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OXIDATIVE STRESS RESPONSES IN THE AQUATIC MACROPHYTE, *CERATOPHYLLUM DEMERSUM* L., AS BIOMARKERS OF METAL EXPOSURE

by

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DECLARATION

I, Judith Lize Arnolds, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

30 November 2017

Signed **Date**

ABSTRACT

Metal pollution in aquatic environments is considered a major environmental concern because of variation in several abiotic factors that impose severe restrictions on organisms living in these areas. *Ceratophyllum demersum* L. (family Ceratophyllaceae), a hornwort or coontail, free floating rootless macrophyte has been suggested a suitable model for investigating metal stress and was used in the current study. This study assessed the use of selected biological responses, namely antioxidant responses and changes in chlorophyll concentration in *Ceratophyllum demersum* L., as biomarkers of metal exposure, and also investigated the field application of these responses in the Diep River. The ultimate aim was also to determine the usefulness of *C. demersum* as model of metal contamination and as phytoremediator after a pollution event. An investigation of metal bioaccumulation in this macrophyte exposed to different concentrations of a combination of metals over a five-week exposure period in a greenhouse, was undertaken, as well as a field study in the Diep River, Milnerton, Cape Town and a pond (reference site) at the Cape Peninsula University of Technology, Cape Town, to validate experimental results. In the laboratory study the water was contaminated once off at the beginning of the study, to simulate a pollution event. The metal concentrations in the water and plants were measured in the four treatments and the control every week over a five-week exposure period. The samples were acid-digested and analysed with an Inductively-Coupled Plasma-Mass Spectrophotometer (ICP-MS). The results showed that concentrations of the metals in the water varied in all treatments over time with no specific patterns amongst the treatment groups. This macrophyte proved highly effective in the bioaccumulation of these metals at all four exposure concentrations. The metals bioaccumulated rapidly in the plants after the water was spiked.

The main focus of the study was to investigate the possible use of biochemical responses in *C. demersum* as possible biomarkers for metal exposure. A range of antioxidant/oxidative stress parameters were measured in the plant exposed to a combination of metals (Al, Cu, Fe, Zn) in four different treatments over the five week exposure period. Total antioxidant

capacity (TAC) was measured using Total Polyphenols (TP), Ferric Reducing Antioxidant Power (FRAP) and Oxygen Radical Absorbance Capacity assay (ORAC), enzyme activity was determined using Catalase (CAT), Superoxide Dismutase (SOD), Ascorbate Acid (AsA) and Total Glutathione (GSHt) and lipid peroxidation was measured by using Thiobarbituric Acid Reactive Substances (TBARS) and Conjugated Dienes (CDs). The cocktail of the four metals induced significant changes in the antioxidant defence system of *C. demersum*, including the antioxidant enzyme activities. The different metal exposures disturbed the cellular redox status in the plant. The current study has demonstrated that this macrophyte shows tolerance to metal-induced oxidative stress and that it can survive under relatively high concentrations of these metals by adapting its antioxidant defence strategies.

Chlorophyll was extracted in 80% chilled acetone in the dark and the absorbance values were determined using a spectrophotometer. Chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and total chlorophyll (chl *t*) contents were measured under different exposure concentrations of metals in the macrophyte. The results of this study indicated that chlorophyll contents were variable over the exposure period and no significant differences in chlorophyll concentrations were found between weeks.

A field study in the Diep River and the pond located at the CPUT campus (reference site) was conducted to validate experimental results. Plants in a polluted section of the Diep River were shown to bioaccumulate metals to high concentrations. Bioaccumulation of metals in *C. demersum* might have induced oxidative stress, and other environmental factors such as temperature- and chemical stress might have caused chlorophyll degradation. The chlorophyll concentrations in the plants of the pond (reference site) might also have been affected by temperature and chemical stress of the water. Significantly higher AsA, CAT, ORAC, SOD and TBARS concentrations in the Diep River plants might be an indication that the plants in the river might be well adapted to the constant exposure to metals and that the plants might have developed a tolerance mechanism to cope with oxidative stress compared to those of the pond.

The results show that metals are bioaccumulated quickly by *C. demersum* after the water is contaminated with metals, i.e. after the "pollution event". However, over time, metals are continuously exchanged between the plants and the water, accounting for the fluctuations in metal concentrations observed over time.

This study has shown that *C. demersum* has phytoremediation potential because it was able to remove high concentrations of metals from the contaminated water. Therefore, *C. demersum*, can be applied as a model for metal contamination and a phytoremediator after a pollution event. The potential to antioxidant responses and chlorophyll content as biomarkers of metal exposure in *C. demersum* have been demonstrated.

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DEDICATION

To GOD, the Almighty

If Heaven Was Never Promised

You may ask me why I serve the Lord,
Is it just for Heaven's gain.
Or to walk those mighty streets of gold,
And to hear the angels sing.

Is it just to drink from the fountain,
That never shall run dry.
Or just to live forever, ever, and ever,
In that sweet, sweet bye and bye.

Chorus

But if heaven never was promised to me,
Neither God's promise to live eternally.
It's been worth just having the Lord in my life.
Living in a world of darkness,
You came along and brought me the light.

If there were never any streets of gold,
Neither a land where we'll never grow old,
It's been worth just having the Lord in my life.
You've been my closest friend down through the years,
And every time I cry You dry my tears.

It's been worth just having the Lord in my life.
Living in a world of darkness,
living in a world of darkness,
You came along and brought me the light.

(Pastor Andrae Crouch, 1942-2015)

AND TO

My sister, Vivian M. Pretorius,

My brothers Trevor P. Anderson and Errol A. Anderson

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GLOSSARY

Terms/Acronyms/Abbreviations	Definition/Explanation
A	
6-HD	6-hydroxydopamine
AA	L-Ascorbic acid
AAE	Ascorbic Acid Equivalents
AAPH	2,2'azobis (2-amidinopropane) dihydrochloride
AsA	Ascorbic Acid
ATP	Adenosine triphosphate
C	
CAT	Catalase
CD	Conjugated diene
D	
DDT	Dichlorodiphenyltrichloroethane
DETAPAC	Diethylenetriaminepentaacetic acid
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
E	
EDTA	Ethylenediaminetetra-acetic acid
F	
FI	Fluorescein sodium salt
FRAP	Ferric reducing antioxidant power
G	
GR	Glutathione reductase
GSH	Reduced glutathione
GSHt	Total glutathione

GSSG	Oxidised glutathione
GSX-PX	Glutathione peroxidase
H	
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
L	
LP	Lipid peroxidation
M	
M2VP	1-methyl-2-vinylpyridinium trifluoromethanesulphonate
MDA	Malondialdehyde
MT	Metallothioneins
N	
NADPH	Nicotinamide adenine dinucleotide phosphate
ND	Not detected
NO	Nitric oxide
O	
O-PA	Orthophosphoric acid
ORAC	Oxygen radical absorbance capacity
P	
PBS	Phosphate buffer saline
PCA	Perchloric acid
PCB	Polychlorinated biphenyl
R	
ROS	Reactive oxygen species

S

SD	Standard deviation
SOD	Superoxide dismutase

T

TAC	Total antioxidant capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
t-BHP	Tertiary-butyl hydroperoxide
TE	Trolox equivalents
TPTZ	2,4,6-Tripyridyl-s-triazine

LIST OF TERMS AND CONCEPTS

Antioxidants	Compounds that act as scavengers of free radicals. Molecules that prevent oxidation of other molecules (Gupta & Sharma, 2006).
Bioaccumulation	The increase in concentration of a substance in exposed organisms over time (usually increasing over time and with age) (Wright & Welbourn, 2002).
Bioindicator	An organism or part of an organism that contains information on the quality of its environment (Siebert *et al*., 1995).
Biomarker	Any biological response to an environmental chemical below individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism (Van Gestel & Van Brummelen, 1996).
Biomonitor	An organism or part of an organism that quantifies the quality of its environment. This is done by reacting to certain changes in the environment which can be measured, like changes in its morphology or physiology of its metabolism. The organism reflects the exposure of the contaminant in the environment (Wright & Welbourn, 2002).
Free radical	Any molecule capable of independent existence that contains one or more unpaired electrons (Lobo *et al.,* 2010).
Lipid peroxidation	Oxidative degradation of lipids and propagating lipid chain breaking reaction initiated by the attack of free radicals (Mylonas & Kouretas, 1999).
Oxidative stress	Metabolic imbalance between the production of free radicals and their scavenging counteract antioxidants in favour of free radical overload and subsequent cellular changes (Betteridge, 2000).
Phytoremediation	The removal of contaminants and toxic waste from the environment by plants. The plants can then be harvested and discarded or metals can be extracted for a specific use (Salt *et al.,* 1995).
Reactive oxygen species	Any compound derived from oxygen which contains one or more unpaired electrons (Ray *et al.,* 2012).

CHAPTER 1: INTRODUCTION

1.1. Pollution in aquatic ecosystems

Water is one of the most important natural resources, and there are different demands upon it. The water molecule is comprised of two atoms of hydrogen and one of oxygen. Water moves from solid to liquid to gas depending on temperature. High evaporation and transpiration reduces availability of water and could cause water scarcity. Water covers 70% of the total surface area of the earth and a small amount of it is directly available as freshwater (Agnew & Anderson, 1992). Water is a limited resource and is becoming scarce in South Africa each day. According to Allanson (1995) it is estimated that by the year 2020 demand for water will probably exceed supply and the resources from well-watered countries will need to be handled with care. Careful management of our water resources especially in South Africa is needed if they are to be used for agriculture, domestic and industrial supply, commercial and sport fisheries, waste disposal and electricity supply (Rashed, 2008). Aquatic ecosystems such as rivers and oceans are the dumping grounds for the wastes of our industrialized society. These ecosystems form an essential part of our water resources.

Industrial development and agricultural activities in the last few decades caused huge loads of pollutants to be added to our rivers through anthropogenic activities such as over abstraction and disposing waste water into the water resources in such a way that the water resources become degraded and rendered unsafe for use (Kaushik *et al.,* 2009). South Africa has only a few rivers which are not over utilized, degraded or polluted. According to Ndiitwani (2004) perennial rivers like the Diep River, Western Cape, Republic of South Africa, have previously been over utilized that they now only flow seasonally and have reduced water quality. Water pollution means the change of the physical, chemical and/or biological properties of a water resource to make it unfit for use. Signs of water pollution are sometimes noticeable, even to the casual observer. Examples of water pollution could be the bad taste of drinking water, uncontrolled growth of aquatic weeds in water bodies, the decline of freshwater fish numbers and the odours that rivers and lakes emit from being polluted (Ndiitwani, 2004).

Pollution is the damage that results because of the presence of a substance or substances where they would not normally be found or because they are present in larger than normal quantities. Polluting substances may occur as a solid, liquid or gas. Pollution of the aquatic environment occurs from many different sources. When pollution comes from a single

source, such as an oil spill, it is called point-source pollution. Most types of pollution affect the immediate area surrounding the source. In some instances the pollution may affect the environment hundreds of miles away from the source, e.g. nuclear waste, and this is called transboundary pollution (EPA, 2012). Point source and non-point (i.e. diffuse) source are the main types of aquatic pollution. Point source type of pollution is caused by runoff generated by sewage treatment works and industries, as well as leachates generated by waste disposal sites or mines. These points are mostly in the form of a pipeline or discharge point, and are easily detectible. Non-point pollution sources occur when water flows over the surfaces collecting particles and dissolved material from the rocks and plant cover and releases this into the river (Allanson, 1995). This form of pollution is mainly from storm water runoff from towns, informal settlements, villages, agricultural areas, and through dumping waste directly into the water. This is essentially connected to the pollution of organic waste, siltation, nutrients and pesticides (Shieh *et al.,* 1999). According to Stephens and Bredenkamp (2002) irrigation return flows might become contaminated with fertilizers and salts into the water resources. When wastewater is used for irrigation, some may leach through the soil and ions from wastewater may contaminate the groundwater or runoff into surface water (DWAF, 1995; Pearce & Schumann, 2001).

1.2. Metal pollution in aquatic ecosystems and aquatic plants

Environmental pollution is known to be one of the major problems in urban areas, and metals, depending on their oxidation state, can be highly reactive and can consequently be toxic to most organisms (Radwan *et al.,* 2010). Metals are released by a variety of anthropogenic sources such as industrial activities, traffic and the burning of fossil fuels which suggests an increasingly important role for metal pollution (Pinto *et al.,* 2003). They have long residence times in soils and are able to continue exerting harmful effects on the environment (Menon *et al.,* 2007). Metals represent a potential threat to human health (Jarup, 2003) long after the source of pollution has ceased to operate (Radwan *et al.,* 2010).

Over the past few decades more and more concerns have been raised about the occurrence and adverse effects of metal pollution in aquatic systems (Guecheva *et al.,* 2003; Zhou *et al.,* 2008). Rapid industrialization and urbanization have caused elevated levels of metals in the biosphere (Lu *et al*., 2004). Metal pollution in aquatic environments is considered a significant environmental issue because of a variation in several abiotic factors that impose severe restrictions to organisms living in these areas (Matthiesen & Law, 2002). Metal pollution is of major concern because some may change into persistent metallic compounds with high toxicity and can be bioaccumulated in aquatic organisms and be increased in the

food chain and could threaten human health (Jin, 1992; Zhou *et al.,* 2008; Kaushik *et al.,* 2009). Numerous harmful effects such as fetal abnormalities, reproduction failure and immunodeficiency have been demonstrated due to aquatic metal exposure (Chang, 2000; Zhou *et al.*, 2008). Therefore, research, monitoring and prevention of metal contamination of aquatic systems are some of the biggest concerns for environmentalists today. Metals from geological origin can naturally enter the river system by weathering or erosion (Zhang *et al.,* 1993), or be produced by the slow leaching from soil/rock water at low concentrations with no serious toxic effects on human health (Zhou *et al.,* 2008). They can also be produced through anthropogenic activities due to industrial processing, mining, agricultural, urban activities and sewage disposal containing contaminants such as sewage, fertilizers, and metals that have proven to be very damaging to aquatic habitats and species (Abbasi *et al.,* 1998). High concentrations of mercury (Hg), chromium (Cr), lead (Pb), copper (Cu), zinc (Zn) and nickel (Ni) in aquatic systems are indicators of metal pollution (Liang *et al.,* 2004). An amount of trace metals is utilized by living organisms to stabilize protein structures, facilitate electron transfer reactions and catalyze enzymatic reactions (Ash & Stone, 2003; Torres *et al.,* 2008). Copper, zinc and iron (Fe) are essential constituents of catalytic sites for several enzymes, while other metals such as lead, mercury and cadmium (Cd) may displace or substitute for essential trace metals and interfere with correct functioning of enzymes and associated cofactors (Ash & Stone, 2003). Elements such as As, Cd, Co, Cu, Cr, Hg, Mn, Ni, Pb, Se and Zn are major environmental pollutants. These elements are considered to be potentially cytotoxic, mutagenic and carcinogenic although a few of them are essential for vital metabolic processes (Hadjiliadis, 1997; Devi & Prasad, 1998; Zhou *et al.,* 2008).

The bio-concentration of metals by aquatic macrophytes is of special concern to human health and for environmental protection and conservation (Ornes & Sajwan, 1993). The organisms that are most directly and adversely affected by toxic pollutants consist of organisms that live at the surface or near the bottom of aquatic habitats where pollutants tend to settle (EPA, 2008). In an aquatic system fast removal of these metals from the water to sediments may occur by settling particles, while other pollutants can be mobilized by accumulating into the biota from the sediments (Kaushik *et al.,* 2008). Macrophytes can cover large areas and is the dominant primary producers in aquatic environments. Submerged macrophytes growing in polluted water bodies can absorb the toxic xenobiotics which enter the food chain, posing a serious threat to human health (Gupta & Chandra, 1998). *Ceratophyllum demersum* L. (family Ceratophyllaceae), a hornwort or coontail, is a submerged, free floating rootless macrophyte. It is a perennial plant, of cosmopolitan distribution and grows rapidly in shallow, muddy, quiescent water bodies at low light

intensities. As *Ceratophyllum demersum* L. is rootless, it is therefore advantageous for use in laboratory bioassays as this would eliminate the complication of soil-root-continuum and shoot-root metal partitioning. It thus serves as a suitable model system for investigating metal stress (Aravind *et al.,* 2009).

Macrophytes are considered to be important components of the aquatic ecosystem, not only as a food and oxygen source, and habitat for aquatic invertebrates and fish, but as efficient accumulators of metals (Rai, 2009). Aquatic macrophytes play an essential role in structural and functional aspects of aquatic ecosystems in various ways. The ability of these plants to absorb metals makes them interesting research candidates especially for treatment of industrial effluent and sewage waters through the process of phytoremediation (Andra *et al*., 2010). Submerged macrophytes possess significant potential to bio-accumulate metals due to their bigger surface area compared to non-submerged plants (Sinha *et al*., 1997; Dhir *et al*., 2009). Several submerged macrophyte species, such as *Ceratophyllum demersum* (Keskinkan *et al*., 2004), *Myriophyllum spicatum* (Keskinkan, 2005), *Potamogeton spp.* (Fritioff & Greger, 2006; Peng *et al*., 2008; Monferrán *et al*., 2012) have been used to test their accumulation potential. In the aquatic environment, macrophytes are seldom exposed to a single metal and in most cases the stress of pollution may be attributed to the effect of a combination of metals (Sinha *et al*., 2003). Therefore, there must be several differences in the accumulation capacity of submerged macrophytes after exposure to a single metal or a combination of different metals. Bioavailability of metals is the proportion of total metals that are available for incorporation into biota (bioaccumulation). There are several factors that may affect metal bioavailability to aquatic organisms and plants. The most important factors seem to be the metal concentrations of solutions, solute metal speciation, temperature, pH and redox potential (Louma, 1983). Therefore, bioaccumulation of metals depend on numerous biotic and abiotic factors, such as temperature, pH and dissolved ions in water and bioavailability (Xing *et al*., 2013). According to Demirezen and Aksoy (2004) there is a relationship between cadmium concentration in *Potamogeton pectinatus* and water pH. Several studies conducted on aquatic plants have indicated that the aquatic plant often accumulates much higher concentrations of metals than the surrounding medium (Demirezen & Aksoy, 2006). Soares *et al*. (2008) reported on *Salvinia auriculata* (a non-submerged macrophyte), which has the capacity to bio-accumulate large concentrations of chromium in its leaves.

1.3. Plants as biomonitors of metal pollution

The application of aquatic plants as biomonitors constitutes a tool for investigation in ecological research, applied to the conservation of coastal or littoral ecosystems. Currently, studies in both the laboratory and the field have provided positive insights into the capacity of aquatic plants to act as biomonitors of environmental quality, through the use of biomarkers (Rainbow & Phillips, 1993; Ferrat *et al.,* 2003). Plants play an essential role in ecosystems and these organisms have been underemployed for the diagnosis or prediction of the deleterious consequences of human activities, although physiological processes, biochemical response and mechanisms of adaptation or mortality can be employed to evaluate the quality of a medium (Vangronsveld *et al.,* 1998; Ferrat *et al.,* 2003). Plants are sedentary, sensitive to environmental changes and respond, as primary stages of the food chain, more rapidly to the presence of pollutants than organisms living at higher stages (Lovett *et al.,* 1994). Aquatic plants can play a significant role in metal removal via filtration, absorption, cation exchange, and through plant-induced chemical changes in the rhizosphere (Dunbabin-Bowmer, 1992; Wright & Otte, 1999).

The application of macrophytes is significant in the biomonitoring of metal contamination (e.g. *Fucus vesiculosus* (L.), *Ascophyllum nodosum* (L.) Le Jol., *Sargassum* sp., *Ulva lactuca*) (Ferrat *et al.,* 2003). The mechanisms of accumulation of these metals have been studied under laboratory conditions (e.g. *Padina gymnospora* (Kutzing) Vickers and *Ulva lactuca*) (Amado-Filho *et al.,* 1997), and under natural conditions: e.g. *Caulerpa taxifolia* (Vahl) C. Agardh (Gnassia-Barelli *et al.,* 1995); *Cystoseira* sp. (Catsiki & Bei, 1992); *Fucus vesiculosus* (Ostapczuk *et al.,* 1997); *Padina pavonica* (Campanella *et al.,* 2001); *Ulva lactuca* (Catsiki & Papathanassiou, 1993); *Ulva rigida* C. Agardh (Favero *et al.,* 1996). Macrophytes accumulate pollutants via their below ground biomass submerged in sediments (Biernacki *et al.,* 1996; Salt, 1998) and absorb chemicals from the water through their leaves (Biernacki *et al.,* 1996). These plants can be reliable indicators of metal pollution in freshwater ecosystems (Ray & White, 1976; Franzin & McFairlane, 1980). The degree of metal uptake by some plants is dependent on the type of metal and plant species involved (Mortimer, 1985). Species differ inherently in their sensitivity to toxicants and these differences have been recognized as useful tools for determining environmental quality standards and for use in ecological risk assessment (Posthuma *et al.,* 2002). Organisms living in chronically polluted sites may be exposed to low concentrations of pollutants for long periods in the natural environment. Persistent hydrophobic chemicals and metals may accumulate in aquatic organisms through different mechanisms, by uptake directly from water, through uptake of suspended particles or by the consumption of lower trophic level

organisms (Torres *et al.,* 2008). These species play a significant role in biogeochemical cycling of toxic elements and are being increasingly considered for environmental phytomanagement (Prasad *et al.,* 2006).

1.3.1. Macrophytes as biomonitors in freshwater ecosystems

Research into the accumulative properties of water plants have been conducted for many years in order to use them in biomonitoring and phytoremediation of waters contaminated by heavy metals. The research has focused on sorption mechanisms, factors influencing the process of kinetics and equilibrium and structure and habitat (Krems *et al.,* 2013). Several studies have concentrated on *Ceratophyllum demersum* L. as bioaccumulator of metals (Gupta & Chandra, 1996; Keskinkan *et al*., 2004; Kumar & Prasad, 2004; Mishra *et al*., 2006; Erasmus, 2012; Fawzy *et al.,* 2012). Many biomonitoring studies of fresh water ecosystems have been done with the use of submerged or floating macrophytes such as *C. demersum* L., *Lemna minor*, *Potamogeton pectinatus* and *Myriophyllum spiactum* as indicators of aquatic pollution (Krems *et al.,* 2013). These plants accumulate metals in their organs and show the physical condition of the environment. According to Krems *et al.* (2013) bioaccumulation is a slow process that can last many days. Studies of metal accumulation by *Potamogeton pectinatus* and *Potamogeton malaianus* showed that they accumulate on average 92% Cd, 70% Cu and 67% Zn in the initial solution (Peng *et al.,* 2008). *C. demersum* kept in Selene solution with concentration of 0.13 mmol/dm^3, after 31 days bioaccumulated 0.0062 ±0.0011 mmol/g d.m., whereas *Myriophyllum spicatum* after 13 days bioaccumulated 0.0027 ±0.0001 mmol/g d.m. of the analyte (Mechora *et al.*, 2011). Aquatic plants are therefore suitable organisms for biomonitoring of metal pollution in aquatic ecosystems. Macrophytes are visible, abundant, sedentary and easy to collect. These plants are able to bioaccumulate and tolerate high concentrations metals in their anatomical parts.

1.4. Phytoremediation

Phytoremediation can be defined as the use of green plants to remove pollutants from the soil and waters or to render them harmless (Salt *et al*., 1995; Lone *et al*., 2008). This technology also referred to as the green technology, can also be applied to both organic and inorganic contaminants in the soil, water and air (Salt *et al*., 1998; Gratao *et al*., 2005). Plants can thus be compared to solar driven pumps capable of removing and concentrating certain

elements from their environment (Salt *et al*., 1995). Phytoremediation has gained increasing attention as an emerging and cheaper technology (Lone *et al*., 2008). Pollution of the aquatic and terrestrial environments by metals is of serious concern to the developing world (Mohamed *et al*., 2012). Huge efforts have been made in the last two decades to reduce sources of contamination and remedy the polluted soil and water resources by developing techniques that are easy to use, sustainable and economically feasible (Lone *et al*., 2008). Several complications for remediation at a large scale were experienced because of high costs and side effects. This study partly aims to demonstrate the phytoremediation potential of *Ceratophyllum demersum* L. exposed to different metals. This effect was studied with reference to selected biochemical parameters and physico-chemical parameters in a laboratory experiment and in the water from the Diep River.

1.5. Biomarkers

Van Gestel and Van Brummelen (1996) defined a biomarker as any biological response to an environmental chemical below individual level, measured inside an organism or in its products (urine, faeces, hairs, feathers, etc.), indicating a departure from the normal status, that cannot be detected from the intact organism. Biomarker applications in monitoring programmes for environmental quality is increasingly common (Amiard *et al.,* 1998; Ferrat *et al.,* 2003) and are essential tools for exposure identification. Evaluating the risk of pollutant exposure in wildlife and human populations involves the measurement of specific chemical deposits in soil, water or air or in tissues of habituating populations, which is time consuming and often not a good indicator of the bioavailability of a chemical. In contrast to the simple measurement of contaminants accumulating in tissues, biomarkers can provide more comprehensive and biologically more relevant information on the potential impact of toxic pollutants on the health of organisms (van der Oost *et al.,* 1996; Ferrat *et al.,* 2003; Kakkar & Jaffery, 2005). Biomarkers can be used as early warning signals for general or particular stress (Vangronsveld *et al.,* 1998). Early laboratory and field studies have indicated that several biomarkers are sensitive indicators of stress conditions resulting from contaminant exposure in organisms (Reinecke *et al.,* 2007). Biomarkers are used in an attempt to define and measure the effects of pollution, for example metals in rivers. Biomarker response can be regarded as biological or biochemical effects after exposure to a toxicant, which theoretically makes them useful indicators of exposure and effects (Van der Oost *et al.,* 2003).

When organisms are exposed to environmental contaminants, molecular, biochemical, and/or physiological, compensatory mechanisms may become operative and can result in inhibition or facilitation of one or more physiological processes or functions and/or structural changes. Changes in a range of biochemical and physiological parameters at sub-organismal level could be useful for identifying and predicting the impact of pollutants and variations in biomarker responses to pollutant exposure and have been demonstrated in several studies (Black *et al.,* 1996). There is no single species or monitoring system most sensitive or suitable for the detection of all potential toxicants (Kramer & Botterweg, 1991; Forbes & Forbes, 1994) in polluted aquatic or terrestrial systems. Biomarker response can mirror the stress in organisms and thus act as more precise indicators of the environmental status than that of chemical analysis. Therefore, chemical measurements need to be complemented with biochemical assays in a multidisciplinary approach to assess water contamination (Hallare *et al.,* 2005).

1.6. Metal-induced oxidative stress in plants

Plants are exposed to natural climatic or edaphic stresses, for example high irradiation, heat, chilling, late frost, drought, flooding and nutrient imbalances. Several of these stress factors may fluctuate significantly in intensity and duration on time scales of hours, days, seasons, or years. Some may change slowly and gradually affect plant growth conditions. Plants have limited mechanisms for stress prevention because they are sessile and they need flexible methods for acclimation to changes in environmental conditions (Schützendübel & Polle, 2002).

Oxidative stress is defined as an imbalance between oxidants and antioxidants and can potentially lead to damage in organisms (Sies, 1997). Oxidants are produced as a normal product of aerobic metabolism but can be formed at high levels under stressful physiological conditions. Antioxidant defense is in part, able to adapt to changing needs (Sies, 1997). Oxidative stress responses against environmental stress in organisms are considered early warning indices of pollution in the environment (Maity *et al.,* 2008). It is important to understand the mechanisms contributing to stress tolerance to improve the protection of the plant (Schützendübel & Polle, 2002). The redox state of the cell is mainly dependent on an iron (and copper) redox couple and is maintained within strict physiological limits (Park *et al*., 2009). Recent studies have found that transition metals act as catalysts in the oxidative

reactions of biological macromolecules and as a result the toxicities associated with these metals might be due to oxidative tissue damage.

Redox-active metals such as iron (Fe), copper (Cu) and chromium (Cr) undergo redox cycling whereas redox-inactive metals, such as lead (Pb), cadmium (Cd), mercury (Hg) and others deplete cells' major antioxidants, especially thiol-containing antioxidants and enzymes (Ercal *et al*., 2001). Homeostasis of metal ions is maintained by tightly regulated mechanisms of uptake, storage and secretion and is therefore critical for life and is maintained within strict limits (Bertini & Cavallaro, 2008). Metal ion carriers participate in maintaining the required levels of the various metal ions in the cellular compartments (Rolfs & Hediger, 1999). Several studies in the past have indicated that redox active metals such as Fe, Cu, Cr, Co and other metals undergo redox cycling reactions and have the ability to produce reactive radicals such as superoxide anion radical and nitric oxide in biological systems. Fenton-like reactions appear to play a major role in oxidative stress experienced in redox-metal toxicity (Liochev, 1999). Disturbance of metal ion homeostasis could lead to oxidative stress, a state where increased formation of ROS overpowers body antioxidant protection and consequently induces DNA damage, lipid peroxidation, protein alteration and other effects (Jomova & Valko, 2011). The process of breakdown of metal-ion homeostasis has caused a lot of diseases (Halliwell & Gutteridge, 1990; Matés *et al*., 1999; Valko *et al*., 2005).

Iron is essential for cell growth, oxygen utilization, several enzymatic activities and responses of immune systems. Regardless of this iron is an abundant metal in food, but more than 2 billion people worldwide suffers from anemia (Stoltzfus, 2001). According to Toyokuni (1996) iron deficiency results in impaired production of iron-containing proteins, the most important of which is hemoglobin. Cellular iron shortage inhibits growth and as a results leads to cell death. Redox-inactive toxic metals, Pb, Hg and Cd all have electron-sharing affinities that can result in the formation of covalent attachments (Bondy, 1996). These attachments are generally formed between metals and sulfhydryl groups of proteins (Quigg, 1998). Lead cannot willingly undergo changes. The mechanisms that enable lead to induce oxidative stress are not clear (Gurer & Ercal, 2000). In an earlier study several metals were shown to increase the rate of essential fatty acid oxidation. In this study lead was found to be ineffective (Willis, 1965), and in a later study lipid peroxidation was examined by malondialdehyde (MDA) analysis and was found to be increased by Pb (Gerber *et al*., 1978; Rehman, 1984; Sandhir & Gill, 1995; Yin & Lin, 1995).

Cadmium (Cd) is a nonessential metal. This metal can be found in foods (vegetables, grains and cereals), water and tobacco leaves and is also a product of zinc and lead mining and

smelting (Stohs *et al*., 2000). Cadmium is widespread in nature, it can be ingested or inhaled but since Cd is not a redox-active metal like lead, its oxidant role is not clear. This metal has a long biological half-life (10-30 years) and is excreted very slowly from the body (Jones & Cherian, 1990). The mechanisms responsible for Cd-induced toxicity may be multifactorial. Proposed mechanisms for Cd-induced oxidative stress can be examined in three groups: 1) Adverse effects of cadmium on cellular defense systems and thiol status, 2) Enhancement of lipid peroxidation by cadmium, 3) Toxic effects of cadmium on cellular enzymes. Several isoforms of metallothioneins (MT's), which are known to protect cells from oxidative stress, exist. MT's are quite cysteine-rich and metals have a high affinity for thiols. MT's are known to sequester metals (Simpkins, 2000). Metals (especially Cd) are stored as a Cd-MT complex in the liver (Klaassen & Liu, 1997). A few studies have indicated that Cd changes GSH levels. GSH protects cells against oxidative stress and any change in GSH levels (increase or decrease) indicates an unstable oxidant status (Ercal *et al*., 2001).

Sulfhydryl reactivity is one of the most important mechanisms for Hg-induced oxidative damage. Both Hg^{2+} and MeHg form covalent bonds with GSH and the cysteins residues of proteins once it is absorbed in the cell. The primary intracellular antioxidant and the conjugating agent, GSH, were shown to be depleted and to have decreased function in Hg toxicity. One Hg ion can bind to and cause irreversible excretion of up to two GSH molecules (Quig, 1998). GSH functions as a primary line of cellular defense against Hg compounds. The release of Hg ions from complexes with GSH and cysteine causes greater activity of the free Hg ions disturbing GSH metabolism and injuring cells.

Continuous exposure to metals by aquatic biota can cause problems that have harmful effects on the exposed organisms. Mortality tests are important in acute toxicity and the observation of physiological or morphological changes at cellular or organ level for chronic exposure is used to accompany chemical analysis. Should the target molecule be a part of the defense, repair or detoxification apparatus of the cell, it becomes a direct and specific marker of exposure and effect (biomarker) (Guecheva *et al.,* 2003). Little information is available on the physiological effects of induced oxidative stress on *C. demersum* by a combination of metals.

Currently research focuses on the identification of stress biomarkers in aquatic plants. Totally or partially submerged aquatic plants have been studied principally from lagoon or estuarine ecosystems, under stress of various origin such as light, hydric/haline stress, herbicides, metals and organic contaminants (Ferrat *et al.,* 2003). The whole plant or leaves are being used for research on biomarkers. There is however very little information on the stress

response in different parts of the plant. Stress responses can vary according to the degree of exposure and the physiological role of the different parts of the plant (Pflugmacher *et al.,* 1999a). Principal biomarkers are used to test 'measurable responses' that takes place during photosynthetic activity, enzymatic processes of nutrition, secondary metabolite synthesis, oxidative stress and/or detoxication mechanisms (Ferrat *et al.,* 2003). In contaminated aquatic ecosystems, macrophytes act as biofilters of contaminants (Doust *et al.,* 1994; Ribeyre *et al.,* 1994) and they are reported to accumulate trace metals (thousand to several thousand folds) that are toxic to organisms when present in easily available form in the interstitial waters (Devi & Prasad, 1998). In this study the tolerance capacity of *C. demersum* to a combination of metals were tested.

1.7. Oxidative stress biomarkers

The use of oxidative stress biomarkers is of potential interest for assessing the impact of contaminants or seasonal variation in animals (Regioli & Principato, 1995; Verlecar *et al.,* 2008) or plants under field conditions. Significant changes in activities of antioxidant defense systems have been found in many species of animals in response to several factors other than metal pollutants. These include physiological stress of anoxia (Hermes-Lima, 2004), estivation (Nowakowska *et al*., 2009), extended heat stress (Luschak & Bagnyukova, 2006), chilling (Joanisse & Storey, 1996), and seasonal changes (Verlecar *et al.,* 2008). The relationship between metals and the mechanisms of the antioxidant defense systems plays a significant role in the eco-toxicological response of an organism to its environment (Regoli *et al.,* 2006). Therefore, studies on the relationships are important and they are suitable for identifying biomarkers that can serve as early warning systems for environmental monitoring. The evaluation of oxidative stress biomarkers is a key question in the study of oxidative stress in organisms (Luschak, 2011). Molecular biomarkers of oxidative stress found widespread applications in mechanisms of environmental toxicity and eco-toxicity in aquatic organisms exposed to a variety of chemical pollutants (Livingstone, 2001). Molecular biomarkers are used to test oxidative damage in biomolecules and various aspects of oxidative stress by free radicals in experimental animals. In addition to using primary and secondary products of free radical damage, biomarkers can monitor the status of various antioxidant defense mechanisms against free radicals. Living organisms have the ability to synthesize and control specific enzymatic systems which can be used for repair and removal of damaged proteins, lipids and DNA (Fenech & Ferguson, 2001). Also, since oxidative stress levels may vary from time to time, organisms are able to adapt to such fluctuating

stresses by inducing the additional synthesis of antioxidant enzymes to regulate oxidative damage (Young & Woodside, 2001; Martins *et al.,* 1991). Several studies have indicated that aquatic plants serve as suitable models for the assessment and monitoring of metal toxicity in plants (Aravind & Prasad, 2003; Kara, 2005; Hou *et al*., 2006 Mishra & Tripathi, 2007). Polyphenols have antioxidant activities and act as antioxidants *in vitro* by sequestering metal ions and by scavenging reactive oxygen and nitrogen species (Wiseman *et al*., 1997; Frei & Higdon, 2003). Polyphenols are secondary metabolites found in plants and protects the plants against ultraviolet radiations or against attack by pathogens (Beckman, 2000). In the following section the role of enzymatic and non-enzymatic antioxidants in plants will be discussed.

1.8. The role of enzymatic and non-enzymatic antioxidants in plants

1.8.1. Enzymatic antioxidants

1.8.1.1. Superoxide dismutase (SOD)

Metalloenzyme, SOD, is the most efficient intracellular enzymatic antioxidant which is abundant in all aerobic organisms and in all subcellular compartments prone to ROS-mediated oxidative stress. The SODs (Table 1.1) are the first enzymes in the ROS detoxifying process that converts O_2^- to H_2O_2 in the cytosol, chloroplast and mitochondria. SOD plays an axial role in cellular defense mechanisms against the risk of OH^- formation (Salin, 1998; Gratão et al., 2005). This was first validated in maize which comprised six genetically and biochemically clear isozymes (Scandalios, 1i993). It is well documented that several environmental stresses often lead to the increased generation of ROS, where, SOD has been suggested to be important in plant stress tolerance and provide the first line of defense against the toxic effects of elevated levels of ROS (Gill & Tuteja, 2010). SOD catalyse the disproportionation of superoxide radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and O_2 and prevent oxidative damage to organisms and consequently are important for plant stress tolerance (Bowler *et al.,* 1994). The SODs remove O_2^- by catalyzing its dismutation, one $O_2^{\cdot-}$ being reduced to H_2O_2 and another oxidized to O_2 (Table 2). It removes $O_2^{\cdot-}$ and consequently decreases the risk of OH^- formation via the metal catalyzed Haber-Weiss-type reaction (Scheme 1).

Scheme 1:

Haber-Wiess reaction:

$H_2O_2 + .O^{2-} \rightarrow .OH + OH^- + O_2$

Fenton reaction:

$H_2O_2 + Fe2^+(Cu^+) \rightarrow Fe^{3+}(Cu^{2+}) + .OH + HO^-$

$O2 + Fe3^+(Cu2^+) \rightarrow\bullet\ Fe2^+(Cu^+) + O_2$

The Haber- Weiss reaction has a 10,000 fold faster rate than spontaneous dismutation (Gill & Tuteja, 2010).

Table 1.1. Major reactive oxygen species scavenging antioxidant enzymes (Gill & Tuteja, 2010)

Enzymatic antioxidants	**Enzyme code**	**Reactions catalyzed**
Superoxide dismutase (SOD)	EC 1.15.1.1	$O_2^{\bullet-} + O_2^{\bullet-} + 2H^+ \rightarrow 2H_2O_2 + O_2$
Catalase (CAT)	EC 1.11.1.6	$H_2O_2 \rightarrow H_2O + ½\ O_2$
Ascorbate peroxidase (APX)	EC 1.11.1.11	H_2O_2 +AA $\rightarrow 2H_2O$ + DHA
Guaicol peroxidase (GPX)	EC 1.11.1.7	H_2O_2 + GSH $\rightarrow H_2O$ + GSSG
Monodehydroascorbate reductase(MDHAR)	EC 1.6.5.4	MDA + NAD (P)H $\rightarrow$ AA $NAD(P)^+$
Dehydroascorbate reductase (DHAR)	EC 1.8.5.1	DHA + 2GSH $\rightarrow$ AA + GSSG
Glutathione reductase (GR)	EC 1.6.4.2	GSSG + NAD(P)H $\rightarrow$ 2GSH + $NAD(P)^+$

Three classes of SODs are known in plants. SODs are classified by their metal cofactors into: the copper/zinc (Cu/Zn-SOD) (Table 1.2), the manganese (Mn-SOD) and the iron (Fe-

SOD) SODs, which is localized in different cellular compartments (Mittler, 2002). The CuZnSODs are localized in the cytosol, chloroplasts, nucleus and apoplast; the MnSODs in the mitochondria and peroxisomes; and the FeSODs in the chloroplasts (Kliebenstein *et al.,* 1998; Moran *et al.,* 2003). Moran *et al*. (2003) found that FeSOD (VuFeSOD) is also localized in the cytosol of cowpea root nodules and may become an important defensive mechanism against oxidative stress associated with senescence of nodules. Chloroplast CuZnSOD and FeSOD were proposed to catalyse the same chemical reaction but to be functionally different. FeSOD has been suggested to protect chloroplasts from superoxide radicals produced by the photosynthetic electron chain, while CuZnSOD has been associated with the protection from radicals produced during dark metabolism or chloroplast biogenesis (Kurepa *et al.,* 1997). FeSOD class occurs in some plant families such as Aceraceae, Gingkoaceae, Nymphaceae and Cruciferaceae, but there is no evidence on the presence of FeSOD in either rice or maize (Niewiadomska *et al.,* 1997; Alscher *et al.,* 2002). Among a number of theories proposed to explain the apparently random occurrence of FeSOD, the most plausible theory is that the SODB gene (encoding FeSOD) exists in all plant species, but it is not expressed constitutively, and environmental determinants can lead to preferential expression of one of the plastid-located SODs (Kurepa *et al.,* 1997).

Table 1.2. Different superoxide dismutase's in cell organelles (Gill & Tuteja, 2010)

SOD isozymes	**Location**	**Resistant to**	**Sensitive to**
Fe-SOD	Chloroplast	KCN	H_2O_2
Mn-SOD	Mitochondria and Peroxisomes	KCN and H_2O_2	-
Cu/Zn-SOD	Chloroplast and Cytosol	-	H_2O_2 and KCN

Studies dealing with changes in SOD activity under metal stress have been restricted to mostly the determination of the total activity of the enzyme (Rucin´ska *et al.,* 1999). Metal stress has been restricted to mostly the determination of the total activity of the enzyme (Rucin´ska *et al.,* 1999; Schickler & Caspi, 1999; Lidon & Teixeira, 2000; Dra¸zkiewicz *et al.,*

2004; Cho & Seo, 2005). Data on the response of activities of SOD classes to metals are non-synonymous. For example, a strong reduction of CuZnSOD activity was found in pea plants exposed to Cd (Sandalio *et al.,* 2001), while an opposite effect was exhibited in radish seedlings (Vitória *et al.,* 2001). The increase of CuZnSOD activity under Cu treatment occurred in tobacco cell cultures (Bueno & Piqueras, 2002). However, in leaves of *Pisum sativum* Cu excess did not affect CuZnSOD activity (Palma *et al.,* 1987). Earlier studies indicated that exposure of *Arabidopsis thaliana* plants to Cd and Cu excess resulted in fluctuating changes of total SOD activity in leaves, depending on the metal concentration (Dra˛zkiewicz *et al.,* 2004).

1.8.1.2. Catalases (CAT)

Catalases are one of the most important components of the plant's protective mechanisms that exist in the mitochondria and peroxisomes (Gupta *et al.,* 1993). This enzyme has an important role in the scavenging of free radicals especially H_2O_2 generated during photorespiration (Bowler *et al.,* 1992). CAT catalyzes H_2O_2 to H_2O and O^2 (Table 1.1) by two-electron transfer and prevents the generation of $OH^{\cdot}$ and protect proteins, nucleic acids and lipids against ROS (Imlay & Linn, 1988; Rastgoo & Alemzadeh, 2011). Catalases do not require a reducing substrate for their activity (Inzé & Van Montagu, 1995) and they are tetrameric heme-containing enzymes with the potential to directly dismutate H_2O_2 to H_2O and O_2 (Table 1) and is essential for ROS detoxification during stressed conditions (Grag & Manchanda, 2009). CAT has one of the highest yields for all enzymes: one molecule of CAT can transform ≈6 million molecules of H_2O_2 to H_2O and O_2 per minute (Gill & Tuteja, 2010). It is important in the removal of H_2O_2 generated in peroxisomes by oxidases involved in ß-oxidation of fatty acids, photorespiration and purine catabolism. Catalase isozymes have been studied widely in higher plants (Polidoros & Scandolios, 1999) e.g. 2 in *H. vulgare* (Azevedo *et al.,* 1998), 4 in *Helianthus annuus* and as many as 12 isozymes in *Brassica* (Frugoli *et al.,* 2007).

Catalases in plants can be classified into three classes: class I are most noticeable in photosynthetic tissues, and are involved in the elimination of H_2O_2 generated through the process of photorespiration; class 2 catalases are formed in the vascular tissues and may play a role in lignification. The exact role in biology is unclear. Class 3 catalases are abundant in seeds and young plants and their activity is related to the elimination of excessive water formed during fatty acid degeneration in the glyoxylate cycle in glyoxisomes (Willekens *et al*., 1994; Ahmad *et al.,* 2010). Maize has three isoforms/differentially regulated

catalases (CAT1, CAT2 and CAT3) that are found on separate chromosomes. According to Scandalios (1990) and Inzé & Van Montague (1995) the situation appears to hold true in dicotyledonous plants. The functional relationship between catalases of monocotyledonous and dicotyledonous plants is currently not clear and, as such, the nomenclature is arbitrary. CAT1 and CAT2 are localised in peroxisomes and the cytosol, whereas, CAT3 is found in the mitochondria (Willekens et al. 1994). Ali and Alqurainy (2006) reported that apart from reacting with H_2O_2, CAT also react with some hydroperoxides such as methyl hydrogen peroxide.

The varying response of CAT activity has been observed under metal stress. CAT activity declined in *Glycine max* (Balestrasse *et al.,* 2001), *Phragmites australis* (Ianelli *et al.,* 2002) and *Arabidopsis thaliana* (Cho & Seo, 2005), while CAT activity increased in *Oryza sativa* (Hsu & Kao, 2004) and in *Brassica juncea* (Mobin & Khan, 2007) under cadmium stress. A decrease of CAT activity was reported in *Anabaena doliolum* under NaCl and Cu^{2+} stress (Srivastava, 2005).

1.8.2. Non-enzymatic antioxidants

1.8.2.1. Ascorbic acid (AsA)

Ascorbic acid (AsA) is one of the most significant, abundant and water soluble antioxidants to prevent or minimize the damage caused by ROS in plants (Smirnoff, 2005; Athar *et al.,* 2008). It occurs in all plant tissues, and is usually higher in photosynthetic cells and meristems (and some fruits). The AsA concentration is reported to be highest in mature leaves with fully developed chloroplasts and highest chlorophyll concentration. It has been reported that ascorbic acid mostly remains available in the reduced form in leaves and chloroplast under normal physiological conditions (Smirnoff, 2000). According to Foyer and Noctor (2005) about 30 to 40% of the total ascorbate is found in the chloroplast and stromal concentrations and concentrations as high as 50 mM have been reported. AsA is one of the most essential antioxidants in plants and animals. It detoxifies ROS either directly or through the glutathione-ascorbate cycle. Ascorbate is involved in redox signalling, modulation of gene expression and the regulation of enzymes (Noctor, 2006; Foyer & Noctor, 2009). Ascorbate appears in a reduced form (ascorbic acid or vitamin C) and two oxidized forms (mono- and dehydro-ascorbic acid). The ratio between reduced and oxidized ascorbate is important for the ability of the plant to fight oxidative stress (Zechmann, 2011). The mitochondria in plants play an essential role in the metabolism of ascorbic acid. The mitochondria not only

synthesize ascorbic acid by the process of L-galactono-g-lactone dehydrogenase but also play a role in the regeneration of AsA from its oxidised forms (Smirnoff, 2000).

Regeneration of AsA is vital because fully oxidized dehydroascorbic acid has a short half-life and would be lost unless it is reduced. Ascorbic acid is considered as a very powerful ROS scavenger because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions. It can provide protection to membranes by directly scavenge $O_2^{\cdot-}$ and $OH^{\cdot}$ and by regenerating α-tocopherol from tocopheroxyl radicals. In chloroplasts, AsA acts as a cofactor of violaxantin de-epoxidase thus sustaining dissipation of excess excitation energy (Smirnoff, 2000). In addition to the importance of AsA in the ascorbic-glutathione (ASA-GSH) cycle, it also plays an important role in preserving the activities of enzymes that contain prosthetic transition metal ions (Noctor & Foyer, 1998). The AsA redox system consists of L-ascorbic acid, monodehydroascorbate (MDHA) and dehydroascorbate (DHA). Both oxidized forms of AsA are relatively unstable in aqueous environments while DHA can be chemically reduced by GSH to AsA (Foyer & Halliwell, 1976). Proof to support the actual role of dehydroascorbate reductase DHAR, GSH and glutathione reductase (GR) in maintaining the foliar AsA pool has been observed in transformed plants overexpressing GR (Foyer *et al.,* 1995). *Nicotiana tabacum* and *Populus* X *Canescens* plants have higher foliar AsA contents and improved tolerance to oxidative stress (Aono *et al.,* 1993; Foyer *et al.,* 1995). When ROS are formed inside plant cells during environmental stress situations, large amounts of dehydroascorbic acid can be produced by oxidation of ascorbic acid which moves the ascorbate pool more towards the oxidative state and decrease the oxidative capacity of plants (Zechmann, 2011). Furthermore, environmental stress conditions can change total ascorbate contents in plants which makes ascorbate an important biomarker of stress during abiotic and biotic stress situations (Vanacker *et al.,* 1998; Ratkevicius *et al.,* 2003; Bartoli *et al.,* 2006; Collin *et al.,* 2008).

