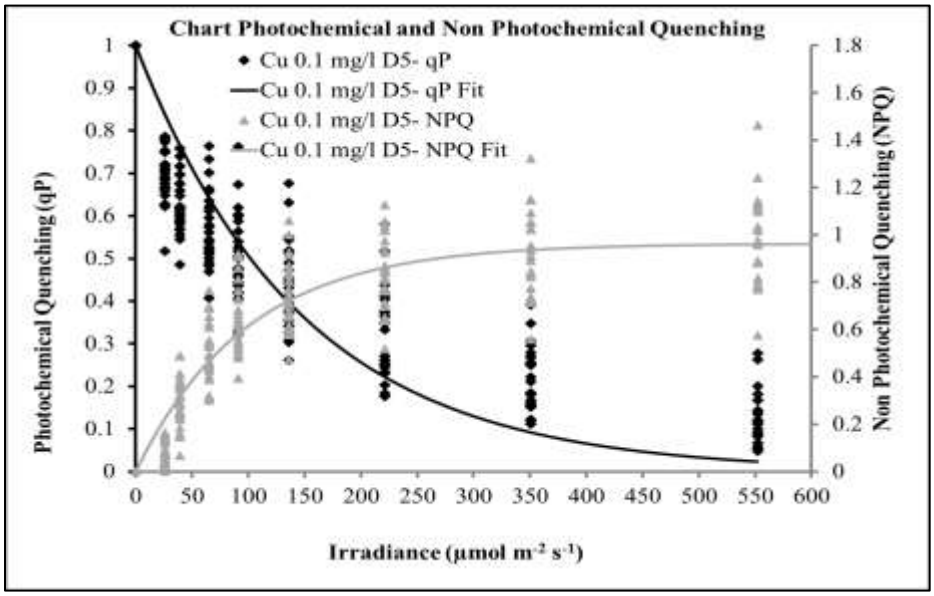

Figure A.16 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu on day 5.

Photosynthesis parameters of *C. demersum* under 0.1 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.63 ± 0.02 and electron transport rate (ETR_{max}) is 14.55 ± 0.46 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.15).

Figure A.16 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu treatment on day 5. The half point of Photochemical Quenching (qP) is 101.86 ± 6.66 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 67.89 ± 7.89 µmol photon m^{-2} s^{-1} on day 5.

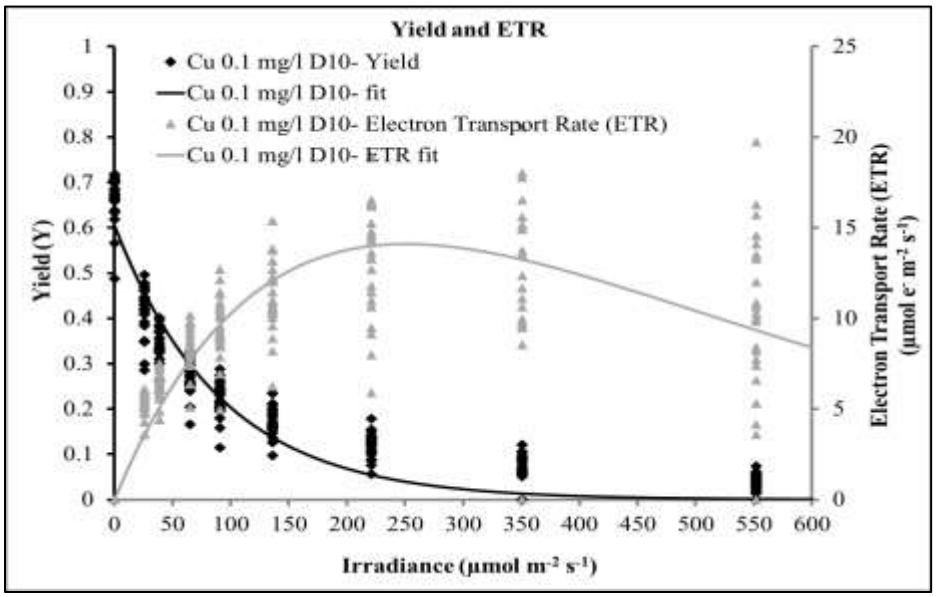

Figure A.17 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l Cu on day 10.

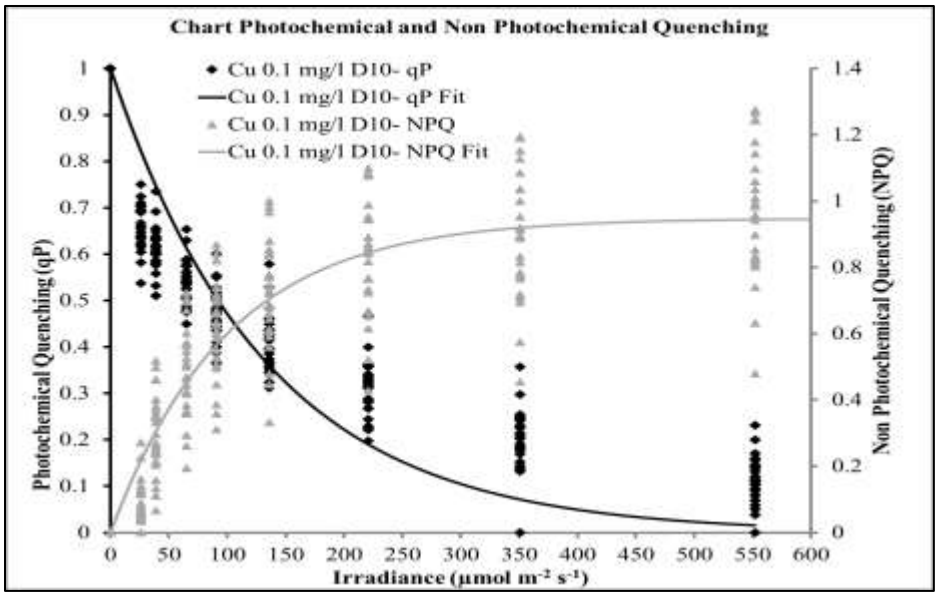

Figure A.18 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu on day 10.

Photosynthesis parameters of *C. demersum* under 0.1 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.61 ± 0.02 and electron transport rate (ETR_{max}) is 14.11 ± 0.52 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.17).

Figure A.18 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu treatment on day 10. The half point of Photochemical Quenching (qP) is 92.18 ± 5.62 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.15 ± 8.70 µmol photon m^{-2} s^{-1} on day 10.

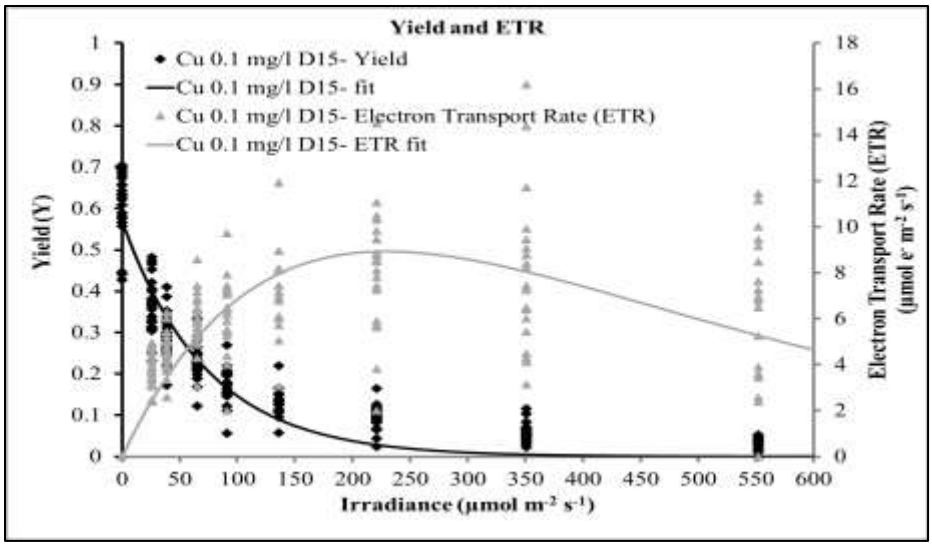

Figure A.19 Yield (Y), irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.10 mg/l Cu on day 15.

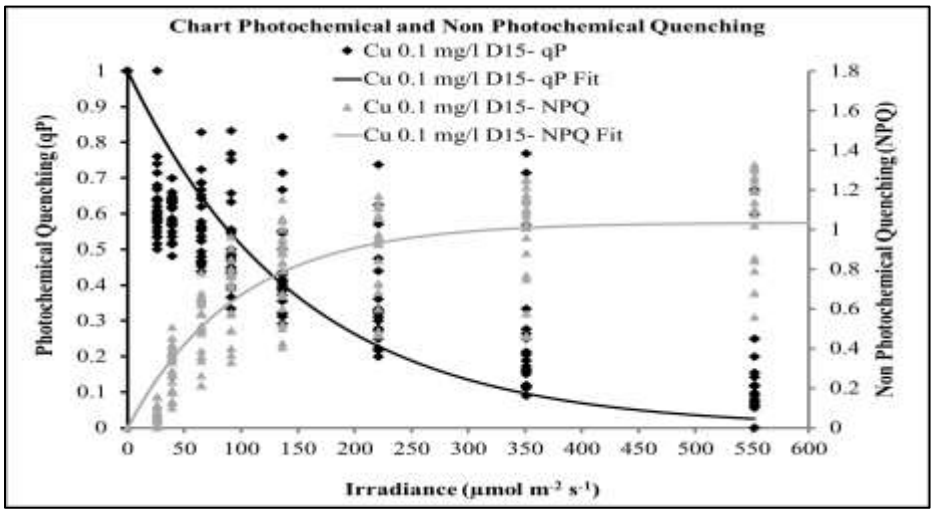

Figure A.20 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu on day 15.

Photosynthesis parameters of *C. demersum* under 0.1 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and electron transport rate (ETR_{max}) is 8.93 ± 0.42 µmol e^{-} m^{-2} s^{-1} on day 15 (Figure A.19).

Figure A.20 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l Cu treatment on day 15. The half point of Photochemical Quenching (qP) is 103.75 ± 10.16 µmol photon m^{-2} s^{-1} and the half point for Non Photochemical Quenching (NPQ) is 66.50 ± 9.69 µmol photon m^{-2} s^{-1} on day 15.

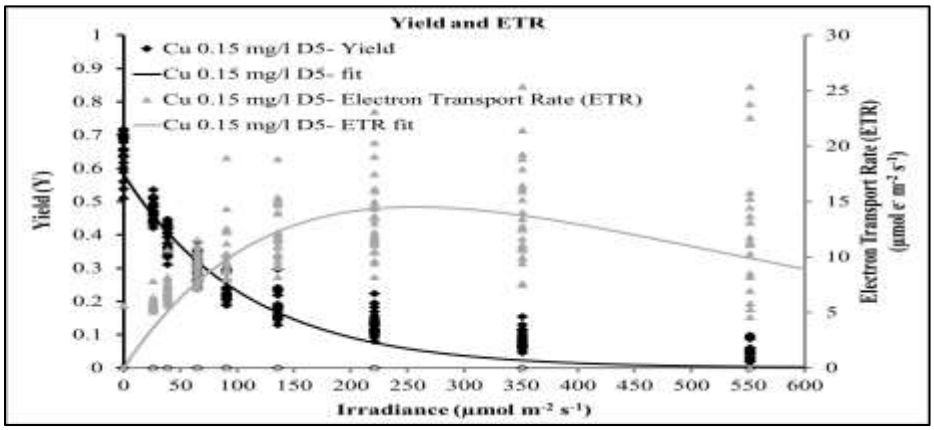

Figure A.21 Yield (Y), irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.15 mg/l Cu on day 5.

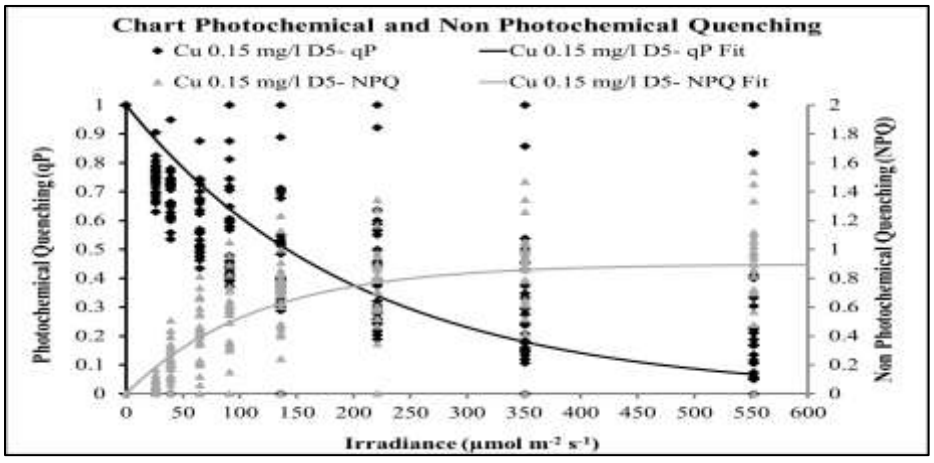

Figure A.22 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu on day 5.

Photosynthesis parameters of *C. demersum* under 0.15 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.58 ± 0.02 and electron transport rate (ETR_{max}) is 14.52 ± 0.74 µmol e^- m^{-2} s^{-1} on day 5 (Figure A21).

Figure A.22 shows Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu treatment on day 5. The half point of Photochemical Quenching (qP) is 141.73 ± 15.72 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 79.85 ± 17.97 µmol photon m^{-2} s^{-1} on day 5.

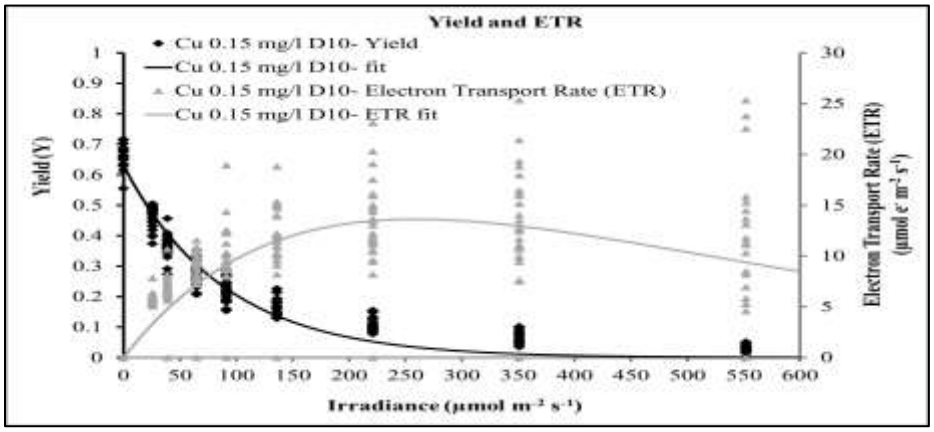

Figure A.23 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.15 mg/l Cu on day 10.

