CHAPTER I:
GENERAL INTRODUCTION
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Heavy metals

Heavy metals are stable naturally occurring substances, that cannot be degraded or destroyed and often tend to accumulate in the environment (soil, water and sediments) at low levels (Vardanyan et al., 2008). Indiscriminate anthropogenic activities have altered their geological and biological redistribution resulting in an increase in their concentrations in the environment (Singh et al., 2011a). Such alterations influence the metal toxicity by accumulation throughout the foodchain in plants and animals (Malik, 2004).

Heavy metals are defined as those metals whose density exceeds 5 g per cubic centimeter (Barakat, 2011). However, it has been suggested that in the context of plants, the definition of the term ‘heavy metal’ should not be based on the density of the metal in elemental form, as the elemental form of the metal is not accessible to plants. Instead, it should be based on the periodic table as the position in the periodic table better defined the chemical properties of the element (Appenroth, 2010). Some of the heavy metals such as Cu, Mn, Fe, Zn, Ni and others are considered to be essential micronutrients for plant growth and development (Uysal and Taner, 2007) but have the potential to become toxic to plants when present in bioavailable forms at excessive levels (Reichman, 2002; Tuna et al., 2002; Chen et al., 2009). On the contrary, other heavy metals such as Hg, As, Cd, etc. have no known beneficial role in living organisms and are toxic even at very low concentrations (Sandalio et al., 2001; Järup, 2003; Göthberg and Greger, 2006; Shaibur et al., 2009).

Sources of heavy metal pollution in the environment include both natural as well as anthropogenic activities. Some of the natural sources of heavy metals are weathering of minerals, erosion and volcanic activity, while anthropogenic sources include mining, smelting,
energy and fuel production, electroplating, sludge dumping, use of pesticides and fertilizers as well as diffuse sources such as combustion by-products and vehicle emissions and also application of commercial inorganic fertilizers in the field, industrial discharge, urban runoff, sewage effluents, industrial chimney gases, domestic wastes and garbage etc. (Tuna et al., 2002; Modaihsh et al., 2004; Chehregani and Malayeri, 2007; Vardanyan et al., 2008; Fulekar et al., 2009; Wuana and Okieimen, 2011; Shagal et al., 2012).

Copper

Copper (Cu) is the 29th element of the periodic table and is found in +1 and +2 oxidation states in biological systems (Yruela, 2005; Rusjan, 2012). Several compounds of Cu have been used in the manufacture of wires for electrical appliances, manufacture of alloys, fungicides, herbicides and pesticides, electroplating of iron, as wood preservatives, as a pigment for ceramics, and as an antirust protection agent for marine paints (Shrivastava, 2009; Rusjan, 2012). Besides this there are also other sources of Cu which would lead to the contamination of the environment (water bodies) such as plastic industry, blast furnace, agro-based industry, and uncontrolled waste dumps (Gowd and Govil, 2008).

Cu is an essential trace metal required by plants for their normal growth metabolism. It has a regulatory role in photosynthetic electron transport, oxidative phosphorylation, catalyzing redox reaction in mitochondria, chloroplast, cell wall, and in the cytoplasm of plant cells (Maksymiec, 1997; Fargašová, 2004; Yruela, 2005). Its deficiency causes chlorosis, reduces respiration rates, reduces phenol oxidase activity leading to the distortion of leaves and stems, and inadequate auxin supply leading to the lack of germination (Rusjan, 2012). Cu deficiency in humans also causes several brain diseases such as Menkes and Wilson’s diseases, premature births, chronic diarrhoea, stomach diseases (Krupanidhi et al., 2008; Rusjan, 2012). Cu, despite
being an essential trace nutrient, can be highly toxic to both plants and animals at elevated level. Cu is mainly found in the environment in water-soluble and ethanol-soluble form, and is consequently easily translocated in plants (Cheng, 2003). Cu is transported into the cytoplasm and to intracellular compartments of the plant cells by several metal chaperones which bind the Cu ions (Yruela, 2005).

Plants, especially algae and aquatic plants are highly sensitive to Cu because they are directly exposed to its toxic effects in contrast to the land plants where the harmful effects of Cu are reduced because of its strong binding to organic and inorganic colloids (Fernandes and Henriques, 1991). Several studies have been conducted on the toxic effects of Cu on plant growth (Alaoui-Sossé et al., 2004), photosynthetic pigment concentration (Singh et al., 2007), deformation of root structures (Sheldon and Menzies, 2005), cytogenetical and ultrastructural alterations (Küpper et al., 2009), and changes in antioxidant enzyme activities (Devi and Prasad, 2005).