Hydrogen peroxide (H_2O_2) within the plant cell can be detoxified by ascorbate peroxidase (APX). In this reaction the reduced form of ascorbate (Asc) is oxidized to monodehydroascorbate (MDHA). MDHA is then either reduced by monodehydroascorbate reductase (MDHAR) to Asc or, since very unstable, reacts to dehydroascorbate (DHA). DHA is reduced by dehydroascorbate reductase (DHAR) to Asc. In this reaction the reduced form of glutathione (GSH) is oxidized to glutathione disulfide (GSSG). GSSG is then reduced by glutathione reductase (GR) to GSH. The electron acceptor NADP is regenerated during the reduction of MDHA and GSSG by the respective enzymes. Asc and GSH are additional able to detoxify reactive oxygen species by direct chemical interaction. Thus, besides the total ascorbate level their redox state (reduced vs. oxidized state) which depends on the activity of

the described enzymes (grey boxes) is also very important for successful plant protection (Zechmann, 2011).

1.8.2.2. Glutathione (GSH)

Glutathione is one of the most important metabolites in plants which are considered essential in intracellular defenses against ROS-induced oxidative damage. Glutathione is freely available in the reduced form (GSH) in plant tissues and is localized in all cell components like cytosol, ER, vacuole, mitochondria, chloroplasts, peroxisomes as well as in the apoplast (Mittler & Zilinskas, 1992; Jimenez *et al.,* 1998). GSH plays a valuable role in various physiological processes such as regulation of sulphate transport, signal transduction, and conjugation of metabolites (Xiang *et al.,* 2001). It is well documented that GSH also plays a central role in several growth and development processes in plants which includes cell differentiation, cell death and senescence, pathogen resistance and enzymatic reaction (Rausch & Wachter, 2005). The synthesis of glutathione occurs in two ATP-dependent steps. First, glutamate-cysteine ligase (GCL) catalyzes formation of ɣ-glutamylcysteine from Cys and Glu which is thought to be the rate limiting step of the pathway. Second, glutathione synthetase (GS) adds Gly to g-glutamylcysteine to yield GSH. As synthesized, GSH provides a substrate for multiple cellular reactions that yield GSSG (i.e., two glutathione molecules linked by a disulfide bond). The balance between the GSH and GSSG is a central component in maintaining the cellular redox state (Foyer & Noctor, 2005). The function of GSH is to maintain the normal reduced state of cells to reduce the inhibitory effects of ROS-induced oxidative stress (Meyer, 2008). Furthermore, GSH plays a key role in the antioxidative defense system by regenerating another potential water soluble antioxidant, ascorbate, via the AsA-GSH cycle (Rauasch & Wachter, 2005). Several studies have indicated that when the intensity of a stress increases, GSH concentrations usually decline and the redox state becomes more oxidized, leading to the deterioration of the system (Tausz *et al.,* 2004). The role of glutathione in the antioxidant defense system provides a strong basis for its use as a stress biomarker. GSH levels have a major effect on the antioxidant function and it varies considerably under abiotic stresses. Strong evidence has indicated that elevated levels of GSH concentration are correlated with the ability of plants to withstand metal-induced oxidative stress (Gill & Tuteja, 2010). Studies have indicated that high antioxidant activity in leaves and chloroplast of *Phragmites australis* was associated with a large pool of GSH, which resulted in protecting the activity of photosynthetic enzymes against thiophilic bursting of Cd (Pietrini *et al.,* 2003).

1.8.2.3. Phenolic compounds

Polyphenols are diverse secondary metabolites such as flavonoids, tannins and lignin and are abundant in the tissues of plants. Phenolics have a perfect structural chemistry for free radical scavenging activity, and have proven to be more effective antioxidants *in vitro* than tocopherols and ascorbate (Schroeter *et al*., 2002; Ahmad et al., 2010). These compounds represent one of the most commonly occurring and abundant groups of plant metabolites which is an essential part of the human diet (Schroeter *et al*., 2002).

The antioxidative properties of polyphenols result from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (the chain-breaking function) and also from their capacity to chelate transition metal ions (the termination of the Fenton reaction) (Rice-Evans *et al.,* 1997). Then ability of flavonoids to change peroxidation kinetics by the modification of the lipid packing order and to limit the fluidity of the membranes is another mechanism triggering the antioxidative properties of phenolics (Schroeter *et al*., 2002). These modifications might sterically impede diffusion of free radicals and inhibit peroxidative reactions. These compounds that act as antioxidants could perform as terminators of free radical chains and chelators of redox-active metal ions that are capable of catalyzing lipid peroxidation (Schroeter et al., 2002; Ahmad *et al.*, 2010).

Phenolics could perform as terminators of the chain reaction by cooperating with other free radicals. Under certain circumstances for example, a high concentration of phenolic antioxidants, the presence of redox-active metals such as copper and iron and a high pH, phenolics may act as pro-oxidants. It was reported that phenolic compounds can be involved in the hydrogen peroxide scavenging cascade in plant cells (Ahmad *et al.,* 2010).

1.9. Oxidative stress biomarkers/parameters

Oxidative stress is described as an imbalance between pro-oxidative factors and reactive oxygen species (ROS) (Scandalios 1993). A number of parameters and biomarkers exist to determine the oxidative stress status, but the section below will focus on the biomarkers used in the current study. Total glutathione (GSHt), TBARS, CD's, ORAC, CAT, SOD and AsA were used to assess their usability as biomarkers of oxidative stress in this study.

1.9.1. Lipid peroxidation

Lipid peroxidation (LP) can be described as the oxidative deterioration of lipids containing a number of carbon-carbon double bonds (C=C) (Rice-Evans & Burdon, 1993). Lipid peroxidation disrupts biological membranes and is harmful to the functioning and structure of the membranes (Yoshikawa *et al.,* 2003). A large number of toxic by-products are formed during LP and affects a site away from their generation. These by-products can be measured by different assays. Thiobarbituric acid (TBA) is widely used to measure thiobarbituric acid-reactive substances (TBARS) of lipid peroxidation (Buege & Aust, 1978; Gray, 1978). Malondehaldehyde (MDA) and TBARS assays have been used extensively since the 1950's to determine peroxidation of lipids in membrane and biological systems (Sinnhuber *et al.,* 1958, Blokhina, 2003; Prasad, 2013). TBARS can easily be measured by spectrophotometry. MDA is formed through auto-oxidation and enzymatic degradation of polyunsaturated fatty acids in cells. TBA reacts with MDA, a product of lipid peroxidation, to give a red fluorescent 1:2 chromagen with maximum absorbance at 532 nm (Kappus, 1985; Janero, 1990). Although this method has been criticized for its lack of specificity and its tendency to miscalculate MDA content, it has been shown to be sensitive to small TBARS changes in animal and plant tissue (Scholz *et al.,* 1990; Landry *et al.,* 1995). The TBARS assay remains popular due to its simplicity, cost effectiveness and rapidity with which large numbers of samples can be processed with minimal manipulation (Hodges *et al.,* 1999). However, concerns have been raised that non-MDA substances may inflate readings, resulting in overestimation of lipid oxidation (Janero, 1990; Valenzuela, 1991). The aim of TBARS assay is to determine lipid peroxidation in plant or animal tissue. Wherever possible, the TBARS assay should be combined with other assays for lipid peroxidation such as conjugated dienes (CDs) (Devasagayam *et al.,* 2003) to provide a more accurate account of the oxidative damage measured. In a study by Yang *et al*. (2012) it was reported that MDA concentrations (TBARS) increased under high cadmium concentrations over a long exposure period in germinating soybean seeds. Howlett and Avery (1997) have reported that conjugated diene levels increased with the unsaturation index in copper exposed cells of *Saccharomyces cerevisiae* (yeast). The concentrations of CDs increased in *Raphanus sativus* growing under Cu stress (Sgerri, 2003).

1.9.2. Antioxidant content and -capacity

1.9.2.1 Total phenolic content (TP)

Metals can severely impair central metabolic processes in plants and other organisms. The photosynthetic apparatus is one of the key target areas of metal damage. Polyphenolics have been defined as electron-donating agents and is able to act as antioxidants (Michalak, 2006), acting as reducing agents, hydrogen donors, and preventing the evolution of oxidant-free radical and reactive species derived from, and preventing the evolution of oxidant-free radical and reactive species derived from metal catalysis by Fenton-like reactions (Lopes *et al.,* 1999; Schroeter *et al.,* 2002). Plant TP has shown a variety of properties including plant resistance against pathogens, solar radiation and metal stress. TP metabolism stimulates in response to metal stress in plants for the protection of plants and recovery from metal injury (Poonam *et al.,* 2015). Previous studies have suggested that polyphenols may act as biomarkers of metal exposure (Balońska *et al*., 2007).

Metals can obstruct photosynthesis at structural and metabolic level (Schroeter *et al.,* 2002). In *Jatropha curcas* L. (physic nut) TP concentrations showed positive and negative correlations between metal uptake and antioxidant activity (Chinmayee *et al.,* 2014). In a study by Márquez-García *et al.* (2012) it was found that cadmium increased the TP levels and the total antioxidant capacity under laboratory conditions in *Erica andevalensis* (heather).

1.9.2.2. Reduced glutathione (GSH)

As previously mentioned, glutathione (GSH) is a tripeptide widely distributed in both plants and animals (Arias and Jakoby, 1976). It serves as a nucleophilic co-substrate to glutathione transferases in the detoxification of xenobiotics and is an essential electron donor to glutathione peroxidases in the reduction of hydroperoxides and is also involved in amino acid transport and maintenance of protein sulfhydryl reduction status (Arias & Jakoby, 1976; Baillie & Slatter, 1991). GSH is easily oxidized to the disulfide dimer GSSG. GSSG is produced during the reduction of hydroperoxides by glutathione peroxidase. GSSG is reduced to GSH by glutathione reductase and it is the reduced form that exists mainly in biological systems. In a study by Nadgórska-Socha *et al.* (2013) it was found that GSHt related positively with zinc (Zn) and cadmium (Cd) concentrations in *Cardaminopsis arenosa* and with lead (Pb) concentrations in *Plantago lanceolate.* Boojar and Tavakkoli (2011) have reported that a pioneer plant species, *Zygophyllum fabago* in comparison to *Peganum harmala*, grown in tailings of a Pb and Zn mine, showed an increase in GSH concentrations

in aerial plants. Apel and Hirt (2004) have shown that plants increase the activity of GSH levels in response to biotic and abiotic stresses.

1.9.2.3 Oxygen Radical Absorbance Capacity (ORAC)

The oxygen radical absorbance capacity (ORAC) assay method has been used extensively in the field of antioxidant and oxidative stress to determine the antioxidant capacity. It uses fluorescein as probe for oxidation by peroxyl radicals (Prior *et al*., 2005). Hundreds of reports have been published on the use of this method to determine antioxidant capacity in food and biological samples (Nkhili & Brat, 2011). The ORAC method is a simple, sensitive, and reliable way to measure the peroxyl radical absorbing capacity (with 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH)) of antioxidants and serum or other biological fluids. Hydroxyl radical absorbing capacity of serum has been performed successfully using the ORAC method with H_2O_2-Cu^{2+}. This fluorescence-based method was first developed by Glazer *et al.* in 1998 and is based on the discovery that the fluorescence of phycoerythrin (PE) changes with respect to time upon damage caused by peroxyl or hydroxyl radical attack. In a study by Milne *et al*. (2012) it was reported that ORAC increased under applications of silicon (Si) in lettuce (*Lactuca sativa*). ORAC determinations were also performed on *Arabidopsis thaliana* under low antioxidant concentrations by Brosché and Kangasjärvi (2011).

1.9.2.4. Ferric reducing ability of plasma (FRAP)

The FRAP assay was developed by Benzie and Strain in 1996. The FRAP assay gives fast, reproducible results with plasma, with single antioxidants in pure solution, and with mixtures of antioxidants in aqueous solution (Benzie & Strain, 1996). The FRAP assay uses an oxidation/reduction reaction to measure the ability of a sample to reduce Fe^{3+} to Fe^{2+}. An antioxidant donates electrons in the same manner as a reductant in an oxidation/reductions, so it is assumed that the FRAP assay is a method for evaluating antioxidant capacity. FRAP values are obtained by comparing the absorbance change at 593 nm in test reaction mixtures with those containing ferrous ions in known concentration (Benzie & Strain, 1996). However, it does not directly measure the antioxidant capacity of a potential antioxidant. Also, since there are no free radicals introduced into the system, there is no way of comparing the antioxidant capacity towards different kinds of radicals (Benzie & Strain, 1996). Gjorgieva *et al*. (2013) reported that results from the FRAP assay indicated that metals induce oxidative stress [*Urtica dioica* (Nettle)] in samples exposed to high metal

concentrations. In a study by Szöllösi´ *et al*. (2011) it was found that FRAP levels decreased in the seeds of Indian mustard (*Brassica juncea* L.) under high Cu and Zn concentrations. Yang *et al.* (2012) reported that FRAP concentrations decreased with time in germinating soybean seeds during exposure to high concentrations of cadmium (Cd).

1.10. Changes in photosynthetic activity due to metal pollution

The quantity and distribution of aquatic plants is directly correlated with the amount of light available. Light is fundamental to the survival of endogenous tissues, because they depend on the oxygen supply from photosynthesis performed by epigenous tissues (Baker, 2008). Inhibition of the activity of photosystem II (PSII) is the result of the exposure of photosynthetic organisms to strong light (Aisen *et al.,* 2001; Agnisola, 2005; Ahmad *et al.,* 2006). This phenomenon is termed photo inhibition. Light energy is the driving force for photosynthesis and photo-inhibition is unavoidable in photosynthetic organisms (Arillo & Melodia, 1990; Apel & Hirt, 2004). Chlorophyll fluorescence measurement is a tool to evaluate the biochemical and physiological state of plants. It is a reliable technique, easy to carry out, non-destructive and rapid (Kramer *et al.,* 1987; Walker (1990) in Vangronsveld *et al.,* 1998). Chlorophyll fluorescence and chlorophyll content are used to highlight stress due to a single environmental factor or to a combination of different environmental factors, but they also constitute potential biomarkers of anthropogenic stress (Ferrat, 2003).

Analysis of photosynthetic pigment concentration generally confirms the results obtained by chlorophyll fluorescence measurements. The magnesium ion (Mg^{+}) can be substituted by metals in the chlorophyll molecule, leading to the failure to catch photons and thus leads to a decrease in photosynthetic activity (Ferrat, 2003). In general, stressed plants increase their carotenoid concentration to provide protection against the formation of free radicals. A decrease in total chlorophyll content and a decrease in ratio chlorophyll to carotenoids are often observed. Changes in photosynthetic pigments exposed to metals and herbicides have been observed for several species, eg. *Halophila ovalis* (Ralph & Burchett, 1998; Ralph, 2000; Ferrat *et al.,* 2003). Contamination by Cr reduces all photosynthetic pigments and even carotenoids (Nichols *et al.,* 2000). Fargašová (1999) observed a decrease in chlorophyll *a* in planktonic diatoms caused by oxidative stress due to Cu, and a decrease in carotenoids due to Zn (Rijstenbil *et al.,* 1994). Chlorophyll pigment biosynthesis and enzymes involved in this process may be inhibited by metals. The same trend is observed

with exposure to high irradiance, whereby photosynthetic pigments (chlorophyll *a* and *b*) decrease (Yakovleya & Titlyanoy, 2001).

1.10.1. Chlorophyll degradation as a consequence of metal exposure in plants

Chlorophyll is an essential component in the process of photosynthesis, which enables plants to convert carbon dioxide and water in the presence of energy from the sun to produce carbohydrates (Hopkin, 1993; Walker *et al.,* 2006). Chlorophyll is the most widely distributed natural pigment and occurs in the leaves and other parts of almost all plants (Humphrey, 2004) and plays an important role in the plants' growth and development processes and has a distinct green colour. Chlorophylls and carotenoids are the primary light capturing pigments in higher plants, and are located in thylakoid membranes of the chloroplast (Humphrey, 2004).

The main function of pigments is to absorb light energy for photosynthesis, and protect the photosynthetic apparatus from excess light. Excess light can create a surplus of excited electrons, which exceeds the capacity of the photosynthetic electron transport chain, leading to the formation of reactive oxygen species (ROS) (Buchanan *et al*., 2001; Brain & Cedergreen, 2009). Quantification of photosynthetic pigments is typically measured spectrophotometrically after extraction with organic solvents such as acetone, ethanol, methanol or diethyl ether (Arnon, 1949; Greenberg *et al.,* 1992; Porra, 2002). Absorbance of whole plant extracts is calculated from the ratio of extract to reference blank, using various equations (Arnon, 1949; Lichtenthaler, 1987; Porra *et al.,* 1989). Absorbance of chlorophyll *a* and *b* are measured at wavelengths of 645 and 663 nm. Metabolic processes in plants are affected by stress and may produce reactions that could be detected by using specialised methods and equipment.

Principal biomarkers tested are significant responses that take place during photosynthetic activity, enzymatic processes of nutrition, secondary metabolite synthesis, oxidative stress and/or detoxification mechanisms (Ferrat *et al.,* 2003; Vangronsveld & Clijsters, 2004). Stressed plants in general increase their carotenoid concentration to provide against the formation of free radicals. Decreases in total chlorophyll concentration and in the chlorophyll/carotenoids are often observed. Variations in photosynthetic pigments that have been exposed to metals and herbicides have been observed for various species for example, *Halophila ovalis* (Ralph & Burchett, 1998; Ralph, 2000), *Salvinia minima* and plankton

diatoms. All photosynthetic pigments and carotenoids are reduced in *Salvinia minima* during Cr contamination (Nichols *et al.,* 2000). In a study by Fargašová (1999) planktonic diatoms showed a decrease of chlorophyll a, caused by oxidative stress due to Cu and a decrease of chlorophyll *c* due to Zn (Rijstenbil *et al.,* 1994). Iron toxicity in tobacco, canola, soybean and *Hydrilla verticillata* are accompanied with reduction of plant photosynthesis and yield and the increase in oxidative stress and ascorbate peroxidase activity (Sinha *et al.,* 1997). Bibi *et al*. (2010) reported negative effects of high levels of metals (Cd, Cr, and Zn) on the freshwater macrophyte, *Nitella graciliformis* J. by decreasing the chlorophyll content and exhibiting poor plant growth.

High concentrations of most metals in plants will interfere with chlorophyll concentration and will induce chlorosis (Padmaja *et al.,* 1990). A series of studies, mainly on metals (Powell *et al.*, 1996; Lagriffoul *et al.,* 1998; Qi *et al*., 2006; Appenroth *et al*., 2010) indicated that chlorophyll and carotenoid content to be as or more sensitive stress indicators than biomass or relative growth rate. According to Kushwana and Bhowmik (1999) measuring chlorophyll *a*, *b* and carotenoid content in cucumber cotyledons treated with isoxaflutole, demonstrated nearly double the sensitivity compared to fresh weight. Advantages of pigment concentration are that it can be an easy-to-measure and robust biomarker, applicable to both laboratory and field-based investigations. Inhibition of pigment content may signify modes of action, if the contaminant disrupts photosynthesis or pigment biosynthesis. Pigment content can be a more sensitive effect indicator than growth, especially where the pigment biosynthetic pathway or photosynthetic apparatus is targeted directly by bleaching herbicides and PSII inhibitors (Brain & Cedergreen, 2009).

Chlorophyll fluorescence is another technique for measuring chlorophyll concentration in plants. Analysis of photosynthetic pigment concentration usually confirms the results obtained from chlorophyll fluorescence measurements. Metals can substitute for the magnesium ion in the chlorophyll molecule, leading to the inability to catch photons and therefore lead to a reduction in photosynthetic activity (Ferrat *et al.,* 2003). Chlorophyll fluorescence is affected by chemicals that interfere directly with the photosystem II (PSII) electron transport chain (mainly herbicides), or otherwise increase production of ROS that are damaging to PSII (Brain & Cedergreen, 2009). Metals including Cr, Cu, Cd and Zn, have shown effects on chlorophyll fluorescence; only Cr, however, shows such effects at concentrations lower than those affecting growth (Appenroth *et al*., 2001; Drinovec *et al.*, 2004). Chlorophyll fluorescence is a fast, cheap, non-destructive biomarker for a large range of chemicals; effects can also be detected at an earlier stage than by measuring growth

rates. Chlorophyll fluorescence has demonstrated comparable or greater sensitivity than growth endpoints for a number of contaminants, depending on the mechanism of action (Brain & Cedergreen, 2009). Chlorophyll fluorescence is a non-destructive measure; the kinetics of the toxic effect on photosynthesis can be measured over time, making it a powerful tool for assessing uptake rates, effects, internal transportation and recovery in plants (Abbaspoor *et al.,* 2006; Cedergreen *et al*., 2004).

Chlorophyll content and chlorophyll fluorescence are indicators of stress due to a single environmental factor or to a combination thereof, but they also represent potential biomarkers of anthropogenic stress (Ferrat *et al.,* 2003). It can be concluded that metals may inhibit chlorophyll pigment biosynthesis and enzymes involved in this process. Therefore, chlorophyll concentration in plants could be used as a potential biomarker of stress in ecotoxicological studies (Stoltzs & Gregor, 2002; Bragato *et al.,* 2006).

1.10.2. Changes in photosynthetic activity due to excessive exposure to metals in plants

The distribution and abundance of aquatic plants is directly correlated with the amount of light available. Light is important for the survival of endogenous tissues, as they depend on the oxygen supply from photosynthesis performed by epigenous tissues (Ferrat *et al.,* 2003). Metals can affect the physiological processes in plants such as photosynthesis which is essential for growth and development (Clijsters & Van Assche, 1985; Hopkin, 1993; Walker & Hopkin, 2006).

Inhibition of photosynthesis take place at several levels for example, carbon dioxide fixation, stomatal conductance, chlorophyll synthesis, electron transport and enzymes of the Calvin cycle (Prasad & Strzalka, 2000; Monnet *et al.,* 2001; Shanker *et al.,* 2004). Photosynthesis in plants is affected by exposure to excessive metals through several mechanisms. In a study by Singh *et al.* (2010) it was reported that chlorosis and fragmentation of leaves with mucilaginous discharge occurred in *Najas indica* plants exposed to a high level of Pb. A range of other studies have indicated that high concentrations of Cu affected the oxidative enzymes in wheat, oat and bean leaves, thus affecting photosynthesis (Shainberg *et al.,* 2001). The chlorophyll formation process might be influenced by high metal concentrations which could have an adverse effect on the plants' photosynthetic activity and thus affecting plant growth (Padmaja *et al.,* 1990; Jonak *et al.,* 2004).

1.11. Bioaccumulation and effects of selected metals in plants

Macrophytes are considered to be important components of the aquatic ecosystem, not only as a food and oxygen source, and habitat for aquatic invertebrates and fish, but as efficient accumulators of metals (Rai, 2009). Aquatic macrophytes play an essential role in structural and functional aspects of aquatic ecosystems in various ways. The ability of these plants to absorb metals makes them interesting research candidates especially for treatment of industrial effluent and sewage waters through the process of phytoremediation (Andra *et al*., 2010). Submerged macrophytes possess significant potential to bioaccumulate metals due to their bigger surface area compared to non-submerged plants (Sinha *et al.*, 1997; Dhir *et al.*, 2009). Several submerged macrophyte species, such as *Ceratophyllum demersum* (Keskinkan *et al.,* 2004), *Myriophyllum spicatum* (Keskinkan, 2005) and *Potamogeton spp.* (Fritioff & Greger, 2006; Peng *et al.,* 2008; Monferrán *et al.,* 2012) have been used to test their accumulation potential.

In the aquatic environment, macrophytes are seldom exposed to a single metal and in most cases the stress of pollution may be attributed to the effect of a combination of metals (Sinha *et al.,* 2003). Therefore there must be several differences in the accumulation capacity of submerged macrophytes after exposure to a single metal or a combination of different metals. Most bioaccumulation studies have been conducted under strict laboratory conditions (Deng *et al.,* 2005; Pilon-Smuts, 2005; Dhir *et al.,* 2009; Rai, 2009; Monferrán *et al*., 2012; Xue & Yan, 2011; Singh *et al.,* 2011).

Bioaccumulation of metals depend on numerous biotic and abiotic factors, such as temperature, pH and dissolved ions in water (Xing *et al.,* 2013). According to Demirezen and Aksoy (2004) there is a relationship between cadmium concentration in *Potamogeton pectinatus* and water pH value. Several studies conducted on aquatic plants have indicated that the aquatic plant often accumulates much higher concentrations of metals than the surrounding medium (Demirezen & Aksoy, 2006). Soares *et al*. (2008) reported on *Salvinia auriculata* (a non-submerged macrophyte), which has the capacity to bioaccumulate large concentrations of chromium in its leaves.

1.11.1. Aluminium (Al)

Aluminium is the most abundant and the third most common element in the earth's crust (Panda & Matsumoto, 2007), but is not considered as an essential nutrient. At low

concentrations it can sometimes increase plant growth or induce other desirable effects (Foy *et al.,* 1978; Foy & Flemming, 1982; Foy, 1983). Aluminium is not a transition metal and cannot catalyze redox reactions, therefore the involvement of Al toxicity in oxidative stress has been proposed (Boscolo *et al*, 2003). Aluminium is a major component of soil and as a result plants grow in soil environments in which the roots are potentially exposed to high levels of Al (Dipierro *et al.,* 2005). Aluminium toxicity is an important growth-limiting factor for plants in acidic soils with a pH below 5.0 but can occur at pH levels as high as 5.5 in mine spoils (Alam & Adams, 1979; Severi, 1997). Inhibition of root growth is the most easily recognized symptom of Al toxicity and is a widely accepted measure of Al stress in plants. According to Delahaize and Ryan (1995) micromolar concentrations of Al can begin to inhibit root growth within 60 minutes in simple nutrient solutions.

Exposure to Al was found to increase oxidative stress and was an important event in the inhibition of cell growth (Pereira *et al.,* 2010). The relationship between ROS and the enhancement of lipid peroxidation and small increases in enzyme activities such as SOD peroxides suggests a generation of ROS caused by Al (Cakmak & Horst, 1991). However, many studies have focused on the aspect of toxicity and various mechanisms of action have been suggested, but the causes of Al, have been poorly understood (Pereira *et al.,* 2010).

1.11.2. Copper (Cu)

Copper is an important micronutrient for normal plant growth and development (Jonak *et al.,* 2004) and is a component of several enzymes that mainly participate in electron flow and catalysing the redox reactions (Fernandes & Henriques, 1991; Devi & Prasad, 1998). Cu is a cofactor for many physiological processes, including photosynthesis, respiration, superoxide scavenging, ethylene sensing and lignification (Jonak *et al.,* 2004). However, when in excess, copper interferes with several physiological processes in the plant (Devi & Prasad, 1998). It is known to damage cell membranes by binding to sulfhydryl groups of membrane proteins and by inducing lipid peroxidation (de Vos *et al.,* 1992). Shuping *et al*. (2011) and Erasmus (2012) have found that Al, Cu, Fe and Zn were the most dominant metals in the Diep River, Milnerton. According to Shuping *et al.,* (2011) metal concentrations of aluminium and zinc in the lower reaches of the Diep River, were well over The Target Quality Guidelines for Aquatic Ecosystems (TWQR), set out by the Department of Water Affairs and Forestry (DWAF, 1996).

Copper is a vital micronutrient essential for normal plant growth and development (Thomas *et al.,* 1998), and plays an important role in carbon dioxide assimilation and ATP synthesis (Yadav, 2010). However, copper in excess is harmful (Jonak *et al.,* 2004) and is an efficient generator of toxic oxygen species such as $O_2^{\bullet-}$, H_2O and HO• in Fenton-type reactions (Aust, 1985; Kappus, 1985; Kurepa *et al.,* 1997; Drażkiewicz *et al.,* 2004). Copper is also a component of primary electron donor in photosystem I (PS I) of plants. It can readily gain and lose an electron. Copper is a cofactor of oxidase, mono- and di-oxidase (e.g. amine oxidases, ammonia monoxidase, ceruloplasmin, lysyl oxidase) (Nagatjyoti *et al.,* 2010). An important characteristic of Cu toxicity is the initiation of oxidative stress in plants (Luna *et al.,* 1994; Allen, 1995). Industrial and mining activities have contributed to the increasing occurrence of Cu in ecosystems. Copper is added to soils from different anthropogenic activities including mining and smelting of Cu-containing ores. Mining activities generate a huge amount of waste rocks and tailings, which get deposited at the surface. High levels of Cu in soil play a cytotoxic role, induce stress and causes injury to plants (Yadav, 2010). This leads to retardation in plant growth and leaf chlorosis (Lewis *et al.,* 2001).

1.11.3. Iron (Fe)

While iron is an essential nutrient for plants, its accumulation within cells can be toxic. Fe functions to accept and donate electrons and plays essential roles in the electron transport chains of photosynthesis and respiration. It is toxic when accumulating in high levels in plants. Fe is a constituent of antioxidant enzymes such as catalase, ascorbic peroxidase, guaiacol-peroxidase and ferro-superoxide dismutase. When plants are exposed to various unfavourable conditions, including chilling, high light, drought, paraquat and oxidative stress, it is primarily due to the decrease antioxidant defences but also due to the increase in free-radical production mediated by catalytic Fe54 (Arora *et al.,* 2002). Plants respond to Fe stress in terms of both iron deficiency and iron excess (Connolly & Guerinot, 2002). Iron deficiency symptoms are interveinal chlorosis in young leaves caused by inhibition of chloroplast development. Iron toxicity can cause browning of the leaves, known as ‘bronzing’ (Mengel & Kirkby, 1987). The symptoms are diverse among plant species and Fe toxicity is difficult to identify from the outer appearance of the plants (Foy *et al.,* 1978).

1.11.4. Zinc (Zn)

Zinc is an essential micronutrient for the plant system. It has been reported that Zn deficiency in animals induce oxidative stress to all cellular components and changes the antioxidant enzyme activity, disturbs cellular homeostasis and induce severe oxidative damage to macromolecules (Bray *et al.,* 1990). Compared with the knowledge of the role of Zn as an antioxidant in experimental studies in animals, relative little information is available using model plant systems (Aravind *et al.,* 2008). Zn participates in the maintenance of the normal function and structure of membranes (Verstraeten *et al.,* 2004) and is present in various enzymes (Broadley *et al.,* 2007). It has been suggested that Zn plays a role in protecting DNA and membranes from damage caused by reactions with ROS (Cakmak, 2000), and Zn supplementation has been shown to protect plants from oxidative stress induced by other metals (Aravind & Prasad, 2005). Zinc contamination in freshwater bodies has been reported to exceed the environmental limit by up to100 times (Srikanth *et al*., 1993; Pistelok & Galas, 1999; Shikazono *et al*., 2008). Stunted growth, chlorosis and necrosis are some of the visible symptoms indicating severe metal phytotoxicity. General symptoms of zinc toxicity are turgor loss, necrosis on older leaves, and reduced growth. At high concentrations Zn inhibit root growth (Hagermeyer, 2004).

1.12. Metal pollution in the Diep River, Western Cape

The Diep River is one of the major catchments which fall within the Berg River Water Management Area (WMA) (Figure 2.1). The Diep River rises in the Perdeberg and Riebeek-Kasteel Mountains, north-east of the catchment, and then flows in a south-western direction through Malmesbury (Brown & Magoba, 2009; Water Institute of Southern Africa, 2009). The Diep River discharges into Table Bay in the Atlantic Ocean, north of Cape Town, and has a total length of about 86 km. The catchment has a total area of about 1 495 km^2. The Diep River Catchment is low lying and flat with isolated mountains on its eastern boundary, namely the Perdeberg, Kasteelberg and Paarlberg (IWQS, 2000). The Mosselbank River, which drains the catchment areas north of Durbanville and Kraaifontein, forms the major tributary to the Diep River with the Diep- Mosselbank River System eventually discharging into Rietvlei.

Rietvlei falls within a Nature Conservation Area (Table Bay Nature Reserve) and is of ecological importance. The Mosselbank River has tributaries called the Klapmuts River and Platklip River (IWQS 2000). Other tributaries include the Riebeek River, Groen River, Sout River and Philadelphia stream (DWAF, 2002; Brown & Magoba, 2009). An Estuary

Management Plan for the Diep Estuary prepared for the City of Cape Town in 2011, highlighted problems of pollution in the lower parts of the river. It stated that the main sources of pollution came from the various waste water treatment works along the river, of which Malmesbury did meet the required standards at the time of the study. The report mentioned that storm water from urban areas, agricultural activities such as fertilizer and pesticide runoff and cattle manure, and mining were the main sources of pollution. Urbanisation in Cape Town led to increase in farming, resulting in the construction of more dams in the upper reaches. Dredging and industrial activities have changed the characteristics of the Diep River over time and influenced its general structure (Coastal & Environmental Consulting, 2011).

The rivers in urban areas or cities of South Africa are being polluted by metals, pesticides and industrial waste. According to Brown & Magoba (2009) the lower Diep River, the Milnerton lagoon area, is directly affected by sewage effluent from the Potsdam Waste Water Treatment Works, which is situated close to the industrial area of Montague Gardens. A few studies have been done on metal concentrations in South African rivers (Okonkwo *et al.,* 2005). Previous studies have indicated that the Diep River is polluted in terms of metals (Ayeni *et al*., 2010; Shuping *et al*., 2011; Erasmus, 2012). According to Shuping *et al.* (2011) metal concentrations of aluminium and zinc in the lower reaches of the Diep River were well over The Target Quality Guidelines for Aquatic Ecosystems (TWQR), set out by the Department of Water Affairs and Forestry (DWAF, 1996). It was also found that concentrations of copper were high during summer. Human activities, such as mining, agriculture and other industries, increase metal concentrations in a river (Smol, 2002). Shuping (2008) indicated that the lower Diep River has been subjected to deterioration in water quality over decades due to bad farming practices and other land uses.

Land use in the upper catchment is mainly agriculture, while in the lower catchment it is largely residential (formal and informal settlements) and industrial. Jackson *et al.* (2009) found the lower Diep River to be polluted with a variety of metals. The concern is that industrial and household effluents could be discharging substantial quantities of metals into the Diep River which may be damaging to wetland plants, microorganisms, human health and ecosystem health in general.

1.13. Statement of the research problem

The Diep River, Milnerton, Western Cape, is known to be polluted with metals, notably Al, Cu, Zn and Fe (Ayeni *et al*., 2010; Shuping *et al*., 2011; Erasmus, 2012). Erasmus (2012) found strong metal bioaccumulation and some resultant effects on chlorophyll content in *Ceratophyllum demersum* L. exposed to Diep River water. However, it is unknown as to what the most effective biomarker/-s of metal exposure and metal stress may be, using this plant species in the Diep River. A field study alone will not provide this answer, therefore an exposure experiment under controlled laboratory conditions is needed, in order to study the toxicity of bioaccumulated metals in this plant species, so as to have a clearer indication of cause and effect.

It is also unknown as to how quickly metals are bioaccumulated in this species, and to what degree, as well as whether there is a pattern of metal exchange between plant and water over time. The time factor is a particularly poignant question, when considering a pollution event in a river. Previous laboratory studies with aquatic plants have all been conducted over short exposure periods (e.g. 15 days (Rai *et al*., 1995) or 7 days (Malar *et al.,* 2014)) but in the present study the exposure period is 5 weeks, and the water is only contaminated once, so as to simulate a pollution event and to study the metal exchange between the plants and the water, long after the “event”. This has not been attempted in a laboratory study before to the author’s knowledge.

1.14. Main research aim

The main aim of this study is to investigate the use of selected biological responses, namely antioxidant responses and changes in chlorophyll concentration in *Ceratophyllum demersum* L., as biomarkers of metal exposure, as well as to investigate the field application of these responses in the Diep River. Ultimately the aim is also to determine the usefulness of *C. demersum* as model of metal contamination and as phytoremediator after a pollution event.

The objectives of the research:

- The first objective is to determine the degree of metal bioaccumulation in *Ceratophyllum demersum* L. exposed to different concentrations of metals under laboratory conditions over a five week exposure period.

- The second objective is to determine if antioxidant responses can be applied to evaluate the effects of metal-induced stress in *C. demersum* L.

- The third objective is to investigate the effects of accumulated metals on chlorophyll content of *C. demersum* L.

- The fourth objective is to investigate the field application of antioxidant responses as biomarkers of metal exposure *in C. demersum* in the Diep River

- The final objective is to determine whether *C. demersum* L., is an effective model of metal stress in the laboratory and Diep River and whether it can be applied as a suitable biomonitor species for phytoremediation after a pollution event.

CHAPTER 2: MATERIALS AND METHODS

2.1. Study site and test species selection

The field study was conducted along the banks of the lower Diep River. This river is located in Cape Town, Western Cape, South Africa. The Diep River originates from the Riebeek-Kasteel and Perdeberg Mountains north east of Malmesbury and flows in a south-westerly direction towards Table Bay, where it flows into the Atlantic Ocean (Brown & Magoba, 2009) (Figure 2.1).

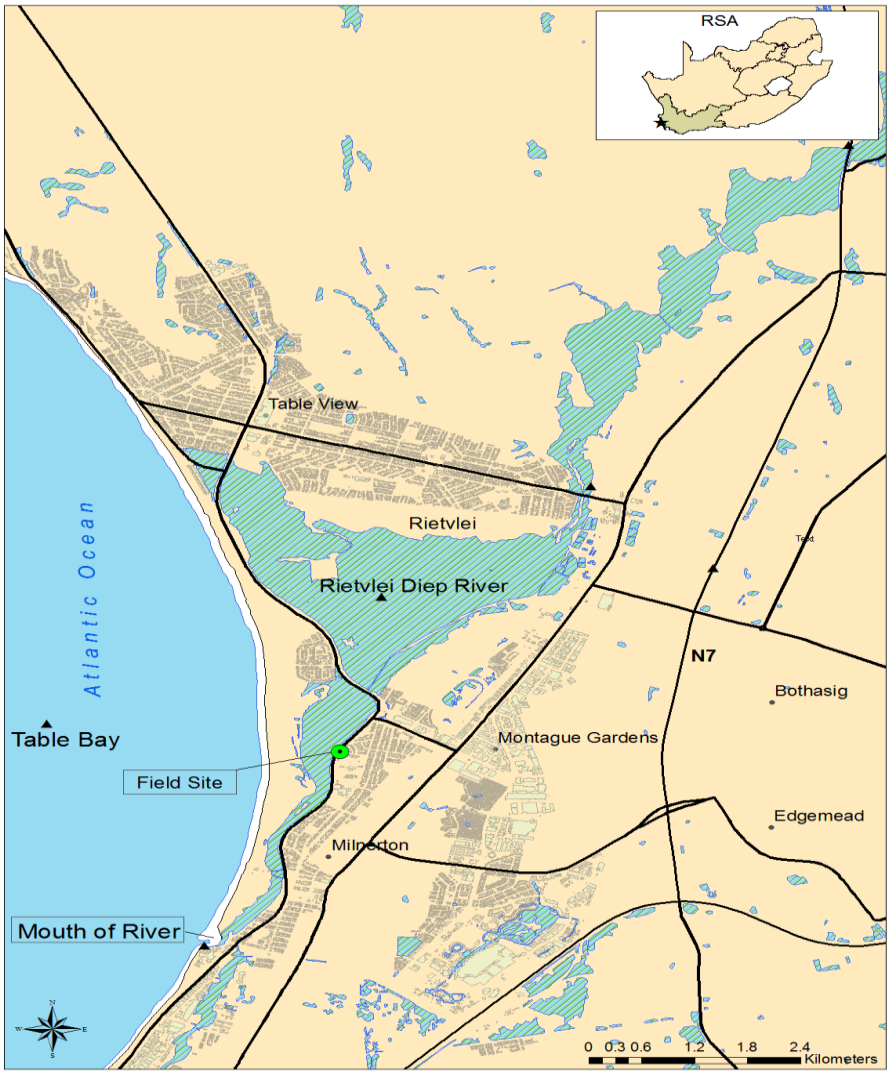

Figure 2.1. Diep River sampling site and surrounding areas (Source: Mpfunzeni Tsindane, 2016)

The Diep River catchment, approximately 65 km in length, is located in the South Western Cape Region and is surrounded by industrial and residential areas. The estuary is about 900 ha and consists of the Milnerton Lagoon and the Table Bay Nature Reserve and Boating Club (Lochner *et al.,* 1994). The Diep River-Rietvlei system has silted up significantly over the past few years which has resulted in extensive erosion (Grindley & Dudley, 1988) and can therefore be regarded as a storage area for sediment-rich water during floods. The sedimentation rate is increased by vegetation in the vlei, mainly where treated sewage water is being discharged (Paulse *et al*., 2009). The river is surrounded by various industrial establishments ranging from spray painting to chemical manufacturers as well as a wastewater treatment plant and an oil refinery, which could all have a major impact on the water source and the surrounding environment (Paulse *et al*., 2009). Earlier studies have shown that the Diep River is polluted in terms of metals (Ayeni *et al*., 2010; Shuping *et al*., 2011).

A suitable plant species was required to be tested for oxidative stress responses and as a potential indicator for metal pollution in the lower Diep River. An aquatic species, *Ceratophyllum demersum* L. was found growing in the lower reaches of the Diep River. This test species was found growing abundantly (GPS co-ordinates S 33^0 $56^{'}$ $20.3^{"}$ & 18^0 $31^{'}$ $01.9^{"}$) in the relatively slow moving to stagnant water body near Gill Road, Table View, behind a garden centre (Erasmus, 2012). According to Shuping *et al*. (2011) this site is contaminated with above average levels of zinc, copper, aluminium and iron. The water at the site is dark and muddy and the test species is well established and appears to be healthy.

For the greenhouse study *C. demersum* L. plants were collected from the reasonably clean and unpolluted fishpond at the greenhouse nursery, situated on the Cape Town campus of the Cape Peninsula University of Technology (CPUT), where it flourishes in a pond community with other macrophytes and fish. These plants occupy most of the available pond space and appear to be healthy.

Ceratophyllum demersum L. (Figure 2.2) is a rootless, submerged, perennial aquatic macrophyte with a cosmopolitan distribution. It has a wide ecological tolerance. When water is disturbed, it is quite common for native species to increase their growth and become a threat for human use of the water body (Cook, 1990). Frequent disturbance of the bed of the water body or soils in the catchment results from an increase in the trophic level of the water or the substrate. *C. demersum* has become locally troublesome on several occasions (Cook, 1990). It is one of the 26 aquatic vascular plant species that Cook (1985) characterized as 'very widespread', and is unlikely to be native throughout its whole range of occurrence.

This macrophyte occurs in quiet or slow flowing, hard calcareous, nutrient-rich or eutrophic waters of streams, ditches, canals, ponds and lakes as a near free-floating aquatic plant where it may form large masses. It is especially favoured by nitrate-rich conditions where it grows in greater abundance (Goulder & Boatman, 1971; Toetz, 1971; Best, 1980; Kulshreshta, 1982).

Ceratophyllum demersum L. will normally grow with the base of its stem buried in sandy or salty substrates. It does not form roots. It is prone to dislodgement, and its buoyant stems may become free-floating. *C. demersum* L. is regarded as being a good water oxygenator and provides a protective environment for fish eggs and is an ideal habitat and food source for small snails and insects. It is common throughout freshwater rivers and lakes of the world and prefers stagnant slow moving water bodies. *C. demersum* L. can form a dense subsurface canopy and can reach a height of 5-6 m and frequently grow as a mono-specific community. It can form modified leaves when growing near the lake's bottom, which it employs to anchor the plant in the sediment (Keskinkan *et al*., 2004).

Common names of *Ceratophyllum demersum* L. are coontail and common hornwort. *Ceratophyllum demersum* L. is endemic to North America. It now has a global distribution, at least in part due to the aquarium and pond trade. It is a submerged aquatic plant which has the potential to form dense mono-specific beds. This plant causes problems to recreational activities on waterways and in some cases causing blockages at hydroelectric power stations. *C. demersum* can spread rapidly and grows in a large range of aquatic environments (Keskinkan *et al*., 2004). The taxonomic classification of *Ceratophyllum demersum* L. is tabled below (Table 2.1).

Table 2.1. Classification of *Ceratophyllum demersum* L. (Zhuang, 2013).

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Nymphaeales
Family	Ceratophyllaceae
Genus	*Ceratophyllum*
Species	*Ceratophyllum demersum* L.

Figure 2.2. *Ceratophyllum demersum* L. (Source: Author, 2012)

2.2. Experimental design and growing conditions

2.2.1. Greenhouse environment

2.2.1.1. Photosynthetic photon flux density (PPFD)

The net assimilation rate of many sun and shade plants is linearly related to the logarithm of the light intensity up to maximum daylight (Blackman & Wilson, 1951). Since the light intensity in a greenhouse may be reduced and its quality affected according to the alignment of the greenhouse and the type and cleanliness of the glaze, it was measured at different positions in the greenhouse. Measurements of PPFD were taken at solar noon (13h00 SAST) in the greenhouse at its northern, central, southern, eastern and western extremities and in the outdoor environment with a quantum sensor (LiCor 189, Li-COR, Lincoln, NE, USA).

2.2.1.2. Air temperature

Photosynthesis and growth of plants are affected by the air temperature (Chabot, 1977). Since air temperature may vary at different positions in a greenhouse, these were monitored

daily in the greenhouse at its southern, central and northern extremities and in the outdoor environment.

2.2.2. Experimental design

Ceratophyllum demersum L. plants (150 plants) were collected from the fish pond at the Cape Peninsula University of Technology in Cape Town, South Africa. Plants were carefully removed from the pond and washed in deionised water to remove any debris that could be attached to the plants. Excess water was shaken off and each plant was weighed on a two decimal point Mettler balance (PC2200). Each plant was weighed (± 8.5 g) and placed in a 68 L hydroponic container (thirty plants per container-total of 5 containers) filled with 10% Hoagland solution for 5 weeks. Before metal treatment, plants were acclimatized for one week under laboratory conditions. The metals used (aluminium, copper, iron and zinc) were selected for this study as they were the most abundant metals measured by Shuping (2008) and Erasmus (2012) in the lower Diep River. Plants were treated with different concentrations of aluminium ($AlSO_4$), iron ($FeSO_4$), copper ($CuSO_4$) and zinc ($ZnSO_4$) in combination and were maintained in 10% Hoagland solution (Hoagland & Arnon, 1950) in containers under laboratory conditions for a period of 5 weeks. There were four treatments (Table 2.2). A fifth group served as the control. Aeration in containers was achieved by using small pumps. There were no overcrowding in the containers and the initial volume was 68 L of water. Additional stress factors were kept at a minimum. The water was only spiked once with metals and the water levels and water chemistry was not adjusted weekly, in order to simulate a single pollution event and to monitor the interactions between plant and water over the test period.

Table 2.2. Concentrations of metals provided to containers with T1 indicating concentrations measured in the Diep River (Shuping, 2008)

Treatments (T)	**T¼**	**T½**	**T 1**	**T2**
$AlSO_4$ (mg/L)	25.5	51.0	102.0	204.0
$CuSO_4$ (mg/L)	0.85	1.7	3.4	6.8
$FeSO_4$ (mg/L)	93.5	187.0	374.0	748.0
$ZnSO_4$ (mg/L)	8.5	17.0	34.0	68.0

Abbreviations: T1 = Average of sediment metal concentrations measured by Shuping (2008) in the Diep River (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L).

2.3. Sampling procedures

2.3.1. In the greenhouse

Samples were collected for this study every week for five weeks from the containers in the greenhouse. Plants were acclimatised during week 0. Pre-exposure and during exposure pH, temperature and electrical conductivity of the water in each container were measured with a handheld multi-parameter instrument (Eutech PCSTEST35-01X441506 / Oakton 35425-10). The water in the four containers were spiked (except for the control) only once to simulate a pollution event and to investigate changes in the water and plants.

Every week five plants were randomly harvested per treatment and 5 plants from the controls for each analysis (metals, chlorophylls and antioxidants). The plants were individually washed in 500 ml deionised water, blotted with paper and placed in labelled plastic bags. The samples in the greenhouse were immediately placed in 5L flasks containing liquid nitrogen and transported to a -80^0 C freezer where it was stored until analysed. During each sampling occasion, water samples were taken from each container for metal analysis. Water samples were placed in plastic bottles and labelled. These samples were labelled and stored in a -4 °C freezer until all sampling occasions had been completed.

2.3.2. Field sampling

Samples for this study were collected in the lower reaches of the Diep River (referred to as the field site) (Figure 2.1) which is located at the end of Gill Road, Table View, behind a garden centre (GPS co-ordinates S 33° 56' 20.3" & E 18° 31' 01.9") where *Ceratophyllum demersum* L. grows in abundance. Five plants were collected for each analysis (metals, chlorophyll and antioxidants) during spring (September) as the rainfall of the region mainly falls in winter and the river flows well and is accessible during spring and summer. Before harvesting, pH, temperature and electrical conductivity of the water were measured with a handheld multi-parameter (Eutech PCSTEST35-01X441506 / Oakton 35425-10). The five plants that were collected were individually washed in 500 ml deionised water, blotted with paper and placed in labelled plastic bags. The samples from the field were immediately placed in 5L flasks containing liquid nitrogen and transported to a -80^0 C freezer where it was stored until analysed. During sampling, five water samples were taken for metal analysis. Water samples were taken from one meter from the river edge and placed in a plastic water bottle and labelled. These samples were labelled and stored in a -80 °C freezer until all sampling occasions had been completed. The Diep River is much polluted and does not

have a reliable reference site. The pond at the Cape Peninsula University of Technology (CPUT) was used as a reference site for comparison. Plants were collected from the pond as comparison and were treated in the same manner.

