The effect of elevated NH_4-N concentrations under gradient oxygen levels on growth, nutrient up take and some biochemical parameters in submerged macrophytes *(E. nuttallii* and *P. pectinatus)*

異なる酸素条件下で沈水植物（コカナダモ、エビモ）の生長、栄養塩吸収および いくつかの生化学的パラメータに対するアンモニウムイオン濃度の影響について

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Abstract

Submerged macrophytes are important functional and structural elements of aquatic ecosystems, and fulfill several important functions in these systems. They can be regarded as key species; changes in the macrophyte community can have major consequences for the aquatic ecosystems. Many different types of submerged aquatic macrophytes have been identified globally. Most submerged aquatic macrophytes belong to the families Ceratophyllaceae, Haloragaceae, Hydrocharitaceae, Nymphaeaceae and Potamogetonaceae. Submerged macrophytes are unique among rooted aquatic plants in linking the water column and the sediment through their physical structure and are capable of taking up nutrients from both water column and sediment. Decline of aquatic vegetation, especially submersed macrophytes, may be critical for the phase changes of shallow lake ecosystems from clear water state toward turbid state. Widespread reduction in the abundance of submerged macrophytes has been reported over the past several decades in many areas of the world. The decline can be related to water and sediment characteristics, reduction of water clarity by phytoplankton and suspended particles in excessive nutrient loading and eutrophication induced low light stress. Under natural conditions, aquatic plants frequently encounter combinations of stress factors like mechanical and resource stress. In such conditions, plant growth is altered in a complex interactive manner that cannot be simply predicted from the responses to each stress factor when considered independently. Consequently, the individual ability to tolerate multiple stresses through morphological adjustments is a major feature that determines species survival and colonization, and hence the ecological breadth of the species. The abiotic factors include organic substances, pH, temperature, alkalinity and hardness, inorganic ligands, interactions, sediments, redox status etc. It was reported that biomass growth of aquatic weeds decreased at high nutrient and organic contents of the sediment.

Lack of oxygen or anoxia is a common environmental challenge which submerged

plants have to face throughout their life. Submerged plants subjected to gradient redox conditions, such as they occur in flooded soil, eutrophic lakes, and waste water. Anoxic conditions result in a number of ecological processes that can degrade water quality. A primary concern related to anoxic conditions in lakes is the release of phosphorus from reduced sediments. Other water quality impacts related to hypolimnetic anoxia include hypolimnetic accumulation of various reduced species, including ammonia, iron, manganese, and sulfide. Sediment anoxia affects plants by regulating respiration and phytotoxin production as the result of anaerobic degradation of organic matter. In these aquatic environments, due to the cessation of ammonium nitrification, NH_4–N level increases. Increased ammonium concentration and low redox status (reduced condition) in the natural habitat (due to pollution or eutropication) are two prominent characteristics associated with eutropic lakes, such as Plesne Lake in Central Europe. Furthermore, under reduced environment different oxidize elements become available in the surrounding environment. Literature on the effect of reduce environment on aquatic macrophyte is very scanty. Various wetland plant response to flooded soil conditions have been reported in numerous publications in late 80's and 90's. Little is known about the relationship between soil oxidation-reduction and aquatic macrophyte functioning. Therefore, present attention has focused on the effects of submersed macrophytes on sediment redox, nutrient statues and the effects of sediment organic composition on macrophyte growth and biochemical activities.

The influence of sediment anoxia on the growth and production of submerged macrophytes in the freshwater environment is poorly understood, and information and quantitative data are scarce. So far, the few studies that have been conducted on freshwater macrophytes have been mostly based on the natural environment or field observation. It is often difficult to explain underlying mechanisms and to make reliable conclusions based on field observation data because many ecological processes occur concurrently. Thus, laboratory studies under controlled conditions are necessary to fully understand these phenomena.

Therefore, the aim of the present work was to investigate the effect of diversified sediment redox state on various physiological and biochemical parameters in relation to oxidative stress and to evaluate the tolerant capability in *P. pectinatus* and *Elodea nittallii* . Both the species of plants are known to be stress resistance. Their physiology, morphology and biochemical response are taken into account. This study will be beneficial for the selection of macrophyte species for habitat restoration. The enzymes, physiology, gene involved in such adaptation in different species of *Potamogeton* have been widely studied since the last three decades. The nutritional quality and quantity, as well as antioxidant response under oxygen deprived states not been evaluated.

Bioavailability and bioaccumulation of heavy metals in aquatic ecosystems is gaining tremendous significance globally. It has been suggested that pH and redox conditions are the factors that most affect the chemistry of metals in soils, and their uptake by organisms. The uptake of metals increases with increasing external metal concentration, but this is not a linear correlation. Aquatic macrophytes take up metals from the water, producing an internal concentration several fold greater than their surroundings. The metals are thereby made available to grazing moluscs and, thus, reintroduced into the food web via fish to birds and humans. These elements which are not biodegradable, in excess concentration might cause deleterious effect by disordering physiological and biochemical processes in the plant cells and might contributes to the food chain. Generation of free radicals and reactive oxygen species (ROS) is an established impact of stresses and their synthesis is stimulated in the presence of heavy metals in plants. Therefore, it is imperative to estimate the effect of soil properties on the availability and the uptake of heavy metals by plants to minimize the toxic effects and the translocation to food chains.

Exposure to stress can lead to the disruption of cellular and molecular processes. Oxidative damage in particular is associated with many types of stress. Under environmental

stress, changes in free radical processes are expected to occur and these are in turn to affect the radical scavenging ability of a plant. Anoxic stress leads to hydrogen peroxide formation in plant cells. Direct and indirect evidence has accumulated on the involvement of ROS (Reactive Oxygen Species) in the anoxic stress response and excessive generation of ROS is the first sign of oxidative stress. Formation of ROS occurs through several reducing steps, yielding first the superoxide anion, then hydrogen peroxide and the hydroxyl radical and finally water. Among the chemical species only hydrogen peroxide is relatively stable and able to penetrate the plasma membrane as an uncharged molecule, thus it could act as a second messenger under stress conditions. H_2O_2 is starting to be accepted as a second messenger for signals generated by means of ROS because of its relatively long life and high permeability across membranes. When ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and significant oxidative damage to a plant. These cytotoxic ROS can strongly disrupt normal metabolism through oxidative damage of chlorophyll, lipids, protein, and nucleic acids. To maintain its functional and structural integrity, a plant organism has to be resistant towards unfavourable factors. If a stress factor surpasses this range the plant has to trigger additional energy and physiological–biochemical mechanisms to survive under unfavourable conditions. To protect them against oxidative stress, plant cells produce both antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) etc. The regulation of carbohydrate and energy metabolism seems to be important for the adaptation to oxygen shortage. Heavy metals suppress the functioning of essential biological components of plants as they tend to bind with sulphydryl groups of enzymes. Thus the balance between producing and removing of free oxygen radicals is damaged in both experimental plants (*Elodea nuttallii* and *Potamogeton pectinatus*). When the anoxic condition exceeded the tolerance level of the plants under present experimental condition, plants showed reductions in growth, retarded metabolic and physiological processes as supported by the previous study. *E. nuttallii* under reduced

conditions even treated with suitable concentration of NH_4-N (2.5 ppm) showed retarded growth, decreased levels of photosynthetic pigments. Both *E. nuttallii* and *P. pectinatus* effected nutrient and ion concentrations of surface water and sediment pore water.

Hypoxia along with elevated concentration of NH_4-N act as the important factor in distribution and abundance of these species and submerged macrophyte *E. nuttallii* and *P. crispus* are poorly tolerant of anoxia in terms of cell detoxification response. Oxygen deprived reduced conditions and with elevated NH_4-N concentration retarded growth, significantly affected the photosynthetic apparatus as well as C-N balance in plants. H_2O_2 was found to promote senescence based on chlorophyll, protein degradation, decreased IAA, MDA and proline content, a decrease in membrane stability, which were partially regulated in the presence of free radical scavengers. Some essential macro and micro elements in plants were found below critical limit for plant survival. *P. crispus* were found to be more tolerant than *E. nuttallii* under such adverse conditions. The combined effect of elevated ammonium concentration under various redox levels, on *Elodea nuttallii* were studied for the implication of a suitable phytoremediation technologies. Heavy metal toxicity along with oxygen deprived conditions were manifested in a reduction of biomass, photosynthetic pigments and biochemical disorders such as excess generation of ROS, lipid peroxidation and reduction of major macro elements. The BCF sequence for micro and non-essential elements was Cu>Mn>Zn>Al>Cd>Fe>Pb in both conditions under reduced treatments. The combination of low redox state and high ammonium concentration has stronger physiological impact on submerged macrophytes than the two factors acting alone. Of the two factors, low redox status had greater effects on macro-micro nutrient balance than did the high concentration on ammonium. Based on the present results it can be suggested that *E. nuttallii* can be a useful tool not for all phytoremediation technologies but for phytoextraction.

Long-term ecological records of the decline of submersed macrophytes in the progress of

eutrophication have suggested that sediment may play an important role for the decline due to its close relations to macrophyte growth and distribution. In eutrophic water, only the canopy growth form and fertile resistant species are able to sustain. Therefore, process and mechanism studies on the decline of the vegetation will provide scientific basis for the management of the shallow lake ecosystems of this area.

Publication List

● International peer reviewed journals

1. Effects of NH_4–N concentrations and gradient redox level on growth and allied biochemical parameters of *Elodea nuttallii* (Planch.)

   Tanjeena Zaman and Takashi Asaeda

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   Tanjeena Zaman and Takashi Asaeda

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3. Effect of different stages of trial impoundment on organic matter flux and downstream macroinvertebrate community during construction of a large dam in the mountainous area

   Takashi Asaeda**, Tanjeena Zaman** and Yoichi Takahashi

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● Book Chapters

1. *Book Title*: Biochemistry of Plant Metal Interactions

   Book Chapter: Heavy metal uptake and tolerance of charophytes (Chapter 6)

Takashi Asaeda and **Tanjeena Zaman**

Springer Publications

2. *Book Title*: Peroxidases: Biochemical Characteristics, Functions and Potential Applications

   Book Chapter: Biochemical responses of aquatic macrophytes to two abiotic stresses

Takashi Asaeda and **Tanjeena Zaman**

Nova Science Publishers, Inc. New York

● **Conference proceedings**

The Effect of Sediment Redox conditions on Growth and Hormonal activity of *E. nuttallii*

ELR2012, Tokyo University of Agriculture

● **Articles under review**

1. Assessment of macro and microelements accumulation capabilities of *Elodea nuttallii* under oxygen stress and elevated NH_4-N concentration

   Tanjeena Zaman and Takashi Asaeda

2. Physiological responses of *Potamogeton pectinatus* and *Elodea nuttallii* to various sediment redox states

   Tanjeena Zaman and Takashi Asaeda

3. The effects of flow turbulence on growth, morphology and some biochemical parameters in three submerged aquatic macrophytes: *Myriophyllum spicatum* (L), *Nymphoides peltata* (Kuntze) and *Trapa japonica* (Flerow)

   Takashi Asaeda* **Tanjeena Zaman** & Takahito Igura,

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Chapter 1. **GENERAL INTRODUCTION & LITERATURE REVIEW**

The intensity of soil reduction can be rapidly characterized by the oxidation–reduction potential (Eh), which is a measure for the electron availability and allows the prediction of the stability and availability of various metals in floodplain soils and sediments. The dynamics of the hydrological and redox regime have been numerously reported (Du Laing et al., 2009, Rinklebe, 2007). The factors governing contaminant release in the field are also effective when studying the release by laboratory methods (where artificial factors are additionally operative). Du Laing et al. (2009) reported that the effects were less pronounced in the field. In addition, the occurrence of pH/Eh changes, microorganisms activity and organic matter play an important role in driving the biogeochemical processes (María-Cervantes et al., 2010). Anoxic conditions result in a number of ecological processes that can degrade water quality. A primary concern related to anoxic conditions in lakes is the release of phosphorus from reduced sediments (Boström et al., 1988).

Aquatic macrophyte

Submersed macrophytes are important functional and structural elements of aquatic ecosystems, and fulfill several important functions in these systems (Scheffer, 1998). They can be regarded as key species; changes in the macrophyte community can have major consequences for the aquatic ecosystems. It plays an important role in water purification and the restoration of degraded shallow lakes (Lauridsen et al., 1994). Aquatic macrophytes are taxonomically closely related to terrestrial plants, but are aquatic phanerogams, which live in a

completely different environment. Their characteristics to accumulate metals make them an interesting research objects for testing and modeling ecological theories on evolution and plant succession, as well as on nutrient and metal cycling (Förstner and Wittmann, 1979). Therefore, it is very important to understand the functions of macrophytes in aquatic ecosystem. Totally submerged plants are the true water plants or hydrophytes. Because they are truly aquatic they have the greatest number of adaptations to life in water. Submerged plants lack the external protective tissues required by land plants to limit water loss. The epidermal (outermost) layer shows very little, if any, sign of cuticle formation. All the surface cells appear to be able to absorb water, nutrients and dissolved gases directly from the surrounding water. As a result, the internal system of tubes (xylem) which normally transports water from the roots to all parts of the plant is often greatly reduced, if not absent. Thus, if these plants are removed from the water, they wilt very quickly, even if the cut stems are placed in water as because the normal water transport system is poorly developed. As might be expected, there are also no stomata (breathing pores) on the leaves. Submerged vascular macrophytes influence redox conditions in the sediment by releasing oxygen through their roots.

Stress and Response

Under natural conditions, plants frequently encounter combinations of stress factors like mechanical and resource stress (Bazzaz, 1996, Sultan et al., 1998). Consequently, the individual ability to tolerate multiple stresses through morphological adjustments is a major feature that determines species survival and colonization, and hence the ecological breadth of the species (Bazzaz, 1996, Sultan et al., 1998). Studies on plant responses to multiple stresses often deal only with stresses linked to limitations in several resources (nutrients, inorganic carbon or water availability, light quality or quantity; e.g. (Sack, 2004), and focus on the optimization of resource acquisition and allocation (Fig.1.1).

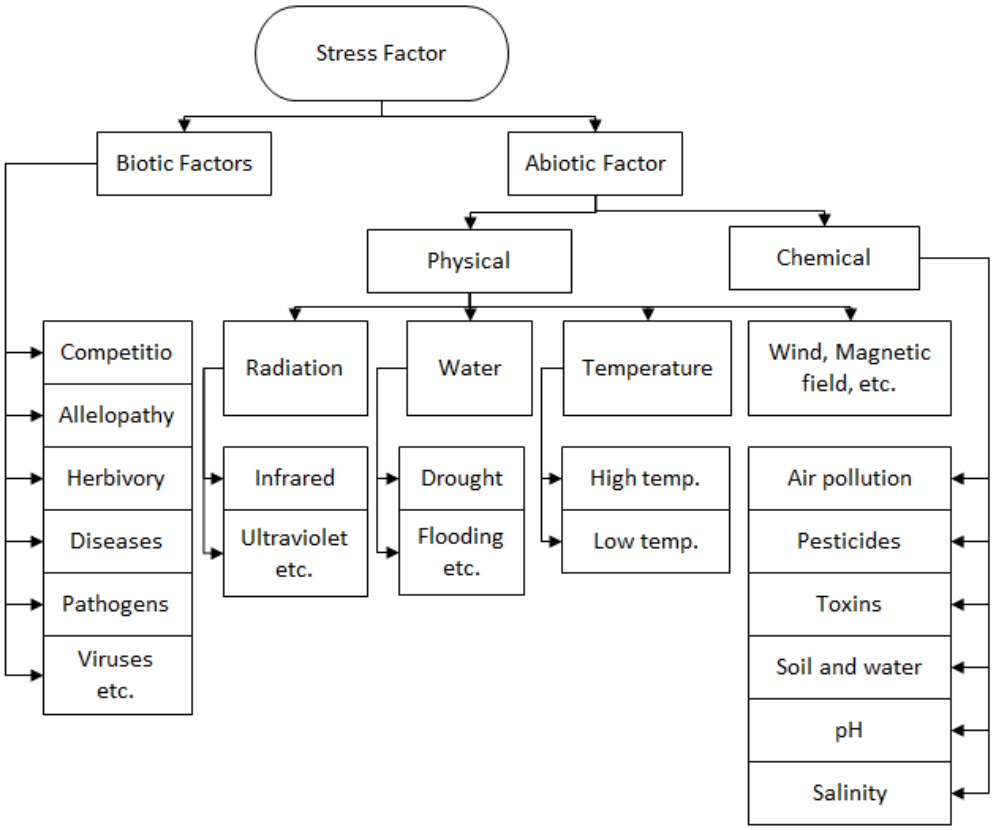

Figure 1.1 The sources of environmental stress in plants

Exposure to stress can lead to the disruption of cellular and molecular processes. Oxidative damage in particular is associated with many types of stress (Blokhina et al., 2003). However, stress can also boost the stress tolerance of the plant through induction of acclimation responses. Tolerance can be linked to an array of morphological, physiological and biochemical responses that decrease stress exposure limit damage or facilitate repair of damaged systems. Some responses protect against specific stresses only, whereas the antioxidant defenses prevent damage by Reactive Oxygen Species (ROS) in general, and offer a degree of cross-protection against mechanistically distinct stresses (Blokhina et al., 2003). Stress-induced morphogenic responses are observed in plants exposed to a variety of distinct abiotic stresses. SIMRs (Stress induced morphogenic responses) comprise three components: (a) inhibition of cell elongation, (b) localized stimulation of cell division and (c)

alterations in cell differentiation status (Potters et al., 2009). Plants use morphogenic responses to decrease stress exposure. The similarities between morphogenic responses induced by distinct stresses reflect common molecular effects, such as increased ROS-production and altered auxin metabolism. Environmental disturbances may alter baseline ecosystem conditions, modify the gradients and facilitate the establishment of invasive/exogenous plant species which, otherwise would not grow in a non disturbed scenario (Tiner, 2004).

Sediment Anoxia

Lack of oxygen or anoxia is a common environmental challenge which plants have to face throughout their life. Low O_2 concentration can be a normal attribute of a plants natural environment. Under natural conditions anoxic stress includes several transition states (hypoxia, anoxia and re-oxygenation) characterized by different O_2 concentrations.

Table 1.1 Range of redox potentials required to reduce oxidized forms of the various redox couples in soil and wetland environments

Redox Couple in Wetland Soils (*Ox*→ *Red*)	Range of Measured Redox Potential (mV)
$O_2 \rightarrow H_2O$	+400 to +350
$NO_3^- \rightarrow N_2$	+250 to +200
$Mn_4^+ \rightarrow Mn_2^+$	+200 to +150
$Fe^{3+} \rightarrow Fe^{2+}$	-25 to -75
$SO_4^{2-} \rightarrow S^{2-}$	-125 to -175
$CO_2 \rightarrow CH_4$	-200 to -250

Source: Patrick et al. (1996)

Anoxic conditions result in a number of ecological processes that can degrade water quality. A primary concern related to anoxic conditions in lakes is the release of phosphorus from reduced sediments (Boström et al., 1988). Other water quality impacts related to hypolimnetic anoxia include hypolimnetic accumulation of various reduced species, including ammonia, iron, manganese, and sulfide (table 1.1). These compounds may exacerbate eutrophication,

complicate potable water treatment, and/or exhibit toxicity to aquatic biota. Previous research has demonstrated that sulfide is a dominant ligand for a number of metals in anoxic environments and can potentially control metal speciation and toxicity under low oxygen environments as well (Peter M Chapman et al., 1998). When present under anoxic conditions, dissolved sulfide readily reacts with reduced iron (Fe^{2+}) to form various iron mineral phases (Fig.1.2).

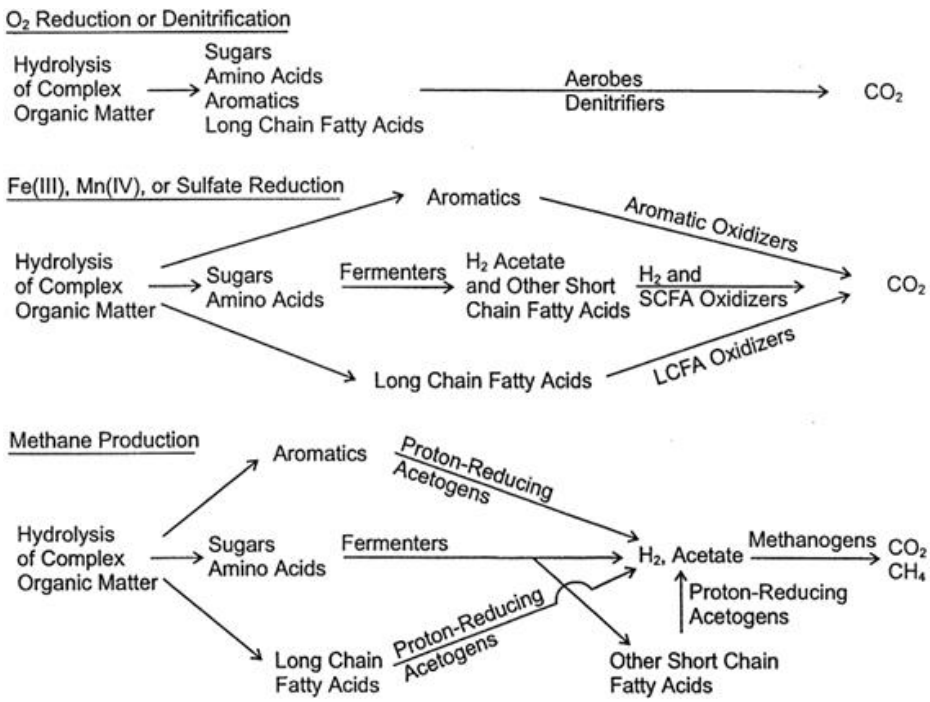

Figure 1.2 Pathways of microbial decomposition of organic matter in various redox environments [from (Lovely and Chapelle, 1995)]

As a general rule, hydromorphy in a well buffered contaminated soil at a first step should increase the mobility of divalent trace metals, by decreasing pH and reducing Mn and Fe oxides, but prolonged flooding can lead to fix trace metals again, rather by re-adsorption or precipitation phenomena than by formation of insoluble sulphides. Zinc may have a strong environmental impact; due particularly to its phytotoxicity above a certain threshold, and it is

generally associated with cadmium, whose critical concentration is much lower. However, concentration is not the only factor to be considered. The speciation of the elements (their physicochemical forms and associations with soil constituents) also affects their toxicity and their mobility (Kabata-Pendias and Pendias, 1992). Mobility of most trace metals is low in well-buffered soils.

It has been suggested that pH and redox conditions are the factors that most affect the chemistry of metals in soils, and their uptake by organisms (Förstner, 1991). There has been considerable research on the effect of soil pH on the mobility or the availability of trace metals, and less on the effects of reducing conditions on their behaviour in soils. However, alternate aerobic and anaerobic conditions lead to changes in both pH and redox potential (Ponnamperuma, 1972), and affect most of the processes regulating the speciation of any metal in soils (Calmano W, 1993):

- sorption/desorption onto the different solid components;
- adsorption/coprecipitation onto hydrous oxides of Fe and Mn;
- formation/decomposition of soluble and insoluble metal organic complex compounds;
- dissolution of carbonates, metal oxides or hydroxides;
- precipitation as insoluble sulphides under highly reducing conditions and their dissolution as sulphates under oxic conditions.

The other important process often called to mind is adsorption-desorption of trace metals on Fe and Mn oxides (Förstner, 1991) which are affected by reducing conditions. Several researcher (Reddy and Patrick, 1977) evoked this process to explain that solubility of Co, Pb, and Zn respectively, increased in soils at low Eh; on the contrary, lu et al. (1981) and Ghanem

and Mikkelsen (1987) found that retention of Cu and Zn increased under reducing conditions and attributed this to transformations of Fe and Mn oxides. A.J. and C.J. (1990) reported that Cd, Cr, Ni, Pb, and Zn co-precipitated with synthetic goethite can be solubilized under anoxic conditions by microbial action.

Plant nutrients

Sixteen chemical elements are known to be important to a plant's growth and survival. The sixteen chemical elements are divided into two main groups: non-mineral and mineral. The 13 mineral nutrients, which come from the soil, are dissolved in water and absorbed through a plant's roots. The mineral nutrients are divided into two groups: Macronutrients and micronutrients.

Macronutrients can be broken into two more groups: primary and secondary nutrients. The primary nutrients are nitrogen (N), phosphorus (P), and potassium (K). These major nutrients usually are lacking from the soil first because plants use large amounts for their growth and survival.

Micronutrients: Micronutrients are those elements essential for plant growth which are needed in only very small (micro) quantities. The micronutrients are boron (B), copper (Cu), iron(Fe), chloride (Cl), manganese (Mn), molybdenum (M_0) and zinc (Zn). These elements are sometimes called minor elements or trace elements, but use of the term micronutrient is encouraged by the American Society of Agronomy and the Soil Science Society of America.

Toxic Metals: According to Wood (1974) toxic elements are classified into three groups on the basis of their pollution potential; (i) non critical (Fe, Rb, Sr, Al); (ii) toxic but very insoluble or very rare (Ti, Hf, W, Zr, Ta, Nb, Re, Ga, La, Os, Rh, Ir, Ru, Ba); (iii) very toxic and relatively accessible (Be, Co, Ni, Cu, Zn, Sn, As, Se, Te, Pb, Ag, Cd, Pt, Au, Hg, Pb, Sb, Bi). Other elements such as Mn, Cr, etc. fit into more than one category depending on their

specific mobility or chemical form. The effects of trace elements and other toxic substances in an aquatic ecosystem can be assessed by changes in the community structure, physiological activity and ultra-structural components of macrophytes (Guilizzoni, 1991).

Organic matter content and metal translocation

Apart from soil pH, organic matter content in soil is also one of the most important soil properties affecting heavy metal availability. Organic matter is a major contributor to the ability of soils for retaining heavy metals in an exchangeable form. In addition, organic matter also supplies organic chemicals to the soil solution that can serve as chelates and increase metal availability to plants (McCauley et al., 2009). The role of organic matter on metal availability has been extensively investigated. It was reported that heavy metal adsorption onto soil constituents declined with decreased organic matter content in soils (Hettiarachchi G.M. et al., 2003, Antoniadis et al., 2008). Moreover, the dissolved organic matter in soils could increase the mobility and uptake of heavy metals to plant roots (Du Laing et al., 2009). Several researchers found positive correlation with organic matter contents in soils and concentration of different metals (Dai et al., 2004, Rai, 2008). Limiting steps

- Slow transport kinetics can become more important in controlling the uptake process than diffusion of the species from a solution to the cell due to limited space available in plasma membrane.
- Another rate limiting step is the adsorptive processes on the cell membrane when the metals can be simply adsorbed on cell walls and cell membranes without being transported into the cytoplasm, or undergo a chemical reaction (e.g. enzymatic and non-enzymatic metal reduction) at the cell surface.
- Simkiss and Taylor (1989) proposed different routes for the transport of trace metals across the cytoplasmic membrane, which includes-

1. Passive diffusion of non-polar lipid soluble metal forms, such as alkyl-metal compounds,

and small water soluble solutes.

