ACCUMULATION AND TOXICITY OF HEAVY METALS, PARTICULARLY CADMIUM, IN LEMNA POLYRRHIZA L. AND AZOLLA PINNATA R. Br.

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PREFACE

In the aftermath of Minamata and Nigata incidents in Japan, a lot of concern has been aroused about the harmful effects of heavy metal pollution in aquatic environment. This led to a surge in studies concerning metal pollution effects on aquatic organisms. Although a good deal of information has been generated on metal effects and accumulation in algae, fungi and bacteria, higher plants, including aquatic macrophytes, have been little explored in this regard. Macrophytes have great relevance in such kinds of investigations because they are important primary producers in ponds and lakes. Tremendous potential exists in using these plants for metal toxicity bioassays, and also for the reclamation of metal-enriched wastewaters. These considerations prompted me to take up this work. Lemna polyrrhiza L. and Azolla pinnata R.Br. which grow abundantly in pools, ponds and lakes in India, were selected as the test organisms in the present study.

The thesis is organised into six chapters with 'Summary' at the end. The Chapter I 'General Introduction' reviews the current status of the subject and gives the reasons for embarking upon this study. The second chapter deals with toxicity and accumulation of Cd, Co, Cr, Cu, Ni, Pb and Zn in test plants. The kinetics of extracellular and intracellular uptake of Cd are described in Chapter III. This is followed by investigations on influence of cations and metals on Cd uptake

in Chapter IV. The next chapter (Chapter V) discusses the influence of physico-chemical characteristics of the environment on Cd toxicity and accumulation. Although each chapter includes discussion, a brief 'General Discussion' (Chapter VI) has also been included. A list of papers cited in the text is given at the end in 'Literature Cited'.

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Chapter-I

GENERAL INTRODUCTION

The term 'heavy metal' although often not rigidly defined, is generally held to those metals having specific gravity greater than 5 g cm^{-3}, about 40 elements in all (Passow et al. 1961). Nieboer and Richardson (1980) suggested the abandonment of the term 'heavy metal', and its replacement with a classification separating metals and metalloids into class A (oxygen-seeking), class B (nitrogen/sulfur-seeking) or borderline (intermediate between A and B) category. This classification is related to atomic properties such as electronegativity and ionic radius, and solution chemistry of metal ions. Class A ions include the alkali metals and alkaline earths, notably the biologically essential K^{+}, Na^{+}, Mg^{2+} and Ca^{2+}. Class B ions, in contrast, include Cu^{+}, Hg^{2+}, Ag^{2+} and Pb^{2+} which are extremely toxic and for the most part non-essential. Borderline ions include Fe^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+}, which have biological roles. Whitton (1984), however, advocates that the term 'heavy metal' should continue to be used in pollution studies. Tiller (1989) pointed out that 'heavy metal' may be a useful umbrella for metals classed as environmental pollutants. These heavy metals constitute a very heterogenous group of elements which greatly differ in their chemical properties and biological functions. However, high concentrations of all heavy metals are toxic to living organisms.

Low concentrations of some heavy metals, such as, Cu, Fe, Mn and Zn, are essential for the metabolic machinery of plants. Nevertheless, metals are toxic to microbes, plants and animals at higher concentrations. The essential heavy metals serve as co-factors and activators in enzymatic reactions, e.g., in forming enzyme/substrate/metal complexes (Mildvan 1970), or they exert their catalytic properties as a prosthatic group in metallo-proteins. Most of these essential metals function by valency change (Sändmann and Boger 1983). Copper is an essential element for plants, and is a component of metalloenzymes and respiratory pigments, such as laccase, ascorbate oxidase, plastocyanin, tyrosinase and amine oxidases (NAS 1977). Manganese has an important role in the reactions of some enzymes (e.g., malic dehydrogenase and oxalosuccinic decarboxylase) in the Krebs cycle (Devlin 1979). It is also needed for water-splitting activity of PS II and for superoxide dismutase. Nickel is essential for plant growth as it acts as a co-factor for the urease enzyme system (Dixon et al. 1975, Polacco 1977). It is also needed for the activity of hydrogenase. In plants, Zn is an essential component of several enzymes such as carbonic anhydrase, alcohol dehydrogenase, and glutamic dehydrogenase. Zinc catalyzes oxidation process in plant cells, participates in the formation of carbohydrates and helps in the absorption of water (Lindsay 1972, Farnworth and Kline 1973). Although regarded as non-essential, Cd has been recently found to replace Zn in some of its functions in the marine diatom <u>Thalassiosira weissflogii</u> (Price and Morel 1990a). The actual site(s) of replacement remains to be elucidated, but in suspension-cultured

tobacco cells, stimulation of biomass production by Cd is correlated with an increase in RNA synthesis (Hirt et al. 1989), suggesting that Cd acts at the level of transcription.

Heavy metal input into aquatic environments occurs from two sources: (i) natural (geologic weathering processes), and (ii) man-made (anthropogenic). One can be misled into believing that metal pollution arises only through anthropogenic means, but geothermal discharges, especially if they are saline or at low pH or high temperature, can contain considerable levels of metals (Smith 1986). Geothermal springs can contaminate natural waterways and algae growing in hot mud pools (Förstner and Wittmann 1981), and submarine geothermal activity is no doubt a source of trace metals in oceanic systems. Volcanic action is a major natural source of Cd (Hutton 1987), and high background levels of As and Hg in streams resulting from weathering of rocks and soil have also been recorded (Förstner and Wittman 1981).

The main sources of metals from a variety of anthropogenic inputs on a global basis are atmospheric fall out, manufacturing processes of metals, chemicals, domestic wastewater, dumping of sewage, sludge, etc. Lead contamination in freshwater and marine systems is unusual in that it arises mainly by atmospheric transport. Organolead comes from volatilization of gasoline additives, but inorganic Pb is a more general problem (Jaworski 1987). An inventory for Lake Erie (Nriagu et al. 1979) revealed that atmospheric inputs accounted for 20 and 50% of Cu and Pb, respectively. Atmospheric dispersion of Hg is probably via Hg^{o}.

Metal contamination from urban run off and erosion is significant (Jaworski 1987). Organometallic compounds, such as tributyl tin used as antifouling paints on pleasure crafts, can lead to reduced primary productivity in estuaries and marina (e.g., Langston 1990).

Heavy metals in aquatic environments include metals in the sediment, in suspended particulates (>0.45 µM), adsorbed onto oxides and humic colloides, and in true solution. Particles arising from dead biomass are particularly effective in scavenging metals from solution, a high percentage of metals is usually carried by rivers to the oceans in particulate matter (Martin and Whitfield 1983). The enrichment of metals in the dissolved phase (from interaction with sediments and particulate matter) may be more dependent on local natural hydrochemical conditions, than on further anthropogenic inputs. Thus dissolved Cd levels along rivers can fluctuate by as much as a factor of eight depending on local conditions of pH, salinity, chloride ions and alkaline earth elements (Meybeck et al. 1989). Estuaries can be considered to be zones of metal deposition because of reduced flow rates, changes in pH and flocculation of negatively charged particles and colloidal aggregates of Fe oxides and organic materials at the freshwater-seawater interface (Moore and Ramamoorthy 1984). Bryan et al. (1985) listed some of the more common metal species present in aquatic systems, but there is an almost endless array of metal species possible in polluted and unpolluted waters as inorganic complexes, as complexes derived from synthetic and natural chelators present in water, as

organometallic compounds of both anthropogenic and natural origins. The biomethylation of Hg, As, and Sn have been shown to occur (e.g., Smith 1986).