2.4. Metal analysis

2.4.1. Determination of metal concentrations in water medium and plants in the greenhouse

Water samples from the containers in the greenhouse were tested for aluminium, copper, iron and zinc. These metals were selected as they were the most prominent metals measured by Shuping (2008) in the lower Diep River. Metal analysis was performed according to the method described by Shuping *et al.* (2011) for all water samples. Five ml of 55% nitric acid (HNO_3) was added to each 10 ml water sample and 5 ml nitric acid was prepared as a blank. The samples were then heated in a Grant UBD dry block heater in a fume cabinet, at 40 ^{0}C for 1 hour. After 1hr the temperature was increased to 120 ^{0}C for a further 3 hours. After digestion, the samples were left to cool. After cooling, the samples were filtered through 90 mm Whatman filter paper (Whatman International Ltd, Maidstone, England) and then filtered using 0.45 μm cellulose nitrate membrane filter paper using a sterilized needle and syringe. Finally, the samples were diluted to 100 ml with distilled water. Samples were then transferred into polyethylene plastic containers and stored at 4 ^{0}C until ICP-MS (Inductively Coupled Plasma- Mass Spectrophotometer) analyses.

Five plants of *C. demersum* L. were harvested every week for five weeks from the containers in the greenhouse. The samples were frozen in individually labelled bags after collection. Thawed *C. demersum* plants (having been stored in a freezer) were weighed in the petri dishes after thawing. Five replicates of the whole plant were used for analyses. Whole plant samples were dried in an oven for 48 h at 60 ^{0}C to obtain the dry weight. The sampling procedure and methods set out by Shuping *et al.* (2011), using nitric acid digestion was applied.

Metal concentrations were determined using the ICP-MS (Inductively Coupled Plasma-Mass Spectrophotometer) at Stellenbosch University. ICP results were then converted using the following formula:

$$\text{For plants:} \quad \frac{(\text{ICP reading} - \text{Blank}) \times 100}{\text{mass (g)}}$$

For water samples: [ICP reading – Blank] X 10

The plant metal concentrations were expressed as mg/kg and all water metal concentrations as mg/L. Due to a calibration error in ICP analyses no experimental data for Fe in water medium are available and because of the cost implication the samples could not be re-analysed.

2.4.2. Determination of metal concentrations in water and plants from the field site (Diep River).

Water and plant samples from the lower Diep River sampling site were tested for aluminium, copper, iron and zinc. Five plants of *C. demersum* L. were collected from the lower Diep River. The same procedures for metal analyses as in 2.4.1 were followed in the samples from the Diep River.

2.5. Chlorophyll content

2.5.1. Determination of chlorophyll content in C. demersum L.

Five samples of *C. demersum* L. were collected from the containers every week for six weeks. Chlorophyll analyses were performed according to the method described by Arnon (1949) for all plant samples. The fresh leaf mass (± 250 mg) was determined for the leaf samples prior to chlorophyll measurement. Chlorophyll was extracted in 80% chilled acetone in the dark. A 3 ml sample of chlorophyll extract was transferred into a small glass cuvette for absorbance determination. Absorption of the extracts at wavelengths of 663 nm (D_{663}) and 645 nm (D_{645}) were measured with a Beckman (DU 640) spectrophotometer. Concentrations of chlorophyll a (Chl-a), chlorophyll b (Chl-b), and total chlorophyll (Chl-t) were calculated using the following equations (Arnon, 1949):

$$\text{Chl-a} = 12.25A_{663} - 2.79A_{645}$$

$$\text{Chl-b} = 21.5A_{645} - 5.10A_{663}$$

$$\text{Chl-t} = 20.2\,A_{645} + 7.15A_{663}$$

Total chlorophyll content was expressed in milligrams per litre (mg/L).

2.5.2. Determination of chlorophyll content in C. demersum L. in the field (Diep River)

Five plants of *C. demersum* L. were collected from the lower Diep River for chlorophyll analyses. The same procedures for chlorophyll analyses as in 2.5.1 were followed in the samples from the Diep River.

2.6. Biochemical analyses

2.6.1. Chemicals and equipment

Sodium di-hydrogen orthophosphate mono hydrate ($NaPO_4$), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), 6-hydroxydopamine (6-HD), diethylenetriaminepenta-acetic acid (DETAPAC), 5,5'-dithio-bis-(2-nitrobenzoic acid) reagent (DTNB), ethylenediaminetetra-acetic acid (EDTA), fluorescein sodium salt (Fl), glacial metaphosphoric acid (MPA), glutathione reduced (GSH), glutathione reductase (GR), L-ascorbic acid (AA), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), iron chloride hexahydrate and 2,4,6-tri[2-pyridyl]-s-triazine (TPTZ), malondialdehyde (MDA) standard, 1-methyl-2-vinylpyridinium trifluoromethanesulphonate (M2VP), orthophosphoric acid (O-PA), perchloric acid (PCA), potassium phosphate (KH_2PO_4), reduced β-nicotinamide adenine dinucleotide phosphate (NAD(P)H), sodium azide, sodium hydroxide (NaOH), iron(III) chloride ($FeCl_3$), sulphuric acid, superoxide dismutase standard, tertiary-butyl hydroperoxide (t-BHP), thiobarbituric acid (TBA) and trisodium citrate was purchased from Sigma-Aldrich (Johannesburg, SA). All solvents used were of analytical reagent grade. Acetic acid, chloroform, glacial acetic acid, hydrochloric acid (HCl), isopropanol, methanol, perchloric acid (PCA) 70%, sodium acetate and trifluoroacetic acid (TFA), 6-hydroxydopamine (6-HD), were purchased from Merck (Johannesburg, SA). Hydrogen peroxide (H_2O_2) was purchased from BDH AnalaR®. Ultrapure MilliQ water (Millipore) was used throughout the study. Reactions for ORAC were measured and read in Nunc black 96-well flat bottom fluorescence microplates (Sigma–Aldrich,Johannesburg, South Africa) using a Fluoroskan Ascent analyser (Thermo Electron Corporation, Finland). All other reactions were measured and read in clear Greiner 96-well flat bottom and Costar 96-well UV flat bottom microplates (Sigma-Aldrich, South Africa) in a Multiskan spectrophotometer (Thermo Electron Corporation, Finland). All centrifugations were performed using a refrigerated bench top centrifuge (Eppendorf 5810R, Eppendorf, Germany).

2.6.2. Plant sampling and preparation

Five plants of *Ceratophyllum demersum* L. were collected each week from each container. These samples were rinsed with distilled water, blotted dry with paper and placed in individually labelled plastic bags and immediately transferred into a 5 L flask with liquid nitrogen. Samples were then stored at -80^0 C until analyses were performed. All samples (±250 mg) were homogenized with 6 mL of 25 mM HEPES-KOH buffer containing 0.2 mM EDTA and 2% PVP (pH 7.8), on ice. The homogenate was split into 3 x 2 mL microcentrifuge tubes and centrifuged at 15 000 *g* for 10 minutes at 4 °C. The resulting supernatant was transferred to new 2 mL microcentrifuge tubes and stored at -80 °C until needed.

2.6.3. Evaluation of antioxidant content and -capacity

2.6.3.1. Total Polyphenol determination (TP)

The total phenolic content was determined as described by Waterhouse (2005). Fresh plant tissue samples (± 250 mg) were homogenized in 10 mL 80% methanol (CH_3OH) in 15 mL test tubes. The samples were further extracted by placing it on a tube rotator (Intelli mixer) for 15 minutes at 35 rpm and centrifuged for at 15 000 *g* for 10 minutes at 4 ^{0}C. The samples (25 µL) were added in triplicate to a 96-well plate followed by the addition of 125 µL Folin-Ciocalteau phenol reagent (0.2N) and 100 µL sodium carbonate solution (Na_2CO_3) (7.5%, w/v). Gallic acid was dissolved in 10% ethanol (CH_2OH) (200 mg/L) and used as the control. Plates were incubated for two hours at room temperature before read in a UV/VIS spectrophotometer at 280 nm. Results were expressed as mg gallic acid equivalents (GAE) per gram plant material.

2.6.3.2. Oxygen Radical Absorbance Capacity (ORAC)

Plant samples, reagents and standards were prepared in phosphate buffer (75 mM, pH 7.4, ORAC buffer) and centrifuged at 4 000 rpm for 10 minutes at 4^0 C. The ORAC assay was performed according to the method of Cao & Prior (1999) in a 96-microwell Nunc plate using a Fluoroscan Ascent (Thermo Electron Corporation) fluorescence spectrophotometer. All samples were done in triplicate. The reaction consisted of 12 µL of diluted sample (1:4) and 138 µL fluorescein (final concentration 14 µM per well) that were mixed in a black Nunclon 96-well plate. Stock solution of 2.2´ - azobis (2-amidinopropane) dihydrochloride (AAPH) (500 µM) was prepared and 50 µl was added to the plate before readings. The standards

were prepared within a range of 0 – 417 µM Trolox. The fluorescence (emission 530 nm, excitation 485 nm) was recorded every 5 minutes for 2 hours. The ORAC values were calculated using a regression equation ($Y = a + bx + cx^2$) between Trolox concentration (Y) (µM) and the net area under the fluorescence decay curve (x). The ORAC values were calculated and expressed as micromoles of Trolox equivalents (TE) per milligram of sample (µmole TE/g) fresh weight of the plant.

2.6.3.3. Ferric Reducing Ability of Plasma (FRAP)

The homogenized plant material (1.5 µL) were added in triplicate to a 96-well plate followed by the addition of 300 µL of FRAP reagent which consisted of 30 mL acetate buffer (300 mM, pH 3.6), 3 mL TPTZ (2,4,6-tri[2-pyridyl]-s-triazine) 10 mM solution, 3 mL $FeCl_3$ (Iron (III) chloride hexahydrate) 20 mM (F2877) solution and 6.6 mL distilled water. The blank was prepared using the same chemical reagents excluding the extract. L-Ascorbic acid 400 µM was used as the control and 10 µL was added in the control wells. The final volume of the assay was 310 µL. The plate was incubated for 30 minutes in a 37 ^{0}C water bath. The change in absorbance was then recorded on a spectrophotometer at 593 nm in a Multiskan reader. Final results were obtained by comparison to the calibration curve standard using a regression equation ($y = a + bx$). The results were expressed as µmole ascorbic acid equivalents (AAE)/g.

2.6.3.4. Ascorbic acid (AsA)

The assay for ascorbic acid (AsA) was done in the same way as the assay for FRAP. The homogenized plant material (1.5 µL) were added in triplicate to a 96-well plate followed by the addition of 300 µL of FRAP reagent. The blank was prepared using the same chemical reagents excluding the extract. L-Ascorbic acid 400 µM was used as the control and 10 µL was added in the control wells. The final volume of the assay was 310 µL. The plate was incubated for 30 minutes in a 37 ^{0}C water bath before readings. Another 96-microwell plate was prepared in the same manner as above with the addition of 10 µL D-ascorbic acid (DAA). The change in absorbance was recorded at 593 nm using a Multiskan reader. Final results were obtained by comparison to the calibration curve standard using a regression equation ($y = a + bx$). The results were expressed as µmole ascorbic acid equivalents (AAE)/g.

2.7. Evaluation of antioxidant defense system

2.7.1. Superoxide Dismutase (SOD)

Superoxide dismutase activity was determined by a modified method from Ellerby and Bredesen (2000). The assay was performed by adding 170 µl DETAPAC solution (0.1 mM) in an SOD assay buffer ($NaHPO_4$, 50 mM, pH 7.5) to a 96-microwell plate. A sample volume of 12 µL was added to a 96-microwell plate. The samples were diluted 1:10 (v:v) homogenate to buffer and the SOD buffer was added to the wells to make up a final volume of 200 µL. Fifteen microliters of stock 6-HD (1.6 mM) to initiate the reaction, where after the combined solution was mixed and the amount of protein used that resulted in 50% inhibition of auto oxidation of the 6-HD was measured at 490 nm for 4 min at 1 min intervals in a Multiskan reader.

2.7.2. Catalase activity (CAT)

Catalase activity was determined by the modified method of Ellerby and Bredesen (2000). The homogenates were thawed on ice and diluted (1:5 v:v) homogenate to buffer. To a 96-microwell plate, an assay mixture containing 170 µL of phosphate buffer (50 mM KPO_4 buffer, pH 7.0) and 10 µL of the diluted homogenate (0.1 µg/µL), in triplicate, was added. Thereafter 75 µL of H_2O_2 stock solution (30% v/v) was added. The plate was gently shaken to ensure mixing, where after CAT activity was determined spectrophotometrically at 240 nm by monitoring the decomposition of H_2O_2. A linear absorbance at 240 nm decrease/min was read for at least 1 minute in 15 second intervals. The activity was expressed (equation 1) µmoles/min/µg protein using the millimolar extinction coefficient of 0.000394 mM^{-1}. cm^{-1}.

Activity = [(A1-A2)/ε] x 0.5 / µg protein = µmole/min/µg …….1

Equation 1: Calculation of catalase activity in plant material

2.7.3. Determination of Total Glutathione (GSHt) concentrations

Reduced and oxidized glutathione (GSH:GSSG) levels were determined according to Tietze (1969). In this assay glutathione reductase is added and hence both GSH and GSSG measured, which indicates total glutathione presence. For the GSSG determination, the frozen plant tissue were homogenised using 500 mM NaPO4 with 1 mM EDTA (pH 7.5), containing M2VP and centrifuged at 10000 x g for 5 minutes at 4 °C. GSH determination was

done on previously homogenised frozen plant samples without M2VP. This enabled conjugation of GSH for the determination of GSSG. Reduced glutathione and GSSG standards (50 µL) were prepared in triplicate and added to 96-microwell plates. To these wells, 50 µL (0.3 mM) DTNB and thereafter 50 µL of GR (1U/50µL) were added. The microwell plates were then mixed and incubated for 5 minutes at 25 ºC. To initiate the reaction, 50 µL of 1 mM NADPH was added to each well. The total content of glutathione was quantified using a spectrophotometer which monitored the reduction of DTNB at 412 nm within 2 min. Each sample was run in triplicate and final results were obtained by comparison to the calibration curve standard using a regression equation (y = a + bx). Calibration curves for GSSG and GSH were determined separately and the GSH:GSSG ratios calculated by dividing the difference between GSH and GSSG concentrations by the concentrations of GSSG. GSHt concentration was expressed as µmole/g.

2.8. Evaluation of oxidative damage

2.8.1. Lipid peroxidation evaluation

2.8.1.1. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content following Heath and Packer (1968) with slight modification. Plant samples were stored at -80 ^{0}C prior to assay. Plant material (± 0.250 g) was homogenized in 6 mL of 80% methanol. The supernatant (50 µL) was mixed with 6.25 µL of 0f 4 mM butylated hydroxytoluene/ethanol and 50 µL ortho-phosphoric acid. The resultant homogenate was combined with 6.25 µL of 0.67% thiobarbituric acid (TBA) solution and incubated at 90 ^{0}C for 45 minutes then cooled in ice water. The mixture was allowed to cool to room temperature and then mixed with 500 µL n-butanol and 50 µL saturated NaCl. The samples were vortexed and then centrifuged at 12 000 rpm for 2 minutes at 4 ^{0}C. From this mixture, 150 µL of the supernatant was added to a 96-microwell plate (in triplicate). The absorbance was measured at 532 nm using a Multiskan plate reader. Lipid peroxidation was expressed as nmole TBARS per mg protein.

2.8.1.2. Determination of Conjugated Dienes levels (CDs)

Plant conjugated dienes levels were estimated using a ultraviolet spectrophotometric-modified method by Recknagel and Glende (1984). A 2:1 solution of chloroform ($CHCl_3$) was prepared of which 400 µL was added to 50 µL of tissue sample in an Eppendorf tube. The

mixture was vortexed for 60 seconds and centrifuged at 10 000 *g* for 15 minutes at 4 ^{0}C. After centrifugation, the mixture separated in three layers, namely a top aqueous layer, a protein layer and a lipid layer at the bottom. The top aqueous layer was removed and discarded. The lipid layer was collected by inserting a pipet tip very gently along the wall of the tube. The lipid phase was then transferred to a new eppendorf tube and dried under nitrogen gas. One millilitre of cyclohexane (C_6H_{12}) was added to the dried tube and vortexed for 30 sec. The aqueous supernatant was discarded 300 µl of this solution was transferred into a 96-microwell UV Costar plate and read at 234 nm using a Multiskan spectrophotometer. The samples were done in duplicate. The CDs were expressed as µmole/g plant material using a molar extinction coefficient of 26550 M^{-1} cm^{-1}.

2.9. Biochemical analyses of plants from the Diep River

Plants of *C. demersum* L. and water were collected from the lower Diep River. Methods for biochemical analyses were performed as specified in 2.6 to 2.8.

2.10. Data synthesis and statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Kruskall-Wallis non-parametric test was used to test for significant differences in metal concentrations over time The Kruskall-Wallis test is a typical 'rank' test, which means that the raw data are converted into ranks before the test is carried out. The advantage of this is that it is ideal for situations where the highest value went off the scale or if extreme values are present, as these have a disproportionate influence on the results of parametric tests (Dytham, 2003). Post hoc ANOVA analyses were done using the Student-Newman-Keuls (SNK) Test to determine statistically significances between groups over time ($P<0.05$). Statistical analysis of control and exposure groups was performed by Student's t-test. Differences were considered significant at $P<0.05$. The MediCalc Version 15.2.2 (1993-2015) software package was used for all statistical evaluations.

CHAPTER 3: RESULTS AND DISCUSSION: EXPOSURE EXPERIMENT: Metal analysis of water medium and plants (*Ceratophyllum demersum* L.)

3.1. Results: Water medium

3.1.1. Physico-chemical parameters

Conductivity, pH, salinity and temperature of the water were measured in each treatment during each sampling occasion. These parameters are tabulated in Table 3.1.

3.1.1.1. Water pH

According to Hoagland and Arnon (1950) the pH of Hoagland solution is 6.0. During the five week experimental period the lowest mean water pH of the control was 6.39 ± 0.01 and the highest mean was 8.44 ± 0.06. The lowest mean water pH of treatment T¼ was 6.46 ± 0.01 and the highest mean water pH was 8.42 ± 0.08 over the experimental period. The lowest mean water pH for treatment T½ was 6.56 ± 0.09 and the highest mean water pH was 7.9 ± 0.08. The lowest water pH of treatment T1 was 6.50 ± 0.00 and the highest mean water pH was 6.90 ± 0.01. For treatment T2 the lowest water pH was 6.56 ± 0.09 and the highest water pH was 6.91 ± 0.01 (Table 3.1).

3.1.1.2. Water temperature

The lowest mean water temperature of the control was 21.26 ± 0.04 °C and the highest mean water temperature was 29.0 ± 0.06 ˚C during the five week experimental period. The lowest mean water temperature for treatment T¼ was 20.84 ± 0.08 ˚C and the highest mean water temperature was 28.70 ± 0.10 ˚C and for treatment T½ the lowest mean water temperature was 20.84 ± 0.08 ˚C and the highest mean was 28.26 ± 0.15 ˚C. The lowest mean water temperature for treatment T1 was 21.30 ± 0.13 ˚C and the highest mean was 28.08 ± 0.07 ˚C and for treatment T2 the lowest mean water temperature was between 21.38 ± 0.04 ˚C and the highest mean was 27.96 ± 0.05 ˚C (Table 3.1).

3.1.1.3. Conductivity

Electrical conductivity (EC) is a useful indicator of the salinity or total salt content in a water sample (Anon, 1996). The mean electrical conductivity was measured in each container over

the five week experimental period and the lowest mean conductivity was 0.000 ± 0.00 and the highest mean was 511.12 ± 0.07 mS/cm for the control; the lowest mean conductivity was 0.000 ± 0.00 and the highest mean was 525.85 ± 0.75 mS/cm for treatment T¼; the lowest mean conductivity was 0.000 ± 0.00 and the highest mean was 453.63 ± 2.33 mS/cm for treatment T½; the lowest mean was 0.000 ± 0.00 and the highest mean was 535.41 ± 0.80 mS/cm for treatment T1 and the lowest mean was 0.000 ± 0.00 and the highest mean was 487.00 ± 0.89 for treatment T2 (Table 3.1).

3.1.1.4. Salinity

The mean salinity was measured in each container over the six week experimental period and the lowest mean salinity was 150.40 ± 0.49 ppm and the highest mean was 201.22 ± 1.60 ppm in the control; the lowest mean salinity was 156.60 ± 0.49 and the highest mean salinity was 196.61 ± 1.02 ppm for treatment T¼; the lowest mean salinity was 150.2 ± 0.40 and the highest mean was 196.84 ± 1.47 ppm for treatment T½; the lowest mean salinity was 152.33 ± 0.47 and the highest mean was 196.80 ± 1.47 ppm for treatment T1 and the lowest mean salinity was 153.20 ± 0.84 and the highest mean was 199.65 ± 1.62 ppm for treatment T2 (Table.3.1).

Table 3.1. Conductivity, pH, salinity and temperature, measured in water in each experimental treatment during each sampling occasion.

Sampling occasion	Conductivity (mS/cm)	pH	Salinity (ppm)	Temperature (^{0}C)
w0				
Control	0.00	6.39	150.40	24.56
T¼	0.00	6.46	153.20	24.72
T½	0.00	6.40	150.26	24.52
T1	0.00	6.50	152.33	24.28
T2	0.00	6.52	153.20	24.20
w1				
Control	137.20	6.51	178.01	29.06
T¼	123.01	6.54	176.25	28.71
T½	133.28	6.71	154.84	28.35
T1	125.31	6.64	167.03	28.15
T2	128.25	6.6 2	165.83	28.04
w2				
Control	451.22	6.45	186.48	25.62
T¼	450.75	6.47	196.61	26.13
T½	387.61	6.44	194.01	25.90
T1	400.04	6.49	188.00	25.24
T2	422.02	6.33	187.23	25.00
w3				
Control	469.05	8.34	199.03	23.50
T¼	486.01	8.45	187.27	25.13
T½	413.61	7.91	199.03	26.02
T1	479.07	6.83	196.84	24.47
T2	442.05	7.01	199.65	24.69
w4				
Control	454.61	8.41	201.22	21.32
T¼	446.40	8.34	179.41	21.43
T½	388.20	7.90	189.25	21.36
T1	478.22	6.92	176.45	21.38
T2	419.83	6.83	194.00	21.47
w5				
Control	511.12	7.20	191.63	21.21
T¼	525.85	7.34	179.02	20.91
T½	453.63	7.11	182.80	20.82
T1	535.41	6.91	181.10	21.44
T2	487.00	6.92	181.82	21.40

Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.

3.1.2. Comparisons of aluminium (Al) concentrations between weeks in water samples

Comparisons of the mean Al concentrations in water, measured between weeks, are illustrated in Table 3.2 and Figure 3.1.

Control (baseline): When compared to week 0, the Al concentrations were significantly ($P<0.05$) lower during weeks 1, 2, 4 and 5, the latter indicating an overall decrease in Al from the start to the end of the experiment. Between consecutive weeks a significant increase in Al was found between week 2 and week 3, while significant decreases in Al concentrations were recorded between week 0 and week 1 and between week 3 and week 4 ($P<0.05$).

Treatment T¼: When compared to week 0, the Al concentrations were significantly ($P<0.05$) lower during weeks 1, 2 and 3. Between consecutive weeks a significant ($P<0.05$) decrease in Al concentrations was shown between week 0 and week 1 and a significant increase in Al concentration was found between week 4 and week 5 ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T½: When compared to week 0, the Al concentrations were significantly ($P<0.05$) higher during week 1. Between consecutive weeks Al concentrations increased significantly ($P<0.05$) between week 0 and week 1 and indicated a significant decrease in Al concentrations between week 1 and week 2, while recovering to the same baseline concentrations between weeks 2 and 3; between weeks 3 and 4 and between weeks 4 and 5 ($P>0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T1: When compared to week 0, Al concentrations were significantly ($P<0.05$) higher during weeks 2 and 3. Between consecutive weeks significant ($P<0.05$) increases in Al concentrations were indicated between week 2 and week 3 and between week 4 and week 5, while Al concentrations between week 3 and week 4 recovered to the same level as that of the baseline ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T2: When compared to week 0, Al concentrations were significantly ($P<0.05$) higher during week 1, but significantly ($P<0.05$) lower during weeks 2, 3, and 4. Between consecutive weeks significant ($P<0.05$) increases in Al concentrations were indicated between week 0 and week 1, between week 2 and week 3 and between week 4 and week 5. A significant decrease in Al concentration was found between week 1 and week 2 ($P<0.05$).

Table 3.2. Mean (±SD) aluminium (Al) concentrations (mg/L), measured in water medium from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}1.54^{a}$ ±0.22	$^{a}1.11^{b}$ ±0.48	$^{a}0.00^{c}$ ±0.00	$^{a}0.00^{c}$ ±0.00	$^{a}0.77^{d}$ ±0.10
1	$^{*b}0.00^{a}$ ±0.00	$^{*b}0.01^{b}$ ±0.01	$^{*b}2.06^{c}$ ±0.23	$^{a}0.76^{d}$ ±0.27	$^{*b}2.02^{e}$ ±0.28
2	$^{*c}0.03^{a}$ ±0.04	$^{*b}0.10^{a}$ ±0.04	$^{c}0.00^{a}$ ±0.00	$^{*b}0.90^{b}$ ±0.09	$^{*c}0.17^{b}$ ±0.05
3	$^{c}2.07^{a}$ ±0.40	$^{*b}0.01^{b}$ ±0.01	$^{c}0.01^{b}$ ±0.03	$^{*c}1.84^{c}$ ±0.19	$^{*d}0.22^{d}$ ±0.03
4	$^{*d}0.00^{a}$ ±0.00	$^{b}0.04^{a}$ ±0.08	$^{c}0.00^{a}$ ±0.00	$^{d}0.00^{a}$ ±0.00	$^{*d}0.00^{a}$ ±0.00
5	$^{*e}0.02^{a}$ ±0.05	$^{c}2.62^{b}$ ±0.46	$^{c}0.00^{c}$ ±0.00	$^{d}0.40^{d}$ ±0.12	$^{e}1.37^{e}$ ±0.19
Pooled data for entire experimental period	**0.61^{a} ±0.11**	**0.66^{a} ±0.18**	**$^{b}0.35^{b\#}$ ±0.04**	**0.65^{c} ±0.11**	**0.76^{d} ±0.11**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

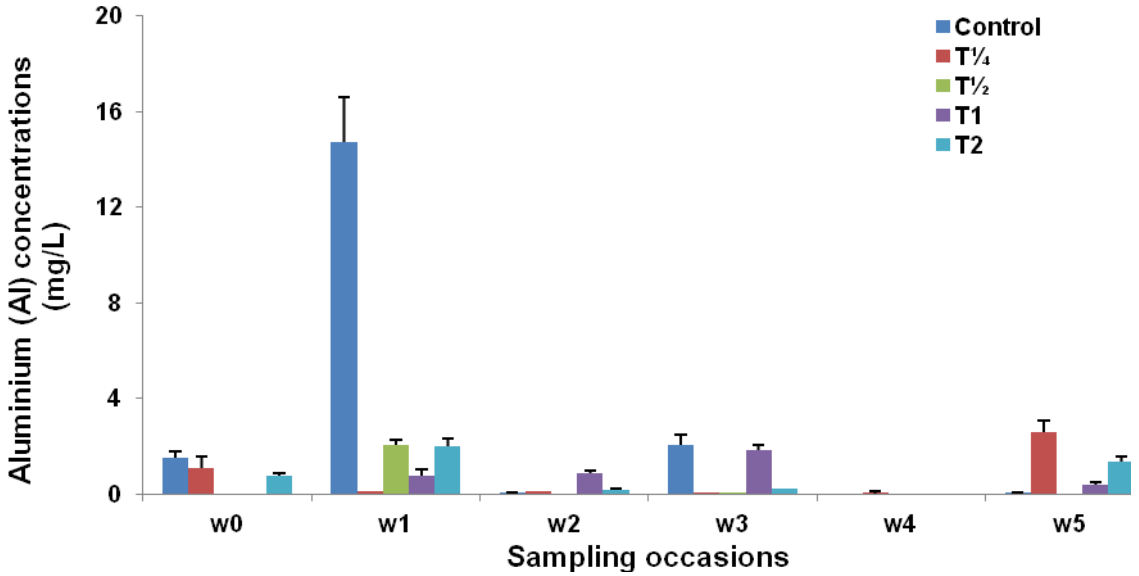

Figure 3.1. Mean (±SD) Aluminium (Al) concentrations (mg/L), measured in water medium per week in experimental treatments. *Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure.*

3.1.3. Comparisons of aluminium (Al) concentrations between treatments per week in water samples

Table 3.2 shows comparisons of Al concentrations in water samples between treatments per week as well as comparisons of pooled data.

Week 0: The Al concentrations measured for treatments T¼, T½, T1 and T2 were all significantly lower compared to the control (baseline) ($P<0.05$). Between treatments: The Al concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T¼; the Al concentrations of treatment T1 were significantly higher compared to the Al concentrations of treatment T½ and the Al concentrations of treatment T2 were significantly higher compared to the Al concentrations of treatment T1 ($P<0.05$).

Week 1: The Al concentrations of treatments T¼, T½, T1 and T2 were all significantly higher compared to Al concentrations of the control (baseline) ($P<0.05$). Between treatments: The Al concentrations of treatment T¼ were significantly higher compared to the concentrations of the control; the Al concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T¼; the Al concentrations of treatment T1 were significantly

lower compared to the Al concentrations of treatment T½, while the Al concentrations of treatment T2 were significantly higher compared to treatment T1 (P<0.05).

Week 2: The Al concentrations of treatment T1 were significantly higher compared to those of the control (P<0.05). Between treatments: The Al concentrations of treatment T1 were significantly higher compared to the Al concentrations of treatment T¼ (P<0.05).

Week 3: The aluminium concentrations of treatments T¼, T½ and T2 were significantly lower compared to those of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly lower compared to those of the control; the Al concentrations of treatment T1 were significantly higher compared to treatment T½ and the Al concentrations of treatment T2 were significantly lower compared to those of treatment T1 (P<0.05).

Week 4: No significant differences in Al concentrations were found between the treatments and the control and also between treatments (P>0.05).

Week 5: The Al concentrations of treatments T¼ and T2 were significantly higher and the Al concentrations of treatment T1 were significantly lower compared to those of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly higher compared to those of the control; the Al concentrations of treatment T½ were significantly lower compared to those of treatment T¼; the Al concentrations of treatment T1 were significantly higher compared to those of treatment T½ and the concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 (P<0.05).

Pooled data: The Al concentrations for treatment T½ were significantly lower (P<0.05) compared to the control (baseline), and the Al concentrations for treatments T¼, T1 and T2 indicated no significant difference compared to the control (P>0.05). Between treatments: The Al concentrations of treatment T1 were significantly higher compared to treatment T½ (P<0.05). In treatments T½ and T1 there were an increase in Al concentrations from the start to the end of the experiment. Treatment T¼ and the control indicated decreases in Al concentrations from the start to the end of the experiment and the Al concentrations of treatment T2 remained at the baseline concentration.

3.1.4. Comparisons of copper (Cu) concentrations between weeks in water samples

Comparisons of the mean Cu concentrations in water, measured between weeks, are illustrated in Table 3.3 and Figure 3.2.

Control (baseline): When compared to week 0, the Cu concentrations were significantly ($P<0.05$) higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Al concentrations from the start to the end of the experiment. Between consecutive weeks a significant ($P<0.05$) increase in Cu concentrations were found between week 0 and week 1 and between week 2 and week 3. Significant decreases in Cu concentrations were found between week 1 and week 2 and between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, the Cu concentrations were significantly ($P<0.05$) lower during weeks 1, 2, 3 and 4. Between consecutive weeks significant decreases in Cu concentrations were shown between week 0 and week 1 and between week 3 and week 4 and a significant increase was found between week 2 and week 3 ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T½: When compared to week 0, the Cu concentrations were significantly lower during weeks 2, 3 and 4. Between consecutive weeks Cu concentrations decreased significantly between week 1 and week 2, between week 2 and week 3 and between week 3 and week 4, while increasing significantly between week 4 and week 5 ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T1: When compared to week 0, the Cu concentrations were significantly lower during weeks 1, 2, 3 and 4. Between consecutive weeks significant decreases in Cu concentrations were found between week 0 and week 1, between week 1 and week 2 and between week 3 and week 4. Significant increases in Cu concentrations were indicated between week 2 and week 3 and between week 4 and week 5 ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Treatment T2: When compared to week 0, the Cu concentrations were significantly higher during weeks 2, 3 and 4. Between consecutive weeks a significant increase in Cu concentration was indicated between week 1 and week 2 ($P<0.05$). There was however no significant difference when comparing week 0 and week 5 over the entire experimental period.

Table 3.3. Mean (±SD) copper (Cu) concentrations (mg/L), measured in water medium from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}0.00^{a}$ ±0.00	$^{a}1.79^{b}$ ±0.26	$^{a}0.55^{c}$ ±0.04	$^{a}1.95^{d}$ ±0.04	$^{a}0.05^{e}$ ±0.03
1	$^{*b}1.59^{a}$ ±0.05	$^{*b}0.07^{b}$ ±0.08	$^{a}0.49^{c}$ ±0.06	$^{*b}0.37^{d}$ ±0.07	$^{a}0.05^{e}$ ±0.04
2	$^{*c}0.36^{a}$ ±0.42	$^{*b}0.00^{a}$ ±0.00	$^{*b}0.28^{a}$ ±0.09	$^{*c}0.00^{a}$ ±0.00	$^{*b}0.42^{a}$ ±0.07
3	$^{*d}1.38^{a}$ ±0.67	$^{*c}0.49^{b}$ ±0.00	$^{*c}0.01^{c}$ ±0.03	$^{*d}0.92^{d}$ ±0.08	$^{*b}0.67^{e}$ ±0.12
4	$^{*d}0.90^{a}$ ±0.71	$^{*d}0.00^{b}$ ±0.00	$^{*d}0.31^{c}$ ±0.08	$^{*e}0.46^{d}$ ±0.16	$^{*b}0.71^{e}$ ±0.10
5	$^{*e}0.37^{a}$ ±0.08	$^{d}0.00^{b}$ ±0.00	$^{e}0.01^{b}$ ±0.01	$^{f}0.99^{c}$ ±0.11	$^{b}0.79^{d}$ ±0.07
Pooled data for entire experimental period	**0.77^{a} ±0.45**	**$^{b}0.39^{b\#}$ ±0.06**	**$0.27^{c\#}$ ±0.05**	**0.78^{d} ±0.08**	**0.45^{d} ±0.07**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between treatments per week and significant statistical differences between the control and treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

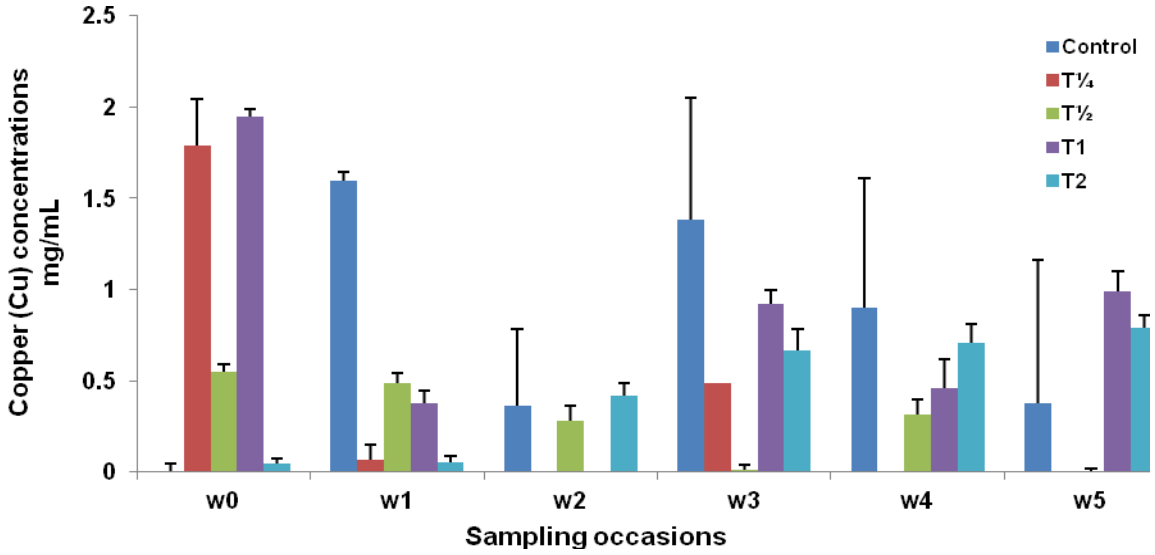

Figure 3.2. Mean (±SD) copper (Cu) concentrations (mg/L), measured in water medium in experimental treatments per week. *Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure.*

3.1.5. Comparisons of copper (Cu) concentrations between treatments in water samples

Table 3.3 and Figure 3.2 show comparisons between treatments per week, as well as comparisons of pooled treatment data.

Week 0: The Cu concentrations measured for treatments T¼, T½, T1 and T2 were all significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: Cu concentrations of treatment T¼ was significantly higher compared to the control, Cu concentrations of treatment T½ was significantly lower compared to treatment T¼, and Cu concentration treatment T1 was significantly higher compared to the Cu concentration of treatment T½, while the Cu concentrations of treatment T2 were significantly lower compared to treatment T1 ($P<0.05$).

Week 1: The Cu concentrations measured for treatments T¼, T½, T1 and T2 were all significantly lower compared to the Cu concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to those of the control, the Cu concentrations of treatment T½ were significantly higher

compared to those of treatment T¼, the Cu concentrations of treatment T1 were significantly lower than the Cu concentrations of treatment T½ and the Cu concentrations of treatment T2 were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Week 2: No significant differences in Cu concentrations were indicated between the treatments and the control ($P>0.05$). Between treatments: No significant differences in Cu concentrations were found between treatments ($P>0.05$).

Week 3: The Cu concentrations measured for treatments T¼, T½, T1 and T2 were significantly lower compared to the Cu concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to those of the control, the Cu concentrations of treatment T½ were significantly lower compared to those of treatment T¼, the Cu concentrations of treatment T1 were significantly higher than the Cu concentrations of treatment T½ and the Cu concentrations of treatment T2 were significantly lower compared to the Cu concentrations of treatment T1 ($P<0.05$).

Week 4: The Cu concentrations of treatments T¼, T½, T1 and T2 were all significantly lower compared to the Cu concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to those of the control, the Cu concentrations of treatment T½ were significantly higher compared to those of treatment T¼, the Cu concentrations of treatment T1 were significantly higher than the concentrations of treatment T½ and the Cu concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 5: No significant differences in Cu concentrations were indicated between the treatments and the control ($P>0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to the Cu concentrations of the control, the Cu concentrations of treatment T1 were significantly higher compared to the Cu concentrations of treatment T½ and the Cu concentrations of treatment T2 were significantly lower compared to the Cu concentrations of treatment T1 ($P<0.05$).

Pooled data: The Cu concentrations for treatments T¼ and T½ were significantly lower compared to the control (baseline). Treatments T1 and T2 indicated no significant differences compared to the Cu levels of the control ($P>0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to the control, the Cu concentrations of treatment T¼ were significantly lower compared to the Cu concentrations of treatment T½. The Cu concentrations of treatment T1 were significantly higher compared to the Cu concentrations of treatment T½ ($P<0.05$). In treatments T¼, T½ and T1 there were

a decrease in Cu concentrations from the start to the end of the experiment. Treatment T2 and the control indicated increases in Cu concentrations from the start to the end of the experiment.

3.1.6. Comparisons of iron (Fe) concentrations between weeks in water samples

Due to a technical error (see Chapter 2, section 2.4.1) no experimental data for Fe in water is available.

3.1.7. Comparisons of iron (Fe) concentrations between treatments in water samples

Due to a technical error (see Chapter 2, section 2.4.1) no experimental data for Fe in water is available

3.1.8. Comparisons of zinc (Zn) concentrations between weeks in water samples

Comparisons of the mean Zn concentrations in water, measured between weeks, are illustrated in Table 3.4 and Figure 3.3.

Control (baseline): When compared to week 0, the Zn concentrations were significantly lower during week 1, and were significantly higher at week 2. Between consecutive weeks the Zn concentrations decreased between week 0 and week 1 and between week 1 and week 2. Week 5 indicated and overall significant increase in Zn concentration from the start to the end of the experiment ($P<0.05$).

Treatment T¼: When compared to week 0, the Zn concentrations were significantly higher during weeks 1 and 4 ($P<0.05$). Between consecutive weeks a significant increase in Zn concentration was found between week 0 and week 1 and significant decreases were found between week 1 and week 2 and between week 4 and week 5 ($P<0.05$). No significant difference in Cu concentrations was found overall from the start to the end of the experiment ($P>0.05$).

Treatment T½: When compared to week 0, the Zn concentrations were significantly higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Zn concentrations from the start to the end of the experiment. Between consecutive weeks Zn concentrations increased significantly between week 0 and week 1 and between week 2 and week 3. Zn concentrations decreased significantly between week 1 and week 2 and between week 3 and week 4 ($P<0.05$).

Treatment T1: When compared to week 0, the Zn concentrations were significantly higher during weeks 2, 3, 4 and 5, the latter indicating an overall increase in Zn concentrations from the start to the end of the experiment. Between consecutive weeks Zn concentrations increased significantly between week 1 and week 2 and between week 4 and week 5. Zn concentrations decreased significantly between week 2 and week 3 and between week 3 and week 4 ($P<0.05$).

Treatment T2: When compared to week 0, the Zn concentrations were significantly lower during weeks 2 and 4. Week 5 indicated and overall increase in Zn concentration from the start to the end of the experiment but it was not significant. Between consecutive weeks Zn concentrations decreased significantly between week 1 and week 2 and between week 3 and week 4. Zn concentrations increased significantly between week 2 and week 3 and between week 4 and week 5 ($P<0.05$).

Table 3.4. Mean (±SD) zinc (Zn) concentrations (mg/L), measured in water medium from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}0.29^{a}$ ±0.06	$^{a}0.38^{a}$ ±0.14	$^{a}0.03^{b}$ ±0.02	$^{a}0.11^{c}$ ±0.02	$^{a}0.61^{d}$ ±0.13
1	$^{*b}0.15^{a}$ ±0.04	$^{*b}3.58^{a}$ ±0.73	$^{*b}0.88^{b}$ ±0.06	$^{a}0.18^{c}$ ±0.09	$^{a}0.71^{d}$ ±0.07
2	$^{*c}0.56^{a}$ ±0.12	$^{c}0.33^{a}$ ±0.03	$^{*c}0.13^{b}$ ±0.05	$^{*b}1.27^{c}$ ±0.08	$^{*b}0.21^{d}$ ±0.12
3	$^{c}0.29^{a}$ ±0.01	$^{c}0.54^{a}$ ±0.06	$^{*d}2.09^{a}$ ±0.15	$^{*c}0.39^{a}$ ±0.06	$^{c}0.58^{a}$ ±0.35
4	$^{c}0.32^{a}$ ±0.07	$^{*c}1.00^{a}$ ±0.52	$^{*e}0.60^{b}$ ±0.17	$^{*d}0.22^{c}$ ±0.08	$^{*d}0.00^{d}$ ±0.00
5	$^{*d}0.31^{a}$ ±0.23	$^{d}0.05^{a}$ ±0.00	$^{*e}0.66^{a}$ ±0.17	$^{*e}0.81^{a}$ ±0.18	$^{e}0.33^{a}$ ±0.10
Pooled data for entire experimental period	**0.32^{a} ±0.09**	**0.98^{a} ±0.25**	**0.48^{a} ±0.29**	**0.50^{a} ±0.09**	**0.41^{a} ±0.13**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between treatments per week and significant statistical differences between the control and treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

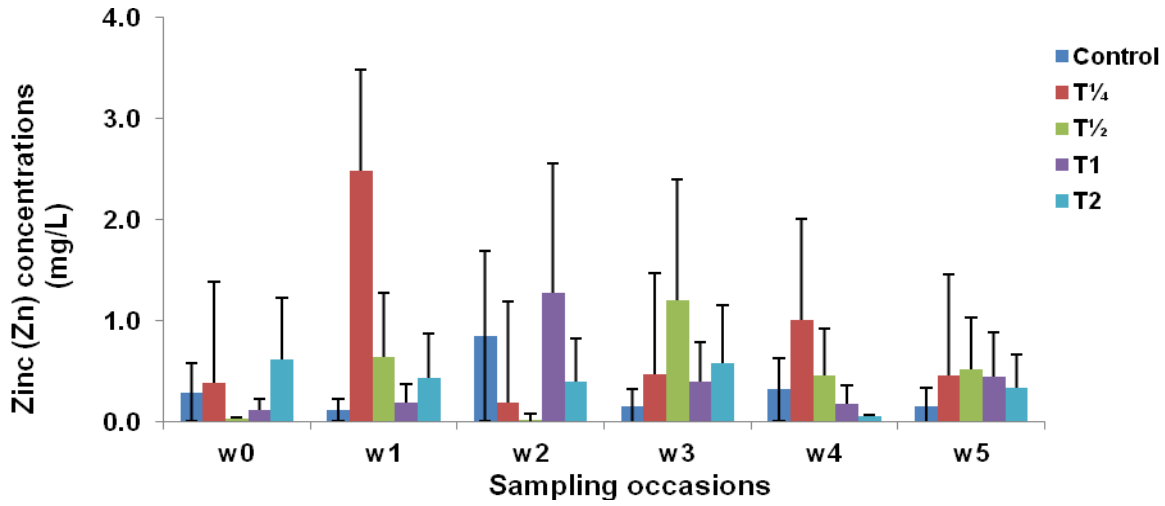

Figure 3.3. Mean (±SD) zinc (Zn) concentrations (mg/L), measured in water medium per week between experimental treatments**.** *Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure.*

3.1.9. Comparisons of zinc (Zn) concentrations between treatments per week in water samples

The comparisons of the concentrations of Zn in water samples of the different treatments are illustrated in Table 3.4 and Figure 3.3.

Week 0: The Zn concentrations measured for treatments T½ and T1 were significantly lower, while the Zn concentrations of treatment T2 were significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: Zn concentrations of treatment T½ was significantly lower compared to treatment T¼, and Zn level treatment T1 was significantly higher compared to the Zn level of treatment T½, while the Zn level of treatment T2 were significantly higher compared to treatment T1 at week 0 ($P<0.05$).

Week 1: The Zn concentrations measured for treatments T½ and T1 were significantly higher compared to the Zn concentrations of the control ($P<0.05$). Between treatments: The Zn concentrations of treatment T½ were significantly lower compared to those of treatment T¼, the Zn concentrations of treatment T1 were significantly lower than the concentrations of treatment T½ and the Zn concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 2: The Zn concentrations measured for treatments T½ and T2 were significantly lower; the Zn concentrations of treatment T1 were significantly higher compared to the Zn concentrations of the control ($P<0.05$). Between treatments: The Zn concentrations measured for treatment T½ were significantly lower compared to those of treatment T¼, the Zn concentrations measured for treatment T1 were significantly higher than the concentrations of treatment T½ and the Zn concentrations measured for treatment T2 were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Week 3: No significant differences in Zn concentrations were indicated between the treatments and the control ($P>0.05$). Between treatments: No significant differences in Zn concentrations were found between treatments ($P>0.05$).

Week 4: The Zn concentrations of treatment T½ were significantly higher compared to the control and the Zn concentrations of treatments T1 and T2 were significantly lower compared to the Zn concentrations of the control ($P<0.05$). Between treatments: The Zn concentrations measured for treatment T½ were significantly lower compared to those of treatment T¼, the Zn concentrations measured for treatment T1 were significantly lower than the Zn concentrations measured for treatment T½ and the Zn concentrations measured for treatment T2 were significantly lower compared to the Zn concentrations measured for treatment T1 ($P<0.05$).

Week 5: No significant differences in Zn concentrations were indicated between the treatments and the control. Between treatments: No significant differences in Zn concentrations were found between treatments ($P>0.05$).

Pooled data: No significant differences in Zn concentrations were indicated between the treatments and the control ($P>0.05$). Between treatments: No significant differences in Zn concentrations were found between treatments ($P>0.05$). In treatments T¼, T½ and T1 there were an increase in Zn concentrations from the start to the end of the experiment. Treatment T2 and the control indicated a decrease in Zn concentrations from the start to the end of the experiment.

3.2. Results: Plants

3.2.1. Comparisons of aluminium (Al) concentrations in *Ceratophyllum demersum* L. between treatments

Comparisons of the mean Al concentrations in plants measured between weeks are illustrated in Table 3.5 and Figure 3.4.

Control (baseline): When compared to week 0, the Al concentrations were significantly higher during week 4 and significantly lower at week 5, the latter indicating an overall decrease in Al concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks a significant increase in Zn level was found between week 3 and week 4 and decreased significantly between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, the Al concentrations were significantly higher during week 3 and significantly lower during weeks 2 and 4 ($P<0.05$). Between consecutive weeks significant decreases in Al concentrations were found between week 1 and week 2 and between week 3 and week 4. Significant increases in Al concentrations were indicated between weeks 2 and week 3 and between week 4 and week 5 ($P<0.05$). Week 5 remained at the baseline concentration with no significant differences in Al concentrations indicated from the start to the end of the experiment ($P<0.05$).

Treatment T½: When compared to week 0, the Al concentrations were significantly higher during weeks 1, 3 and 5, the latter indicating an overall increase in Al concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Al concentrations increased significantly between week 0 and week 1, between week 2 and week 3 and between week 4 and week 5. Al concentrations decreased significantly between week 1 and week 2 and between week 3 and between week 4 ($P<0.05$).

Treatment T1: When compared to week 0, the Al concentrations were significantly higher during weeks 1, 2, 4 and 5, the latter indicating an overall increase in Al concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Al concentrations increased significantly between week 0 and week 1($P<0.05$).