2. Transport via carrier mediated proteins or ion channels where metals are transported down a concentration gradient within proteins with hydrophilic cores by passive diffusion.
3. Active transport via ion pumps (channels) when a metal ion binds with a membrane protein and is transported across the membrane against a concentration gradient.
4. Endocytosis.

After entering the cell, the subsequent fate of the trace metal depends on the particular physiology of the organism and type of the metal. Depending on whether or not the metal is used for essential metabolic purposes, it can become available to bind to sites where it can play an essential role, be bounded to another biomolecule or complexing agent and stored within the cell, be transported to subcellular compartments, excreted, or even gains access to the 'wrong' biomolecule and thus exerts a toxic effect.

Nonessential metals entering the cell may exert a toxic effect due to

1. Metal binding to physiologically important sites leading to blocking functional groups of biomolecules (often thiols),
2. Displacement of essential metals from their normal sites within biomolecules
3. Resulting in changed conformation (and activity) of biomolecules.

Metals bind to negatively charged binding sites in the cell walls (Cutler and Rains, 1974) which consists primarily of polygalacturonic acids. The affinity of metals to these acids differs between metals (Ernst and Van der Werff, 1978) and consequently they can be bound to cells walls either hard and non-exchangeable, or more loosely and exchangeable (Ernst and Van der Werff, 1978). Plants may respond to metal exposure by somehow restricting further uptake into the cell, which could be done by producing binding sites in response to metal exposure. The uptake of metals increases with increasing external metal concentration, but this is not a linear correlation. With time, the metal concentration in the tissue increases,

which causes saturation and the effective uptake, will decrease. The uptake of heavy metals may decrease due to the toxic effect caused by metals. It was hypothesized that plants with high metal concentration in their tissue would have a lower net uptake of metals than those with a lower tissue concentration of metals based on the theory concerning effective uptake of metals. *E. canadensis* accumulates high amounts of all three investigated metals (Cu, Cd, Zn) in its tissue, which is supported by its definition as an accumulator for certain metals (Kähkönen et al., 1997, Fritioff et al., 2005). Even with a high tissue concentration of metals the plants showed no elevated stress levels in Cu and Cd treated plants. Whilst in the cell, Zn and Cu are quickly subjected to enzyme synthesis, needed for other physiologically active molecules, or incorporated in membranes or other cellular components and can then not easily leak out of the cell as a response. The results might also indicate that the apoplastic pole has a higher capacity for binding of metals to the cell wall and consequently more metals can be taken up and bind to the apoplast before one might be able to measure a decreasing uptake of metals.

The division of labour between uptake by root and shoot is probably associated with the anatomy and morphology of the different taxa (cuticle thickness, leaf cell layers) as well as by the sorptive capacities characteristics of species differing in growth rate, surface to volume ratio and physiological condition of individual plants. Accumulation rates of different metals var by the sampling period, the physiological status of macrophytes and species, their tissue age. For example, *Elodea canadensis* accumulates about four times as much Hg as *Egeria densa* in laboratory conditions (Mortimer, 1985). Macrophytes take up heavy metals mainly through the root, although uptake through the leaves may also be of significance. As the macrophytes die and decay, the accumulated metals in the decaying macrophytes can increase in the concentration of heavy metals in the sediments. Aquatic plants often grow more vigorously where nutrient loading is high. They are capable of removing water soluble

substances from solution and temporarily immobilize them within the system (Untawale et al., 1980). Bioavailability and bioaccumulation of heavy metals in aquatic ecosystems is gaining tremendous significance globally. Several of the submerged, emergent and free-floating aquatic macrophytes are known to accumulate and bioconcentrate heavy metals (Rai and Tripathi, 2007). Aquatic macrophytes take up metals from the water, producing an internal concentration several fold greater than their surroundings. Many of the aquatic macrophytes are found to be the potential scavengers of heavy metals from water and wetlands (K.L. et al., 1979). Results confirm that aquatic plants can play an important role as a transportation link for metals from the sediments up into shoots. The metals are thereby made available to grazing moluscs and, thus, reintroduced into the food web via fish to birds and humans. In addition, macrophytes in shallow coastal zones function as living filters for nutrients and metals that become bound to living plant material. Additionally, vascular aquatic macrophytes are involved in the biogeochemical cycles of nutrient and non-essential elements in many aquatic ecosystems. These plants often take up elements in excess of need and can accumulate essential as well as non-essential elements to concentration many times higher than those of the surrounding waters.

Phosphorus release

Phosphorus release is influenced by a variety of environmental factors, including water temperature, pH, form of phosphorus, dissolved oxygen (DO), nitrate, redox potential, and hydrological conditions. Gao et al. (2005) and Ishii et al. (2005) shown that the DIP release flux was observed to decrease with an increase in the rate of nitrate supply. The cause of phosphorus enrichment can be denoted under the following major points-

1.1.1. Soil itself is a sink for P

The lowered redox potential is usually followed by a release of P to the water column.

Therefore, the sediments can either act as a source or a sink (mineralization/sedimentation) for P in the lake ecosystem. According to the classic model of P release from sediment, Kopáèek et al. (2004) proposed that, during low redox potential P released from the sediment and sediment is considered as the main source of P.

1.1.2. Microbial activity

Microbes may also cause P release via cleavage of polyphosphate molecules. During times of anoxic stress, it is proposed that bacteria hydrolyze polyphosphates providing a source of P flux from the sediments (Wetzel et al., 1991). Therefore, microbially-incorporated P may be a possible source of P flux from anoxic sediments.

1.1.3. Organic matter concentration in sediment

Phosphorus associated with organic matter (org-P) may also have a role in P dynamics in the surficial sediments of anoxic lakes, since it is mostly subject to diagenesis. Org-P is considered to be mainly phospholipids, mono-ester and di-ester P compounds. The lower release of P is linked to a lower decrease of bacterial activity (Montigny and Prairie, 1993).

Phytoremediation and phytomanagement

Ion exchange, reverse osmosis, electrolysis, precipitation, and adsorption are a number of methods available to remove toxic metals from water. However, they are expensive, relatively inefficient, and in most cases, they generate a great amount of waste that is difficult to dispose of (Rai and Tripathi, 2007). Furthermore, from the economic point of view, the need of an alternative cost-effective technology is recommended as the cleanup of hazardous wastes by conventional technologies is projected to cost at least $400 billion in the USA alone, based on estimates obtained from various institutions (Salt et al., 1995). Methods using living aquatic plants to remove metals from water can be a viable alternative process. Rai (2008) extensively

reviewed the utility of macrophytes in heavy metals removal from polluted aquatic ecosystems. The heavy metal pollution of aquatic ecosystems is often most obviously reflected in high metal levels in sediments and macrophytes than in elevated concentrations in water. Studies and experiments using radioactive tracers have established that most rooted aquatic plants can take up chemicals primarily from the sediment pore water (Jackson, 1998). The concentrations of metals in aquatic plants can be more than 100,000 times greater than in associated water column (Jackson, 1998). Aquatic plants thus accumulate metals that they take from the environment and concentrate within the trophic chains causing an accumulative effect. However some macrophytes possess exclusion strategy leading to metal tolerance, for example, Deng et al. (2004) also observed accumulation of Cu along with Pb and Cd and showed that metals accumulated by wetland plants were mostly distributed in root tissues. A higher concentration of metals in the plants usually, but not always, indicates a proportional increase in element levels in the water and/or sediments (Guilizzoni, 1991). Competition between elements and factors, indirectly influencing metal uptake such as, for example, light conditions and eutrophication, seem to limit in some cases the utility of using macrophytes as bio-indicators.

Since preservation and restoration of polluted wetland ecosystems must be compatible with a decrease in the environmental risks, the application of non-invasive remediation/management options such as phytomanagement appears as a key-tool. Phytomanagement consists on the engineering or manipulation of soil–plant systems to control pollutant fluxes in the environment, decreasing the environmental risks posed by elevated concentrations of these elements in the soil. The role of vegetation in a contaminated site under phytomanagement is to add value to the land and possibly mitigate the negative effects associated with soil contamination. Low plant metal uptake is critical to the success of using the vegetation in this kind of projects in order to enhance biodiversity.

Elevated concentrations of metal (loid)s in shoots not only increase the likelihood of their entry into the food chain, but may also result in an accumulation of metal(loid)s on the soil surface, as leaf litter is deposited (Robinson et al., 2009). Successful examples of phytomanagement have been carried out in the South Spain in 1998 areas affected by the mine tailings-dam failure in Doñana (Dominguez et al., 2008) .

Oxidative Stress

Free radicals and ROS are produced under normal physiological condition and can be detected in all plant tissues. Under environmental stress, changes in free radical processes are expected to occur and these are in turn to affect the radical scavenging ability of a plant (Fig.1.3). Namely, when ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and significant oxidative damage to a plant. These cytotoxic ROS can strongly disrupt normal metabolism through oxidative damage of chlorophyll, lipids, protein, and nucleic acids (Herbinger et al., 2002). To protect them against oxidative stress, plant cells produce both antioxidant enzymes (table-1.2), such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), glutathione-S-transferase (GST), and non-enzymatic antioxidants such as glutathione and ascorbate. Some of these constitute good molecular bioindicators for contaminant- mediated oxidative stress. Also, in vivo free radical formation has been cited as a contributing factor to the deleterious effects of many chemical pollutants. Free radical reactive intermediates react directly or indirectly with molecular oxygen to form ROS. Superoxide anion (O_2^{-2}), produced by xanthine oxidase, tryptophan dioxygenase, diamine oxidase and activated neutrophils, can be a source of additional harmful ROS.

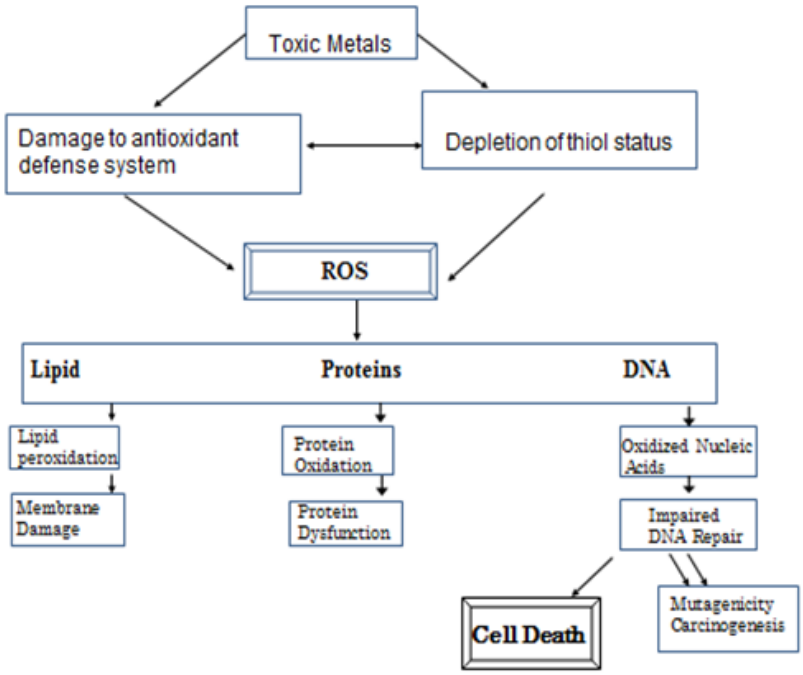

Figure 1.3 Flow diagram showing the effect of toxic metals on excess ROS generation, causing cell death.

The one electron reduction of molecular oxygen results in the form of superoxide radical anion as an intermediate. O_2^{-2} radical is toxic byproducts of oxidative metabolism and can interact with H_2O_2 to form highly reactive hydroxyl radicals (OH^-), which is thought to be primarily responsible for oxygen toxicity in the cell.

Table 1.2 ROS scavenging and detoxifying enzymes

Enzyme	EC number	Reaction catalyzed
Superoxide dismutase	1.15.1.1	$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \leftrightarrow 2H_2O_2 + O_2$
Catalase	1.11.1.6	$2H_2O_2 \leftrightarrow O_2 + 2H_2O$
Glutathione peroxidase	1.11.1.12	2GSH + PUFA-OOH ↔ GSSG + PUFA + $2H_2O$
Glutathione S-transferases	2.5.1.18	RX + GSH ↔ HX + R-S-GSH*
Phospholipid-hydroperoxide glutathione peroxidase	1.11.1.9	2GSH + PUFA-OOH (H_2O_2) ↔ GSSG + $2H_2O$**
Ascorbate peroxidase	1.11.1.11	AA + H_2O_2 ↔ DHA + $2H_2O$
Guaiacol type peroxidase	1.11.1.7	Donor + H_2O_2 ↔ oxidized donor + $2H_2O$***
Monodehydroascorbate reductase	1.6.5.4	NADH + 2MDHA ↔ NAD+ + 2AA
Dehydroascorbate reductase	1.8.5.1	2GSH + DHA ↔ GSSG + AA
Glutathione reductase	1.6.4.2	NADPH + GSSG ↔ NADP+ + 2GSH

*R may be an aliphatic, aromatic or heterocyclic group; X may be a sulfate, nitrite or halide group.

**Reaction with H_2O_2 is slow.

*** AA acts as an electron donor.

Plant Hormone and adaptive response of *Potamogeton* species

The plant hormone auxin functions as a signaling molecule and a driver of growth and developmental processes. Auxin metabolism is itself regulated by the availability of free sugars. The regulation of the biosynthesis and degradation of the main auxin, indole-3-acetic acid (IAA), by sugars requires changes in the expression of multiple genes and metabolites linked to several IAA biosynthetic pathways (Sairanen et al., 2012). Plants response to anoxic stress: Prolonged periods of oxygen deficiency inevitably lead to cell death, and the extent to which plant tissues can tolerate the absence of oxygen varies widely between species and during development. The absence of oxygen inhibits oxidative phosphorylation, and so anoxic tissues are forced to rely on fermentation for the production of ATP. Fermentation only produces a small fraction of the ATP that would be obtainable from oxidative phosphorylation, and it can also impair pH regulation, leading to a potentially lethal acidification of the cytoplasm (Xia and

Roberts 1994). It is therefore remarkable that some plant tissues are able not only to maintain cellular functions, but also to support the elongation of new tissue under these extreme conditions. All such tissues contain substantial carbohydrate reserves as well as the enzymatic machinery to mobilize these reserves for fermentation (Harada et al., 2005). In addition, ethanolic fermentation, which reduces the accumulation of protons that can contribute to cytoplasmic acidosis, is predominant and lactate fermentation is minimal or absent. Dixon et al. (2006) proposed the regulation and tolerance mechanism in *Potamogeton pectinatus* mainly by two processes-supply of ATP for the biosynthesis of macromolecules and generation of turgor pressure.

Some physiological adaptation of this species includes:

1. Over-winters as a tuber with a pre-formed shoot, and both the tuber and shoot are rich in starch, providing ample substrate for prolonged fermentation.
2. The presence of a pre-formed shoot means that growth can be supported by expansion of existing cells rather than cell division. Cell expansion is a considerably less energetic process than cell division, and this growth mechanism has a clear advantage when ATP is a limited commodity and cell expansion and elongation allow rapid extension of the stem tissue, which can be viewed as a physiological adaptation to escape the anoxic environment.
3. The presence of ADH (alcohol dehydrogenase) and PDC (pyruvate decarboxylase) in preformed shoots, ensures a rapid fermentative response at the onset of anoxia.
4. *P. pectinatus* has the capacity to degrade starch via starch phosphorylase, which avoids the need to use ATP for the subsequent phosphorylation of glucose. As starch is the main source of carbohydrate for fermentation, the ability to maintain a high rate of starch breakdown under anoxia, and to do so via an ATP-efficient pathway, may be a key factor in the survival of *P. pectinatus* under anoxia.

5. Anoxia do not affect the ability to regulate cytoplasmic pH, due to interplay between lactate and ethanol production as these appears to be a key factor in achieving satisfactory pH regulation under anoxia.

Nevertheless, sufficient amounts of carbohydrates must be available for energy production, enabling the plants to survive. In the absence of oxygen the energy supply may result from fermentation processes, connected with accelerated substrate consumption (Pasteur effect)

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Chapter 2. **EXPERIMENT 1: EFFECTS OF NH_4–N CONCENTRATIONS AND GRADIENT REDOX LEVEL ON GROWTH AND ALLIED BIOCHEMICAL PARAMETERS OF *ELODEA NUTTALLII* (PLANCH.)**

Abstract

Aquatic plants frequently encounter multiple stresses under natural conditions. Nuttall's water weed, *Elodea nuttallii* (Planch.) is a submerged aquatic macrophyte which has flexible ability to use different nutrient sources from various environments. However, recently the growth of *E. nuttallii* has been declining in waters of Japan and in the Chesapeake Bay, a large estuary in the United States. In the present experiment, we studied growth and survival capabilities of the plant under a gradient of redox conditions; from highly oxic (+400 to +440 mV) to extremely reduced (−180 to −120 mV) conditions. Reduced environment was prepared by adding glucose to growth medium and nitrogen gas bubbling, while the oxic environment was brought about by atmospheric air bubbling. In comparison to the oxic environment, growth rate and carbon–nitrogen content of the plants were significantly affected negatively at hypoxic and anoxic conditions. In hypoxic and anoxic environments, indole acetic acid (IAA), tissue nitrogen and chlorophyll levels were down-regulated, whereas hydrogen peroxide (H_2O_2), indole acetic acid oxidase (IAAO) and peroxidase (POD) levels were up-regulated. It was also found that high NH_4–N concentrations (10–40 ppm) affect the growth rate and biochemical parameters of the plant; however, in hypoxic and anoxic treatments the effects were more severe. We conclude that *E. nuttallii* is poorly tolerant to hypoxia/anoxia. Moreover, oxygen stress combined with high ammonium concentration act as important factors influencing distribution and abundance of this species.

2.1 Introduction

The principal stress factor for plants in flooded soil is biochemical reduction, the intensity of which is measured as redox potential (Lissner et al., 2003). A reduced redox potential dramatically alters the chemistry and microbial metabolism at the sediment–water interface; this is a common phenomenon in water-logged soil. The reducing chemicals and biogeochemical processes induced by oxygen (O_2) depletion accelerate the sediment's oxygen

demand and lower its redox potential to values at −300 mV (DeLaune et al., 1990). Wetland soils are characterized by gradients of redox conditions from totally oxidized to extremely reduced state. A lack of O_2 may negatively affect plant metabolism, including nitrogen uptake and assimilation (Gibbs and Greenway, 2003). Water-logging of soil and consequent hypoxia induce complex morphological responses in plants. Under reducing conditions, NH_4^+ is the available form of N in environment. Several previous studies have shown that NH_4^+ enrichment can directly cause decline in aquatic plant populations in natural water (Cao et al., 2007, Goldyn, 2010). The mechanisms include the formation of reactive oxygen species (ROS), induced by NH_4^+ metabolism by the plants (Nimptsch and Pflugmacher, 2007) and the imbalance of C–N reserves in plants stemming from the incorporation of NH_4^+ into free amino acids accompanied by consumption of soluble carbohydrates (Cao *et al.*, 2007). Mittler (2002) proposed that oxidative damage in plants is associated with many types of stresses (biotic and abiotic), and plant hormones play a major role in signaling responses and regulations of developmental processes. The common reactive oxygen species (ROS) such as the superoxide anion ($O_2^{-\bullet}$), H_2O_2, and the hydroxyl radical ($HO^{\bullet}$) can damage biological molecules (DNA, RNA, and proteins) and membranes by inducing lipid peroxidation (Weckx and Clijsters, 1996). However, ROS scavenging mechanisms exist, and it is crucial to identify key components involved in plant tolerance to strong oxidative conditions (Sinha and Saxena, 2006). On the other hand, the phytohormone indolyl acetic acid (IAA) and also H_2O_2 are considered to regulate plant growth especially in stressful environments (Pasternak et al., 2005).

Submerged macrophytes are unique among rooted aquatic plants in linking the water column and the sediment through their physical structure and are capable of taking up nutrients from both water column and sediment (Ottosen et al., 1999). *Elodea nuttallii* (Planchon), an exotic submerged aquatic macrophyte, has undergone explosive growth in

Japan since the early 1960s, although this trend seems to be diminishing (Nagasaka, 2004). The population of *Elodea* spp. in the Chesapeake Bay, USA, has displayed similar trends (Stevenson et al., 1979). Researchers have suggested different causes for such decline, including progression of eutrophication (Riis et al., 2000), climatic factors (Hamabata and Kobayashi, 2002) and lack of genetic variability (Kadono et al., 1997).

E. nuttallii is often subjected to gradient redox conditions, such as they occur in flooded soil, eutrophic lakes, and waste water. In these aquatic environments, due to the cessation of ammonium nitrification, NH_4–N level increases, as it was described, for example, in Plesne Lake in Central Europe (Kopáèek et al., 2004). Chlorosis of leaves (brown–black discoloration of the leaves), suppression of growth rate, decreased photosynthetic rates, etc., were reported as NH_4^+ toxicity symptoms for *E. nuttallii* (Dendène et al., 1993), suggesting that increased NH_4–N concentrations affect negatively growth and distribution of the plant. However, it is still unknown whether this species is affected directly by gradients in oxygen levels/ redox potentials or gradients of redox potentials in combination with high NH_4–N concentrations simultaneously affect the growth and distribution of the species. In the present study, *E. nuttallii* was subjected to different oxygen levels and NH_4–N concentrations, and growth and related biochemical parameters of the plants responding to these conditions were investigated.

2.2. Objectives of the study

High NH_4–N and low sediment redox state were found to inhibit the growth of aquatic macrophytes. Although these two stressful factors always coexist in eutrophic natural waters, little is known about the combined effects of the two factors on biochemistry of macrophytes.

- Study the variations of plant growth and physiochemical processes under different treatments and conditions

- Study the changes of plant hormones and enzymes under different treatments and conditions

The results of the present study revealed the combined effects of low redox and high ammonium stresses on macrophytes trigeer changes in C-N balances and induction of oxidative stress in *E. nuttallii.*

2.3. Materials and Methods

2.3.1. Collection of sediment and plants

The sediment was collected from a pond in Oaso Park, 60 km northwest from Tokyo, in December, 2011. The sediment was derived from the top surface (<15 cm depth) of the pond sediments. Soil material was homogenized, air-dried and sieved to <2 mm. The sediment contained 5.4 ± 0.2% (n=4) organic matter content. *E. nuttallii* plants were collected from Motoarakawa River, close to the park. About 10cm apical tips of the plants were incubated for two weeks in a growth chamber at a temperature of 25 °C, with a relative air humidity of 90 % and a photon flux density of approximately 100 µmol m^{-2} s^{-1}, which was provided by fluorescent lamp tubes in a 12:12 h light:dark cycle.

Figure 2.1 Experimental plant *Elodea nuttallii*

This species is native to North America, particularly mid- and north-eastern U.S.A. and Canada; exotic in Japan, where it has displayed explosive growth since in the early 1960s (Nagasaka, 2004).

2.3.2. *Experimental set-up*

E. nuttallii was subjected to different redox potentials under different NH_4-N concentrations. Since it was difficult to keep a constant redox potential throughout the experimental period, certain range of redox potential was maintained. The three levels of redox potential (Eh values) applied were (i) +400 mV to +440 mV (Oxic; O_1), (ii) -5 mV to 5 mV (hypoxic/moderately reduced; O_2) and (iii) -180 mV to -120 mV (anoxic/highly reduced; O_3): Fig 2.1. Regarding the nitrogen source, the suitable NH_4-N concentration for *E. nuttallii* is 2.5 ppm (Ozimek et al., 1993). In eutrophic environment the maximum reported NH_4-N concentration was 50 ppm (Kopáèek et al., 2004). In our study, four different concentrations of NH_4-N [2.5 (N1), 5 (N2), 10 (N3) and 40 (N4)] were used (Fig.2.2). The experiment was conducted in microcosms (MCs), each consisting of a 6 L (15.7 cm ×15.7 cm × 24.5 cm) glass vessel which was hermetically sealed with an air-tight lid. Each MC was filled with 600 g of air-dried soil and deionized water in a 1:5 ratio. The growth medium was a 5 % Hoagland nutrient solution (Hoagland and Arnon, 1950) and ammonium sulphate was added to adjust the required NH_4-N concentration. For control, 5% Hoagland nutrient medium was used without any further treatment. Highly reduced and moderately reduced microcosms were prepared following the method developed by Yu et al. (2007b). Glucose, a simple carbon source, was used in this experiment during the 22 day incubation period. At the beginning of incubation, 8.16 g glucose was added to each reduced (MC 3) and highly reduced microcosms (MC 4) at 1st and 3rd day and twice that amount was repeated at 5th day. At 14th day, again 8.16 g glucose was added to MC 4. Continuous flushing of N_2 gas was carried out for last 3 and 7 days to accelerate the Eh values to reduce

approximately -5 and -180 mV for hypoxic/moderately reduced (MC3) and anoxic/highly reduced (MC4) conditions, respectively.

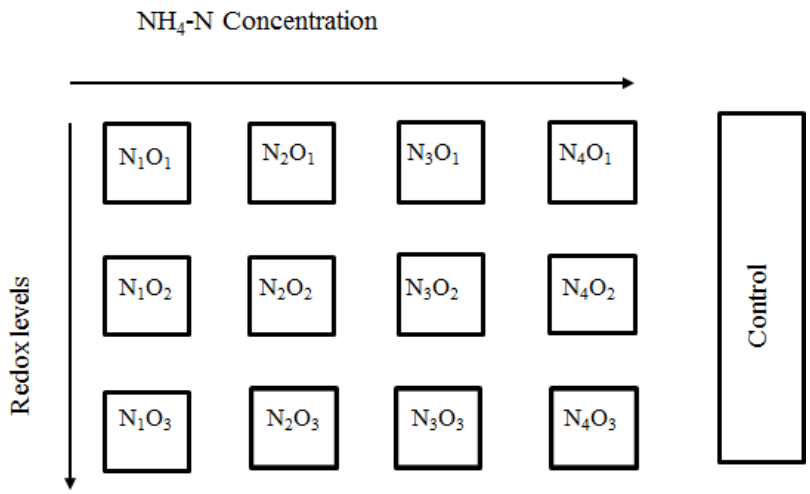


Figure 2.2 Layout of the experimental set-up (13 microcosms/treatment). NH_4–N concentration and redox levels are presented as N and O, respectively. Microcosms were randomly distributed with equal spacing in the growth chamber.

For oxic treatments, continuous bubbling with atmospheric air was used. Redox potential (Eh) and pH were measured four times a day using four portable pH/ORP meter (POT-101M, SIBATA, Japan). The temperature was maintained at 23 ± 2 °C in a room with fluorescent lighting. No attempt was made to control the pH of the sediment suspensions. The total experimental period was 14 days. In total three treatments, each with 13 microcosms were used (Fig.2.2). In each microcosms eight plants (12-14 cm) were planted. Eight (around 12-14 cm) plants were planted in each microcosm after 22 days of incubation period. We suspected the growth might be hampered under extreme condition (under 10 and 40 ppm), thus we selected plants for particular chemical analysis for example, 1st pair for growth study, 2nd pair for photosynthetic parameters and % of C and N study, 3rd pair for hormone and enzyme study and 4th pair for MDA and Proline content study.