Industrial and mining effluents can contain extremely high concentrations of metals, which can persist despite remedial works to stabilize spoil heaps. For example, in Australia, the concentrations of Zn in the river immediately below a mine were still very high (up to 25.3 mg l^{-1}) twenty years after the mine had closed (Kelly 1988). Heavily contaminated sites such as these are generally impoverished or completely denuded in fauna. Resulting changes in species abundances of both algae and fauna have been described at length by Kelly (1988).

Upon exposure to high levels of heavy metals, algae and higher plants accumulate these pollutants to a dangerous extent. A great deal of information has been generated on algae, whereas higher plants, including macrophytes, have been little explored in this regard. *Lemna trisulca* could concentrate Cd in its tissues upto 24,800 times in comparison to the growth medium (Huebert and Shay 1991). *Lemna valdiviana* accumulated Cu more than 10,000 times the concentration in the culture solution, and the concentration appeared to increase at the higher levels of metal in solution (Hutchinson and Czyrska 1975). The water hyacinth can accumulate upto 500 ppm of Cd, Pb and Hg (Chigbo et al. 1982). *Azolla filiculoides* accumulated Cd, Cu, Ni and Zn at concentrations 500 to 1,000 fold higher than in the growth medium (Sela et al. 1989). *Agrostis* grown in culture solution,

containing 6 ppm Cu accumulated 3,500 ppm metal in roots (Wu et al. 1975). In freshwater plants the concentration of Cd ranges between 0.15 and 342 $\mu g\ g^{-1}$ (Moore and Ramamurthy 1984).

In higher plants, roots are the first organs to come in contact with the toxic metals, and roots usually accumulate significantly higher metal levels than the aboveground plant parts (Breckle 1989). Koeppe (1977) observed that roots generally contain two-fold cadmium concentration in comparison to the top portion. Accumulation of As in the root of Silene vulgaris is much higher than the shoot (Paliouris and Hutchinson 1991). In Eichhornia crassipes and Pistia stratiotes the concentration of As in the roots was an order higher than that in the leaves (Lee et al. 1991). High quantities of Cd, Cu and U were localized within the cell wall in the shoot and root of Azolla (Sela et al. 1988), and as much as 70% of the total content of Cd taken up by beans was stored in the cytoplasmic fraction in roots (Weigel and Jager 1980). All these reports tend to indicate that roots can immobilize heavy metals and raise a very efficient barrier against heavy metal translocation within the plant. Leita et al. (1991) also indicated the existence of a physiological barrier for Cd in roots and stems of Phaseolus vulgaris.

It would be tempting to assume that because primary producers are at the bottom of the trophic ladder, herbivory would lead to the biomagnification of metals within higher trophic levels. Although biomagnification, the process whereby a

substance is found at higher total body tissue concentrations at successively higher trophic levels, may be true for certain organic pollutants. The case of heavy metals points to the reverse (Moore and Ramamoorthy 1984). In general, with the possible exception of Hg and As (e.g., Kneip and Laver 1973, Förstner and Wittmann 1981, Langston and Bryan 1984, Prahalad and Seenayya 1988), heavy metals are not biomagnified from algae because algae contain higher concentrations in polluted and unpolluted situations than the next member in the food chain (Kneip and Laver 1973, Förstner and Wittmann 1981). Some recent examples which have largely confirmed the absence of biomagnification from algae include studies on As, Cr, Mn, Fe, Cd, Cu, Pb and Zn (Moore and Ramamoorthy 1984, Tateda et al. 1985, Romeo and Nicolas 1986, Prahalad and Seenayya 1986, Seenayya and Prahalad 1987, Sanders et al. 1989).

The process of heavy metal uptake by organisms is very complex, and dependent on the metal ion and the biological system in question. In aquatic plants the uptake occurs in two stages, an initial rapid uptake (passive uptake) followed by a much slower uptake (active uptake). During the passive uptake the metal ions adsorb onto the surface of the cells within a relatively short span of time (few seconds or minutes). This includes the physical adsorption, ion exchange and chemosorption. In the second stage, the metal ions are transported across the cell membrane into the cytoplasm. The surface adsorption does not involve any metabolic process or require any expenditure of energy by the cells, while the membrane transport is dependent on

cell energetics and metabolism. Two phases can also be distinguished in the process of Cd uptake by algae. The first fast phase is Cd adsorption on the cell surface and the second slow phase is the energy-dependent transport (Skowronski 1984a and 1984b). Similarly, Skipnes et al. (1975) have shown that the uptake of Zn in <u>Ascophyllum</u> <u>nodosum</u> occurs through a fast reversible process and a relatively slow irreversible process. In water hyacinth, Turnquist et al. (1990) found a rapid initial Ni uptake extending through approximately the first 4 h at lower concentration, but of some what shorter duration at higher concentrations. The uptake of Ni was increased by an increase in the root mass. The uptake of Cd by <u>Chlorella</u> also follows two phases, the initial was not affected by temperature or by light, whereas the following phase was light- and temperature-dependent, and the absorbed Cd was firmly bound to the cells (Sakaguchi et al. 1979, Gipps and Coller 1980a). Thus, the initial phase may be considered as adsorption at the cell surface or penetration into the free space. The following phase has characteristics of a carrier-mediated transport and may represent an uptake into the cells with binding to cell membrane or intracellular components (Parry and Hayward 1973, Sakaguchi et al. 1979, Stacey and Klaassen 1980).

Adsorption and uptake of metal ions will, however, depend upon the nature and chemical composition of cell surface in direct contact with metal ions. Cell surfaces are known to consist of a mosaic of interspersed cationic and anionic exchange

sites, with the net charge on the cell wall being dependent on the extent to which these sites are occupied by the anions or cations (Davies 1978). Phytoplankton cells exhibit large surface areas containing various functional groups, such as, carboxylic, amino, thio, hydroxo, and hydroxy-carboxylic, that can interact coordinately with heavy metals (Crist et al. 1981). Among the metal binding chemical groups present in the bacterial cell wall (carboxyl, phosphate, amine and hydroxyl), the carboxyl groups seems to be of great importance (Bauda and Block 1990). The alteration of carboxyl groups in cell wall of gram positive bacteria and _E. coli_ severely limited binding of a large number of metals (Doyle et al. 1980, Bauda and Block 1990). It seems that the carboxyl-metal interaction is directly influenced by the electric charge. Introduction of positive charges into the cell walls results in a decrease of the metal binding. Decreased pH diminishes the affinity of the wall for metal, suggesting that metallic cations and protons compete for the same binding site (Doyle et al. 1980).