Treatment T2: When compared to week 0, the Al concentrations were significantly higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Al concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Al concentrations increased significantly between week 0 and week 1; between week 2 and

week 3 and between week 4 and week 5. A significant decrease in Al concentration was indicated between week 3 and week 4 ($P<0.05$).

Table 3.5. Mean (±SD) aluminium (Al) concentrations (mg/kg), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}3054.50^{a} ±1336.74	^{a}3983.20^{a} ±812.47	^{a}2511.75^{a} ±678.35	^{a}2764.82^{a} ±703.12	^{a}2462.83^{a} ±359.19
1	^{a}2820.05^{a} ±536.75	^{a}4155.08^{b} ±939.55	^{*b}5311.53^{b} ±1345.83	^{*b}4048.75^{b} ±2036.90	^{*b}4457.82^{b} ±1982.52
2	^{a}2145.77^{a} ±334.35	^{*b}2506.37^{a} ±613.09	^{c}2536.65^{a} ±376.74	^{*b}4826.92^{b} ±1170.06	^{*b}4476.17^{b} ±721.55
3	^{b}3056.20^{a} ±1521.92	^{*c}6026.11^{b} ±1807.53	^{*d}4279.51^{b} ±743.88	^{b}3580.79^{b} ±1924.17	^{*c}9245.92^{c} ±2175.70
4	^{*b}5067.31^{a} ±1021.68	^{*d}2291.91^{b} ±589.05	^{e}2189.13^{b} ±218.60	^{b}5677.85^{c} ±1386.06	^{*d}2376.46^{d} ±659.99
5	^{*c}1348.85^{a} ±261.65	^{e}3903.26^{b} ±1065.56	^{*f}4434.84^{b} ±854.32	^{*b}4722.93^{b} ±1381.44	^{*e}7153.18^{c} ±1005.67
Pooled data entire experimental period	**2915.45^{a} ±1130.10**	**3810.99$^{b\#}$ ±971.21**	**3543.90^{b} ±702.95**	**4270.34$^{c\#}$ ±1433.63**	**5028.73$^{c\#}$ ±1150.67**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

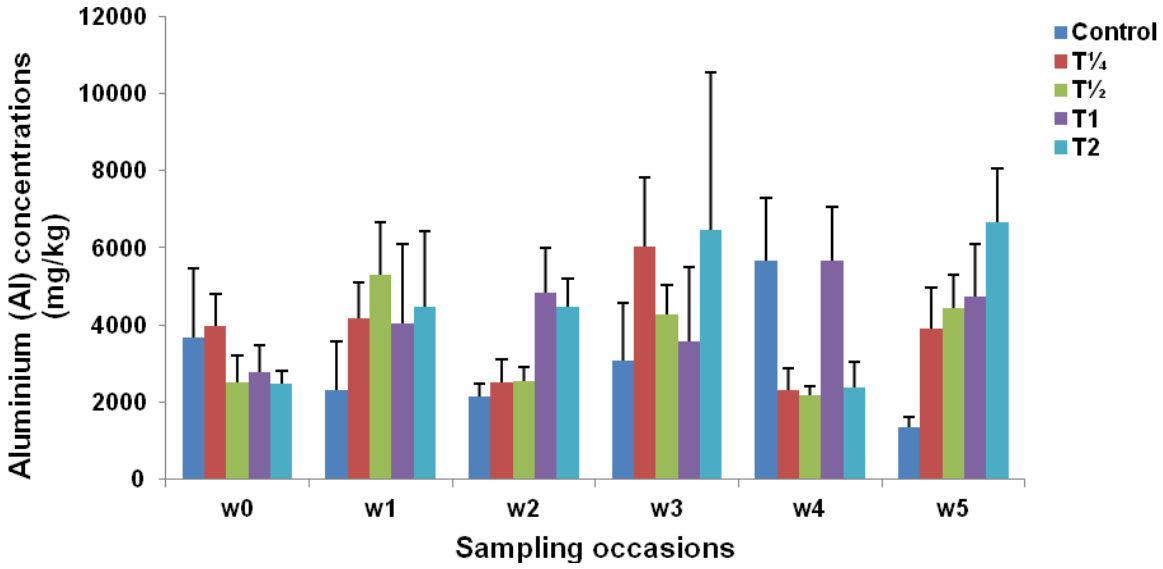

Figure 3.4. Mean (±SD) aluminium (Al) concentrations (mg/kg), measured per week in experimental treatments in *Ceratophyllum demersum* L. *Abbreviations: T1=treatment at environmentally relevant metal concentrations (mg/L); T¼= quarter T1 exposure concentrations (mg/L); T½= half T1 exposure concentrations (mg/L); T2=double T1 exposure concentrations (mg/L). w0= week 0 (start of experiment/baseline); w1= week 1 of exposure; w2= week 2 of exposure; w3= week 3 of exposure; w4= week 4 of exposure; w5= week 5 of exposure.*

3.2.2. Comparisons of aluminium (Al) concentrations in *Ceratophyllum demersum* L., between treatments per week

The comparisons of the concentrations of Al in plant samples of the different treatments are illustrated in Table 3.5 and Figure 3.4.

Week 0: No significant differences in Al concentrations were indicated between the treatments and the control ($P<0.05$). Between treatments: No significant differences in Al concentrations were found between treatments ($P<0.05$).

Week 1: The Al concentrations of treatments T½, T1 and T2 were significantly higher than the Al concentrations of the control ($P<0.05$). Between treatments: Al concentrations of treatment T¼ was significantly higher compared to the control. No other significant differences in Al concentrations were indicated for the other treatments ($P<0.05$).

Week 2: The Al concentrations of treatments T1 and T2 were significantly higher compared to the Al concentrations of the control ($P<0.05$). Between treatments: The Al concentrations of treatment T1 were significantly higher compared to those of treatment T½ ($P<0.05$).

Week 3: The Al concentrations of treatments T¼ and T2 were significantly higher compared to the Al concentrations of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly higher compared to those of the control and the Al concentrations of treatment T2 were significantly higher than the Al concentrations of treatment T1 (P<0.05).

Week 4: The Al concentrations of treatments T¼ and T2 were significantly lower compared to the Al concentrations of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly lower compared to those of the control; the Al concentrations of treatment T1 were significantly higher compared to those of treatment T½ and the Al concentrations of treatment T2 were significantly lower than the concentrations of treatment T1 at week (P<0.05).

Week 5: The Al concentrations of treatments T¼, T½, T1 and T2 were significantly higher compared to the concentrations of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly higher compared to those of the control and the Al concentrations of treatment T2 were significantly higher than the concentrations of treatment T1 (P<0.05).

Pooled data: The Al concentrations of treatments T¼, T1 and T2 were significantly higher compared to the Al concentrations of the control (P<0.05). Between treatments: The Al concentrations of treatment T¼ were significantly higher compared to the Al concentrations of the control and the Al concentrations of treatment T1 were significantly higher compared to the Al concentrations of treatment T½ (P<0.05).

3.2.3. Comparisons of copper (Cu) concentrations in *Ceratophyllum demersum* L. between weeks

Comparisons of the Cu concentrations between weeks, per treatment, are illustrated in Table 3.6 and Figure 3.5.

Control (baseline): When compared to week 0, the Cu concentrations were significantly higher during weeks 1, 2, 3 and 5, the latter indicating an overall decrease in Cu concentrations from the start to the end of the experiment (P<0.05). Between consecutive weeks significant decreases in Cu level were indicated between week 0 and week 1, between week 2 and week 3 and between week 4 and week 5. A significant increase in Cu

concentrations was shown between week 1 and week 2 and between week 3 and week 4 ($P<0.05$).

Treatment T¼: When compared to week 0, the Cu concentrations were significantly lower during week 4 and significantly higher during week 5, the latter indicating an overall increase in Cu concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Cu concentrations increased significantly between week 2 and week 3 and between week 4 and week 5. The Cu concentrations decreased significantly between week 3 and week 4 ($P<0.05$).

Treatment T½: When compared to week 0, the Cu concentrations were significantly higher during weeks 1, 2, 3, 4 and week 5, the latter indicating an overall increase in Cu concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Cu concentrations increased significantly between week 0 and week 1, between week 2 and week 3 and between week 4 and week 5. The Cu concentrations decreased significantly between week 1 and week 2, between week 3 and week 4 ($P<0.05$).

Treatment T1: When compared to week 0, the Cu concentrations were significantly higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Cu concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Cu concentrations increased significantly between week 0 and week 1, between week 1 and week 2 and between week 3 and week 4. The Cu concentrations decreased significantly between week 2 and week 3 ($P<0.05$).

Treatment T2: When compared to week 0, the Cu concentrations were significantly higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Cu concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Cu concentrations increased significantly between week 0 and week 1, between week 1 and week 2 and between week 3 and week 4. Cu concentrations decreased significantly between week 2 and week 3 ($P<0.05$).

Table 3.6. Mean (±SD) copper (Cu) concentrations (mg/kg), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0	^{a}1237.74^{a} ±261.59	^{a}607.66^{b} ±170.34	^{a}315.78^{c} ±82.74	^{a}333.65^{c} ±85.49	^{a}204.72^{d} ±32.35
1	^{*b}291.29^{a} ±49.32	^{a}487.44^{b} ±112.831	^{*b}1485.83^{c} ±262.23	^{*b}694.91^{d} ±68.91	^{*b}1258.83^{e} ±45.86
2	^{*c}539.62^{a} ±84.42	^{a}481.79^{a} ±116.41	^{*c}845.33^{b} ±72.53	^{*c}2729.60^{c} ±750.56	^{*c}3190.04^{c} ±540.19
3	^{*d}305.48^{a} ±28.37	^{b}672.92^{b} ±115.84	^{*d}1158.31^{c} ±216.625	^{*d}749.70^{d} ±100.41	^{*d}1170.39^{e} ±272.40
4	^{e}755.82^{a} ±55.82	^{*c}310.52^{b} ±79.50	^{*e}479.59^{c} ±120.04	^{*e}1594.33^{d} ±426.87	^{*e}2564.83^{e} ±351.23
5	^{*f}222.52^{a} ±39.55	^{*d}2259.71^{b} ±345.91	^{*f}644.34^{c} ±120.86	^{*e}2001.79^{d} ±539.20	^{*e}2083.86^{d} ±293.01
Pooled data for entire experimental period	**558.75^{a} ±86.51**	**803.34^{a} ±156.81**	**821.53$^{a\#}$ ±145.84**	**1350.66$^{a\#}$ ±270.09**	**1745.44$^{a\#}$ ±255.84**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

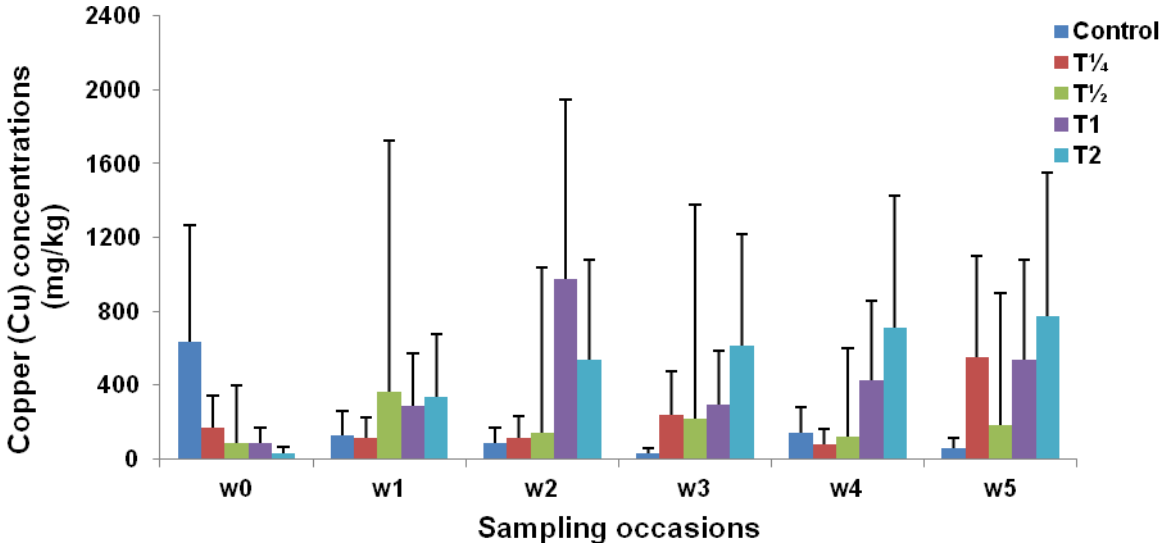

Figure 3.5. Mean (±SD) copper (Cu) concentrations (mg/kg), measured per week in experimental Cu treatments in *Ceratophyllum demersum* L. *Abbreviations: T1=treatment at environmentally relevant metal concentrations (mg/L); T¼= quarter T1 exposure concentrations (mg/L); T½= half T1 exposure concentrations (mg/L); T2=double T1 exposure concentrations (mg/L). w0= week 0 (start of experiment/baseline); w1= week 1 of exposure; w2= week 2 of exposure; w3= week 3 of exposure; w4= week 4 of exposure; w5= week 5 of exposure.*

3.2.4. Comparisons of copper (Cu) concentrations in *Ceratophyllum demersum* L. between treatments per week

The comparisons of the concentrations of Cu in water samples of the different treatments are illustrated in Table 3.6 and Figure 3.5.

Week 0: The Cu concentrations of treatments T¼, T½, T1 and treatment T2 were significantly lower compared to the concentrations of the control ($P<0.05$). Between treatments: Cu concentrations of treatment T¼ was significantly lower compared to the control; the Cu concentrations of treatment T½ was significantly lower compared to treatment T¼, while the Cu concentrations of treatment T2 were significantly compared to treatment T1 at week 0 ($P<0.05$).

Week 1: The Cu concentrations of treatments T¼, T½, T1 and treatment T2 were significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: Cu concentrations of treatment T¼ was significantly higher compared to the control; the Cu concentrations of treatment T½ was significantly higher compared to treatment T¼; the Cu concentrations of treatment T1 were significantly lower compared to treatment T½, while the Cu concentrations of treatment T2 were significantly higher compared to treatment T1 at week 1 ($P<0.05$).

Week 2: The Cu concentrations of treatments T½, T1 and treatment T2 were significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T½ was significantly higher compared to treatment T¼; the Cu concentrations of treatment T1 were significantly lower compared to treatment T½. No significant difference was found in Cu concentrations between treatment T1 and T2 ($P<0.05$).

Week 3: The Cu concentrations of treatments T¼, T½, T1 and treatment T2 were significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly higher compared to the Cu concentrations of the control; treatment T½ was significantly higher compared to treatment T¼; the Cu concentrations of treatment T1 were significantly lower compared to treatment T½, while the Cu concentrations of treatment T2 were significantly higher compared to treatment T1 at week 3 ($P<0.05$).

Week 4: The Cu concentrations of treatments T¼ and T½ were significantly lower, while the Cu concentrations of treatments T1 and T2 were significantly higher compared to the concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly lower compared to the Cu concentrations of the control; the Cu concentrations of treatment T½ was significantly higher compared to treatment T¼; the Cu concentrations of treatment T1 were significantly higher compared to treatment T½, while the Cu concentrations of treatment T2 were significantly higher compared to treatment T1 ($P<0.05$).

Week 5: The Cu concentrations of treatments T¼, T1 and T2 were significantly higher, compared to the Cu concentrations of the control ($P<0.05$). Between treatments: The Cu concentrations of treatment T¼ were significantly higher compared to the Cu concentrations of the control; the Cu concentrations of treatment T½ was significantly lower compared to treatment T¼, The Cu concentrations of treatment T1 were significantly higher compared to the Cu concentrations of treatment T½ ($P<0.05$).

Pooled data: The Cu concentrations of treatments T½, T1 and T2 were significantly higher compared to the Cu concentrations of the control ($P<0.05$). Between treatments: No significant differences in Cu concentrations were indicated between treatments ($P>0.05$).

3.2.5. Comparisons of iron (Fe) concentrations in *Ceratophyllum demersum* L. between weeks

Comparisons of the Fe concentrations between weeks, per treatment, are illustrated in Table 3.7 and Figure 3.6.

Control (baseline): When compared to week 0, the Fe concentrations were significantly higher during week 2 and significantly lower during weeks 1 and 5, the latter indicating an overall decrease in Fe concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Fe concentrations decreased significantly significant between week 0 and week 1, between week 2 and week 3 and between week 4 and week 5. The Fe concentrations increased significantly between week 1 and week 2and week 4 and ($P<0.05$).

Treatment T¼: When compared to week 0, the Fe concentrations were significantly higher during weeks 2, 3 and 5, the latter indicating an overall increase in Fe concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Fe concentrations increased significantly between week 1 and week 2, between week 2 and week 3 and between week 4 and week 5. Fe concentrations decreased significantly between week 3 and week 4 ($P<0.05$).

Treatment T½: When compared to week 0, the Fe concentrations were significantly higher during weeks 1, 2, 3, 4 and week 5, the latter indicating an overall increase in Fe concentrations from the start to the end of the experiment ($P<0.05$). Between consecutive weeks, the Fe concentrations increased significantly between week 0 and week 1, between week 2 and week 3 and between week 3 and week 4. The Fe concentrations decreased significantly between week 1 and week 2 and between week 4 and week 5 ($P<0.05$).

Treatment T1: When compared to week 0, the Fe concentrations were significantly higher during weeks 1, 2 and 4 ($P<0.05$). There was no significant difference in Fe concentrations between week 0 and week 5 through the entire experimental period ($P>0.05$). Between consecutive weeks, the Fe concentrations increased significantly between week 0 and week 1 and between week 4 and week 5. The Fe concentrations decreased significantly between week 2 and week 3 ($P<0.05$).

Treatment T2: When compared to week 0, the Fe concentrations were significantly higher compared to weeks 2, 3 and 5, the latter indicating an overall increase in Fe concentrations from the start to the end of the experiment ($P<0.05$). Fe concentrations were significantly lower during week 4 ($P<0.05$). Between consecutive weeks, the Fe concentrations increased

significantly between week 1 and week 2 and between week 4 and week 5. A significant decrease Fe in concentration was indicated between week 3 and week 4 ($P<0.05$).

Table 3.7. Mean (±SD) iron (Fe) concentrations (mg/kg), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}13990.89^{a} ±3902.34	^{a}5386.76^{b} ±446.04	^{a}4658.64^{c} ±456.72	^{a}23279.93^{d} ±2036.78	^{a}53157.70^{e} ±3133.71
1	^{*b}3655.79^{a} ±2010.41	^{a}4714.17^{a} ±1083.94	^{*b}22004.83^{b} ±3086.52	^{*b}38148.50^{c} ±4298.36	^{a}36566.10^{c} ±7570.51
2	^{*c}23032.37^{a} ±3721.88	^{*b}8805.24^{b} ±2044.51	^{*c}8846.20^{b} ±1219.16	^{*b}60394.92^{c} ±11375.34	^{*b}83477.99^{c} ±13030.70
3	^{d}11600.61^{a} ±1155.08	^{*c}17727.02^{b} ±3195.70	^{*d}14850.89^{c} ±2598.52	^{c}30999.90^{d} ±2545.52	^{*c}85113.64^{e} ±18975.31
4	^{d}16346.82^{a} ±539.94	^{d}4006.86^{b} ±1323.99	^{*e}21299.99^{c} ±1480.24	^{*c}33720.96^{d} ±2545.52	^{d}26661.26^{e} ±2723.00
5	^{*e}3810.46^{a} ±1014.09	^{*e}42174.17^{b} ±6052.30	^{*f}6994.89^{b} ±1412.63	^{d}8748.41^{b} ±916.00	^{*e}68129.73^{d} ±10124.3
Pooled data for entire experimental period	**12212.88^{a} ±1859.78**	**13802.37^{a} ±2357.75**	**13109.24^{a} ±1708.97**	**32548.77$^{b\#}$ ±4515.85**	**58851.07$^{c\#}$ ±9259.59**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

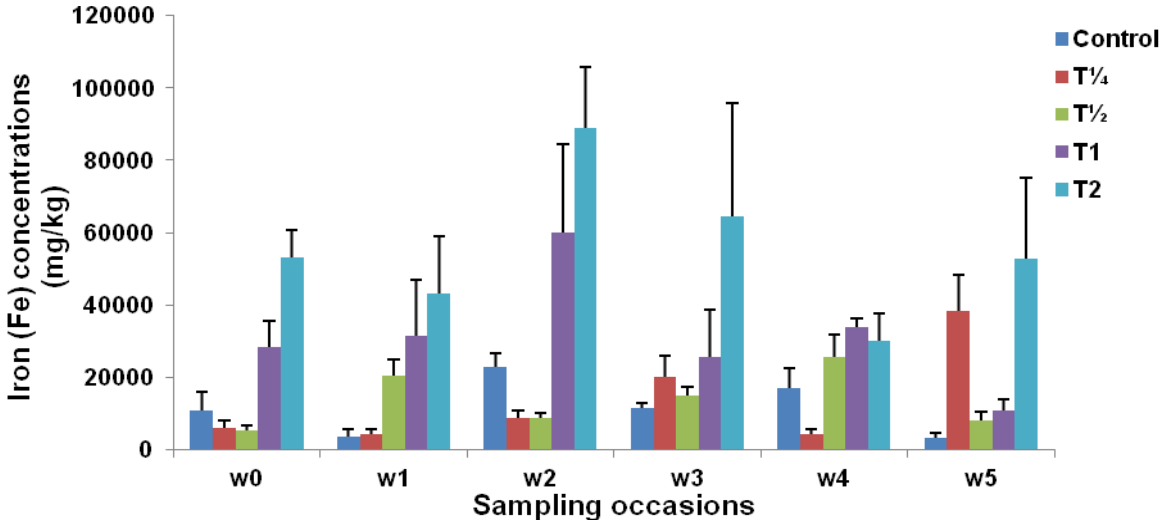

Figure 3.6. Mean (±SD) iron (Fe) concentrations (mg/kg), measured per week in experimental treatments in *Ceratophyllum demersum* L. *Abbreviations: T1=treatment at environmentally relevant metal concentrations (mg/L); T¼= quarter T1 exposure concentrations (mg/L); T½= half T1 exposure concentrations (mg/L); T2=double T1 exposure concentrations (mg/L). w0= week 0 (start of experiment/baseline); w1= week 1 of exposure; w2= week 2 of exposure; w3= week 3 of exposure; w4= week 4 of exposure; w5= week 5 of exposure.*

3.2.6. Comparisons of iron (Fe) concentrations between treatments in plant samples

The comparisons of the concentrations of Fe in plant samples of the different treatments are illustrated in Table 3.7 and Figure 3.6.

Week 0: The Fe concentrations of treatments T¼ and T½ were significantly lower, and the Fe concentrations of treatments T1 and T2 were significantly higher compared to the Fe concentrations of the control ($P<0.05$). Between treatments: Fe concentrations of treatment T¼ was significantly lower compared to that of the control; the Fe concentrations of treatment T½ was significantly higher compared to treatment T¼, while the Fe concentrations of treatment T1 were significantly higher compared to the Cu concentrations of T½; the Cu concentrations of treatment T2 were significantly higher compared to treatment T1 ($P<0.05$).

Week 1: The Fe concentrations of treatments T½, T1 and treatment T2 were significantly higher compared to the concentrations of the control. Between treatments: The Fe concentrations of treatment T½ was significantly higher compared to treatment T¼ and the

Fe concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ (P<0.05).

Week 2: The Fe concentrations of treatments T¼ and T½ were significantly lower, and the Cu concentrations of treatments T1 and T2 were significantly higher compared to the Cu concentrations of the control (P<0.05). Between weeks: The Fe concentrations of treatment T¼ were significantly lower compared to the Fe concentrations of the control and the Fe concentrations of treatment T1 were significantly higher compared to the Fe concentrations of treatment T1. No significant difference in Fe concentrations were found between treatment T1 and T2 (P>0.05).

Week 3: The Fe concentrations of treatments T¼, T½, T1 and treatment T2 were significantly higher compared to the concentrations of the control (P<0.05). Between treatments: The Fe concentrations of treatment T¼ were significantly higher compared to the Fe concentrations of the control; the Fe concentrations of treatment T½ was significantly lower compared to treatment T¼; the Fe concentrations of treatment T1 were significantly higher compared to treatment T½, while the Fe concentrations of treatment T2 were significantly higher compared to treatment T1 (P<0.05).

Week 4: The Fe concentrations of treatments T¼ were significantly lower, while the Fe concentrations of treatments T½, T1 and T2 were significantly higher compared to the concentrations of the control. Between treatments: The Fe concentrations of treatment T¼ were significantly lower compared to the Fe concentrations of the control; the Fe concentrations of treatment T½ was significantly higher compared to treatment T¼; the Fe concentrations of treatment T1 were significantly higher compared to treatment T½, while the Fe concentrations of treatment T2 were significantly higher compared to treatment T1 at week 4 (P<0.05).

Week 5: The Fe concentrations of treatments T¼, T1 and T2 were significantly higher, while the Fe concentrations of treatment T½ were significantly lower compared to the concentrations of the control at week 5. Between treatments: The Fe concentrations of treatment T¼ were significantly higher compared to the Fe concentrations of the control; the Fe concentrations of treatment T½ was significantly lower compared to treatment T¼; the Fe concentrations of treatment T1 were significantly higher compared to treatment T½, while the Fe concentrations of treatment T2 were significantly higher compared to treatment T1 at week 5 (P<0.05).

Pooled data: The Fe concentrations of treatments T1 and T2 were significantly higher compared to the Fe concentrations of the control. No significant difference was indicated between treatments T¼ and T½ and the control (P>0.05). Between treatments: The Fe concentrations of treatment T1 were significantly higher compared to the Fe concentrations of T½ and the Fe concentrations of treatment T2 were significantly higher compared to the Fe concentrations of treatment T1 (P<0.05).

3.2.7. Comparisons of zinc (Zn) concentrations in *Ceratophyllum demersum* L. between weeks

Comparisons of the Zn concentrations between weeks, per treatment, are illustrated in Table 3.8 and Figure 3.7.

Control (baseline): When compared to week 0, the Zn concentrations were significantly lower during weeks 1, 3, 4 and 5, the latter indicating an overall decrease in Zn concentrations from the start to the end of the experiment (P<0.05). Between consecutive weeks, significant decreases in Zn concentrations were indicated between week 0 and week 1 and between week 3 and week 4. Significant increases in Zn concentrations were shown between week 1 and week 2 (P<0.05).

Treatment T¼: When compared to week 0, the Zn concentrations were significantly higher during weeks 2, 3, and 5, the latter indicating an overall increase in Zn concentrations from the start to the end of the experiment (P<0.05). Zn concentrations were significantly lower compared to week 0 during week 4 (P<0.05). Between consecutive weeks, the Zn concentrations decreased significantly between week 1 and week 2 and between week 3 and week 4. The Zn concentrations increased significantly between week 2 and week 3 and between week 4 and week 5 (P<0.05).

Treatment T½: When compared to week 0, the Zn concentrations were significantly higher during weeks 1, 2, 3 and 4 (P<0.05). Zn concentrations at week 5 remained at the baseline and indicated no significant differences from the start to the end of the experiment (P>0.005). Between consecutive weeks, the Zn concentrations increased significantly between week 0 and week 1 and between week 2 and week 3. The Zn concentrations decreased significantly between week 1 and week 2, between week 3 and week 4 and between week 4 and week 5 (P<0.05).

Treatment T1: When compared to week 0, the Zn concentrations were significantly higher during weeks 1, 2, 4 and week 5, the latter indicating an overall increase in Zn concentrations from the start to the end of the experiment (P<0.05). Between consecutive weeks, the Zn concentrations increased significantly between week 0 and week 1, between week 1 and week 2, between week 3 and week 4 and between week 4 and week 5. The Zn concentrations decreased significantly between week 2 and week 3 (P<0.05).

Treatment T2: When compared to week 0, the Zn concentrations were significantly higher during weeks 1, 2, 3, 4 and 5, the latter indicating an overall increase in Zn concentrations from the start to the end of the experiment (P<0.05). Between consecutive weeks, the Zn concentrations increased significantly between week 0 and week 1, between week 1 and week 2 and between week 4 and week 5. A significant decrease Zn concentration was indicated between week 2 and week 3 in treatment T2 (P<0.05).

Table 3.8. Mean (±SD) zinc (Zn) concentrations (mg/kg), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion: n=5

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}23761.87^{a} ±4682.74	^{a}4682.74^{b} ±1562.26	^{a}4002.61^{c} ±1079.67	^{a}6577.51^{d} ±513.38	^{a}4549.14^{e} ±435.56
1	^{*b}4778.22^{a} ±840.04	^{a}7170.24^{b} ±958.83	^{*b}12825.28^{c} ±1984.40	^{*b}10776.28^{c} ±1183.40	^{*a}7887.63^{d} ±609.60
2	^{c}13789.37^{a} ±1469.12	^{*b}2833.40^{b} ±509.10	^{*c}5618.93^{c} ±826.41	^{*c}20943.08^{d} ±5677.53	^{*b}11548.90^{e} ±1501.15
3	^{*c}9770.83^{a} ±3122.60	^{*c}8404.34^{a} ±1435.38	^{*d}10586.33^{a} ±1806.18	^{d}5897.71^{a} ±2754.29	^{*c}9136.63^{a} ±200.74
4	^{*d}4447.00^{a} ±539.48	^{*d}3037.53^{a} ±871.17	^{*e}6480.33^{b} ±1574.26	^{*e}8319.49^{c} ±165.59	^{*d}8274.97^{c} ±937.16
5	^{*d}5064.29^{a} ±920.22	^{*e}16279.74^{b} ±2315.86	^{f}4331.57^{c} ±1019.55	^{*e}12847.43^{d} ±1619.00	^{*e}16518.26^{d} ±2344.67
Pooled data for entire experimental period	**10268.60^{a} ±1929.03**	**5227.45^{a} ±1275.43**	**7307.51^{a} ±1381.74**	**10893.58^{b} ±1985.53**	**9652.60^{b} ±1004.81**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between*

the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0= week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.

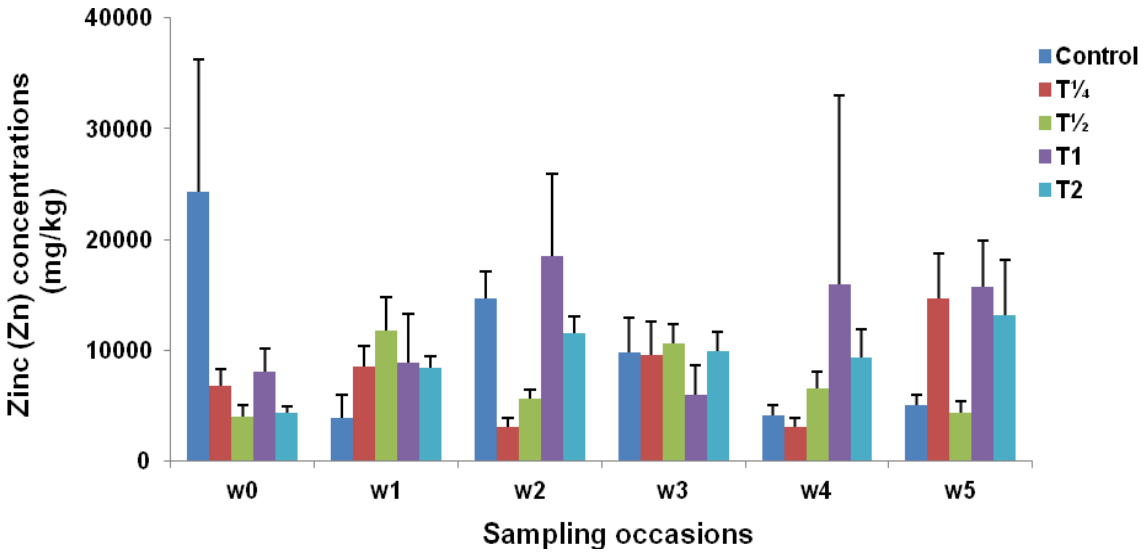

Figure 3.7. Mean (±SD) zinc (Zn) concentrations (mg/kg), measured per week in experimental treatments in *Ceratophyllum demersum* L. *Abbreviations: T1=treatment at environmentally relevant metal concentrations (mg/L); T¼= quarter T1 exposure concentrations (mg/L); T½= half T1 exposure concentrations (mg/L); T2=double T1 exposure concentrations (mg/L). w0= week 0 (start of experiment/baseline); w1= week 1 of exposure; w2= week 2 of exposure; w3= week 3 of exposure; w4= week 4 of exposure; w5= week 5 of exposure.*

3.2.8. Comparisons of zinc (Zn) concentrations between treatments in plant samples.

The comparisons of the concentrations of Zn in plant samples of the different treatments are illustrated in Table 3.8 and Figure 3.7.

Week 0: The Zn concentrations of treatments T¼, T½, T1 and T2 were significantly lower compared to the Zn concentrations of the control (P<0.05). Between treatments: Zn concentrations of treatment T¼ was significantly lower compared to the Zn concentrations of the control; the Zn concentrations of treatment T½ were significantly lower compared to the Zn concentrations treatment T¼, while the Zn concentrations of treatment T1 were significantly higher compared to the Zn concentrations of T½; the Zn concentrations of treatment T2 were significantly lower compared to treatment T1 (P<0.05).

Week 1: The Zn concentrations of treatments T¼, T½, T1 and treatment T2 were significantly higher compared to the Zn concentrations of the control at week 1 (P<0.005). Between treatments: The Zn concentrations of treatment T¼ was significantly higher compared to the Zn concentrations of the control; the Zn concentrations of T½ were significantly higher compared to the concentrations of treatment T¼; the Zn concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T½ and the Zn concentrations of treatment T2 were significantly lower compared to the Zn concentrations of treatment T1 at week 1(P<0.05).

Week 2: The Zn concentrations of treatments T¼, T½ and T2 were significantly lower, and the Cu concentrations of treatments T1 were significantly higher compared to the Zn concentrations of the control (P<0.05). Between treatments: The Zn concentrations of treatment T¼ was significantly lower compared to the Zn concentrations of the control; the Zn concentrations of T½ were significantly higher compared to the concentrations of treatment T¼; the Zn concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ and the Zn concentrations of treatment T2 were significantly lower compared to the Zn concentrations of treatment T1 (P<0.05).

Week 3: No significant differences in Zn concentrations were indicated between the treatments and the control (P>0.05). Between treatments: No significant differences in Zn concentrations were found between treatments (P>0.05).

Week 4: The Zn concentrations of treatment T½ and T1 were significantly higher compared to the Zn concentrations of the control (P<0.05). Between treatments: The Zn concentrations of treatment T¼ was significantly higher compared to the Zn concentrations of treatment T½; the Zn concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T½ and the Zn concentrations of treatment T2 were significantly higher compared to the Zn concentrations of treatment T1 (P<0.05).

Week 5: The Zn concentrations of treatment T¼; T1 and T2 were significantly higher compared to the Zn concentrations of the control (P<0.05). Between treatments: The Zn concentrations of treatment T¼ were significantly higher compared to the concentrations of the control; the Zn concentrations of treatment T½ were significantly lower compared to the Zn concentrations of treatment T¼, while the Zn concentrations of treatment T1 were significantly higher compared to treatment T½ (P<0.05).

Pooled data: No significant differences in Zn concentrations were indicated between the treatments and the control (P>0.05). Between treatments: The Zn concentrations of

treatment T1 were significantly higher compared to the Zn concentrations of treatment T½ ($P<0.005$). No other significant differences between treatments were detected ($P>0.05$).

3.3. Discussion

3.3.1. Metals in water medium

The highest mean water temperature for the control (29 °C) was found during week 1 and the highest mean water temperature for treatment T¼ (28 °C) was found during week 1. The highest mean water temperature for treatments T½, T1 and T2 were also recorded during week 1 (Table 3.1). According to DWAF (1996) the increase in water temperature does not affect pH values. The pH of the water varied between slightly acidic in the control to slightly alkaline in the control and in treatments T¼ and T½ over the five week experimental period. The higher dosage treatments (T1 and T2) were slightly acidic over the five week period. The pH is generally acknowledged as the main factor that governs the concentration of soluble and plant available metals (Malviya & Rathore, 2007).

The present study indicates that under low exposure metal concentrations the pH varied from slightly acidic to slightly alkaline. Under high exposure concentrations the pH was slightly acidic to neutral over the experimental period (Table 3.1). Marshner (1995) indicated that in hydroponics, metal uptake usually increases with increasing pH and because of high pH fewer protons will compete with metal ions at uptake sites. In the present study Al and Cu concentrations decreased in the water under higher pH in the low exposure treatments and the control and increased under higher exposure treatments. Zn concentrations increased under lower pH levels and higher exposure treatments and varied under higher pH and lower exposure treatments and the control (Tables 3.2, 3.3, 3.4). A possible explanation for the results of this study could be that *C. demersum* L. is tolerant to pH variations and that a regulating mechanism exists in the plants to deal with different pH levels in the water (Javed, 2011). In a study by Nyquist and Greger (2009) it was reported that pH increase of the surrounding medium increased the cadmium (Cd) uptake by shoots of *Elodea canadensis* and *Carex rostrate* probably due to diminished interactions between H^+ and Cd^{2+}.
In the present study salinity has increased in all the treatments and the control over the 5 week exposure period. Salinity can affect the accumulation of metals in plants. An increase in salinity resulted in an increase in the accumulation of metals, as e.g. in *Aster tripolium* (Fitzgerald *et al*., 2003) and in *Bolboschoenus maritimus* (Shuping *et al*., 2011). According to

Hinchman *et al.* (1998) high salinity may exert harmful effects on aquatic life due to changes in the osmotic pressure.

Electrical conductivity (EC) is the capacity of material to carry current. In water it is generally used as a measure of the mineral or ionic concentration. The EC in all the treatments and the control increased over the five week exposure period. In the present study the highest mean EC for the control was 511.12 (±0.084) mS/cm during week 5 and the highest EC for treatment T¼ was 525.85 (±0.084) during week 5 (Table 3.1). The highest EC for treatments T½, T1 and T2 were also found during week 5. Michaud (1991) indicated that evaporation and loss of fresh water will increase the conductivity and salinity of a water body. The increase of salinity and EC on this study could be a result of evaporation and the combination of high concentrations of metals found in the waters of the different treatments over the exposure period.

3.3.1.1. Aluminium

Aluminium is released to the environment mainly by natural processes such as erosion of rock and then via industries that process or use aluminium. There are several factors that influence aluminium mobility and subsequent transport within the environment, i.e. chemical speciation, hydrological flow paths, soil–water interactions, and the composition of the underlying geological materials. Acidic environments caused by acid mine drainage or acid rain can cause an increase in the dissolved aluminium content of the surrounding waters (ATSDR, 1992; WHO, 1997). Aluminium is a toxic trace metal and is probably not an important nutrient in any organism. The bioavailability of aluminium is strongly pH-dependent and its toxicity depends on the chemical species involved (DWAF, 1996). Al can occur in a number of different forms in water. It can form monomeric and polymeric hydroxy species, colloidal polymeric solutions and gels, and precipitates, all based on aquated positive ions or hydroxylated aluminates. Al can also form complexes with various organic compounds such as humic or fulvic acids and inorganic ligands (e.g. fluoride, chloride, and sulfate), most but not all of which are soluble. The chemistry of aluminium in water is complex, and many chemical parameters, including pH, determine which aluminium species are present in aqueous solutions. In pure water, Al has a minimum solubility in the pH range 5.5–6.0. The concentrations of total dissolved aluminium increase at higher and lower pH values (CCME, 1988; ISO, 1994).

In the present study, aluminium concentrations varied in all the treatments in the water medium over all five weeks of exposure. However, no significant differences in Al concentrations were found between the start and the end of the experimental period in the different treatments. Only in the control, a significant decrease was found between the start and the end of the experiment. Weekly variations did however occur. Significant increases in Al concentrations were detected in treatments T½ and T2 and a significant decrease in Al concentrations were detected in treatment T¼ during week 1 (Table 3.2). Plant data (Section 3.2) is needed to determine the "fate" of the metal. After week 1 the Al concentrations decreased and increased on a weekly basis between in the different treatments and the control. The Al concentrations of the pooled data also differed in the treatments. The highest Al concentrations were found in the highest dosage treatment (T2) and the lowest Al concentrations were found in treatment T½ (Table 3.2) in the water.

3.3.1.2. Copper

Although copper is one of the world's most commonly used metals (Anon, 1996), it is regarded as a potential hazard (Anon, 2003). Copper is also an essential micronutrient required by all organisms and is rapidly accumulated in bodies of plants and animals (Anon, 1996). Copper is found naturally in the environment and is a sought after metal in industry and agriculture, thus it is released both naturally and from human activities into the environment. Copper is associated with mines, industry, landfills and waste disposal. Most water–soluble copper is due to agricultural runoff, as copper is an important ingredient of many fungicides (Walker *et al*., 2006). Cu is found in surface water, groundwater, seawater and drinking-water, but it is primarily present in complexes or as particulate matter (ATSDR, 2002).

Copper concentrations in surface waters ranged from 0.0005 to 1 mg/litre in several studies in the USA; the median value was 0.01 mg/litre. Cu concentrations in drinking-water vary widely as a result of variations in water characteristics, such as pH, hardness and copper availability in the distribution system (ATSDR, 2002). Copper in drinking water, is highly toxic in high concentrations to both animals and humans.

In the present study, the copper concentrations varied in all the treatments in the water medium over all five weeks of exposure. However, no significant differences in Cu concentrations were found between the start and the end of the experimental period in the

different treatments, except for the control where a significant increase in Cu concentration was found between the start and the end of the experiment. Significant increase in Cu concentrations was found in the control during week 1 and significant decrease in Cu concentrations in treatments T¼ and T2. After week 1 the Cu concentrations decreased and increased between weeks in the different treatments and the control. The pooled data also indicated differences in Cu concentrations in all treatments compared to the control (Table 3.3).

According to Jones and Belling (1967) Cu is mostly more soluble in acidic waters at pH values below 6.5. A possible explanation for the variation in the results of the present study could be that the pH of the water in the control and different treatments changed from slightly acidic during week 0 to slightly alkaline during week 3 and to more neutral during week 5 and maybe affecting bioavailability. The uptake of metals by plants is dependent on the bioavailability of the metal in the water phase. Bioavailability of metals is dependent on the retention time of the metal and also the interaction with other elements and materials in the water (Tangahu *et al.,* 2011).

3.3.1.3. Zinc

Zinc is an essential micronutrient for living organisms because it forms part of the active site in several metalloenzymes. The zinc (II) as oxidation state of zinc is toxic to aquatic biota at relatively low concentrations in most waters. High concentrations of dissolved Zn occur at low pH, low alkalinity and high ionic strength solutions. Adsorption of Zn by hydrous metal oxides, clay minerals and organic material is an essential process in aquatic ecosystems since it affects the bioavailability and toxicity of Zn (Anon, 1996).

In the present study, the zinc concentrations decreased significantly in the control and increased significantly in the low dosage treatments during week 1 (Table 3.4). No significant differences in Zn concentrations were found in the higher dosage treatments during week 1. After week 1 Zn concentrations fluctuated between weeks in the control and different treatments. Significant differences were found between the start and the end of the experiment after the five week exposure period. The Zn concentrations of the pooled data differed with an increase in Zn dosage compared to the control (Table 3.4). The Zn concentration of treatment T¼ were higher than the concentrations of the three higher

dosage treatments compared to the control in the pooled data. The plant data will reveal the movement of the metal (Zn) in this study (Section 3.2.7).

From the pooled data of the control, the concentrations of the metals from highest to lowest in the water were Al>Cu>Zn. In treatment T¼ the concentrations of the metals from highest to lowest were Zn>Al>Cu and in treatment T½ the concentrations were Zn>Al>Cu. In treatment T1 the metal concentrations in the water were from highest to lowest Cu>Al>Zn and in treatment T2 the metal concentrations were Al>Cu>Zn. Concentrations of Al, Cu and Zn in water medium varied in all treatments over time, with no specific patterns emerging amongst treatment groups. The fluctuation of metal concentrations between weeks in the treatments could be attributed to the leaching of metals in and out of the plants to maintain homeostasis. Leaching of metals from the plants into the water could be a mechanism to regulate internal metal concentrations.

It is evident from other related studies that metals could have adverse effects on the composition and the presence of several stream biota. Changes in the pH of water can have a direct bearing on the water solubility of metals and also on the deposition capacity of such metals in the substrata of stagnant and flowing water ecosystems (Van der Merwe *et al*., 1990). Interactions between a combination of metals in solution are often complex, and they are dependent on the metal concentration and pH of the growth medium (Balsberg-Påhlsson, 1989). In this study the pH fluctuated in the control and all the other treatments (Table 3.1). A significant correlation between low water pH and high aluminium concentration has been reported in fresh water, where Al may reach levels of 0.3 -1.6 mM (Dickson, 1978) and cause severe metabolic disruption in the food chain (Petterson, *et al*., 1985; Gesemer & Playle, 1999). Water pH affects many chemical and biological processes in the water e.g., low water pH can allow toxic elements and compounds to become mobile and available for uptake by aquatic plants (Anon, 2003). A water pH reading below 6.5 generally considered as being acidic could cause problems of metal toxicity (Anon, 1993 a). Readings ranging between 6.5 and 7.5 are considered neutral and suitable for plant growth (Parkpain *et al.*, 2000). Aluminium bioavailability, and in result, toxicity, is mostly restricted to acidic environments (Silva, 2012). The availability of the metals in the water of the present study could also have been influenced by chemical speciation, organic chelators, the presence of other metals and anions, ionic strength, light intensity, temperature and oxygen level (Greger, 1999).

In this study, the higher temperature readings could be attributed to high temperatures inside the greenhouse during the experimental period. According to Fritioff *et al*. (2005) water temperature may influence water chemistry, metal solubility and metal uptake by plants and

also affect plant growth. Seasonal variation in water temperature has no direct effect on the solubility of metal in water (Zumdahl, 1992). In this study zinc concentrations (Table 3.4) in water medium increased with higher temperature during week 1 in treatments T¼, treatment T½ and treatment T1 and concentrations declined with lower temperature. The higher temperature during week 1 might have caused evapotranspiration in treatments T¼, T½ and T1 and have led to the higher Zn concentrations.

The mixture of metals in the water and their interaction with each could have influenced the bioavailability of Zn in the water.

3.3.2. Metals in *Ceratophyllum demersum* L.

The concentrations of aluminium, copper, iron and zinc detected in the plants of the different treatments were much higher than the concentrations detected in the water. Bioaccumulation of metals in aquatic macrophytes is known to produce significant physiological and biochemical responses in terms of the growth of roots, stems and leaves (Chandra & Kulshreshtha, 2004; Shankers *et al*., 2005). Several studies have examined bioaccumulation of metals by aquatic macrophytes (Kleiman & Cogliatti, 1998; Deng *et al*., 2008; Peng *et al.,* 2006; Kumar & Oommen, 2012). The accumulation of metals in various parts of higher plants is often accompanied by a generation of a variety of cellular changes, some of which directly contribute to metal tolerance capacity of the plants (Devi & Prasad, 1998). Rooted macrophytes have been shown to be more sensitive to metals than the floating macrophytes, such as the duckweeds, which are commonly used as a biomonitor (Lovett-Doust *et al*., 1994; Lewis, 1995). High temperature has a profound effect on plant growth rates and higher temperatures will thus result in greater biomass production and distribution of submerged macrophyte communities (Marschner, 1995; Rooney & Kalf, 2000; Fritioff *et al*., 2005). Plants with higher biomass may have a greater metal uptake capacity. This could be the result from lower metal concentration in its tissue because of a growth rate that exceeds its uptake rate (Ekvall & Greger, 2003). Changes in the composition of the plasma membrane lipids could be a result of changes in temperature. This alters plant membrane fluidity at low temperatures and lower metal uptake (Marschner, 1995). Metal concentrations (Al, Cu) in the control in water medium increased with higher temperatures (29.06 ^{0}C) (Table 3.1) at week 1 and decreased with lower water temperatures (Table 3.2 and Table 3.3). Metal concentrations (Al, Cu, Fe and Zn) in *Ceratophyllum demersum* L. fluctuated with increase and decrease in temperature in all the treatments (Table 3.1, Table 3.5, Table 3.6; Table 3.7 and Table 3.8).

3.3.2.1. Aluminium

Aluminium is a nonessential element for metabolic processes (Fodor, 2002) and it is well known for its toxic effect on plant growth and metabolism but their toxicity thresholds are highly variable (Umebese & Motajo, 2008). Aluminium has been shown to inhibit the absorption and transport of some essential nutrients as well as with cell division in roots, to increase cell wall rigidity (cross-linking pectins), to alter plasma membrane, and to change activities of many enzymes and metabolic pathway involved in repair mechanisms (Rout *et al*., 2001). A significant correlation between low pH and high Al concentration has been reported in freshwater, where Al could reach levels of 0.3 -1.6 mM (Dickson, 1978) and could trigger serious metabolic disruption in the food chain (Petterson *et al*., 1985; Gensemer & Playle, 1999).