2.3.3. *Plant growth study*

At 14 DAT (day after beginning of treatment) two plants from each tank were harvested, cleaned and the fresh weight and length were measured after blotting with laboratory tissue. The average relative growth (RGR) was calculated using the following equation

$$RGR = \frac{(\ln W_2 - \ln W_1)}{T_2 - T_1} \qquad [1]$$

where RGR is the average weight of specific growth rate (g DW/g/day) and W_1 and W_2 are plant weights at time (days) T_1 and T_2 respectively. Shoot elongation was calculated by:

$$SGR = \frac{(L_2 - L_1)}{T_2 - T_1} \qquad [2]$$

Where, SGR is the shoot growth rate (cm/day) L_1 and L_2 are the initial and final shoot lengths (cm) at the time (days) T_1 and T_2 respectively.

2.3.4. *Chlorophyll content, carotenoid content and chlorophyll fluorescence*

Contents of chlorophylls and carotenoids in fresh leaves were estimated by the method of Lichtenthaler (1987). Leaf samples (50 mg) were mashed with a mortar and pestle and extracted with 80% acetone (v/v) in dark for 24 h. After wards the sample was centrifuged for 10 min at 8000 × g. The supernatant was collected and the light absorption read at wavelengths 665 and 649 nm for chlorophyll a (chl a) and chlorophyll b (chl b), respectively, and at 470 nm for the carotenoid content, using a spectrophotometer (Shimadzu UV-1700, Japan).

Chl a fluorescence measurements were performed with a handy flurocam (FC 1000-H, Photon Systems Instruments, Czech Republic) using auto image segmentation. The plants were incubated in dark for 15 min before the measurements were taken in order to allow complete oxidation of the photosystem II (PSII) reaction centers. Maximum

photochemical efficiency of PSII (Fv/Fm), the activity of PSII (Fv/Fo) and the electron transport rate (ETR) through PSII (Fm/Fo) were determined and used as stress indicators for plants.

2.3.5. Total carbon (TC) and nitrogen (TN)

After the above measurements plants were separated into leaves, shoots and roots and were dried at 60° C to constant weight in an oven drier for 72 h. Then dry samples were reweighed (for dry weight) and homogenized by grinding into a fine powder using a mortar and pestle. Powdered samples were stored in airtight vials for subsequent analysis. TN, TC of powdered plant samples were measured by CHN coder (YANACO MT-3).

2.3.6. Hormone and enzyme analysis

For hormone analysis two plants from each tank were harvested, cleaned, dried and powdered. To compare the hormonal change over time, a base line was established by analyzing the initial condition of plants from each microcosm. The IAA concentrations in the tissues were measured using Salowski reagent (Gordon and Weber, 1951). For the analysis of endogenous H_2O_2 concentrations, samples were extracted with cold acetone and the method was followed after (Cervilla et al., 2007). Phosphate buffer (0.1 mol L^{-1}) at pH 6 was used to make extracts for the measurements of POD and IAAO activities. IAA destruction was measured to determine IAAO activity (Zhang et al., 2009). POD was determined after Goel et al. (2003) and the absorbance differences at 420 nm were plotted every 30 s for 3 min (Chanjirakul et al., 2006).

2.3.7. Lipid peroxidation and proline concentration

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA), a product of lipid peroxidation in the plant samples estimated by thiobarbituric acid (TBA) reaction (Heath

and Packer, 1968). Plant samples (500 mg) were homogenized in 3 ml of 0.5% TBA in 20% trichloroacetic acid (TCA) (w/v). The mixture was heated at 95°C for 30 min and quickly cooled in an ice bath. The samples were centrifuged at 10,000×g for 10 min and absorbance was measured at 450, 532 and 600 nm (Ultrospec 3000, Pharmacia Biotech, England). The concentration of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and by correcting for the specific absorbance at 600 nm (532–600 nm).

The concentration of proline was measured after Bates et al. (1973). Plant material was homogenized with 10 ml of 3% (v/v) sulfosalicylic acid. The homogenate was centrifuged at 800×g for 15 min. Free proline present in the supernatant was treated for 1 h at 80 °C with acid-ninhydrin. The reaction was terminated in an ice bath and the colored complex was extracted in toluene. Its absorbance was measured spectrophotometrically at 520 nm. The standard curve for proline was prepared by dissolving proline in 3% (v/v) sulfosalicylic acid covering the concentration range 0.1-5.0 µg mL^{-1}.

2.3.8. Light microscopic study

Bright field microscopy (Lecia MZ FLIII) was done to see chloroplasts at longitudinal apical sections (unstained, wet mount) of the *E. nuttallii* shoot at 40× magnification.

2.3.9. Statistics

All data are presented as the mean ± SD (n=4). Statistical analyses were performed using the SPSS for Windows (Release 13, SPSS INC., Chicago, IL) statistical software package. The data were tested by both one-way analysis of variance (ANOVA). Before performing a statistical analysis, data were checked for normal distribution. Statistical differences between treatments were identified by the Tukey HSD's test at a 5% significance level. Pearson's correlation analysis was carried out to explore the correlations between the treatments,

chlorophyll content and hormone levels.

2.4.Results

2.4.1. Plant growth and shoot elongation

Plants subjected to high concentration of NH_4–N along with hypoxic/anoxic treatments showed brown-black discoloration of the leaves. Increment of ammonium in oxic treatment had little effect on RGR (Fig. 2A) but significantly decreased SGR (Fig. 2B).

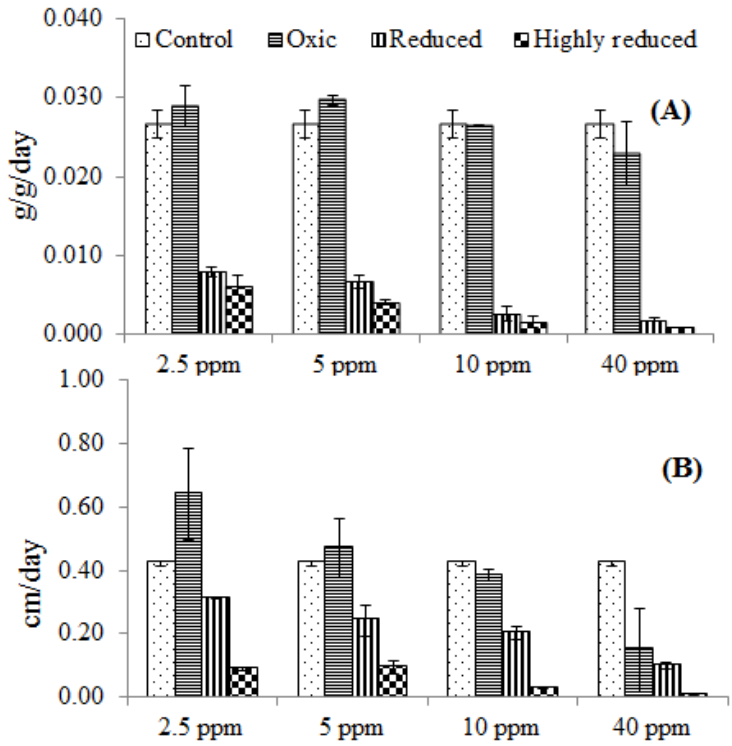


Figure 2.3 Effects of NH_4–N concentrations and oxygen treatments on the growth of Elodea nuttallii. (A) Relative growth rate (g/g/day, RGR), (B) shoot growth rate (cm/day, SGR) of the plant (mean ± SD).

When oxygen level decreased, both RGR and SGR were significantly affected at all ammonium levels, however, more effect was observed at 40 ppm NH_4–N than at the other NH_4–N levels. RGR and SGR of plants varied between treatments ($P < 0.001$) and were

significantly affected ($P < 0.01$) by different NH_4–N concentrations (Fig. 2.3). The highest RGR (at 2.5 ppm, 0.03 ± .002 day^{-1}) and SGR (at 2.5 ppm, 1.4 ± 0.1 cm day^{-1}) values were found in oxic treatments, and lowest values were found in highly reducing conditions.

2.4.2. Chlorophyll and carotenoid content, and electron transport rate (ETR)

Photosynthetic pigments, Chl a, Chl b and carotenoid content, showed similar decreasing trends with the increment of NH_4–N concentration in oxic treatments (Fig. 2.4 A–C). Increment of ammonium accelerated the effect of hypoxia as both chlorophyll and carotenoid rigorously declined at higher ammonium concentrations under hypoxic and anoxic conditions. Moreover, carotenoids seem to be affected more severely and were almost completely absent in the highly reduced treatment at 10 and 40 ppm NH_4^+ concentrations (Fig. 3C). The values of the maximum quantum yield of PSII are shown in Fig. 3D. Among different NH_4–N concentrations, the Fv/Fm ratio and electron transport rate (ETR) did not differ significantly in plants under oxic treatment, but were significantly lower for plants grown in the reduced and highly reduced treatments (Fig. 3D and E). Thus both oxygen treatments and elevated levels of NH_4–N concentration significantly affect Fv/Fm ratios, ETR, chlorophyll and carotenoid content of *E. nuttallii*. The figures of different values of chlorophyll transport rate though Flurocam are presented in Annex 2.

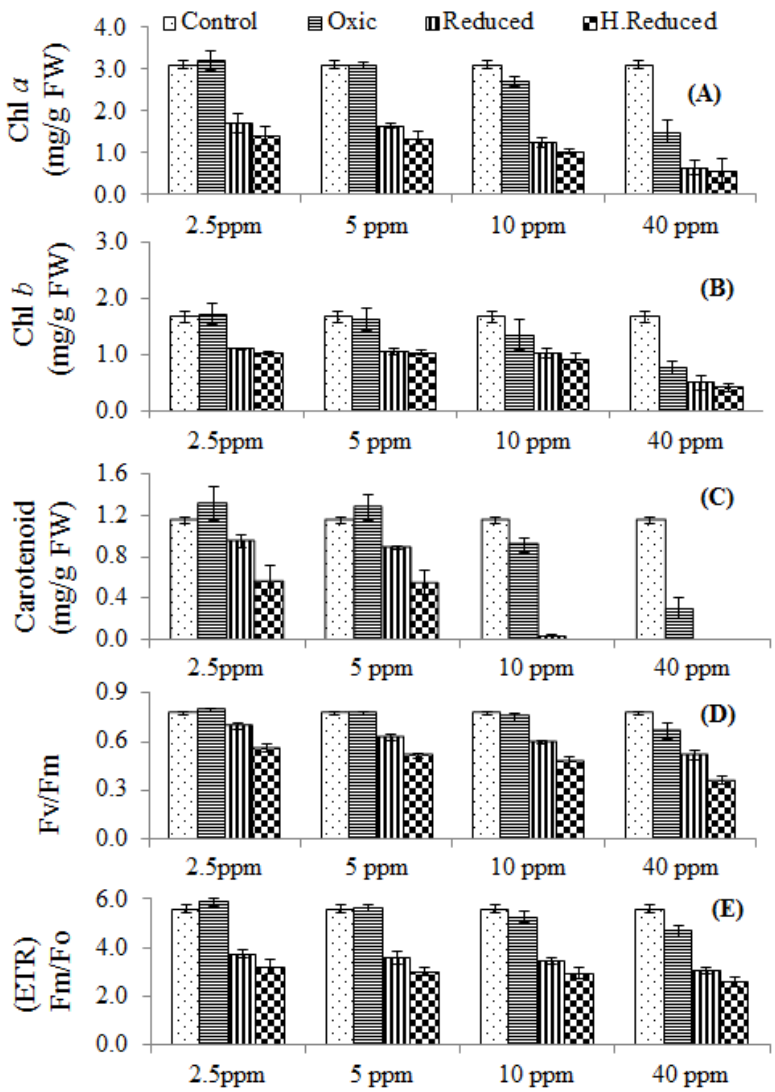


Figure 2.4 Effects on plant pigments. (A) Chl a level (mg/g FW), (B) chl b level (mg/g FW), (C) carotenoid level (mg/g FW), (D) Fv/Fm ratio, (E) Fm/Fo (ETR) of *Elodea nuttalli* (mean ± SD) grown under different NH_4–N concentrations and oxygen treatments.

2.4.3. Carbon and Carbon and nitrogen

In all treatments (oxic, reduced and highly reduced), the increment of ammonium concentrations declined both carbon (C) and nitrogen (N) levels (Table 2.1). With concern to the redox situation in comparison to oxic treatment C and N levels were significantly decreased

in reduced and highly reduced treatments (Table 2.1).

Table 2.1 Carbon (C%) and nitrogen (N%) content (mean ± SD) of *Elodea nuttallii* grown under different concentration of NH_4-N (conditions) and at different levels of redox state (treatments). The superscripts letters indicate significant difference between treatment

Redox State	NH_4^+ conc.	C%			N%		
		Leaf	Shoot	Root	Leaf	Shoot	Root
Control		36.6±1.0^{a}	34.6±0.7^{a}	28.3±1.6^{a}	3.5±0.2^{a}	3.2±0.2^{a}	3.3±0.3^{a}
Oxic	2.5 ppm	39.4±0.8^{a}	37.6± 1.5^{a}	30.8 ± 1.6^{a}	3.8 ± 0.1^{a}	3.4 ± 0.5^{a}	3.0 ± 0.2^{a}
	5 ppm	36.7±1.4^{a}	35.3± 1.2^{a}	27.8±1.7^{ab}	3.7 ± 0.1^{a}	3.4 ± 0.5^{a}	2.9 ± 0.2^{a}
	10 ppm	31.5± 2.4^{ab}	30.6 ± 1.6^{b}	24.2± 2.2^{ab}	3.2 ± 0.2^{ab}	3.4 ± 0.4^{a}	2.6 ± 0.3^{b}
	40 ppm	29.7± 1.1^{ab}	29.1 ± 1.1^{b}	23.7± 0.6^{ab}	3.1 ± 0.5^{ab}	2.4 ± 0.3^{c}	2.4 ± 0.4^{b}
Reduced	2.5 ppm	29.7 ± 1.6^{b}	27.4 ± 3.0^{bc}	23.6 ± 1.7^{ab}	2.7 ± 0.1^{b}	2.6 ± 0.2^{bc}	2.0 ± 0.2^{c}
	5 ppm	28.9 ± 1.7^{c}	27.2 ± 1.9^{bc}	21.3 ± 1.4^{b}	2.6 ± 0.1^{b}	2.4 ± 0.2^{c}	1.9 ± 0.2^{c}
	10 ppm	25.9 ± 1.5^{d}	23.4 ± 1.8^{c}	19.8 ± 1.3^{b}	2.5 ± 0.3^{b}	2.1 ± 0.6^{cd}	1.7 ± 0.2^{cd}
	40 ppm	23.7 ± 2.8^{de}	21.4 ± 0.7^{c}	19.82 ± 1.3^{b}	2.3 ± 0.2^{b}	2.0 ± 0.1^{cd}	1.6 ± 0.2^{cd}
Highly Reduced	2.5 ppm	22.2 ± 1.3^{de}	21.5 ± 2.1^{c}	21.0 ± 1.5^{b}	2.3 ± 0.2^{b}	2.0 ± 0.2^{cd}	2.0 ± 0.3^{c}
	5 ppm	21.4 ± 1.9^{de}	19.2 ± 2.6^{c}	18.7 ± 2.4^{bc}	2.1 ± 0.2^{bc}	1.8 ± 0.2^{d}	1.9 ± 0.3^{c}
	10 ppm	19.7 ± 1.0^{e}	18.2 ± 2.2^{cd}	17.3 ± 1.6^{bc}	2.2 ± 0.2^{bc}	1.9 ± 0.1^{d}	1.7 ± 0.2^{cd}
	40 ppm	18.9 ± 0.9^{e}	18.3± 1.6^{cd}	14.8 ± 0.4^{c}	1.9 ± 0.3^{c}	1.6 ± 0.2^{e}	1.4 ± 0.2^{e}
F-ratio		29.2***	32.1***	17.6**	3.1***	4.5***	8.0**

These together showed that both high NH_4^+ concentration and hypoxia affect negatively C and N levels in the plants. Leaves, shoots and roots seem to be similarly affected by higher NH_4–N concentrations and hypoxia/anoxia.

2.4.4. IAA concentration and IAA catabolism

Endogenous IAA is a major growth hormone, the concentration of which was significantly

decreased (Fig. 2.5 A) in the plants when exposed to reduced and highly reduced treatments and normal to high NH_4^+ concentrations available in the surrounding water (Fig. 2.5 A). When NH_4^+ concentration was increased under oxic conditions, the IAA level declined as well. However, hypoxia seems to affect IAA level more significantly than higher ammonium concentration does. On the other hand, IAAO activity (Fig. 2.5 C) was significantly ($P < 0.001$) elevated in plants under oxygen deprived conditions. Both IAA concentration and IAAO activity of *E. nuttallii* in oxic treatment were affected by high NH_4^+ concentrations, being more severely affected when oxygen level was decreased. This suggests that high NH_4^+ concentrations accelerate IAAO activities and reduce IAA concentration in plant under oxygen deprived condition. The IAA concentration was negatively correlated with IAAO activity in all treatments and conditions (oxic, $r = -0.908$, $n = 16$, $P < 0.01$; reduced, $r = -0.952$, $n = 16$, $P < 0.001$ and highly reduced $r = -0.981$, $n = 16$, $P < 0.001$).

2.4.5. ROS production and POD activity

A significantly higher H_2O_2 concentration ($P < 0.05$) was found throughout the experimental period in plants under reduced and highly reduced treatments (Fig. 2.5 B). Also a 10 and 40 ppm NH_4^+ concentration under oxic treatments, plants exhibited significant increase of H_2O_2 generation. POD activity was also up-regulated throughout the experimental period in reduced and highly reduced treatments (Fig. 2.5 D). H_2O_2 concentration and POD activity were positively correlated in all treatments and conditions (oxic, $r = 0.847$, $n = 16$, $P < 0.01$; reduced, $r = 0.948$, $n = 16$, $P < 0.001$, and highly reduced $r = 0.929$, $n = 16$, $P < 0.001$).

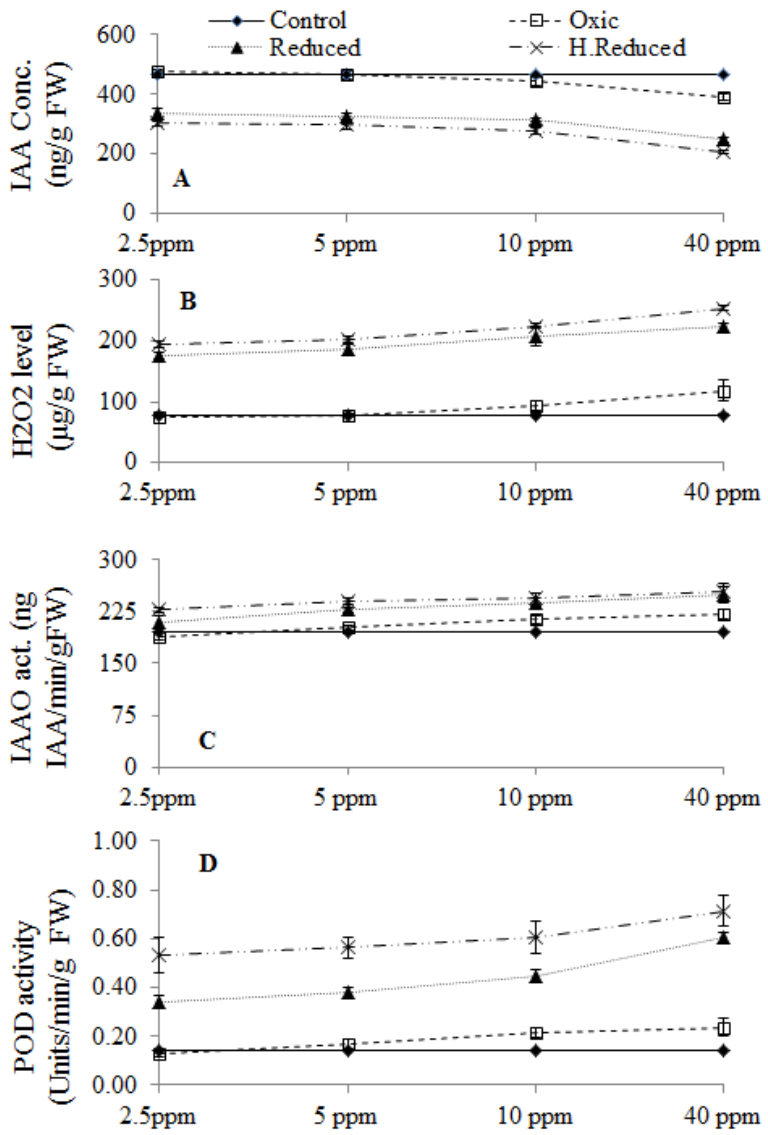


Figure 2.5 Effects on hormone and enzymes activities of the plant. (A) IAA concentration (ng/g FW), (B) H_2O_2 concentration (µg/g FW), (C) IAAO activity (ng IAA/min/g FW), (D) POD activity (units/min/g FW) of *Elodea nuttalli* (mean ± SD) grown under different concen

2.4.6. Lipid peroxidation rate and proline content

Lipid peroxidation rate was measured as MDA content. MDA content was significantly increased (Fig. 2.6 B) in plants that were exposed to reduced treatments ($P < 0.01$). The highest increment of MDA content was observed in plants exposed to highly reduced condition

throughout the experiment but was decreased at 40 ppm NH_4–N concentration. In all treatments (oxic, hypoxic and anoxic), increments of NH_4^+ concentration reduced the proline levels in plants (Fig. 2.6 A), suggesting that this latter is down-regulated by high ammonium concentrations. In reduced and highly reduced treatments, the proline content seemed to be negatively affected more significantly (at 40 ppm, under highly reduced treatment about 90.8% comparing to control).

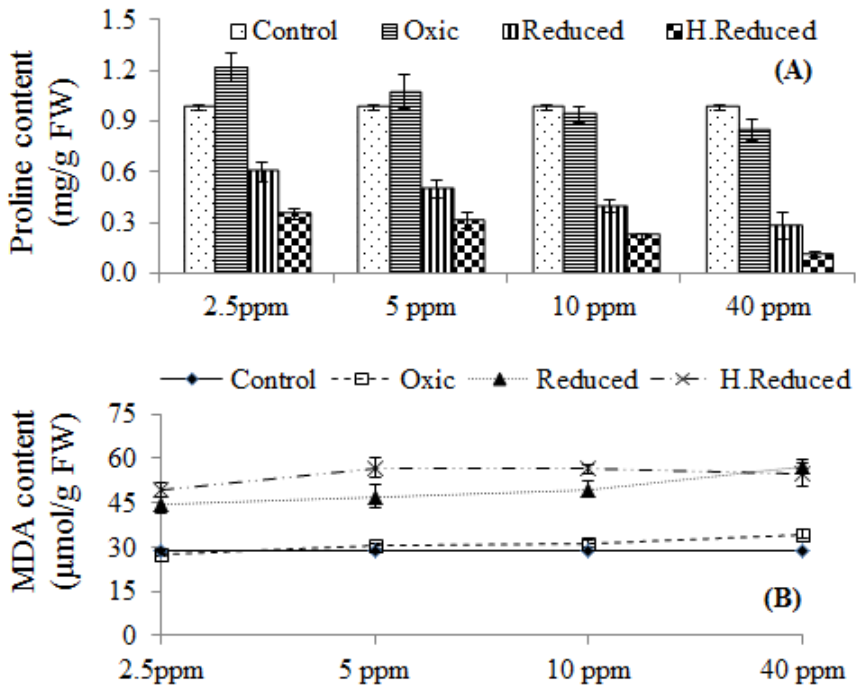


Figure 2.6 Effects on proline and MDA content of the plant. (A) Proline concentration (mg/g FW), (B) MDA content (µmol/g FW) of *Elodea nuttalli* (mean ± SD) grown under different concentrations of NH_4–N and diversified redox states (oxic, reduced, highly reduced).

2.4.7. Chloroplast number and appearance

In the oxic treatment, the number of chloroplasts was not noticeably affected at 2.5 and 5 ppm

NH_4^+ concentrations. However, at higher NH_4^+ concentrations (10 and 40 ppm) the chloroplast number in the cells became drastically reduced, with brown discoloration at the center of the chloroplast (Fig. 2.7). In plants subjected to high NH_4–N concentration along hypoxic/anoxic treatments chloroplasts appeared disrupted, with brown-black discoloration of the leaves. For this reason it was impossible to count the chloroplast numbers in such leaves.

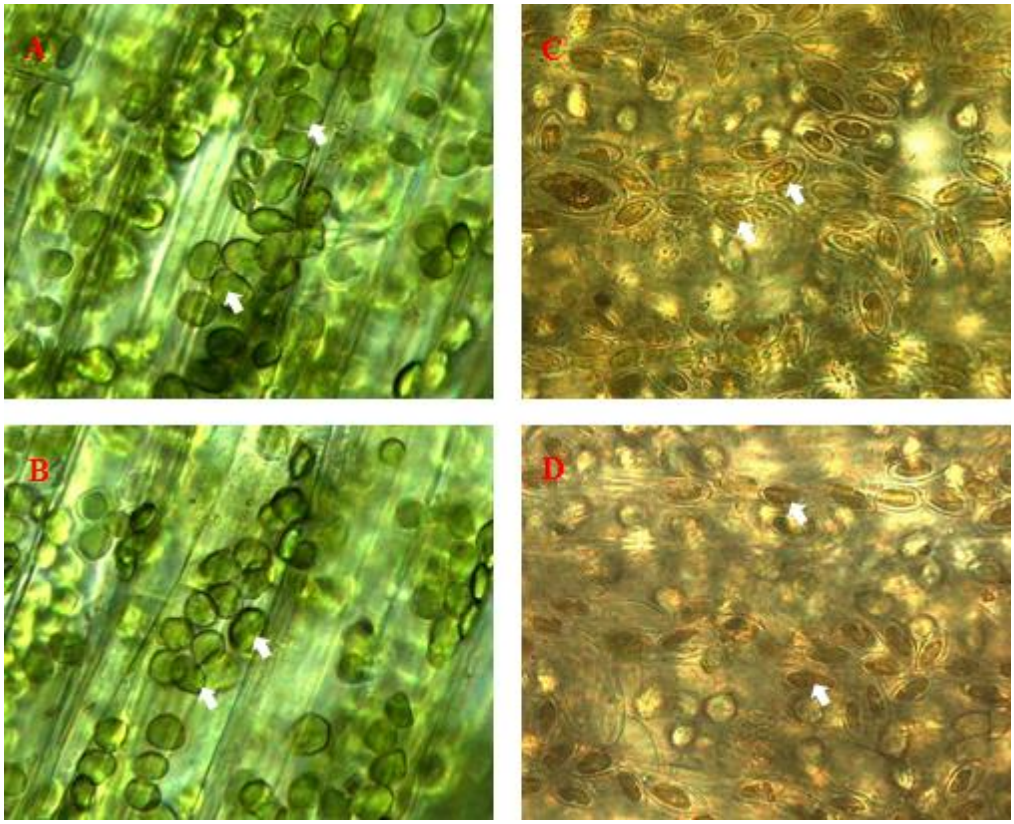


Figure 2.7 Microscopic appearance of the longitudinal apical section of shoots of *Elodea nuttallii* at (A) 2.5 ppm, (B) 5 ppm, (C) 10 ppm and (D) 40 ppm NH_4^+ concentrations. White arrows point to chloroplasts.