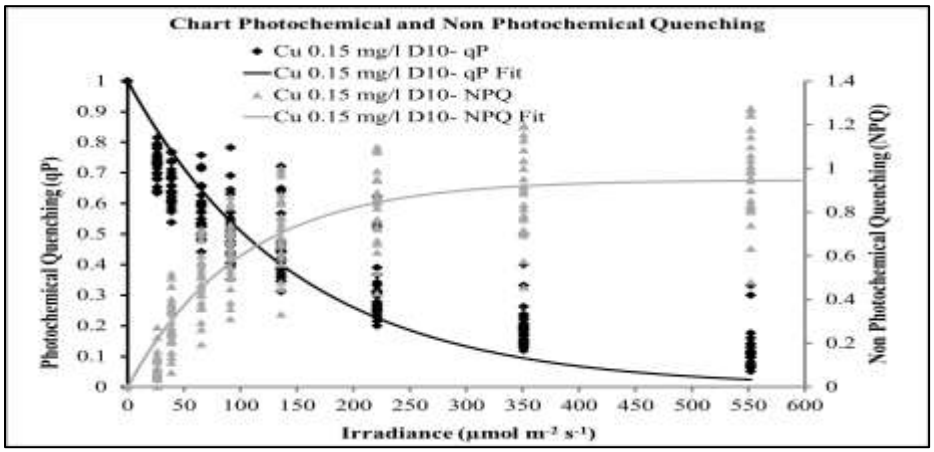

Figure A.24 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu on day 10.

Photosynthesis parameters of *C. demersum* under 0.15 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.63 ± 0.02 and electron transport rate (ETR_{max}) is 12.47 ± 0.43 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.23).

Figure A.24 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu treatment on day 10. The half point of Photochemical Quenching (qP) is 103.02 ± 6.24 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.15 ± 8.70 µmol photon m^{-2} s^{-1} on day 10.

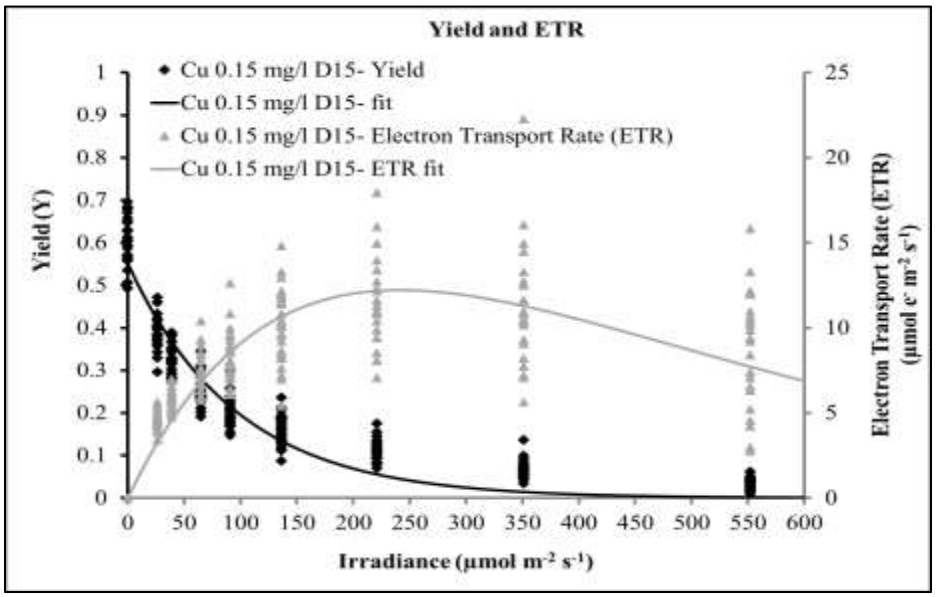

Figure A.25 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.15 mg/l Cu on day 15.

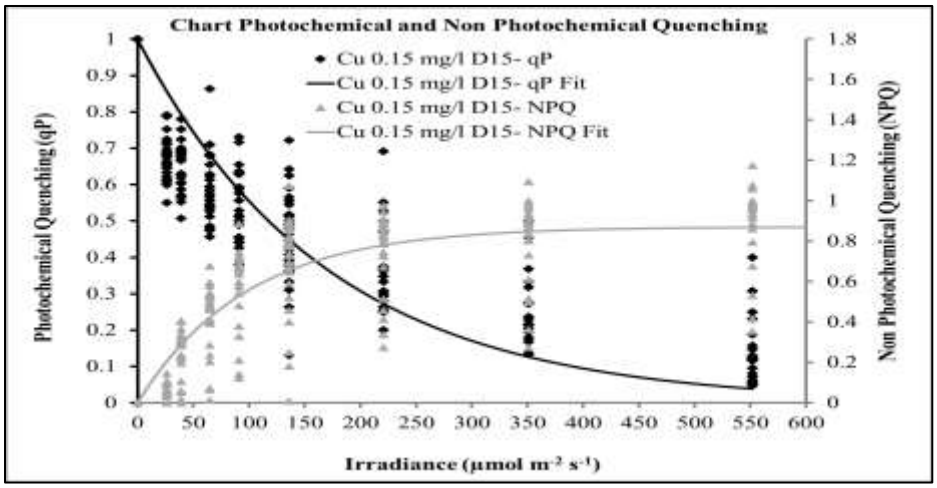

Figure A.26 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu on day 15.

Photosynthesis parameters of *C. demersum* under 0.15 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.55 ± 0.02 and electron transport rate (ETR_{max}) is 12.22 ± 0.43 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.25).

Figure A.26 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.15 mg/l Cu treatment on day 15. The half point of Photochemical Quenching (qP) is 117.65 ± 8.40 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 67.59 ± 11.40 µmol photon m^{-2} s^{-1} on day 15.

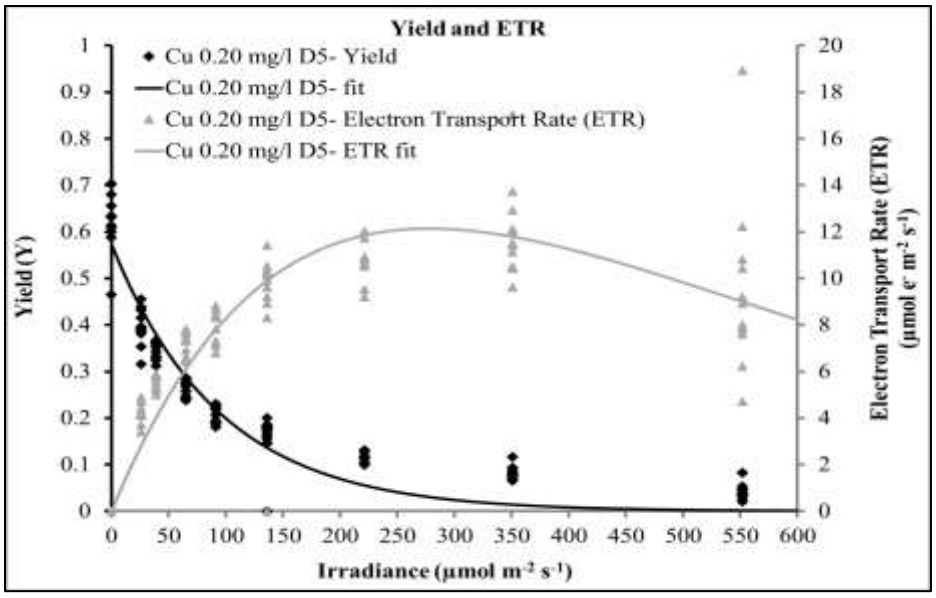

Figure A.27 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR),of *C. demersum* in the 0.15 mg/l Cu on day 5.

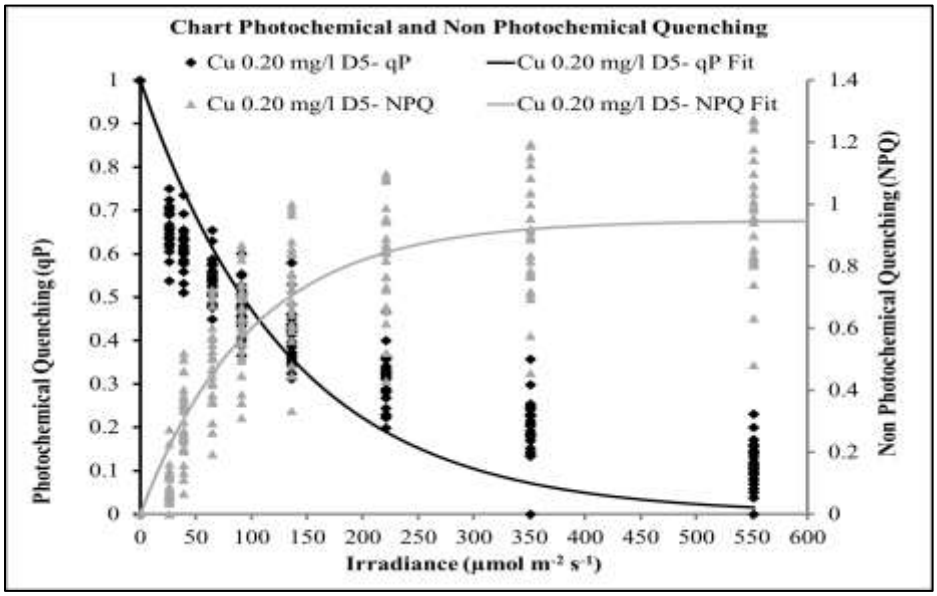

Figure A.28 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu on day 5.

Photosynthesis parameters of *C. demersum* under 0.20 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.57 ± 0.02 and electron transport rate (ETR_{max}) is 12.14 ± 0.62 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.27).

Figure A.28 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu treatment on day 5. The half point of Photochemical Quenching (qP) is 92.18± 5.62 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.15 ± 8.70 µmol photon m^{-2} s^{-1}on day 5.

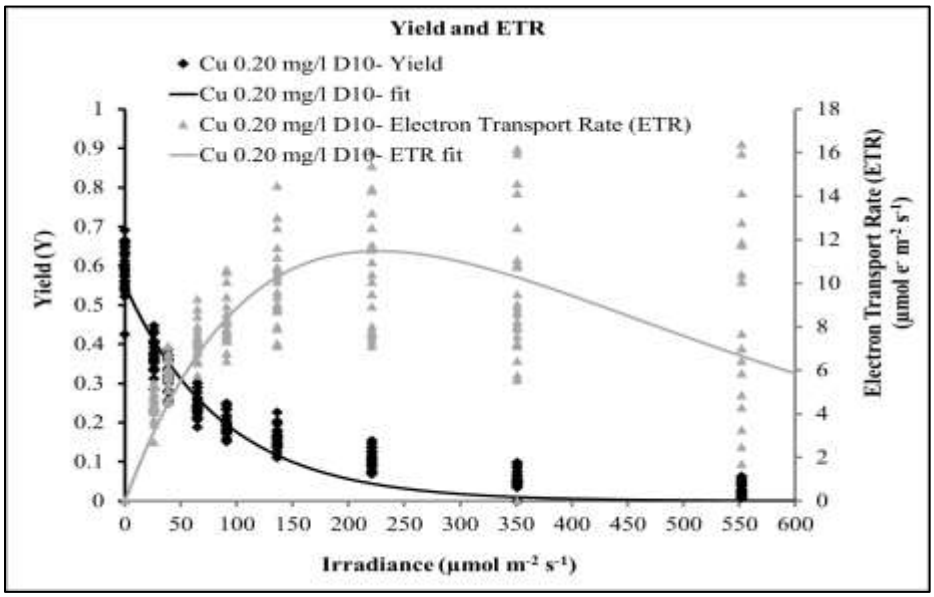

Figure A.29 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR),of *C. demersum* in the 0.15 mg/l Cu on day 10.

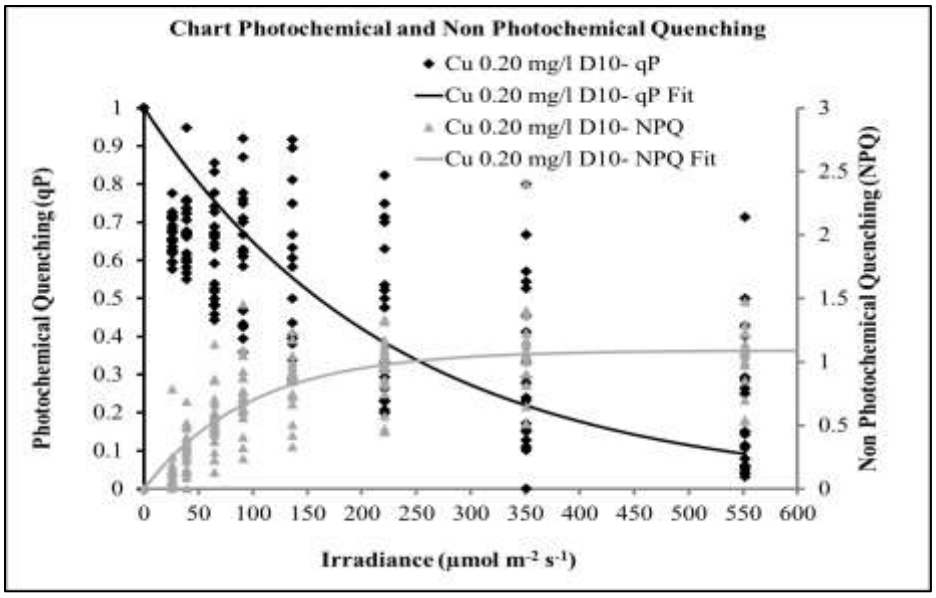

Figure A.30 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu on day 10.

Photosynthesis parameters of *C. demersum* under 0.20 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.55 ± 0.02 and electron transport rate (ETR_{max}) is 11.49 ± 0.50 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.29).

Figure A.30 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu treatment on day 10. The half point of Photochemical Quenching (qP) is 160.15 ± 18.54 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 67.39 ± 11.75 µmol photon m^{-2} s^{-1} on day 10.

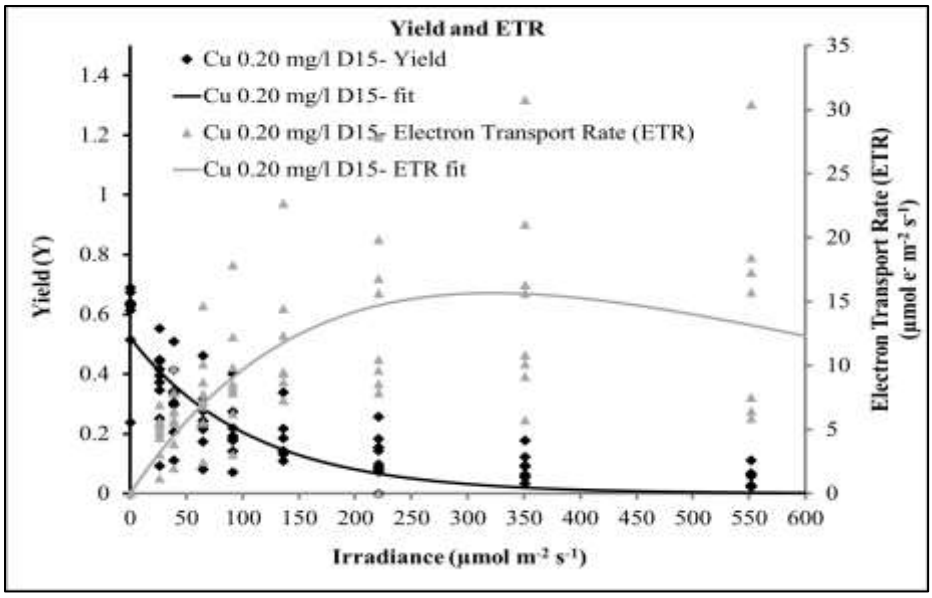

Figure A.31 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.20 mg/l Cu on day 15.