In this study the Al concentrations in the plants were much higher than the Al concentrations in the water medium during week 0 (Tables 3.2 and 3.5). A possible explanation for the high concentrations of Al in the plants could be that the pond where the plants were sampled was not free from Al. Cation exchange between water and sediment could have taken place. The Al concentrations increased in all the treatments and decreased in the control during week 1 in the plants, and fluctuated thereafter between the weeks during the experimental period in the plants. In the water the Al concentrations decreased in the control and treatment T¼ and increased in the higher dosage treatments during week 1 and fluctuated thereafter between weeks during the experimental period. In all exposure treatments Al concentrations fluctuated between weeks (Table 3.5 & Figure 3.4). This trend corresponds with that of the water concentrations between weeks (Table 3.3 & Figure 3.1). Some metals within the water or sediment, depending on the mode of uptake by the plant, especially that of the bioavailability of metals to plants.

The bioavailability of Al could have been influenced by the pH of the water. The Al concentrations declined after week 1 and increased significantly at week 3 in the control. The Al concentrations in all the other treatments varied between the weeks. The highest accumulation occurred during week 3 in treatment T2 (9245.92 ± 2175.70 mg/kg). Significant increases in Al concentrations were indicated between the start and the end of the experiment (between week 0 and week 5) in treatments T½, T1 and T2. A significant decrease in Al concentrations was found between week 0 and week 5 in the control. According to these results *C. demersum* was able to remove a large amount of Al from the water during the experimental period. The Al concentrations in the plants and water samples between the treatments per week also showed fluctuation.

The pooled data indicated that accumulation in plants were higher with higher Al dosage. In this study aluminium accumulated in *C. demersum* which is similar to the results found in common buckwheat (*Fagopyrum esculentum*) (Ma *et al*., 1997) and also in both tartary buckwheat (*Fagopyrum tataricum Gaertn cv. Rotundatum*) and wild buckwheat (*Fagopyrum homotropicum Ohnishi cv. Mianshawan*) (Wang *et al*., 2015). Short-term exposure to aluminium resulted in accumulation of Al to concentrations >1 mg/g in buckwheat leaves. The present study has indicated high Al accumulation and could possibly be attributed to the rapid uptake and xylem loading of aluminium (Wang *et al*., 2015). Radić *et al*. (2010) reported a 25- to 43-fold increase in uptake of metals (Al) and 27- to 66-fold increase in Zn following duckweed (*Lemna minor* L.) exposure to Zn and Al. In tomato cultivars, Al exposure decreased the content of Fe, and Zn in roots, stems, and leaves (Simon *et al*., 1994). Variations in Al uptake could be influenced by chemical speciation of the metal, organic chelators, the presence of other metals and anions, ionic strength, light intensity, temperature and oxygen level (Greger, 1999). Variation in present results could possibly be attributed to the difference in the plant growth rate and in the efficiency towards metal absorption. Results after three weeks of exposure indicated that the plants in all four exposure treatments had high concentrations of Al.

After five weeks of exposure to Al the plants in three of the treatments (T½, T1 and T2) showed significantly higher concentrations compared to the control plants. In all exposure treatments Al concentrations fluctuated between weeks (Table 3.5 & Figure 3.4). This trend corresponds with that of the water concentrations in all the treatments between weeks (Table 3.3 & Figure 3.1). Some metals within the sediment or water, depending on the mode of uptake by the plant, could influence the concentration of metals the plant is able to absorb (Robinson *et al*., 2003). Many variables are involved regarding the uptake and storage of metals within plants, especially that of the bioavailability of metals to plants. Biologically available metals are those that occur in a form that are assimilable by living organisms (bioavailable), as metals occur in various forms and are not all bioavailable to plants (Wright & Welbourn, 2002). The bioavailability of Al could have been influenced by the pH of the water medium. Another explanation could be that after reaching a certain threshold concentration in the plant, the Al is eliminated by physiological mechanisms because plants might not have a proper mechanism to regulate Al (Wright & Welbourn, 2002). In the present study the growth rate of *C. demersum* L. might have been more important than the accumulation of Al and could be a possible explanation for the fluctuation of Al concentrations in the plants after week 1. In a study by Koo *et al.* (2013) it was found that

metal uptake decreased with growth, and the kinetics of metal uptake were essentially of first order during 4 weeks of growth as indicated by accumulation in corn shoots.

Several studies involving submerged macrophytes as bioaccumulators of metals within their tissues were conducted by for example Cardwell *et al*. (2002); Duman *et al*. (2006); Fritioff & Greger (2006); Deng *et al*. (2008) and Peng *et al.* (2008). Babovic *et al*. (2010) found that *C. demersum* L. accumulated the highest amount of zinc, copper and iron in its tissues compared to other macrophytes used in the study of a fishpond in Serbia. Rashed (2002) found that of the three aquatic plants from the Nile River that were studied, *C. demersum* L. accumulated most of the metals that were tested and was considered to be an excellent biomonitor of metal pollution.

3.3.2.2. Copper

Copper is an essential trace element and is needed by plants as a micronutrient (Brown & Rattigan, 1979). It is a constituent of hormones, vitamins, enzymes and nucleoprotein complexes. It is also a biocide and has been shown to be one of the most toxic metal ions when present in high concentrations. Copper is regulated by living organisms because of this element could cause toxicity (Phillips, 1977; Brown & Rattigan, 1979; Devi & Prasad, 1998). Cu participates in electron flow and catalyses redox reactions (Fernandes & Henriques, 1991; Ouzounido, 1991). Copper concentrations in natural environments, and its biological availability, are important. Naturally occurring concentrations of copper have been reported from 0.03 to 0.23 µg/L in surface seawaters and from 0.20 - 30 µg/L in freshwater systems (Bowen, 1985). Copper is probably the most immobile of the micronutrients and various factors affect the availability of copper to the plant such as pH, organic matter, lack of oxygen, lack of nitrogen and balances between copper and other elements like zinc, nitrogen and phosphorus (Salisbury & Ross, 1985). When in excess, Cu ions interfere with several physiological processes. Cu damages cell membranes by binding to the sulphydryl groups of membrane proteins and by inducing lipid peroxidation (De Vos *et al*., 1989).

The copper concentrations varied between weeks in all the treatments and the control. The highest copper concentrations were found during week 2 in treatment T2 (3190.04 ± 540.19 mg/kg). The same trend is also found in the different treatments per week where the Cu concentrations fluctuated between treatments. Significant increases in Cu concentrations were found between the start and the beginning of the experiment in all treatments (between

week 0 and week 5). A significant decrease in Cu concentrations was found between week 0 and week 5 in the control (Table 3.6).

The pooled data indicated an increase with higher Cu dosage compared to the control. The uptake of Cu in the plants in all the treatments was directly related to the dosage concentrations of the water. Cu uptake in the plants rose sharply with high dosage treatments (treatments T1 and T2) compared to the control. Cu uptake by *C. demersum* L. could depend on passive diffusion and active uptake in this study. In the present study *C. demersum* L. could be identified as a hyper accumulator of Cu. Significant accumulation of copper has also been observed in other macrophytes like *Lemna trisulca* (Prasad *et al.,* 2001), *Vallisneria spiralis* (Vajpayee *et al*., 2005), *Potamogeton pectinatus* and *Potamogeten malaianus* (Peng *et al.,* 2008). The findings in this study indicate that *C. demersum* L. can be used for extraction of copper from contaminated waters. The results of Cu concentrations in the water medium also indicated variation. During week 1 in the water medium the mean Cu concentrations decreased in treatments T¼, T½ and T1. The highest mean Cu concentration in the water medium was found during week 1 in the control (1.59 ±0.05) and the lowest mean Cu concentration (0.00 ±0.00 mg/L) in the control during week 1. During week 1 the Cu concentrations in treatments T½, T1 and T2 increased significantly in the plants. In the plants the mean Cu concentrations for the control decreased significantly from 1237.74 (±261.59) mg/kg to 291.29 (±49.32) mg/kg during week 1 (Table 3.6).

A slight decrease in Cu concentrations was found in treatment T¼ during week 1 but it was not significant. The copper concentrations in the treatments and control fluctuated after week 1 between weeks. The lowest mean copper concentration was found in treatment T2 (204.72 ±32.35 mg/kg) during week 0 and highest mean Cu concentration was found in treatment T2 during week 2 (3190.04 ±540.19 mg/kg) (Table 3.6).

The amount of Cu in the plants was much higher than that of the water. Several studies have shown that aquatic plants are capable of removing metals from water through biosorption and metabolism-dependent accumulation (Sivaci *et al*., 2004; Fritioff *et al.*, 2005). Copper occurs naturally and is essential for cell metabolism. The uptake of Cu is variable and is dependent on the kinetics and excretion. Accumulation of metals in various parts of higher plants is often accompanied by an induction of a variety of cellular changes, some of which directly add to metal tolerance capacity of plants (Phillips, 1976). The different Cu concentrations between the weeks per treatment and between treatments per week in *C. demersum* L. in the present study could be indicative of not only bioaccumulation but also the plants releasing Cu into the water through the leaves over time or when decaying started.

Pollutants may enter plants both by root uptake from the sediment and by absorption from the water column through leaves (Welsh & Denny, 1980; Biernacki & Lovett-Doust, 1997). Compounds that are not able to move through the plant, such as some metals, are presumed to have strong localized effects in plants, while mobile compounds will have more general effects. Copper appears to be mobile in plants (Mal *et al*., 2002). Macrophytes not only absorb pollutants, they also release them into the water column when they decay (Kähkönen & Manninen, 1998) and through leaching or diffusion into the water from the plant. It is possible that lower ion concentrations in the plants at week 0 could have facilitated rapid ion uptake during week 1 in the plants and also in the higher exposure treatments (T1 and T2) during week 2. A possible reason for the rapid uptake was that the plants were not fully adapted to the polluted environment and was more vulnerable into taking up copper during the first week. After week 1 the plants might have adapted to the environment and could regulate the uptake of copper into the plants. Dickinson *et al*. (1991) discussed many examples of how plants survive polluted environments and how plants adapt genetically to their environment by natural selection over time (generations). A possible reason for the fluctuation could be that a down regulation mechanism in copper uptake might exist. Some metals such as copper negatively affect cell membrane integrity causing an increase in electrical conductivity of water (de Vos *et al*., 1989; Devi & Prasad, 1998; Kumar & Prasad, 2004). Another reason for the fluctuations of the Cu concentrations between weeks in the different treatments might be due to the plant trying to internally regulate concentrations of the metal over time to reduce the toxicity. The toxicity of Cu in combination with other metals in water can cause oxidative stress over time, resulting in lipid peroxidation and ion leakage (Devi & Prasad, 1998).

In the present study copper was detected in the water and plant samples (Table 3.3. and 3.6). The results from this study indicate that the conductivity did increase from the start to the end of the experiment (Table 3.1). This could be an indication that copper in combination with the other metals (Al, Fe and Zn) could have negatively affected cell membrane integrity. In the present study, plants exhibited high concentrations of copper, and it can be deduced that the copper concentrations could have caused oxidative stress in the plants.

The subcellular distribution of metals provides a better understanding of the metal tolerance in plants. According to Neumann *et al*. (1997) the cell wall could play a role in metal tolerance and accumulation of copper was mainly in the cell walls (MacFarlane & Burchett, 2000). Ke *et al*. (2007) have found that more than 50% of copper was bound in the cell walls of *Daucas carota* leaves. Allen and Jarrel (1989) and Liu *et al*. (2009) have reported that the cell wall can allow big amounts of functional groups (e.g. hydroxide, carboxyl and amidogen),

that might interact with metal ions to isolate metals within the cell wall. This interaction could reduce their cross-membrane transport and minimize the metal concentration in the protoplast, which is the metabolism nucleus for the cell. High amounts of copper ions that are bound to the cell wall could subsequently avoid excess Cu toxicity in the protoplast (Yan & Xue, 2013). This mechanism might partly explain the tolerance for Cu in *C. demersum* L. but it would need further investigation.

3.3.2.3. Iron

Metals such as Fe are needed in appropriate concentrations for structural and catalytic components of proteins and enzymes as co-factors, and are essential for normal growth and development of plants (Singh & Sinha, 2004; Bouazizi, 2010). However, accumulation of these metals within cells can be toxic (Connely & Guerinot, 2002). Iron is the fourth most abundant element in the earth's outer crust. Iron is released into the environment through natural processes, such as erosion of sulphide ores and igneous rock, sedimentary and metamorphic rocks and by human activities such as the burning of coal, acid mine drainage, mineral processing and corrosion of iron and steel (Anon, 1996). Iron concentrations in water are low because of low solubility (Molot & Dillon, 2003; Shaked *et al*., 2004; Xing & Liu, 2011). Chemical behaviour of iron in the aquatic environment is determined by oxidation-reduction reactions, pH and the presence of coexisting inorganic and organic agents (Anon, 1996). Fe toxicity is a complex condition that can affect different physiological aspects of a plant. Excessive iron accumulated in a plant can lead to the enhancement of oxidative stress, as it increases the production of reactive oxygen species (Fang *et al*., 2001; Majerus *et al*., 2009). It unleashes disorder on most metabolic processes, including photochemical or biochemical obstructions of photosynthesis, with a resulting reduction in the rate of carbon assimilation (Suh *et al*., 2002; Nenova, 2009; Pereira *et al*., 2013). Anatomical alterations of cellular constituents are the result of iron toxicity and affects plant performance (Zhang *et al*., 2011).

In the present study iron accumulated fast in *C. demersum* L. from experimental treatments. Although Fe concentrations varied between weeks in all the treatments, the highest concentration of Fe was 85113.64 ± 18975.31 mg/kg found at week 3 in treatment T2 and the lowest Fe concentration was recorded at week 1 (3655.79 ± 2010.41 mg/kg) in the control plants. These results indicate that *C. demersum* L. plants were able to bioaccumulate high concentrations of iron from the water in all treatments between weeks. A possible reason could be due to the leaves having more stomata present than the stems. Uptake of

metals in the leaves occur through the ectodesmata which are situated in the epidermal cell walls (Franke, 1961). It is believed that when leaves have large surface areas and are more exposed to iron containing water they would accumulate more iron than the stems (Cardwell *et al.*, 2002; He & Yongfeng, 2009; Shuping *et al*., 2010). In a study by Xing *et al.* (2009), *Spirodela polyrrhiza* (L.) Schleid (duckweed) exhibited high accumulation of iron to a 100 mg/L concentration. Nutrient concentrations in aquatic plants are much higher than necessary for metabolism due to active absorption (Tanner & Beevers, 2001). Accumulation of metals in macrophytes is often accompanied by several morphological and physiological changes, some of which directly contribute to the tolerance capacity of plants (Prasad *et al*., 2001; Ding *et al.,* 2007).

The variation in Fe concentrations in the majority of the treatments of the present study could be attributed to an internal regulation mechanism to maintain homeostasis (metabolism) within the plants to resist the Fe toxicity. In the present study the concentrations of Fe in *C. demersum* L. increased with exposure time and dosage treatment. The pooled data also indicated under higher treatment concentrations the accumulation in plants were also higher. Basiouny *et al*. (1977) pointed out that the contents of iron and chlorophyll in *Hydrilla verticillata* (L.f.) Royle increased with the increase of iron exposure. According to Xing *et al*. (2010) the activities of antioxidative enzymes in aquatic plants, such as *Elodea nuttallii* (Planch.) H. St. John, are seriously inhibited by high iron concentrations. In aquatic plants, long-term accumulation and iron toxicity could alter the physiology and ecology of plants, such as morphology, anatomy, life-history traits, species composition and community dynamics (Xing & Liu, 2011). Stanković *et al.* (2000) evaluated iron contents in the most common submerged and floating aquatic plants (*Ceratophyllum demersum* L., *Myriophyllum spicatum* L., and *Nymphoides flava* Hill.) of Lake Provala, and have found that the iron contents in submerged species were considerably higher than in floating ones, and this could be a reason for degradation of submerged macrophytes. Van der Welle *et al*. (2007a; 2007b) have found that iron severely influences species composition and distribution of wetland plants. Therefore further investigations are required into the effects of iron toxicity and accumulation on species distribution and composition in *C.demersum* L.

3.3.2.4. Zinc

Zinc is an essential element for plant metabolism although high levels of Zn inhibit many plant metabolic functions. This can result in limited growth and cause senescence (Rout & Das, 2003). Zn is released into the environment in considerable amounts by both natural

processes and anthropogenic activities (Gensemer & Plale, 1999; Shikazono *et al.,* 2008). Zinc is required in plants to produce auxins, regulates sugars and activates enzymes, forms starch, influences seed and stalk maturation, is involved in the formation of chlorophyll and carbohydrates and assists plants in surviving low temperatures (Salisbury & Ross, 1985). Zinc availability is affected by pH and balances between itself and certain elements like phosphorus, copper, manganese, magnesium and arsenic (Landner & Reuther, 2004). Free zinc (Zn^{2+}) is found to be one of the most common phytotoxic elements under acidic conditions (Stephan *et al*., 2008). Zinc pollution in freshwater bodies has been reported to exceed the environmental limit by up to 100 times (Srikanth *et al.,* 1993; Pistelok & Galas, 1999; Shikazono *et al*., 2008).

In the present study the zinc concentrations in the plants were far higher than the concentrations measured in the water (Tables 3.4 and 3.8). Zn concentrations differed in all the treatments between weeks. The lowest mean Zn concentration was found in week 2 (2833 ± 509.10 mg/kg) in treatment T¼ and the highest mean concentration in week 2 (20943 ± 5677.53 mg/kg) in treatment T1 in the plants (Table 3.8 and Figure 3.7). The Zn concentrations in all the treatments except for the control increased during week 1 and then fluctuated in all the other consecutive weeks compared to week 0. Zn concentrations varied per week in the different treatments compared to the control. Significant increases between the start and the end of the experiment were found in treatments T¼, T1 and T2. A significant decrease between the start and the end of the experiment was found in the control. There is a possibility that the bioavailability of zinc decreased over the study period. This decrease could have been because of the effect of a combination of metals in solution, pH, temperature, evaporation, salinity and also the effect the metals could have had on each other in the water (Salisbury & Ross, 1985). This initial accumulation of zinc by the plant in the treatments (T¼, T½, T1 and T2) except for the control could be related to the need of the plant to absorb zinc as a micronutrient to facilitate normal metabolic processes (Salisbury & Ross, 1985). The plants might have had a zinc deficiency originally.

The pooled data indicated higher Zn dosages lead to greater bioaccumulation. Greater accumulation of Zn than Al in *Lemna minor* was found in a study by Radić *et al*. (2010). The results of this study are similar to the results indicated by these authors. In studies by Srivastava *et al*. (2007) and Bakar *et al*. (2013) a significant Zn accumulation in *Hydrilla verticillata* plants were found.

It is well known that metal concentrations in aquatic plants vary considerably according to the plant part as well as the metal (Larsen & Shierup, 1981; Stoltz & Greger, 2002). Sensitivity of

plants to metals depend on an interrelated network of physiological and molecular mechanisms such as: (i) uptake and accumulation of metals through the binding to extracellular exudates and cell wall components; (ii) efflux of metals from cytoplasm to extranuclear compartments including vacuoles; (iii) complexation of metal ions inside the cell by various substances, for example, organic acids, amino acids, phytochelatins and metallothioneins; (iv) accumulation of osmolytes and osmoprotectants and induction of antioxidative enzymes; (v) activation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures (Kabata-Pendias & Pendias, 2001; Cho *et al*., 2003; Brahim & Mohamed, 2011).

A comparison of week 0 and week 5 of metal concentrations indicated that bioaccumulation took place within *C. demersum* L. Aluminium and Zn increased in all the treatments and decreased in the control in the plants during the first week and fluctuated during the other weeks of the experimental period. Copper and Fe increased in treatments (T½, T1 and T2) and decreased in the control and treatment T¼ during week 1 and fluctuated during the other weeks of the experimental period (Tables 3.5, 3.6, 3.7, 3.8). A possible reason for the fluctuations could be explained by the bioremoval or accumulation process in the macrophytes. The bioremoval process using macrophytes contains two uptake processes: initial fast, reversible, metal-binding processes (biosorption); and a slow, irreversible, ion-sequestration step (bioaccumulation) (Salisbury & Ross, 1995; Keskinkan *et al*., 2004). The results of this study of *C. demersum* L. correspond with the findings of Keskinkan *et al*. (2004). It was reported that biosorption may be classified as being: extracellular accumulation/precipitation, cell surface sorption/precipitation, and intracellular accumulation (Veglio & Beolchini, 1997) and can occur by complexation, co-ordination, chelation of metals, ion exchange, adsorption and micro precipitation (Wang *et al*., 1996). The experimental plants were not modified genetically to limit metal uptake, due to their short term exposure to the waters. According to Dickson *et al*. (1991) plants need a long period of exposure to an external stimulus before they show any signs of genetic modification to overcome any setbacks to their metabolism by that external stimulus.

3.4. Conclusion

Earlier studies indicated that macrophytes are capable of removing metals from water through biosorption and metabolism-dependent uptake (Fritioff *et al*., 2005). In time-dependent kinetic studies on metal uptake by aquatic plants, an initial rapid accumulation was detected, followed by a slower linear phase of accumulation. It was proposed that the

initial phase represented a rapid, reversible, metal binding process (biosorption) and that the subsequent slower phase was due to transport across the plasma membrane into the cytoplasm (bioaccumulation) (Veglio & Beolchini, 1997). In the current study the significant high concentrations of metals by *C. demersum* L. indicated that this aquatic plant species was capable of removing metals directly from water via the biosorption process. During the first week the plants in this study accumulated metals (Al, Fe, Zn) quite rapidly in the higher dosage treatments and then the excessive concentrations of metals in the plants might have leached into the water because of the long exposure period, damage to the cell membranes and oxidative stress. A downregulation mechanism might exist that regulate the accumulation of metals and could maybe explain the varying results of this study. Another reason for the variation of accumulation between weeks could be the combination of metals in the water and the effect that the metals could have had on each other (Shanmugam, 2011).

Ceratophyllum demersum L. was tested for accumulation of four metals, Al, Cu, Fe and Zn over a 5 week period. This macrophyte proved to be highly effective in the uptake of these metals at all four exposure concentrations. The plant accumulated metals in the order: Fe>Zn>Al>Cu. The results have indicated that the species can be effectively used for removal of metals (for all metals, except Fe) from a solution of different metals. The characteristics of high metal accumulation capacity and easy harvest make this plant an ideal candidate to be used in cleaning up metal-contaminated water bodies. This study has demonstrated that macrophytes are biological filters that rehabilitate water bodies by accumulating metals. The results of this study also confirm the findings of an earlier study by Erasmus (2012) that *C. demersum* L. plants are able to adapt to metal contaminated environments and is able to rapidly bioaccumulate relatively large concentrations of metals within a short period of time. The results also showed *C. demersum* L. to accumulate various concentrations of the metals and thus show potential to be used as a biomonitor of metal exposure. Further investigation into mechanisms of uptake of metals under oxidative stress in *Ceratophyllum demersum* L. is needed and will be elucidated in the following chapter.

CHAPTER 4: RESULTS AND DISCUSSION: EXPOSURE EXPERIMENT: Oxidative stress redox status of *Ceratophyllum demersum* L.

4.1. Results

4.1.1. Comparisons of Total Polyphenols (TP) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of total polyphenols (TP) measured between different weeks in the experimental plants are shown in Table 4.1 and Figure 4.1.

Control (baseline): When compared to week 0, the TP concentrations decreased significantly ($P<0.05$) in the control after week 1 and week 2, while recovering to the same level as week 0 after weeks 3 and 4, with an increase in concentration during week 5. The latter indicate an overall increase in TP concentrations from the start to the end of the experiment ($P<0.05$). A significant increase ($P<0.05$) in TP concentrations were found between week 2 and week 3.

Treatment T¼: When compared to week 0, the TP concentrations were significantly ($P<0.05$) lower during week 2. The TP concentrations decreased significantly between week 1 and week 2 and increased significantly between week 2 and week 3. No significant difference in TP concentrations were found between week 0 and week 5 ($P>0.05$).

Treatment T½: When compared to week 0, no significant differences were found in TP concentrations during weeks ($P>0.05$). No significant differences in TP concentrations were found between weeks ($P>0.05$).

Treatment T1: When compared to week 0, the TP concentrations were significantly lower during week 3 and significantly higher during week 4 ($P<0.05$). The TP concentrations decreased significantly between week 2 and week 3 and increased significantly between week 3 and week 4 ($P<0.05$). No significant difference in TP concentrations were found between week 0 and week 5 ($P>0.05$).

Treatment T2: When compared to week 0, the TP concentrations were significantly higher during weeks 2, 3, 4 and 5, with the latter indicating a significant increase between the start and the end of the experiment ($P<0.05$). The TP concentrations increased significantly between week 1 and week 2 ($P<0.05$).

4.1.2. Comparisons of Total Polyphenols (TP) concentrations between treatments per week in *Ceratophyllum demersum L.*

The comparisons of the concentrations of total polyphenols (TP) in experimental plant samples of the different treatments are illustrated in Table 4.1 and Figure 4.1.

Week 0: No significant differences in TP concentrations were found in treatments T¼, T½, T1 and T2 compared to the control ($P>0.05$). No significant differences in TP concentrations were found between treatments ($P>0.05$).

Week 1: The TP concentrations of treatments T¼, T½, T1 and T2 were significantly higher compared the control ($P<0.05$). The TP concentrations of treatments T2 were significantly lower compared to concentrations of treatment T1 ($P<0.05$).

Week 2: The TP concentrations of treatments T¼, T½, T1 and T2 were significantly higher compared the control ($P<0.05$). The TP concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T¼. The TP concentrations of treatment T½ were significantly lower compared to the TP concentrations of treatment T1 and the TP concentrations of treatment T2 were significantly higher compared to the TP concentrations of treatment T1 ($P<0.05$).

Week 3: The TP concentrations of treatment T1 were significantly higher compared to the concentrations of the control ($P<0.05$). The TP concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T1 and the concentrations of T2 were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 4: No significant ($P>0.05$) differences in TP concentrations were found between the control and the different treatments. No significant ($P>0.05$) differences in TP concentrations were found between weeks in the different treatments.

Week 5: No significant ($P>0.05$) differences in TP concentrations were found between the control and the different treatments. No significant ($P>0.05$) differences in TP concentrations were found between weeks in the different treatments.

Pooled data: No significant differences in TP concentrations were indicated between the treatments and the control ($P>0.05$). No significant differences in TP concentrations were found between treatments ($P>0.05$).

Table 4.1. Mean (±SD) Total Polyphenol (TP) concentrations (mg/g), measured in *Ceratophyllum demersum* L. from experimental treatments: n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}4.870^{a} ±1.180	^{a}4.710^{a} ±1.550	^{a}5.241^{a} ±0.603	^{a}3.757^{b} ±1.254	^{a}2.650^{a} ±1.690
1	^{*b}1.030^{a} ±0.603	^{a}5.990$^{b\#}$ ±0.686	^{a}5.500$^{b\#}$ ±0.740	^{a}4.540$^{b\#}$ ±1.711	^{a}2.231$^{c\#}$ ±0.963
2	^{*c}0.727^{a} ±0.182	^{*b}2.827$^{b\#}$ ±1.015	^{a}5.387$^{c\#}$ ±0.633	^{a}4.254$^{d\#}$ ±0.798	^{*b}5.400$^{e\#}$ ±0.770
3	^{a}4.884^{a} ±1.198	^{a}5.302^{a} ±0.775	^{a}5.060^{a} ±0.530	^{*b}0.732$^{b\#}$ ±0.161	^{*c}4.276$^{c\#}$ ±1.580
4	^{a}5.632^{a} ±1.213	^{a}4.940^{a} ±0.855	^{a}5.569^{a} ±0.745	^{*c}5.510^{a} ±0.362	^{*d}5.270^{a} ±1.240
5	^{*d}6.602^{a} ±0.954	^{a}5.263^{a} ±0.760	^{a}4.480^{a} ±0.890	^{a}5.260^{a} ±1.330	^{*e}5.592^{a} ±0.631
Pooled data for entire experimental period	4.870^{a} ±2.470	4.710^{a} ±1.076	5.241^{a} ±0.400	3.757^{a} ±1.730	2.650^{a} ±1.470

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

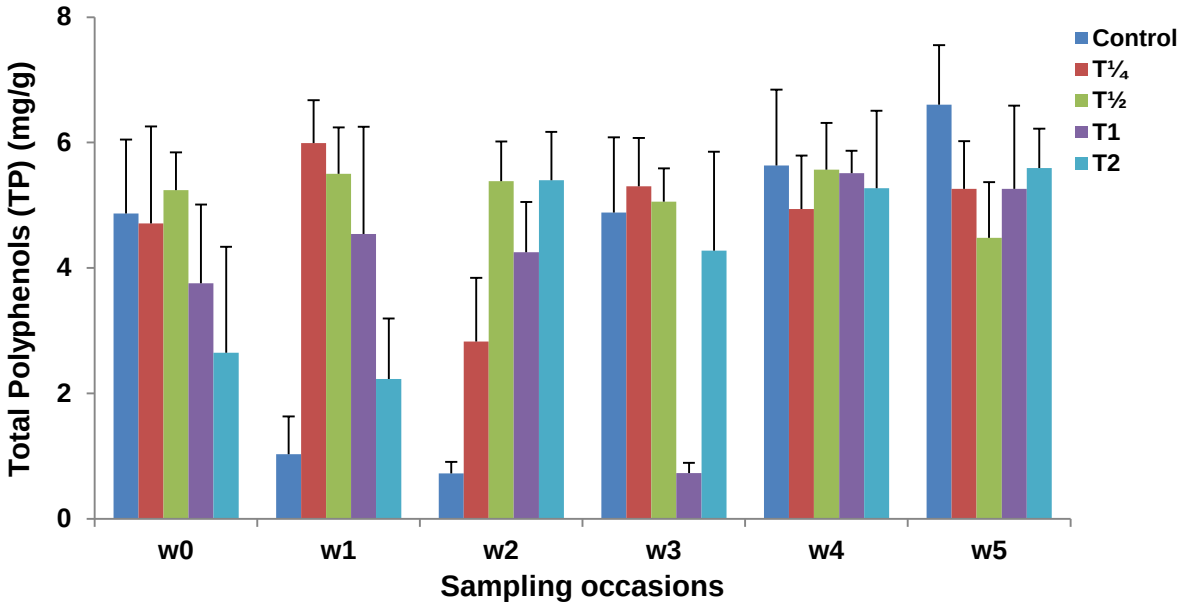

Figure 4.1. Mean (±SD) Total Polyphenol concentrations (TP) (mg/g), measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.3. Lipid peroxidation

4.1.3.1. Comparisons of Conjugated Dienes (CDs) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of conjugated dienes (CDs) measured between different weeks in the experimental plants are shown in Table 4.2 and Figure 4.2.

Control (baseline): When compared to week 0, the CD concentrations increased significantly ($P<0.05$) in the control during week 3 and week 5 ($P<0.05$). The latter indicate an overall increase in CD concentrations from the start to the end of the experiment ($P<0.05$). A significant increase ($P<0.05$) in CD concentrations was found between week 2 and week 3 and a significant decrease in CD concentrations were found between week 4 and week 5 $P<0.05$).

Treatment T¼: When compared to week 0, the CD concentrations were significantly ($P<0.05$) lower during weeks 1, 2, 3, 4 and 5 ($P<0.05$). The latter indicate an overall increase in CD concentrations from the start to the end of the experiment ($P<0.05$). The CD concentrations decreased significantly between week 4 and week 5 ($P<0.05$).

Treatment T½: When compared to week 0, a significant decrease in CD concentrations were found during week 1 ($P<0.05$). A significant increase in CD concentrations was found between week 1 and week 2 ($P<0.05$). A significant decrease in CD concentrations was found between week 0 and week 5 ($P<0.05$).

Treatment T1: When compared to week 0, the CD concentrations were significantly lower during week 1 and significantly higher during weeks 3 and 4 ($P<0.05$). The CD concentrations increased significantly between week 1 and week 2, and decreased significantly between week 4 and week 5 ($P<0.05$). No significant decrease in CD concentrations was found between week 0 and week 5 ($P>0.05$).

Treatment T2: When compared to week 0, the CD concentrations were significantly higher during weeks 2, 3 and 4 ($P<0.05$). The CD concentrations decreased significantly between week 4 and week 5 ($P<0.05$). No significant decrease in CD concentrations was found between week 0 and week 5 ($P>0.05$).

4.1.3.2. Comparisons of Conjugated Dienes (CDs) concentrations between treatments per week in *Ceratophyllum demersum L.*

The comparisons of the concentrations of conjugated dienes (CDs) in experimental plant samples of the different treatments are illustrated in Table 4.2 and Figure 4.2.

Week 0: The CD concentrations of treatments T¼ and T½ were significantly higher compared to the control ($P<0.05$). The CD concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 1: The CD concentrations of treatment T1 were significantly lower compared the control ($P<0.05$). The CD concentrations of treatment T¼ were significantly higher compared to treatment T½ and the concentrations of treatment T1 were significantly lower compared to treatment T2 ($P<0.05$).

Week 2: The CD concentrations of treatment T2 were significantly higher compared the control ($P<0.05$). The CD concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 3: No significant ($P>0.05$) differences in CD concentrations were found between the different treatments and the control. No significant differences were found between the different treatments ($P>0.05$).

Week 4: No significant ($P>0.05$) differences in CD concentrations were found between the different treatments and the control. No significant differences were found between the different treatments ($P>0.05$).

Week 5: The CD concentrations of treatments T¼, T1 and T2 were significantly lower compared to the control ($P<0.05$). The CD concentrations of treatment T¼ were significantly lower compared to treatment T½ and the concentrations of treatment T½ were significantly higher compared to treatment T1 ($P<0.05$).

Pooled data: No significant differences in CD concentrations were indicated between the treatments and the control ($P>0.05$). No significant differences in CD concentrations were found between treatments ($P>0.05$).

Table 4.2. Mean (±SD) Conjugated Dienes (CDs) (µmol/g), measured in *Ceratophyllum demersum* L. from experimental treatments: n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}1.774^{a} ±0.561	^{a}3.467^{b} ±0.234	^{a}3.133^{c} ±0.699	^{a}1.910$^{a\#}$ ±0.363	^{a}1.849^{a} ±0.406
1	^{a}1.904^{a} ±0.632	^{b}2.356^{a} ±0.247	^{b}1.667$^{a\#}$ ±0.194	^{b}1.335^{b} ±0.390	^{a}2.404$^{a\#}$ ±0.713
2	^{a}1.974^{a} ±0.754	^{c}2.644^{a} ±0.374	^{*a}2.689^{a} ±0.175	^{*a}2.016^{a} ±0.634	^{b}2.779$^{b\#}$ ±0.657
3	^{b}2.855^{a} ±0.536	^{d}2.863^{a} ±0.346	^{a}2.904^{a} ±0.203	^{c}2.459^{a} ±0.493	^{c}2.559^{a} ±0.613
4	^{a}2.397^{a} ±0.443	^{e}2.789^{a} ±0.201	^{a}2.859^{a} ±0.332	^{d}2.570^{a} ±0.446	^{d}2.614^{a} ±0.579
5	^{c}2.181^{a} ±0.512	^{*f}1.878^{b} ±0.564	^{a}2.856$^{a\#}$ ±0.381	^{*a}1.909$^{c\#}$ ±0.259	^{*a}1.745^{d} ±0.149
Pooled data for entire experimental period	2.317^{a} ±0.383	2.728^{a} ±0.435	2.685^{a} ±0.519	2.108^{a} ±0.445	2.459^{a} ±0.322

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

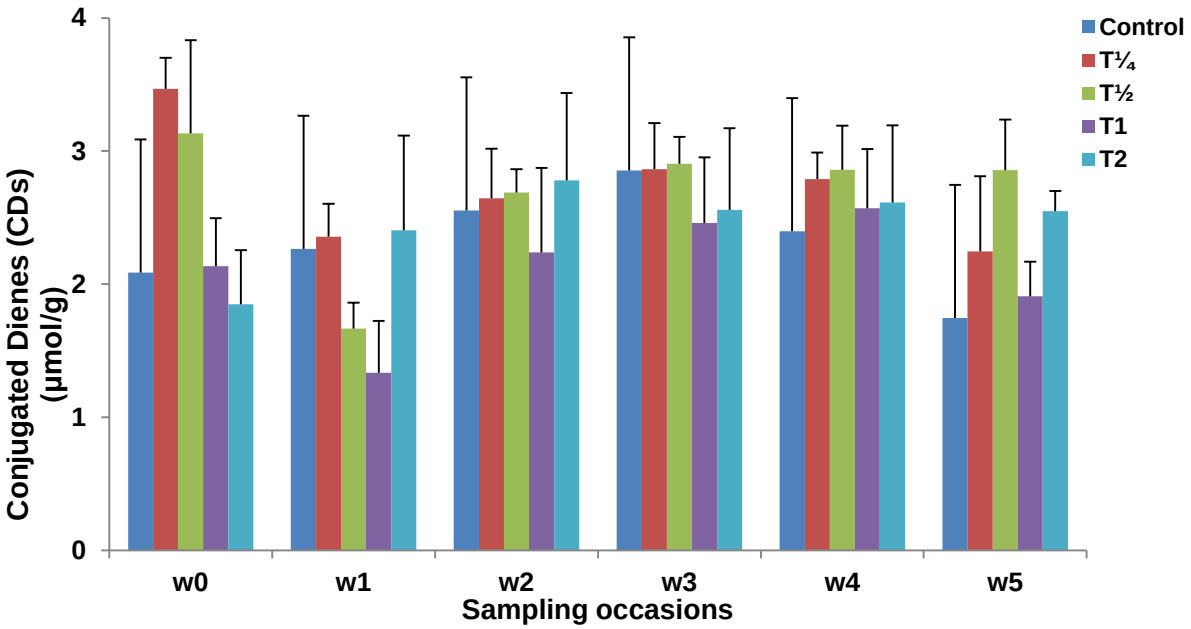

Figure 4.2. Mean (±SD) Conjugated Dienes (CDs) concentrations (µmol/g), measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.3.3. Comparisons of Thiobarbituric Acid Reactive Substances (TBARS) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured thiobarbituric acid reactive substances (TBARS) between different weeks in the experimental plants are shown in Table 4.3 and Figure 4.3.

Control (baseline): When compared to week 0, the TBARS concentrations increased significantly in the control during weeks 1, 2, 3, 4 and 5 ($P<0.05$). The latter indicate an overall increase in TBARS concentrations from the start to the end of the experiment ($P<0.05$). A significant decrease ($P<0.05$) in TBARS concentrations were found between week 1 and week 2 and between week 3 and week 4 and a significant increase in TBARS concentrations were found between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, the TBARS concentrations were significantly higher during week 1 and significantly lower during weeks 3 and 5 ($P<0.05$). The latter indicate an overall decrease in TBARS concentrations from the start to the end of the experiment ($P<0.05$). The TBARS concentrations decreased significantly between week 1 and week 2, between week 2 and week 3 and between week 4 and week 5 ($P<0.05$). The TBARS concentrations increased significantly between week 3 and week 4 ($P<0.05$).

Treatment T½: When compared to week 0, significant decreases in TBARS concentrations were found during weeks 1, 2, 3 and 5 ($P<0.05$). The latter indicate an overall decrease in TBARS concentrations from the start to the end of the experiment ($P<0.05$). A significant increase in TBARS concentrations were found between week 3 and week 4 and a significant decrease was found between week 4 and week 5 ($P<0.05$).

Treatment T1: When compared to week 0, significant decreases in TBARS concentrations were found during weeks 1, 2, 3 and 5 and a significant increase was found during week 4 ($P<0.05$). The latter indicate an overall decrease in TBARS concentrations from the start to the end of the experiment ($P<0.05$). A significant increase in TBARS concentrations were found between week 3 and week 4 and a significant decrease was found between week 4 and week 5 ($P<0.05$).

Treatment T2: When compared to week 0, the TBARS concentrations were significantly higher during week 4 ($P<0.05$). The TBARS concentrations decreased significantly between week 4 and week 5 ($P<0.05$). No significant difference in TBARS concentrations was found between week 0 and week 5 ($P>0.05$).

4.1.3.4. Comparisons of Thiobarbituric Acid Reactive Substances (TBARS) concentrations between treatments per week in *Ceratophyllum demersum L.*

The comparisons of the concentrations of thiobarbituric acid reactive substances in experimental plant samples of the different treatments are illustrated in Table 4.3 and Figure 4.3.

Week 0 (baseline): The TBARS concentrations of treatments T¼, T½, T1 and T2 were significantly higher compared to the control ($P<0.05$). The TBARS concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T2 ($P<0.05$).

Week 1: The TBARS concentrations of treatment T¼ were significantly higher compared the control and the concentrations of treatments T½, T1 and T2 were significantly lower compared to the control ($P<0.05$). The TBARS concentrations of treatment T¼ were significantly higher compared to treatment T½ ($P<0.05$).

Week 2: No significant differences in TBARS concentrations in the different treatments were found compared to the control ($P>0.05$). No significant differences in TBARS concentrations were found between the different treatments ($P>0.05$).

Week 3: No significant differences in TBARS concentrations in the different treatments were found compared to the control ($P>0.05$). No significant differences in TBARS concentrations were found between the different treatments ($P>0.05$).

Week 4: No significant differences in TBARS concentrations in the different treatments were found compared to the control ($P>0.05$). No significant differences in TBARS concentrations were found between the different treatments ($P>0.05$).

Week 5: No significant differences in TBARS concentrations in the different treatments were found compared to the control ($P>0.05$). No significant differences in TBARS concentrations were found between the different treatments ($P>0.05$).

Pooled data: No significant differences in TBARS concentrations were indicated between the treatments and the control ($P>0.05$). No significant differences in TBARS concentrations were found between treatments ($P>0.05$).

Table 4.3. Mean (±SD) Thiobarbituric Acid Reactive Substances (TBARS) (µmol/g), measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}37.930^{a} ±3.820	^{a}98.026^{b} ±6.586	^{a}100.020^{c} ±8.090	^{a}95.860^{d} ±4.350	^{a}85.580$^{e\#}$ ±2.744
1	^{b}100.620^{a} ±7.690	^{b}118.680^{b} ±9.625	^{*b}88.130$^{c\#}$ ±6.910	^{*b}81.450^{d} ±5.080	^{a}87.250^{e} ±3.490
2	^{*c}81.401^{a} ±7.170	^{*a}88.821^{a} ±3.966	^{*c}80.642^{a} ±5.175	^{*c}77.713^{a} ±7.537	^{a}80.180^{a} ±8.740
3	^{d}81.573^{a} ±5.537	^{*c}73.898^{a} ±9.424	^{*d}81.767^{a} ±4.656	^{*d}71.548^{a} ±6.016	^{a}72.907^{a} ±11.51
4	^{*e}120.078^{a} ±16.517	^{*a}106.030^{a} ±10.260	^{a}102.563^{a} ±6.250	^{*e}113.911^{a} ±11.075	^{*b}109.970^{a} ±5.270
5	^{*f}74.664^{a} ±5.267	^{*d}83.070^{a} ±4.540	^{*e}76.499^{a} ±5.975	^{*f}79.977^{a} ±5.826	^{a}84.797^{a} ± 10.507
Pooled data for entire experimental period	82.711^{a} ±27.532	94.754^{a} ±16.229	88.270^{a} ±10.783	86.743^{a} ±15.540	86.794^{a} ±12.478

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

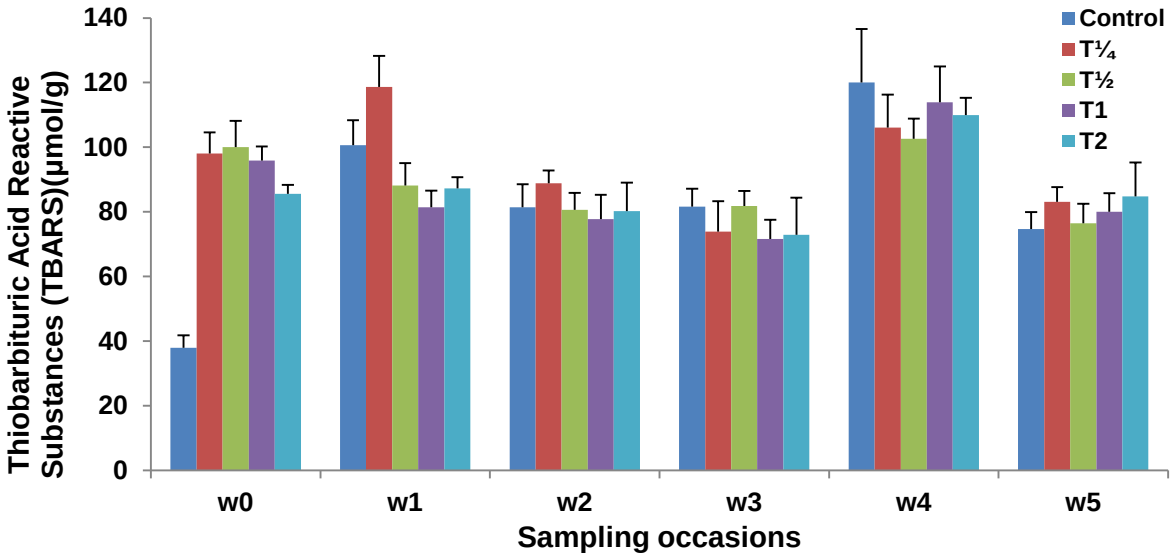

Figure 4.3. Mean (±SD) Thiobarbituric Acid Reactive Substances (TBARS) concentrations (µmol/g) measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.4. Total antioxidant capacity (TAC): Ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity assay (ORAC)

4.1.4.1. Comparisons of Ferric Reducing Antioxidant Power (FRAP) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured ferric reducing antioxidant power (FRAP) between different weeks in the experimental plants are shown in Table 4.4 and Figure 4.4.

Control (baseline): When compared to week 0, the FRAP concentrations decreased significantly ($P<0.05$) in the control during weeks 1, 2, 3, 4 ($P<0.05$). No significant differences ($P>0.05$) in FRAP concentrations were found between consecutive weeks.

Treatment T¼: When compared to week 0, the FRAP concentrations were significantly ($P<0.05$) lower during week 1, 2, 3, 4, 5. The latter indicate an overall decrease in FRAP

concentrations from the start to the end of the experiment (P<0.05). The FRAP concentrations increased significantly between week 2 and week 3 (P<0.05).

Treatment T½: When compared to week 0, no significant differences in FRAP concentrations were found during weeks 1, 2, 3 and 5 (P>0.05). No significant differences in FRAP concentrations were found between consecutive weeks (P>0.05).

Treatment T1: When compared to week 0, a significant increase in FRAP concentrations were found during week 3 and a significant decrease was found during week 5 (P<0.05). The latter indicate an overall decrease in FRAP concentrations from the start to the end of the experiment (P<0.05). A significant increase in FRAP concentrations were found between week 3 and week 4 and significant decreases were found between week 3 and week 4 and between week 4 and week 5 (P<0.05).

Treatment T2: When compared to week 0, the FRAP concentrations were significantly higher during week 3 (P<0.05). The FRAP concentrations increased significantly between week 2 and week 3, and decreased significantly between week 3 and week 4 (P<0.05). No significant difference in FRAP concentrations was found between week 0 and week 5 (P>0.05).

4.1.4.2. Comparisons of Ferric Reducing Antioxidant Power (FRAP) concentrations between treatments per week in *Ceratophyllum demersum L.*

The comparisons of the concentrations of ferric reducing antioxidant power (FRAP) in experimental plant samples of the different treatments are illustrated in Table 4.4 and Figure 4.4.

Week 0 (baseline): The FRAP concentrations of treatments T¼ were significantly higher and the concentrations of treatments T1 and T2 were significantly lower compared to the control (P<0.05). The FRAP concentrations of treatment T¼ were significantly higher compared to the concentrations of treatment T½ (P<0.05).

Week 1: No significant differences in FRAP concentrations in the different treatments were found compared to the control (P>0.05). No significant differences in FRAP concentrations were found between the different treatments (P>0.05).

Week 2: No significant differences in FRAP concentrations in the different treatments were found compared to the control (P>0.05). No significant differences in FRAP concentrations were found between the different treatments (P>0.05).

Week 3: The FRAP concentrations of treatments T1 and T2 were significantly higher compared to the control (P<0.05). The FRAP concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T1 (P<0.05).

Week 4: No significant differences in FRAP concentrations in the different treatments were found compared to the control (P>0.05). The FRAP concentrations of treatment T¼ were significantly lower compared to treatment T½ (P<0.05).

Week 5: The FRAP concentrations of treatment T1 were significantly lower compared to the control (P<0.05). The FRAP concentrations of treatment T½ were significantly higher compared to treatment T1 and the concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 (P<0.05).

Pooled data: No significant differences in FRAP concentrations were indicated between the treatments and the control (P>0.05). No significant differences in FRAP concentrations were found between treatments (P>0.05).