2.5. Discussion

In the middle of the 20th century, an almost explosive spread of *Elodea nuttallii* occurred in Japan and had a profound influence on the other hydrophytes and the plant communities of the inland waters (Kadono et al., 1997). Since the late 20th century, noticeable declines of *E.*

nuttallii populations in Japan have been reported repeatedly (Nagasaka, 2004). A similar rapid decline was also observed for the alien *Elodea canadensis* in Europe in the middle of the 20th century (Sculthorpe, 1967), and scientists have suggested different stress (biotic and abiotic) factors causing this decline (Kadono et al., 1997). Exposure to various stress factors can lead to the disruption of cellular and molecular processes in plants, which ultimately negatively affects plant growth. In our study, both reduced and highly reduced water conditions drastically reduced RGR and SGR of *E. nuttallii*, with higher NH_4^+ concentrations even accelerating these effects. Brix and Sorrell (1996) also reported that wetland plants grown in reducing aquatic environments stopped growing and some lost biomass. Marschner (1995) suggested that the physiological mechanism of ammonium toxicity in plants lies in the ability of ammonia to uncouple photosynthetic electron transport, which may lead to necrosis through inhibition of photosynthesis. Moreover, a secondary toxic effect may result from an inability to buffer the protons, released from ammonium assimilation, and ultimately, affecting the function of many enzymes and membrane processes.

In the oxic treatment of present study, when NH_4^+ concentration was high, and in oxygen deprived treatments at low to high NH_4^+ concentrations, chlorophyll synthesis was significantly affected in a negative way. Loss of chlorophyll may have disturbed the photosynthetic machinery, so that the electron transport rate of PSI and PSII could not work regularly, leading to generation of ROS. By feedback effect, ROS accumulation can further impede the chlorophyll biosynthesis, either directly or indirectly by inhibiting the activity of the photosynthetic machinery [Fig. 2.7; see also Asada (1994) and Dominguez et al. (2008)]. The damages of the chloroplast structure that occurred during the experimental period in oxygen deprived treatments imply functional changes of the photosynthetic process. In comparison to chlorophyll, carotenoids were even more severely reduced by higher NH_4^+ concentrations. Carotenoids are highly effective in quenching of chlorophyll triplet states and

singlet oxygen (Lichtenthaler, 1987). This highly cytotoxic singlet oxygen ($O_2^{-\bullet}$) can seriously disrupt normal metabolism through oxidative damage to the photosynthetic apparatus (Chen and Cheng, 2003). Carbohydrate levels are determined by the balance between production of carbohydrates in photosynthesis and access to storage and consumption in respiration or fermentation and for growth. Because all the plants showed retarded root elongation under hypoxic treatment, the accumulation is probably due to a decreased incorporation of carbohydrates in growth processes. The degree of damage to the photosynthetic apparatus resulting from anoxia was determined by chlorophyll fluorescence of PSII in dark adapted leaves, where the Fv/Fm values considerably decreased with increased oxygen stress (to values <0.4). The Fv/Fm ratio, the maximum quantum yield of PSII photochemistry, is frequently used as an indicator of photoinhibition or of other kinds of stress to photosystem II (Calatayud and Barreno, 2004).

Apart from the photosynthetic apparatus, the cell membrane which is constituted by lipids, often becomes the primary target for the action of stress factors (Chirkova, 1997). MDA content of plant is an indicator of the lipid peroxidation which can occur there. It increased in the *E. nuttallii* leaves in reduced treatments but decreased at final harvest in highly reduced treatments, which might have resulted from the plant senescence that restricts further generation of MDA. We found a positive correlation ($r = 0.87$, $n = 64$, $P < 0.001$) between H_2O_2 level and MDA content, which goes along with the findings of (Yang and Miao, 2010) who repoted about similar results when drought stress affected two *Populus* species. On the other hand, chl a ($r = -0.94$, $n = 64$, $P < 0.001$), chl b ($r = -0.81$, $n = 64$, $P < 0.001$) and carotenoid contents ($r = -0.85$, $n = 64$, $P < 0.001$) showed a negative correlation with H_2O_2 concentration.

POD is an essential component of the anti-oxidative defense system and considered to be a stress marker enzyme with a high affinity for H_2O_2 [Fig. 2.7; see also

Andrews et al. (2002)]. It is activated as a short-term stress response (Domínguez et al., 2010). The high concentration of cellular H_2O_2 and the elevated POD activity in our experimental plants suggest that the ROS scavenging system was activated there under the given stress conditions. Similarly, Wang et al. (2008) found the increase of POD in Vallisneria under high ammonium concentration, findings that support our results with Elodea. We can assume that the observed H_2O_2 accumulation in the tissues may promote the loss of leaves, since high H_2O_2 accumulation has been identified as an important mechanism of leaf senescence (Wang et al., 2008).

Phytohormones can regulate the synthesis of basic antioxidant enzymes, and some of the isoforms of antioxidant enzymes are also implicated in phytohormone catabolism (Fig. 2.7). Therefore, these hormones play a major role in the regulation of developmental processes and in signaling networks under stress conditions. IAA is one of the major growth hormones related to shoot elongation and cell division and it may be the dominant factor controlling shoot elongation in *E. nuttallii* (Ellawala et al., 2011). We found a strong negative correlation between IAA and H_2O_2 concentration ($r = -0.96$, $n = 64$, $P < 0.001$) which suggested that H_2O_2 concentrations in the tissues alter the levels and distribution of IAA (Pasternak et al., 2005). Oxidative stress affects auxin by oxidizing IAA via induction of POD activity (Kawano, 2003). Furthermore, IAAO and POD activities are known to catabolize IAA (Hiraga et al., 2001) and to reduce auxin-induced growth of plants; similar phenomena became obvious also in our experiment. Kovtun et al. (2000) proposed with regard to such metabolic interactions that the regulation of auxin transport and auxin-dependent activities of H_2O_2 may shift the energy utilization toward stress protection and survival. The data (concentration of IAA and H_2O_2) gained in our experiment support the concept that stress-induced alterations in plant growth are not simply a cessation of growth but involve specific reorientation of growth, that is mediated by metabolism of ROS and by an auxin

induced morphogenetic growth response (Potters et al., 2009). The dramatic loss of nitrogen found in reduced and highly reduced treatments (in all NH_4^+ concentrations), suggests a direct effect of the decreased IAA concentration.

Lower values for carbon were also found in plants under reducing environment in all conditions and at 10 and 40 ppm NH_4^+ levels under oxic treatments, which supports the hypothesis that carbohydrate limitation may contribute to the toxicity syndrome in plants feed with high levels of NH_4^+ (Kronzucker, 1998). Under eutrophic conditions, high amounts of carbon and energy are needed for NH_4^+ extrusion and NH_4^+ assimilation and hence, it is difficult for submerged species (*Elodea*, *Potamogeton*, *Hydrilla*, *Vallisneria*) to survive at such conditions (Litav and Lehrer, 1978). Loss of carbon may also have resulted from the catabolism of sugar into energy when the plants were stressed. On the other hand, proline accumulation is considered to be involved in stress resistance mechanisms (Lutts et al., 1999). At higher concentration of NH_4^+ under oxygen deprived treatments the concentration of proline decreased, which might be due to the inability in highly stressed treatments to further produce the protective molecules.

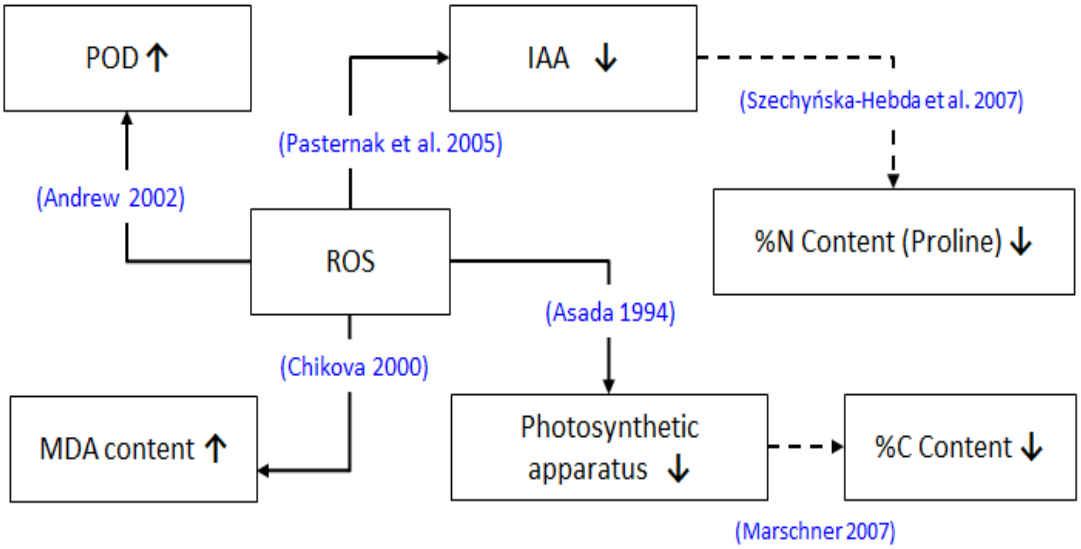


Figure 2.8 A schematic diagram of the effects of reactive oxygen species

(ROS) on hormonal activities of *Elodea nuttallii* (upward or downward arrow in a block indicates that the corresponding parameter is affected positively or negatively, respectively by ROS).

2.6. Conclusion

Our data reconfirmed the previous record that NH_4^+ toxicity results in chlorosis of leaves (brown–black discoloration of the leaves), suppressed growth, and decreased photosynthetic rates in *E. nuttallii* (Dendène et al., 1993). In addition, we found oxygen deprivation stress conditions to affect negatively in a still higher degree the growth, photosynthetic apparatus as well as the C–N balance in plant, and high NH_4^+ concentrations in the surrounding water accelerate and intensify these toxic effects of hypoxia. Overall in became evident that the submerged macrophyte *Elodea nuttallii* is rather poorly tolerant to hypoxia/anoxia in terms of cell detoxification responses. Therefore, reduced redox states of the sediment in combination with high NH_4^+ concentrations of the water play an important role in distribution and abundance of this species. Further studies are recommended to evaluate the macromicro elements accumulation capabilities of *E. nuttallii* under such oxygen-altered environments under aspects of the load of industrial and domestic wastewaters with these, in some cases even at low concentrations toxic ions and possible accumulator or avoider traits of this submerged macrophyte.

Chapter 3. **2ND EXPERIMENT: ASSESSMENT OF MACRO AND MICROELEMENTS ACCUMULATION CAPABILITIES OF *ELODEA NUTTALLII* UNDER OXYGEN STRESS AND ELEVATED NH_4-N CONCENTRATION**

Abstract

Reducing soil conditions comprehend soil oxygen deprivation, at the same time producing various compounds in soil, many of which are considered highly phytotoxic. *Elodea nuttallii* (Planch.) is a submerged aquatic macrophyte, has flexile ability to use different nutrient source from various environments. In the present study, *Elodea nuttallii* was subjected to various redox conditions at both low and high ammonium concentration, and evaluated for macro and microelement accumulation. Reduced environment was prepared by adding glucose to growth medium and nitrogen gas bubbling, whereas oxic treatment was made by atmospheric air bubbling. Plants under oxygen deprived conditions were manifested with heavy metal (HM) toxicity; reduction of biomass and photosynthetic pigments, biochemical disorders such as excess generation of ROS, lipid peroxidation and reduction of major macro elements. In reduced treatments, BCF sequence for micro and non-essential elements was Cu>Mn>Zn>Al>Cd>Fe>Pb at both low and high NH_4^+ conditions. Proline level was found to decline in plants under oxic treatments by high NH_4^+ concentrations however, in reduced treatments the proline content did not decline significantly with the increment of NH_4-N concentration. The combined effect of low redox state and high ammonium concentration has strong physiological impact on the submerged macrophyte than the two factors acting separately. However, macro and micronutrients accumulation was more significantly affected by reduced environment than the high NH_4-N concentration (as for oxic treatments).

3.1. Introduction

Generally in wetland soils, plants are faced with not only the lack of oxygen but a substantial

demand for oxygen in the sediment (DeLaune et al., 1990, Kludze and Delaune, 1996b) that is represented by the reducing conditions in the soil (low soil Eh). Extensive field data on the relationship between redox potential and community distribution in saltmarshes were presented by (Armstrong and Beckett, 1985). Metal mobility and metal (loid) fate in sediment and in wetlands is governed by a number of soil factors and processes; e.g., adsorption/desorption reactions, precipitation/dissolution and complexation /decomplexation, salinity, organic matter content, sulphur (S) and carbonates, plant growth, pH and redox potential (Eh) as well as microorganisms activity (Du Laing et al., 2009, María-Cervantes et al., 2010). Oxidation and reduction process subsequently affect pH (Yu et al., 2007b) which directly related to stability and solubility of various metal and nutrient elements in soils and sediments, and their availability to plants (Reddy and Patrick, 1977). According to Devai and DeLaune (1995) redox potentials of soils can range from -300 to + 700 mV and anaerobic soils exhibit redox potentials from + 350 mV to as low as - 250 to -300 mV. Soils tend to undergo a series of sequential redox reactions in a homogenous environment when soil redox status changes from aerobic (high Eh) to anaerobic (low Eh) conditions and vice versa. Major reactions include, in order of Eh from high to low, nitrification, denitrification, manganic manganese [Mn (IV)] reduction, ferric iron [Fe (III)] reduction, sulfate (SO_4^{2-}) reduction, and methanogenesis (Patrick et al., 1996). With the reduction of oxides and microbial activity elements that are fixed in them such as phosphorus (P), molybdenum (Mo), cobalt (Co), copper (Cu), Zn are often transformed to a more mobile and plant available form (Jackson et al., 1993, A.J. and C.J., 1990) under anoxic condition. Lower sediment pH under mildly oxic redox conditions increase the bioavailability of Al, Cu, Fe, Mn and Zn to rooted aquatic plants (Jackson et al., 1993). Submerged aquatic plants are adapted to detoxify reduced elements by releasing root oxygen to the rhizosphre, but this phenomenon is also governed by Eh condition and microbial oxygen demand (Laskov et al., 2006). High contents of heavy metals in soils would increase the potential uptake of these

metals by plants. The concentration of metals in plants can be more than 100,000 times greater than in the associated water (Albers and Camardese, 1993) . Furthermore, accumulation of various soil phytotoxins, that are by-products of soil reduction, may lead to injury to certain species. Potential toxicity of trace metals result from the fact that they are transitional elements able to form stable coordinated compounds with a range of both organic and inorganic ligands. From a physiological-ecology standpoint, the knowledge of soil Eh represents an indication of the status of various soil compounds (Pezeshki, 2001). Heavy metals in soils may be present in several forms with different levels of solubility as follows: (i) dissolved (in soil solution), (ii) exchangeable (in organic and inorganic components), (iii) structural components of the lattices in soils and (iv) insolubly precipitated with other soil components (Aydinalp and Marinova, 2003). Usually, only the first two forms are able to be absorbed and utilized by plants. Therefore, plant uptake of a metal is mainly dependent on the metal mobility and availability in soils. Recent studies on HM toxicity revealed that these metals may cause molecular damage to plant cells either directly or indirectly through the excess generation of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$) and superoxide radicals ($O_2^{\cdot -}$). These can damage membrane and inactive several enzymes by reacting very rapidly with DNA, lipids, pigments and proteins (Weckx and Clijsters, 1996). Thus low redox conditions exert substantial influence on physiological processes of plants.

Elodea nuttallii is submerged aquatic rooted macrophytes can absorb nutrients either by roots or shoots or by both together in varying proportions (Barko et al., 1991). This species is previously well known as hyper accumulator of various metals and elements as well as stress resistance to various environmental factors (Mishra and Tripathi, 2008). Submersed macrophyte effects on sediment dynamics have been characterized by sediment-oxidation status in both field and laboratory studies (Boon and Sorrell, 1991, Jaynes and Carpenter, 1986). The capability of submerged macrophytes shoots and roots to

accumulate trace metals, depending on the element concentrations in the sediments and/ or water, allows their use in trace element biomonitoring in lake ecosystems (Baldantoni et al., 2004). Physical factors which fluctuate temporarily including pH, redox potential, temperature, salinity or light as well as presence of other metal ions in the surrounding aquatic environment strongly affect metal uptake by submerged plants (Fritioff et al., 2005). The comprehensive analysis of macro-micro nutrients transformation in this study represent the main factors regulating the major biochemical processes under moderately reduced to oxic environment.

3.2. Objectives

Soil redox status and its effect in wetlands plants and crops have been vigorously studied in the last three decades. The effect of reduce environment on aquatic macrophyte is very scanty (DeLaune et al., 1999, Jackson et al., 1993). Increased ammonium concentration and low redox status (reduced condition) in the natural habitat (due to pollution or eutropication) are two prominent characteristics associated with eutropic lakes, such as Plesne Lake in Central Europe (Kopáèek et al., 2004). Furthermore, under reduced environment different oxidize elements become available in the surrounding environment. The accumulation of heavy metals in the submerged plant, *Elodea* has been studied by Brown and Rattigan (1979). These elements which are not biodegradable, in excess concentration might cause deleterious effect by disordering physiological and biochemical processes in the plant cells (Lu et al., 2007) and might contributes to the food chain. Therefore, it is imperative to estimate the effect of soil properties on the availability and the uptake of heavy metals by plants to minimize the toxic effects and the translocation to food chains. The effect of reduced environment along with high NH_4-N concentrations on nutrient uptake of freshwater macroalgae and submerged macrophytes is needed to be investigated. In viewing of the effect of Eh and ammonium concentration on the solubility and availability of heavy metals in soils, the current experiments

were conducted to

(i) determine extractable contents of chromium (Cr), copper (Cu), iron (Fe), manganese (Mn), lead (Pb) and zinc (Zn) in soils and water,

(ii) their concentration and translocation in *Elodea nuttallii* and

(iii) the oxidative damage caused by these elements under normal and high NH_4-N concentrations. This study might be helpful for phytoremediation technologies in terms of selection of hyper accumulator.

3.3. Materials and Methods

3.3.1. Experimental Design

E. nuttallii was subjected to gradient redox potentials under normal and high NH_4-N concentrations. Since it was difficult to keep a constant redox potential throughout the experiment period, a range of potential was maintained. Three levels of redox potential were used, as (i) +400 mV ~ +440 mV, (Oxic; O_1), (ii) -5 mV ~ +5 mV (hypoxic/moderately reduced; O_2) and (iii) -180 mV ~ -120 mV (anoxic/highly reduced; O_3) (Fig. 3.1).

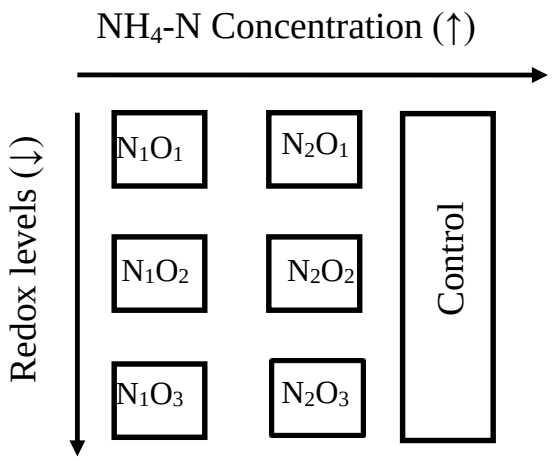


Fig. 3.1. Layout of the experimental set-up (7 microcosms/treatment). NH_4-N Concentration and redox levels are presented as N and O, respectively. Microcosms were randomly distributed with equal spacing in growth chamber.

In case of nitrogen source, the suitable NH_4-N concentration for the plant is 2.5 ppm (Ozimek et al. 1993). Here, two different NH_4-N concentrations {2.5 (N_1) and 10 (N_2) ppm} were used as two different conditions (Fig. 3.1).

3.3.2. Soil, plant and water analysis

Soil samples were air-dried, homogenized and sieved to <2 mm. The particle sizes of the soil samples (in terms of D50) were determined using sieves according to the American Society for Testing and Materials protocol (ASTM D422-63, 2002). Plants were carefully washed using tap water and finally with distilled water, and were separated into leaves, shoots and roots. Plants materials were dried using oven drier at 60 ^{0}C until constant weight. Plants materials were reweighed (for dry weight), homogenized by grinding into fine powder using a mortar and pestle. Powdered samples were stored in airtight vials for subsequent analysis. TN, TC of powdered plant samples were measured by CHN coder (YANACO MT-3). About 10 mg of dried plant sample and 200 mg of dried soil sample were digested at 200 ^{0}C with di-acid mixture (nitric acid : perchloric acid; 1 : 2) until evolution of nitrous gas had stopped and the digest became clear. The digests were diluted with distilled water to a total of 100 ml and passed through Whatman 42 filter paper. Organic matters in the soil were measured by Walkley-Black method (1934). The concentration of following elements were measured in the soil and plant samples: Fe, Mn, Zn, Pb, Ca, Mg, Cu and K with atomic absorption spectrophotometer (AAS; Shimadzu AA-660G) using direct air-acetylene flame method, and the concentration of Al and Cd were determined with graphite furnace atomizer (GFA-4B), according to standard procedures and using commercial laboratory standards. Total sulphur (S) was measured after barium chloride method respectively. Replicate samples were analyzed separately, analyses were done in duplicate and results for plant materials and sediments were calculated on dry weight basis. Water samples were collected at 7 days interval and were passed through

Whatman glass microfibre filters GF/C and stored at 4°C until analysis. The concentration of Fe, Mn, Zn, Pb, Ca, Mg, Cu, K, Al, Cd and S of water sample were measured following the methods as were used for soil and plant sample analysis.

3.3.3. Biomass increment

At 14th DAT, two plants from each tank was harvested, cleaned with tap water and fresh biomass was measured after blotting with laboratory towel. The fresh biomass increment was calculated as the percent increment of plant mass relative to initial fresh mass at the time of transplanting, using the following equation (Eq.1)

$$B_t = (F_t - F_0) \times |(100/F_0)| \qquad [3]$$

Where Bt is the increased biomass (% relative to initial fresh biomass) at the 14th DAT, Ft is the fresh biomass at 14th DAT and Fo is the initial fresh biomass of the plant.

3.3.4. Bioconcentration factor (BCF) and Translocation factor (TF)

The bioconcentration factor (BCF) is an index to express the ability of plant to accumulate metal with respect to metal concentration in substrate. BCF was calculated by the following formula

$$BCF = \frac{\mathrm{P}\ (ppm)}{\mathrm{T}\ (mg/kg)} \qquad [4]$$

Where, P represents the trace element concentration in plant tissues (ppm), T represents the residual concentration in sediment (mg/Kg dry weight).

Translocation factor is an indication of the ability of the plant to translocate metals from the roots to the aerial parts of the plant. TF was calculated by the following formula:

$$TF = \frac{\mathrm{M_a}}{\mathrm{M_r}} \qquad [5]$$

TF= (Metal concentration in aerial parts)/(Metal concentration in roots)

Translocation factors (TF) for trace elements between sediment and roots and within a plant were expressed by the ratios of [Trace element] $_{sediment}$/ [Trace element] $_{root}$ and [Trace element] $_{root}$/ [Trace element] $_{(shoot + leaves)}$ to show trace elements translocation properties from sediment to roots and from roots to shoots.

3.3.5. Biochemical parameters and Chlorophyll content study

The biochemical parameters such as H2O2, POD, MDA , Proline and Chlorophyll contents (Chla, Chlb and carotenoid) were studied and the methods are as same as mentioned in Experiment-1.

3.3.6. Statistical Analysis

The experiment was set up as a completely randomised design, with four replications of each treatment. Data were analysed statistically, using the SPSS 13.0 software package, by ANOVA and by Tukey's multiple range test, to determine differences between means. Before performing a statistical analysis, data were checked for normal distribution. Pearson correlation coefficients were calculated to determine the relationship between sediment pH, Eh and sediment metal contents.

3.4. Results

3.4.1. Biomass increment

Plants subjected to high concentration of NH_4-N along with hypoxic/anoxic treatments showed brown-black discoloration of the leaves, and biomass increment values were negative (at 10 ppm). At 2.5 ppm NH_4-N nutrition condition by hypoxic and anoxic treatment, the fresh biomass

was declined 73.02 and 80%, respectively. Increment of ammonium in oxic treatment considerably declined biomass too (Fig. 3.2). When oxygen level decreased biomass was more affected at both ammonium levels. Biomass increment varied between different redox treatments ($P < 0.001$) and was significantly affected ($P < 0.01$) by high NH_4-N concentration.

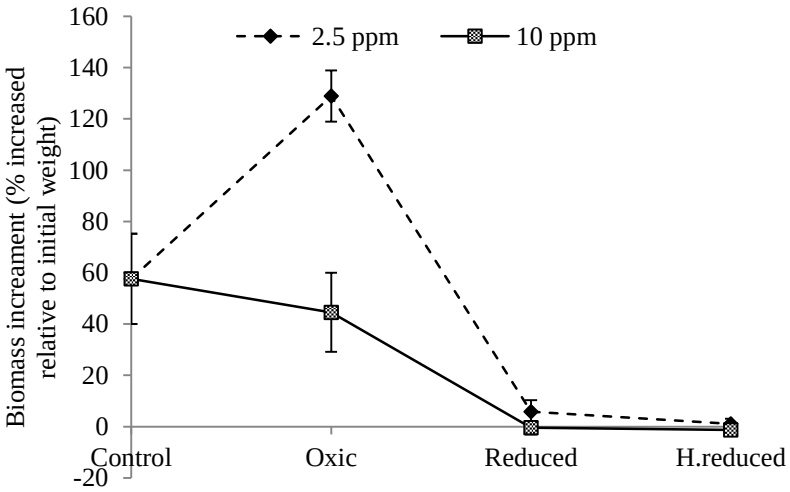


Figure 3.2 Effect of different concentrations of NH_4-N under various redox conditions on biomass in *Elodea nuttallii*. The data are presented as the mean ± SD.