Heavy metals disrupt many physiological processes in plants. Metals like Cd, Ag, Pb are phytotoxic even in very small amounts (Sandmann and Böger 1983). These metals have a strong affinity for acidic and thiol groups of proteins and nucleotides, and thus interfere with the function of these biologically-important macromolecules.

Cadmium ions at low concentrations can uncouple mitochondrial phosphorylation. Cadmium has a tendency to replace

Zn in certain enzymes, altering their stereostructures, and impairing their catalytic functions. It also has an affinity for sulfur and carboxylate sites (Carty et al. 1976). Cadmium interacts with phospholipid monolayer, and this may affect the biological membranes (Wong et al. 1980). Simpson (1981) reported that sublethal concentrations of cadmium suppress photosynthesis in marine algae by uncoupling the photosystem II, electron transport system. Cadmium is also known to disrupt normal cell division process (Nakamo et al., 1978, De Fillipis et al. 1981). Chromium is considered to be toxic to plants interfering with the uptake by root of some essential elements, e.g., Ca, K, P and their translocation (NRC 1976). Excess of copper results in an inhibition of photosynthetic electron transport (Shioi et al. 1978, Bohner et al. 1980). When present at comparatively high levels within the chloroplast, the redoxactive copper ions compete with catalase for hydrogen peroxide and other peroxo compounds. Lead inhibits photosynthesis and ATP synthesis (Silverberg 1975). Green house experiments have shown that lead decreased Ca, Mg, K and P uptake by corn plants, and also reduced their growth (Walker et al. 1977). Chlorosis at excessive levels of Zn, Cu, Ni and Cd appears to be due to a direct or an indirect interaction with foliar Fe (Chaney et al. 1975). Tyler (1981) reported that inactivation of enzymes by heavy metals could be a result of masking of active groups, protein denaturation, effect on enzyme conformation and competition with activating cations involved in the formation of enzyme-substrate complexes.

Heavy metals are known to interfere with acquisition and assimilation of certain nutrients. Alkaline phosphate, an ecto-enzyme needed for utilizing dissolved organic phosphorus, has been shown to be inhibited by Cu (Rueter 1983). Inhibition occurs at concentrations that do not inhibit the growth rate of the organism (Brand et al. 1986), when orthophosphate is the phosphorus source. Certain inorganic metal complexes interfere with the transport and assimilation of major nutrients. Arsenate, a structural analogue of PO_4^{3-}, competitively inhibits P transport in a marine yeast (Button et al. 1973), and inhibits growth and P uptake of phytoplankton (Planas and Healey 1978). On the other hand, growths of some As-resistant phytoplankton cultured under P limitation are actually stimulated by arsenate addition (Creed et al. 1990). This perhaps represents As substitution for P in certain metabolic functions. In light of the two contrasting responses, the importance of As/P interactions seems to be difficult to predict at this stage.

The transport and metabolism of essential heavy metals may be inhibited by high concentrations of other heavy metals. A high concentration of Cu exerts its toxic effect by interfering with Mn metabolism (Sunda et al. 1981). As Cu concentration increases the intracellular Mn level decreases and the growth rate is reduced. Cadmium exerts its toxic effects on *Thalassiosira weissflogii* by inhibiting Fe transport and by interfering with Fe metabolism (Harrison and Morel 1983). At low levels of ferric ions, Fe uptake rates are competitively inhibited by Cd, resulting in decreased intracellular Fe

concentration and growth. It has been further pointed out by Harrison and Morel (1983) that Cd blocks or interferes with Fe assimilation and creates a condition of Fe deficiency in these cells in spite of high intracellular Fe levels.

The order of toxicity of heavy metals has been found to vary from organism to organism. Among the most toxic heavy metals are Hg, Cd and Ag, whereas Pb and Zn are the least toxic (Hutchinson 1973, Rosko and Rachlin 1975, Gächter 1976, Rai et al. 1981a, Fisher et al. 1984, Kapur and Chopra 1989, Sela et al. 1989, Wong and Chang 1991).

Plants are known to synthesize metal-binding proteins, popularly known as phytochelatins, in response to heavy metal stress. These polypeptides are composed of the repeating dipeptide units of gamma-glutamylcysteinyl with a single carboxyl terminal glycine residue - (gamma EC)$_{n}$G (Robinson 1989). Grill et al. (1985, 1987, 1988) found that phytochelatins (oliogo-peptides capable of binding heavy metal ions via thiolate coordination) are the principal metal binding components of plants. Low molecular weight, cysteine-rich, soluble, metal-binding proteins, similar to the metallothioneins studied extensively in animals (Webb 1975) have been found in resistant plant cells (see Steffen 1990). In <u>Datura innoxia</u> cells that are resistant to Cd, such a metal binding protein has been found and de novo synthesis of this protein has been found to be induced by Cd (Delhaize et al. 1989). A similar Cu-binding metallothionein-like protein has been found in the roots of <u>Mimulus gattatus</u>

(Salt et al. 1989). In Scenedesmus acutiformis and C. fusca the synthesis of (gamma EC)$_n$G has been shown to increase following the exposure to Cd, Pb, Zn, Ag, Cu and Hg (Gekeler et al. 1988). On the basis of the results obtained, Leita et al. (1991) confirmed that the synthesis of Cd-associated polypeptides with low molecular weight was induced in leaves, stems and roots. In addition roots were able to synthesize another specific protein fraction with higher apparent molecular weight, and this can contribute to elucidate the higher ability of roots to retain Cd. They also showed that Cd ions can induce the biosyntesis of both metallothionein and phytochelatin in roots of bushbean. Various metal binding substances have been isolated from fungi, including the Cd and Cu-metallothioneins from Saccharomyces cerevisiae, animal-like Cu-metallothioneins from Neurospora crassa (Lerch 1980) and Agaricus bisporus (Münger and Lerch 1985), and also Cd-cadystins (phytochelatins) induced by Cd^{2+} and other metal ions (Grill et al. 1985, Robinson and Jackson 1986, Reese and Wagner 1987).

Phytochelatins are distinctive in that heavy metals are the primary inducers. Cadmium, Pb, Zn, Sb, Ag, Ni, Hg, Cu, Sn, Au, Bi, Te, and W induced phytochelatins (Grill et al. 1987). Among the common metals, Cd is the strongest inducer, while Zn appears to be the weakest, requiring very high levels for induction (Steffens 1990), and phytochelatin biosynthesis is tightly regulated by the availability of metal ions. Biochemical analysis of tissue samples showed that acclimation to Cd by

Salvinia minima parents led to increases of phytochelatins and thiols in daughter ramets (Outridge and Hutchinson 1991). Increased accumulation of (gamma EC)$_n$G occurs very rapidly following exposure to elevated concentrations of metals (Grill et al. 1986). In one higher plant cell line increased accumulation of (gamma EC)$_n$G was detected as early as 5 min after exposure to Cd (Robinson et al. 1988).

The phytochelatin response or synthesis of heavy metal-binding polypeptides, in plants is an adaptive response. The extent to which this response accounts for the differential tolerance is not clear (Steffens 1990). However, it is evident that phytochelatins play a major role in the detoxification of excess metals. Phytochelatins are also involved in trace metal homeostasis, and their participation in detoxification of excess metals may be a consequence of this homeostasis.