Table 4.4. Mean (±SD) Ferric Reducing Antioxidant Power (FRAP) (µmole/g) concentrations measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}8.105^{a}$ ±1.403	$^{a}10.706^{b}$ ±2.381	$^{a}8.467^{a\#}$ ±1.646	$^{a}6.080^{c}$ ±1.520	$^{a}5.600^{d}$ ±1.424
1	$^{b}6.453^{a}$ ±1.375	$^{*b}5.620^{a}$ ±0.970	$^{a}6.595^{a}$ ±2.027	$^{a}7.170^{a}$ ±1.181	$^{a}3.560^{a}$ ±1.910
2	$^{c}5.880^{a}$ ±1.330	$^{*c}3.797^{a}$ ±1.040	$^{a}5.957^{a}$ ±1.196	$^{a}5.193^{a}$ ±2.478	$^{a}5.300^{a}$ ±1.730
3	$^{d}5.490^{a}$ ±1.374	$^{*d}5.608^{a}$ ±0.986	$^{a}6.107^{a}$ ±1.251	$^{*b}9.528^{b\#}$ ±1.434	$^{*b}8.180^{c}$ ±2.320
4	$^{e}6.030^{a}$ ±1.870	$^{*e}4.813^{a}$ ±0.507	$^{a}7.760^{a\#}$ ±1.730	$^{*a}6.650^{a}$ ±1.570	$^{*a}6.340^{a}$ ±1.210
5	$^{a}6.626^{a}$ ±1.223	$^{*f}6.147^{a}$ ±0.951	$^{a}5.840^{a}$ ±1.690	$^{*c}3.736^{b\#}$ ±0.740	$^{a}6.490^{c\#}$ ±1.452
Pooled data for entire experimental period	6.431^{a} ±0.916	6.035^{a} ±2.422	6.788^{a} ±1.082	6.393^{a} ±1.954	5.912^{a} ±1.527

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

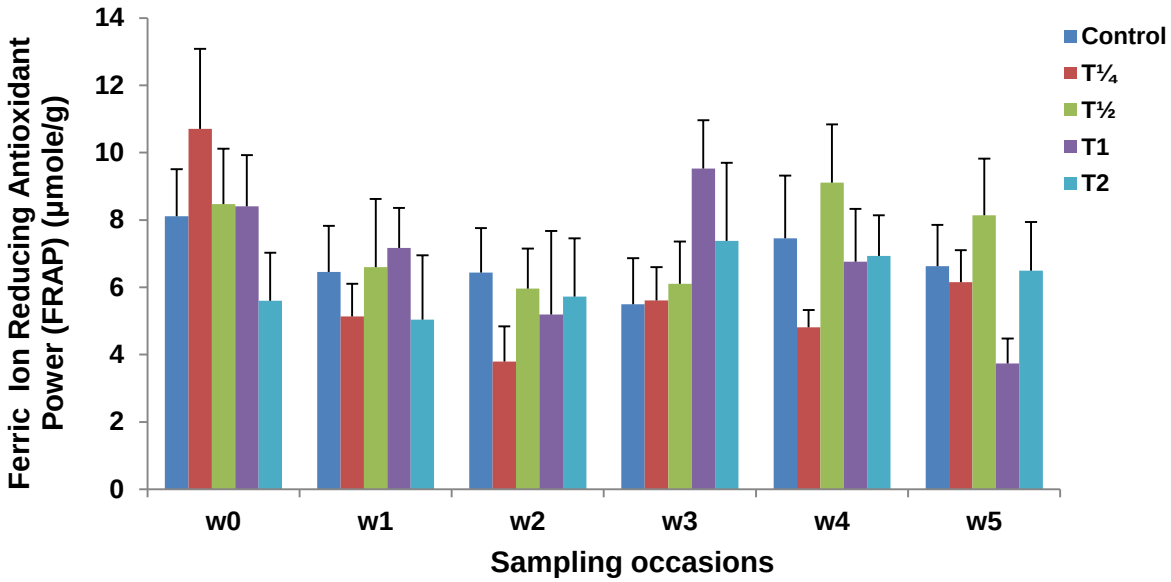

Figure 4.4. Mean (±SD) Ferric Reducing Antioxidant Power (FRAP) (µmole/g), measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.4.3. Comparisons of Oxygen Radical Absorbance Capacity Assay (ORAC) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured oxygen radical absorbance capacity (ORAC) between different weeks in the experimental plants are shown in Table 4.5 and Figure 4.5.

Control (baseline): When compared to week 0, no significant differences in ORAC concentrations were found in the control ($P>0.05$). A significant increase in ORAC concentrations were found between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, the ORAC concentrations were significantly ($P<0.05$) higher during weeks 4, 5. The latter indicate an overall increase in ORAC concentrations from the start to the end of the experiment ($P<0.05$). The ORAC concentrations increased significantly between week 3 and week 4 ($P<0.05$).

Treatment T½: When compared to week 0, no significant differences in ORAC concentrations were found during weeks 1, 2, 3 and 5 (P>0.05). A significant decrease in ORAC concentrations were found between week 1 and week 2, and a significant increase was found between week 2 and week 3 (P>0.05).

Treatment T1: When compared to week 0, a significant increase in ORAC concentrations were found during week 1 (P<0.05). No significant differences in ORAC concentrations were found between consecutive weeks (P>0.05).

Treatment T2: When compared to week 0, the ORAC concentrations were significantly higher in the control during weeks 1 and 3 (P<0.05). The ORAC concentrations decreased significantly between week 1 and week 2, and increased significantly between week 2 and week 3 (P<0.05).

4.1.4.4. Comparisons of Oxygen Radical Absorbance Capacity Assay (ORAC) concentrations between treatments per week in *Ceratophyllum demersum* L.

The comparisons of the concentrations of ferric reducing antioxidant power (FRAP) in experimental plant samples of the different treatments are illustrated in Table 4.5 and Figure 4.5.

Week 0 (baseline): The ORAC concentrations of treatments T½ were significantly higher compared to the control (P<0.05). The ORAC concentrations of treatment T¼ were significantly lower compared to the concentrations of treatment T½, and the concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T1 (P<0.05).

Week 1: The ORAC concentrations of treatments T½ and T1 were significantly higher compared to the control (P<0.05). No significant differences in ORAC concentrations were found between the different treatments (P>0.05).

Week 2: A significant decrease in ORAC concentrations were found in treatment T2 compared to the control (P<0.05). The ORAC concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 (P<0.05).

Week 3: The ORAC concentrations of treatment T½ were significantly higher compared to the control (P<0.05). The ORAC concentrations of treatment T¼ were significantly lower

compared to the concentrations of treatment T½, and the concentrations of treatment T½ were significantly higher compared to treatment T1 ($P<0.05$).

Week 4: The ORAC concentrations of treatments T¼ and T½ were significantly higher compared to the control ($P<0.05$). The ORAC concentrations of treatment T½ were significantly higher compared to treatment T1 ($P<0.05$).

Week 5: The ORAC concentrations of treatment T½ were significantly higher compared to the control ($P<0.05$). The ORAC concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Pooled data: The ORAC concentrations for treatment T½ were significantly higher compared to the control ($P<0.05$). No significant differences in ORAC concentrations were found between treatments ($P>0.05$).

Table 4.5. Mean (±SD) Oxygen Radical Absorbance Capacity Assay (ORAC) (µmol TE/g) concentrations measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}14.400^{a}$ ±1.762	$^{a}12.544^{a}$ ±4.248	$^{a}18.831^{b\#}$ ±3.718	$^{a}14.791^{a\#}$ ±2.021	$^{a}11.220^{a}$ ±2.840
1	$^{a}14.630^{a}$ ±2.960	$^{a}16.880^{a}$ ±1.930	$^{a}18.760^{b}$ ±2.830	$^{b}18.930^{c}$ ±2.900	$^{b}16.920^{a}$ ±2.530
2	$^{a}13.690^{a}$ ±1.640	$^{a}15.318^{a}$ ±2.423	$^{*a}14.890^{a}$ ±2.336	$^{a}17.280^{a}$ ±3.330	$^{*a}10.010^{b\#}$ ±1.960
3	$^{a}14.770^{a}$ ±3.180	$^{a}15.588^{a}$ ±1.766	$^{*a}21.280^{b\#}$ ±2.710	$^{a}13.340^{c\#}$ ±1.602	$^{*c}13.340^{a}$ ±1.600
4	$^{a}11.100^{a}$ ±2.290	$^{*b}18.700^{b}$ ±1.850	$^{a}17.100^{c}$ ±2.120	$^{a}13.940^{a\#}$ ±1.950	$^{a}12.800^{a}$ ±2.530
5	$^{*a}15.800^{a}$ ±1.370	$^{c}19.480^{a}$ ±2.180	$^{a}20.920^{b}$ ±2.800	$^{a}15.850^{a\#}$ ±2.550	$^{a}13.100^{a}$ ±3.860
Pooled date for entire experimental period	14.065^{a} ±1.604	16.418^{a} ±2.518	18.630^{b} ±2.394	15.688^{a} ±2.119	12.898^{a} ±2.349

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 =*

week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.

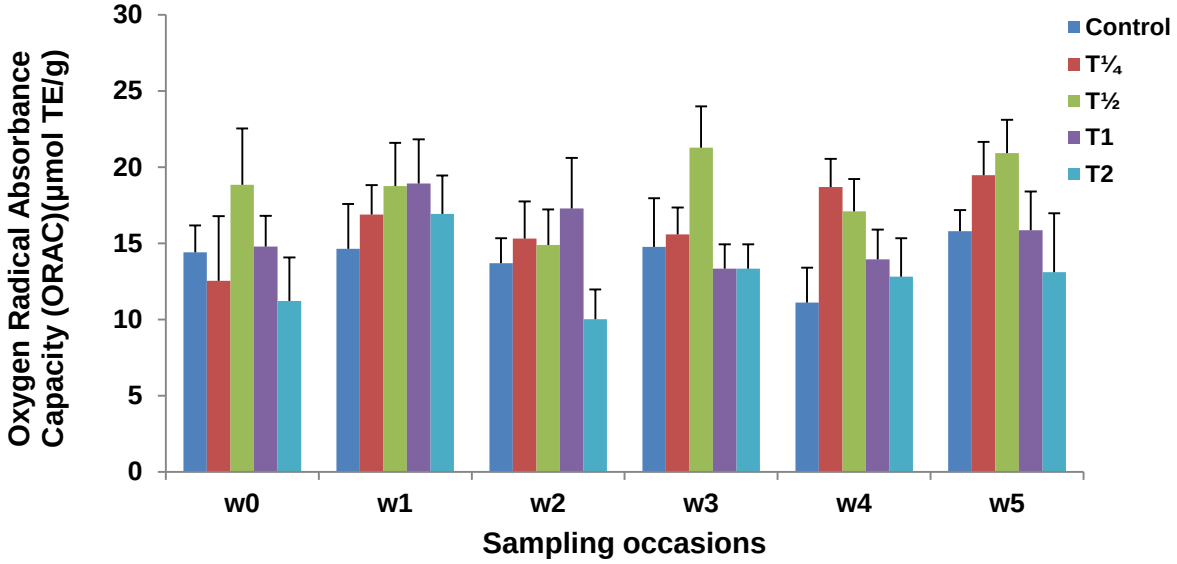

Figure 4.5. Mean (±SD) Oxygen Radical Absorbance Capacity Assay (ORAC) (µmol TE/g) concentrations measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.5. Antioxidant enzymes

4.1.5.1. Comparisons of Catalase (CAT) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured catalase (CAT) between different weeks in the experimental plants are shown in Table 4.6 and Figure 4.6.

Control (baseline): When compared to week 0, a significant decrease in CAT concentrations was found during week 1 and a significant increase was found during week 5. The latter indicate an overall increase in CAT concentrations from the start to the end of the experiment ($P<0.05$). A significant increase in CAT concentrations was found between week 1 and week 2 ($P<0.05$).

Treatment T¼: When compared to week 0, the CAT concentrations were significantly ($P<0.05$) higher during weeks 1, 3 and 5. The latter indicate an overall increase in CAT concentrations from the start to the end of the experiment ($P<0.05$). The CAT concentrations decreased significantly between week 1 and week 2 and between week 3 and week 4, and the CAT concentrations increased between week 2 and week 3 and between week 4 and week 5 ($P<0.05$).

Treatment T½: When compared to week 0, significant increases in CAT concentrations were found during weeks 1, 2, 3, 4 and 5. The latter indicate an overall increase in CAT concentrations from the start to the end of the experiment ($P<0.05$). A significant decrease in CAT concentrations was found between week 1 and week 2 ($P>0.05$).

Treatment T1: When compared to week 0, significant increases in CAT concentrations were found during weeks 1 and 5. The latter indicate an overall increase in CAT concentrations from the start to the end of the experiment ($P<0.05$). A significant decrease in CAT concentrations was found between week 4 and week 5 ($P<0.05$).

Treatment T2: When compared to week 0, the CAT concentrations were significantly lower during weeks 3 and 4 ($P<0.05$). The CAT concentrations were significantly higher between week 4 and week 5 ($P<0.05$). No significant difference in CAT concentrations was found between week 0 and week 5 ($P>0.05$).

4.1.5.2. Comparisons of Catalase (CAT) concentrations between treatments per week in *Ceratophyllum demersum* L.

The comparisons of the concentrations of catalase (CAT) in experimental plant samples of the different treatments are illustrated in Table 4.6 and Figure 4.6.

Week 0 (baseline): The CAT concentrations of treatment T½ were significantly lower and the concentrations of treatment T2 were significantly higher compared to the control ($P<0.05$). The CAT concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½, and the concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T2 ($P<0.05$).

Week 1: The CAT concentrations of treatments T¼, T½, T1, T2 were significantly higher compared to the control ($P<0.05$). The CAT concentrations of treatment T½ were significantly

higher compared to the concentrations of treatment T¼ and the concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T½ ($P<0.05$).

Week 2: No significant differences in CAT concentrations were found in the control ($P>0.05$). The CAT concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T¼, and the concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T½ ($P<0.05$).

Week 3: The CAT concentrations of treatments T¼ and T½ were significantly higher compared to the control ($P<0.05$). The CAT concentrations of treatment T¼ were significantly higher compared to the concentrations of treatment T½, and the concentrations of treatment T½ were significantly higher compared to treatment T1 ($P<0.05$).

Week 4: The CAT concentrations of treatment T½ were significantly higher compared to the control ($P<0.05$). The CAT concentrations of treatment T½ were significantly higher compared to treatment T¼ and the concentrations of T½ were significantly higher compared to the concentrations of T1 ($P<0.05$).

Week 5: No significant differences in CAT concentrations were found in the control of the different treatments ($P>0.05$). No significant differences in CAT concentrations were found between the treatments ($P>0.05$).

Pooled data: The CAT concentrations for treatment T½ were significantly higher compared to the control ($P<0.05$). No significant differences in CAT concentrations were found between treatments ($P>0.05$).

Table 4.6. Mean (±SD) Catalase (CAT) (mmole/µg) concentrations measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}7.140^{a} ±1.350	^{a}6.373^{a} ±1.362	^{a}4.180^{b} ±0.580	^{a}6.930$^{a\#}$ ±1.910	^{a}9.360$^{c\#}$ ±2.000
1	^{b}5.070^{a} ±1.880	^{b}11.928^{b} ±1.975	^{b}26.363$^{c\#}$ ±4.791	^{b}10.103$^{d\#}$ ±0.284	^{a}9.435^{e} ±2.656
2	^{*a}8.890^{a} ±1.970	^{*a}1.520^{a} ±1.240	^{*c}12.333$^{a\#}$ ±4.078	^{a}6.540$^{a\#}$ ±1.580	^{a}10.560^{a} ±2.120
3	^{a}7.170^{a} ±1.180	^{*c}15.510^{b} ±1.720	^{d}12.140$^{c\#}$ ±2.360	^{a}4.730$^{a\#}$ ±1.340	^{b}5.060^{a} ±2.560
4	^{a}7.620^{a} ±2.850	^{*a}7.233^{a} ±2.810	^{e}12.035$^{b\#}$ ±2.560	^{a}5.450$^{a\#}$ ±1.710	^{c}5.170^{a} ±1.570
5	^{c}12.930^{a} ±1.530	^{*d}14.437^{a} ±1.485	^{f}12.398^{a} ±2.576	^{*c}10.588^{a} ±3.315	^{*a}12.075^{a} ±1.396
Pooled data for entire experimental period	8.137^{a} ±2.651	9.500^{a} ±5.385	13.241^{a} ±7.190	7.390^{a} ±2.423	8.610 ±2.881

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

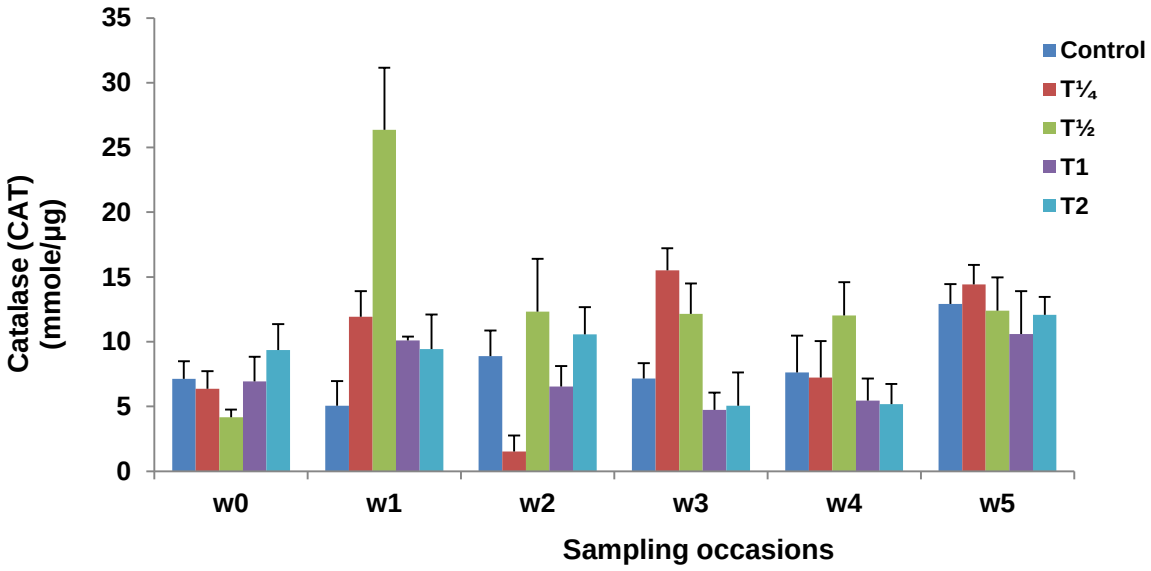

Figure 4.6. Mean (±SD) Catalase (CAT) mmole/µg, measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.5.3. Comparisons of Superoxide Dismutase (SOD) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured superoxide dismutase (SOD) between different weeks in the experimental plants are shown in Table 4.7 and Figure 4.7.

Control (baseline): When compared to week 0, significant decreases in SOD concentrations were found during weeks 1, 2 and significant increases were found during weeks 2, 4 and 5. The latter indicate an overall increase in SOD concentrations from the start to the end of the experiment ($P<0.05$). Significant decreases in SOD concentrations were found between week 1 and week 2 and between week 4 and week 5, significant increases were found between week 2 and week 3 and between week 3 and week 4 ($P<0.05$).

Treatment T¼: When compared to week 0, the SOD concentrations were significantly lower during weeks 1, 2 and 3 ($P<0.05$). The SOD concentrations increased significantly between week 3 and week 4 ($P<0.05$). No significant difference in SOD concentrations was found between week 0 and week 5 ($P>0.05$).

Treatment T½: When compared to week 0, significant decreases in SOD concentrations were found during weeks 1 and 2 ($P<0.05$). A significant increase in SOD concentrations were found between week 2 and week 3 ($P<0.05$). No significant difference in SOD concentrations was found between week 0 and week 5 ($P>0.05$).

Treatment T1: When compared to week 0, significant decreases in SOD concentrations were found during weeks 1, 2 and 4 ($P<0.05$). Significant increases in SOD concentrations were found between week 1 and week 2 and between week 2 and week 3 ($P<0.05$). No significant difference in SOD concentrations was found between week 0 and week 5 ($P>0.05$).

Treatment T2: When compared to week 0, the SOD concentrations were significantly lower during weeks 1, 2, 4 and 5 ($P<0.05$). The SOD concentrations increased significantly higher between week 1 and week 2 and between week 2 and week 4. The SOD concentrations decreased significantly between week 3 and week 4 and between week 4 and week 5 ($P<0.05$). No significant difference in SOD concentrations was found between week 0 and week 5 ($P>0.05$).

4.1.5.4. Comparisons of Superoxide Dismutase (SOD) concentrations between treatments per week in *Ceratophyllum demersum* L.

The comparisons of the concentrations of superoxide dismutase (SOD) in experimental plant samples of the different treatments are illustrated in Table 4.7 and Figure 4.7.

Week 0 (baseline): The SOD concentrations of treatments T¼, T1 and T2 were significantly higher compared to the control ($P<0.05$). The SOD concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½, and the concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T2 ($P<0.05$).

Week 1: The SOD concentrations of treatment T½ were significantly higher and the concentrations of treatment T2 were significantly lower compared to the control ($P<0.05$). The SOD concentrations of treatment T½ were significantly higher compared to the concentrations of treatment T¼ and the concentrations of treatment T2 were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Week 2: The SOD concentrations of treatments T¼, T½, T1 and T2 were significantly higher compared to the control ($P<0.05$). The SOD concentrations of treatment T1 were significantly

higher compared to the concentrations of treatment T½, and the concentrations of treatment T2 were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Week 3: The SOD concentrations of treatment T¼ were significantly lower compared to the control ($P<0.05$). The SOD concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 4: The SOD concentrations of treatments T¼, T½, T1 and T2 were significantly lower compared to the control ($P<0.05$). No significant differences in SOD concentrations were found between the treatments ($P>0.05$).

Week 5: The SOD concentrations of treatment T2 were significantly lower compared to the control ($P<0.05$). The SOD concentrations of treatment T2 were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Pooled data: The SOD concentrations for treatment T½ were significantly higher compared to the control ($P<0.05$). No significant differences in SOD concentrations were found between treatments ($P>0.05$).

Table 4.7. Mean (±SD) Superoxide Dismutase (SOD) (U/mg) concentrations measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	^{a}31.202^{a} ±3.879	^{a}38.745^{b} ±3.642	^{a}39.643^{a} ±0.413	^{a}52.250$^{c\#}$ ±6.359	^{a}41.389$^{d\#}$ ±5.620
1	^{*b}21.163^{a} ±5.823	^{b}19.403^{a} ±2.791	^{b}25.835$^{b\#}$ ±3.826	^{b}23.874^{a} ±3.307	^{b}13.402$^{c\#}$ ±1.504
2	^{*c}14.531^{a} ±1.520	^{c}25.219^{b} ±0.852	^{c}27.367^{c} ±2.019	^{*c}32.465$^{d\#}$ ±3.187	^{*c}26.830$^{e\#}$ ±4.810
3	^{*d}39.259^{a} ±5.556	^{d}29.491^{b} ±7.805	^{*a}34.690^{a} ±2.629	^{*a}43.976$^{a\#}$ ±7.624	^{*a}41.585^{a} ±6.103
4	^{*e}46.297^{a} ±5.969	^{*a}38.945^{b} ±3.375	^{a}32.889^{c} ±6.493	^{d}38.727^{d} ±6.048	^{*d}34.594^{e} ±3.747
5	^{*f}36.566^{a} ±3.304	^{a}35.010^{a} ±3.360	^{a}39.476^{a} ±4.530	^{a}40.347^{a} ±3.621	^{*e}27.712$^{b\#}$ ±4.866
Pooled data for entire experimental period	31.503^{a} ±11.830	31.136^{a} ±7.865	33.317^{a} ±5.855	38.607^{a} ±9.724	30.919^{a} ±10.684

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

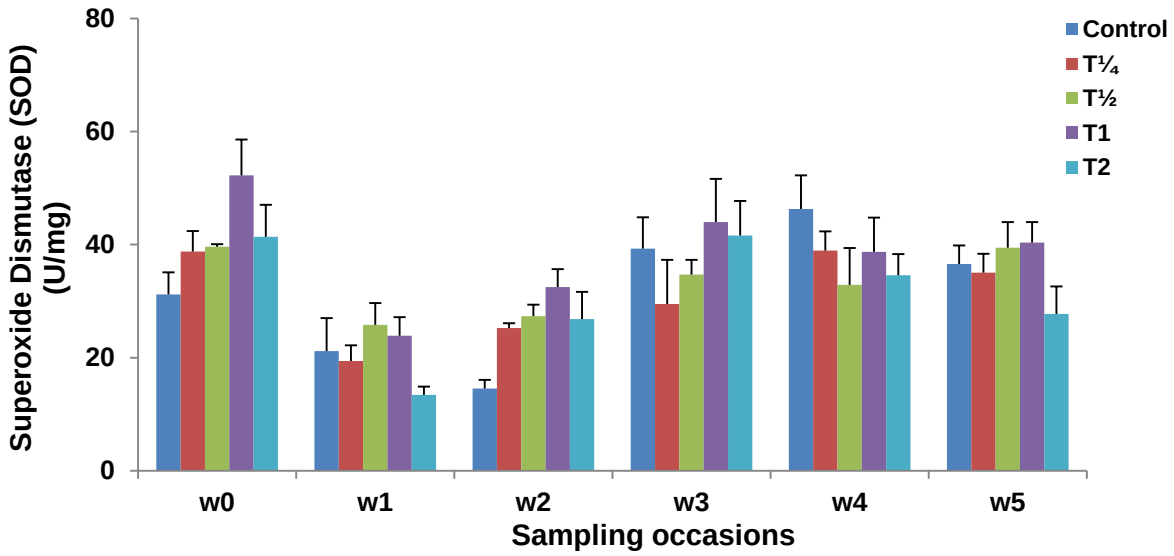

Figure 4.7. Mean (±SD) Superoxide Dismutase (SOD) (U/mg) concentrations measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.5.5. Comparisons of Total Glutathione (GSHt) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured total glutathione (GSHt) between different weeks in the experimental plants are shown in Table 4.8 and Figure 4.8.

Control: When compared to week 0, significant decreases in GSHt concentrations were found during weeks 2 and 5. The latter indicate an overall decrease in GSHt concentrations from the start to the end of the experiment ($P<0.05$). Significant decreases in GSHt concentrations were found between week 1 and week 2 and between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, no significant differences in GSHt concentrations were found in T¼ ($P>0.05$). No significant differences in GHSt concentrations were found between the weeks ($P>0.05$).

Treatment T½: When compared to week 0, no significant differences in GSHt concentrations were found in T½ ($P>0.05$). No significant differences in GHSt concentrations were found between weeks ($P>0.05$).

Treatment T1: When compared to week 0, significant decreases in GSHt concentrations were found in T1 during weeks 2, 3 and 5 ($P<0.05$). The latter indicate an overall decrease in GSHt concentrations from the start to the end of the experiment ($P<0.05$). Significant decreases in GSHt concentrations were found between week 1 and week 2 and between week 4 and week 5 ($P<0.05$).

Treatment T2: When compared to week 0, the GSHt concentrations were significantly lower in T2 during weeks 2 and 5 ($P<0.05$). The latter indicate an overall decrease in GSHt concentrations from the start to the end of the experiment ($P<0.05$). The GSHt concentrations decreased significantly between week 1 and week 2 and between week 4 and week 5. The GSHt concentrations increased significantly between week 3 and week 4 ($P<0.05$).

4.1.5.6. Comparisons of Total Glutathione (GSHt) concentrations between treatments per week in *Ceratophyllum demersum* L.

The comparisons of the concentrations of total glutathione (GSHt) in experimental plant samples of the different treatments are illustrated in Table 4.8 and Figure 4.8.

Week 0 (baseline): The GSHt concentrations of treatment T½ were significantly lower compared to the control ($P<0.05$). The GSHt concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 1: The GSHt concentrations of treatments T¼ and T½ were significantly lower compared to the control ($P<0.05$). The GSHt concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 2: The GSHt concentrations of treatments T¼ and T½, were significantly lower compared to the control ($P<0.05$). The GSHt concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 3: The GSHt concentrations of treatments T¼ and T½, were significantly lower compared to the control ($P<0.05$). The GSHt concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 4: The GSHt concentrations of treatments T¼ and T½ were significantly lower and the concentrations of treatment T2 were significantly higher compared to the control ($P<0.05$). The GSHt concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ and the concentrations of treatment T2 were significantly higher compared to treatment T1 ($P<0.05$).

Week 5: No significant differences in GSHt concentrations were found between the control and the different treatments ($P>0.05$). No significant differences in GSHt concentrations were found between the treatments ($P>0.05$).

Pooled data: The GSHt concentrations for treatments T¼ and T½ were significantly lower ($P<0.05$) compared to the control. The GSHt concentrations of treatment T1 were significantly higher compared to treatment T½ ($P<0.05$).

Table 4.8. Mean (±SD) Total Glutathione (GSHt) (µmol/g) concentrations measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}0.045^{a}$ ±0.020	$^{a}0.041^{a}$ ±0.044	$^{a}0.022^{b}$ ±0.001	$^{a}0.052^{a\#}$ ±0.024	$^{a}0.057^{a}$ ±0.024
1	$^{a}0.054^{a}$ ±0.023	$^{a}0.019^{b}$ ±0.002	$^{a}0.017^{c}$ ±0.008	$^{a}0.062^{a\#}$ ±0.028	$^{a}0.062^{a}$ ±0.032
2	$^{*b}0.032^{a}$ ±0.008	$^{a}0.020^{b}$ ±0.003	$^{a}0.020^{c}$ ±0.002	$^{*b}0.035^{a\#}$ ±0.012	$^{*b}0.034^{a}$ ±0.014
3	$^{a}0.037^{a}$ ±0.013	$^{a}0.018^{b}$ ±0.001	$^{a}0.018^{c}$ ±0.001	$^{c}0.043^{a\#}$ ±0.016	$^{a}0.044^{a}$ ±0.017
4	$^{a}0.049^{a}$ ±0.017	$^{a}0.018^{b}$ ±0.001	$^{a}0.017^{c}$ ±0.001	$^{a}0.051^{a\#}$ ±0.020	$^{*a}0.068^{d\#}$ ±0.034
5	$^{*c}0.018^{a}$ ±0.001	$^{a}0.019^{a}$ ±0.001	$^{a}0.019^{a}$ ±0.001	$^{*c}0.019^{a}$ ±0.002	$^{*c}0.018^{a}$ ±0.002
Pooled data for entire experimental period	0.039^{a} ±0.013	0.022^{b} ±0.009	0.019^{c} ±0.002	0.044^{a*} ±0.015	0.047^{a} ±0.019

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

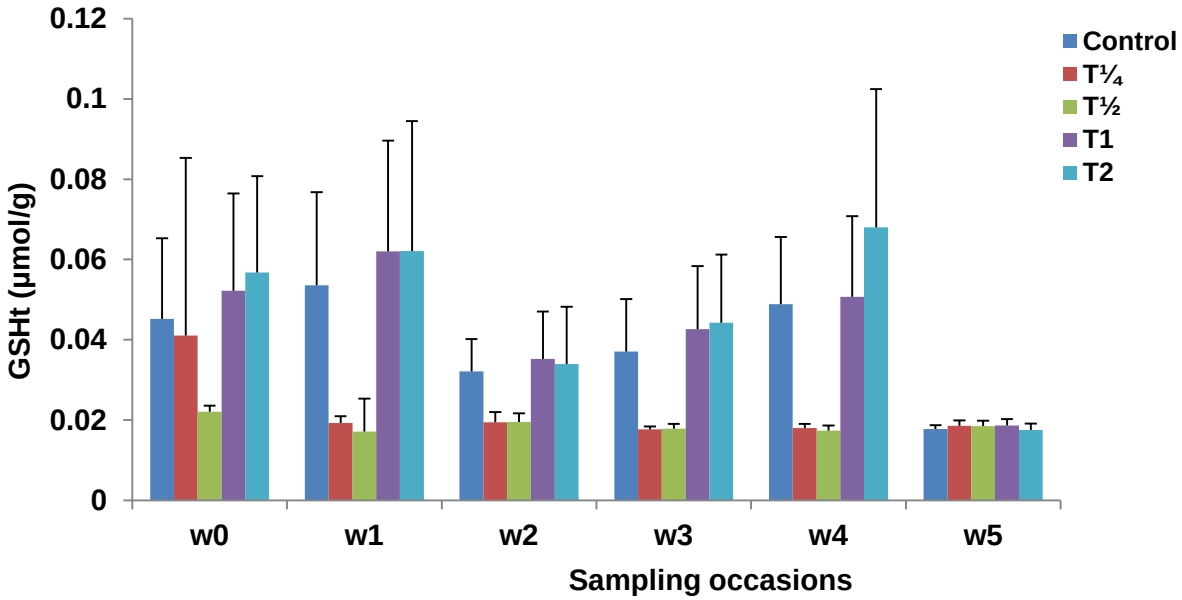

Figure 4.8. Mean (±SD) Total Glutathione (GSHt) (µmol/g), measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.1.5.7. Comparisons of Ascorbic Acid (AsA) concentrations between weeks in *Ceratophyllum demersum* L.

Comparisons of the mean (±SD) concentrations of measured ascorbic acid (AsA) between different weeks in the experimental plants are shown in Table 4.9 and Figure 4.9.

Control: When compared to week 0, significant decreases in AsA concentrations were found during weeks 1, 2, 4 and 5. The latter indicate an overall decrease in AsA concentrations from the start to the end of the experiment ($P<0.05$). Significant increases in AsA concentrations were found between week 2 and week 3 and between week 3 and week 4. Significant decreases in AsA concentrations were found between week 2 and week 3 and between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, significant decreases in AsA concentrations were found during weeks 1, 2, 3 and 4 ($P<0.05$). A significant increase in AsA concentrations were

found between week 4 and week 5 (P<0.05). No significant difference in AsA concentrations was found between week 0 and week 5 (P>0.05).

Treatment T½: When compared to week 0, a significant increase in AsA concentrations were found during week 3 (P<0.05). Significant decreases in AsA concentrations were found between week 3 and week 4 and a significant increase in AsA concentrations were found between week 4 and week 5 (P<0.05). No significant difference in AsA concentrations was found between week 0 and week 5 (P>0.05).

Treatment T1: When compared to week 0, significant increases in AsA concentrations were found in T1 during weeks 1, 2, 4 and 5 (P<0.05). The latter indicate an overall increase in AsA concentrations from the start to the end of the experiment (P<0.05). Significant increases in AsA concentrations were found between week 1 and week 2 and between week 3 and week 4. Significant decreases in AsA concentrations were found between week 2 and week 3 and between week 4 and week 5 (P<0.05).

Treatment T2: When compared to week 0, the AsA concentrations were significantly higher in T2 during weeks 1, 2, 4 and 5 (P<0.05). The latter indicate an overall increase in AsA concentrations from the start to the end of the experiment (P<0.05). Between consecutive weeks: The AsA concentrations decreased significantly between week 2 and week 3 and between week 4 and week 5. The AsA concentrations increased significantly between week 3 and week 4 (P<0.05).

4.1.5.8. Comparisons of Ascorbic Acid (AsA) concentrations between treatments per week in *Ceratophyllum demersum* L.

The comparisons of the concentrations of ascorbic acid (AsA) in experimental plant samples of the different treatments are illustrated in Table 4.9 and Figure 4.9.

Week 0 (baseline): The AsA concentrations of treatments T½ and T2 were significantly lower compared to the control (P<0.05). The AsA concentrations of treatment T¼ were significantly higher compared to the concentrations of treatment T½, the concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T1 and the concentrations of treatment T2 were significantly lower compared to treatment T1 (P<0.05).

Week 1: The AsA concentrations of treatments T¼, T½, T1, T2 were significantly higher compared to the control (P<0.05). The AsA concentrations of treatment T¼ were significantly lower compared to treatment T½, the concentrations of treatment T½ were significantly lower

compared to the concentrations of treatment T1, and the concentrations of treatment T2 were significantly higher compared to the concentrations of treatment T1 ($P<0.05$).

Week 2: The AsA concentrations of treatments T¼ and T½, were significantly lower compared to the control ($P<0.05$). The AsA concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ ($P<0.05$).

Week 3: The AsA concentrations of treatments T¼, T1, T2 were significantly lower compared to the control ($P<0.05$). The AsA concentrations of treatment T½ were significantly higher compared to treatment T¼, and the concentrations of treatment T1 were significantly lower compared to the concentrations of treatment T½, the concentrations of treatment T2 were significantly higher compared to treatment T1 ($P<0.05$).

Week 4: The AsA concentrations of treatments T¼, T½, T1 were significantly lower compared to the control ($P<0.05$). The AsA concentrations of treatment T1 were significantly higher compared to the concentrations of treatment T½ and the concentrations of treatment T2 were significantly higher compared to treatment T1 ($P>0.05$).

Week 5: The AsA concentrations of treatments T¼, T½, T1, T2 were significantly higher compared to the control ($P<0.05$). The AsA concentrations of treatment T¼ were significantly higher compared to treatment T½, the concentrations of treatment T½ were significantly lower compared to the concentrations of treatment T1 ($P<0.05$).

Table 4.9. Mean (±SD) Ascorbic Acid (AsA) concentrations (µg/g) measured in *Ceratophyllum demersum* L. from experimental treatments. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}25.640^{a}$ ±3.270	$^{a}27.550^{a}$ ±1.820	$^{a}18.780^{b\#}$ ±2.610	$^{a}26.750^{a\#}$ ±2.360	$^{a}19.280^{c\#}$ ±1.430
1	$^{*b}6.227^{a}$ ±2.295	$^{b}13.926^{b}$ ±1.884	$^{a}21.830^{c\#}$ ±3.940	$^{*b}35.360^{d\#}$ ±1.410	$^{b}94.650^{e\#}$ ±13.270
2	$^{*c}92.550^{a}$ ±0.797	$^{c}14.780^{b}$ ±1.160	$^{a}18.430^{c}$ ±1.900	$^{*c}91.380^{a\#}$ ±9.120	$^{c}104.900^{a}$ ±6.280
3	$^{*a}24.904^{a}$ ±3.700	$^{d}12.322^{b}$ ±3.168	$^{b}24.960^{a\#}$ ±2.580	$^{*d}4.690^{c\#}$ ±0.740	$^{*a}16.215^{d\#}$ ±2.067
4	$^{*d}75.440^{a}$ ±3.340	$^{e}16.870^{b}$ ±3.190	$^{*a}16.670^{c}$ ±1.640	$^{*e}48.014^{d\#}$ ±5.249	$^{*d}79.270^{a\#}$ ±6.130
5	$^{*e}1.580^{a}$ ±0.640	$^{*a}30.800^{b}$ ±3.920	$^{*a}23.320^{c\#}$ ±1.670	$^{*f}34.130^{d\#}$ ±2.790	$^{*e}32.790^{e}$ ±2.852
Pooled data for entire experimental period	37.723^{a} ±37.516	19.375^{a} ±7.800	20.680^{a} ±3.183	40.054^{a} ±28.922	57.851^{a} ±39.688

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment and significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week and significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test ($p<0.05$)). Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

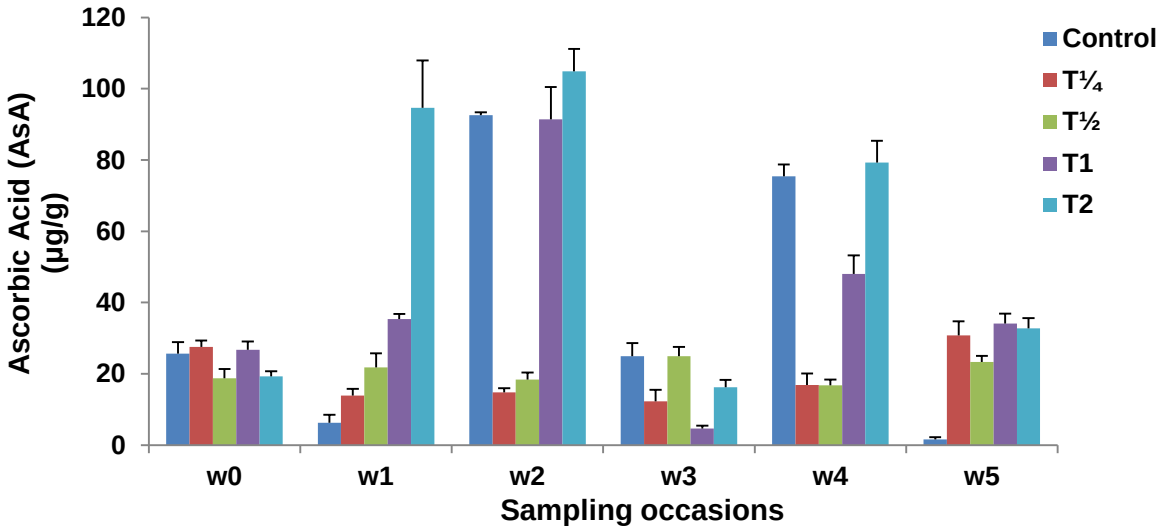

Figure 4.9. Mean (±SD) Ascorbic Acid (AsA) (µg/g), measured per week in experimental treatments in *Ceratophyllum demersum* L. *(Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

4.2. Discussion

Cho *et al*. (2003) reported that the sensitivity of plants to metals and the potential of plants to accumulate these metals depend on an interrelated network of physiological and molecular mechanisms such as: uptake and accumulation of metals through binding to extracellular exudates and cell wall constituents, accumulation or modification of plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged cell structures, accumulation of osmolytes and osmoprotectants and induction of antioxidant enzymes, among others. Metal toxicity includes inactivation of biomolecules by either blocking essential functional groups or by displacement of essential metal ions (Goyer, 1997).

Oxidative stress damages plant growth and development when antioxidant capacity and ROS are unbalanced (Munns & Tester, 2008; Ellouzia *et al.,* 2011). Antioxidant enzymes and certain metabolites play a significant role in adaptation and ultimate survival of plants during periods of stress. Activities of antioxidant enzymes are inducible by oxidative stress (Baisak

et al., 1994; Foyer *et al.,* 1994), which suggests that a general strategy is required to overcome stress (Shah *et al*., 2001). The accumulation of metals in plants causes oxidative stress due to interruption of various metabolic processes. One of the most important oxidative biomarker responses of metals is the production of large quantities of reactive oxygen species (ROS), which can cause damage to proteins, lipids and DNA (Schùtzendùbel & Polle, 2002; Apel & Hirt, 2004; Verbruggen *et al*., 2009, Singh & Prasad, 2014). Over production of ROS by the Fenton reaction may cause cellular injury (Stohs & Bagchi, 1995) or cell death (Mittler, 2002), when metal toxicity stress point is reached at the toxic threshold level of the metal in the tissues of the plants (Bhaduri *et al*., 2012). Metals that are redox-active, such as Fe, Cu and Cr, undergo redox cycling and produces ROS, whereas redox-inactive metals, such as Pb, Cd, Hg, and others, reduce the cell's major antioxidants and enzymes (Shah *et al*., 2001; Maheshwari & Dubey, 2009). If ROS production induced by metals is not sufficiently counterbalanced by cellular antioxidants, oxidative damage of lipids, proteins and nucleic acids, follows (Sharma & Dubey, 2009; Mishra *et al.,* 2011; Srivastava & Dubey, 2011). A significant enhancement in lipid peroxidation and decrease in protein thiol contents were observed when rice seedlings were subjected to Al, Ni and Mn toxicity (Maheshwari & Dubey, 2009; Sharma & Dubey, 2009). Plants therefore need ways to detoxify ROS.

4.2.1. Total Polyphenols (TP)

All plants produce a remarkable diversity of secondary metabolites. One of the most important groups of these metabolites is the phenolic compounds (Michalak, 2006). Induction of phenolic compounds biosynthesis was observed in wheat in response to nickel toxicity (Diáz *et al.,* 2001) and in maize in response to aluminium (Winkel-Shirley, 2002). Little attention has been given towards the influence of metals on the polyphenol metabolism in plants (Deval *et al*., 2012). According to Parida *et al*. (2002) accumulation of polyphenols play a key role in plants with regards to stress. In the present study total polyphenol levels declined significantly in the experimental plants in the control at weeks 1 and 2 and increased significantly at week 5, while weeks 3 and 4 remained at the same levels as week 0. During week 2, polyphenol levels decreased significantly when exposed to treatment T¼ and recovered to week 0 levels during weeks 1, 3, 4 and 5. No changes were indicated during weeks 1, 2, 3, 4 and 5 in treatment T½ compared to week 0. During week 3 in treatment T1, TP levels decreased significantly and during week 5 the TP levels increased significantly relative to week 0. TP levels increased significantly during weeks 2, 3, 4 and 5 relative to week 0 levels in treatment T2. Increased levels of polyphenol concentrations,

especially at week 4, in treatment T1 and at weeks 2, 3, 4 and 5 in treatment T2 might have induced accumulation of secondary metabolites in *C. demersum* L. to tolerate the metal stress conditions. Total polyphenols increased significantly between the control and treatments during week 1, 2 and between the control and treatment T1 during week 3. Increases in polyphenol concentrations in almost all the weeks of treatment T2 might be due to the protective function of these compounds against metal stress by metal chelation and ROS scavenging (Brown *et al*., 1998; Lavid *et al*., 2001; Rastgoo *et al*., 2011). Increases in phenolic content indicate antioxidant activity for these compounds under stress conditions. Previously it was shown that increases in phenolics corresponded to the increase in the activity of enzymes involved in phenolic compound metabolism and a *de novo* synthesis of phenolics under metal stress was proposed (Parry *et al*., 1994). Earlier studies indicated that phenolic compounds, beside ascorbate, can protect the cell against oxidative stress by phenol-coupled ascorbate peroxidase (APX) reactions (Polle *et al*., 1997). Several studies have reported that the antioxidant properties of phenolic components is due to their ability to chelate the transition metal ion, and the inhibition of superoxide-driven Fenton reaction (Rice-Evans *et al.,* 1997; Arora *et al*., 1998) and membrane stability by decreasing membrane fluidity (Blokhina *et al.,* 2003). The increase in total polyphenol (TP) concentrations may also be related to the modified tolerance mechanism adopted by the plants for overall growth and development.

4.2.2. Lipid peroxidation

The main area of attack by any redox active metal in a plant cell is generally the cell membrane. Metals cause severe lipid peroxidation due to the removal of hydrogen by ROS from unsaturated fatty acids leading to lipid radical formation (Aravind *et al.,* 2003). This formation leads to a cascade of cyclical reactions which leads to a repetitive formation of short chain alkanes and lipid acid aldehydes which totally destroy the lipid structure. This leads further to dimerization and polymerization of proteins, which are considered to be most damaging to membranes (Logani & Davies, 1980).

4.2.2.1. Conjugated Dienes (CDs)

Conjugated dienes (CDs) and thiobarbituric acid reactive substances (TBARS) were used to evaluate lipid peroxidation damage over the course of the experimental period. Each of the assays evaluated the damage at a different stage of the lipid oxidative damage process. CDs

characterized the initial product of radical attack and a rearrangement of double bonds in unsaturated fatty acids (Pannunzio & Storey, 1998).

A lipid peroxyl radical is formed when the free electron on a CD react with oxygen. The lipid peroxyl removes a hydrogen ion from lipid hydroperoxide. Lipid peroxidation could be changed under both abiotic and biotic stresses (Hildebrand *et al.,* 1988; Leone *et al.,* 2001). Formation of conjugated dienes occurs when free radicals attack the hydrogens of methylene groups separating double bonds and leading to a rearrangement of the bonds (Recknagel & Glende, 1984). A few studies have investigated the effect of metals on lipid peroxidation occurrence and activity. Membrane-bound lipid peroxidation activity was unchanged in the roots of wheat growing under Cu deficiency (Quartacci *et al.*, 2001). Lipoxygenase (LOX) activity increased in the leaves exposed to Fe, Cd, Cu and Pb both in short-term and long-term experiments (Gallego *et al.,* 1996; Djebali *et al.*, 2005).

The results of the current study indicated that the CD levels increased significantly at weeks 3 and 5 compared to week 0 and weeks 1, 2 and 4 recovered to the week 0 levels. Significant decreases in CD levels were shown at weeks 1, 2, 3, 4 and 5 compared to week 0 in treatment T¼. A significant decrease in CD level was detected at week 1 in treatment T½ and the same CD levels were shown for weeks 2, 3, 4 and 5 compared to week 0. Significant increases in CD levels were found between the control and weeks 3 and 4 in treatments T1, a significant decrease were shown at week 1 and week 5 recovered to the week 0 levels. CD levels increased significantly in treatment T2 at week 2, 3 and 4 compared to week 0 and CD levels of week 5 recovered to the week 0 levels. This could be an effect of plants growing under high metal concentrations as CDs declined in the lower exposure concentrations. The amount of CDs increased in *Raphanus sativus* (radish) growing under high Cu concentrations (Sgherri *et al.,* 2003). In the current study, CD concentrations declined significantly between the control and T¼, between the control and T1, and between the control and T2 during week 5. By week 5 the CD levels decreased in treatments T¼, T½ and T2 to lower than the start of the experiment, suggesting that this might be a sign of adaptation by *C. demersum* L. and that the plants were able to deal with the stress.

4.2.2.2. Thiobarbituric Acid Reactive Substances (TBARS)

The plasma membranes of plants are considered a primary target for metal toxicity in both leaves and roots (Sytar *et al*., 2013). In most studies the level of non-enzymatic lipid peroxidation, expressed as a level of malondialdehyde (MDA), was determined in plants

treated with metals. The thiobarbituric acid reactive substances (TBARS) assay measures one of the terminal products in the peroxidation consequence of the breakdown of lipids, known as malondialdehyde (MDA) and this assay is one of the basic methods of the research process to determine lipid peroxidation (LP) in biological systems (Pannunzio & Storey, 1998; Sytar *et al.,* 2013). LP causes membrane damage and changes in LP concentrations serve as an indicator of the extent of oxidative damage under stress (Halliwell & Gutteridge, 1993).