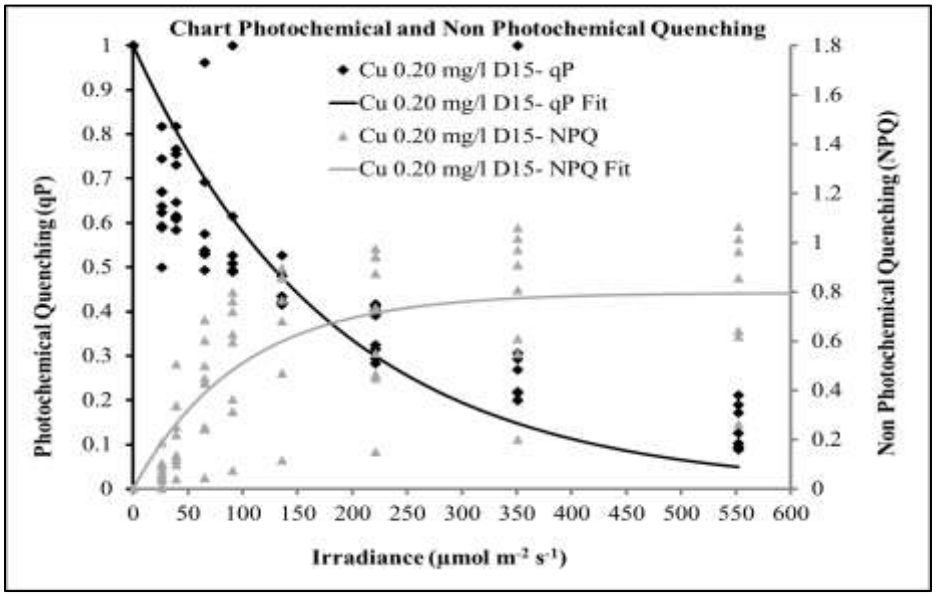

Figure A.32 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu on day 15.

Photosynthesis parameters of *Ceratophyllum demersum* under 0.20 mg/l Cu treatment including the maximum yield (Y_{max}) is 0.52 ± 0.06 and electron transport rate (ETR_{max}) is 15.67 ± 1.99 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.31).

Figure A.32 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.20 mg/l Cu treatment on day 15. The half point of Photochemical Quenching (qP) is 127.09 ± 21.76 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 67.36 ± 25.16 µmol photon m^{-2} s^{-1} on day 15.

According to this study, toxic effect of Cu as $CuSO_4$ to *C. demersum* was significant when exposed to increasing concentrations and toxicity increased over time. All of the concentration treatments showed a similar impact to the plants within 5 days, however up to 10 days to 15 days of the experiment *C. demersum* seem to show more effects on photosynthesis. Overall, this study showed that Cu increased in toxicity to *C. demersum* over time. Up to 15d were needed to show significant toxicity effects.

A.3Zinc Effects

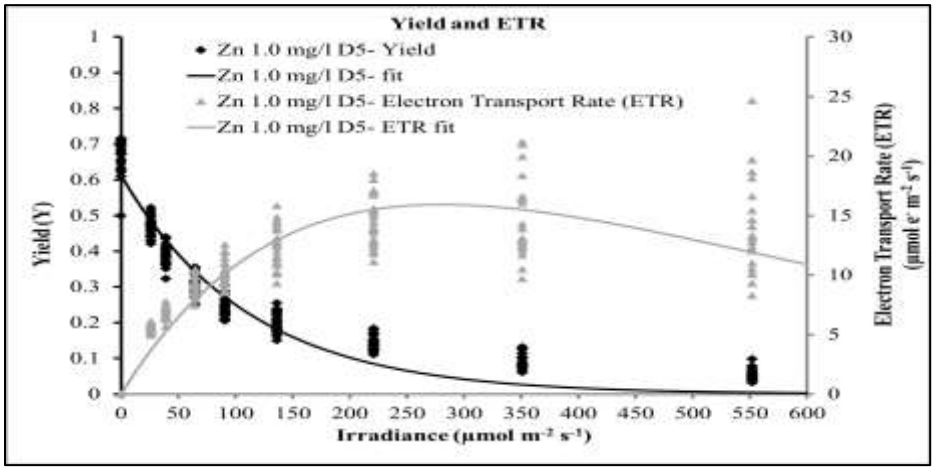

Figure A.33 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 1.0 mg/l Zn on day 5.

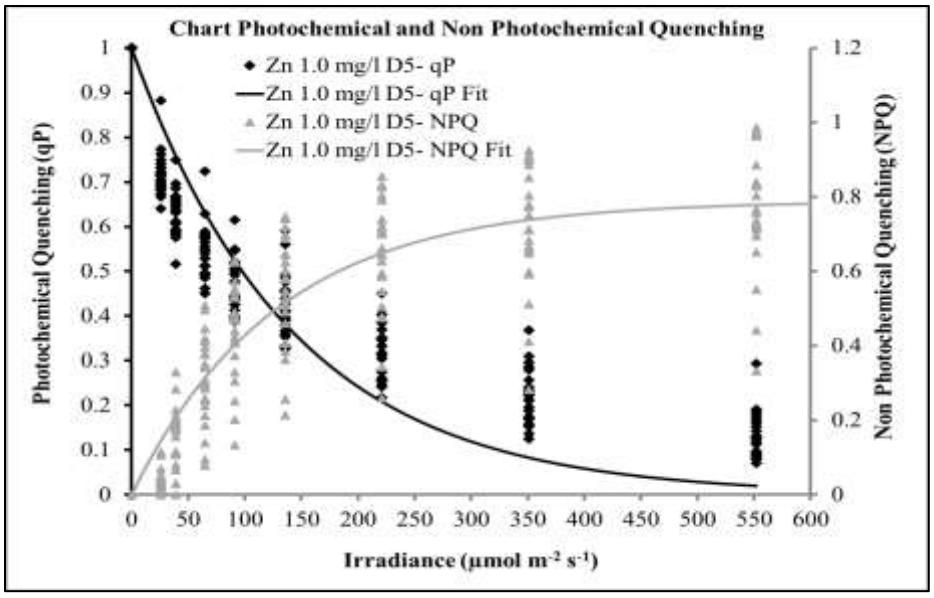

Figure A.34 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn on day 5.

Photosynthesis parameters of *C. demersum* under 1.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.61 ± 0.02 and electron transport rate (ETR_{max}) is 15.93 ± 0.45µmol e^- m^{-2} s^{-1} on day 5 (Figure A.33).

Figure A.34 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn treatment on day 5. The half point of Photochemical Quenching (qP) is 97.75 ± 5.72 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 88.59 ± 12.68 µmol photon m^{-2} s^{-1} on day 5.

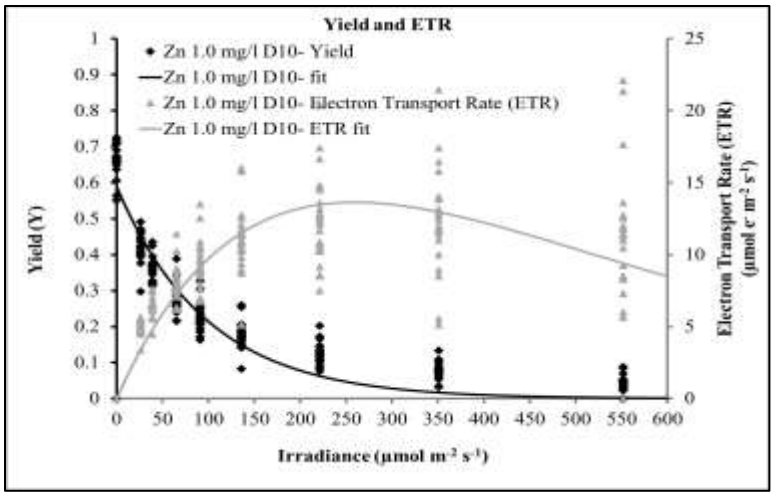

Figure A.35 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 1.0 mg/l Zn on day 10.

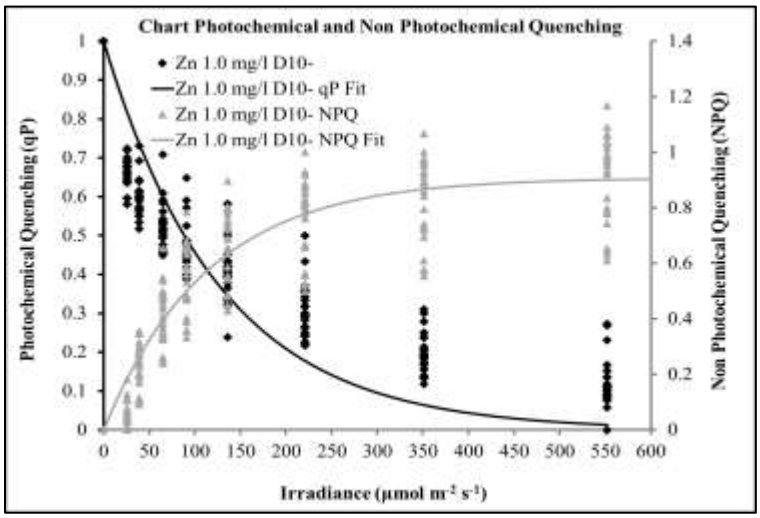

Figure A.36 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn on day 10.

Photosynthesis parameters of *C. demersum* under 1.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.59 ± 0.02 and electron transport rate (ETR_{max}) is 13.64 ± 0.52µmol e^- m^{-2} s^{-1} on day 10 (Figure A.35).

Figure A.36 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn treatment on day 10. The half point of Photochemical Quenching (qP) is 88.54 ± 5.53 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 78.80 ± 9.21 µmol photon m^{-2} s^{-1} on day 10.

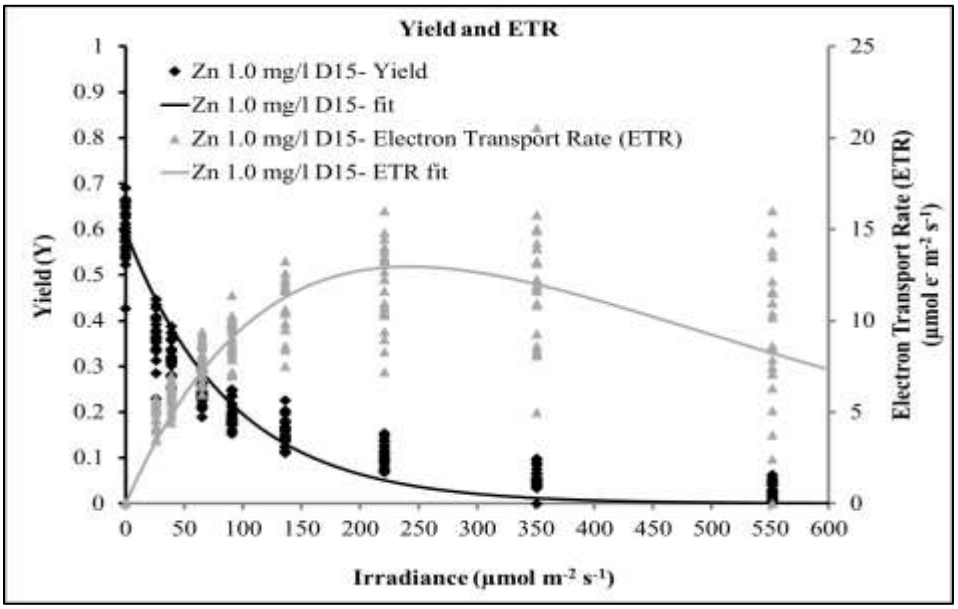

Figure A.37 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 1.0 mg/l Zn on day 15.

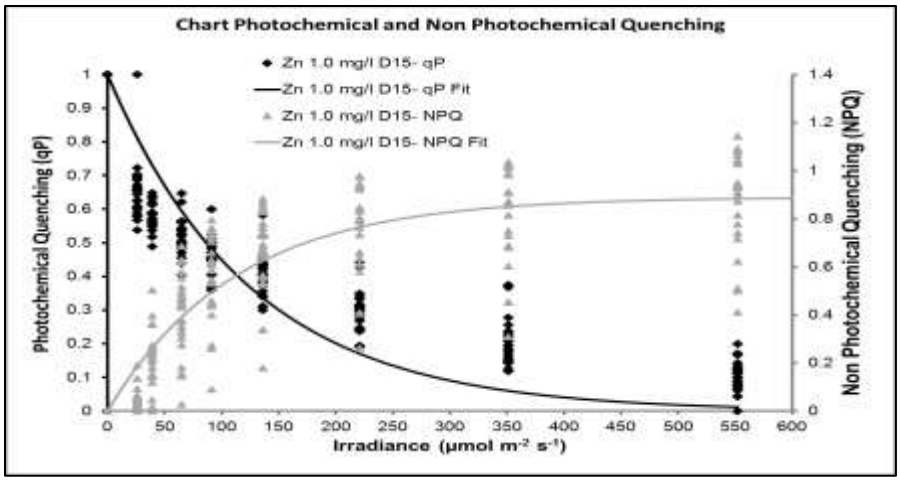

Figure A.38 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn on day 15.

Photosynthesis parameters of *C.demersum* under 1.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.59 ± 0.02 and electron transport rate (ETR_{max}) is 12.92 ± 12.96 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.37).

Figure A.38 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 1.0 mg/l Zn treatment on day 15. The half point of Photochemical Quenching (qP) is 86.52 ± 5.66 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 77.73 ± 11.93 µmol photon m^{-2} s^{-1} on day 15.

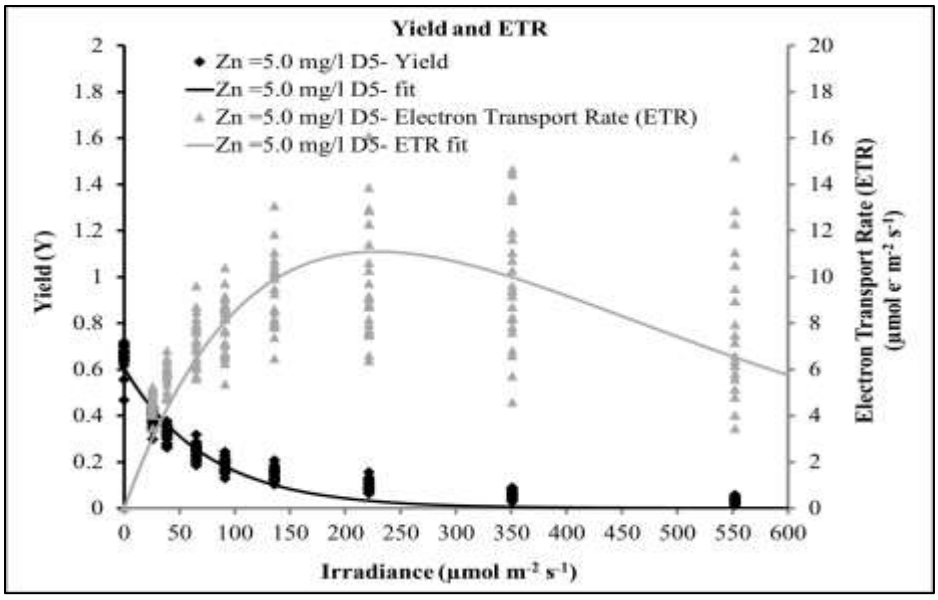

Figure A.39 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 5.0 mg/l Zn on day 5.