Exclusion is yet another mechanism adopted by plants for resisting heavy metals. The mechanism(s) responsible for the exclusion of the metalloid As from the shoots of tolerant *Silene vulgaris* individuals is perhaps located in the roots and it may be any one of the following: Cell wall binding, complexation with organic acids, and complexation with metal binding proteins (Paliouris and Hutchinson 1991). In *Plectonema boryanum*, Jensen et al. (1982a) have found that a range of heavy metals, including Zn, are taken up and sequestered in sectors of the cell with polyphosphate bodies.

Harding and Whitton (1981) reported genetic adaptation to high zinc levels of a natural population of Anthoxanthum odoratum. They found that filamentous green algae from high Zn site were more tolerant than those from low zinc site. Heavy metals affect the number, species diversity and productivity of microbiota in aquatic ecosystems. Decreased growth of a natural phytoplankton community occurred in estuarine waters supplemented with 10 µM Hg or 100 µM Cd, Pb or Zn (Hollibaugh et al. 1980). Blue green and diatoms appear to be less tolerant than green algae, and metal-contaminated waters favour filamentous green algae (Whitton 1970). Shifts in the species of phytoplankton and decreases in the numbers of species were evident in lake waters amended with combinations of Hg, Cu, Cd, Zn and Pb (Gächter and Mares 1979). Primary productivity of natural communities is also affected by heavy metal contaminants. Mercury, Cu or Cd at 10 ppb inhibited photosynthesis of a phytoplankton community collected in the west Caspian Sea, but 10 ppb Zn stimulated photosynthesis by 15% (Babich and Stotzky 1985). Williams and Mount (1965) determined that 9 mg Zn l^{-1} causes shift from predominantly autotrophic to heterotrophic communities of epilithon. Colwell et al. (1989) also found changes in epilithon community dosed with 1 mg Zn l^{-1}. Kumari et al. (1991) reported a direct relationship of phytoplankters with Fe, Mn, Co and an inverse relationship with Zn, Cu, Pb and Ni. Aquatic plants growing in pools contaminated with mine debris can partially reflect the concentration of metals in the water (Lee et al. 1991).

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The physico-chemical characteristics of an environment into which heavy metals are deposited determine the chemical speciation forms and hence the bioavailability and toxicity of heavy metals to indigenous biota (Babich and Stotzky 1985). The interactive effect of environmental factors on the toxicity of heavy metals is therefore extremely important for realistic interpretations. Rates of uptake, translocation, accumulation and concentration or retention of heavy metals could be influenced by temperature, light, pH and ionic nature of the metal, type of toxicant combinations, level of metals in the medium, existence of competing metal chelators and the physiological state and type of the organism (Ting et al. 1989). Accumulation of zinc and copper is temperature-dependent in *Fucus serratus*, but temperature-independent in *Potella vulgata* (Miramand and Bentley 1992). Cadmium transport into *Stichococcus bacillaris* greatly depended on temperature and pH (Skowroński 1986b). The uptake of Cd is also affected by temperature in *Lemna minor* (Kwan and Smith 1991). It is generally observed that free ions predominates at low pH in solutions (Darimont and Frenay 1990). At high pH, the complexes such as carbonates, oxides, hydroxides, and silicates are more stable and thus prevail. Soeder et al. (1978) also demonstrated an increase in the uptake of Cd by algal cells at lower pH. The interaction intensities are pH-dependent for Cd in oxidative medium; in alkaline conditions insoluble hydroxides are formed and become unavailable to test plants (Kwan and Smith 1991). In a mixture of two or more metal species in solutions the synergistic or antagonistic

interactions occurring between the metal ions may affect the uptake of individual metals. The response of organisms to mixtures of metals may show antagonistic or synergistic interactions. The most logical reason for antagonistic action was claimed to be the competition for adsorption sites on the cells and competititon for transport across the membrane with the more efficient competitor preventing the uptake of the other metal. Another mechanism of antagonism between heavy metals may involve the sorption of one heavy metal to the amorphous complex of the other metal. Synergistic effect of two metals on organisms may result from the adsorption of both metals on the surface of the cell, with the adsorption of one metal increasing the permeability to the second metal.

A perusal of literature suggests that effect of metals on floating aquatic plants has not been adequately studied, although some preliminary reports have appeared during the last five years (Wang 1986a, Charpentier et al. 1987, Sela et al. 1988, Sela et al. 1989, Outridge and Hutchinson 1990, Wang 1990). Not much is known about the effects of heavy metals on the physiology of aquatic vascular plants (Porter and Francko 1991). On the other hand, the corpus of information on algae seems quite impressive (Rai et al. 1981a, Davies 1983, Stokes 1983, Whitton 1984, Vymazal 1987, Gadd 1988).

Algae and aquatic plants growing in metal-enriched wastewaters tend to concentrate metals to exceptionally high

levels. A direct relationship between metal content in milieu and organisms has been reported, and suggestions have been put forth to use metal content of plants for biomonitoring of metals in aquatic environments. In order to make most effective use of metal accumulation in a particular species as a means of monitoring aqueous metal concentration, the following should be known: relationship between concentration in plants and water; influence of environmental factors on this relationship; rate of loss following environmental downshift in metal concentration or uptake when there is an upshift.

Jennet et al. (1977) have expressed the possibility of using algae to glean heavy metals from metalliferous effluents. However, outdoor cultivation and harvesting of algae present formidable difficulties (Benemann et al. 1977). Aquatic plants may serve the purpose provided they can accumulate high concentrations of heavy metals. However, not much has been done in evolving a macrophyte-based system for the biological treatment of metalliferous wastewaters. In order to achieve this objective a thorough investigation about the mechanism of metal uptake and accumulation by floating plant species should be conducted. It is also necessary to study the role of environmental factors and nutrient ions on metal accumulation by aquatic plants.

Present Study

It aims at examining the interaction of heavy metals,

particularly Cd, with Lemna polyrrhiza and Azolla pinnata. The species of Lemna, commonly known as duckweeds, are small free-floating plants capable of fast growth under wide ranging environmental conditions, and these could be employed for stripping nutrients from wastewaters (Oron et al. 1984), and are ideal for toxicological studies (Huebert and Shay 1991). Azolla, a widely-distributed water fern commonly occurring in paddy fields, stagnant waters or ponds and at wide-range of altitudes, has tremendous capacity for vegetative multiplication (Jamir 1982). Water hyacinth (Eichhornia crassipes) can accumulate very high levels of heavy metals. However, it has not been included in the present study due to its following characteristics: inability to grow in cold climate, high rate of transpiration and loss of huge amounts of water, porous foliage providing excellent conditions for mosquito larvae development, and notoriety for creating serious ecological problems (Oron et al. 1984). If species of Lemna and Azolla could take up and accumulate high concentrations of heavy metals, it may become possible to use them for removing metals from polluted waters. The recovery of precious metals (like, Ag) may also become feasible once we know the mechanism of their uptake and accumulation by these organisms. Metal toxicity bioassays often use algae as the test organisms despite the fact that the culturing of algae requires sophisticated laboratory facilities. Floating plants are relatively easier to handle and have all important characteristics needed to become ideal test organisms for toxicity bioassays. Their routine application in laboratory bioassays would,however,

require a more thorough understanding of the effects of heavy metals on them.