**Nickel**

Nickel (Ni) is the 28th element of the periodic table. It is a silver-white metal which occur in several oxidation states (1 to +4) and commonly in the oxidation state of +2 in biological systems (Ilic et al., 2007). Ni is used in the manufacture of alloy and stainless steel, in dye manufacture, electroplating, and in porcelain enameling (Das et al., 2008; Padmavathy, 2008; Sreekanth et al., 2013). However, other anthropogenic activities increase the level of Ni in the environment (water bodies) which include municipal sewage sludge, sewage treatment plant contaminants, groundwater near landfill sites, and tannery effluent (Gowd and Govil, 2008; Tariq et al., 2005).
Ni is essential for plants whose deficiency can lower urease activity, affect citric acid cycle, disrupt amino acid and carbon metabolism, failure to produce viable seeds, reduced Fe uptake, and chlorosis in leaf and meristem tissues (Brown et al., 1987a; Bai et al., 2006; Ahmad and Ashraf, 2011). Soluble Ni form enters the plant bodies either through cation transport system or by forming metal-ligand complex with chelators such as nicotianamine, histidine and organic acids (Chen et al., 2009). It has a role in maintaining the levels of urea in the plant tissues and in the absence of Ni, lower root and shoot weights were observed in barley plants (Brown et al., 1987b). Ni is essential for the conversion of arbuscular mycorrhizal urea to root cell ammonia in nitrogen poor soils for better growth of plants (Polacco et al., 2013). In case of bacteria it is required in hydrogen metabolism, biosynthesis of carbon monoxide dehydrogenase, and acetogenesis (Anke et al., 1984; Ahmad and Ashraf, 2011). The importance of Ni in human health has not been well specified (Nielsen, 1991; White and Brown, 2010).

Ni becomes toxic to plants at higher concentrations inhibiting mitotic activities, damaging DNA, interfering in biochemical and physiological processes, and also having adverse effects on fruit yield and quality as well as decreased water and nutrient uptake in plants and antioxidative enzyme activities (Madhava Rao and Sresty, 2000; Seregin and Kozhevnikova, 2006; Gajewska et al., 2006; Yan et al., 2008; Gjorgieva et al., 2013). Ni reach the human body mainly through inhalation and ingestion. Acute poisoning of nickel carbonyl causes frontal headache, vertigo, nausea, vomiting, insomnia and irritability in humans (Ilic et al., 2007). Ni toxicity can also lead to dermatitis, asthma, conjunctivitis, skin allergy reaction, and cardiac arrest. Besides, it can also act as a carcinogenic agent (Anke et al., 1995; Cempel and Nikel, 2006; Das et al., 2008; Mishra et al., 2010).
Biochemical components of the plants and heavy metals

Biochemical composition of the plants plays a major role in promoting growth and development of the plants and is also considered as important parameters commonly assessed for phytotoxicity testing.

Chlorophyll is the main green pigment involved in photosynthesis which allows plants to absorb energy from sunlight. It produces green colour in the leaf and the stem. There are several types of chlorophyll such as chlorophyll \( a \), \( b \) and \( c \). The most common pigment is chlorophyll \( a \), which is present in all plants, followed by chlorophyll \( b \), while chlorophyll \( c \) is mostly found in photosynthetic members of dinoflagellata and chromista. It contains a porphyrin ring with magnesium as the central atom. Chlorophyll metabolism in plants influences processes such as the ‘stay-green’ phenomenon and also chloroplast-nucleus communication (Tanaka and Tanaka, 2006).