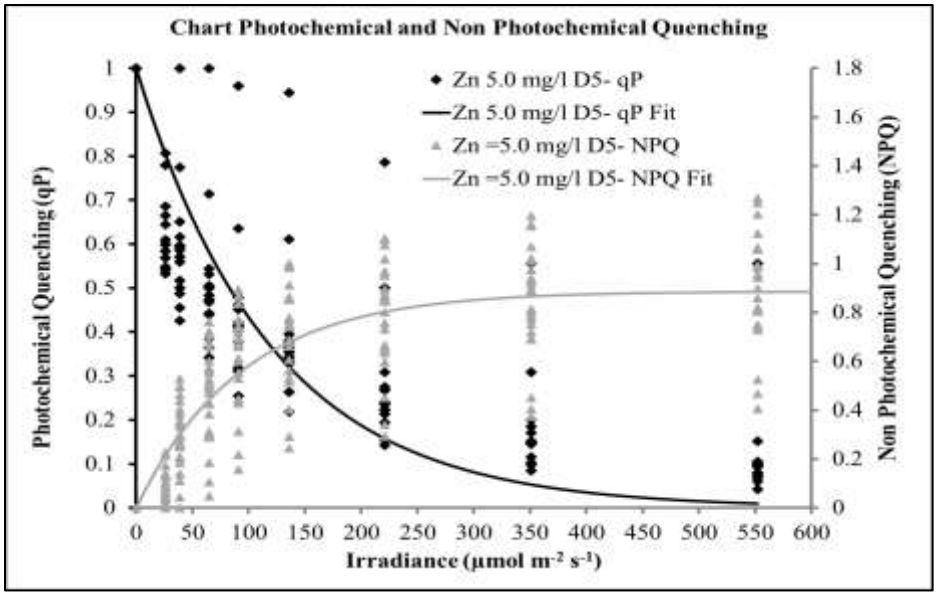

Figure A.40 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn on day 5.

Photosynthesis parameters of *C. demersum* under 5.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.60 ± 0.02 and electron transport rate (ETR_{max}) is 10.46 ± 0.50 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.39).

Figure A.40 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn treatment on day 5. The half point of Photochemical Quenching (qP) is 82.85 ± 9.66 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 68.34 ± 16.83 µmol photon m^{-2} s^{-1} on day 5.

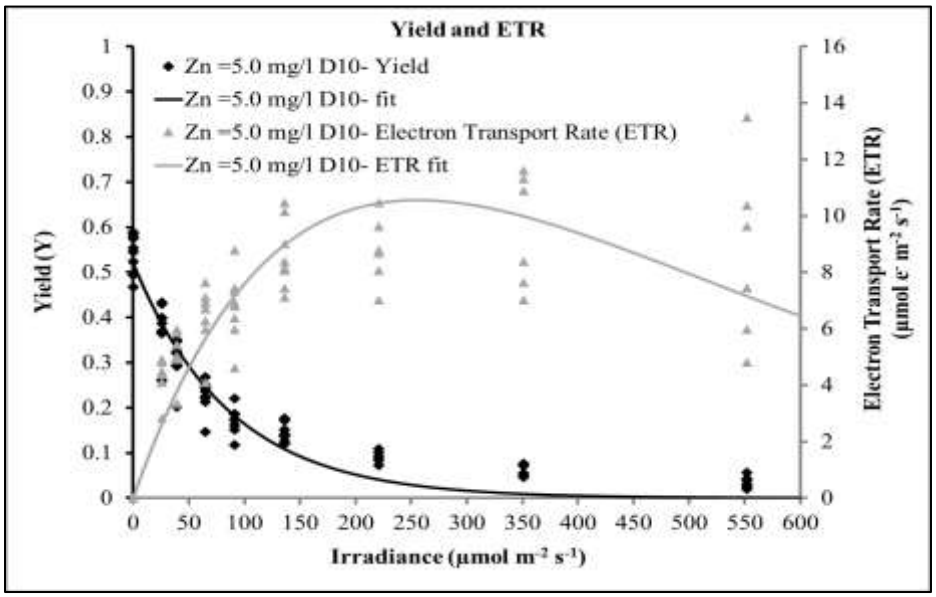

Figure A.41 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 5.0 mg/l Zn on day 10.

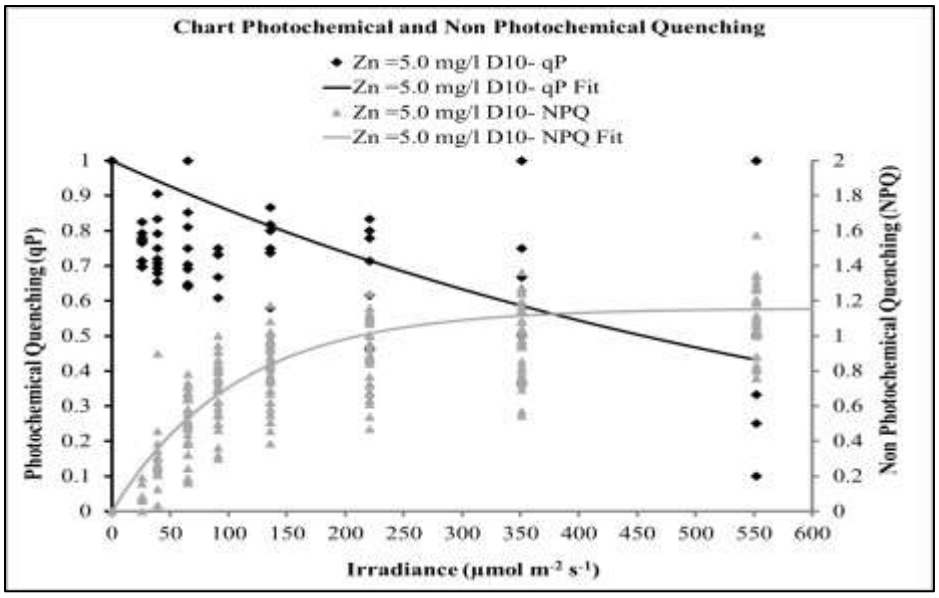

Figure A.42 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn on day 10.

Photosynthesis parameters of *C. demersum* under 5.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.52 ± 0.03 and electron transport rate (ETR_{max}) is 10.56 ± 0.66 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.41).

Figure A.42 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn treatment on day 10. The half point of Photochemical Quenching (qP) is 455.83 ± 129.72 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 73.49 ± 13.56 µmol photon m^{-2} s^{-1} on day 10.

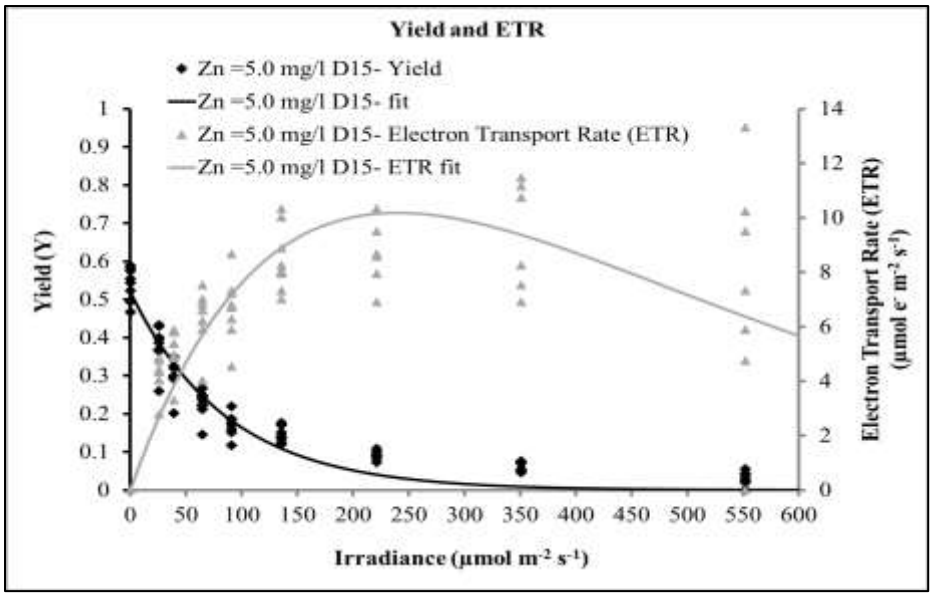

Figure A.43 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 5.0 mg/l Zn on day 15.

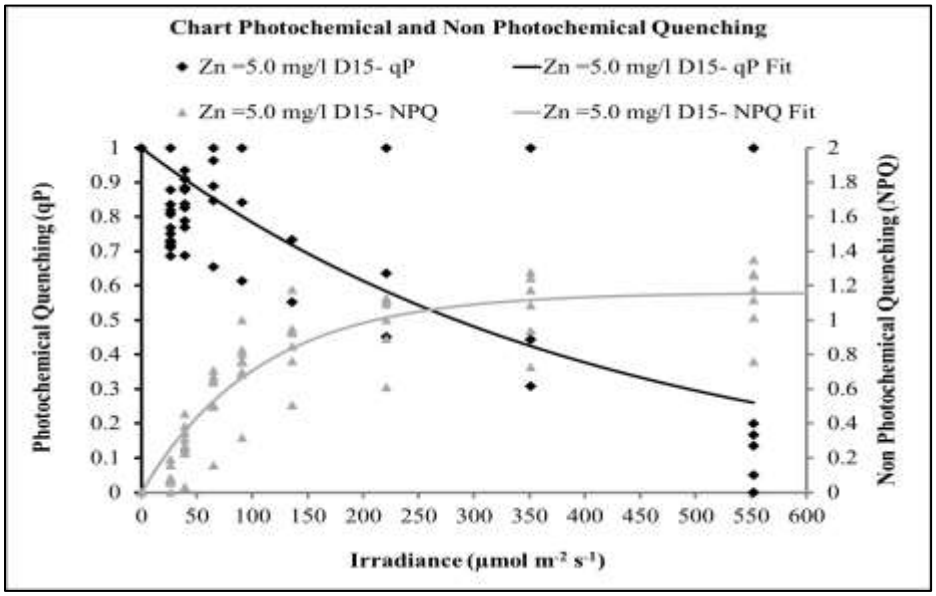

Figure A.44 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn on day 15.

Photosynthesis parameters of *C. demersum* under 5.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.52 ± 0.03 and electron transport rate (ETR_{max}) is 10.18 ± 0.70 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.43).

Figure A.44 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn treatment on day 15. The half point of Photochemical Quenching (qP) is 284.49 ± 76.25 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 73.69 ± 13.18 µmol photon m^{-2} s^{-1} on day 15.

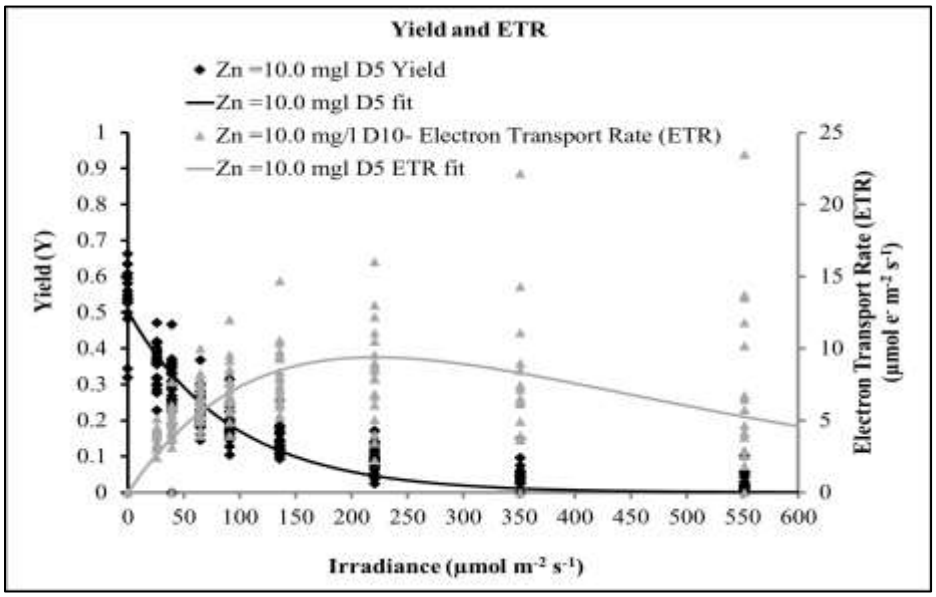

Figure A.45 Yield (Y), irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 10.0 mg/l Zn on day 5.

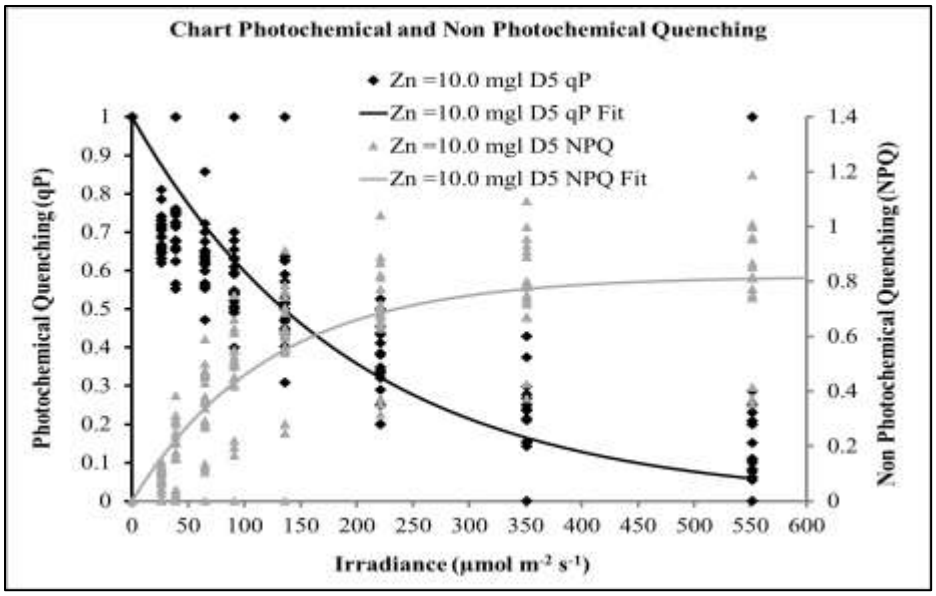

Figure A.46 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn on day 5.

Photosynthesis parameters of *C. demersum* under 10.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.50 ± 0.02 and electron transport rate (ETR_{max}) is 9.40 ± 0.68 µmol e^{-} m^{-2} s^{-1} on day 5 (Figure A.45).