In the present study greater emphasis has been laid upon Cd as it is one of the most toxic and widely distributed heavy metals. However, other metals, such as Co, Cr, Cu, Ni, Pb and Zn were also briefly studied with regard to their toxicity and bioaccumulation. Generally speaking, Cd is a non-essential element which enters into aquatic environments from mines, smelters and industries involved in the manufacture of alloys, paints, batteries, and from burning of fossil fuels (Reeder et al. 1979). On a global scale, the total anthropogenic input of Cd into the aquatic environments ranges between 2.1-17 x 10^6 kg yr^{-1} (Nriagu and Pacyna 1988). Of which, manufacturing processes contribute 0.5-1.8 x 10^6 kg yr^{-1}, whereas atmospheric fall out contributes 0.9-3.6 x 10^6 kg yr^{-1}.

As mentioned earlier, _Lemna polyrrhiza_ L. and _Azolla pinnata_ R. Br. were used as test plants, and the following aspects have been investigated:

(i) Toxic effects of Cd, Co, Cu, Cr, Ni, Pb and Zn on growth and pigment levels of test plants.

(ii) Extent of metal bioaccumulation and changes in the levels of essential cations (Ca^{2+}, K^+ and Mg^{2+}) in test plants.

(iii) Time course study on Cd uptake.

(iv) Mechanisms of extracellular and intracellular uptake of Cd in test plants.

(v) Interactive effects of metal combinations on Cd uptake in test plants.

(vi) The uptake of Cd as influenced by Ca, Mg, K, and Na.

(vii) Effects of environmental factors, namely, pH, temperature and light on the accumulation and toxicity of Cd.

Chapter-II

HEAVY METAL TOXICITY AND ACCUMULATION IN TEST PLANTS

Introduction

Heavy metal toxicity results from a multitude of direct and indirect inhibitory effects on practically all physiological functions of living organisms. A great deal of information has already been gathered about metal toxicity to algae, and a number of review articles are available (Rai et al. 1981a, Davies 1983, Stokes 1983, Whitton 1984, Vymazal 1987). Nevertheless, it is equally important to determine how macrophytes are affected since they are also exposed to heavy metals in polluted waters. Unfortunately, studies on heavy metal interaction with aquatic vascular plants are extremely limited, despite the fact that these plants occur abundantly in many waterbodies. Sculthorpe (1967), van der Werff and Pruyt (1982) and Wang (1990) suggested that aquatic vascular plants may be potentially useful as pollution indicators. Some of them have been shown to concentrate heavy metals to very high levels relative to their environment. Concentration factors of 100-2,000 have been reported (Dietz 1973, Nakada et al. 1979, Aulio and Saling 1982, Sela et al. 1989, Turnquist et al. 1990).

Some workers have carried out preliminary investigations on heavy metal toxicity to macrophytes. Hutchinson and Czyrska (1975) found Cd toxicity to *Lemna minor* and *Salvinia natans* at

50 and 10 ppb, respectively. Wang (1986) found significant decreases in growth of Lemna minor at 50 ppb Cd. Other workers have reported toxic effect of Cd at 112 ppb in Lemna paucicostata (Nasu et al. 1983), 200 ppb in Lemna polyrrhiza (Charpentier et al. 1987), 500 ppb in Eichhornia crassipes (Kay et al. 1984) and at around 1000 ppb in Lemna gibba (Polar and Küçükcezzar 1986) and Spirodella polyrrhiza (Srivastava and Jaiswal 1989). These variabilities may be ascribed to differences in metal chemistry, biology of test plants, and culture media. Mineral nutrients can also affect the toxicity of heavy metals (Seto et al. 1979, Nasu et al. 1984). It is generally accepted that toxicity of heavy metals is related to the activity of the ion and not to the concentration (Borgmann 1983). Hence, alterations in medium composition are expected to change the ionic strength of a given metal. Various workers have employed different test methods thus giving rise to inconsistencies in results. Therefore, it is virtually impossible to compare the results of previous workers, and to have a clearer understanding of heavy metal effects on aquatic plants. Moreover, previous workers have conducted toxicity tests using one or two metals.

The seventeenth edition of 'Standard Methods for Examination of Water and Wastewater' recommends Lemna minor, whereas the American Society for Testing and Materials recommends L. gibba for laboratory bioassay of effluents and toxicants (see Wang 1990). Other species of Lemna (Lemna trisulca, Lemna valdiviana, Lemna paucicostata) have also been evaluated as test organisms for toxicity bioassays.

The species of *Lemna*, as also of *Azolla*, are floating vascular plants capable of fast growth, and are widely distributed all over India. Unlike algae, the species of *Lemna* and *Azolla* may be better test organisms for biomonitoring of effluents. Many industrial and municipal wastewaters are turbid. Such samples must be filtered for conducting algal bioassay; however, filtration might change the characteristics of effluents. The filtration of effluents need not be done if species of *Lemna* or *Azolla* are employed as test organism for bioassay. Nasu et al. (1984) demonstrated certain effects of Cu and Cd on *Lemna paucicostata* that cannot be obtained by algal test. Copper suppressed both frond multiplication and frond growth (wet weight increase of each frond) of *L. paucicostata*, while Cd ion suppressed only frond multiplication and did not affect frond growth. Interesting findings such as these are very important for comparative studies of environmental toxicology.

An attempt has been made to assess the individual effects of seven heavy metals namely Cd, Co, Cr, Cu, Ni, Pb and Zn on two aquatic plants, *Lemna polyrrhiza* and *Azolla pinnata*. The culture medium and test condition were kept similar for various metal treatments so as to obtain comprehensive and comparable information. The effects of heavy metals on test plants were assessed by determining the relative growth rate (RGR), water content, and levels of chlorophyll *a*, chlorophyll *b*, carotenoid and essential cations (Ca, K and Mg). An attempt was also made

to study the efficiencies of test plants in accumulating test metals at their various external concentrations.

Materials and Methods

Test plants

Test plants, Lemna polyrrhiza L and Azolla pinnata R. Br. were collected from paddy fields at Jakhama (Kohima district, Nagaland). Both plants were seen growing together in nature. L. polyrrhiza (a common duckweed) is an aquatic angiosperm, monocotyledon placed in the aroid line of the family Lemnaceae (Hillman 1976). It is a free-floating plant with many adventitous roots. The plant body is small (3-10 mm long) and composed of fronds and roots. L. polyrrhiza grows rapidly in nature (Hillman and Culley 1978). A. pinnata is a free-floating water fern which grows in a wide range of environmental conditions and altitudes, and has tremendous capacity for vegetative multiplication (Jamir 1982). The plant body is small (1-2 cm) and has well defined fronds and roots. A. pinnata contains the filaments of the endosymbiotic cyanobacterium, Anabaena azollae.