Heavy metal accumulation in leaf tissue of plants causes alteration in the functioning of chloroplast membranes and also blocked the synthesis and activities of enzyme proteins responsible for chlorophyll biosynthesis (Prasad and Strzałka, 1999; Jayakumar et al., 2009). Under heavy metal stress, central ion of chlorophyll molecules, Mg ion in both the antenna complex and reaction centers is being replaced by Cu, Hg, Cd, Ni, Zn, Pb ions thereby blocking the electron transport chain in the reaction centres (PSI and PSII) leading to the breakdown in the photosynthesis process of plants (Küpper et al., 1996,1998). A reduction in electron density of chloroplast stroma and the number of grana along with altered structure of thylakoids was observed in plants under Ni stress conditions (Molas, 2002). Heavy metals were found to inhibit or reduce the chlorophyll content of the leaf tissues (Stoeva and Bineva, 2003; Martins and Mourato, 2006; Shakya et al., 2008; Muslu and Ergün, 2013).
Proteins are biological molecules consisting of one or more chains of amino acids. Functions of proteins in plant cells include regulation of primary metabolism, ion transport, cellular trafficking, chloroplast and mitochondrial enzyme activities, and gene transcription (Aducci et al., 2002). Plants contain low levels of proteins compared to animals, as plant structures are mainly composed of structural carbohydrates (Plant and Soil Sciences elibrary, 2014). Amino acids serve as a source of nitrogen for some seedlings that can be catalyzed by enzyme protease exuded from seedling roots. In order to limit the use of inorganic nitrogen fertilizers, the use of organic fertilizers having protein as a nitrogen source can be considered for sustainable agriculture (Adamczyk et al., 2010). However protein function in plants can be altered or inhibited under high concentrations of heavy metals, as heavy metals can bind to the sulfhydryl groups in proteins, thereby inhibiting enzyme activity or protein function (Yruela, 2005). Changes in the expression of photosynthetic protein, inhibition of protein biosynthesis in plants, and reduction in protein content were observed under heavy metal stress (Kumar et al., 2011; Vijayarengan, 2012; Vinod et al., 2012).

Carbohydrates are generally available as an immediate energy source for most animals and plants after having been broken down by enzymes. Plant tissues typically store available carbohydrates in stem, root, tuber, rhizome, etc. This storage ability is useful in evaluating the potential of plants for regrowth and seed production following unfavourable seasons (Madsen, 1997; Taliercio et al., 2009). These storage carbohydrates may also be important in generating the idea of management techniques employed for controlling invasive species, such as harvesting and use of contact herbicides (Madsen, 1997). Under elevated level of heavy metals, reduction in carbohydrate concentration was observed (Singh et al., 2006; Deef, 2007; Bhardwaj et al., 2009; Ezhilvannan et al., 2011; Gubrelay et al., 2013).
Ascorbic acid (vitamin C), a major metabolite in plants, plays a role as an antioxidant, in photoprotection, as enzyme cofactor and in oxalate and tartrate synthesis (Smirnoff, 1996; Conklin, 2001). It is also known to increase the tolerance of plants to oxidative stresses (Khan et al., 2011). In humans, it is involved in transportation of ions, conjunctive tissue formation, conversion of L-phenylalanine into nor-adrenaline, uptake of iron, regulation of blood pressure, and prevention of arteriosclerosis (Barata-Soares et al., 2004; Sarkiyayi and Ikioda, 2010). It is found in a variety of plant tissues commonly associated with chloroplasts and is considered as an excellent reducing agent. Its deficiency causes scurvy in humans (Arya et al., 2000). Ascorbic acid was found to increase in plant tissues under heavy metal stress (Pandey and Tripathi, 2011; Pant et al., 2011; Sinha and Shrivastava, 2012).

Any disturbance in the normal functioning of these biochemical components of the plants can be used as a tool in determining the mechanism and severity of metal toxicity and thus indicating the stress condition (Gubrelay et al., 2013).

**Enzyme activities and heavy metals**

Plants have evolved many mechanisms of defence against toxic metal ions which include binding of metal ions to cell wall or inhibition of long distance transport as an immediate defensive response. If further toxification continues then sequestration and compartmentalization of metal ions into and within the vacuoles as well as metal transportation and chelation continues to isolate the contaminated ones from the sensitive cellular components. This is followed by antioxidative defence mechanisms to scavenge reactive oxygen species (Manara, 2012; Hossain, 2012).

Catalase (CAT) is an iron containing antioxidative enzyme, which exists mainly in the peroxisomes in all aerobic organisms (Mourato et al., 2012). It degrades hydrogen peroxide as:
Thus, it prevents oxidative stress due to metal toxicity (Chen et al., 2000; Mourato et al., 2012). Among the H$_2$O$_2$ degrading enzymes, CAT degrades H$_2$O$_2$ without consuming reducing equivalents thus providing energy efficient mechanisms to remove H$_2$O$_2$. Catalase occurs in multiple enzymatic forms in a variety of plant species suggesting multiple functions of catalase in different plant tissues at various developmental stages and under constantly changing environmental conditions (Scandalios et al., 1997). Under heavy metal stress, CAT activity is stimulated in higher concentrations of metals (Yurekli and Porgali, 2006; Sbartai et al., 2011; Karimi et al., 2012; Sharma and Singh, 2012; Malecka et al., 2012) but the activity is inhibited in lower concentrations of metal (Sbartai et al., 2011).