Figure A.46 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn treatment on day 5. The half point of Photochemical Quenching (qP) is 134.97 ± 12.24 µmolphoton m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 84.31 ± 15.80 µmol photon m^{-2} s^{-1} on day 5.

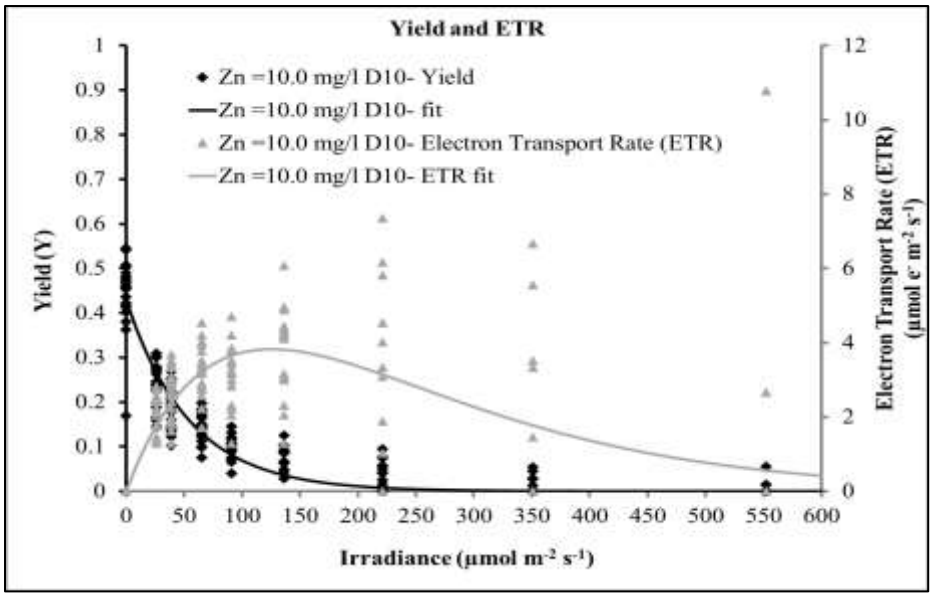

Figure A.47 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 10.0 mg/l Zn on day 10.

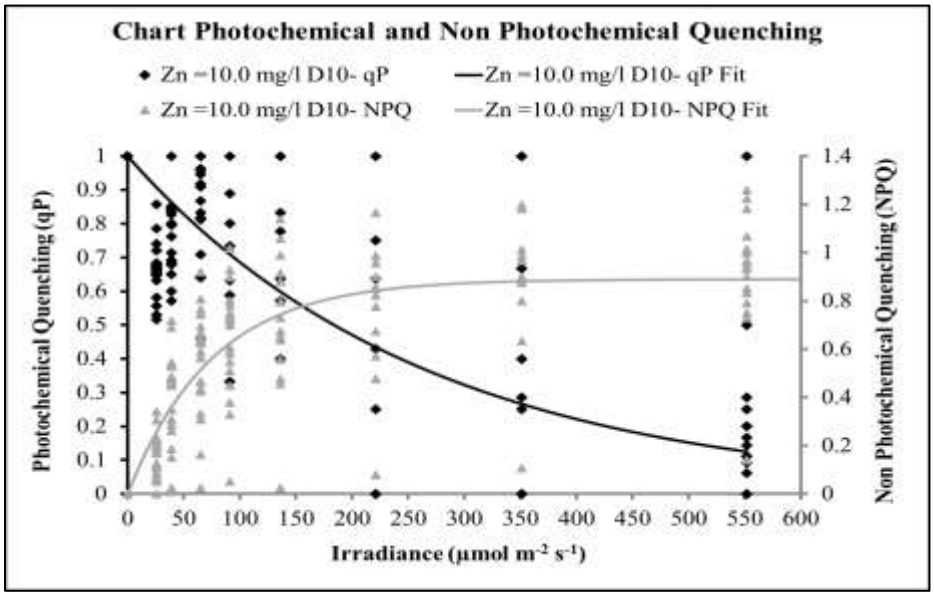

Figure A.48 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn on day 10.

Photosynthesis parameters of *C. demersum* under 10.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.43 ± 0.02 and electron transport rate (ETR_{max}) is 3.82 ± 0.33 µmol e^{-} m^{-2} s^{-1} on day 10 (Figure A.47).

Figure A.48 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 5.0 mg/l Zn treatment on day 10. The half point of Photochemical Quenching (qP) is 184.04 ± 34.90 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 52.18 ± 10.51 µmol photon m^{-2} s^{-1} on day 15.

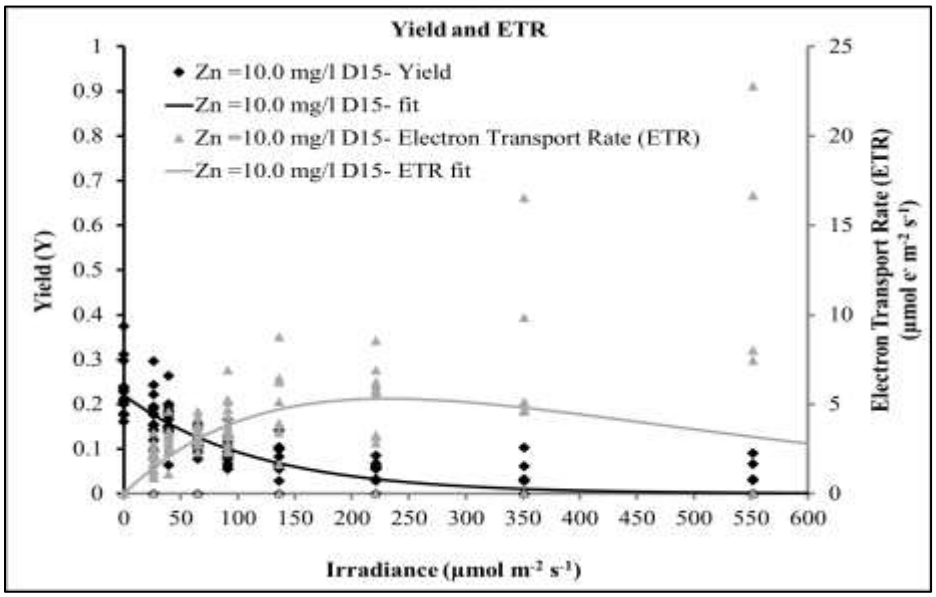

Figure A.49 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 10.0 mg/l Zn on day 15.

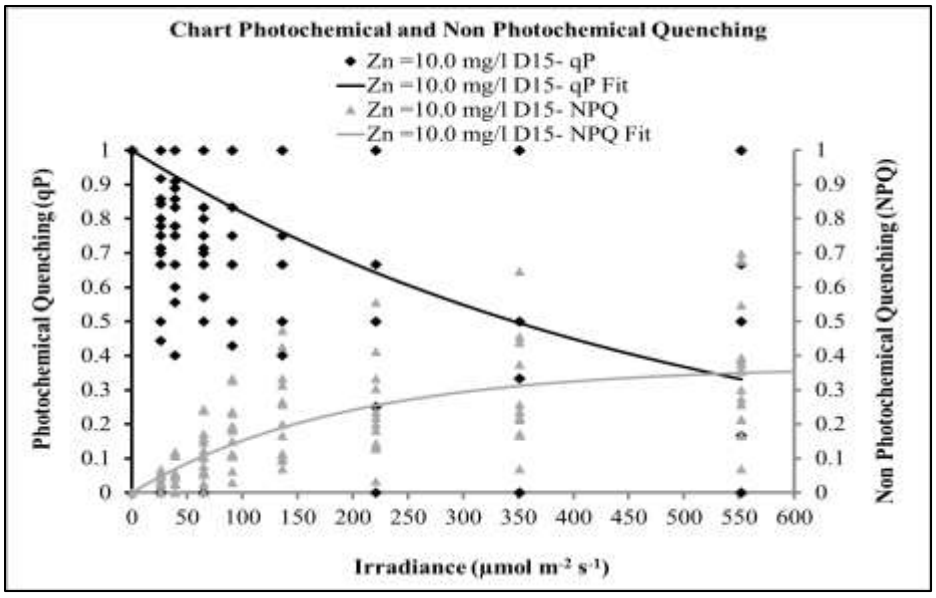

Figure A.50 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn on day 15.

Photosynthesis parameters of *C. demersum* under 10.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.22 ± 0.02 and electron transport rate (ETR_{max}) is 5.31 ± 0.83 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.49).

Figure A.50 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 10.0 mg/l Zn treatment on day 15. The half point of Photochemical Quenching (qP) is 346.49 ± 93.41 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 130.76 ± 53.19 µmol photon m^{-2} s^{-1} on day 15. These plants are very close to death as a result of the exposure to Zn and show abnormal qP & NPQ. Least square fitting of PAM curves on moribund plant material is difficult.

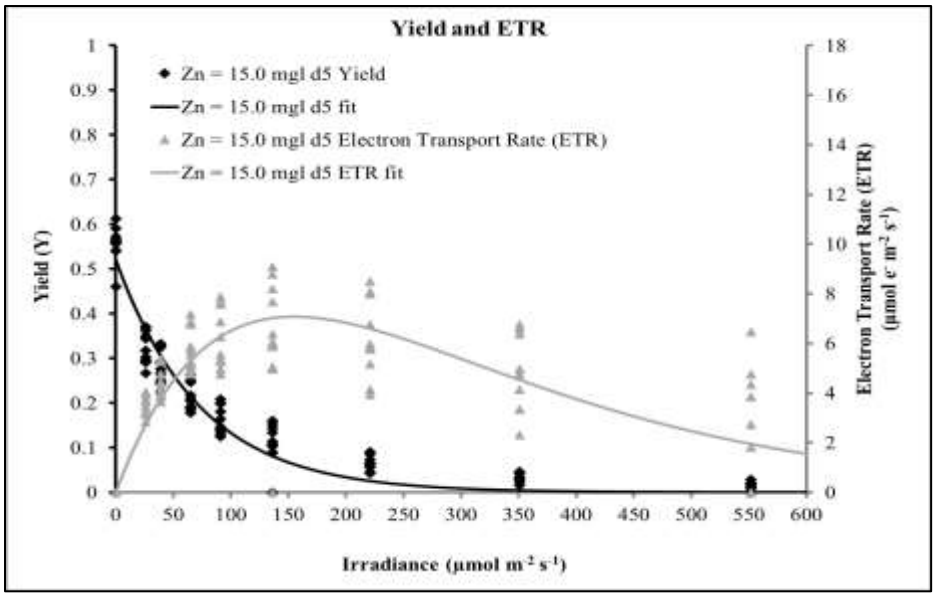

Figure A.51 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 15.0 mg/l Zn on day 5.

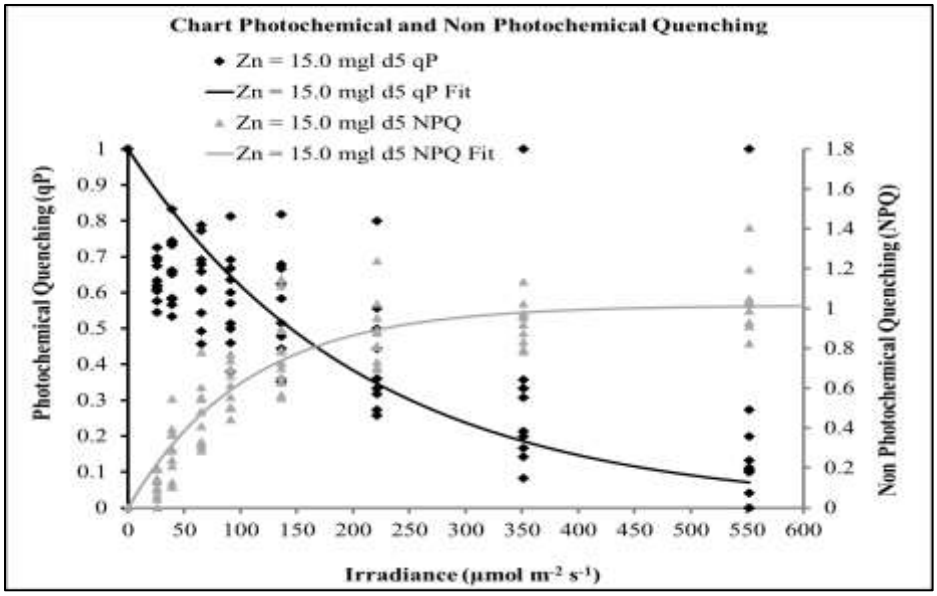

Figure A.52 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 15.0 mg/l Zn on day 5.

Photosynthesis parameter of *C. demersum* under 15.0 mg/l Zn treatment including the maximum yield (Y_{max}) is 0.52 ± 0.02 and electron transport rate (ETR_{max}) is 7.80 ± 0.44 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.51).

Figure A.52 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 15.0 mg/l Zn treatment on day 5. The half point of Photochemical Quenching (qP) is 144.68 ± 24.12 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 72.50 ± 12.10 µmol photon m^{-2} s^{-1} on day 5.As in the previous figures (A.49 &A50), these plants show signs of Zn stress (abnormal qP and NPQ) and low ETR.

The results showed that toxic effect of Zn, as $ZnSO_4$, on *C. demersum* appeared to increase when Zn was increased to high concentrations (10.0 mg/l and 15.0 mg/l) after 10 days of the experiment. This study showed that *C. demersum* could tolerate Zn more than Cu after 15 days of the experiment and responses to Zn were slower than for Cu.

A.4 Copper plus Zinc Combinational Effects

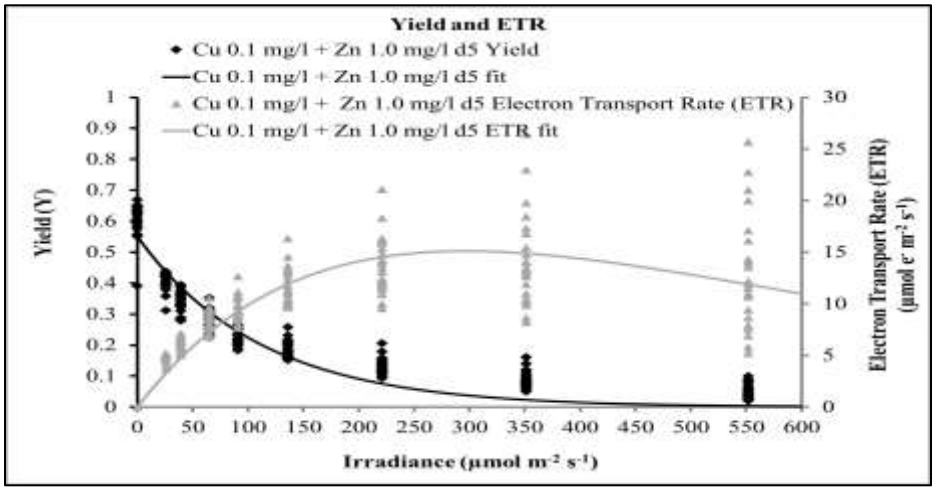

Figure A.53 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 5.