Cultivation of test plants

The cultivation of test plants was carried out under laboratory condition using AAP medium as recommended by Wang (1986a). Table 2.1 shows the composition of nutrient medium. The medium was prepared using double-distilled water. The pH of culture medium was adjusted to 7.0. The stock cultures were

Table 2.1: The composition of growth medium.

Compound	Concentration
A. Macronutrients	(mg l^{-1})
$MgSO_4.7H_2O$	59.64
$MgCl_2.6H_2O$	28.036
K_2HPO_4	6.00
$CaCl_2.2H_2O$	30.00
$NaNO_3$	300.035
$NaHCO_3$	178.55
B. Micronutrients	(µg l^{-1})
H_3BO_3	185.520
$MnCl_2$	264.264
$ZnCl_2$	32.709
$CoCl_2$	0.780
$CuCl_2$	0.009
$Na_2MoO_4.2H_2O$	7.260
$FeCl_3$	96.000

maintained in glass trays. The levels of nutrient medium in these trays was maintained at least 5 cm from the surface to the bottom so that roots could float freely in the medium without touching the bottom of the tray. The stock cultures were grown and maintained at 25±1°C under 14 h light (PAR 45 μmol m^{-2} sec^{-1}) and 10 h dark. The glass trays were replenished with fresh nutrient medium after 7 days of growth. Regularly, some fronds were removed from the stock culture to avoid over crowding and to provide sufficient space for growth and multiplication.

Metals toxicity to test plants

The salts used for various heavy metal toxicity assays were of analytical grade in the following forms: Cd as cadmium acetate, Co as cobalt II chloride, Cu as copper II chloride, Cr as chromium trioxide, Ni as nickel II chloride, Pb as lead II nitrate, and Zn as zinc sulphate. For every experiment, freshly-prepared salt solutions were used, and each metal toxicity assay was carried out separately. As recommended (Wang 1990), assays were carried out in Teflon beakers. Five different concentrations for each metal were added to the medium in separate Teflon beakers. Five replicates were considered for all concentrations. Each beaker (capacity 100 ml) contained 50 ml of medium supplemented with test metal. Ten healthy fronds of Lemna or Azolla were weighed in a Sartorius electronic balance, transferred to beakers containing various concentrations of test metals. All beakers were kept at 25±1°C for 4 days, and were exposed to 14 h light (PAR 45 μmol m^{-2} sec^{-1}) and 10 h dark

periods. After 4-d, the test plants were harvested, kept on a blotting paper for a few seconds and weighed with a Sartorius electronic balance. Five fronds were kept for chlorophyll and carotenoid estimation, and the remaining fronds were oven dried at 60°C till a constant weight was obtained. Chlorophyll extraction was carried out by grinding the whole plants in 90% acetone with the help of a mortar and pestle. The extract was centrifuged, the supernatant was separated and the final volume was adjusted to 5 ml with 90% acetone. Absorbance of the supernatant was measured at different wavelengths using a Hitachi recording spectrophotometer (model 220). The trichromatic method (Strickland and Parsons 1968) was used to calculate chlorophyll a and b, and carotenoid in the extracted samples. The equations for calculating the amount of these pigments are given below:

$$\text{Chlorophyll } \underline{a} \text{ (mg l}^{-1}\text{)} = 11.6E_{665} - 1.3E_{645} - 0.14E_{630}$$

$$\text{Chlorophyll } \underline{b} \text{ (mg l}^{-1}\text{)} = 20.7E_{645} - 4.3E_{665} - 4.42E_{630}$$

$$\text{Carotenoid (mg l}^{-1}\text{)} = \frac{10(E_{480})\,(v)}{(V)\,(Z)}$$

where, E_{665}, E_{645}, E_{630}, and E_{480} are absorbances at 665, 645, 630 and 480 nm wavelengths, respectively, after turbidity correction at 750 nm. The equations gave chlorophyll and carotenoid concentration in unit volume extract. Chlorophyll a may be overestimated without removal of pheophytin, which absorbs nearly the same wavelengths. The samples were corrected for pheophytin a by adding 0.1 ml of 0.1 N HCl to the samples, followed by a thorough mixing and remeasurement of optical

density after 2 min. The corrected chlorophyll a value was thus obtained. The data were subsequently converted to per unit dry weight of test plants.

Relative growth rate and EC_{50}

Growth was measured as an increase in fresh weight. Relative growth rate (RGR) was calculated by the equation (Greger et al. 1991).

$$RGR = (\ln W_2 - \ln W_1)/t$$

where W_1 and W_2 respectively represent the fresh weights at the beginning and end of the time interval t.

The fresh weights were measured and RGR was calculated after performing every experiment. In order to compare the toxicity of test metals on RGR of test plants, the results were transformed to obtain EC_{50}, i.e., the concentration of metals giving 50% effect or inhibition. EC_{50} was extrapolated by fitting a line to the dose (concentration)-response relationship by least squares using $\log_{10}$ of the concentration as the independent variable (Vocke et al. 1980).

Metal accumulation by test plants

Relative efficiencies of L. polyrrhiza and A. pinnata in accumulating test metals were determined after a 4-d exposure. The test plants were treated with different concentrations of metals in a manner similar to that of toxicity bioassays. Five replicates were considered for all treatments. The test plants were harvested after 4-d exposure to metals and dried. The

dried plant materials were subsequently digested. Digestion was done as described by Bates et al. (1982) with slight modification. Pre-weighed and dried samples were taken in Borosil test tubes. To each of these, a 5 ml solution containing concentrated nitric acid , hydrogen peroxide (30% w/v) and double-distilled water in 1:1:3 ratio (v/v/v) was added. Each sample was then gently digested on a hotplate till a clear solution of about 0.5 ml was left in the test tube. This solution was made to 5 ml by the addition of 2% (v/v) nitric acid, and transferred to a plastic tube. The digested samples were taken to the Regional Sophisticated Instrumentation Centre, NEHU, for the analysis of metal content. A Perkin-Elmer atomic absorption spectrophotometer (Model 2380) was used for metal analysis. Metal concentration in test plants was calculated by the equation

$$M = \frac{N \times V}{1000 \times W}$$

where, M is the metal content in test plants (mg g^{-1} dry wt), N is the average reading, V is final volume for estimation and W is the dry weight of the digested sample.

Results

Morphology and growth

Pronounced changes in growth and morphology of test plants were observed after exposure to test metals. Tables 2.2 and 2.3 summarize common morphological symptoms of metal toxicity in *L. polyrrhiza* and *A. pinnata*, respectively. Common morphological

Table 2.2: Morphological symptoms of metal toxicity in *L. polyrrhiza*.

Heavy metal	Symptoms of toxicity
Cadmium	Fronds became fragile and roots shedded at 8.9 µM within 24 h and death occurred subsequently.
Cobalt	Fronds became fragile and dead at 17 µM concentration.
Chromium	No symptoms upto 96 µM concentration, plants died at 192 µM.
Copper	Roots shedded at 8 µM and fronds disintegrated and died at 15.7 µM.
Nickel	Fronds disintegrated and died at 17 µM.
Lead	No symptoms of toxicity even at 48 µM, death occurred at 96 uM.
Zinc	Fronds died at 76 µM.