3.4.2. Elements bioaccumulation and translocation in plant

In terms of bioaccumulation factor (BCF), there was significant difference ($P<0.001$) found among the elements in different treatments at both conditions (Table-3.1). In all treatments K concentration was highest in plants. In control and oxic treatments the higher accumulation was found for macro elements and then essential nutrients (Cu, Mn, Fe and Zn). The non-essential elements (Pb, Cd) in oxic treatments were found with lowest accumulation, which was opposite to the concentration found in reduced treatments.

Table 3.1 Bioaccumulation factor for elements in *Elodea nuttallii* (Mean ± SD) under different condition and treatments. Different letter superscripts at each column

indicate significant differences between treatments.

Elements	2.5 ppm NH_4-N				10.0 ppm NH_4-N		
	Control	Oxic	Reduced	H.reduced	Oxic	Reduced	H.reduced
Ca	3.6±0.6^{a}	3.3±0.3^{a}	0.4±0.1^{b***}	0.2±0.1^{b***}	2.9±0.2^{a}	0.3±0.1^{b***}	0.3±0.1^{b***}
Mg	9.6±3.3^{a}	10.9±3.8^{a}	4.9±1.1^{b**}	3.8±0.8^{b**}	10.1±3.1^{a}	4.2±0.5^{b**}	3.1±0.2^{b**}
K	14.3±4.6^{a}	13.9±4.1^{a}	4.9±1.6^{b**}	4.2±1.1^{b**}	12.6±4.8^{a*}	4.2±1.0^{b**}	3.6±0.8^{b**}
S	2.3±1.1^{b*}	2.6±0.8^{b}	3.9±1.2^{a*}	4.0±1.2^{a*}	2.7±1.0^{b}	3.3±0.8^{a}	3.5±0.6^{a}
Fe	0.3±0.0^{b}	0.3±0.0^{b}	0.7±0.1^{a*}	0.8±0.1^{a*}	0.3±0.0^{b}	0.9±0.1^{a*}	1.0±0.0^{a*}
Cu	0.6±0.1^{c}	0.7±0.2^{c}	4.6±0.5^{b**}	5.9±0.6^{a**}	0.7±0.2^{c}	5.5±0.4^{b**}	6.3±0.8^{a**}
Mn	0.5±0.2^{be}	0.5±0.1^{b}	2.8±0.2^{a**}	2.9±0.4^{a**}	0.5±0.1^{b}	3.4±0.1^{a**}	3.1±0.0^{a**}
Zn	0.8±0.1^{b}	0.9±0.1^{ab}	1.1±0.1^{a}	1.2±0.0^{a}	0.9±0.1^{b}	1.3±0.1^{ab}	1.5±0.2^{a*}
Cd	0.1±0.0^{b}	0.0±0.0^{b}	0.9±0.1^{a***}	1.1±0.1^{a***}	0.0±0.0^{b}	0.8±0.1^{a**}	0.9±0.2^{a**}
Pb	0.1±0.0^{b}	0.1±0.0^{b}	0.5±0.1^{a*}	0.6±0.0^{a*}	0.1±0.0^{b}	0.6±0.1^{a*}	0.6±0.0^{a*}
Al	0.7±0.2^{b}	0.7±0.1^{b}	1.1±0.2^{a*}	1.1±0.2^{a*}	0.7±0.1^{b}	1.1±0.1^{a*}	1.1±0.1^{a*}

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table-3.2 and 3.3 shows the TF of elements in different body parts of *Elodea nuttallii*. Under control and oxic treatments Pb, Cd and Fe showed lower potential to accumulate in plant whereas in reduced treatments, as the metals were bioavailable these elements showed high accumulation in plant root except Pb. In the translocation course from soil to root, the TF of micro and non–essential elements in reduced treatments orders as Zn> Mn>Cu>Cd>Al>Fe>Cd>Pb.

Table 3.2 Translocation factor (sediment/root). Different letter superscripts at each column indicate significant differences between treatments.

Elements	2.5 ppm NH_4-N				10.0 ppm NH_4-N		
	Control	Oxic	Reduced	H.reduced	Oxic	Reduced	H.reduced
Ca	2.9 ± 3.0^{d}	3.0 ± 0.8^{cd}	$13.6\pm9.0^{b**}$	$30.2\pm3.0^{a***}$	3.0 ± 0.6^{c}	$13.6\pm9.0^{b**}$	$39.2\pm9.8^{a***}$
Mg	0.5 ± 0.2^{b}	0.6 ± 0.1^{b}	$1.4\pm0.2^{a**}$	$1.4\pm0.2^{a**}$	0.6 ± 0.1^{b}	$1.6\pm0.3^{a**}$	$1.6\pm0.1^{a**}$
K	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	$0.9\pm0.2^{a*}$	$0.9\pm0.1^{a*}$	0.3 ± 0.0^{b}	$0.9\pm0.0^{a*}$	$1.0\pm0.2^{a*}$
S	1.4 ± 0.5^{a}	1.1 ± 0.1^{b}	1.0 ± 0.2^{b}	1.0 ± 0.2^{b}	1.2 ± 0.3^{a}	1.2 ± 0.3^{a}	1.3 ± 0.2^{a}
Fe	9.3 ± 1.6^{a}	7.9 ± 0.9^{b}	$6.9\pm0.2^{bc*}$	$6.6\pm0.1^{c*}$	7.0 ± 0.0^{a}	5.8 ± 1.3^{b}	5.9 ± 2.1^{b}
Cu	4.8 ± 0.9^{a}	3.2 ± 0.4^{b}	$1.4\pm0.3^{c*}$	$1.2\pm0.1^{c*}$	3.9 ± 1.4^{a}	$1.3\pm0.3^{b*}$	$1.4\pm0.4^{b*}$
Mn	4.9 ± 1.7^{a}	4.9 ± 0.3^{a}	$1.2\pm0.1^{b*}$	$1.1\pm0.1^{b*}$	4.6 ± 0.4^{a}	$1.2\pm0.1^{b*}$	$1.0\pm0.1^{b*}$
Zn	0.7 ± 0.0^{a}	0.7 ± 0.0^{a}	0.6 ± 0.0^{ab}	0.8 ± 0.1^{a}	0.7 ± 0.0^{a}	0.7 ± 0.1^{a}	0.8 ± 0.1^{a}
Cd	$18.6\pm7.6^{b**}$	$23.8\pm4.2^{a**}$	1.9 ± 0.7^{c}	1.5 ± 0.3^{d}	$28.7\pm5.2^{a**}$	1.9 ± 0.4^{b}	1.7 ± 0.1^{b}
Pb	$34.2\pm4.0^{a**}$	$32.2\pm5.7^{a**}$	$24.2\pm2.4^{b*}$	$22.4\pm2.3^{b*}$	31.9 ± 6.1^{a}	$19.8\pm1.1^{c*}$	$23.5\pm2.0^{b**}$
Al	4.0 ± 0.0^{a}	4.1 ± 0.1^{a}	$2.8\pm0.0^{b*}$	$2.8\pm0.0^{b*}$	4.1 ± 0.0^{a}	$3.0\pm0.0^{b*}$	$2.9\pm0.0^{b*}$

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Table 3.3 Translocation factor (root/(shoot+leaf)). Different letter superscripts at each column indicate significant differences between treatments.

Elements	2.5 ppm NH_4-N				10 ppm NH_4-N		
	Control	Oxic	Reduced	H.reduced	Oxic	Reduced	H.reduced
Ca	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	0.3 ± 0.1^{b}	$0.2\pm0.0^{b*}$	0.5 ± 0.0^{a}	0.3 ± 0.1^{b}	$0.1\pm0.0^{c**}$
Mg	0.3 ± 0.1^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.2 ± 0.0^{a}	0.3 ± 0.0^{a}
K	0.4 ± 0.1^{a}	0.4 ± 0.1^{a}	0.3 ± 0.0^{a}	0.3 ± 0.1^{a}	0.3 ± 0.0^{a}	0.3 ± 0.0^{a}	0.3 ± 0.1^{a}
S	0.5 ± 0.3^{a}	0.5 ± 0.2^{a}	0.4 ± 0.1^{a}	0.3 ± 0.0^{b}	0.5 ± 0.2^{a}	0.3 ± 0.1^{b}	0.3 ± 0.0^{b}
Fe	0.7 ± 0.2^{a}	0.6 ± 0.0^{a}	$0.3\pm0.1^{b**}$	$0.3\pm0.0^{b**}$	0.7 ± 0.1^{a}	$0.3\pm0.0^{b**}$	$0.2\pm0.0^{b**}$
Cu	0.5 ± 0.1^{b}	0.7 ± 0.2^{a}	$0.2\pm0.1^{c**}$	$0.2\pm0.0^{c**}$	0.6 ± 0.1^{a}	$0.2\pm0.0^{b**}$	$0.1\pm0.0^{b**}$
Mn	0.8 ± 0.2^{a}	0.6 ± 0.0^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}	0.6 ± 0.0^{a}	0.4 ± 0.0^{b}	0.4 ± 0.0^{b}
Zn	0.3 ± 0.0^{b}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	0.3 ± 0.1^{b}	0.5 ± 0.0^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}
Cd	1.8 ± 1.0^{b}	$0.9\pm0.5^{c**}$	$2.3\pm0.8^{a*}$	1.9 ± 0.2^{b}	$0.8\pm0.3^{b**}$	1.9 ± 0.3^{a}	1.7 ± 0.0^{a}
Pb	0.3 ± 0.0^{a}	0.4 ± 0.1^{a}	$0.1\pm0.0^{b*}$	$0.1\pm0.0^{b*}$	0.4 ± 0.1^{a}	$0.1\pm0.0^{b*}$	$0.1\pm0.0^{b*}$
Al	0.5 ± 0.0^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}	0.5 ± 0.0^{a}	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

3.4. Discussion

Reducing soil condition comprehends soil oxygen deprivation at the same time produce various compounds in soil, many of which considered as highly phytotoxic (Pezeshki and DeLaune, 2012). The results of the present study revealed the combined effects of low redox condition and high ammonium concentration triggered changes in macro-micro nutrient accumulation and the induction of oxidative stress in the plants. In this study, concentration of all elements increased in reduced and highly reduced treatments both in pore water and surface water, as the metal oxides (like Fe and Mn oxides) are reduced to soluble form (Ponnamperuma, 1972). Under reduced treatments the soil pH varied in range 5-4.2. Fe, Mn, Cu, Zn, Cd. Pb and Al were found soluble at low pH under reduced treatments, which was in accordance with the findings of previous studies (Du Laing et al., 2009, Miller et al., 2010). The reduction of Fe

(hydr) oxides also depends on the total Fe content in the bulk soil, which was higher in our experiment. Under oxic conditions the solubility of metals were found low as, hydrous Mn and Fe oxides provides sites for the sorption of other metals which in turn immobilize the heavy metals (Gambrell, 1994). Moreover, the pH value was around 6.5-7.0, in oxic treatments, which might favors to decrease the solubility of these metals (Miller et al., 2010). The concentration of elements under reduced treatments was found to increase at the end of the experiment, which might be due to the re-release of chemicals from plant body into the environment (Mal et al., 2002).

It is crucial to assess heavy metal mobility and heavy metal concentration in plant tissue. Bioavailability depends both on the heavy metal mobility and on the considered plant species (Greger, 2003). Bioconcentration factor (BCF) and translocation factor (TF) were calculated to study the accumulation characteristics of different essential and non-essential elements in different body parts (leaf, shoot and root) of the plants. Excluder plants have TF values $\ll$ 1, whereas for hyper accumulators it is $\gg$1. In general essential nutrients such as Cu, Mn and Zn showed higher translocation factors than the non-essential As, Cd and Pb. Elevated TF implies a potential risk of metal and accumulation of elements on the soil surface. The phyotoxicity due to different element depends on metal type, metal concentration and duration of exposure as proposed by Odjegba and Fasidi (2007). (Nagajyoti et al., 2010) suggested that metals uptake and distribution in submerged plant species vary according to the relative concentration of the element in the environment, the growth form of the plant, type of absorption mechanism, metal speciation, metal stability and constants with ligands, redox potential and pH at water-sediment interface, light, and microbial activity. The final metal concentration in plants under reduced treatments was found significantly larger than in the surrounding water, which was also in agreement with Samecka-Cymerman and Kempers (2007). The presence of high concentrations of heavy metals seems to be directly associated

with the exclusion of essential nutrients. This exclusion applies in our study to the below-critical concentrations of K, Ca and Mg in all plants in reduced treatments under both conditions. The value of BCF < 0.2 normal for plants growing on polluted materials. The BCF sequence for micro and non-essential elements was Cu>Mn>Zn>Al>Cd>Fe>Pb in both conditions under reduced treatments (table-3.2), which was not in agreement with the earlier reports stated by (Rai et al., 1995), on metal accumulation potential of submerged macrophytes. The more mobile elements found in present study were Cu and Mn. Therefore, these two elements might cause the prime metal effect on plant, as the higher the mobility was, the more significant the toxic effect. *Elodea canadensis* and *Elodea nuttallii* are considered as good candidates for metal toxicity investigations (Kähkönen and Kaireselo, 1998) because they are widespread and there is evidence that they accumulate pollutants (Kähkönen et al., 1997). The review of scientific literature over the past 34 years showed that trace metal phytotoxicity followed the general trend (from most toxic to least toxic): Pb, Hg >Cu >CdAs >Co, Ni, Zn >Mn (Kopittke et al., 2010). However these two species are appeared to be very sensitive to copper levels (Nakada et al., 1979, Mal et al., 2002). Metals such as Pb or Cd cause the destruction of the *E. canadensis* protoplast (Stoyanova and Tchakalova, 1999). The TF for macro elements, especially Ca (0.3), were found very low. Trace metal concentrations in aquatic plants vary considerably according to the part of the plant as well as to the chemical characteristics of the elements. Baldantoni et al. (2004) suggested that submerged macrophytes probably take up the elements in the shoots from water; moreover, take up the mobile elements by the roots. In general essential nutrients such as Cu, Mn and Zn showed higher TF likely due to the metabolic use than the non-essential Cd and Pb in oxic treatments. Generally heavy metals are easily retained mainly on exchangeable sites localised in cell walls. However, Cu available in the soils was not sequestered in roots and was quickly translocated into shoots. For Mn, shoot

concentrations were higher than root concentrations. Mn and Zn were also gradually translocated during the experiment. Generally, Mn is known to be rapidly taken up and translocated within plants. However, the Mn content of plants is an effect not only of plant characteristics but also of the pool of available Mn, which is highly controlled by soil properties and particularly by soil acidity and redox conditions (Pendias and Kabata-Pendias, 2000). In reduced treatments due to the bioavailability, these trace and non-essential elements were accumulated directly from the above ground part, which was in accordance to the findings of Guilizzoni (1991). For plants under control and oxic treatments (both 2.5 and 10 ppm NH_4-N) Pb was found higher in shoot than other parts. But in reduced treatments the concentration was found highest in leaves than other parts. The bioavailability of Pb was found lowest, as this element has high affinity to bind with Ca (Pendias and Kabata-Pendias, 2000). Uptake of different metals also depends on protein transporters. Pb, Zn and Cd are taken up at the cell surface through Ca ion channel, so they might compete among others and exclude Ca^{+2}. The toxicity of one trace element is also influenced by other nutrient concentration (Kopittke et al., 2008) on short-term root growth in cowpea (*Vigna unguiculata*) showed that an increase in the activity of Ca^{2+} increased the EC50 of Cu^{2+} activity from c. 0.24 μM to 0.59 μM. (Taylor et al., 1998) suggested that, this cation amelioration of cation toxicity probably does not result from changes in metal-speciation, but is attributable to changes in cation activity both in the bulk solution and, perhaps more importantly, at the root-cell plasma membrane surface.

The concentration of elements in plants was found higher as the concentration of elements in the external medium increased (Guilizzoni, 1991) but was not found as a linear correlation in our experiment. Cu is taken up at the cell surface via Cu-specific transporters, such as Cu^{2+}-ATPase or via Na transporters, such as Na+/K+-ATPase, as an analogue of Na. Though the concentration of Fe was high in the medium under reduced treatments, these

elements were not highly accumulated by the plants, whereas Cu^{+2} was found as highest concentration in plant, which might be due the hypothesis that Fe^{+2} and Cu^{+2} compete with each other for binding sites on cell wall and then be taken into the cell walls of plants. The accumulation of Zn was also low by the plant though this element was bioavailable in the surrounding environment in reduced treatments. The uptake of metals was also found to be pH dependent (Garnham et al., 1991, Marschner, 1995) although in certain cases no pH effect was seen. The concentration of Al and Cd was found to be less accumulated in plants which might due to be the above reason (Nyquist and Greger, 2009). Uptake of the non-essential elements As, Cd, Hg, and Pb into roots may be limited or they may be accumulated in roots due to a reduced transfer into the shoot. These different strategies may explain the frequently low transfer factors of elements such as Cd which are very mobile in the soils, as well as the lower transfer factors of Pb compared to those of Cu, although both revealed a comparable phyto-availability. The uptake of metals is also related to the presence of other metal ions in the surroundings of submerged aquatic macrophytes. Fritioff et al. (2005) demonstrated that at elevated metal concentrations, the uptake of Cu by the roots was somewhat limited, due to competition with other metals. Physical factors, including pH, redox potential, temperature, salinity or light in the surrounding aquatic environment, strongly affect metal uptake by submerged plants (Fritioff et al., 2005). These factors are characterized by temporal fluctuations. The present findings suggest that the soil extractable metal contents could serve as a factor affecting heavy uptake by plants. In fact, numerous researches reported that the uptake of metals was dominated by the mobility and availability of metals. However, apart from physiological functions of plants, soil physico-chemical properties also have a great contribution to the metal uptake and transportation of plants (Adriano, 2001).

In reduced treatments shoot and leaf accumulation of metals were found to be higher than that of roots, which might be the direct uptake by the shoot and leaves or from

root to shoot acropetal transport. Decrease in FW may be the outcome of a decreased water uptake or enhance water loss, both of which may occur following membrane damage since plant cell membranes are generally considered as the primary sites of metal injury Moreover, since roots are degenerated and greatly reduced in size due to metal toxicity (Basiouny et al., 1977), their potential for metal uptake might be limited. While, root as well as shoot elongation was found significantly ($P< 0.001$) inhibited by metal toxicity in reduced treatments and at 10 ppm oxic treatments. Heavy metals could lead to produce oxidative damage to aquatic plants and the ROS generation could be up to 30-fold (Mittler, 2002). This was particularly crucial for photosynthetic organisms which generate reactive oxygen species (ROS) constantly during normal photosynthesis. Chlorophyll concentrations were higher in plants under oxic treatment with low NH_4^+ concentration (2.5 ppm), whereas at higher NH_4^+ concentration chlorophyll level was declined significantly. Conversely, plants under oxygen deprived treatments affected chlorophyll synthesis at low to high NH_4^+ concentration. The loss of chlorophyll contents consequently disrupted the photosynthetic machinery thus the electron transport rates of PSI and PSII were disturbed leading to generation of high concentration of ROS. In the present study reduction of chlorophyll contents was probably achieved both by reaction with constituent biosynthetic enzymes as well as peroxide mediated degradation (Asada, 1994). Pronounced fluorescence decay in plants observed under reduced environments which might be due to the substitution of Mg^{+2} by other metals (eg. Cu, Pb, Cd). The high concentration of cellular H_2O_2 along with elevated POD activity in our experimental plants suggested that the ROS scavenging system was activated under such stressed conditions.

Decrease in proline content in plants under reduced treatments were found which might be the dysfunction of sulphydryl groups during the heavy metal transportation into the plants, which in terns effect on protein synthesis (Nagoor, 1999). Another reason might be the

increased activity of protease or other catabolic enzymes, which are activated by heavy metals and destroy proteins (Gupta et al., 1996). The results of the present study indicates that plants under reduced treatments seemed to be more vulnerable to metal toxicity, by lowering its protein synthesis, as more than one metal was present at toxic level in the reduced experimental conditions. Similar observations were also reported by Monferrán et al. (2012). Lipid peroxidation profoundly alters the structure of membrane and modifies their enzymatic and transport activities. Increased MDA levels in plant tissue indicated an increased lipid peroxidation of cell membrane also experienced in our study. The uptake of heavy metals shown to decrease in previous studies which might be due to the toxic effect caused by metals. A primary phytotoxic effect of Zn, Cu and Cd is oxidative stress (Gallego et al., 1996), which generate the formation of free radicals that in turn cause lipid peroxidation within membranes, ending up in increased membrane permeability. Therefore, heavy metals in excess may lead to membrane leakage of potassium ions and other solutes.

3.5. Conclusion

The symptoms of NH_4^+ toxicity include suppression of growth, chlorosis of leaves, decreased concentrations of mineral cations such as K^+, Ca^{+2} and Mg^{+2} in the tissues and decreased photosynthetic pigments were found for plants under 10 ppm NH_4-N concentration which was in accordance with Britto and Kronzucker (2002). However, it was difficult to distinguish the effect between ammonium and metal toxicity under high ammonium concentration in reduced treatments. Heavy metal toxicity along with oxygen deprived conditions was manifested in a reduction of biomass, photosynthetic pigments and biochemical disorders such as excess generation of ROS, lipid peroxidation and reduction of major macro elements. Studies of the physiological effects of metals under reduced environments in submerged macrophytes, especially test in situ, would enhance the understanding of degradation mechanism for submerged macrophytes and thought to be

beneficial for the ecological risk assessment. However, further studies need to be conducted under field condition (eutrophic shallow lake, water logged aquatic habitat etc.). Based on the present results it can be suggested that *E. nuttallii* can be a useful tool not for all phytoremediation technologies but for phytoextraction. These results indicate that the influence of soil organic matter on plant uptake of a heavy metal is not only dependent on its content, but also its components. Low soil Eh may lead to photosynthetic reduction due to decreased leaf water potential resulting from root dysfunction, reduced activity of major photosynthetic enzymes, disruption in photosynthate transport, alteration in source-sink relationship or reduced sink demand. Furthermore, while root oxygen-deficiency may partially account for the reduction in net photosynthesis, soil phytotoxins produced as by-products of low soil Eh conditions may play a major role in the observed photosynthetic sensitivities.

Chapter 4. **3RD EXPERIMENT: PHYSIOLOGICAL RESPONSE OF *POTAMOGETON PECTINATUS* TO VARIOUS SEDIMENT REDOX STATES**

Abstract

Submerged macrophytes often subjected to gradient redox conditions, such as they occur in flooded soil, eutrophic lakes, and waste water. This study evaluates the combined effects of reduced environment and elevated ammonium concentration on growth, biochemical parameters and macro nutrient concentrations in *Potamogeton pectinatus* (L.). The three levels of redox potential (Eh values, as treatment)) applied were (i) +400 mV to +440 mV (Oxic; O_1), (ii) -5 mV to 5 mV (hypoxic/moderately reduced; O_2) and (iii) -180 mV to -120 mV (anoxic/highly reduced; O_3). One control was used without any treatment. Exposure to reduced environment caused oxidative stress as evident by increased content of malondialdehyde (MDA), Free amino acids (FAA) and decreased contents of chlorophyll, soluble sugar; activities of peroxidase (POD) and superoxide dismutase (SOD), indole acetic acid (IAA), carbon, nitrogen and macronutrients contents in the plant. Photosynthetic efficiency of leaf tissue, as indicated by the quenching of chlorophyll a fluorescence (Fv/Fm, Fm/Fo), decreased significantly. Under low redox potential of soil (Eh) releasing of phytotoxins increased, which impose potential stress to submerged macrophytes.

4.1. Introduction

Plants growing in habitats that carry the risk of oxygen shortage need morphological and physiological adaptation (M.Crawford, 1996). To cope with this environmental stress, carbohydrate supply, regulation and energy metabolism is very important factors. In apparent contradiction to the enhanced carbohydrate consumption in the absence of oxygen (Pasteur-effect), many plants accumulate sugars at low O_2 concentration (Setter et al., 1987). The regulation of carbohydrate and energy metabolism seems to be important for the adaptation to oxygen shortage. The importance of submerged macrophytes is well known in

many aquatic ecosystems such as lakes (Scheffer, 1998) and estuaries (Herbert, 1999). Widespread reduction in the abundance of submerged macrophytes has been reported over the past several decades in many areas of the world. The decline can be related to water and sediment characteristics (Livingston et al., 1998), reduction of water clarity by phytoplankton, suspended particles in excessive nutrient loading (Lauridsen et al., 1994) and eutrophication induced low light stress (Ni et al., 1999).

Submersed macrophytes are rooted in sediment and can complete their growth and reproduction inside the overlying water. Providing a linkage between water and sediment, they have the closest interaction with water environment. This interaction is very complicate partly because sediments are not only served as a base of physical attachment. Recent attention has focused on the effects of submersed macrophytes on sediment redox, nutrient statues and the effects of sediment organic composition on macrophyte growth. Long-term ecological records of the decline of submersed macrophytes in the progress of eutrophication have suggested that sediment may play an important role for the decline due to its close relations to macrophyte growth and distribution. Therefore, more concerns should be given to the inhibitory effects of anoxic sediment on submersed macrophytes when nutrients are excessive for their growth. However, this line of investigation and experiments are relatively weak up-to-date concerning excess nutrients (Ni et al., 1999).

Submerged vascular macrophytes can influence the redox conditions in the sediment by releasing oxygen through their roots. This phenomenon is also governed by redox (Eh) condition and microbial oxygen demand of the sediment (Laskov et al., 2006). Under reduced conditions, NH_4^+ is the available form of N in the environment. Furthermore, under such environment different oxidized elements become available in the surrounding environment, many of which are highly toxic to plants (Pezeshki and DeLaune, 2012). Several previous studies have shown that sediment anoxia affects crop plants by regulating

respiration as well as producing phytotoxic elements resulting from anaerobic degradation of organic matter (Holmer and Bondgaard, 2001, Spencer and Ksander, 1995). FAA (free amino acid) is a physilogical indicator of aquatic macrophytes under various stress factors like ammonium toxicity, water logging, eutrophication and salinity. SC (soluble sugar) is storage in plant, which play a key role in carbon metabolism and function. The ratio between these two parameters (SC/FAA) is used fro the evaluation of growth and resource balance. Angiosperm contains about 15% of the total carbohydrate content (Hendry, 1993). Generation of free radicals and reactive oxygen species (ROS) is an established impact of stresses and their synthesis is stimulated in the presence of heavy metals in plants (Karuppanapandian et al., 2011) and hence normal metabolism is disrupted by lipid peroxidation of membrane system. To mitigate and repair the damage initiated by ROS, plants develop a complex antioxidative system.

Submerged macrophytes are considered as hyper accumulator of various metals and are recommended as bio-monitors to restore water quality (Karuppanapandian et al., 2011). Heavy metals suppress the functioning of essential biological components of plants as they tend to bind with sulphydryl groups of enzymes (Van Assche and Clijsters, 1990). Thus the balance between producing and removing of free oxygen radicals is damaged. *Potamogeton pectinatus*, an aquatic monocot is considered as one of a small number of remarkable plant species has the characteristics to grow and survive under anoxia (Dixon et al., 2006). This species reproduces vegetatively by means of small tubers with a pre-formed shoot. After overwintering, new plants are initiated by elongation of the preformed shoot and remarkably the rate of elongation of the shoot is faster in the absence of oxygen than under aerobic conditions. The physiological response of the pre-formed shoots to anoxia and its hormonal regulation have been examined in detail (Summers et al., 2000) and preliminary consideration has been given to the underlying metabolic adaptation of the plant.