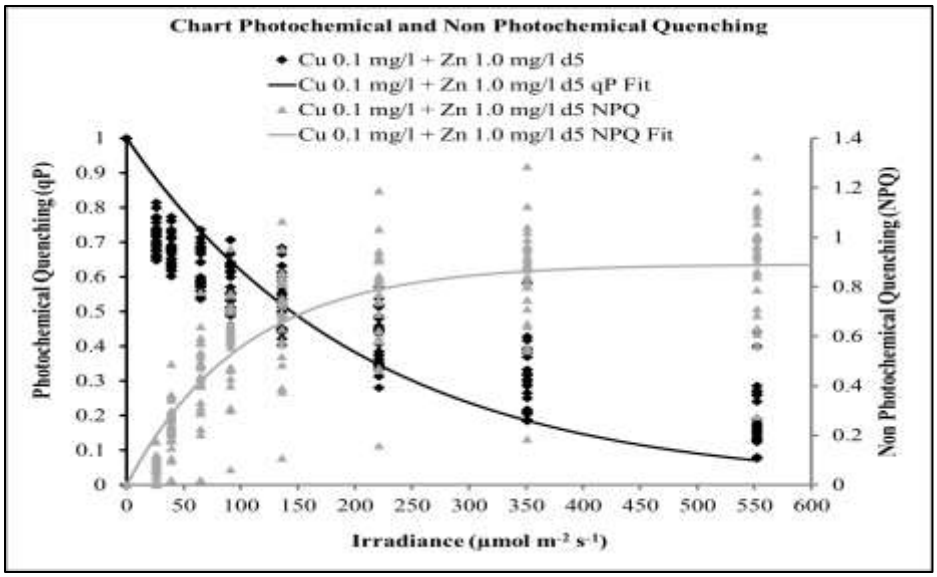

Figure A.54 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 5.

Photosynthesis parameters of *C. demersum* under 0.1 + 1.0 mg/l Cu plus Zn treatment, respectively including the maximum yield (Y_{max}) is 0.55 ± 0.02 and electron transport rate (ETR_{max}) is 15.11 ± 0.67 µmol e^{-} m^{-2} s^{-1} on day 5 (Figure A.53).

Figure A.54 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 5. The half point of Photochemical Quenching (qP) is 144.63 ± 9.81 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 70.79 ± 10.96 µmol photon m^{-2} s^{-1} on day 5.

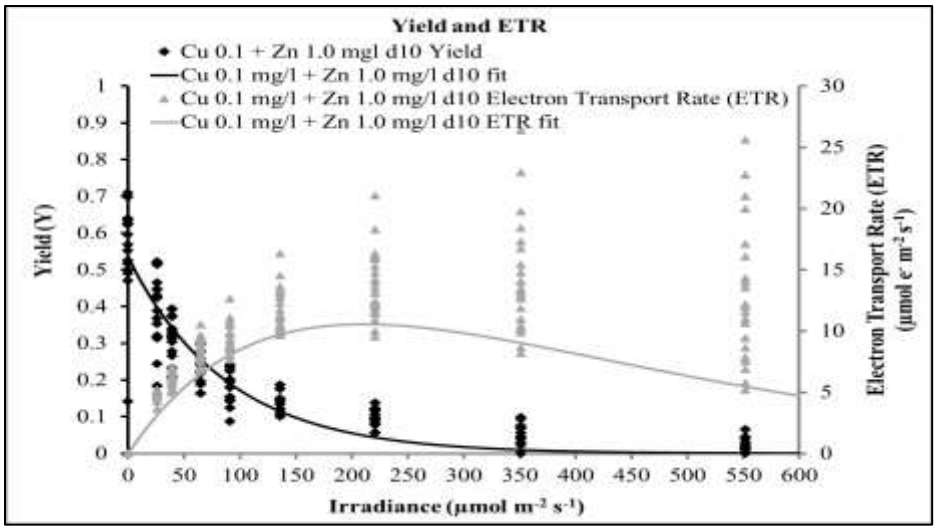

Figure A.55 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 10.

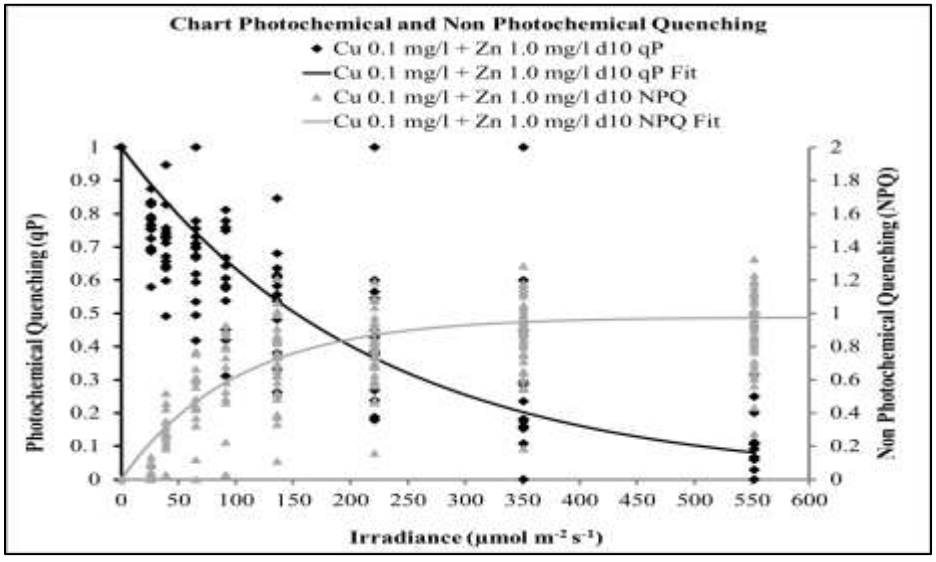

Figure A.56 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 10.

Photosynthesis parameters of *C. demersum* under 0.1 + 1.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.53 ± 0.03 and electron transport rate (ETR_{max}) is 10.56 ± 0.69µmol e^- m^{-2} s^{-1} on day 10 (Figure A.55).

Figure A.56 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 10. The half point of Photochemical Quenching (qP) is 152.23 ± 19.15 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 69.39 ± 14.52 µmol photon m^{-2} s^{-1} on day 5.

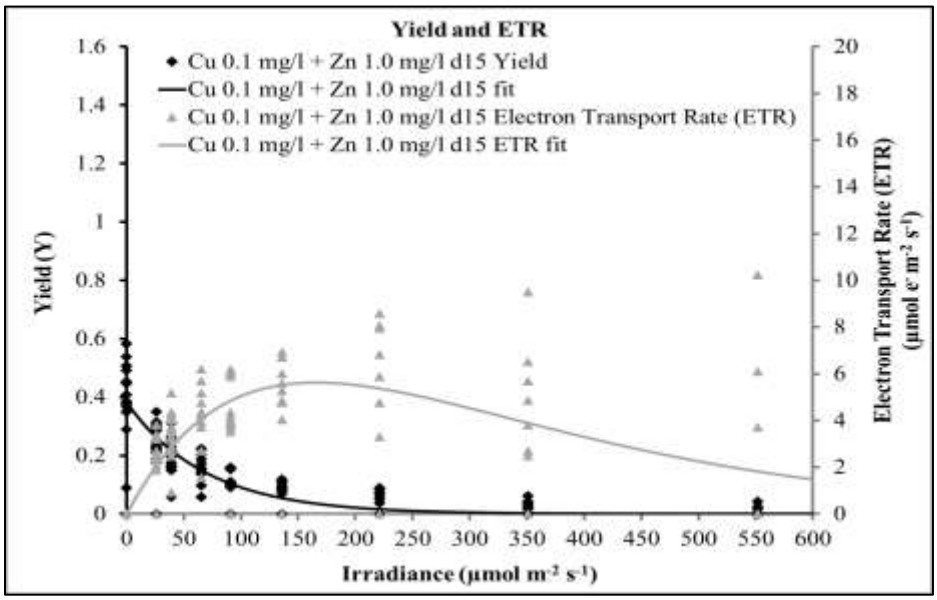

Figure A.57 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 15.

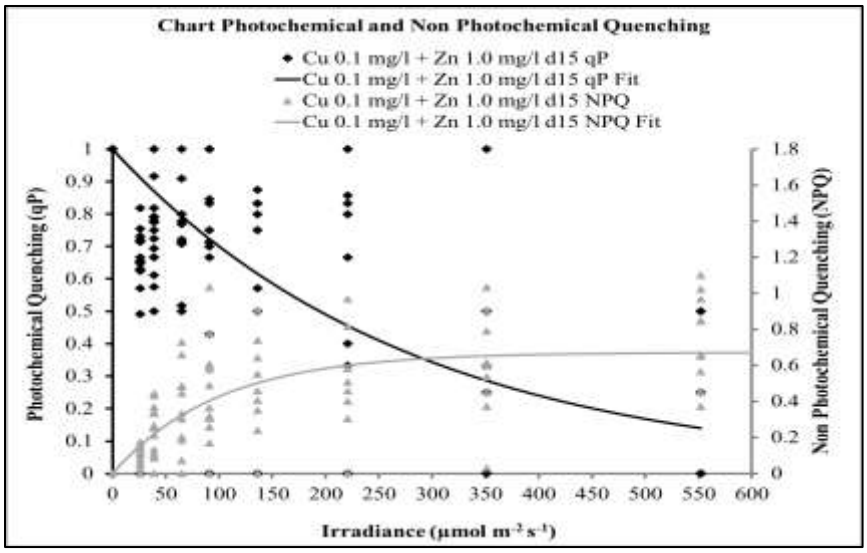

Figure A.58 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu and Zn, respectively on day 15.

Photosynthesis parameters of *C. demersum* under 0.1 + 1.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.38 ± 0.03and electron transport rate (ETR_{max}) is 5.63 ± 0.60 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.57).

Figure A.58 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 15. The half point of Photochemical Quenching (qP) is 194.59 ± 43.43 µmol photon m^{-2} $s^{-1}$and the half point of Non Photochemical Quenching (NPQ) is 68.52 ± 25.78 µmol m^{-2} s^{-1} on day 15.

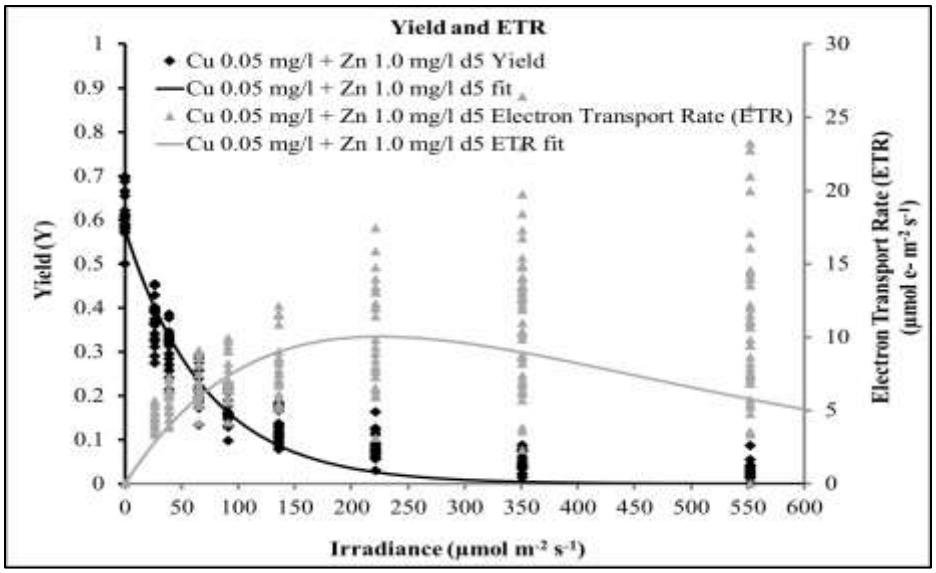

Figure A.59 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 5.

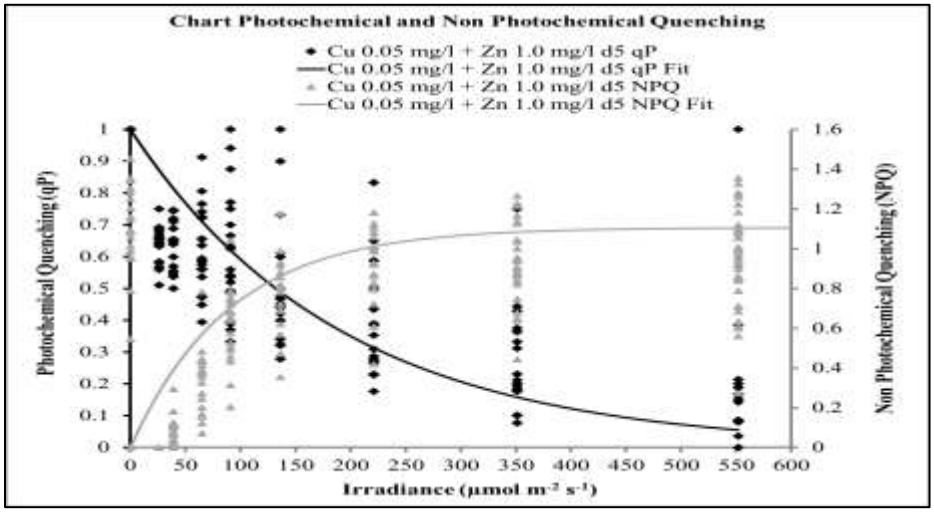

Figure A.60 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 5.

Photosynthesis parameters of *C. demersum* under 0.05 + 1.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.58 ± 0.02 and electron transport rate (ETR_{max}) is 10.04 ± 0.65 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.59).

Figure A.60 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 5. The half point of Photochemical Quenching (qP) is 132.08 ± 16.03 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 62.28 ± 9.12 µmol photon m^{-2} s^{-1} on day 5.

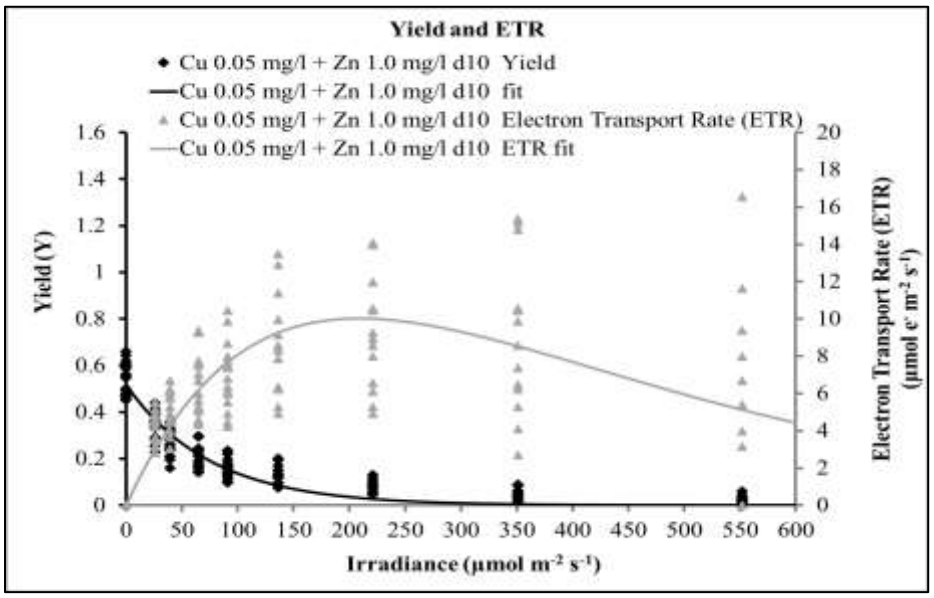

Figure A.61 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 10.