Table 2.3: Morphological symptoms of metal toxicity in _A. pinnata_ during a 4-d incubation period.

Heavy metal	Symptoms of toxicity
Cadmium	Roots shedded at 8.9 µM and plants died at 44 µM.
Cobalt	Fronds became fragile and died at 17 µM.
Chromium	Fronds became fragile and death occurred at 96 µM.
Copper	Roots shedded and fronds became fragile at 7.5 µM, plants died at 15 µM.
Nickel	Fronds died at 85 µM.
Lead	Fronds died out at 96 µM.
Zinc	Fronds became yellow and dead at 76.5 µM.

symptoms of metal toxicity were root shedding, frond fragility and yellowing. Of the various test metals, only Cd (8.89 µM) and Cu (7.5 µM) caused root shedding in both the test plants. At high concentrations of some metals (Cd, Co, Cr), the fronds of *L. polyrrhiza* and *A. pinnata* became fragile and died out. Fronds of test plants became yellowish-green at high concentrations of test metals. As Tables 2.2 and 2.3 reveal the death of test plants occurred at various concentrations of test metals.

The exposure of test plants to heavy metals caused declination of relative growth rate (RGR) in a concentration dependent manner (see Fig. 2.1 for *L. polyrrhiza* and Fig. 2.2 for *A. pinnata*). Cadmium caused most pronounced inhibition of RGR even at its lowest tested concentration (0.44 µM). Other metals, however, elicited toxic effects at much higher concentrations. Lead and Zn were least inhibitory to test organisms. The data relating to RGR of test plants were utilized to calculate metal concentrations causing 50% inhibition. EC_{50} values, regression equations and correlation coefficients for the RGR of *L. polyrrhiza* and *A. pinnata* are respectively presented in Tables 2.4 and 2.5. In all the cases RGR was found to be negatively related with metal concentration in the medium. These data suggest that *A. pinnata* is more sensitive to test metals in comparison to *L. polyrrhiza*. The order of toxicity of metals towards RGR was Cd > Cu = Ni > Co > Cr > Zn > Pb for *L. polyrriza* and Cd > Cr > Co > Cu > Ni > Pb > Zn for *A. pinnata*.

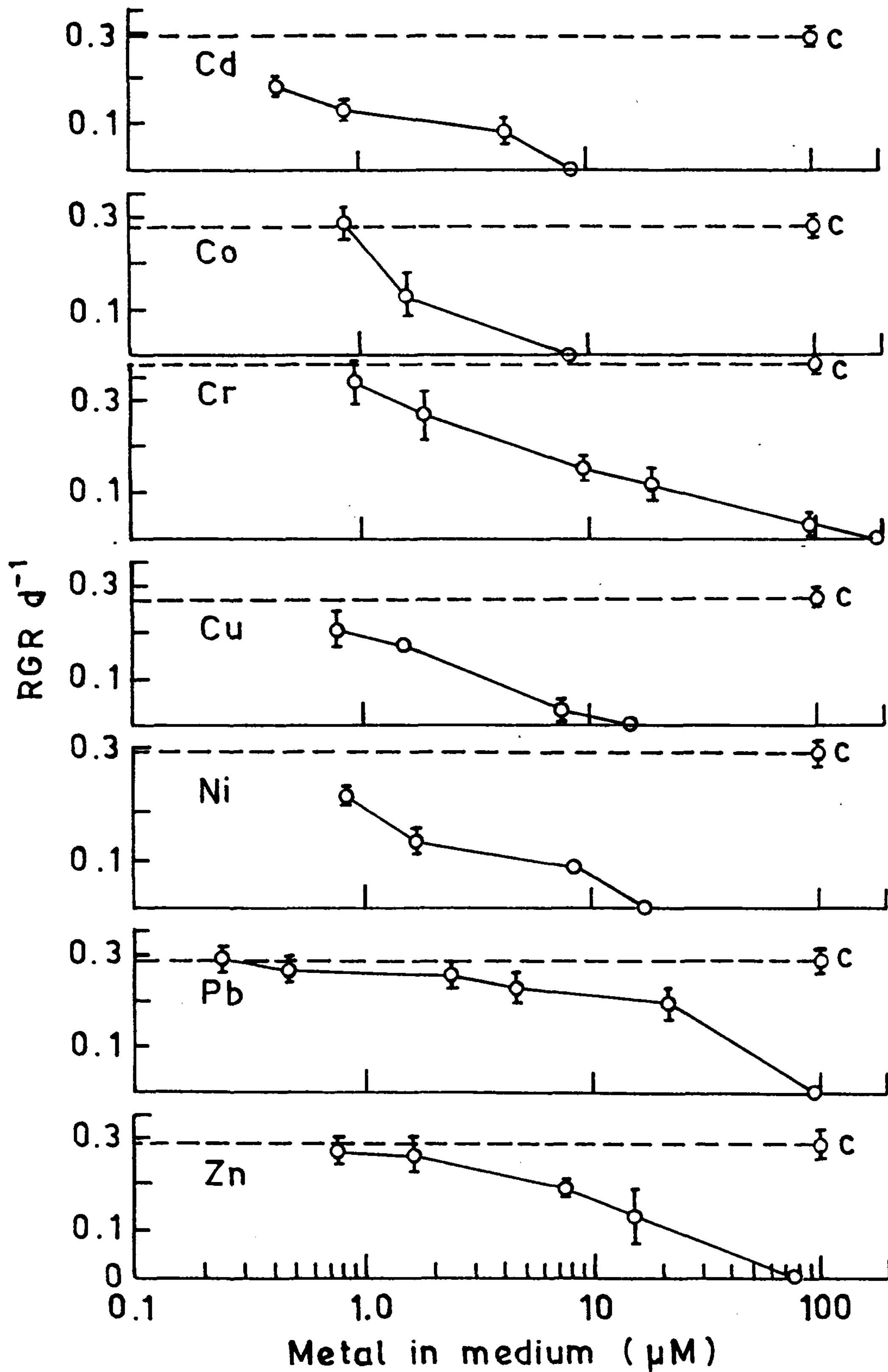


Fig. 2.1: Relative growt rate (RGR) of _L_. _polyrrhiza_ at different concentrations of heavy metals. The broken line shows the control. Error bars represent SD, n = 5.