Radial oxygen loss (ROL) value of this species was measured as 19-38 µmol O_2 h^{-1} g^{-1} DW root (Sand-Jensen et al., 1982). The enzymes, physiology, gene involved in such adaptation in different species of *Potamogeton* have been widely studied since the last three decades (Harada and Ishizawa, 2003, Koizumi et al., 2011). The nutritional quality and quantity, as well as antioxidant response under oxygen deprived states not been evaluated. Soil redox status and its effect in wetlands plants and crops have been vigorously studied in the last three decades. Various wetland plant response to flooded soil conditions have been reported in numerous publications in late 80's and 90's (Vartapetian and Jackson, 1997). Little is known about the relationship between soil oxidation-reduction and aquatic macrophyte functioning. Other consequences of soil reduction processes are changes in availability and/or concentrations of various nutrients that are essential for plant functioning. In addition, soil reduction processes result in the production of a host of compounds, many known to be phytotoxic (Gambrell, 1994, Reddy and Patrick, 1977).

4.2. Objective of the study

The influence of sediment anoxia on the growth and production of submerged macrophytes in the freshwater environment is poorly understood, and information and quantitative data are scarce. So far, the few studies that have been conducted on freshwater macrophytes have been mostly based on the natural environment or field observation (Barko et al., 1991, Lissner et al., 2003). It is often difficult to explain underlying mechanisms and to make reliable conclusions based on field observation data because many ecological processes occur concurrently. Thus, laboratory studies under controlled conditions are necessary to fully understand these phenomena. Therefore, the aim of the present work was to investigate the effect of diversified sediment redox state on various physiological and biochemical parameters in relation to oxidative stress and to evaluate the tolerant capability in *P. pectinatus*. In this

experiment we showed adaptive response of this species under different levels of sediment redox condition. Their physiology, morphology and biochemical response are taken into account. This study will be beneficial for the selection of macrophyte species for habitat restoration.

4.3. Materials and Methods

4.3.1. Treatments and experimental set-up

The plants were collected from the Motoarakawa River, Japan in 2012. The plants were allowed to adapt to the laboratory conditions for 2 weeks in the experimental tanks prior to the start of the experiment. The temperature was maintained at 23 ± 2 ºC in tanks with fluorescence lighting (light intensity 320 µmol cm^{-2}). We used three levels of redox potential in our experiment. Since it was difficult to keep a constant redox potential throughout the experiment period, we maintained a range of the same in each treatment. The levels of the first factor were (i) -5 mV ~ 5 mV (hypoxic/moderately reduced) (ii) -180 mV ~ -120 mV(anoxic/highly reduced) and (iii) +400 mV ~ +440 mV, (Oxic). We used four replications for each treatment.

Experiment was conducted in microcosms (MC) consisted of a 6 L (15.7 ×15.7 × 24.5 cm3) glass vessel which was hermetically sealed with an air-tight lid. Individual MC was allocated to every treatment combinations. MCs were filled with 600 g of air-dried soil and deionized water in a 1:5 ratio. The growth medium was a 5 % Hoagland nutrient solution (Hoagland and Arnon, 1950). Highly reduced and moderately reduced microcosms were prepared following the method developed by Yu et al. (2007b). Glucose, a simple carbon source, was used in this experiment during the 22 day incubation period. At the beginning of incubation, 8.16 g glucose was added to each reduced (MC 3) and highly reduced microcosms (MC 4) at 1st and 3rd day and twice that amount was repeated at 5th day. At

14th day, again 8.16 g glucose was added to MC 4. Continuous flushing of N_2 gas was carried out for last 3 and 7 days to accelerate the EH values to reduce approximately -5 and -180 mV for hypoxic/moderately reduced (MC 3) and anoxic/highly reduced (MC 4) conditions, respectively. For oxic treatments, continuous (MC 2) bubbling with atmospheric air was used. Redox potential (EH) and pH were measured four times a day using four portable pH/ORP meter (POT-101M, SIBATA, Japan). The temperature was maintained at 23 ± 2° C in a room with fluorescent lighting. The light intensity ranged from 240 to 270 µmol m^{-2} s^{-1}, and all microcosms were subjected to a 12 h/12 h light/dark period. No attempt was made to control the pH of the sediment suspensions. The total experimental period was 14 days.

Figure 4.1 Experimental plant *Potamogeton pectinatus*

4.3.2. Shoot length measurement

At 7th and 14th DAT (day after begin of treatment) two plants from each tank were harvested, cleaned and length were measured after blotting with laboratory tissue. Shoot elongation was calculated by Eq. (6)

$$SER = \frac{L_2 - L_1}{T_2 - T_1} \qquad [6]$$

L_1 and L_2 are the initial and final shoot lengths (cm) at the time (days) T_1 and T_2 respectively.

4.3.3. %Carbon, %Nitrogen, Soluble carbohydrate (SC) and free amino acids (FAA) content

Plant total carbon (TC) and total nitrogen (N) content was determined with a CHN Corder Auto sampler (MT-5, Yanaco Co. Ltd., Kyoto, Japan) using 3.0 ± 0.1 mg of dried and pulverized shoot tissues. Plants for SC analyses were oven dried at 70° C to constant weight and grounded into powder. About 50 mg powder was extracted with 5ml 80% ethanol at 80° C for 20 min, then centrifuged at 15,000×g for 10 min. The supernatants were collected, decolorized by 10mg activated charcoal and then filtered through filter papers (micro-void filter film, 20 mm). SC and FAA in the supernatants were determined by the anthrone method (Yemm and Willis, 1954; Yemm and Cocking, 1955) with glucose and alanine as standards respectively. The same extraction procedure was repeated three times, and the supernatant amalgamated to make up to 100 mL. A volume of 0.2 mL supernatant and 1.8 mL distilled water were put into a test tube. After reacting with 0.5 mL anthrone reagent and 5 mL H_2SO_4 in boiling water for 1 min, light absorbance was measured at 630 nm.

4.3.4. Chlorophyll content, carotenoid content and chlorophyll fluorescence study

These parameters were measured and the methodologies applied were discussed on chapter 2.

4.3.5. Macronutrient content study

About 10 mg of dried plant sample and 200 mg of dried soil sample were digested at 200 °C with di-acid mixture (1:2; nitric acid : perchloric acid) until evolution of nitrous gas had stopped and the digest became clear. The digests were diluted with distilled water to a total of 100 ml and passed through Whatman 42 filter paper. The concentration of following parameters were measured in the plant samples: Ca, Mg, K, and K with atomic absorption spectrophotometer (AAS; Shimadzu AA-660G), with direct air-acetylene flame method. Total phosphorus (TP) and S were measured after ascorbic acid method and barium chloride method respectively.

4.3.6. Hormone, Enzyme and Lipid peroxidation concentration study

The IAA, IAAO, H_2O_2, POD, MDA content were analysed by the methods descrived at the materails and method section in Chapter 2.The activity of SOD (EC 1.15.1.1) was measured by the photochemical reduction of nitroblue tetrazolium (NBT) after Stewart and Bewley (1980). One unit of the activity was defined as the amount of enzyme required to inhibit 50% of the initial reduction of NBT under light.

4.3.7. Statistical Analysis

All data are presented as the mean ± SD (n=4). Statistical analyses were performed using the SPSS for Windows (Release 13, SPSS INC., Chicago, IL) statistical software package. The data was tested by both one-way and two-way analysis of variance (ANOVA). Before performing a statistical analysis, data were checked for normal distribution. Statistical differences between treatments were identified by the Tukey HSD's test at a 5% significance level. Pearson's correlation analysis was carried out to explore the correlations between the treatments, chlorophyll content and hormone levels.

4.4. Results

4.4.1. Shoot elongation rate

Plants exposed to reduced treatments showed a significant increase in the SER. In contrast, plants grown under oxic treatments and the control plants showed no significant increase in the SER (Fig. 4.2). The SER of *P. pectinatus*, was found to increase in reduced treatments, but decreased at 14th DAT (Fig. 4.2). At 7th DAT the highest SER was found in highly reduced treatments but after 14th DAT the highest SER was found in reduced treatments.

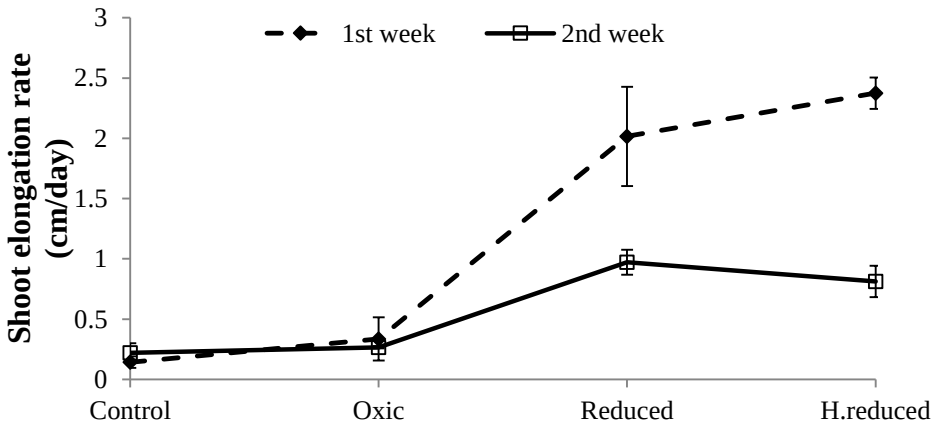


Figure 4.2 Shoot elongation rate of *P. pectinatus* under various redox states

4.4.2. Effect on photosynthetic pigments and chlorophyll fluroscence

Photosynthetic pigments seemed in decreasing trend under reduced treatments. Chl b seemed to be less affected comparing with Chl a, but significantly ($P < 0.01$) affected under reduced treatments which might be due to the presence of different oxidized elements. Carotenoid was found lowest (0.3±0.0) under highly reduced treatments. No significant difference was found in Chl a/b. All measured chlorophyll fluroscence parameters (Fv/Fm, Fm/Fo) were found

significantly affected with sediment anoxia (table-4.1).

Table 4.1 **Photosynthetic parameters and chlorophyll fluorescence parameters (Mean ± SD) under different sediment redox states in *P. pectinatus*. Different letter superscripts at each column indicate significant differences between treatments.**

Redox level	Photosynthetic parameters and chlorophyll fluorescence parameters				
	Chla	Chlb	Carotenoid	Fv/Fm	Fm/F_0
Control	1.9 ± 0.0^{a}	1.0 ± 0.1^{a}	0.8 ± 0.0^{a}	0.8 ± 0.1^{a}	5.8 ± 0.1^{a}
Oxic	2.0 ± 0.1^{a}	1.0 ± 0.1^{a}	0.9 ± 0.0^{a}	0.8 ± 0.0^{a}	6.1 ± 0.1^{a}
Reduced	1.4 ± 0.1^{b}	0.7 ± 0.0^{b}	0.4 ± 0.0^{b}	0.7 ± 0.0^{b}	$4.9\pm0.0^{b*}$
H. Reduced	$1.2\pm0.0^{b**}$	0.6 ± 0.0^{b}	$0.3\pm0.0^{b*}$	0.6 ± 0.0^{b}	$4.8\pm0.1^{c*}$

*, *P*<0.5; **, *P*<0.01; ***, *P*<0.001

4.4.3. Effect on %Carbon, %Nitrogen, Soluble carbohydrate (SC) and free amino acids (FAA) content

The % of N and % of C contents were significantly (P < 0.01) lower in the reduced treatments than in the oxic treatments (table-4.2). The investigated species showed differences in concentration of the soluble carbohydrates. SC content decreased significantly under reduced treatments. The FAA content was found to increase in reduced treatments but significantly (Fig. 4.3 A). The significant increment of FAA/SC was found in plants exposed to reduced treatments (Fig. 4.3 B).

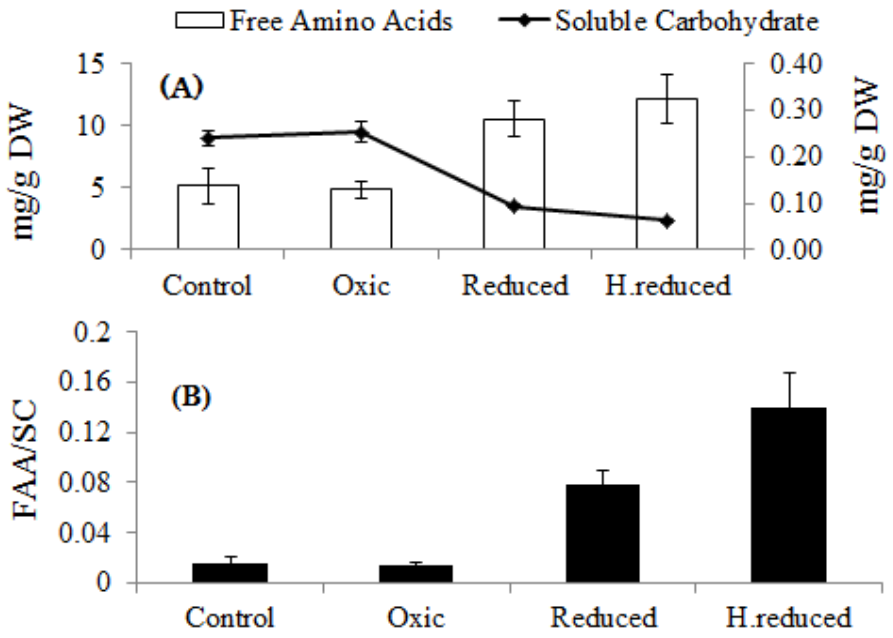


Figure 4.3 The concentration of (A) FAA (column graph) and SC (line graph) and (B) the ratio of FAA/SC in *P. pectinatus*

4.4.4. Effect on macronutrients

All the macronutrients concentrations were found in decreasing trend in plants exposed under reduced treatments. Among the macronutrients K found significantly more effected than other macronutrients (table-4.3).

Table 4.2 Macronutrient contents (%DW) in *P. pectinatus* under different sediment redox conditions. Different letter superscripts at each column indicate significant differences between treatments.

	Macronutrient concentration % Dry Weight				
Redox level	Ca	Mg	K	N	C
Control	1.6±0.2^{a}	0.6±0.1^{a}	3.3±0.3^{a}	3.7±0.4^{a}	36.0±1.1^{a}
Oxic	1.6±0.3^{a}	0.6±0.1^{a}	3.4±0.4^{a}	3.8±0.3^{a}	37.9±1.2^{a}
Reduced	1.1±0.2^{b*}	0.3±0.0^{b*}	2.8±0.2^{b*}	2.9±0.1^{b*}	30.0±2.5^{b}
H. Reduced	0.9±0.1^{b**}	0.3±0.0^{b*}	2.5±0.2^{b*}	2.5±0.1^{b*}	26.1±1.8^{c}

*, $P<0.5$; **, $P<0.01$; ***, $P<0.001$

4.4.5. Hormone, Enzyme and Lipid peroxidation concentration

All the stress enzymes (SOD and POD) increased significantly under low sediment redox treatments in *P. pectinatus*. Compared to the control, SOD and POD activities were enhanced by 77% and 63.8% respectively (Fig. 4.4 A and B). The highest SOD and POD values were found in reduced treatments. Malondealdihide (MDA), known as a product of lipid peroxidation was found highest concentration in highly reduced treatments (14.9 ± 0.7, µ mol/g). The growth hormone IAA found to be increased in reduced treatments (Fig. 4.4 D)

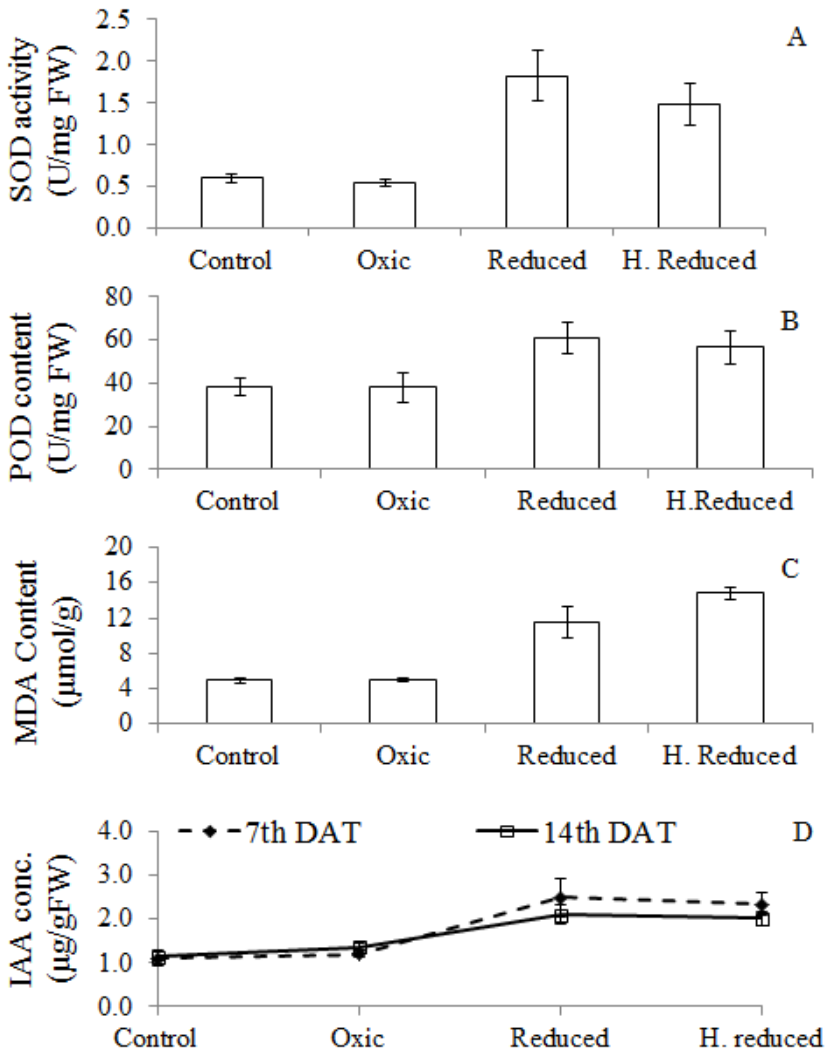


Figure 4.4 The concentration of the stress enzymes SOD and POD (A, B); the

concentration of MDA content (C) and the concentration of growth hormone IAA (D) under different sediment redox states in *P. pectinatus*.

4.5. Discussion

Sediment anoxia affects plants by regulating respiration and phytotoxin production as the result of anaerobic degradation of organic matter (Marguerite et al., 1990). Xie and Yu (2011) found that sediment nutrient content mediates plant vegetative traits and can subsequently affect turion production and reserves in *P. crispus*. Wu et al. (2009) showed that sediment anoxia had greater effect than those of light and suppressed stimulatory effects of intermediate light levels when anoxia was high on turion germination of *P. crispus*. When the anoxic condition exceeded the tolerance level of the plants under present experimental condition, plants showed reductions in growth, retarded metabolic and physiological processes as supported by the previous study of Dixon et al. (2006). *E. nuttallii* under reduced conditions even treated with suitable concentration of NH_4-N (2.5 ppm) showed retarded growth, decreased levels of photosynthetic pigments (Zaman and Asaeda, 2013). Our data indicated that anoxic conditions suppressed net photosynthesis and altered the balance of macronutrients (Table-4.1 and 4.2). Thus, biomass production was inhibited by anoxia. The stem elongation was about twice in first seven days but again declined at 14 DAT in *P. pectinatus*. Summers et al. (2000) suggested that such shoot elongation in anaerobic conditions, are accompanied due to an unusually marked Pasteur effect.

Nutrient levels in sediment have been shown to affect directly the plant growth and development of submersed macrophytes (Chambers and Kalff, 1985). Barko et al., (1991) suggested that the low biomass production of *P. pectinatus* on sediments with high organic matter (>10%) concentration is due to high concentrations of inorganic constituents such as soluble reduced iron and manganese and soluble sulphide or organic constituents such as methane, ethylene, phenols and alcohols formed under anaerobic conditions. Van Wijck et al.

(1992), found that both sulphide and ferrous iron when present in the interstitial water reduced the biomass production of *P. pectinatus*. This species is found to grow better in nutrient poor systems (Van Wijck et al., 1994). It was reported that biomass growth of aquatic weeds decreased at high nutrient and organic contents of the sediment (Barko and Smart, 1983). Eutrophication also caused low resistance of the macrophytes to environmental changes by lowering their tissue carbohydrate reserve at high nutrient supply. Strong tolerance to anaerobic conditions is known to be one of the adaptive characteristics of aquatic plants for their survival in anaerobic environment. However the mechanisms of the tolerance are still not clear (Dixon et al., 2006). Some aquatic macrophytes have been found to exhibit strong tolerance to anaerobic conditions, for example; *Trapa natans* seedlings (Menegus et al., 1992), *P. distinctus* stem and *Sagittaria pygmaea* leaves (Ishizawa et al., 1999). Stem elongation in *P. pectinatus* in the absence of oxygen has been reported to be greater than that in air (Summers and Jackson, 1999).

Studies on the regulation of energy metabolism in anoxia are important to understand how aquatic plants exposed to anaerobic environment. Under anoxic condition ATP production mechanism is also very important to maintain and support anaerobic growth. Anaerobic conditions stimulate glycolysis. Thus alcoholic fermentation is essential for these plants to survive in anoxia. In the contrary Kennedy et al., (1992) concluded that occurrence of Pasteur effect does not correlate with tolerance or intolerance to anoxia in plants. M.Crawford (1996) suggested that the anaerobic tolerant species exhibit a smaller Pasteur effect would contribute to the preservation of respiratory substrates and to the prevention of over-production of toxic end products of fermentation. Ethanol fermentation is the main metabolic pathway for production of ATP which is necessary for anaerobic growth of pond weed turions. Summers et al. (2000) reported that anaerobic shoot elongation of *P. pectinatus* is associated with an unusually large Pasteur effect. They suggested that starch is the source

of energy in anaerobic conditions. The occurrence of the Pasteur effect enhance the CO_2 release under anaerobic conditions. Conversion of starch to sucrose is necessary in order to use it as a substarte for glycolysis. Sugars produced by starch degredation may be transported to a site of glycolysis after sucrose may be involved in a regulation of carbohydrate metabolism, just as a futile cycle of sucrose metabolism operates in sink organs.

Accumulation of FAA and reduction of SC of plants were reported under nutrient enrichment (Marschner, 1995, Cao et al., 2007). In the present study, plants exposed to reduced treatments led to the depletion of SC and slight accumulation of FAA in the tissues of *P. pectinatus* and the consequent high FAA/SC ratios (Fig. 4.3) indicated that the plant carbon and nitrogen metabolisms were severely disturbed. Plants are necessarily highly competitive and have finely tuned mechanisms to adjust growth and development in accordance with opportunities and limitations in their environment. Sugars from photosynthesis form an integral part of this growth control process, acting as both an energy source and as signaling molecules in areas targeted for growth.

P. pectinatus under low sediment redox status showed lower SC contents, than the control plants, suggesting that the plant may not tolerate extended anoxic stress. Chlorophyll a and b are the major pigments participating in photosynthesis. In reduced environments photosynthetic pigments decreased significantly. Under reduced environments the oxides elements might inhibit the enzyme activity associated with chlorophyll biosynthesis (Zengin and Munzuroglu, 2005). Furthermore these elements interfere with chlorophyll synthesis by substituting Mg^{2+} of chlorophyll molecule and resultant inhibition of photosynthesis (Hendrik et al., 1996). This indicates that plants are more susceptible to metal toxicity, expressed by lowering its protein biosynthesis, when more than one toxic metal is present in the system (Choo et al., 2006). In the present study chlorophyll a/b ratio remained

almost unchanged in *P. pectinatus* treated with different sediment redox treatments, indicating their tolerance ability. Furthermore, the Mg^{+2} concentration was found less affected in *P. pectinatus* than that of *E. nuttallii* (Zaman and Asaeda, 2013). Increased chlorophyll a/b ratio was found in *E. nuttallii* which is considered as a stress indicator (Hendry and Price, 1993). This finding supports the flexible ability of Sago pond weed under anoxic condition with maintaining of photosynthesis.

In the present study the NH_4-N concentration in reduced and highly reduced treatments (water column) were found 8.6 and 13.8 mg/L respectively. NH_4-N concentrations inhibit the growth of *P. maackianus* (Katwijk et al., 1997), *P. densus* (Li et al., 2007) and *P crispus* (Ma et al., 2009) are 0.5, 3.7 and 1.56 mg/L respectively. Li et al. (2007) shown in a 6-day experiment, *P. maakianus* that grown in NH_4^+ enriched water showed constant SC levels with a decrease in the starch contents, indicating that the degradation of starch may help the plant capable to tolerate NH_4^+ stress (Li et al., 2007). The reduction of C was found in plants under reduced treatments, which supports the hypothesis that carbohydrate limitation may contribute to the toxicity syndrome in plants fed with high levels of NH_4^+ (Kronzucker, 1998). Under eutrophic conditions, high amounts of carbon and energy for NH_4^+ extrusion and NH_4^+ assimilation are needed (Cao et al., 2009) and it is difficult for submerged species (*Elodea, Potamogeton, Hydrilla, Vallisneria*) to survive at such conditions (Litav and Lehrer, 1978). Cao et al. (2009) suggested that the release of NH_4^+ from the fertile sediment into the water column would inhibit the growth of *Valisneria* genus in eutrophic lake.

Further, Fv/Fm values of the plants exposed to higher turbulences indicated that plants were under stress and the photosynthetic machinery may have been damaged. Usually stress free plants have Fv/Fm values around 0.8. It is apparent that carbohydrate synthesis is reduced due to the damage to photosynthetic machinery (Kasige and Asaeda, 2008). Moreover, stressed plants are observed to utilize more energy for survival than for

growth, hence altering the growth and carbon assimilation patterns (Kasige and Asaeda, 2008, Ellawala et al., 2011).