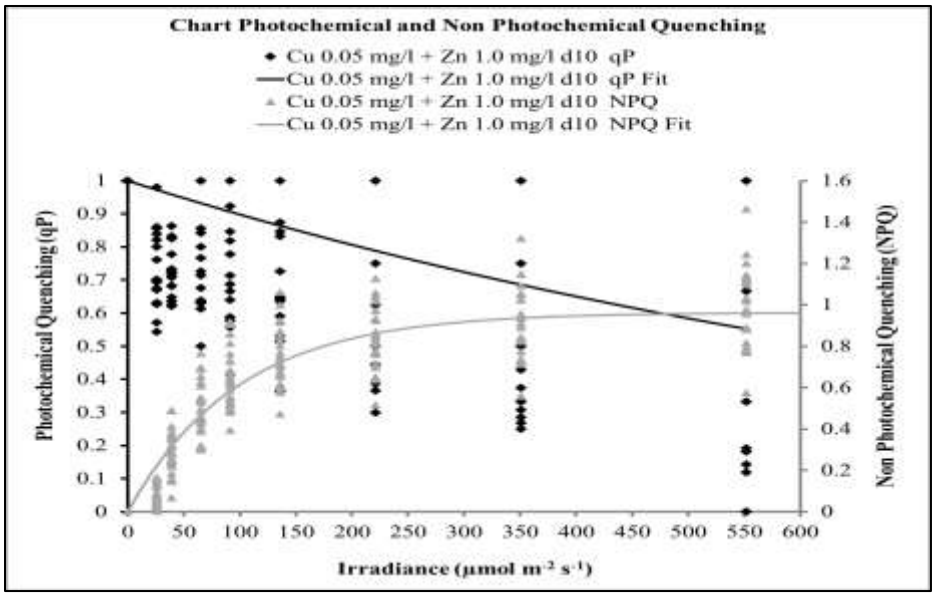

Figure A.62 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 10.

Photosynthesis parameter of *Ceratophyllum demersum* under 0.05 + 1.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.52 ± 0.02 and electron transport rate (ETR_{max}) is 10.02± 0.66 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.61).

Figure A.62 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 10. The half point of Photochemical Quenching (qP) is 260.03 ± 43.91 µmol photon m^{-2} s^{-1} (this is rubbish value) and the half point of Non Photochemical Quenching (NPQ) is 67.99 ± 7.89 µmol photon m^{-2} s^{-1} on day 10.qP (very high ½ point) and NPQ (low) show signs of severe stress by Cu + Zn. ETR is low and replicates are highly variable indicating acute stress and moribund condition of some of the plants.

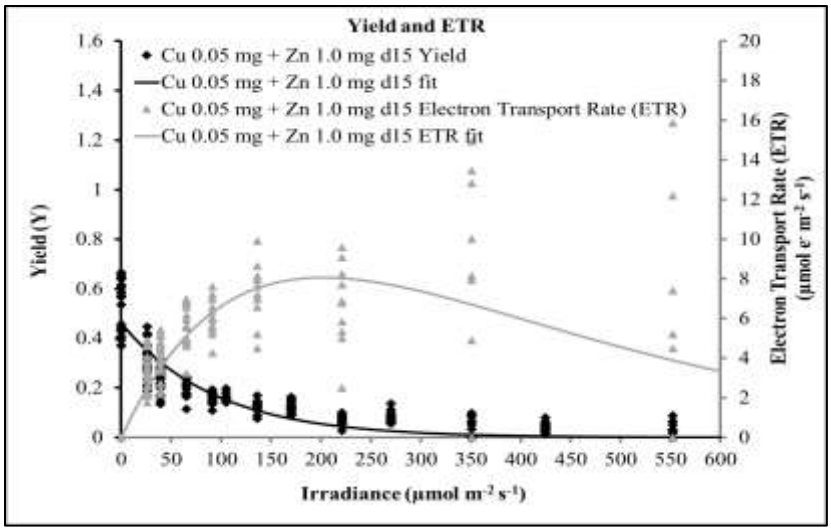

Figure A.63 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 15.

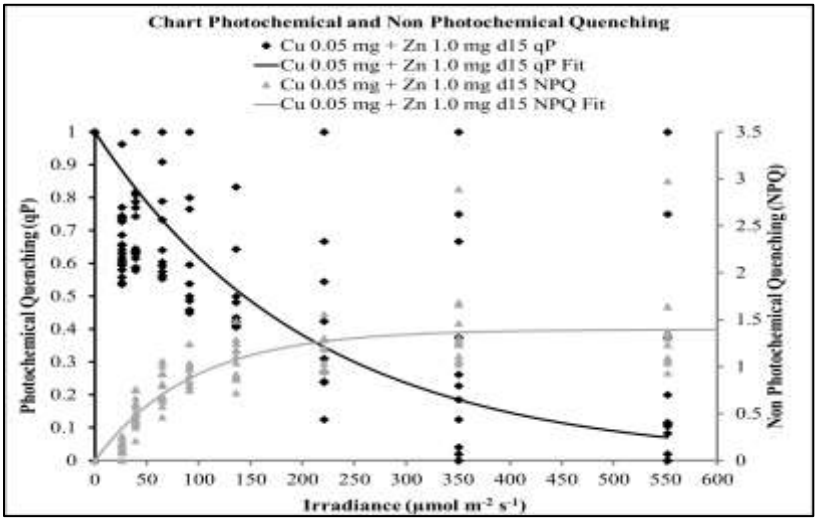

Figure A.64 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu and Zn, respectively on day 15.

Photosynthesis parameters of *C. demersum* under 0.05 + 1.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.46 ± 0.02 and electron transport rate (ETR_{max}) is 8.07 ± 0.82 µmol e^- m^{-2} s^{-1} on day 15 (A.63).

Figure A.64 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 1.0 mg/l Cu plus Zn treatment, respectively on day 15. The half point of Photochemical Quenching (qP) is 144.19 ± 26.46 µmol e^- m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 63.99 ± 11.87 on day 15 µmol photon m^{-2} s^{-1}.

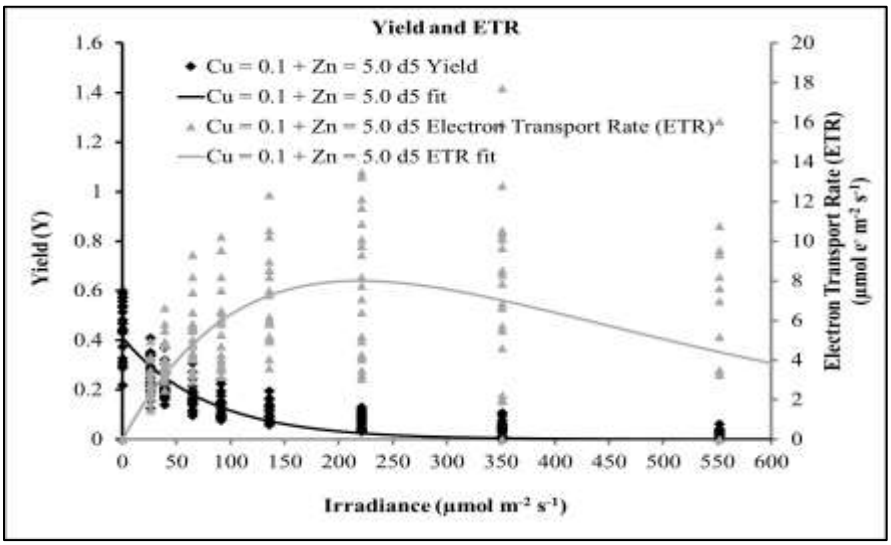

Figure A.65 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.10 mg/l + 5.0 mg/l Cu and Zn, respectively on day 5.

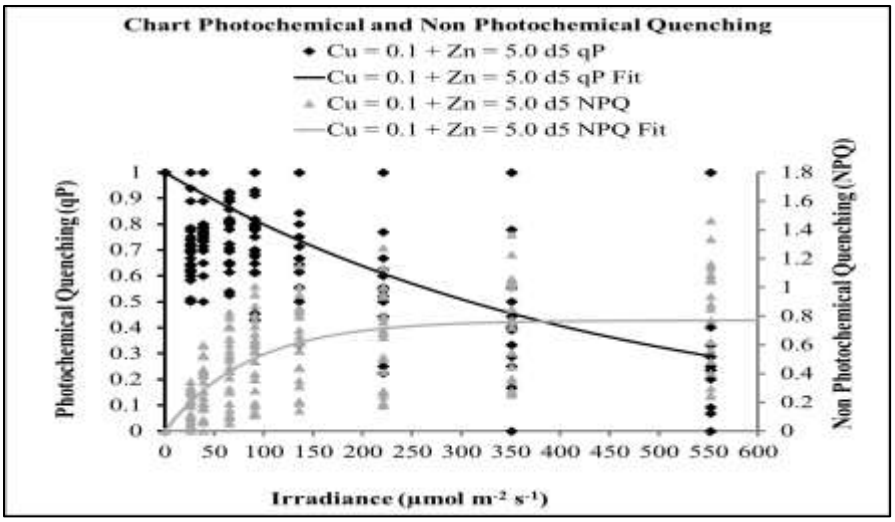

Figure A.66 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 5.

Photosynthesis parameter of *C. demersum* under 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.4 ± 0.02 and electron transport rate (ETR_{max}) is 8.00 ± 0.59 µmol e^- m^{-2} s^{-1} on day 5 (A.65).

Figure A.66 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 5. The half point of Photochemical Quenching (qP) is 308.80 ± 50.64 µmol photon m^{-2} $s^{-1}$and the half point of Non Photochemical Quenching (NPQ) is 58.77 ± 3.49 µmol photon m^{-2} s^{-1} on day 5. High value for qP half-point and low NPQ point due to acute metal stress.

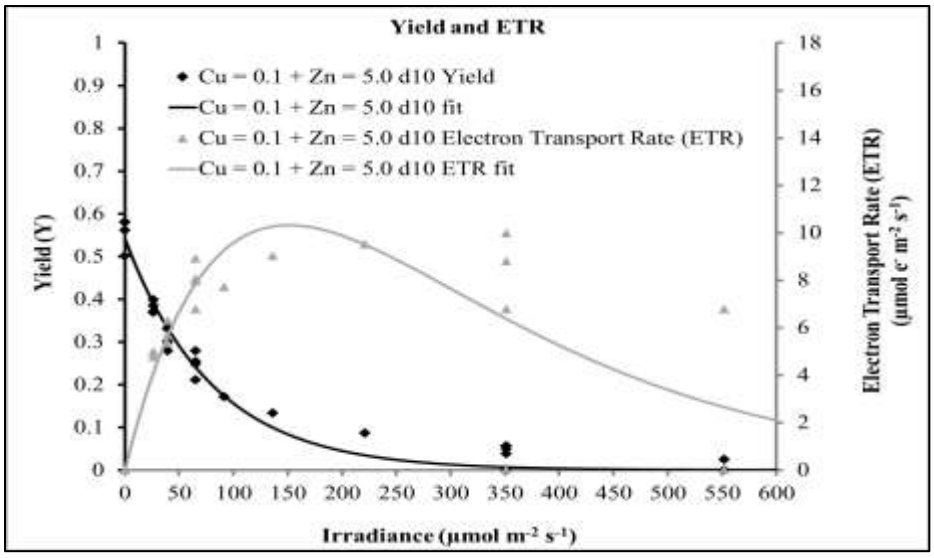

Figure A.67 Yield (Y) and irradiance (E),and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 10.

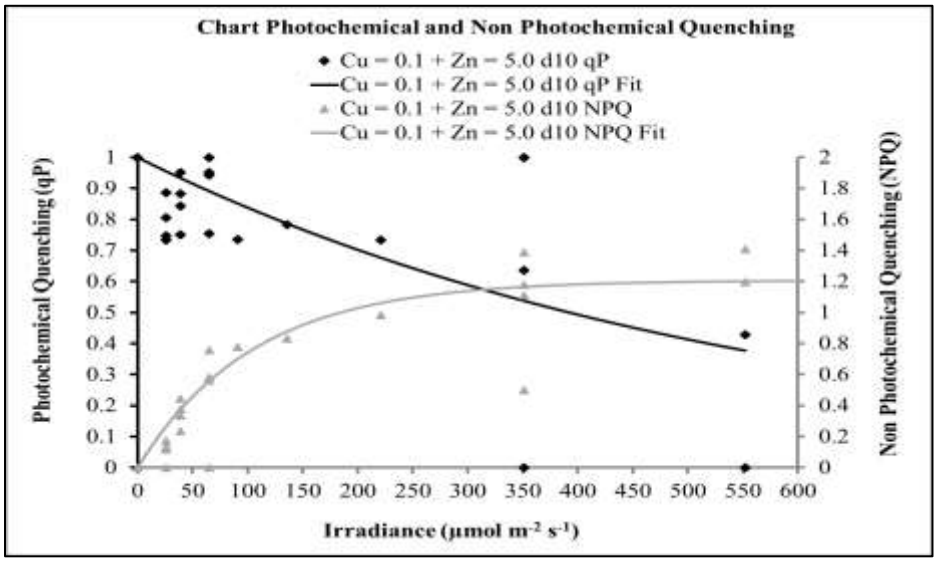

Figure A.68 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 10.

Photosynthesis parameters of *C. demersum* under 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.54 ± 0.03and electron transport rate (ETR_{max}) is 10.32 ± 1.44 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.67).

Figure A.68 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 10. The half point of Photochemical Quenching (qP) is 392.59 ± 181.34 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 72.86 ± 31.49 µmol photon m^{-2} s^{-1} on day 10.

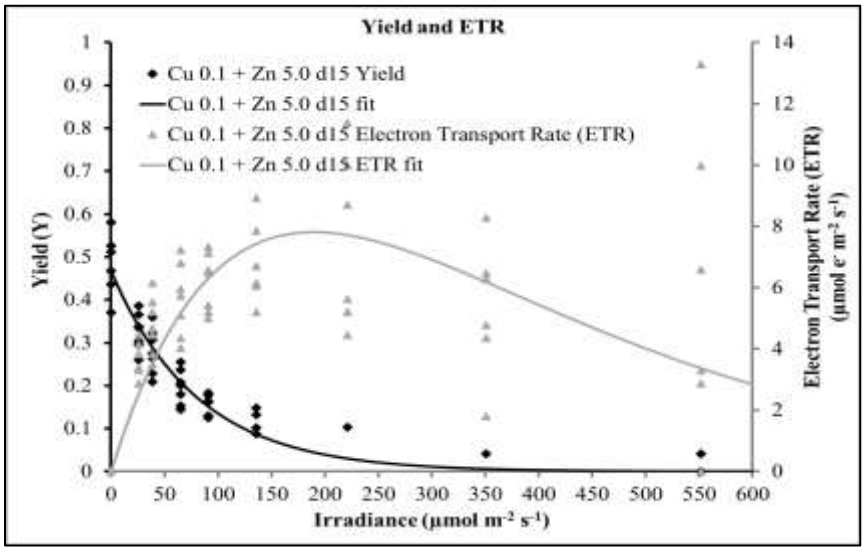

Figure A.69 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 15.