Results of several studies have indicated that under action of metals, plants often activate processes of LP (Dazy *et al.,* 2009; Ann *et al.,* 2011; Kumar *et al.,* 2012). LP is a biomarker for the free radical-mediated damage by production of ROS (Sytar *et al.,* 2013). Free radical reactive intermediates react directly or indirectly with molecular oxygen to form ROS. It is known that when plants are exposed to stress conditions, there is an increase in ROS. Organelles such as the peroxisomes and chloroplast (site of photosynthesis), where ROS are being produced at a relatively high rate, are especially at risk. MDA is a common product of lipid peroxidation and is a sensitive diagnostic indicator of oxidative injury in plant cells (Sun *et al.,* 2008). MDA is thus closely correlated with the level of oxidative stress in plants when exposed to different environmental stress and is a biomarker of lipid peroxidation (Koca *et al.,* 2007). According to Liu (2001) MDA contents in the aquatic plant positively correlated to surfactant concentrations in the solutions and indicated environmental pollution. MDA content in plant tissues is a useful index to evaluate pollution levels and can assess toxic effects of pollutants such as metals and acid rain (Liao *et al.,* 2005).

The results of the present study indicate that significant increases in TBARS levels were found between the control and weeks 1, 2, 3, 4 and week 5. A significant increase in TBARS levels was caused after week 1 of treatment T¼ and significant decreases were found after week 3 and 5 compared to week 0. Significant decreases in TBARS levels were caused after weeks 1, 2, 3, and 5 in treatment T½ compared to week 0. Treatment T1 caused significant decreases in TBARS levels after weeks 1, 2, 3, 5 and a significant increase at week 4 versus week 0. Treatment T2 caused a significant increase in TBARS level after week 4 compared to week 0. The present observation of an increase in TBARS levels in *C. demersum* L. in the control plants and week 1 in treatment T¼ when exposed for 1 to 5 weeks is consistent with those observed in *Pistia stratiotes* (Sinha *et al*., 2003) and *C. demersum* L. (Devi & Prasad, 1998).

Enhanced levels of non-enzymatic lipid peroxidation products were found in *Arabidopsis thaliana* plants under Cd and Cu stress. Under Cu excess, an MDA content increase was

observed (Skòrzyńska-Polit *et al.,* 2004). Studies with increasing Pb concentrations indicated that Pb induced lipid peroxides and oxidative stress in rice (Verma & Dubey, 2003) and in *Talinum triangulare* (a succulent herb) leaves (Kumar *et al*., 2013). Accumulation of Cd and Cu in plant tissues raised LP (Khan *et al.,* 2007; Cuypers *et al*., 2011). An increase in MDA content was observed in Hg- and Cd-treated *Phaseolus aureus* (a wild bean) leaves, but in the Hg treatment the change was more significant. This reaction could be attributed to the direct effect of Hg on photosynthetic electron transport (PET) causing generation of singlet oxygen (Shaw, 1995). The stimulation process of LP might be activated by lipoxygenase (LOX) with the formation of hydroperoxide because the early stress reactions take place at the membrane level (Huang *et al.,* 2012). Studies of Pb with *Potamogeton crispus* (freshwater plant) indicated that the high peroxidases activities and MDA content were detected with an increase in Pb concentration (Hu *et al.,* 2007).

TBARS levels indicated the prevalence of free radicals reactions in plants and membrane lipid peroxidation caused by metal exposure. In the present study it was shown that exposure to a mixture of Al, Cu, Fe and Zn has generally resulted in increased levels of TBARS when exposed to the different treatment concentrations after 4 weeks, while decreasing levels were recorded during the earlier time periods (weeks 1, 2 and 3), and is therefore indicative that the *C. demersum* L. have some antioxidant defences to deal with the increase in ROS associated with the increase in stress caused by metal exposure but the defence declined after longer exposure periods. This metal effect has already been proven (Chaoui *et al.,* 1997; Aravind & Prasad, 2005) and suggests that the primary site of metal injury could probably be at the cell membrane level (Rama *et al.,* 1998). Defence against enhanced ROS generation is accomplished through the activation of antioxidant mechanisms of plants, which includes both enzymatic and non-enzymatic antioxidants. Activity of one or more antioxidant enzymes such as SOD, CAT, APX, and/or GPX generally increases in plants and this elevated activity is usually correlated with increased tolerance (Singh *et al.,* 2010).

4.2.3. Total Antioxidant Capacity (TAC)

4.2.3.1. Ferric Reducing Antioxidant Power (FRAP)

The ability of plants to increase antioxidant protection to combat negative effects of metal stress appears to be limited since many studies indicated that exposure to elevated concentrations of redox reactive metals resulted in decreased and not increased activities of antioxidant enzymes, which could also implicate a threshold-effect to play an important role

in these cases. This is also valid for *C. demersum* L. as indicated in studies by other authors (Meir *et al.*, 1995; Velioglu *et al.,* 1998; Gjorgieva *et al.,* 2013). FRAP and ORAC assays are considered ideal methods to measure total antioxidant capacity (TAC) (Niki, 2010). Both of the methods however do not distinguish between reactivity and concentration and are considered semi-quantitative. According to Cao & Prior (1998), the FRAP assay quantifies the ferric reducing ability of a sample and is different from the ORAC assay because there are no free radicals or oxidants applied in the assay.

FRAP assays only measure non-enzymatic (reductans) antioxidants in a sample and this study indicated an interesting relationship between the metal content and measured FRAP value. In the present study in the control plants significant decreases in FRAP levels were found after weeks 1, 2, 3 and 4, relative to week 0. Treatment T¼ caused significant decreases in FRAP levels after weeks 1, 2, 3, 4 and 5 compared to week 0. Treatment T½ caused decreased trend in FRAP levels in all weeks but the results are not significant. Treatment T1 caused a significant increase in FRAP levels at week 3 and a significant decrease at week 5. A significant increase in FRAP levels was found after week 3 in treatment T2. The higher FRAP levels suggests that other factors such as metal dosages could have been responsible for oxidative stress, including insufficient metal concentrations. The FRAP activity for the other treatments remained constant throughout the experimental period and could be an indication that at that metal concentrations the plants functioned normally. Plants' exposure to metals trigger responses of antioxidative systems, but the direction of response is dependent on the plant species, tissue analysed, the metal used for treatment and also the intensity of the metal stress (van Assche & Clijsters,1990; Shainberg *et al.,* 2000).

4.2.3.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay utilises an inhibition method whereby a sample is added to a free radical-generating system and the free radical is measured. The assay uses AAPH as a free radical, and because of this it measures the capacity of an antioxidant to directly quench free radicals (Cao & Prior, 1998). The results of this study indicate that when considering the control plants, the ORAC levels showed no significant differences between week 0 and weeks 1 to 5. In this study, plants treated with the various concentrations of metals, showed an increased ORAC response at various time points. In this study, no significant differences in ORAC levels were found in weeks compared to the control. Significant increases in ORAC levels were caused after weeks 4 and 5 by treatment T¼ compared to week 0. No significant

differences in ORAC levels were caused by treatment T½ in weeks compared to week 0. A significant increase in ORAC levels was found after week 1 in treatment T1 compared to the baseline. Significant increases in ORAC levels caused by treatment T2 after weeks 1 and 3 compared to week 0.

4.2.4. Antioxidant enzymes

4.2.4.1. Catalase (CAT)

When considering the endogenous antioxidant enzymes in plants, catalase (CAT) is an enzyme which is present in the peroxisomes and mitochondria where it decomposes H_2O_2 into water and oxygen and is one of the main enzymes involved in the removal of toxic peroxides (Lin & Kao, 2000). CAT, APX and peroxidases (POD) are essential enzymes which scavenges the most stable ROS for example H_2O_2 efficiently to prevent oxidative damage to macromolecules (Gill & Tuteja, 2010; Gill *et al.,* 2012; Singh & Prasad, 2014). CAT is often used by cells to rapidly catalyse the decomposition of H_2O_2 into less reactive gaseous oxygen and water molecules (Tayefi-Nasrabadi *et al.,* 2011; Li *et al.,* 2012). Increases in CAT activity can be explained by increases in its substrate i.e. to maintain the level of hydrogen peroxide as an adaptive method of the plants (Reddy *et al.,* 2005). It is well known that CAT plays an important role in reducing oxidative stress by catalysing the oxidation of H_2O_2 (Weckx & Clijsters, 1996).

Catalase accelerates the spontaneous dismutation reaction of hydrogen peroxide. Previously, an increase in CAT activity was indicated in the presence of various pollutants, while peroxidase, glutathione reductase and SOD after 24 hour exposure to organic pollutants were also shown to be significantly increased in *Lemna minor* (Roy *et al.,* 2005). The results of the current study indicate that a significant decrease in CAT level was caused after week 1 and a significant increase was caused at week 5 in the control plants. Treatment T¼ caused significant increases in CAT levels after weeks 1, 3 and 5 compared to week 0. Significant increases in CAT levels were found after weeks 1, 2, 3, 4 and 5 in treatment T½ compared to week 0. Treatment T1 caused significant increases CAT levels at weeks 1 and 5 compared to the control. Compared to week 0, treatment T2 caused significant decreases in CAT levels at weeks 3 and 4. The increase in the activities of the enzyme by the metals suggests increased production of H_2O_2. However, the extent of increase in enzyme activities at treatments T1 and T2 were lower than those of treatments T¼ and T½, which indicate that at higher metal concentrations, CAT may not sufficiently protect plants from oxidative

damage. In *Brassica juncea* grown under excess Zn, increased CAT activity has been reported by Prasad *et al.* (1999). Differences were also observed between the control and treatments T¼ and T½ during week 0.

CAT activity decreases were observed in many plants grown under high Zn concentrations (Andrade *et al.,* 2009). Several studies indicated that low Cu concentration increased CAT activity but high Cu level inhibited this enzyme in *Medicago sativa* (lucerne) (Wang *et al.,* 2011). A decrease of CAT activity was shown in the leaves and shoots of some plants under Cu excess (Bouazizi *et al.,* 2010; Thounaojam *et al.,* 2012). The activity of CAT significantly decreased in rice plants under Fe excess (Mehraban *et al.,* 2008). This decline might be due to inhibition of enzyme synthesis or a change in the assembly of enzyme subunits (Radić *et al.,* 2010; Michelet *et al.,* 2013). Variable responses of CAT activity have been observed under metal stress in several studies. CAT activity declined in soybean (*Glycine max*) (Balestrasse *et al.,* 2001), the common reed (*Phragmites australis*) (Iannelli *et al.*, 2002) and *Arabidopsis thaliana* (a flowering plant) (Cho & Seo, 2005). CAT activity increased in *Oryza sativa* (rice) (Hsu & Kao, 2004), *Brassica juncea* (mustard greens) (Mobin & Khan, 2007), *Triticum aestivum* (common wheat) (Khan *et al*., 2007) under Cd stress. Sharma and Dubey (2005) reported a decrease in CAT activity in rice seedlings under drought stress.

Pan *et al.,* (2006) observed a decrease in CAT activity in *Glycyrrhiza uralensis* (Chinese liquorice) seedlings under the combined effect of salt and drought stress. Inconsistent results regarding CAT activity might be due to differences in the plant organs studied, the durations and concentrations of the metals utilized and the plant species. Differently to these studies, leaf CAT activity was stimulated under all applied Cu concentrations in wheat seedlings, whereas Fe treatment did not affect CAT, indicating that leaf CAT in wheat seedlings is sensitive to Cu stress and appears to be an efficient scavenger of H_2O_2 under Cu treatment. In transgenic tobacco plants, CAT activity indicated accumulation of GSSG and a 4-fold decrease in AsA, which indicates that CAT is vital for maintaining the redox balance during oxidative stress (Willekens *et al.,* 1995).

4.2.4.2. Superoxide Dismutase (SOD)

The metalloenzyme, SOD, is the most effective intracellular enzymatic antioxidant which is abundant in all aerobic organisms and in all subcellular compartments prone to ROS-mediated oxidative stress (Gill & Tuteja, 2010). SOD is considered as the first defence against ROS as it acts on superoxide radicals, which are produced in different compartments

of the cell and act as precursor to other ROS (Alscher *et al.,* 2002; Gill & Tuteja, 2010). SOD is an essential component of plants' antioxidative defence system as it dismutates two $O_2^{\cdot}$ to water and oxygen (Cakmak & Horst, 1991) and thus maintains superoxide radicals at steady state levels (Verma & Dubey, 2003). The upregulation (the process of increasing the response to a stimulus; specifically: increase in a cellular response to a molecular stimulus due to increase in the number of receptors on the cell surface) of SOD's is involved in preventing oxidative stress caused by biotic and abiotic stress and have a critical role in the survival of plants under stressed environments (Gill & Tuteja, 2010).

SOD activity has been reported to be stimulated under a range of stressful conditions including Cu, Al, Mn, Fe and Zn toxicity (Cakmak & Horst, 1991; Prasad *et al.,* 1999). An increase in SOD activity in transgenic plants (transgenic plants are plants that have been genetically engineered, a breeding approach that uses recombinant DNA techniques to create plants with new characteristics), are identified as a class of genetically modified organisms (GMOs), has been shown to give increased protection from oxidative damage (Slooten *et al*., 1995). A significant increase in SOD activity under salt stress was observed in various plants such as mulberry (*Morus* sp.) (Harinasut *et al.,* 2003), chick pea (*Cicer arietinum*) (Kukreja *et al.,* 2005) and *Lycopersicon esculentum* (tomato) (Gapińska & Sklodowska, 2008).

Studies with rice plants showed that an increase in Cd^{2+} levels in the growth medium, while SOD activity was stimulated under a variety of stressful conditions including Cu, Al, Mn, Fe and Zn toxicity (Cakmak & Horst, 1991; Prasad *et al.,* 1999). Demirevska-Kepova *et al.,* (2004) have proven that the decrease in SOD activity under high Cu stress was due to the decline of the MnSOD isoform expression and was linked to a whole cellular metabolism inhibition. Increased SOD activity in transgenic plants has been indicated to give greater protection against oxidative stress (Allen *et al.,* 1997). In the present study superoxide dismutase levels decreased significantly at weeks 1 and 2 and increased significantly at weeks 3 and 4 compared to the control. SOD decreased significantly at weeks 1, 2 and 3 compared to week 0 in treatment T¼. Significant decreases in SOD levels were observed at weeks 1 and 2 compared to week 0 in treatment T½. Treatment T1 caused significant decreases in SOD levels at weeks 1, 2 and 4 compared to week 0. Treatment T2 caused significant decreases in SOD levels at weeks 1, 2, 4 and 5 compared to week 0. Possible explanations of these results could be that the antioxidant enzyme activities may have decreased because of the (i) blocking of essential functional groups in biomolecules or (ii) displacement of essential metal ions from biomolecules by metals (Stroinski & Kozlowska, 1997; Schùtzendùbel & Polle, 2002).

In this study the reason for the decrease in SOD activity might be inactivation of the enzyme by H_2O_2 or binding of metal to the active centre of the enzyme. Increase in SOD activity is attributed to increase in superoxide radical concentration. This is as a result of *de novo* synthesis of enzyme protein (Verma & Dubey, 2003), attributed to superoxide-mediated signal transduction of genes of SOD (Fatima & Ahmad, 2004). Luna *et al.* (1994) observed an increase in SOD activity in Cu-treated *Avena sativa* plants. Srivastava *et al.* (2005) also reported an increase in SOD activity in arsenic-treated *Pteris vittata* plants, which was arsenic tolerant. It is hypothesized that overall activity of SOD enzymes is of more significance in metal stress studies for the maintenance of the overall defense system of plants subjected to oxidative damage (Slooten *et al.,* 1995). The data obtained from the present study can be used to demonstrate how C. demersum L. trigger antioxidant reactions upon exposure to a combination of metals (Al, Cu, Fe and Zn). Increased SOD activity appear to play a key role in the antioxidant defense response of *C. demersum* L. when exposed to a combination Al, Cu, Fe and Zn metal toxicity. These findings clearly show that enhanced antioxidant enzyme mechanisms in coontail to metal stress could help to overcome metal toxicity from ROS detoxification. Interestingly, *C. demersum* L., could serve as serve as an important plant species in phytoremediation of metal polluted rivers.

4.2.4.3. Total Glutathione (GSHt)

Some antioxidants like GSH may also play a role in inducing resistance to metals by protecting macromolecules against attacks by free radicals, formed during various metabolic reactions leading to oxidative stress (Alscher, 1998). Low molecular weight antioxidants AsA and GSH can directly reduce ROS and can serve as co-factors for reactions by ascorbate peroxidase (APX) and glutathione reductase (GR), respectively (Collen & Davison, 1999). GSH acts as a cellular reducing and protective agent against numerous toxic substances (Yin *et al.,* 2007). Total glutathione (GSHt) serve as a protective biological index to show contaminants exposure (Stein *et al.,* 1992) due to its role in resisting reactive oxygen toxicity (Yin *et al.,* 2007). The main obvious effect of certain pollutants is a decrease in thiol levels, i.e., the ratio of reduced to oxidized glutathione (GSH/GSSG), due to either direct radical scavenging or increased peroxidase activity.

The GSH/GSSG ratio could be a useful indicator of the precarious state of the cell (Yin *et al.,* 2007). In the present study, the level of GSHt displayed significant decreases in most of the treatments compared to the control during the exposure period as well as in the treatments per week. The control plants indicated significant decreases in GSHt levels at weeks 2 and 5

compared to week 0. Treatments T¼ and T½ displayed decreased trends in GSHt levels but the results are not significant compared to the week 0. Treatment T1 caused significant decreases in GSHt levels at weeks 2, 3 and 5 compared to the baseline. Significant decreases in GSHt levels were found at weeks 2 and 5 compared to week 0. The GSHt content declined in all treatments over the five week period. In this study the decrease in GSHt could play a contributing role in the oxidative stress status. The ability of plants to cope with oxidative stress depends on the balance between the antioxidant system and the amount of oxidative stress caused by the metal (Mishra *et al.,* 2006).

The decrease in GSHt levels was worsening the toxic effects of metals in *C. demersum* L over the exposure period. De Vos *et al.* (1992) indicated a 50-60% decrease in GSH content after exposure to Cu which correlated with the accumulation of phytochelatins (Grill *et al.,* 1989). The lower levels of such substances can be attributed to the varied level of protection offered by the antioxidant enzymes in protecting GSH from its oxidation (Devi *et al.,* 1998). Depletion of GSH and cellular thiols would increase the plants' susceptibility to free radical damage (De Vos, 1992). Induction of GSH has been reported in *C. demersum* L. exposed to Cu (Devi & Prasad, 1998). Pb induced decrease in GSH have also been reported in *Vicia faba* (broad bean), *Phaseolus vulgaris* (common bean) (Piechalak *et al.,* 2002) and in *Hydrilla* sp, (macrophyte) and *Vallisneria* sp. (macrophyte) (Gupta *et al.,* 1995, 1998).

According to Mishra *et al.* (2006) metal stressed plants maintain a high GSH/GSSG ratio besides induced GSH biosynthesis and rapid reduction of GSSG due to increased activity of GR (glutathione reductase). In a study by Mishra *et al.* (2006) the GSH/GSSG ratio increased up to 10 µM Pb till day 2, which was evidently due to GSH biosynthesis and rapid reduction of GSSG due to increased activity of GR. Once GSH is depleted by any metal, the GSH synthesizing systems begin to produce more GSH from cysteine via the γ-glutamyl cycle. Glutathione is usually not efficient if GSH depletion persists because of chronic metal exposure (Stohs & Bagchi, 1993; Quig, 1998; Hultberg *et al.,* 2001). Several enzymes in the antioxidant defence systems could protect this imbalance. Unfortunately, most of these enzymes become inactive because of direct binding of the metal to the active sites of the enzymes if the sites contain sulfhydryl groups (Quig, 1998). It has been shown that GSH is one of the most effective scavengers of ROS arising as by-products of cellular metabolism or during oxidative stress (Han *et al.,* 2008). Total glutathione (GSHt) serves as a prospective biological index to indicate contaminants exposure (Stein *et al.,* 1992). The most apparent direct effect of certain pollutants is a decrease in thiol status, i.e., the ratio of reduced to oxidized glutathione (GSH/GSSG), as a result of either direct radical scavenging or

increased peroxidase activity. In this study exposure to a combination of metals under different concentrations resulted in a significant decrease in GSHt levels.

4.2.4.4. Ascorbic Acid (AsA)

Ascorbate (AsA) is an essential component of a plant's antioxidant system (Smirnoff & Wheeler, 2000) and plays a protective role in plants against ROS that are produced from photosynthetic and respiratory processes (Guo *et al.,* 2005). AsA is associated with cell growth, and being involved in the cell cycle and other mechanisms of plant cell growth and division as well as acting as a co-factor for many enzymes (Lee & Kader, 2000). Many physiological processes including the regulation of growth, differentiation and metabolism in plants are affected by AsA (Melhorn *et al.,* 1996). Ascorbate is a metabolite and a water soluble antioxidant, which besides positively influencing various aspects in plants also act as a mysterious component of the plant defense system.

AsA is a significant constituent of the ascorbate-glutathione (AsA-GSH) pathway, as it performs multiple essential activities in plants including growth and development by either directly or indirectly breaking down ROS and its products (Anjum *et al.,* 2014). Ascorbic acid (vitamin C) is quantitatively the main antioxidant in plants and is present in subcellular compartments (Ischikawa *et al.,* 2008). Ascorbic acid has been reported to play a role in cell wall biosynthesis, redox signalling and plant response modulation under pathogen (Conklin & Barth, 2004), determination of flowering time (Barth *et al.,* 2006), regeneration of the reduced forms of GSH and $NADP^+$ (e.g., in the highly oxidizing environment of the photosynthesizing chloroplast) (Noctor & Foyer, 1998; Mano *et al.,* 2004; Foyer & Noctor, 2009). AsA also plays a role in the protection of the plasma membrane against oxidative damage (Wang *et al.,* 2010) and ozone (Frei *et al.,* 2012). Protection against enhanced ROS generation is achieved through stimulation of both enzymatic and molecular antioxidants.

AsA in plants stand second to tripeptide glutathione (GSH) in terms of its importance as a key antioxidant metabolite of the antioxidant defense system, redox buffer in plant cells, and also as a major player of key functions in plant growth, metabolism, development, and stress reponses (Noctor & Foyer, 1998; Smirnoff, 2000; Anjum *et al.,* 2014). Ascorbate is found in the cytosol, chloroplasts, vacuoles, mitochondria and cell wall. AsA represents 10% of the total soluble carbohydrate pool in favourable conditions (Noctor & Foyer, 1998; Smirnoff & Wheeler, 2000; Anjum *et al.,* 2014), while a very high level of AsA is present in the cytosol, much lower levels are present in the apoplast and thylakoid lumen (Foyer & Noctor, 2011).

The apoplast (aqueous solution that floods the cell wall) in plants has been shown to display AsA as the major redox buffer (Pignocci *et al.,* 2006), and apoplatic ascorbate has been reported to participate in several physiological phenomena including mitosis (cell division), cell elongation and cell defense (Horemans *et al.,* 2000; Anjun *et al.,* 2014). In the present study, in the control plants, significant decreases in AsA levels were found at weeks 1 and 5 and significant increases were found at week 2 and 4 compared to week 0. Significant decreases in AsA levels were caused by treatment T¼ at weeks 1, 2, 3 and 4 compared to week 0. Treatment T½ caused a significant increase in AsA levels at week 3 compared to week 0. Significant increases in AsA levels were caused by treatment T1 after weeks 1, 2, 4 and 5 and a significant decrease was caused after week 3 compared to week 0. Treatment T2 caused significant increases in AsA levels after weeks 1, 2, 4 and 5 compared to the baseline. Increased ascorbate activity may efficiently scavenge H_2O_2 to protect against oxidative damage.

Plants can show a variety of APX (ascorbate peroxidase) activity when exposed to a single metal or more than two metals at the same time (Aravind & Prasad, 2005; Khan *et al.,* 2007). *Triticum aestivum* (wheat) and *C. demersum* L. exhibited highly increased APX activity under combined Cd and Zn exposure when compared to Cd- or Zn- alone-treated plants, indicating their differential effect on the antioxidant system and ROS scavenging activities by Zn against Cd (Aravind & Prasad, 2005; Khan *et al.,* 2007). *Sesbania drummondii* (perennial shrub or rattlebush) seedlings treated with a combination of metals (Pb, Cu, Ni and Zn) exhibited higher APX activity as compared to those treated with a single metal. Variation of APX activity depends mostly on the different organs of the metal-exposed plant. In nickel-exposed *T. aestivum*, the root and shoot exhibited decreased and increased APX activity (Gajewska & Sklodowska, 2008). According to Yang *et al.,* (2008) high light condition and drought significantly increased the AsA content in *Picea asperata* (dragon spruce) seedlings. Contrarily, a decline in AsA in the roots and nodules of *Glycine max* (soybean) under Cd stress has also been observed (Balestrasse *et al.,* 2001).

SOD, ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) in general show simultaneous induction and decline, which may be due to their co-regulation (Shigeoka *et al.,* 2002). In this study, higher increases in activities of enzymes (SOD, CAT and ascorbate) suggests that there could have been a quick breakdown of superoxide radicals by SOD to keep levels in control at the place of generation and follow up action of ascorbate peroxidase (APX) and guaiacol peroxidase (GPX) along with CAT would have allowed *C. demersum* L. to resist oxidative stress efficiently at least up to average concentrations in the different treatments. Significant increases in the activity of APX, CAT and SOD in response to copper

stress has been reported in *C. demersum* L. by Devi and Prasad (1998). Several groups of workers have reported increased activities of antioxidant enzymes such as GPX, SOD, APX, MDHAR, DHAR and GR as well as nonenzymatic antioxidants in metal-treated plants and suggested involvement of an antioxidant defense system in the adaptive reponse to metal ions (Shah *et al.,* 2001; Maheshwari *et al.,* 2009; Sharma *et al.,* 2012). Concurrent changes in CAT, POD and SOD activities are responsible for the removal and destruction of ROS, and these antioxidant enzymes exhibit important influences on the oxidative damage of membranes in organisms under oxidative stress conditions (Ghnaya *et al.,* 2009; Sytar et al., 2013).

4.3. Conclusion

The current study was conducted to investigate the possible use of *C. demersum* L. biochemical responses as possible biomarkers for metal exposure monitoring. The results suggest the involvement of oxidative stress in the toxicity of mixtures of Al, Cu, Fe and Zn in combination, but also slightly different defense or adaptive strategies in response to the tested metals. According to the results of this study, different metal exposures disturbed the cellular redox status in *C. dem*ersum L. The cocktail of the four metals considered, induced significant changes in the antioxidant defense system, including the antioxidant enzyme activities. The main reason in the variation of activities of the detoxification enzymes (SOD and CAT) may be that they exist in different parts of the cell and having different threshold tolerance to the metals used in this study (Hou *et al.,* 2007).

SOD showed the highest enzymatic activity among the other enzymes, although there is no direct evidence for the role of this enzyme, but it can be explained to its important role in the tolerance mechanism to metal stress. The high SOD activity possibly implies that elevated SOD activity complements the other cellular protective mechanisms of the plant in scavenging free radicals produced due to Fe, Al, Cu and Zn-induced toxicity. Plants are inactive organisms that cannot move in order to find optimal conditions or avoid environmentally generated damage. Most plants possess photosynthetic systems that, when out of control, they may produce a large quantity of ROS. The possibility to regulate ROS influx into the cell and the regulation of cellular antioxidant potential seems critical for survival under continuous exposure to externally induced oxidative stress such as metals (Luschak, 2011).

During week 1 visible increases/decreases in TP, CDs, CAT, SOD concentrations were indicated under different treatments. Increases in phenolic content indicate antioxidant activity for these compounds under stress conditions. Increases in phenolics correspond to the increase in activity of enzymes involved in phenolic compound metabolism (Parry *et al.,* 1994). Decreases CD concentrations is indicative of plants growing in the presence of high metal concentrations (Sgherri *et al.,* 2003). The present study indicated that antioxidant responses can be used as an early warning tool to evaluate the effects of metal-induced stress in *C. demersum* L.

Knowledge of basic procedures related to ROS metabolism in plants and cellular responses to them, open up new possibilities in many fields. In view of the fact that ROS are involved in basic biological processes, such as reproduction, development, aging and many pathologies, new tools are required and developed for fast screening and deciphering of mechanisms of effects potentially useful to prevent and cure these states (Luschak, 2011). The parameters tested characterize different aspects of antioxidant responses to a combination of metals (Al, Cu, Fe, Zn) and are considered to be useful as potential biomarkers of metal pollution. The current study has demonstrated that this macrophyte shows tolerance to different metal-induced oxidative stress and can survive under high concentrations of these metals by adapting its antioxidant defence strategies. It is important to test the field application of biomarkers. In the case of this study the application of biomarkers need to be tested in the Diep River (Chapter 6).

CHAPTER 5: RESULTS AND DISCUSSION: EXPOSURE EXPERIMENT: Chlorophyll content in *Ceratophyllum demersum* L.

5.1. Results

5.1.1. Comparison of chlorophyll *a* (chl *a)* concentrations in *Ceratophyllum demersum* L. between weeks, per treatment

Comparisons of the mean chlorophyll *a* concentrations measured in *C. demersum L* between weeks, for each treatment, are shown in Table 5.1 and Figure 5.1.

Control (baseline): When compared to week 0, the chl *a* concentrations decreased significantly ($P<0.05$) during weeks 1, 2, 3, 4 and 5, the latter indicating an overall decrease in chl *a* concentrations from the start to the end of the experiment. Between consecutive weeks significant decreases were recorded in chl *a* concentrations between week 0 and week 1, between week 2 and week 3, between week 4 and week 5 ($P<0.05$).

Treatment T¼: When compared to week 0, the chl *a* concentrations decreased significantly ($P<0.05$) during weeks, 1, 2, 3, 4 and week 5, the latter indicating an overall decrease in chl *a* concentrations from the start to the end of the experiment. Between consecutive weeks significant decreases in chl *a* levels were recorded between week 0 and week 1, between week 1 and week 2 ($P<0.05$).

Treatment T½: When compared to week 0, the chl *a* concentrations decreased significantly ($P<0.05$) during weeks 1, 2, 3, 4 and 5, the latter indicating an overall decrease from the start to the end of the experiment. Between consecutive weeks a significant decrease in chl *a* concentration was found between week 0 and week 1 ($P<0.05$).

Treatment T1: When compared to week 0, the chl *a* concentrations decreased significantly ($P<0.05$) during weeks 1, 2, 3, 4 and 5, the latter indicating an overall decrease from the start to the end of the experiment. No significant differences in chl *a* concentrations were found between consecutive weeks ($P>0.05$).

Treatment T2: When compared to week 0, significant decreases in chl *a* concentrations were found during weeks, 1, 2, 3, 4 and 5 ($P<0.05$). Between consecutive weeks a significant decrease in chl *a* concentrations was found between week 0 and week 1 ($P<0.05$).

Table 5.1. Mean (±SD) chlorophyll *a* (chl *a*) concentrations (mg/L), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion. n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}2.030^{a}$ ±0.220	$^{a}1.731^{a}$ ±0.347	$^{a}1.737^{a}$ ±0.308	$^{a}1.578^{a}$ ±0.287	$^{a}2.16^{b}$ ±0.181
1	$^{*b}0.017^{a}$ ±0.002	$^{*b}0.016^{a}$ ±0.003	$^{*b}0.016^{a}$ ±0.004	$^{*b}0.012^{a}$ ±0.004	$^{*b}0.016^{a}$ ±0.002
2	$^{*b}0.019^{a}$ ±0.004	$^{*c}0.011^{b\#}$ ±0.002	$^{*b}0.012^{b\#}$ ±0.002	$^{*c}0.013^{b\#}$ ±0.002	$^{*b}0.011^{b\#}$ ±0.001
3	$^{*c}0.012^{a}$ ±0.002	$^{*c}0.012^{a}$ ±0.002	$^{*b}0.013^{a}$ ±0.001	$^{*d}0.01a^{a}$ ±0.002	$^{*b}0.011^{a}$ ±0.002
4	$^{*c}0.012^{a}$ ±0.002	$^{*c}0.011^{a}$ ±0.002	$^{*b}0.011^{a}$ ±0.002	$^{*e}0.012^{a}$ ±0.001	$^{*b}0.013^{a}$ ±0.005
5	$^{*d}0.009^{a}$ ±0.001	$^{*c}0.010^{a}$ ±0.002	$^{*b}0.011^{a}$ ±0.002	$^{*f}0.010^{a}$ ±0.002	$^{*b}0.012^{a}$ ±0.004
Pooled data for entire experimental period	**0.350^{a} ±0.769**	**0.298^{a} ±0.664**	**0.300^{a} ±0.664**	**0.273^{a} ±0.603**	**0.020^{a} ±0.005**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment, while significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week, while significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T0 = control (baseline); T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

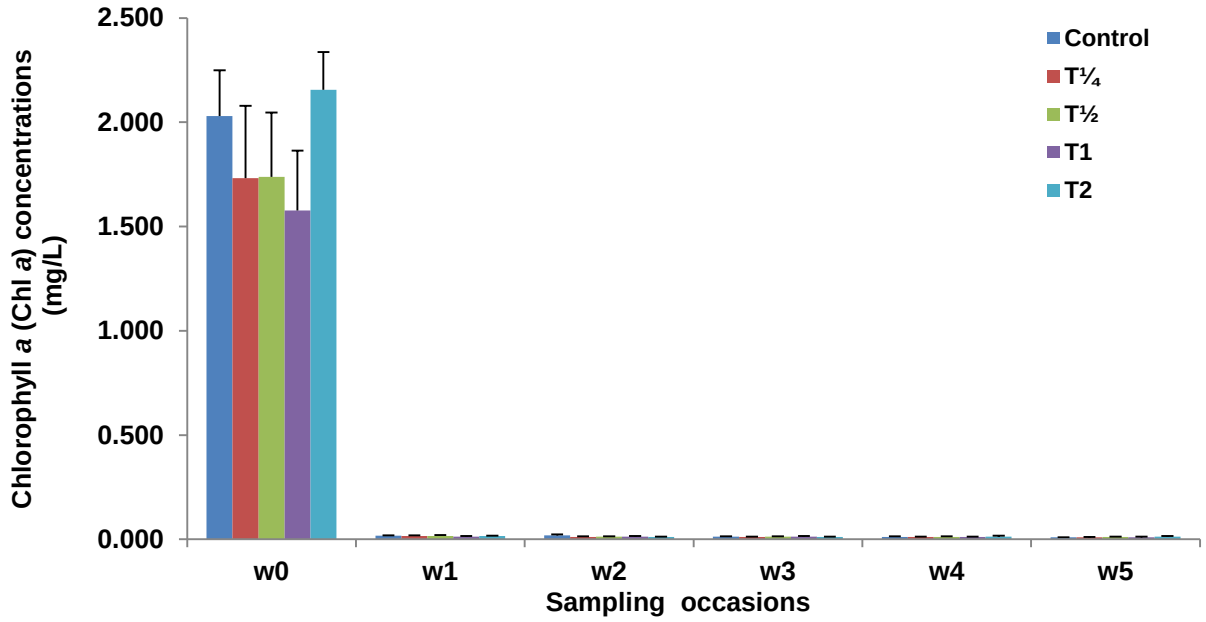

Figure 5.1. Mean (±SD) chlorophyll *a* concentrations (mg/L), measured in plants per week in experimental treatments. (*Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

5.1.2. Comparison of chlorophyll *a* (chl *a)* concentrations between treatments per week in *Ceratophyllum demersum* L.

Comparisons of the mean chlorophyll *a* concentrations in plant samples between treatments per week as well as comparisons of pooled data are illustrated in Table 5.1.

Week 0: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments the chl *a* concentration of treatment T2 were significantly higher compared to the chl *a* concentrations of treatment T1 (P<0.05).

Week 1: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant differences in chl *a* concentrations measured between treatments were detected (P>0.05).

Week 2: The chl *a* concentration measured in treatments T¼, T½, T1 and T2 were all significantly lower compared to the control (P<0.05). Between consecutive treatments the chl

a concentrations of treatment T¼ were significantly lower compared to the chl *a* concentrations of the control (P<0.05).

Week 3: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant differences in chl *a* concentrations measured between treatments were detected (P>0.05).

Week 4: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant differences in chl *a* concentrations measured between treatments were detected (P>0.05).

Week 5: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant differences in chl *a* concentrations measured between treatments were detected (P>0.05).

Pooled data: No significant differences (P>0.05) in chl *a* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant differences in chl *a* concentrations were found between any of the consecutive treatments (P>0.05).

5.1.3. Comparison of chlorophyll *b* (chl *b*) concentrations in *Ceratophyllum demersum* L. between weeks, per treatment

Comparisons of the mean (±SD) concentrations of chlorophyll *b* measured between weeks in *C. demersum L.* are illustrated in Table 5.2 and Figure 5.2.

Control: When compared to week 0, the chl *b* concentrations decreased significantly (P<0.05) during weeks 3 and 5 compared to the control, the latter indicated and overall significant decrease in chl *b* concentrations from the start to the end of the experiment. The chl *b* concentrations of weeks 1, 2 and 4 were the same as the concentrations of the control (P>0.05). Between consecutive weeks: significant decreases in chl *b* concentrations were found between week 2 and week 3 and between week 4 and week 5. A significant increase (P<0.05) in chl *b* level was also found between 3 and week 4.

Treatment T¼: When compared to week 0, chl *b* concentrations decreased significantly (P<0.05) during week 2 compared to the control. Between consecutive weeks a significant (P<0.05) decrease in chl *b* concentrations were found between week 1 and week 2.

Treatment T½: When compared to week 0, no significant differences (P>0.05) in chl *b* concentrations were found compared to the control. Between consecutive weeks no significant differences (P>0.05) in chl *b* concentrations were found between weeks.

Treatment T1: When compared to week 0, the chl *b* concentrations decreased significantly (P<0.05) during week 5, which indicates an overall significant decrease in chl *b* concentrations from the beginning to the end of the experiment. Between consecutive weeks: a significant decrease in chl *b* concentrations was found between week 4 and week 5.

Treatment T2: When compared to week 0, the chl *b* concentrations decreased significantly (P<0.05) during weeks 2, 3, 4 and 5, with the latter indicating an overall significant decrease in chl *b* concentrations from the start to the end of the experiment. Between consecutive weeks: a significant (P<0.05) decrease in chl *b* concentrations were found between week 1 and week 2.

Table 5.2. Mean (±SD) chlorophyll *b* (chl *b*) concentrations (mg/L), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion: n = 5 plants per treatment, per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}0.008^{a}$ ±0.001	$^{a}0.008^{a}$ ±0.002	$^{a}0.008^{a}$ ±0.001	$^{a}0.007^{a}$ ±0.001	$^{a}0.009^{a}$ ±0.001
1	$^{a}0.008^{a}$ ±0.001	$^{a}0.009^{a}$ ±0.001	$^{a}0.009^{a}$ ±0.002	$^{a}0.006^{a}$ ±0.001	$^{b}0.008^{a}$ ±0.001
2	$^{a}0.008^{a\#}$ ±0.002	$^{*b}0.005^{b\#}$ ±0.0001	$^{a}0.006^{b\#}$ ±0.001	$^{a}0.005^{b\#}$ ±0.001	$^{*b}0.005^{b\#}$ ±0.000
3	$^{b}0.005^{a}$ ±0.003	$^{a}0.007^{a}$ ±0.003	$^{a}0.006^{a}$ ±0.001	$^{a}0.007^{a}$ ±0.003	$^{*b}0.006^{a}$ ±0.003
4	$^{*c}0.008^{a}$ ±0.005	$^{a}0.006^{a}$ ±0.002	$^{a}0.007^{a}$ ±0.003	$^{a}0.007^{a}$ ±0.002	$^{*b}0.006^{a}$ ±0.002
5	$^{*d}0.003^{a}$ ±0.001	$^{a}0.006^{a}$ ±0.002	$^{a}0.006^{a}$ ±0.003	$^{*a}0.003^{a}$ ±0.001	$^{*b}0.004^{a}$ ±0.002
Pooled data for entire experimental period	**0.007^{a} ±0.003**	**0.007^{a} ±0.002**	**0.007^{a} ±0.002**	**0.006^{a} ±0.002**	**0.006^{a} ±0.002**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment, while significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week, while significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T0 = control (baseline); T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

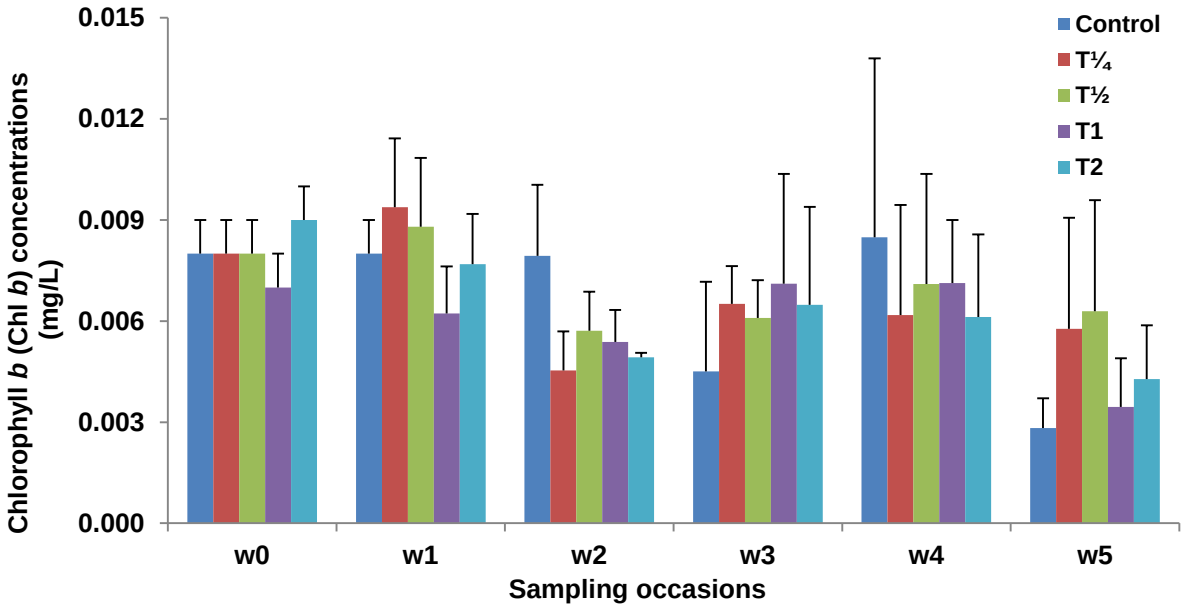

Figure 5.2. Mean (±SD) chlorophyll *b* concentrations (mg/L), measured per week in experimental treatments in *Ceratophyllum demersum L.* (*Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w 4 = week 4 of exposure; w 5 = week 5 of exposure).*

5.1.4. Comparison of chlorophyll *b* (chl *b)* concentrations between treatments per week in *Ceratophyllum demersum* L.

Comparisons of chlorophyll *b* concentrations in plant samples between treatments per week as well as comparisons of pooled data are illustrated in Table 5.2.

Week 0: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

Week 1: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

Week 2: The chl *b* concentrations in treatments T¼, T½, T1 and T2 were all significantly (P<0.05) lower compared to the chl *b* concentrations of the control. Between consecutive

treatments: the chl *b* concentrations of treatment T¼ were significantly (P<0.05) lower compared to the chl *b* concentrations of the control.

Week 3: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

Week 4: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

Week 5: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

Pooled data: No significant differences (P>0.05) in chl *b* concentrations were found between the control and any other treatments. Between consecutive treatments: no significant differences (P>0.05) in chl *b* concentrations were found between treatments.

5.1.5. Comparison of chlorophyll *t* (chl *t*) concentrations in *Ceratophyllum demersum* L. between weeks, per treatment

Comparisons of the mean (±SD) concentrations of total chlorophyll (chl *t*) measured between weeks in *C. demersum* L. are shown in Table 5.3 and Figure 5.3.

Control: When compared to week 0, the chl *t* concentrations decreased significantly (P<0.05) during weeks 3 and 5 compared to the control, the latter indicated and overall significant decrease in chl *t* concentrations from the start to the end of the experiment. The chl *t* concentrations of weeks 1, 2 and 4 recovered to the concentrations of the control (P>0.05). Between consecutive weeks a significant (P<0.05) decrease in chl *t* concentrations was found between week 4 and week 5.

Treatment T¼: When compared to week 0, no significant (P>0.05) differences in chl *t* concentrations were found between weeks and the control. Between consecutive weeks a significant (P>0.05) decrease in chl *t* levels was shown between week 1 and week 2.

Treatment T½: When compared to week 0, no significant differences (P>0.05) in chl *t* concentrations were found between weeks and the control. Between consecutive weeks a significant (P<0.05) decrease in chl *t* levels was shown between week 1 and week 2.

Treatment T1: When compared to week 0, the chl *t* concentrations decreased significantly ($P<0.05$) during week 5, which indicates an overall significant decrease in chl *t* concentrations form the start to the end of the experiment. Between consecutive weeks no significant differences ($P>0.05$) were found in chl *t* concentrations.

Treatment T2: When compared to week 0, the chl *t* concentrations decreased significantly during weeks 2, 3, 4 and 5 compared to the control, the latter indicating a significant decrease in chl *t* concentrations form the beginning to the end of the experiment ($P<0.05$). Between consecutive weeks a significant ($P<0.05$) decrease in chl *t* concentrations was found between week 1 and week 2.

Table 5.3. Mean (±SD) total chlorophyll (chl *t*) content concentrations (mg/L), measured in *Ceratophyllum demersum* L. from experimental treatments, per sampling occasion. n = 5 plants per sampling

Weeks	C	T¼	T½	T1	T2
0 (baseline)	$^{a}0.024^{a}$ ±0.002	$^{a}0.021^{a}$ ±0.004	$^{a}0.021^{a}$ ±0.004	$^{a}0.019^{a}$ ±0.003	$^{a}0.026^{b}$ ±0.002
1	$^{a}0.025^{a}$ ±0.003	$^{a}0.025^{a}$ ±0.004	$^{a}0.025^{a}$ ±0.006	$^{a}0.018^{a}$ ±0.005	$^{a}0.024^{a}$ ±0.003
2	$^{a}0.027^{a}$ ±0.006	$^{b}0.016^{b\#}$ ±0.003	$^{b}0.018^{b\#}$ ±0.001	$^{a}0.018^{b\#}$ ±0.003	$^{*b}0.016^{b\#}$ ±0.002
3	$^{a}0.016^{a}$ ±0.004	$^{b}0.018^{a}$ ±0.003	$^{b}0.019^{a}$ ±0.001	$^{a}0.020^{a}$ ±0.002	$^{*b}0.018^{a}$ ±0.002
4	$^{*a}0.020^{a}$ ±0.006	$^{b}0.017^{a}$ ±0.003	$^{b}0.018^{a}$ ±0.004	$^{a}0.019^{a}$ ±0.002	$^{*b}0.019^{a}$ ±0.004
5	$^{*b}0.012^{a}$ ±0.002	$^{b}0.015^{a}$ ±0.003	$^{b}0.017^{a}$ ±0.004	$^{*a}0.013^{a}$ ±0.004	$^{*b}0.017^{a}$ ±0.005
Pooled data for entire experimental period	**0.021^{a} ±0.006**	**0.019^{a} ±0.005**	**0.020^{a} ±0.004**	**0.018^{a} ±0.004**	**0.020^{a} ±0.005**

*Different letters on the left side of mean values indicate statistical significant differences between consecutive weeks per treatment, while significant differences between week 0 and other weeks are indicated by *. Different letters on the right side of mean values indicate statistical significant differences between consecutive treatments per week, while significant statistical differences between the control and consecutive treatments are indicated by # (Student-Newman-Keuls (SNK) post hoc test (p<0.05)). Abbreviations: T0 = control (baseline); T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). 0 = week 0 (start of experiment/baseline); 1 = week 1 of exposure; 2 = week 2 of exposure; 3 = week 3 of exposure; 4 = week 4 of exposure; 5 = week 5 of exposure.*

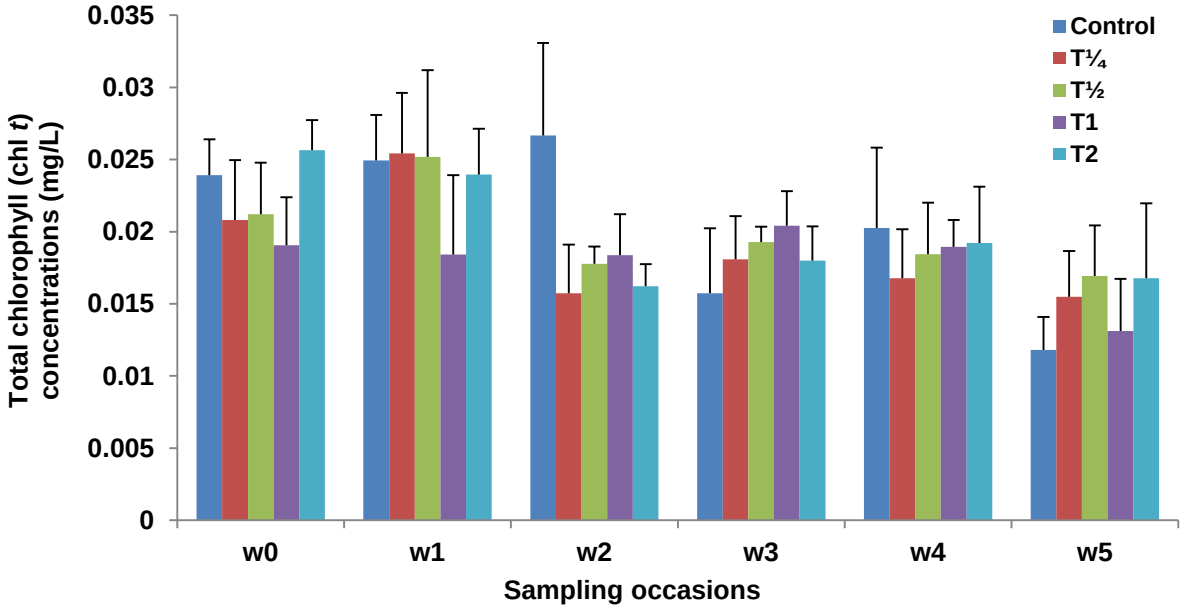

Figure 5.3. Mean (±SD) total chlorophyll concentrations (mg/L), measured per week in experimental treatments in *Ceratophyllum demersum L.* (*Abbreviations: T1 = treatment at environmentally relevant metal concentrations (mg/L); T¼ = quarter of T1 exposure concentrations (mg/L); T½ = half of T1 exposure concentrations (mg/L); T2 = double of T1 exposure concentrations (mg/L). w0 = week 0 (start of experiment/baseline); w1 = week 1 of exposure; w2 = week 2 of exposure; w3 = week 3 of exposure; w4 = week 4 of exposure; w5 = week 5 of exposure).*

5.1.6. Comparison of chlorophyll *t* (chl t*)* concentrations between treatments per week in *Ceratophyllum demersum* L.

Table 5.3 shows comparisons of chlorophyll *t* concentrations in plant samples between treatments per week as well as comparisons of pooled data.