Under flooded environments roots of submerged plants often suffer from energy and carbohydrate shortage (Farrar and Jones, 2000) along with damage caused by reduced components (Mn^{2+}, Fe^{2+} and S^{2-}) and volatile organic acids (propionic and butyric acids) (Gibbs and Greenway, 2003). Under energy crisis, remobilization of stored carbohydrate plays a vital role in providing energy needed for cell and organ maintenance as well as stress responses (Gibbs and Greenway, 2003). It is also crucial to prevent depletion of K^+ in leaves, resulting from cytosolic K^+ leak via outward-rectifying K^+ channels activated by ROS (Demidchik et al., 2010); previous studies have reported a decline in leaf potassium concentration in barley, wheat and corn under flooding conditions (Board, 2008). Additionally, poor sink strength of roots after severe O_2 and energy limitation, even in species having well-developed aerenchyma like *A. philoxeroides* and *H. altissima* (Luo et al., 2009), or effects of toxic components like Mn^{2+}, Fe^{2+} and S^{-2} accumulating in flooded soil (Gibbs and Greenway, 2003). The K^+ concentration was little affected in *P. pectinatus* by anoxic condition in comparison with *E. nuttallii* (Zaman and Asaeda, 2013) as it has adaptive characteristics to prevent depletion of K^+ in leaves, resulting from cytosolic K^+ leak via outward-rectifying K^+ channels activated by reactive oxygen species. Declination of leaf K^+, Ca^{+2} and Mg^{+2} under critical limit were found in *E. nuttallii* which goes along with the results of Board (2008), who observed such declination in crop plants. In reduced environment macro nutrients concentration significantly affected in reduced treatments in both species of plants which might be due to the presence of non-essential oxides elements as these were responsible for ionic imbalance in plants (Yang et al., 2005). In *P. pectinatus* the concentration of macronutrients were found less affected than that of *E. nuttallii*. This might be one of the characteristics of this tolerant species to maintain physiological and metabolic activities under

anoxic environment.

Aquatic plants activate their defense systems against external stressors by triggering the release of various antioxidant enzymes to remove free oxidant radicals (Lee, 2002). Superoxide dismutase is the first enzyme to detoxify highly reactive oxygen species in plants by converting O_2^- radicals to H_2O_2 (Constantine and Ries, 1977). In plants catalase, ascorbate peroxidase and guaicol peroxidise enzymes are considered as of most important in scavenging H_2O_2 (Noctor and Foyer, 1998, Zhang et al., 2007). Peroxidase is one of the principal enzymes involved in the elimination of reactive oxygen species. The activities of SOD and POD in *P. pectinatus* increased simultaneously during growth under reduced environment but decreased under highly reduced treatments. SOD and POD act on scavenging of ROS, thus the increase in their activities is the indication of the oxidative stress in plants (Blokhina et al., 2003). Furthermore, SOD activity was found to increase in *P. crispus* under high ammonium stress (Cao et al., 2004). The presence of toxic elements had great affinity to bind with sulphydryl groups of enzymes and hence suppress the functioning of essential biological components (Van Assche and Clijsters, 1990). Elevation of free radicals and reactive oxygen species (ROS) generation is an established impact of stresses and hence normal metabolism is disrupted by lipid peroxidation of membrane system. Zhanga et al. (2010) showed high correlation with antioxidants engymes and the content of FAA, SC and suggested that antioxidant responses might link to C-N metabolic disorder in *P. crispus*. *E. nuttallii* was found more vulnerable in terms of defense mechanisms under low redox states as the activity of stress enzymes disrupted (Zaman and Asaeda, 2013), which ultimately might break the balance between producing and removing of free oxygen radicals is damaged comparing with *P. pectinatus*.

MDA is routinely produced as a result of lipid peroxidation under stressed conditions and used as an index of stress status due to the elevation of ROS products such as O_2^- and

H_2O_2 accumulation (Zhang et al., 2007). Lipid peroxidation, as an important impact of oxidative stress, also causes degradation of photosynthetic pigments. Increased peroxidation of the membranes, via metal induced production of free radicals, causes membrane destabilization. Thus, exposure to reduced environments is causing oxidative stress in the anoxic stress tolerant *P. pectinatus*.

4.6. Conclusion

P. pectinatus pocesses a special type of adaptive quality under anoxic environment. The growth, biochemical parameters and macro nutrient concentrations in one anoxic stress tolerant submersed species (*P. pectinatus*) studied with various sediment redox states. It is essential to understand the mechanisms behind the oxygen deprived effects in aquatic macrophytes in anoxic sediments, since survival of macrophytes is essential for a successful colonization. Carbon assimilation patterns changed as an adaptation to the oxidative stress responses and damage to photosynthetic machinery. Alterations in growth observed in the plants may be a consequence of altered hormone concentrations, oxidative stress developed in the tissues, altered carbon uptake patterns and metabolism. This study documents that the concentration of growth regulators gets altered; biomass gain reduces when *P. pectinatus* is exposed to reduced conditions for prolonged period of time.

Chapter 5. **EXPERIMENT 4: THE DISTRIBUTION OF MICRONUTRIENT CATIONS IN SOIL UNDER CONDITIONS OF VARYING REDOX POTENTIAL AND PH**

Abstract

A laboratory study was conducted to determine the influence of redox potential and soil pH on the distribution of Fe, Mn, Zn, and Cu in silty clay loam soil and to provide insight into factors affecting micronutrient dissolution and mobility in soil. Generally, greater amounts of Fe, Mn, Zn, and Cu were found in reduced treatments at low pH and Eh than at high pH or Eh (oxic treatments). Among a few published works based upon detailed experimental data on real soil systems, interpretations remain ambiguous or conflicting. Thus the present experiment was designed to focus on the qualitative and quantitative determination of the distribution of micronutrient cations in soil under conditions of varying redox potential and pH. Sieved soil samples were incubated in aqueous suspensions under different redox conditions. Eh and pH, concentration of major and trace elements were measured in the microcosms. Separation of the water-soluble fraction into free ions and those complexed by soluble organic matter indicated that micronutrient cations were complexed by organic matter to a greater extent in reduced soil.

5.1. Introduction

Generally in flood physiology literature, many terms such as ‘flooded’, ‘saturated’, ‘waterlogged’ are used to describe oxygen-deficient root medium. Obviously, these terms do not provide a quantifiable defining of the rhizosphere (DeLaune et al., 1990). In addition, due to the absence of oxygen in most saturated soils, methods used in well-drained soils to quantify oxygen content and oxygen diffusion rate cannot be employed effectively in wet soils (Gambrell, 1994). In contrast, soil Eh is a useful term because it can be measured in laboratory and in the field (Patrick and Delaune, 1997). Furthermore, quantifying soil Eh is particularly advantageous in periodically flooded soils since the range of Eh is much wider, ranging between approximately

−300 to +700 mV, than either aerated (Eh > +400 mV) or permanently waterlogged (Eh < +350 mV) soils (Delaune and Pezeshki, 1991). From a physiological-ecology standpoint, the knowledge of soil Eh represents an indication of the status of various soil compounds, for example- a soil redox potential of zero mV indicates that oxygen and nitrate are not likely to be present and that the bio-reducible iron and manganese compounds are in a reduced state. A redox potential of +400 mV indicates that oxygen may be present despite the presence of excess water in the soil (DeLaune et al., 1990). Thus, soil Eh measurements in wetlands represent an excellent quantifying tool for defining soil chemical status. In a typical series of reductions NO_3^- is reduced to NO_2^-, Mn^{+4} to Mn^{+2}, Fe^{+3} to Fe^{+2}, SO_4^{2-} to H_2S, S^{2-} or HS^- (depending upon pH) and accumulations of acetic and butyric acids produced by microbial metabolism. The result of these transformations is that Eh become more negative (Patrick and Delaune, 1997). Thus, while drained soils have Eh +400 millivolts (mV) waterlogged soils may exhibit Eh as low as −300 mV.

Wetland soil systems are characterized by biological activities, thus, many redox systems are functioning. In these systems, the oxidation-reduction is ordinarily used to denote the intensity of reduction. However, the reduction of inorganic redox systems including oxygen in hydric soils following flooding can be described in 'intensity' and 'capacity' terms (DeLaune et al., 1990) The intensity factor determines the relative ease of the reduction, whereas the capacity factor denotes the amount of the redox system undergoing reduction, e.g. oxygen consumption at root interface (Kludze and Delaune, 1996a). The relationship may be depicted as follows:

$$E_h = E_0 + 2.3\left[RT/nF\right] \times \log e\left[\mathrm{Ox}/\mathrm{Red}\right] \qquad [7]$$

Ox and Red are the concentrations of the oxidized and reduced forms, respectively, of the substance under consideration, Eo is the electrode potential of the 50% oxidized

system specific to that substance, n is the number of moles of electrons transferred, F is the Faraday constant, R is the gas constant, and T is the temperature (K). It follows that Eh is dependent on the ratio of oxidized and reduced forms and not on their absolute quantities. Thus, a 90% oxidized system will have the same electrode potential no matter whether the total concentration is 0.01 or 10%, but the poising (capacity) of the latter will be 1000 times greater. Thus, a system of Eo+0.1 volt will oxidize a system of Eo−0.1 volt, but will be oxidized by a system of Eo+0.3 volts but the extent to which the reactions will take place will depend upon the capacity of the systems.

The redox capacity factor is important although much less is known about whether it is the intensity or the capacity, which are most affecting, plants. As mentioned above, two different soils with the same level of intensity of reduction may differ substantially in the capacity for reduction. Soil reduction capacity can be estimated using measurements of soil respiration and calculating oxygen equivalent by stoichiometry (Kludze and Delaune, 1996a). However, some of the oxygen consumption will be due to microbial respiration and some will be due to direct oxidation of the accumulated reductants. At the present, levels of soil redox capacity may be created and/or manipulated by providing extra carbon and energy source (organic matter) to the soil while maintaining the same redox intensity level. In an experimental set up, reduction capacity may be controlled by adding different amounts of granular D-glucose to the growth medium which is also maintained under reducing conditions. Evaluation of wetland plant responses to soil flooding require that both intensity and capacity of soil reduction are quantified since these two components influence oxygen demand in soil. Kludze and Delaune (1996a) demonstrated that oxygen demand in the root medium governed wetland plant functioning.

5.2. Objectives

Only few studies report on the effects of reduction with respect to metal mobility and emissions

to surrounding groundwater systems. There are some useful studies that include field or laboratory measurements (Du Laing et al., 2009). Moreover, little is known about both the qualitative (mechanisms) and quantitative (concentrations and rates) aspects of this practice. Thus the aim of the present experiment is to focus on the qualitative and quantitative determination of the distribution of micronutrient cations in soil under conditions of varying redox potential and pH.

5.3. Materials and methods

5.3.1. Experimental set up

The experiment was conducted in 6 L glass vessel (15.7 ×15.7 × 24.5 cm^3) which was hermetically sealed with an air-tight lid. MCs were filled with 600 g of air-dried soil and deionized water in a 1:5 ratio. Three different redox levels were maintained (-180 mV to -120 mV, highly reduced; -5 mV to 5 mV, moderately reduced and +400 mV to +440 mV, Oxic) each with 4 replications were used. Highly reduced and moderately reduced microcosms were prepared following the method developed by Yu et al. (2007). Glucose, a simple carbon source, was used in sequential process to create reduced environment. At the beginning of incubation, 8.16 g glucose was added to each reduced (MC 3) and highly reduced microcosms (MC 4) at 1st and 3rd day and twice that amount was repeated at 5th day. At 14th day, again 8.16 g glucose was added to MC 4 and continuous flushing of N_2 gas for 7 days accelerated the Eh values to reduce approximately -180 and -5 mV under highly reduced and reduced MCs respectively. For oxic treatments, continuous bubbling with atmospheric air was used. Redox potential (Eh) and pH were measured four times a day using four portable pH/ORP meter (POT-101M, SIBATA, Japan). The total experimental period was 36 days including 22 days of incubation.

5.3.2. *Dissolved Oxygen Measurement (DO)*:

DO was measured weekly, by Winklers Titration method (APHA, 1976). For fixation of 250 ml sample water 1 ml $MnSO_4$, 1 ml alkaline iodine (KOH-KI) and 1 ml concentrated H_2SO_4 were used. Then 200 ml fixed water was titrated with 0.025 (N) $Na_2S_2O_3$ (sodium thiosulphate). Later 5 ml starch solution was added and again titrated with $Na_2S_2O_3$. Following equation was used to determine the DO in mg/L

(Final reading – Initial reading) × 0.698

5.3.3. Collection of pore water and different component analysis in water, pore water and soil

Pore water was extracted by centrifugation of carefully collected sediment samples. Thawed samples were centrifuged at 3000 rpm for 15 mins at 4^0 C. Following centrifugation, pore water was filtered using Whatman GF/F (47 mm) paper. Auto analyzer (TRAACS 800, Technicon, New York, USA) was used to analyse NH_4-N, NO_3-N and PO4-P in water and pore water.

The concentration of following parameters were measured in the water samples: Fe, Mn, Zn, Ca, Mg, Cu and K with atomic absorption spectrophotometer (AAS; Shimadzu AA-660G), with direct air-acetylene flame method and the concentration of Al were determined with graphite furnace atomizer (GFA-4B), according to standard procedures and using commercial laboratory standards. Total phosphorus (TP) and S were measured after ascorbic acid method and barium chloride method respectively. Phosphate-phosphorus, nitrate nitrogen and ammonium nitrogen were determined by autoanalyzer (Technicon II TRAAC 800).

5.3.4. Statistical analysis

Data were statistically analyzed by ANOVA and MANOVA test using a statistical package,

SPSSversion 17.0 (SPSS, Chicago, IL). For each significant difference of $p<0.05$, a LSD test was performed to locate these differences. Pearson correlation coefficients were calculated to determine the relationship between soil pH value, redox levels and soil heavy metal contents. Single linear regression analysis was employed firstly to examine the effect of soil pH or Eh on the availability of heavy metals in soils and the concentrations of heavy metals in water.

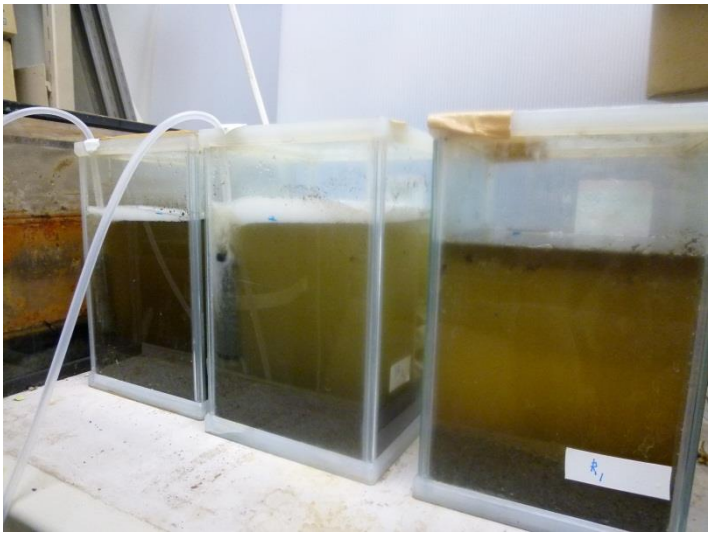


Figure 5.1 Experimental tanks with reduced treatments

Figure 5.2 Highly reduced tanks after 36th DAT

5.4. Results

5.4.1. Sediment characteristics

The sediment is clay-loam, with 5. 11 % OM content and a moderate pH (table-5.1). Fe concentration in the bulk soil was highest and Cd was found at lowest concentration.

Table 5.1 Concentration (mg/Kg) of different elements in soil

Soil properties		
Elements (mg/Kg)	Mean	SD
Ca	3735.5	200.66
Mg	1879.75	229.78
K	4628	452.30
Fe	7121	702.76
Cu	37.5625	11.33
Zn	159	24.90
Mn	1014.25	38.18
S	1767.5	781.08
P	232	35.21
Cd	5.775	2.73
Pb	35.25	10.59
Al	1867.5	511.17
%Organic matter	5.1115	0.23
% of TOC	2.9655	0.131971
Soil texture	Clay-Loam	

5.4.2. DO concentration, pH and Eh values

In highly reduced and reduced treatments the DO value were found 0 ppm and 0.26 ppm at 32th DAT. The DO value was around 8 ppm in oxic treatments (Fig. 5.3). A negative correlation was found (r=- 0.916) between DO and Eh (5.5). In reduced treatments the pH lowered to about 4.

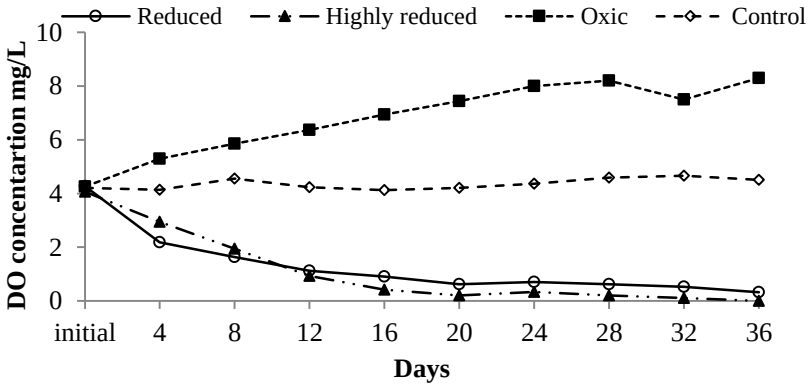


Figure 5.3 DO concentration in the microcosms during the experimental periods including 22 days of incubation period.

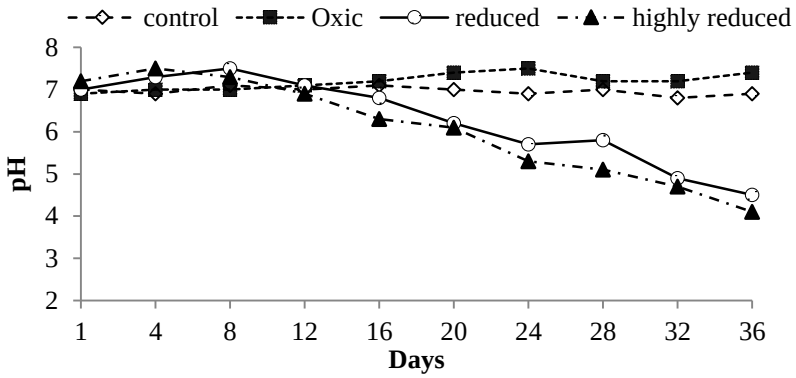


Figure 5.4 pH in the microcosms during the experimental periods including 22 days of incubation period.

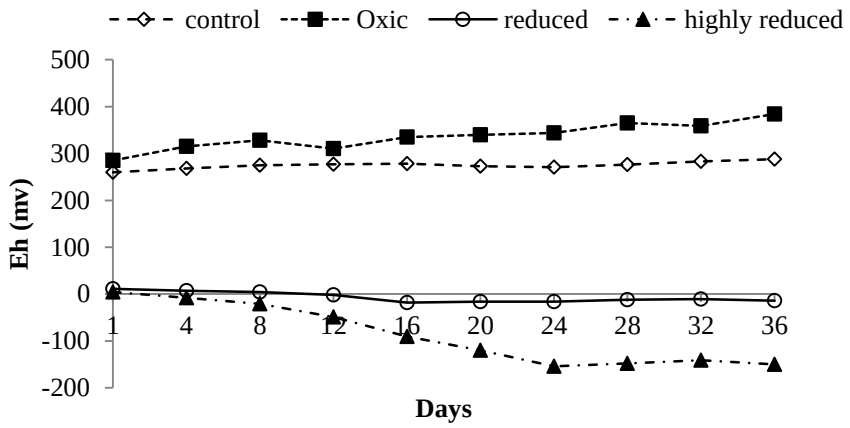


Figure 5.5 Eh in the microcosms during the experimental periods including 22 days of

incubation period.

5.4.3. Pore water quality

The concentration of different elements in pore water is presented in fig 5.6 and table-5.2. The concentration of elements in reduced treatments according to the order of magnitude are as follows Ca>Mg>K>Fe>Al> Mn>S>P>Zn>Cd>Pb.

Figure 5.6 Element concentration in pore water of different treatments.

The concentration of PO_4-P, NH_4-N and NO_3-N in pore water of different treatments are presented in fig 5.7. The concentration of NH_4-N in reduced treatment was found to be high about 78.42% than control. Slight increment of PO_4-P was found in reduced tratemnts.

Table 5.2 Different elements concentration (mg/L) in pore water of different treatments.

Elements	Oxic	reduced	H.reduced
Ca	25.42925	165.574	173.7283
Mg	4.6125	112.89	110.936
K	6.397	88.13525	95.33118
Cu	0.001006	0.673	1.372129
Mn	0.4275	18.69725	20.07434
Zn	0	0.475872	0.945079
Fe	2.9075	65.68945	78.0372
P	0.187	2.1	3.075
S	0.2225	5.035925	6.38855
Al	0.13935	60.0984	62.44195
Pb	0	0.078328	0.29
Cd	0.018	0.24875	0.25

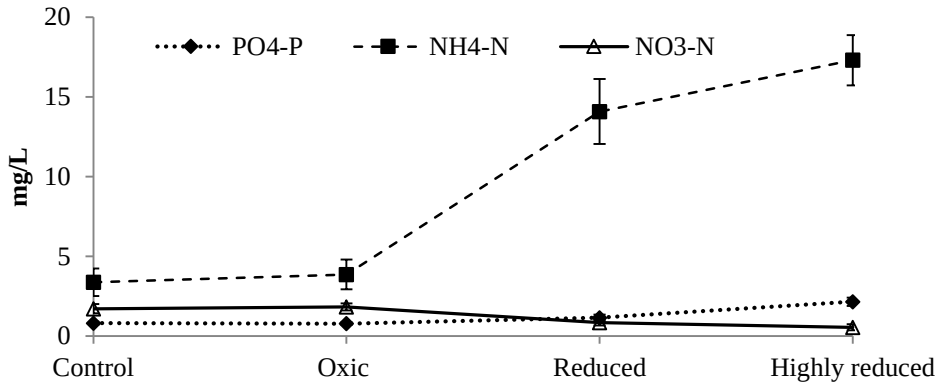
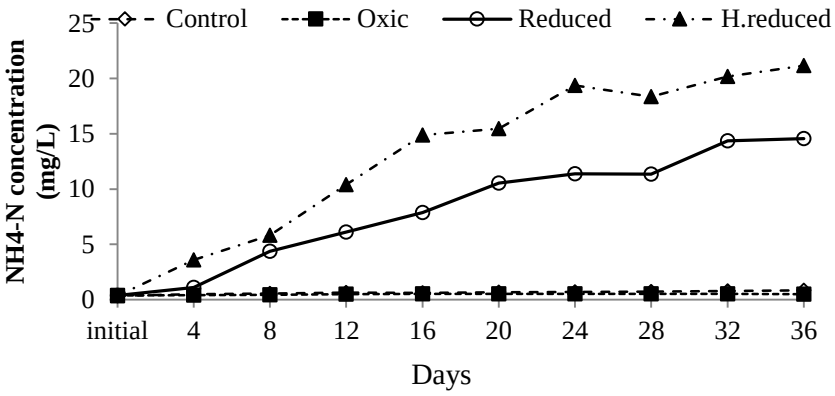


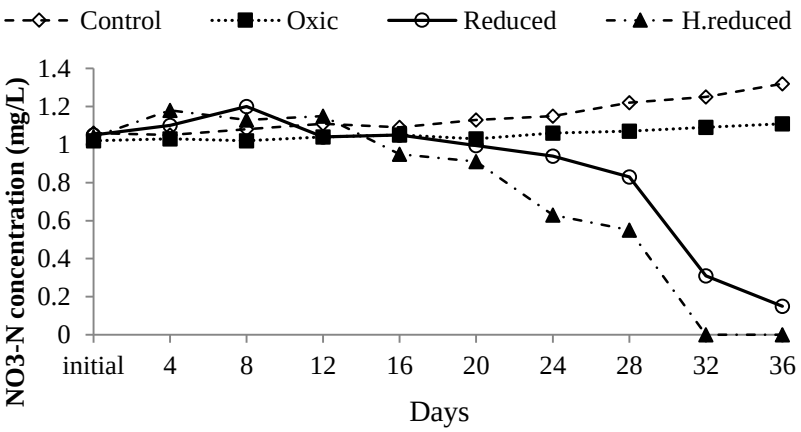
Figure 5.7 Concentration of PO_4-P, NH_4-N and NO_3-N in pore water of different treatments after 36 DAT.

5.4.4. Elements and Concentration of PO_4-P, NH_4-N and NO_3-N in Water column

Dissolved nitrogen and phosphorus in the water of the microcosms, especially NH_4-N and PO_4-P species increased continuously during the period of the study in reduced treatments. The concentration of NO_3-N were found zero in reduced treatments. PO_4-P concentration found almost 3 times higher in reduced treatments than the control (Fig. 5.8). NH_4-N concentration was found almost 80% higher in highly reduced treatment than the control (Fig. 5.8 A). PO_4-P and NH_4-N concentrations were positively correlated.

The different element concentrations in water column in different treatments are shown in table -5.3. Sharp increases of almost all the cations were found in reduced treatments. Zn concentration in the medium was not correlated with pH or Eh.





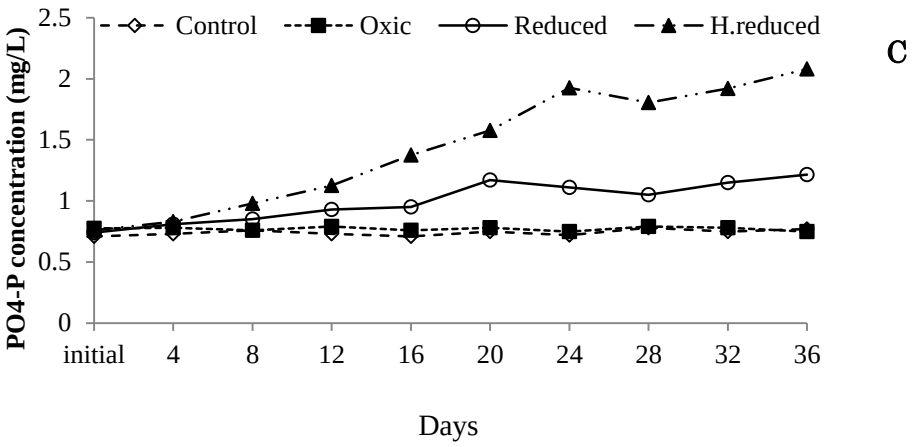


Figure 5.8 Concentration of (A) NH_4-N and (B) NO_3-N and (C) PO*4*-P in water column in different treatments

Table 5.3 Different elements concentration (mg/L) in water column of different treatments.