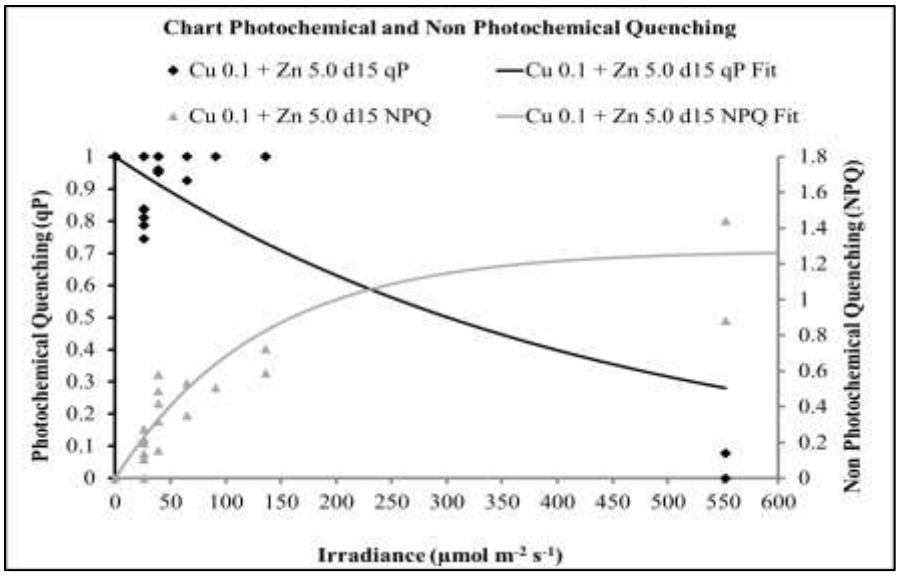

Figure A.70 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu and Zn, respectively on day 15.

Photosynthesis parameter of *C. demersum* under 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.47 ± 0.03 and electron transport rate (ETR_{max}) is 7.81 ± 0.84 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.69).

Figure A.70 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.1 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 15. The half point of Photochemical Quenching (qP) is 301.19 ± 123.69 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 90.24 ± 25.66 µmol photon m^{-2} s^{-1} on day 15. Acute metal stress is evident from the very poor qP results and NPQ. Only a few of the qP and NPQ calculations were valid calculations. A qP value greater than 1 is spurious and the Walz software flags spurious NPQ values. Plants that are nearly dead give very aberrant PAM results.

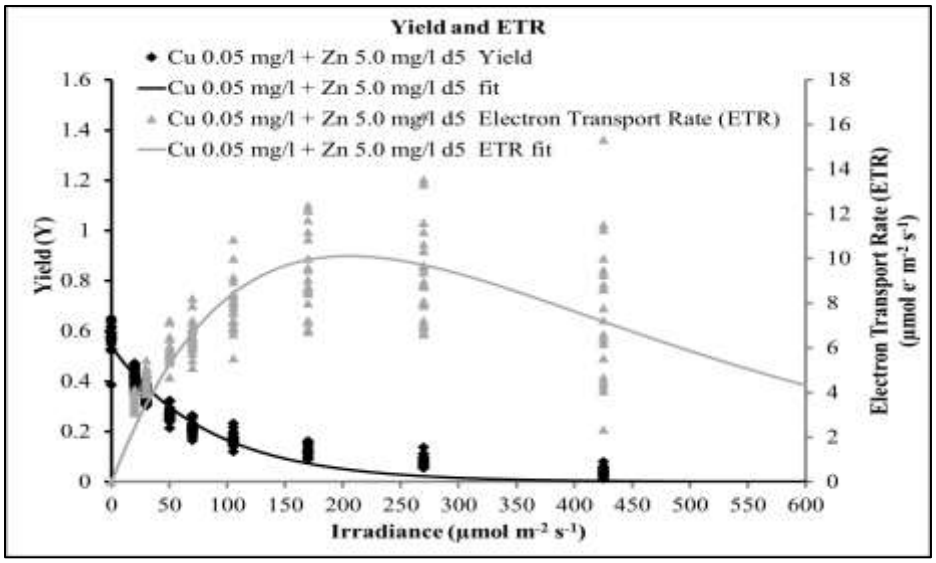

Figure A.71 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 5.

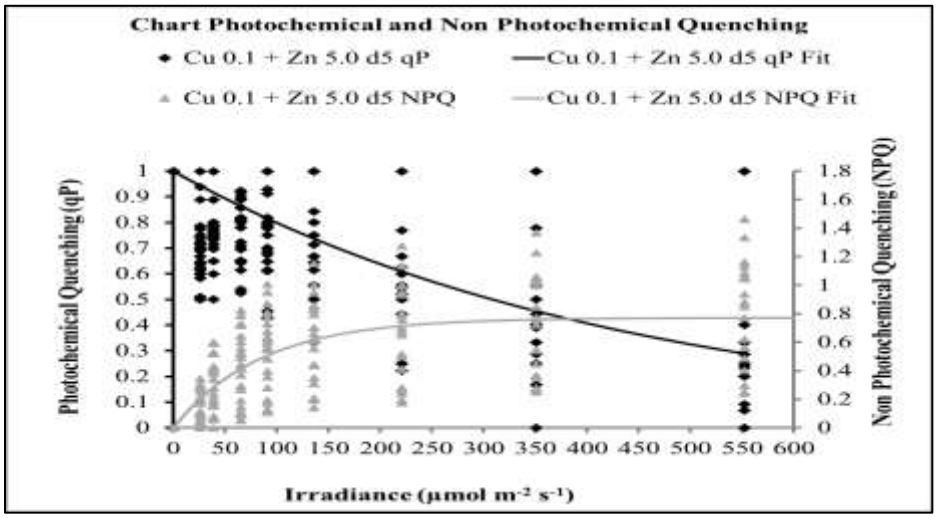

Figure A.72 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 5.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.54 ± 0.01 and electron transport rate (ETR_{max}) is 10.12 ± 0.32 µmol e^- m^{-2} s^{-1} on day 5 (Figure A.71).

Figure A.72 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 5. The half point of Photochemical Quenching (qP) is 113.42 ± 6.93 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 56.55 ± 8.49 µmol photon m^{-2} s^{-1} on day 5.

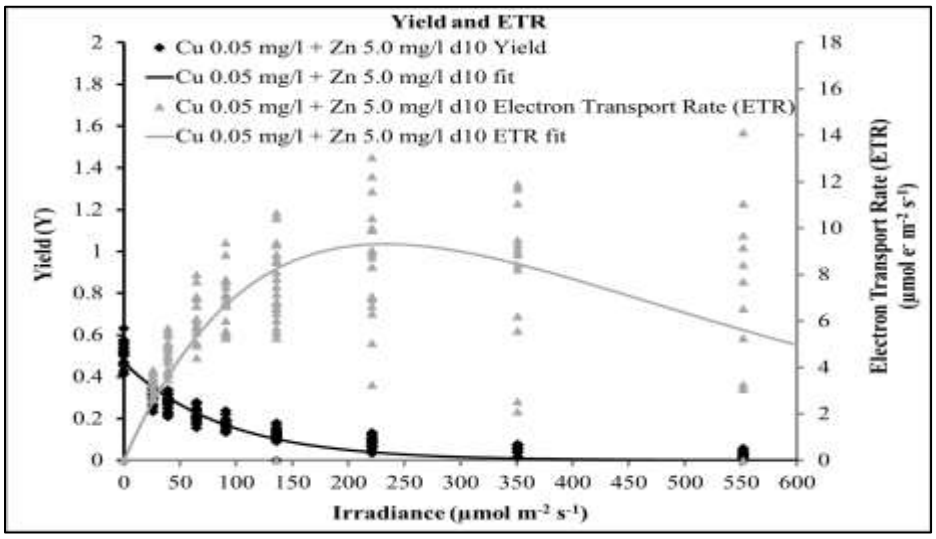

Figure A.73 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 10.

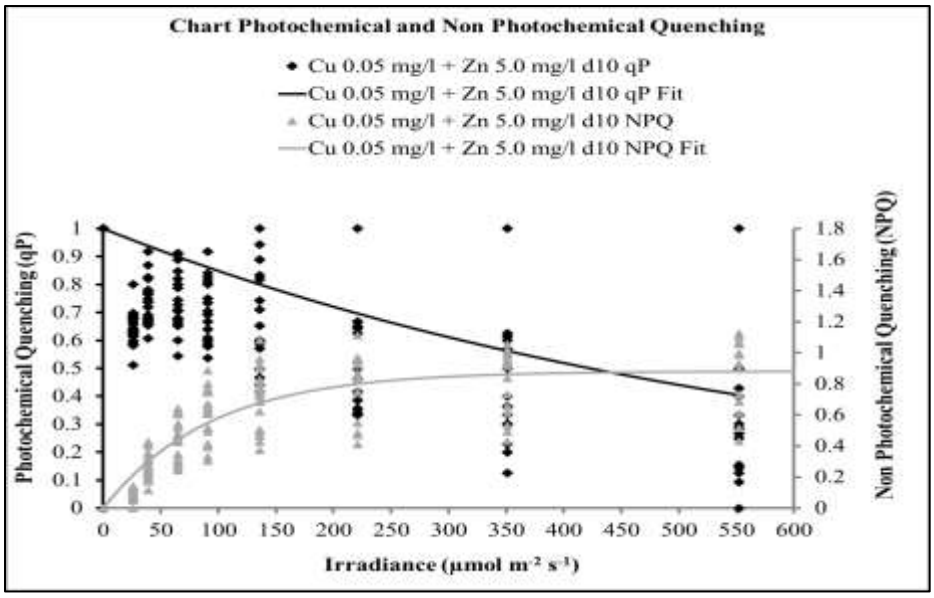

Figure A.74 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 10.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.47 ± 0.02 and electron transport rate (ETR_{max}) is 9.32 ± 0.46 µmol e^- m^{-2} s^{-1} on day 10 (Figure A.73).

Figure A.74 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 10. The half point of Photochemical Quenching (qP) is 243.28 ± 200.01 µmol photon m^{-2} s^{-1} and the half point of Non Photochemical Quenching (NPQ) is 64.48 ± 10.35 µmol photon m^{-2} s^{-1} on day 10.

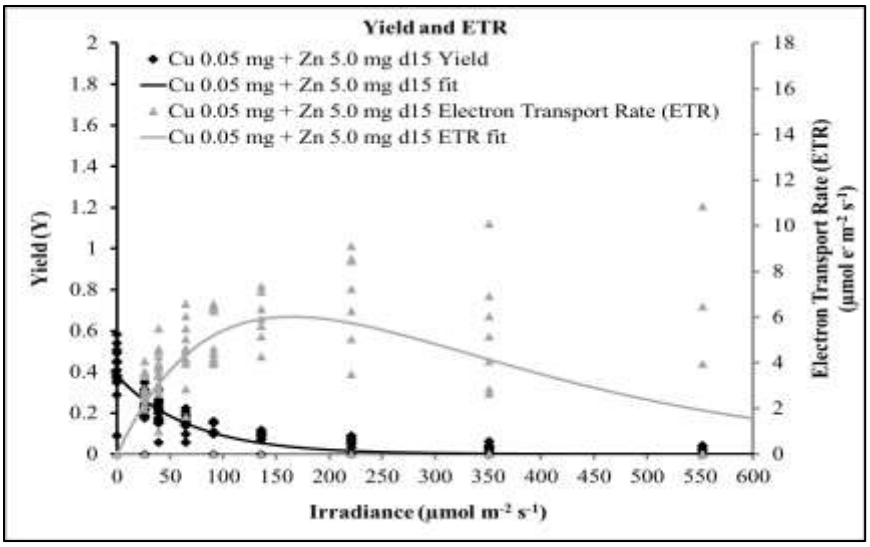

Figure A.75 Yield (Y) and irradiance (E), and the Photosynthetic Electron transport rate (ETR), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 15.

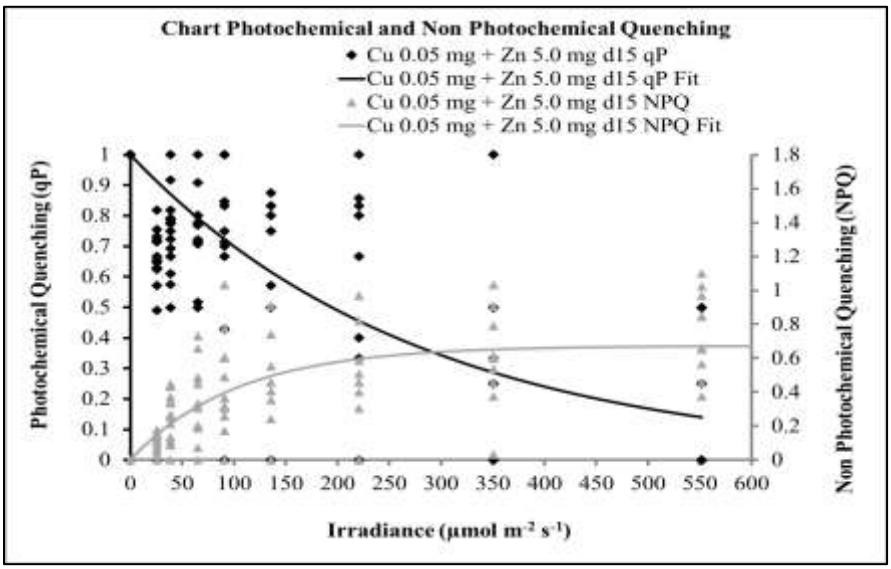

Figure A.76 Yield (Y) and irradiance (E), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu and Zn, respectively on day 15.

Photosynthesis parameters of *C. demersum* under 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment including the maximum yield (Y_{max}) is 0.38 ± 0.03 and electron transport rate (ETR_{max}) is 6.02 ± 0.65 µmol e^- m^{-2} s^{-1} on day 15 (Figure A.75).

Figure A.76 Photochemical Quenching (qP) and Non Photochemical Quenching (NPQ), of *C. demersum* in the 0.05 mg/l + 5.0 mg/l Cu plus Zn treatment, respectively on day 15. The half point of Photochemical Quenching (qP) is 194.59 ± 43.43µmol photon m^{-2} s^{-1} the half point of Non Photochemical Quenching (NPQ) is 70.88 ± 27.29 µmol photon m^{-2} s^{-1} on day 15. Yield values and ETR are both very low indicating acute metal stress. qP had large numbers of spurious values and the ½ point was high. These results indicate acute metal stress.

For this study it was found that, *C. demersum*, there was a more toxic effect by a combination of Cu and Zn at 0.1 mg/l + 5.0 mg/l on day 5 than in plants exposed to Cu or Zn separately. This means that when Cu and Zn were combined, it has more reaction to cause physiological stress in *C. demersum*. Hence, *C. demersum* was less tolerant of Cu and Zn whenCu and Zn were together than when exposed separately.

VITAE

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