Peroxidases are single-polypeptide chain, haem-containing large family of enzymes that are ubiquitous in fungi, higher plants, vertebrates and are involved in removing hydrogen peroxide formed due to induced stress using different reductants (Ghamsari et al., 2007; Mika and Lüthje, 2003; Mourato et al., 2012). They have the general reaction (Bisswanger, 2004):

\[
\text{Donor} + \text{H}_2\text{O}_2 \rightarrow \text{Oxidized donor} + 2\text{H}_2\text{O}.
\]

On the basis of their donor, peroxidases are categorized as guaiacol peroxidases that use guaiacol (o-methoxyphenol) as electron donor or reducing substrate; ascorbate peroxidase that uses ascorbic acid; and glutathione peroxidase that uses glutathione (Mourato et al., 2012; Ghamsari et al., 2007). In case of soyabean, guaiacol peroxidase is mainly localized in seed coat and is a very stable and completely soluble enzyme (Sariri et al., 2003). Guaiacol peroxidases are located in cytosol, vacuole, cell wall, apoplast and extracellular medium (Ghamsari et al., 2007). An alteration in the normal functioning of peroxidase activity was
observed under heavy metal stress condition (Panda et al., 2003; Verma and Dubey, 2003; Singh et al., 2006; Wang et al., 2010).

**Scanning electron microscopy studies and heavy metals**

Plants are the major recipients of various pollutants in the environment and respond directly to any alteration or deterioration in air, water and soil quality. Under different stress conditions they undergo significant morphological and metabolic changes. In case of metal toxicity, visible symptoms in plants like changes in the colour of leaves, stunted growth or even death are considered to be the structural and ultrastructural alterations at the cell, tissue and organ level induced by metals (Ghelich and Zarinkamar, 2013a). Increase in the size of guard cells in both adaxial and abaxial surfaces along with alteration in structure and deposition of epicuticular waxes in leaf tissues under heavy metal stress was observed when analyzed with scanning electron microscope (SEM) (Ghelich and Zarinkamar, 2013b). Disruption and alteration in the orientation and arrangement of vascular as well as associated tissues were observed in stem and crimped structures of xylem and phloem elements occurred in root. Scanning electron microscopic analysis detected reduced frequency of occurrence of stomata, alteration in the structure of guard cells, and closure of stomatal apertures in leaves (Mondal et al., 2013). Scanning Electron Microscope-Energy Dispersive Spectrophotometer (SEM-EDS) technique is also used in quantitative analysis of some elements in the samples (Ramamurthy and Kannan, 2009) and also localization of metals in different cellular structures within the plants was done (Sánchez-Pardo et al., 2012).

**Bioaccumulation of heavy metals**

Bioaccumulation of heavy metals deals with the uptake of metals by organisms from its metal contaminated environment as would occur in nature. Plants have the ability to
bioconcentrate the essential and non-essential metals to a much higher concentration in their roots and shoots than that available in the soil (Raskin et al., 1994). Aquatic plants serve as indicators of heavy metal pollution in aquatic ecosystems by accumulating heavy metals in their bodies and thus showing morphological alterations. Submerged or emergent plants accumulate metals through the root and the shoot system from water and through the root from sediment (Göthberg, 2008). Accumulation of heavy metals in plant bodies depends on the plant species involved, element species as well as environmental conditions (Cheng, 2003). The extent and pattern of accumulation of metals differs in the shoot and root systems. In most plants, metal accumulation is more in root as compared to shoot of the plants. Above all the metal concentration in the plant bodies irrespective of root and shoot increases with increasing metal concentration in the growth medium or the environment (Wu et al., 2004; Israr et al., 2006; Phetsombat et al., 2006; Xiao et al., 2008; Hu et al., 2010). Regarding the bioaccumulation potential of the plants, several studies have been conducted to understand the phytoremediation potential of different species. Phytoremediation was found to be environment-friendly, cost effective and applicable to a wide range of contaminants (Tangahu et al., 2011) as compared to other physical and chemical treatments such as soil washing, immobilization techniques (Wuana and Okieimen, 2011), soil flushing, solidification, vitrification, thermal desorption, encapsulation (Jadia and Fulekar, 2009), and adsorption using activated carbon (Keskinkan et al., 2004).

There are also limitations to phytoremediation techniques which include lengthy period of time. Further, large amount of biomass productivity is required for sufficient removal of toxic element. Yet another factor is the age of the plants, as roots of young plants have greater ability to absorb ions than the older plants of similar size. Other limiting factors include soil
chemistry and level of contamination, root depth, climatic condition, changes in the chemical form of the metal, range of contaminant concentration, and the like. Because of the aforementioned constraints, the maximal metal levels that can be accumulated by plants are far below the optimum requirement of metal removal rate (Tangahu et al., 2011).