Elements	Unit	Control	Oxic	Reduced	H. reduced
Ca	mg/L	11.3±4.1^{c}	10.6±5.8^{c}	19.6±3.2^{b}	27.7±1.9^{a}
Mg	mg/L	9.1±1.3^{c}	10.7±1.1^{c}	16.9±1.7^{ab}	25.2±1.5^{a}
K	mg/L	12.7±5.1^{d}	12.6±5.6^{c}	19.7±3.4^{b}	23.3±1.7^{a}
S	mg/L	15.7±6.2^{c}	15.4±3.8^{c}	29.2±6.0^{b}	36.9±7.9^{a}
TP	mg/L	0.8±1.5^{c}	0.9±2.7^{b}	6.0±2.9^{a}	9.5±3.5^{a}
Cu	mg/L	0.0±0.0^{b}	0.0±0.0^{b}	0.9±0.2^{a}	1.3±0.3^{a}
Mn	mg/L	1.3±0.1^{c}	1.2±0.2^{c}	7.2±0.7^{b}	10.0±1.4^{a}
Zn	mg/L	2.5±0.4^{c}	2.6±0.3^{c}	5.2±1.5^{b}	8.4±1.9^{a}
Fe	mg/L	4.1±1.7^{c}	4.4±1.2^{c}	41.4±9.7^{b}	130.8±10.2^{a}
Al	µg/L	0.0±0.0^{c}	0.0±0.0^{c}	3.2±0.1^{b}	4.3±0.7^{a}
Pb	µg/L	0.0±0.0^{b}	0.0±0.0^{b}	0.2±0.0^{a}	0.3±0.0^{a}
Cd	µg/L	0.0±0.0^{b}	0.0±0.0^{b}	0.1±0.0^{a}	0.2±0.0^{a}
NO_3-N	mg/L	1.2±0.2^{b}	2.1±0.6^{a}	0.0±0.0^{c}	0.0±0.0^{c}
NH_4-N	mg/L	0.4±0.0^{c}	1.8±0.0^{c}	10.4±0.2^{b}	18.2±0.5^{a}
PO_4-P	mg/L	0.7±0.1^{b}	0.8±0.1^{b}	1.1±0.2^{ab}	1.8±0.3^{a}

5.4. Discussion

The mobility of metals in wetlands is determined by a complex of soil factors and processes; e.g., adsorption/desorption reactions, salinity, organic matter content sulphur (S) and carbonates, plant growth, pH and redox potential (Eh). Redox processes are central to the mobility of many metals and metalloids (Du Laing et al., 2009, Burton and Hung, 2003). Whereas the mobilization of cadmium (Cd) and zinc (Zn) primarily depends on soil pH and mobile element content (Kalbitz and Wennrich, 1998). A negative correlation was found between E_H and pH (r^2 = -0.5472) which is similar to the earlier studies by Yu et al., 2007. All major soil redox reactions (such as denitrifi cation, and reduction of Mn, Fe, and SO_4^{2-}) increase soil pH but is limited by the precipitation of Fe(II) and Mn(II) carbonates occurring at about pH 7, and production of CO_2 and organic acids from decomposing OM (Yu et al., 2007a). Initially the mobility of divalent trace metals increased by decreasing pH and reducing Mn and Fe Oxides but later at 36 th DAT trace metal reduces which might be due to the precipitation of insoluble sulphides. The stability of Fe^{2+} and Fe (hydr) oxides primarily depends on a combination of Eh and pH of the sediment. The more amorphous Fe(OH)3 minerals (ferrihydrite) are reduced at a higher Eh for a given pH than the crystalline minerals of FeOOH (goethite) or Fe2O3 (hematite) (Du Laing et al., 2009). The reduction of Fe (hydr) oxides also depends on the total Fe content in the bulk soil, which was higher in our experiment (Table-5.1). It was suggested that the soil contamination may be considered when concentrations of an element in soils were two-three times greater than the average background levels (Logan et al., 1983). Fe can exist only in solution at low Eh (probably mainly as Fe^{2+}) or as soluble organic complexes in oxic soils. Thus, the Fe concentration increased in the biogeochemical microcosm set-up during low Eh values since Fe^{2+} become mobilized. In general, the same can be observed in the lysimeter as well as in the field, although some other factors might have an additional impact. Under oxic conditions Fe will precipitate as Fe oxides; trace metals can

co-precipitate or adsorb to these oxides (Du Laing et al., 2009). The low concentration of Fe and Mn in oxic treatments was found (table-5.3) which might be the result of the formation of Fe (hydr) oxides at high Eh (Yu et al., 2007a) and these oxides are very sparingly soluble (Gambrell, 1994). (van Griethuysen et al., 2005) pointed out that the dynamics of many trace metals in floodplain soils is driven by seasonal redox cycles of S, iron (Fe) and manganese (Mn). At the oxic–anoxic interface and in the anoxic layers of floodplain soils, specific redox-sensitive processes occur which often involve the precipitation and dissolution of metals. The kinetics of these processes are of large importance for these soils as the location of the oxic–anoxic interface is subject to change due to fluctuating water table levels (Du Laing et al., 2009). As a result of temporal inundations, Mn^{2+} and especially Fe^{2+} occur as soluble metal-organic complexes in soil at low Eh and can be translocate in the soil profile in this form. With the reduction of oxides elements that are fixed in them such as phosphorus (P), molybdenum (Mo), cobalt (Co), copper (Cu), Zn are often transformed to a more mobile and plant available form. Reduction and subsequent dissolution of Mn(IV–VII)-oxides and Fe(III)-oxy-hydroxides initially leads to the release of metals.

In general, the mobility and availability of heavy metals are controlled by adsorption and desorption characteristics of soils (Krishnamurti et al., 1999). The adsorption and desorption of heavy metals have been demonstrated to be associated with soil properties, including pH, organic matter content, cation exchange capacity (CEC), oxidationereduction status (Eh), the contents of clay minerals, calcium carbonate, Fe and Mn oxides (Antoniadis et al., 2008). Among these soil properties, soil pH was found to play the most important role in determining metal speciation, solubility from mineral surfaces, movement, and eventual bioavailability of metals, due to its strong effects on solubility and speciation of metals both in the soil as a whole and particularly in the soil solution. A negative correlation between soil pH and heavy metal mobility and availability to plants has been well documented in numerous

studies. For example, with decreased soil pH, the dramatic increases in heavy metal desorption from soil constituents and dissolution in soil solution was observed for Cd, Pb and Zn (Bang and Hesterberg, 2004). The mobility and bioavailability of heavy metals also increase with decreased soil pH. According to Graf et al. (2007) who worked with different soils in the pH range from 5 to 8, Fe (hydr) oxides were able to retain Cu, while Chuan et al. (1996) observed low Cd and Zn solubilities at high Eh at pH 5. They attributed this to adsorption of metals on Fe (hydr) oxides at high Eh. Differences between our data and those reported in literature may be explained by several factors. Metals like Cd, Cu, Ni, and Zn were soluble at low pH, while higher pH values decreased the solubility of these metals. One reason for that can be the desorption of metal cations from organic matter or the surface of clay minerals and other sorbents at low pH. Several authors also proved that acidic conditions mobilize Cd, Zn, and Ni ((Miller et al., 2010). For example, Chuan et al. (1996) detected that Cd and Zn solubilities were significantly higher at pH 5 in comparison to pH 8 and increased drastically at pH 3.3. Furthermore, Cu is reported to be mobile at low pH (Kumpiene et al., 2008) with a mobility threshold of pH 5.5. In addition, there was a report that soil pH appeared to be the greatest determinant of Cr, Pb and Zn solubility and mobility in a light-textured sandy soil. Solubility of divalent trace metals decreases with pH. So, reductive dissolutions of Mn and Fe oxides certainly can lead to increasing solubility if pH is kept constant; if not, these processes could only imply mobilisation without dissolution. The role of Mn reduction often appeared clearer regarding trace metal solubility than the role of Fe. Du Laing et al. (2009) suggested that for many metals (e.g., Cd and Zn), changes of the valence state as a consequence of Eh changes have not been observed in natural sediments and soils. Therefore, we can assume that high concentrations of some heavy metals in the soluble fraction under oxidizing conditions (Zn) might be related to other processes in our study.

DOM is an excellent provider of bonding sites. In most cases, metal concentrations

increased during reduction as a result of association with DOM, in some cases far exceeding quality standards for living organisms (Annex-1). To form complexes with sulfides, metals first need to dissociate from DOM. However, desorption rates are often much slower than sorption rates (Strawn et al., 1998), and sorption reactions of metals to DOM may even be non-reversible. The dark colored sediments and strong unpleasant odors often indicate the sulfide contamination in the aquatic bodies. Metal sulfides are responsible for the dark colour sediments in these reduced treatments while the odor nuisance is due to escape of volatile sulfur containing species including hydrogen sulfides into air. This findings are in agreement with the past researches (Frohne and Rinklebe, 2013). When much C was available (incubation with 3% glucose and NaOH), high concentrations of acetic and butyric acids should have favoured trace metal solubility, e.g., more than 2/3 of Pb in solution was complexed by the organic ligands formed. Considering also the spontaneous acidification in that case, one can conclude that anaerobic transformations of much available C, without other nutriments, lead to mobilisation of trace metals by acidification and complexation, besides the effects of reducing Fe and Mn compounds. Organic carbon also acts as a transporter of trace elements; indeed WSOC is the most mobile fraction of organic ligands that exist in sediments and may assist in the transfer of metal (loids) as organic complexes (Cao et al., 2004). However, organic acids are believed to contribute to the acidity of some wetlands and lakes, which suggests that natural sources of acidity can occur. Previous research shows that the mobility of trace metals from soil has a strong pH dependency. 'V shaped' leaching curves have been identified for nickel (Ni), zinc (Zn), copper (Cu), cadmium (Cd), lead (Pb) (Dijkstra et al., 2004) aluminium (Al) and iron (Fe). The solubility of manganese (Mn) is said to increase 104 times (pH range 7 to 4) and Fe^{2+} 100 times with each pH unit decrease (Xu et al., 1991). In aquatic sediments, reduction rates of sulphates were six times faster, and the release of DOM occurred in lower amounts than in soils from

terrestrial origin.

An important additional parameter potentially governing all processes mentioned above is time because these processes are often not instantaneous, but retarded by reaction kinetics, i.e. they are rate-limited. A number of studies have shown that metal (Cd, Cr, Cu, Ni, Pb and Zn) release from contaminated soil is rate-limited (Sukreeyapongse et al., 2002). Thus, the possibility of rate limitation should be considered when trying to comprehend the fate of trace metals in soil.

PO_4^{-2} in pore water increased which might be due to two main mechanisms: (1) dissolution of inorganic phases such as strengite, $FePO_4$ (Eggleton and Thomas, 2004), and (2) P-release via decomposition of particulate organic matter, which is indicated by the increase of [NH_4^+] (Monbet et al., 2007). They found a 6-fold P-release in sludge that became anaerobic, which they attributed to a reduced phosphate uptake by microorganisms. Due to the reduction of Fe(III) to Fe(II), large amounts of [Fe^{2+}] occurs in solution, and the released phosphate precipitates to form vivianite minerals [$Fe_3(PO_4)_2$]. This subsequently results in P-immobilization. The observed trends give a strong indication that the release of phosphates may boost the mineralization of organic matter, followed by the production of DOM. The dissolution of Mn-oxides may also play a role, since Mn^{2+} is an essential micronutrient in many biochemical reactions. In aerobic conditions, oxides like Mn are poorly soluble, resulting in low concentrations in pore water and hence low bioavailability. Our experiment supports this hypothesis as in pore water we experienced the concentration of phosphate in reduced treatments were getting higher with time. In our study under reduced treatments the PO_4–P in interstitial water increased which might be due to decomposition of particulate organic matter (Monbet et al., 2007).

5.5. Conclusion

For most metals (of all soil types), the greatest mobility was seen under mild to moderately

acidic conditions. Induced changes of pH can significantly affect the speciation and solubility of trace metals, and partly explain observed evolutions when redox conditions are modified (Charlatchka and Cambier, 2000). Variations of pH have been noticed in different directions. Finally all evoked processes may result in increasing or decreasing the mobility of trace metals when soils undergo reducing conditions. Among a few published works based upon detailed experimental data on real soil systems, interpretations remain ambiguous or conflicting.

Chapter 6. **CONCLUSION AND RECOMMENDATION**

6.1. Conclusion

Wetland plant response is dependent on many factors including the species, duration of soil reduction, the timing, the intensity, and the capacity of soil reduction. The reducing condition of the soil is a major factor in wetland ecosystems that influences plant survival, growth, and productivity. Thus, quantifying soil reduction is critical to the understanding and interpretation of wetland plant responses to such conditions. In addition, plant response to low soil Eh conditions also reflects species' ability to respond to such conditions by utilizing rapidly a variety of internal defense mechanisms. Nevertheless, many wetland species including those that possess a wide range of tolerance/avoidance capabilities to cope with low soil redox conditions are impacted negatively. The impact is a reflection of the fact that reducing soil conditions encompass not only soil oxygen deprivation but also production of various compounds, many of which considered highly phytotoxic. Thus, soil reducing conditions exert substantial influence on various critical plant processes including gas exchange, water relations, photosynthate partitioning, translocation, hormonal balance, nutrition, growth, and biomass production. Based on the data presented, both intensity and capacity of reduction appear to influence plant functioning in wetland ecosystems although the roles of both factors need further investigations. In wetland soils, plants are faced with a substantial demand for oxygen, the potential for loss of oxygen to soil, and the adverse effects of soil phytotoxins. Hypoxia along with elevated concentration of NH_4-N act as the important factor in distribution and abundance of these species and submerged macrophyte *E. nuttallii* and *P. pectinatus* are

poorly tolerant of anoxia in terms of cell detoxification response. Oxygen deprived reduced conditions and with elevated NH_4-N concentration retarded growth, significantly affected the photosynthetic apparatus as well as C-N balance in plants. H_2O_2 was found to promote senescence based on chlorophyll, protein degradation, decreased IAA, MDA and proline content, a decrease in membrane stability, which were partially regulated in the presence of free radical scavengers. Some essential macro and micro elements in plants were found below critical limit for plant survival. *P. pectinatus* were found to be more tolerant than *E. nuttallii* under such adverse conditions.

6.2. Recommendation

Based on the four experiments further study required on the following aspects:

- In the first experiment the effect of NH_4-N and different redox levels could not be distinguished. Thus we propose to maintain a controlled medium where the NH_4-N levels and redox levels can be used as a constant.
- The pH levels should be maintain also to examine the effect of various pH levels along with different redox levels on aquatic macrophytes
- Same experiments need to be conducted with the common aquatic macrophytes that are used for the restoration to evaluate their tolerance capacity as well as defense mechanisms under such stress conditions.
- Plant anatomy should be under taken in future experiments.
- The other enzymes related to plant defense should be consider to get complete idea on the defense mechanism under such stress.
- Molecular biomarkers of oxidative stress in aquatic macrophytes in relation to toxic environmental pollutants should be studied.
- The physiological and metabolic adaptation of *P. pectinatus* L. stem tissue in the absence of oxygen is still need to be studied.

- The effects of specific ions should also be considered. For example, phosphate inhibits arsenate uptake due to a competitive interaction hence, the phytotoxicity of As is likely to be underestimated where high P concentrations are used.
- The current results indicate that the movement of metals from soil solution to plant roots and the translocation of metals in plant tissues are complex, and dependent on many soil properties and physiological functions of plants. Therefore, apart from soil pH, redox potential and extractable metal content, as well as other soil properties, such as organic matter, hydrological conditions, salinity, calcium carbonate, cation exchange capacity and gypsum etc. and plant physiological functions should be taken into consideration for precisely predicting heavy metal concentrations in submerged aquatic plants under field conditions.
- The effects of reduction intensity and capacity on plant functioning are clearly common but varies across species. Thus, the need for additional data on various aspects of plant functioning and growth in wetland ecosystems in response to soil redox conditions, both the intensity and the capacity, as well as the specific effects of soil phytotoxins is clear.

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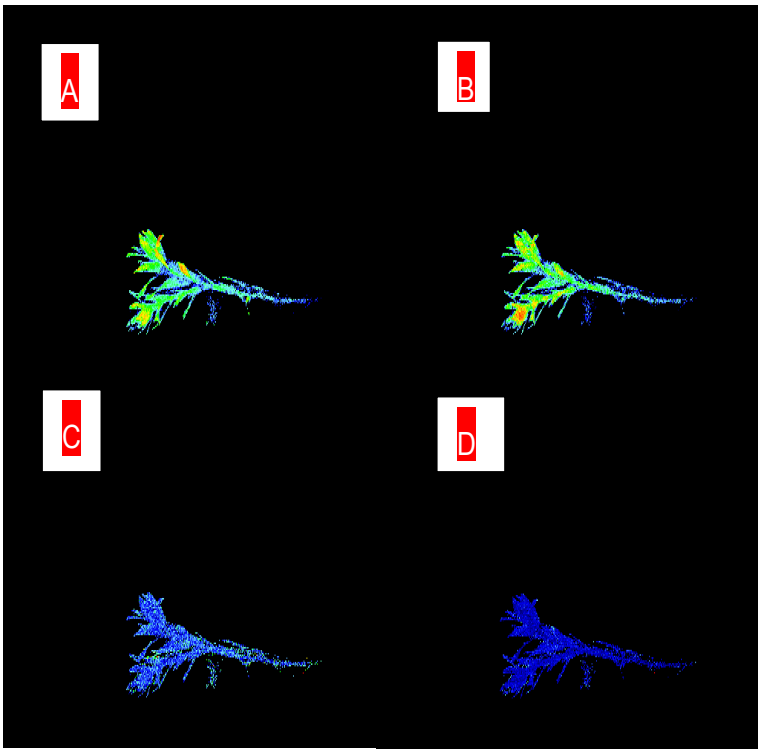
Annex

Annex 1 Water quality for living organisms (Mean heavy metal concentration in water bodies) in India and Japan

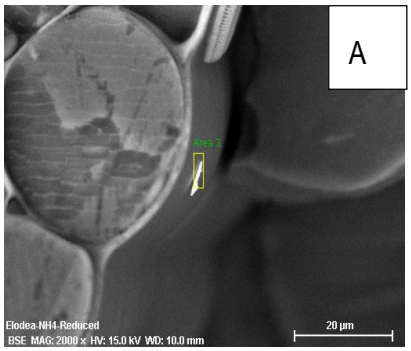
Heavy metals	CPCB, 1993 (mg/L)	MOE, Japan, 2011 (mg/L)
Iron (Fe)	3	10
Copper (Cu)	3	3
Zinc (Zn)	5	2
Manganese (Mn)	2	10
Hydrogen ion activity (pH)	5.5-9	5-8-8.6

Annex 2

A) initial QY max; B) initial fm; C) initial NPQ D) initial npqD1



Annex-3



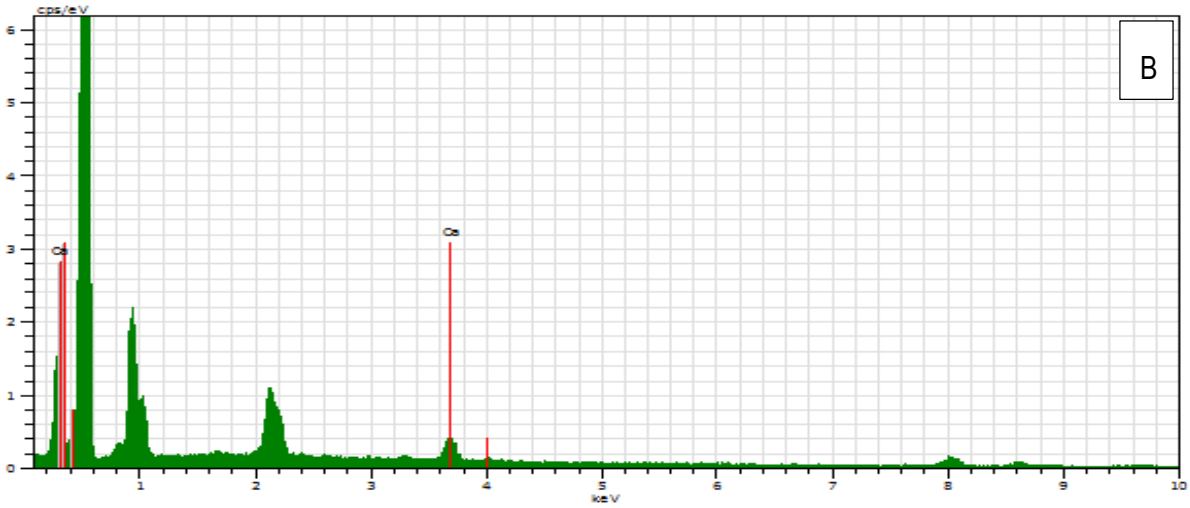


Fig. Scanned electron microscopic view of Ca concentration outer layer of *E. nuttallii* plant cell. A. Cross section of the stem of *Elodea nuttallii.* Map:HV:15.0 kv pulse Throughput:10.29kcps

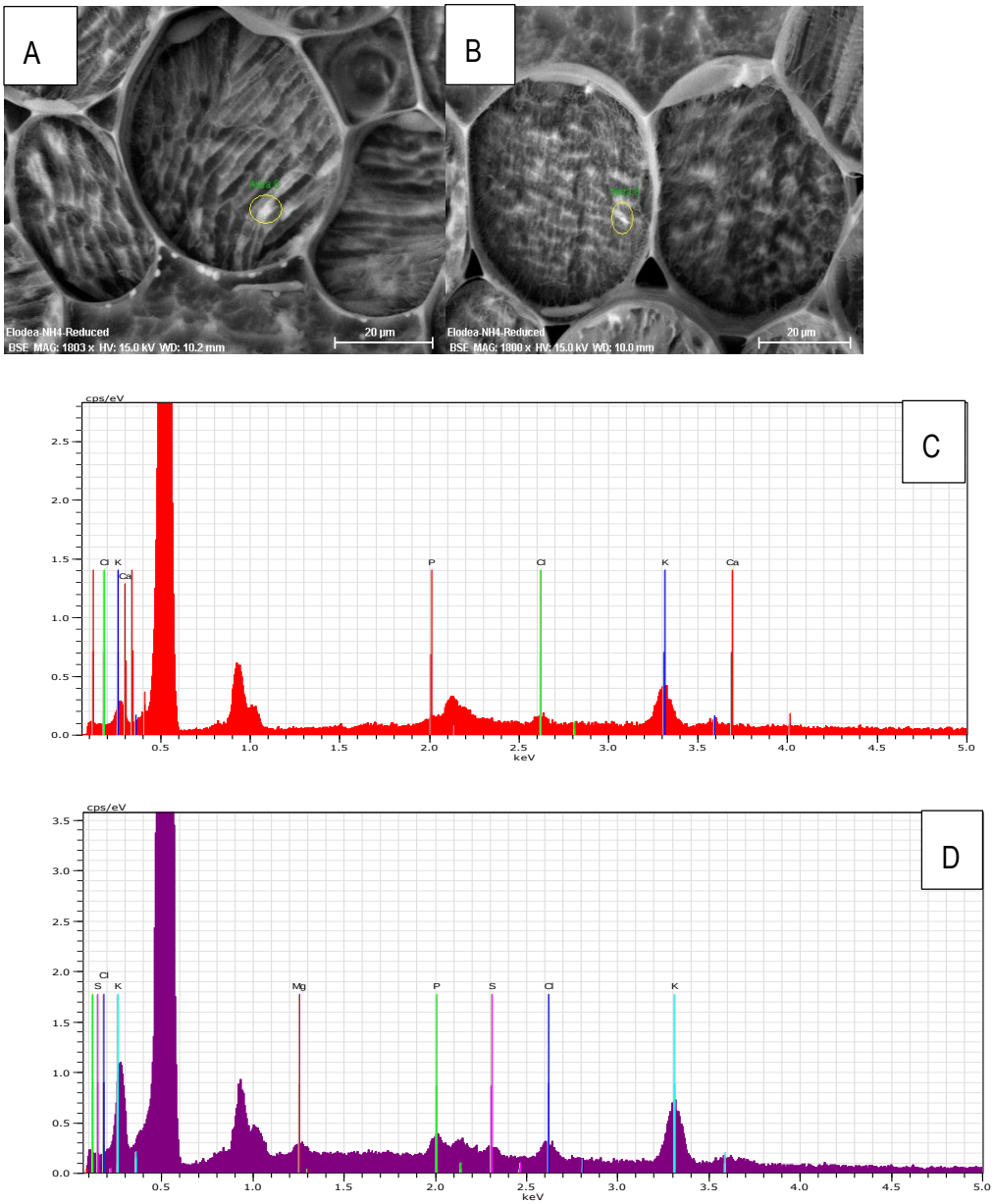
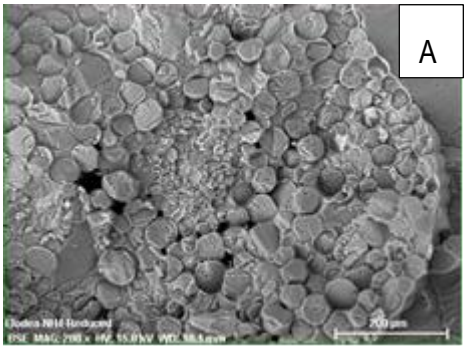
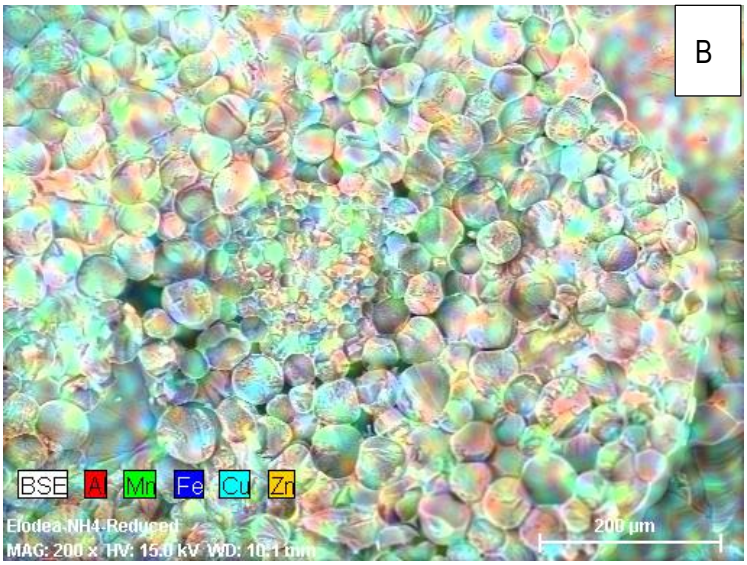


Fig. Scanned electron microscopic view of element concentration in the A and B position in *Elodea nuttallii* plant cell (image size: magnification 512×384:200xHV:15.0kV). Map (C,D): HV:15.0 kV pulse throughput:10.29kcps





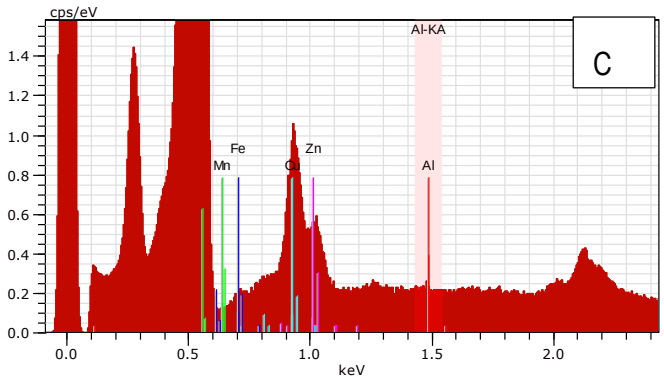


Fig. A. Section of plant stem (under SEM), B. Element distribution on the stem surface and C. Element concentration on the stem surface of *E. nuttallii* plant