Week 0: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments the chl *t* concentrations of treatment T2 were significantly (P<0.05) lower compared to the chl *t* concentrations of treatment T1.

Week 1: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant (P>0.05) differences in chl *t* concentrations were found between treatments.

Week 2: The chl *t* concentrations in treatments T¼, T½, T1 and T2 were all significantly (P<0.05) lower compared to the chl *t* concentrations of the control. Between consecutive treatments the chl *t* concentrations of treatment T¼ were significantly (P<0.05) lower compared to the chl *b* concentrations of the control.

Week 3: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant (P>0.05) differences in chl *t* concentrations were found between treatments.

Week 4: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant (P>0.05) differences in chl *t* concentrations were found between treatments.

Week 5: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant (P>0.05) differences in chl *t* concentrations were found between treatments.

Pooled data: No significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments. Between consecutive treatments no significant (P>0.05) differences in chl *t* concentrations were found between the control and any of the other treatments.

5.2. Discussion

High concentrations of metals in plants are known to affect photosynthesis and chlorophyll production negatively (Küpper *et al*., 1996; Küpper *et al*., 1998; Mukherjee *et al.,* 2004; Myśliwa-Kurdziel & Strzatka, 2002; Shakya *et al.,* 2008). Decreases in the concentrations of photosynthetic pigments, including chl *a* and chl *b*, on exposure to metals have been observed in several laboratory studies (Van Assche & Clijsters 1990; Krupa *et al.,* 1996; Wozny & Krzeslowska 1993; Kastori *et al*., 1998). Toxic metal concentrations have been reported to cause membrane damage, ion leakage, and decreased chlorophyll concentrations in vascular plants (Monni *et al.,* 2001; Patsikka *et al*., 2002) in bryophytes (Brown & Wells 1990; Guschina & Harwood 2002), and in lichens (Garty *et al*., 1992; Chettri *et al*., 1998, Tarhanen *et al*., 1999).

5.2.1. Chl *a*

Results showed that the concentrations of chlorophyll *a* were significantly lower in all the treatments compared to the control. In the control a significant decrease in chl *a* concentrations was found between the start and the end of the experiment. The highest mean chl *a* concentration in the control was found during week 0 (2.030 ±0.220 mg/L) and the lowest mean chl *a* concentration in the control was found during week 5 (0.009 ±0.001 mg/L). Significant decreases in chl *a* from the start to the end of the experiment were found in all treatments (T¼, T½, T1 and T2). The highest mean chl *a* concentration was found during week 0 in treatment T2 (2.16 ±0.181 mg/L) and the lowest mean chl *a* concentration (0.10 ±0.002 mg/L) were found in treatments T¼ during week 5 and treatment T1 during weeks 3 and 5 (Table 5.1). A possible explanation for the decrease in chl *a* concentrations might be the effect of higher temperature and pH (Koca *et al.,* 2007). Chl *a* decreased significantly in all treatments during week 1 and fluctuated between weeks in the control and all treatments during weeks 2, 3, 4 and 5. Shakya *et al*., (2008) have reported that chlorophyll *a* concentrations decreased significantly in *Thuidium sparsifolium* (moss) after accumulation of Cu+Zn+Pb ions in a mixed metal solution. This finding is in agreement with the findings of this study that chl *a* decreased significantly after accumulation of Al+Cu+Fe+Zn ions in a mixed metal solution. This could indicate a damaging effect of Cu on the chlorophyll contents of *C. demersum*. In a study by Temper *et al*., (2004) on the moss, *Rhytidiadelphus squarrosus*, a significant decrease in chl *a* concentrations was found after exposure to Cu. A possible explanation for the decrease in chl *a* in this study might be the effect of metal toxicity (Shakya *et al*., 2008). The activities of several photosynthetic enzymes and chlorophyll biosynthesis can be inhibited by metal ions. Metals can affect the photosynthetic electron transport processes and cause damage to the chloroplast membrane system (Aggarwal *et al.,* 2011).

5.2.2. Chl *b*

The concentrations of chlorophyll *b* decreased significantly in the control between week 0 and week 4 and between week 0 and week 5. Significant decreases in chl *b* between the start and the end of the experiment in treatments T1 and T2 were found. Chl *b* concentrations in all other treatments between weeks and the control remained at baseline level (Table 5.2). Significant differences ($P<0.05$) in chl *b* concentrations were found between all treatments and the control during week 2. A possible explanation for the reduction in Chl *b* could be associated with the alteration in composition of photosynthetic pigments that possesses lower level of light harvesting chlorophyll proteins (LHCPS) (Loggini *et al*., 1999;

Gill *et al*., 2012). A reduction in the level of LHCPS is an adaptation defence mechanism of leaves and plants, helping them survive under adverse conditions. Photosynthesis in higher plants is more sensitive to metal treatments, affecting biosynthesis of cholorphyll and accessory pigments (Mobin & Khan, 2007, Ahmad & Khan 2009, Iqbal *et al*. 2010, Gill *et al*. 2012). According to Piotrowska *et al*. (2009) it can be assumed that lead (Pb) may inhibit chlorophyll biosynthesis by impairing the uptake of essential photosynthetic pigment elements such as magnesium, potassium, calcium and iron. According to the results in this study the chl *b* concentrations decreased slower than the concentrations of chl *a* (Tables 5.1 and 5.2) under a different concentrations of a mixture of metals in combination (Al+Cu+Fe+Zn). In this study it was found that the effect of metals is greater on chl *a* than chl *b*. This is consistent with similar findings by Jayasri and Suthindhiran (2016) where chl *b* degradation was slower than that of chl *a* in in *Lemna minor* under different Zn and Cd concentrations.

5.2.3. Chl *t*

The total chlorophyll (chl *t*) concentrations decreased significantly between the start and the end of the experiment in the control and treatments T1 and T2 (Table 5.3). The significant decrease in chl t concentrations from the beginning to the end of the experiment in Table 5.3 could be as a result of increased oxidative stress caused by chlorophyll degradation. The chl *t* concentrations fluctuated between weeks in the different treatments and between weeks in the control. The highest mean chl *t* concentration (0.027 ±0.006 mg/L) was found in the control during week 2 and the lowest mean chl *t* concentration (0.012 ±0.002 mg/L) was found in the control during week 5 (Table 5.3). Significant differences in chl *t* concentrations were found between all treatments and the control during week 2. The pooled data indicated no significant differences in chl *t* concentrations between the control and all other treatments.
With the accumulation of a combination of metals (Al, Cu, Fe and Zn) in *C. demersum* L., in this study, negative effects on the photosynthetic pigments and decreased levels of chlorophyll content was observed. Similar reductions in the levels of photosynthetic pigments, chl- *a* and *b* after exposure to metals has been observed in many plant species (Mishra *et al*., 2007; Piotrowska *et al*., 2009; Singh *et al*., 2010). It has also been reported that alterations in photosynthetic activity and the absorption and distribution of essential nutrients lead to reduced plant growth. In this study the decreased rate of photosynthetic pigment concentrations in association with a combination of metals might be the consequence of peroxidation of chloroplast membranes due to increased levels of ROS

generation. This result is consistent with the enhanced level of H_2O_2 and peroxide production in water hyacinth plants treated with lead. The localization of Pb ions mainly observed in the root xylem suggests that it is the main pathway of Pb transport from root to shoot (Malar *et al.,* 2014). Similar observations were also reported by Sharma *et al*. (2004). Metal toxicity can have harmful effects on the content and functionality of the photosynthetic pigments (Broadley *et al*., 2007). This can be caused by direct oxidative damage to the pigments (Oláh *et al.,* 2010). Chlorophyll concentrations was reduced in *Triticum aestivum* L. (Gajewska *et al.,* 2006) exposed to nickel , and in *Phaseolus vulgaris* L. cv. *Anupama* exposed to cobalt (Co) (Chatterjee *et al.,* 2006).

Chlorophyll concentration is a unifying parameter that indicates the effect of specific interventions. It is essential though to record changes in the two components of chlorophyll (chl *a* and chl *b*) and particularly their ratio. This is due to the fact that metals could affect each component at a different level and cause changes in some part of the plants physiology and not in others (Manios *et al*., 2003). Li *et al*. (2012) have indicated increases in chl *t* including chl *a* and chl *b* in wheat seedlings exposed to increasing Fe concentration. In contrast to these results, 100 μM Cu led to significant increases in the concentrations of chl *b* and chl *t*, whereas wheat seedlings displayed notable decreases in chl *a* and chl *t* contents in response to the highest Cu concentration. These findings are consistent with the present results: chl *t* and chl *a* levels decreased significantly in *C. demersum* L. under a combination of Al, Cu, Fe and Zn, while most of the changes in chl *b* levels were not significant. According to Muradoglo *et al*. (2015) the chlorophyll content in strawberry plant organs decreased under Cd (cadmium) treatment. A reduction attributable to Cd application was found in chl *a* and chl *b* in the *Camarosa* (strawberry) cultivar. The chl *a* concentrations were noticeably higher compared to the ratio of chl *b* concentrations. There were 5, 15, 25, and 30% decrease in chlorophyll *a* and 3, 11, 15 and 18% decrease in chlorophyll *b* when Cd applications were increased from 0 to 60 mg kg^{-1} respectively. Results of the present study have also indicated higher chl *a* content compared to the chl *b* content. Yang *et al*. (2011) reported that leaves of *Potamogeton crispus* under Cd stress displayed decreased chl *a* (35.8%) and chl *b* (26.7%) levels. The decrease in chlorophyll content under metal treatment might have been caused by inhibition of chlorophyll biosynthesis. Such decreases in the concentrations of photosynthetic pigments, including chlorophyll *a* and *–b* on exposure to metals have been well documented (Van Ascche & Clijsters, 1990; Krupa *et al*., 1996; Wozny & Krzeslowska, 1998). The decreases in chl *t* can be regarded as general responses associated with metal toxicity (Rout *et al*., 2001; Rout & Das, 2003). If metals accumulated in the tissue of *C. demersum* L., the leaves or stems had crossed the tolerance level, then there

must have been some decrease of the total chlorophyll concentration (Gadallah, 1994; Sharma & Gaur, 1995). Decreases in chl *t* content have also been reported in several plants under metal stress by cadmium, copper, mercury, magnesium and nickel (Mocquot *et al*., 1996; Panda & Patra, 1998; Panda *et al*., 2003, Choudry & Panda, 2004). Chlorophyll pigments appear to be one of the main sites of metal injury in plants (Shakya *et al*., 2008). Species such as *Cyperus difformis* L. (sedge) (Ewais, 1997) and *Digitaria sanguinolis* L. (grass) and *Lemna polyrrhiza* (duckweed) (Sharma & Gaur, 1995), two algae species (*Chlorella fusca* and *Kirchneriella lunaris*) (Abdel-Basset et al., 1995) were used to evaluate the effect of metals in total chlorophyll concentration. All the investigators were in agreement that metal accumulation is responsible for the reduction of chl *t* concentration and also had a similar negative effect in the ratio of chl *a* and chl *b*. This result occurred due to a faster hydrolysis ratio of chl *a* compared with chl *b* when plants are under stress (Schoch & Brown, 1987; Drazkiewicz, 1994; Abdel-Basset *et al.,* 1995).

All plants have an optimal pH for their growth. For survival all plant cells must maintain a near neutral pH in the cytoplasm (Saygideger *et al*., 2004). In the control medium and treatments chl *a* and chl *t* displayed decreases with an increase in water pH, while chlorophyll *b* was not so much affected. The mean water pH varied between 6.9 and 8.4 during the experimental period (Table 3.1). A possible reason for a reduction in pigment accumulation in the present study could be that the pH of the water changed from neutral (6.9) to more alkaline (8.4).

Temperature is a main component among the factors that determine the rate of metabolic processes in plants (Berry & Björkman, 1980; Larcher, 1995; Madsen & Brix, 1997). Physiological responses to temperature differ between species as does the temperature dependence on growth. As a result, temperature has profound effects on species distribution within the temperature range at which plant life can thrive. Most freshwater submerged macrophytes appear to be eurythermic and are able to flourish within a wide range of temperatures (Madsen & Brix, 1997). In the present study mean temperatures in the control and treatments varied between 20.8 and 29 ^{0}C. A combination of parameters such as growth medium, temperature, pH, light and oxidative stress could thus have resulted in the decrease of chlorophyll concentrations.

Metal accumulation in the tissue of different plants causes a decrease of the biomass and chlorophyll content in the leaves or stems (Burzynski & Buczek, 1989; Ouzounidou *et al.,* 1992; Abdel-Basset *et al.,* 1995; Sharma & Gaur, 1995). Metals in plants apply their toxic

action mostly by damaging chloroplasts and disturbing the process of photosynthesis. The inhibition of photosynthesis is the result of interference of metal ions with photosynthetic enzymes and chloroplast membranes (Aggarwal *et al.,* 2012). It has been suggested that Al toxicity can lead to several biochemical and physiological changes in plants (Vitorello *et al*., 2005; Ali *et al*., 2011) such as cellular and ultrastructural modifications in leaves (Vitorello *et al.,* 2005), changes in chloroplasts' form and arrangement of the granum (Moustakas *et al*., 1997), reduction of stomatal openings and decreased photosynthetic activity (Vitorello *et al.,* 2005), damage of the outer membrane of chloroplasts (Hampp & Schnabi, 1975) and cell membrane lipid peroxidation (Yamamoto *et al.,* 2001). Several studies on chloroplast ultrastructure have reported deformation of the chloroplast ultrastructure under metal treatment (Choudry & Panda, 2005). In the case of Pb complete distortion of the chloroplast membrane was seen, while minor changes was observed under Cr, thereby reducing chl *t*, photosynthetic efficiency and productivity of *Taxithelium nepalense* (Schwaeger) (Bassi *et al.*, 1990; Moustakas *et al.,* 1994; Sandalio *et al.,* 2001).

In the present study the Al concentrations in the plants were much higher compared to the concentrations in the water (Tables 3.2 &3.5) and the chlorophyll concentrations decreased significantly in the plants (Tables 5.1-5.3). In this study the Al concentrations increased significantly under the high exposure treatments (T1 and T2) in the plants (Table 3.5). Chl *a* concentrations decreased significantly between weeks in all treatments and the control (Table 5.1). Chl *b* and – *t* concentrations showed significant decreases between weeks and the control after week 1 under high exposure (treatment T2) (Tables 5.2-5.3). These findings are consistent with Ohki (1986) and Ali *et al*. (2011) who reported reduced photosynthesis and chlorophyll content in wheat and sorghum with increasing Al concentration. In a study by Hoddinott and Richter (1987) it was found that direct injection of Al into xylem in beans caused a significant reduction in photosynthetic pigments.

In a study by Van Assche and Clijsters (1986) it was found that zinc (Zn) preferentially accumulates in the chloroplast where it can directly interact with the thylakoid membranes (Szalontai *et al*., 1999). In the present study Zn accumulated significantly in *C. demersum L.* in all treatments (Table 3.8). The Zn concentrations in the water were much lower compared to the Zn concentrations in the plants (Tables 3.4 & 3.8). The photosynthetic pigment concentrations decreased significantly over the experimental period. Chl *a* decreased significantly in all treatments between weeks and compared to the control (Table 5.1). Chl *b* and – *t* showed significant decreases in concentrations between weeks and the control during week 1 under treatment T2 (Tables 5.2 & 5.3). The results of this study are similar to

the findings of Shakya *et al*. (2008) who indicated that under high Zn concentrations chlorophyll production in plants are inhibited. Several studies have reported degradation of chlorophyll content under high Zn exposure (McGrath, 1982; Panda & Patra, 1998; Vajpayee *et al.* 2000; Panda *et al.*, 2003). Zn in combination with other metals such as copper (Cu) is known to replace the central magnesium ion in the chlorophyll molecule, mainly in aquatic plants. Replacement of magnesium affects the harvesting of light and causes an interruption in the photosynthesis process (Küpper *et al*., 1996, 1998). Reduction in chlorophyll content may be attributed to impaired uptake of essential elements such as Mn and Fe, damage of photosynthetic apparatus or due to chlorophyll degradation by increased chlorophyllase activity (Sharma & Dubey, 2005). The high Fe concentrations in the plants as reported in Chapter 3 might also have lessened the toxic effects of Zn in the plants, as indicated in a study by Fontes & Cox (1998), where high Fe concentrations prevented most of the toxic effects of excess Zn.

Copper is an essential element in chlorophyll production but in excess Cu inhibits chlorophyll production by changing cell membrane properties and affecting the enzymes that promote chlorophyll production (Shakya *et al*., 2008). In this study, the Cu concentrations in the plants were much higher compared to those concentrations of the water (Tables 3.3 & 3.6). According to Chettri *et al*. 1998 and Panda & Choudhury (2005) significant decreases in chlorophyll *a*, chlorophyll *b*, total chlorophyll, and the chlorophyll a to -b ratio with an increase in Cu accumulation reflects the inhibitory effect of copper on pigment biosynthesis, which may be a metal specific action. Copper can substitute for cofactors of various enzymes and degrade their activities (Nieboer & Richardson, 1980; Quartacci *et al.* 2001). The phospholipid structure can also be degraded and thereby change the membrane structure and function (Quartacci, *et al*. 2001). Furthermore, it can block the photosynthetic electron transport chain and thus degrade chlorophyll (Quartacci, *et al*., 2001; Patsikka *et al*., 2002). In a study by Monferrán *et al*. (2009), bioaccumulation of copper in the macrophyte, *Potamogeton pusillus*, resulted in significant changes in the plant's physiology. Symptoms of changes in the photosynthetic apparatus were shown in *P. pusillus* after exposure to Cu. These symptoms included decreases in chl *a* and chl *b*. These changes suggest the intensity and diversity of the conditions generated by Cu ions in cell metabolism (Monferrán *et al.,* 2009). The loss of photosynthetic pigments is a common response of plants to stress (heat, diseases and pollution) and has been observed after copper treatment in several aquatic plants such as *Eichornia crassipes* and *Hydrilla verticillata* (Lewis, 1993), *Chlorella pyrenoidosa* (Vavilin *et al*., 1995), and *Lemna* sp. (Filbin & Hough, 1979). These studies attributed the decline in chlorophyll to copper-induced modification of chlorophyll degradation as well as to structural and functional damage (Prasad *et al*., 2001).

An excess of Fe affects chemical processes within the plant cells that produce proteins crucial for plant metabolism. Iron is not a component of the chlorophyll molecule itself and its exact role in the chlorophyll synthesis has not been determined yet, however small quantities of Fe is required by the plant for chlorophyll production (Aggarwal *et al*., 2012). Essential redox enzymes involved in photosynthesis include the haem-containing cytochrome and non-haem iron-sulfur protein. Iron is reversibly reduced from Fe^{3+} to Fe^{2+} state during the course of electron transfer (Aggarwal *et al*., 2012). An excess of Fe could change the chlorophyll in such a way that the plant struggles to photosynthesise (Kampfenkel *et al*., 1995). In the present study, Fe accumulated in *C. demersum* L. significantly in the control and treatments (Table 3.7). The results of this study are in contrast with the findings of Nenova (2006), which indicated that Fe deficiency resulted in a decrease of photosynthetic pigments in pea plants, but excess Fe resulted in an increase of pigment concentrations. No definite evidence exist that any of the enzymes involved in the chlorophyll synthesis are Fe dependent, but the iron requirement could be related to a more general need for the synthesis of the chloroplast constituents, especially e-transport proteins (Aggarwal *et al*., 2012). According to Aggarwal *et al*. (2012) Fe deficiency leads to a simultaneous loss of chlorophyll and degeneration of chlorophyll structure. The destruction of photosynthetic pigments by metals could be a result of impairment of the electron transport chain, replacement of Mg^{2+} ions associated with the tetrapyrrole ring of chlorophyll molecules, inhibition of important enzymes (Van Assche & Clijsters, 1990) associated with chlorophyll biosynthesis or peroxidation processes in chloroplast membrane lipids by reactive oxygen species (ROS) (Sandalio *et al*., 2001). The results of this study can be explained by the study of Van Assche and Clijsters (1990) indicating that Fe could reduce chlorophyll content by inhibiting the pigment biosynthesis and decreasing the photosynthetic transport. Furthermore, chlorophyll loss can be related to membrane oxidative damage produced by oxidative stress (Aarti *et al*., 2006). Ahmed *et al.* (2002) have found that oxygen radicals play a major role in chlorophyll destruction and this cause waterlogging in mung bean leaves *Vigna radiate* (L.) Wilczek. It was also reported in a study by Liu *et al*. (2015) that chlorophyll significantly decreased in rice leaves under submergence stress. Lower chlorophyll concentrations would turn leaves yellow, resulting in a decrease in photosynthesis and photosynthetic products that affects the physiological metabolism of the plant (Zahed *et al*., 2009).

5.3. Conclusion

Results of this study indicate that chl *t* was affected under different metal concentrations during the five week exposure period. Significant decreases in chl *t* concentrations were found during the exposure period. There was some inhibition in plant growth (detected by the reduction in chl *a* to chl *b* ratio) in plants grown under different metal concentrations. Chl *a* seems to be one of the most essential centre pigments in photosynthesis and a decreased amount of chl *a* can reduce photosynthesis greatly (Jayasri & Suthindhiran, 2016). The mechanism of effect of metals on plant level of photosynthetic pigments may be due to three reasons. First, metals enter leaf chloroplast (Sandalio *et al.*, 2001) and may get over accumulated locally that could cause oxidative stress that will cause damage such as lipid peroxidation of chloroplast membranes (Puertas *et al.*, 2004). Metals can also directly destroy the structure and function of chloroplast by binding with –SH group of the enzyme and may also inhibit the overall chlorophyll biosynthesis by targeting Mg^{2+} and Fe^{2+} (Jayasri & Suthindhiran, 2016). Secondly, metal ions inhibit uptake and transportion of other metal elements such as Mn and Fe by antagonistic effects and therefore, the leaves lose their capacity to produce pigments (Das *et al.,* 1997). Thirdly, metals may activate pigment enzyme and accelerate the breakdown of pigment (Wenhua *et al.,* 2007).

Loss of chlorophyll *a, b* and *t* in *C. demersum* L. may have been caused by the cumulative effect of Al, Cu, Fe and Zn in combination. Chlorophyll pigments seem to be one of the main areas where metal injury occurs in plants (Muradoglu, 2015). Several environmental factors could affect plant growth and chlorophyll production and could have affected the chlorophyll results of this study. These factors include light intensity, pH of the water which influences metal bioavailability, metal interactions within the water, temperature and metal toxicity to the plants (Salisbury & Ross, 1985). More experimental studies are highly recommended because they might shed more light on the degradation of chlorophyll under toxic metal concentrations. The application of various concentrations and combinations of metals in a controlled environment for example laboratory studies, can determine a stronger link between cause and effect. The monitoring of total chlorophyll concentration and chl *a* to chl *b* ratio can be used as early warning systems for the toxic effect of metals accumulation in plants. In this study, the loss of chlorophyll *a, b* and *t* in the plants could be an indication that there was some growth inhibition.

CHAPTER 6: RESULTS AND DISCUSSION: Field study, Diep River, Milnerton, Western Cape

6.1. Results

6.1.1. Water chemistry

The metal concentrations (Al, Cu, Fe, Zn) in the water of the Diep River and the CPUT pond (reference site) were below detectable levels.

6.1.2. Comparison of the metal concentrations in *Ceratophyllum demersum* L. from the CPUT pond reference site and the Diep River

The Al, Cu, Fe and Zn concentrations in the plants from the Diep River were all significantly higher compared to the concentrations in the plants from the reference site (Table 6.1) ($P<0.05$).

Table 6.1. Mean (±SD) metal concentrations in *Ceratophyllum demersum* L. from the pond (reference site) and the Diep River. n = 5 plants per site

Metal	Pond (mg/kg)	Diep River (mg/kg)
Aluminium (Al)	764.279* ±35.678	358.32* ±369.831
Copper (Cu)	293.590* ±13.062	746.738* ±102.814
Iron (Fe)	912.277* ±65.912	2065.566* ±101.691
Zinc (Zn)	2708.650* ±249.689	8192.02* ±1363.36

*Significant differences between the metal concentrations in the plants of the pond (reference site) and the Diep River are indicated by *.*

6.1.3. Comparison of oxidative stress parameters of *Ceratophyllum demersum* L. growing in the pond (reference site) and the Diep River

Comparisons of antioxidative stress parameters measured in plants of the pond and Diep River are shown in table 6.3.

The TP, FRAP, ORAC and GSHt concentrations in the plants from the pond were significantly higher compared to the concentrations measured in the plants from the Diep River (Table 6.3) ($P<0.05$).

C. demersum L. plants from the Diep River showed significantly higher SOD, CAT, TBARS and AsA concentrations compared to the plants from the pond (Table 6.3) ($P<0.05$).

Table 6.2. Antioxidant stress status results measured for *Ceratophyllum demersum* L. from the CPUT pond (reference site) and the Diep River. n = 5 plants per site

Parameter	Pond	Diep River
Total Polyphenols (TP) (mg/g)	4.537* ±1.967	0.098* ±0.013
Ascorbic Acid (AsA) (µg/g)	1.431* ±0.000	23.954* ±2.892
Catalase (CAT) (mmole/µg)	5.117* ±0.534	14.076* ±2.073
Conjugated Dienes(CDs) (µmol/g)	1.270 ±0.155	2.187 ±0.235
Ferric Reducing Antioxidant Power (FRAP) (µmole/g)	12.919* ±1.985	4.402* ±0.655
Oxygen Radical Absorbance Capacity (ORAC) µmol TE/g	22.541* ±3.896	16.266* ±1.732
Superoxide Dismutase (SOD) (U/mg)	27.044* ±2.456	97.586* ±2.027
Thiobarbituric Reactive Substances (TBARS) (µmol/g)	41.898* ±2.891	228.315* ±12.265
Total Reduced Glutathione (GSHt) (µmol/g)	0.034* ±0.012	0.016* ±0.001

*Significant differences in concentrations of antioxidant parameters in the plants of the pond (reference site) and the Diep River are indicated by *.*

6.1.4 Comparison of chlorophyll concentrations in *Ceratophyllum demersum* L. growing in the pond (reference site) and the Diep River

Comparisons of the mean (± SD) concentrations of chlorophyll measured in plants of the pond (reference site) and Diep River are shown in Table 6.4.

No significant differences in chlorophyll concentrations were found between the plants from the pond (reference site) and Diep River (Table 6.4).

Table 6.3. Chlorophyll concentrations (mg/L) measured in *C. demersum* L. from the CPUT pond (reference site) and the Diep River. n = 5 plants per site

Parameter	**Pond**	**Diep River**
Chl *a*	0.010 (±0.003)	0.017 (±0.008)
Chl *b*	0.005 (±0.002)	0.007 (±0.005)
Chl *t*	0.015 (±0.004)	0.024 (±0.013)

6.2. Discussion

In the present study the metal concentrations (Al, Cu, Fe, Zn) in the water of the Diep River and the pond (reference site) were below detectable levels. In previous studies by Shuping *et al.* (2011) and Erasmus (2012) the water of the Diep River was tested and it was also found that the water contained low concentrations of metals. The sampling in the Diep River in the present study happened at the end of the local rainy season (September). This could have influenced the concentrations of the metals because of dilution of the river water due to high rainfall during the winter season.

The present study indicates that the plants of the Diep River had high metal concentrations (Table 6.1). These results are in agreement with previous results obtained from the Diep River by Shuping *et al.* (2011) and Erasmus (2012). The results of this study showed that *C. demersum* L. exhibited high accumulation capability for metals. In the present study the plants in the Diep River had 358.32 ±369.831 mg/kg Al, 746.74 ±102.814 mg/kg Cu, 2065.566 ±101.691 mg/kg Fe and 8192.02 ±1363.360 mg/kg Zn (Table 6.2). These

concentrations are significantly higher compared to the concentrations found in the plants of the pond (reference site). These results are an indication that metals in the Diep River are highly bioavailable, despite being present in the water in very low concentrations Shuping *et al.* (2011) and Erasmus (2012) found that the sediment of the Diep River is polluted with metals and contains high concentrations of metals. This could explain the high concentrations of metals in the plants in the present study. Metals are released by the sediment into the water and taken up by the plant through the process of cation exchange. Cation exchange capacity is an important factor for regulating metal bioavailability (Barbafieri *et al.,* 1996). The present study showed a high concentration of Al in the water of the pond (reference site). This might be a result of higher availability of Al in the sediment and the leaching of Al into the water of the pond.

Many submerged macrophyte species are able to accumulate high amounts of Cu and Zn (Kamal *et al*., 2004; Srivastava *et al*., 2006; Dhir *et al*., 2009; Monferrán *et al*., 2012). The results of this study are in agreement with those of previous studies. Peng *et al*. (2008) indicated maximum Cu and Zn concentrations of 1130 and 1320 mg/kg in *Potamogeton pectinatus*, while 945 and 1230 mg/kg were reported in *Potamogeton malaianus*. *Ceratophyllum demersum* L. was tested for accumulation of four metals, Al, Cu, Fe and Zn over a 5 week period (Chapter 3).

The sensitivity of plants to metals and the potential of plants to accumulate these metals depend on an interrelated network of physiological and molecular mechanisms such as: uptake and accumulation of metals through binding to extracellular exudates and the cell wall constituents (Chapter 4) (Cho *et al*., 2003). Plants produce a diversity of secondary metabolites and one of the main groups of these metabolites are phenolic compounds (Michalak, 2006). In the present study the concentrations of total polyphenols (TP) in the plants of the pond were significantly higher (4.537 ±1.967 mg/g) compared to the TP concentrations of the plants in the Diep River (0.098 ±0.013 mg/g). Higher TP concentrations could indicate induced accumulation of secondary metabolites in *C. demersum* in the pond (reference site) to tolerate the overall environmental stress conditions such as temperature, pH, salinity and high metal concentrations. The concentrations of metals (Al, Cu, Fe and Zn) in the plants of the pond (reference site) were significantly lower than in the plants from the Diep River (Table 6.2). The combination of different metals as well as concentrations of these metals might have played a role in the TP concentrations in the pond (reference site) and the Diep River. Increases in polyphenol concentrations might be due to the protective

function of these compounds against metal stress by metal chelation and ROS scavenging and may indicate antioxidant activity for these compounds under stress conditions (Brown *et al*., 1998; Lavid *et al.,* 2001; Rastgoo *et al.,* 2011). Higher concentrations of TP in the pond may be related to the modified tolerance mechanism adopted by plants for overall growth and development (Blokhina *et al.,* 2003) in the pond compared to the plants of the Diep River. The plants in the Diep River might be more adapted to the polluted environment compared to the plants in the less polluted pond. Total polyphenols can chelate transition metal ions, they can directly scavenge molecular species of active oxygen, and can inhibit lipid peroxidation by trapping the lipid alkoxyl radical. They also modify lipid packing order and decrease fluidity of the membranes (Arora *et al.,* 2000). These changes could strictly hinder diffusion of free radicals and restrict peroxidative reactions. Some evidence exists of the induction of phenolic metabolism in plants as a response to multiple stresses (Michalak, 2006). Janas *et al.,* (2009) observed that ROS could serve as a common signal for acclimation to Cu^{2+} stress and could cause accumulation of total phenolic compounds in dark-grown lentil roots. There might have been other unknown stress factors involved that could have influenced the results of this study. The plants of the pond (reference site) might have experienced temperature or chemical stress (evident in the Diep River) apart from the metal stress. These factors might have influenced the field results of this study.

Superoxide dismutase (SOD) is the most effective intracellular enzymatic antioxidant which is abundant in all aerobic organisms and in all subcellular compartments prone to ROS-mediated oxidative stress (Chapter 4) (Gill & Tuteja, 2010). SOD is considered as the first defence against ROS as it acts on superoxide radical (Alscher *et al.,* 2002; Gill & Tuteja, 2010). In the present study the SOD concentrations of the plants of the Diep River (97.586 ±2.027 U/mg) were significantly higher compared to the concentrations of the plants in the pond (27.044 ±2.456 u/mg). The high concentrations of SOD in the plants of the Diep River could be an indication of induced stress via metal toxicity. SOD activity has been reported to be stimulated under a range of stressful conditions including Cu, Al, Mn, Fe and Zn toxicity (Cakmak & Horst, 1991; Prasad *et al*., 1999). SOD's are involved in preventing oxidative stress caused by biotic and abiotic stress and have a critical role in the survival of plants under stressed environments (Gill & Tuteja, 2010). The plants of the pond indicated a lower SOD concentration which is consistent with lower metal concentrations (Table 6.2). The plants in the Diep River might be well adapted to the polluted environment and are capable of handling the oxidative stress compared to those plants in the pond.

Catalase (CAT) plays and important role in reducing oxidative stress by catalysing the oxidation of H_2O_2 (Chapter 4) (Weckx & Clijsters, 1996). In the present study the CAT concentrations in the plants of the pond were significantly lower (5.117 ±0.534 mmole/µg) compared to the CAT concentrations found in the plants of the Diep River (14.076 ±2.073 mmole/µg). High CAT activity can be explained by increases in its substrate i.e. to maintain the level of hydrogen peroxide as an adaptive method of the plants (Reddy *et al.*, 2005). The present study suggests that the high concentrations of metals in the plants caused ROS-induced stress situation and that the high CAT concentrations could be an adaptive method of the plants to lower the level of hydrogen peroxide. The lower CAT concentrations in the pond could indicate less ROS-induced stress due to metals (Table 6.2).

Ferric reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) assays are considered ideal methods to measure total antioxidant capacity (TAC) (Niki, 2010). In the present study the FRAP concentrations in the plants of the pond (12.919 ±1.985) were much higher compared to the concentrations in the plants in the Diep River (4.402 ±0.655) (Table 6.3). According to Cao and Prior (1998), the FRAP assay quantifies the ferric reducing ability of a sample. The significantly lower FRAP concentrations in the plants of the Diep River could be an indication of high concentrations of metals and high metal stress, while the FRAP concentrations of the pond indicate the opposite: lower metal concentrations and lower metal stress. The ORAC assay uses AAPH as a free radical and because of this it measures the capacity of an antioxidant to directly quench free radicals (Chapter 4) (Cao & Prior, 1998). The results of the present study indicate that the ORAC concentrations in the plants of the Diep River (16.266 ±1.732 µmol TE/g) were significantly lower compared to the ORAC concentrations of the pond (22.541 ±3.896 µmol TE/g). The Diep River showed lower ORAC concentrations compared to those of the pond. The lower ORAC concentrations in the polluted Diep River indicates lower antioxidant scavenging activity against the peroxyl radical compared to the higher activity in a less polluted pond (Michalak, 2006).

The ability of plants to cope with oxidative stress depends on the balance between the antioxidant system and the amount of oxidative stress caused by the metals (Mishra *et al*., 2006). In the present study the total reduced glutathione (GSHt) concentrations in the plants of the pond were significantly higher compared to the concentrations found in the plants of the Diep River (Table 6.3). The significantly lower concentrations of GSHt in the plants of the Diep River can be indicative of an increased oxidative stress status as a result of the higher

concentrations of metals in the plants and also the effects of the combination of metals in the plants. The opposite is true for the plants in the pond. GSHt serves as a protective biological index (thus lower oxidative stress status) to indicate contaminant exposure (Stein *et al.,* 1992).

The thiobarbituric reactive substances (TBARS) assay measures one of the terminal products in the peroxidation consequence of the breakdown of lipids, known as malondialdehyde (MDA) and this assay is one of the basic methods to determine lipid peroxidation (LP) in biological systems (Pannunzio & Storey, 1998; Sytar *et al.,* 2013). LP causes membrane damage (Halliwell & Gutteridge, 1993). In the present study the TBARS concentrations in the plants of the Diep River (228.315 ±12.265 µmol/g) are significantly higher compared to the TBARS concentrations found in the plants of the pond (41.898 ±2.891 µmol/g), a clear indication of enhanced lipid oxidative damage in Diep River plants. Changes in lipid peroxidation levels (LP) serve as an indicator of the extent of oxidative damage under stress (Halliwell & Gutteridge, 1993). MDA is a common product of LP and is a sensitive diagnostic indicator of oxidative injury in plants cells (Sun *et al.*, 2008). The higher TBARS and CD concentrations found in the plants of the Diep River are suggesting an increased oxidative stress status of those plants, when compared to the plants from the pond.

Ascorbate (AsA) plays a protective role in plants against reactive oxygen species (ROS) that are produced from photosynthetic and respiratory processes (Guo *et al.,* 2005). AsA is quantitatively the main antioxidant in plants and is present in subcellular compartments (Ischikawa *et al.,* 2008). In the present study the AsA concentrations in the plants of the Diep River (23.954 ±2.892 µg/g) were significantly higher compared to the AsA concentrations in the plants of the pond (1.431 ±0.000 µg/g) (Table 6.3). AsA plays a role in the protection of the plasma membranes against oxidative damage (Wang *et al*., 2010). A possible reason for the higher AsA concentrations in the plants of the Diep River could be the high metal concentrations and the effect of the combination of the metals on the plant. The river is much more polluted than the pond and AsA could play a role in the protection of the plasma membrane against oxidative damage (Wang *et al*., 2010). The results of this study gives an indication of how well adapted *C. demersum* is in the Diep River.

The high concentrations of metals found in the plants of the pond and the Diep River could have influenced chlorophyll production and photosynthesis. Previous studies have indicated that excessive metals (such as Zn, Cd, Ni, Al, Cu) in plant tissue negatively affected

chlorophyll synthesis and photosynthesis process (Gobold, 1984; Rai *et al.,* 1991; Hussain *et al.,* 1991; Vangronsveld & Clijsters, 1992; Kalavrouziotis *et al.,* 2007).

In an aquatic environment the metals and other chemicals present interact with each other and concentrations could change continuously and affect the metabolic processes within plants. An excess of iron could change the chlorophyll in such a way that the plant struggles to photosynthesise (Kampfenkel *et al*., 1995). Several studies have reported reduced chlorophyll content because of excessive metal concentrations, in particular zinc and copper, in various plant species, as well as for metals such as cadmium, lead, nickel and mercury. These metals caused the inhibition of the biosynthesis of photosynthetic pigments and resulted in a decrease in chlorophyll content (Myśliwa-Kurdziel & Strzatka, 2002). No significant differences in chlorophyll concentrations in *C. demersum* L. were found between the pond and the Diep River, despite the fact that the concentrations for Cu, Fe and Zn in reference site plants were significantly lower compared to those of the Diep River. *Ceratophyllum demersum* L. is an invasive alien species and appears to be well adapted to polluted environments. This plant might have developed an internal regulating mechanism to deal with metal toxicity and oxidative stress. This internal mechanism might be able to regulate biosynthesis of photosynthetic pigments in the plant (Prasad *et al.,* 2001).

6.2. Conclusion

The high concentrations of metals in *C. demersum* L. sampled from the Diep River compared to those concentrations in the plants from the pond may be attributable to the pollution in the river by effluents originating from industries, domestic activities and sewerage plants in the area and to high bioavailability of metals. The present study indicated that *C. demersum* L. accumulated more metals in the Diep River compared to the plants of the pond. Accumulation of metals in C*. demersum* L. might have induced stress and could have caused chlorophyll degradation in the plant. The concentrations of metals accumulated in *C. demersum* L. in the pond were lower compared to those of the Diep River. Plants in the pond and the Diep River might have developed an internal mechanism to regulate specific metals to the disadvantage of other functions such as chlorophyll synthesis (Dickinson *et al.,* 1991). There are no significant differences in the chlorophyll results of the pond (reference site) and the Diep River (Table 6.4). The chlorophyll concentrations of the pond, Diep River and the laboratory are low. According to Zengin (2005), Lamhamdi (2013) and Muradoglu (2015) chlorophyll concentrations decrease under high metal stress. The plants in the Diep River and laboratory experiment experienced high metal concentrations that might have caused

the decline in chlorophyll concentrations. The chlorophyll concentrations in the plants of the pond (reference site) might also have been affected by temperature and chemical stress of the water.

The significantly higher AsA, CAT, ORAC, SOD and TBARS concentrations in the Diep River plants might be an indication that the plants in the river might be well adapted to the constant exposure to metals and that the plants might have developed a tolerance mechanism to cope with oxidative stress. The plants in the pond (reference site) might not be as well adapted to the stressful conditions such as temperature, chemicals and high metal concentrations (such as Al) and therefore showed higher TP concentrations. There were no significant differences in the chlorophyll concentrations of the plants of the pond (reference site) compared to the plants of the Diep River (Table 6.4). These results correspond with the results of the laboratory study (Chapter 5). Further studies to monitor *C. demersum* L. over an extended period of time to observe chlorophyll degradation over time would be suggested.

The results of the field study (Diep River) is in agreement with those of the laboratory where *C. demersum* bioaccumulated significantly high concentrations of metals. These aquatic plants were capable of removing metals directly from the water. In the laboratory study the macrophyte proved to be highly effective in the uptake of the metals at all four exposure concentrations. The chlorophyll concentrations of both the laboratory and field studies were more or less the same under the high metal concentrations.

The results of both the field and laboratory studies suggest the involvement of oxidative stress in the toxicity of mixtures of Al, Cu, Fe and Zn in combination, but also slightly different defense or adaptive strategies in response to the tested metals. Metal exposures disturbed the cellular redox status in *C. demersum* L. Physiological and antioxidative responses to metal contamination may therefore be used as biomarkers in a biomonitoring and phytoremediation programme, especially in Western Cape rivers such as the Diep River. Further research is needed for this species.

CHAPTER 7: GENERAL CONCLUSIONS

In the present study *Ceratophyllum demersum* was tested for the bioaccumulation of metals (Al, Cu, Fe, Zn) over a 5 week period under laboratory conditions. Contrary to other experimental exposure studies on aquatic plants, found in the literature, the water was contaminated once off in the beginning of the experiments to simulate a pollution event. The 5 week exposure period was also longer than in most other laboratory studies in order to investigate metal bioaccumulation, plant-medium interaction and metal toxicity over time. This has not been done in exposure experiments for *C. demersum* before, to the author's knowledge.

This macrophyte proved highly effective in the accumulation of these metals at all four exposure concentrations. Metals were accumulated soon after exposure started. The results showed that concentrations of the metals in the water varied in all treatments over time with no specific patterns emerging amongst the treatment groups. The metal concentrations in the plants were much higher compared to the metal concentrations in the water. The metal bioaccumulation in *C. demersum* was variable between consecutive weeks per treatment and between consecutive treatments per week over a five week exposure period. There was no clear statistical pattern that revealed an increase in metal concentrations as exposure concentrations or exposure time increased. It therefore seems that metals were continuously exchanged between the plants and the water medium. This may be due to *C. demersum* being able to regulate Cu, Fe and Zn throughout the exposure period. The metals except Al used in this study are part of the normal metabolism of plants and can therefore be effectively regulated. The plant accumulated metals in the order: Fe>Zn>Al>Cu. It was useful to do a longer exposure study because the results of this showed that the exchange of metals between the plants and the water occurred continuously and that *C. demersum* we able to regulate Cu, Fe and Zn.

The cocktail of the four metals induced significant changes in the antioxidant defense system of *C. demersum,* including the antioxidant enzyme activities. The different metal exposures disturbed the cellular redox status in *C. demersum* The parameters tested characterize different aspects of antioxidant responses to a combination of metals (Al, Cu, Fe, Zn) and are

considered to be useful as potential biomarkers of metal exposure. The current study has demonstrated that this macrophyte shows tolerance to metal-induced oxidative stress and can survive under relatively high concentrations of these metals by adapting its antioxidant defence strategies. Although the metal concentrations in *C. demersum* were found to be rather variable, significant antioxidant responses were still found relative to week 0 and the control plants. It was useful to do a longer exposure study because significant antioxidant responses were found during the five week exposure period.

Chlorophyll contents were measured under different exposure concentrations of metals in the macrophyte. Results of this study indicated that chl *t* was affected under different metal concentrations during the five week exposure period. Significant decreases in chl *t* concentrations were found during the exposure period. The loss of chlorophyll *a, b* and *t* in *C. demersum* may have been caused by the cumulative effect of Al, Cu, Fe and Zn in combination under different treatments. However, as in the case of metal accumulation, the chlorophyll contents were variable over the exposure period.

A field study in the Diep River and a pond located on the CPUT campus was conducted to validate laboratory results and explore the field application of the selected physiological and biochemical responses as biomarkers in a biomonotoring programme. The high concentrations of metals in *C. demersum* L. sampled from the Diep River compared to those in the plants from the pond (reference site) may have been attributable to the pollution in the river by effluents originating from industries and various other sources in the area. Bioaccumulation of metals in *C. demersum* might have induced oxidative stress, and other environmental factors such as temperature- and chemical stress (chemicals found in the water) might have caused chlorophyll degradation. The amount of metals found in the plants in the pond and the Diep River might have developed an internal mechanism to regulate specific metals to the disadvantage of other functions such as chlorophyll synthesis (Dickinson *et al.,* 1991).

Antioxidant responses in *C. demersum* were determined from samples collected from the pond (reference site) and the Diep River. The results of this study showed significant antioxidant responses at the pond (reference site) and the Diep River. These antioxidant responses can be applied as biomarkers of metal exposure in *C. demersum* in the Diep River.

The significantly higher AsA, CAT, ORAC, SOD and TBARS concentrations in the Diep River plants might be an indication that the plants in the river might be well adapted to the constant exposure to metals and that the plants might have developed a tolerance mechanism to cope with oxidative stress. The plants in the pond (reference site) might not be as well adapted to the stressful conditions such as temperature, chemicals and high metal concentrations (such as Al) and therefore showed higher TP concentrations. Higher TP concentrations could indicate induced accumulation of secondary metabolites in *C. demersum* in the pond (reference site) to tolerate the overall environmental stress conditions such as temperature, pH, salinity and high metal concentrations.

The plants in the Diep River and the pond showed no statistical differences in chlorophyll concentrations. The results of this study indicate that the long exposure to high metal concentrations might have played a significant role in the low chlorophyll concentrations of both the pond (reference site) and the Diep River. This is also evident from this laboratory study where under different metal concentrations the chlorophyll concentrations were also low (Chapter 5). According to Zengin (2005), Lamhamdi (2013) and Muradoglu (2015) chlorophyll concentrations decrease under high metal stress. The plants in the Diep River and laboratory experiment experienced high metal concentrations that might have caused the decline in chlorophyll concentrations. The chlorophyll concentrations in the plants of the pond (reference site) might also have been affected by temperature and chemical stress of the water. Chlorophyll concentrations as a biomarker for metal concentrations in *C. demersum* can be recommended.

Finally, the present study has shown that under controlled laboratory conditions and the application of various concentrations of a cocktail of metals, *C. demersum* bioaccumulate metals to relatively high concentrations. The longer exposure period of *C. demersum* to the high metal concentrations have been very useful. It has shown us what happened to the metals in the plants over a longer period. The concentrations of the metals varied over time. The plants were able to regulate the metal concentrations inside the plant possibly through exchanging of metal ions between the plant and the water. In a short term exposure study this regulation of the metals by the plants might not have been recorded because of the exposure time. The present study serves as a basis for future studies to investigate the usefulness of *C. demersum* as a biomonitor plant in biomonitoring programmes for freshwater systems. The potential to use antioxidant responses and chlorophyll content as

biomarkers of metal exposure was demonstrated in this study. However, to assess their practical use and reliability in biomonitoring programmes, further investigation is required.

The results show that metals are bioaccumulated quickly by *C. demersum* after the water is contaminated with metals, i.e. after the "pollution event". However, over time, metals are continuously exchanged between the plants and the water, accounting for the fluctuations in metal concentrations observed over time. Therefore, the author is of the opinion that should this species be used as phytoremediator (e.g. in the Diep River), the plants need to be removed from the river shortly after (2-3 weeks) a known pollution event or after summer, when metal concentrations are more concentrated.

This study has shown that *C. demersum* has phytoremediation potential because it was able to remove high concentrations of metals from the contaminated water. Therefore, *C. demersum*, can be applied as a model for metal contamination and a phytoremediator after a pollution event.

CHAPTER 8: REFERENCES

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