**Ipomoea aquatica** Forsk. and heavy metals

*Ipomoea aquatica* (English: Water spinach; Hindi and Bengali: Kalmi) is considered to be a native of Africa, Asia and southwestern Pacific Islands (Austin, 2007). *I. aquatica* is a semi-aquatic, trailing or floating perennial herb with long, prostrate, hollow stem. Leaves are broad, acute cordate or hastate, entire with long petiole. It is fast growing and is propagated mainly by fragmentation; the roots are readily produced from the nodes producing new plants when segmented. Flowers are infundibuliform, solitary, consisting of five free sepals and five united pale purple petals (Austin, 2007; Göthberg, 2008; Manvar and Desai, 2013).

**Taxonomic classification (USDA)**

- **Kingdom:** Plantae
- **Subkingdom:** Tracheobionta
- **Superdivision:** Spermatophyta
- **Division:** Magnoliophyta
- **Class:** Magnoliopsida
- **Subclass:** Asteridae
- **Order:** Solanales
- **Family:** Convolvulaceae
- **Genus:** *Ipomoea*
- **Species:** *aquatica* Forsk.
The tender shoots and leaves of *I. aquatica* are mainly consumed as green vegetable by humans throughout northern India, Bangladesh and southeast Asia and is used as fodder as well. In countries like Malaysia, China, Singapore, Hong Kong, Indonesia, Vietnam and Thailand, the plant is being cultivated commercially for human consumption. In Manipur, North East India, the boiled extract of tender shoot is also used to treat the excessive thirst in diabetic patients (Khan and Yadava, 2010). Thus this plant is a valuable biological resource in a large part of Asia where it meets the nutritional and nutraceutical needs of the people. On the contrary, it is also known to behave as an invasive species outside its native range (Varshney et al., 2008). However, *Ipomoea* habitats in south and southeast Asia are increasingly receiving sewage and other wastewater with possible metal contamination of its biota. *I. aquatica* collected from several sites near Bangkok revealed accumulation of Cd, Pb and Hg (methylmercury) with the latter reaching concentrations high enough to cause health concerns (Göthberg et al., 2002). In addition, *I. aquatica* has been proposed to be a potential bioremediator of several metals such as Zn, Cu, Pb, Ni, Co, Cd in metal contaminated sites in Gujarat, India (Kumar et al., 2008). The phytoremediant ability of this plant has also been reported for metals Cr, As, Cd and also for plasticizer or ectoparasiticide, di-n-butyl phthalate (Weerasinghe et al., 2008; Lee et al., 1991; Kashem et al., 2008; Cai et al., 2008). It is also considered to be one of the most useful plant species in phytoremediation studies due to its ability to accumulate heavy metals in high concentration in the roots (Kumar et al., 2008). This corresponds to the property of floating aquatic hyperaccumulating plants that absorb or accumulate metals by its roots and that of the submerged plants, which accumulate by their whole body (Rahman and Hasegawa, 2011).
Several studies have been conducted on plants regarding the toxic effects of heavy metals but similar investigations on *I. aquatica* are relatively rare in India. The availability of heavy metals Cu and Ni has been frequently reported in different parts of India including North east India. The present study was conducted to investigate the response of *I. aquatica* to Cu and Ni in relatively short- and long-term exposures at higher and lower concentrations of these two metals. While the main aim of the short-term study was to identify the acutely toxic concentrations of the two metals, the changes in the biochemical along with enzymatic activities were studied in the long-term exposure. Further, responses of the plant to the respective metals have been studied in terms of its morphological changes. The metal accumulation potential of this plant has also been studied in the present investigation.
OBJECTIVES

1. To estimate the acute toxicity of copper and nickel on *I. aquatica* Forsk.

2. To investigate the chronic toxicity of copper and nickel on *I. aquatica* Forsk. against several toxicological endpoints such as growth (shoot height, number of nodes, number of leaf, number of tiller, width of leaf blade and fresh weight).

3. To investigate the changes in biochemical constituents and enzymes activities.

4. To investigate the appearance of stem tissue necrosis in metal-exposed plants and to study the associated biochemical changes.

5. To observe the changes induced by copper and nickel on the ultrastructural morphology of stem tissues using scanning electron microscopy.

6. To study the bioaccumulation of copper and nickel in *I. aquatica* Forsk.