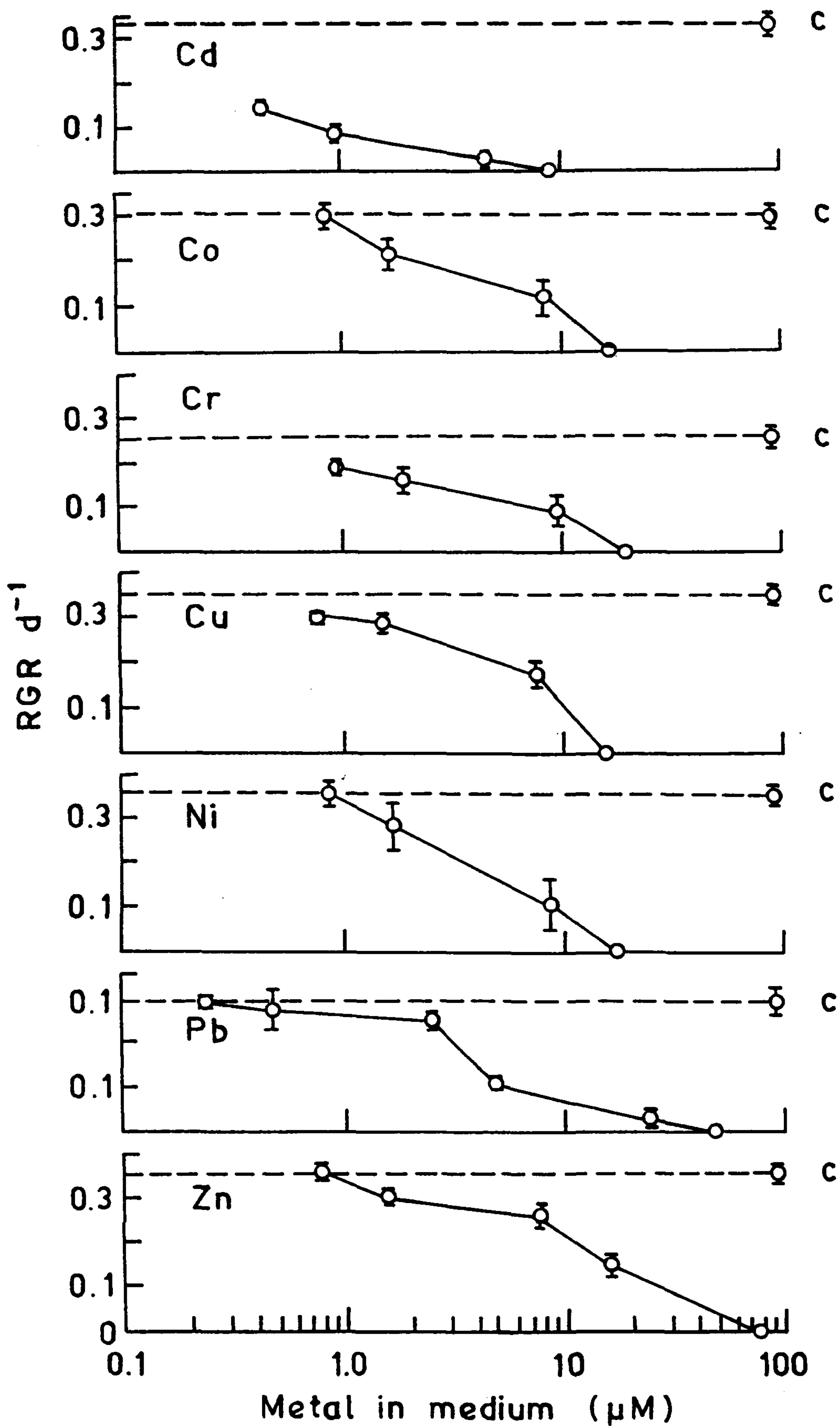


Fig. 2.2: Relative growth rate (RGR) of *A. pinnata* at different concentrations of heavy metals. The broken line shows the control. Error bars represent SD, n = 5.

Table 2.4: Regression equations, correlation coefficients and EC_{50} for the relative growth rate (RGR d^{-1}) of _L. polyrrhiza_ treated with heavy metals.

Heavy metal	Regression equation	r	EC_{50} in µM (Mean ± SD)
Cd	$Y = -0.1\ (\log_{10}x)^{*}+0.12$	-0.89	0.83±0.04
Co	$Y = -0.27\ (\log_{10}x)+0.24$	-0.96	2.29±0.11
Cr	$Y = -0.15\ (\log_{10}x)+0.32$	-0.99	7.08±0.11
Cu	$Y = -0.12\ (\log_{10}x)+0.17$	-0.96	1.80±0.09
Ni	$Y = -0.13\ (\log_{10}x)+0.21$	-0.97	1.80±0.10
Pb	$Y = -0.09\ (\log_{10}x)+0.26$	-0.87	18.00±0.09
Zn	$Y = -0.12\ (\log_{10}x)+0.27$	-0.98	14.30±0.06

*Concentration (µM) of heavy metal in the medium.

All values of r are significant ($p<0.001$).

Table 2.5: Regression equations, correlation coefficients and EC_{50} for the relative growth rate (RGR d^{-1}) of *A. pinnata* treated with heavy metals.

Heavy metal	Regression equation	r	EC_{50} in µM (mean $\pm$ SD)
Cd	$Y = -0.19 (\log_{10}x)^{*}+0.09$	-0.94	0.45 ± 0.10
Co	$Y = -0.2 (\log_{10}x)+0.28$	-0.98	4.11 ± 0.10
Cr	$Y = -0.11 (\log_{10}x)+0.17$	-0.96	2.50 ± 0.08
Cu	$Y = -0.21 (\log_{10}x)+0.31$	-0.93	4.18 ± 0.10
Ni	$Y = -0.45 (\log_{10}x)+0.50$	-0.93	5.06 ± 0.20
Pb	$Y = -0.13 (\log_{10}x)+0.24$	-0.94	5.30 ± 0.09
Zn	$Y = -0.14 (\log_{10}x)+0.34$	-0.94	14.50 ± 0.08

*Concentration (µM) of heavy metals in the medium.

All values of r are significant ($p<0.001$).

Figs. 2.3 and 2.4 present data relating to concentration of chlorophylls in *L. polyrrhiza* and *A. pinnata*, respectively. In general, all test metals caused lowering of chlorophyll *a* and *b* in a concentration-dependent fashion. The inhibition of chloropyll *a* was more pronounced than that of chlorophyll *b*. Lead and Zn were least effective in depressing the levels of chlorophylls in test plants. EC_{50} values of test metals were also computed on the basis of chlorophyll *a* and chlorophyll *b* for *L. polyrrhiza* (Table 2.6) and *A. pinnata* (Table 2.7). Highly significant negative relationship was evident between metal concentration in the medium and the level of chlorophyll *a* or chlorophyll *b* in test plants. EC_{50} values derived from chlorophyll data suggest the following hierarchies of metal toxicity

Cd > Ni > Co > Cu > Zn > Cr > Pb for chlorophyll *a* in *L. polyrrhiza*,

Cd > Ni > Co > Zn > Cu > Cr > Pb for chlorophyll *b* in *L. polyrrhiza*,

Cd > Cu > Co > Ni > An > Cr > Pb for chlorophyll *a* in *A. pinnata*, and

Cd > Cu > Co > Ni > Cr > Zn > Pb for chlorophyll *b* in *A. pinnata*.

Changes in carotenoid content of test plants after heavy metal treatment have been shown in Fig. 2.5. In comparison to chlorophylls the concentration of carotenoids generally did not decrease much after metal treatment. In fact, treatment of test plants with Zn, Pb, Cu, and Cr did not cause a significant change in the levels of carotenoids. On the contrary Cd or Ni

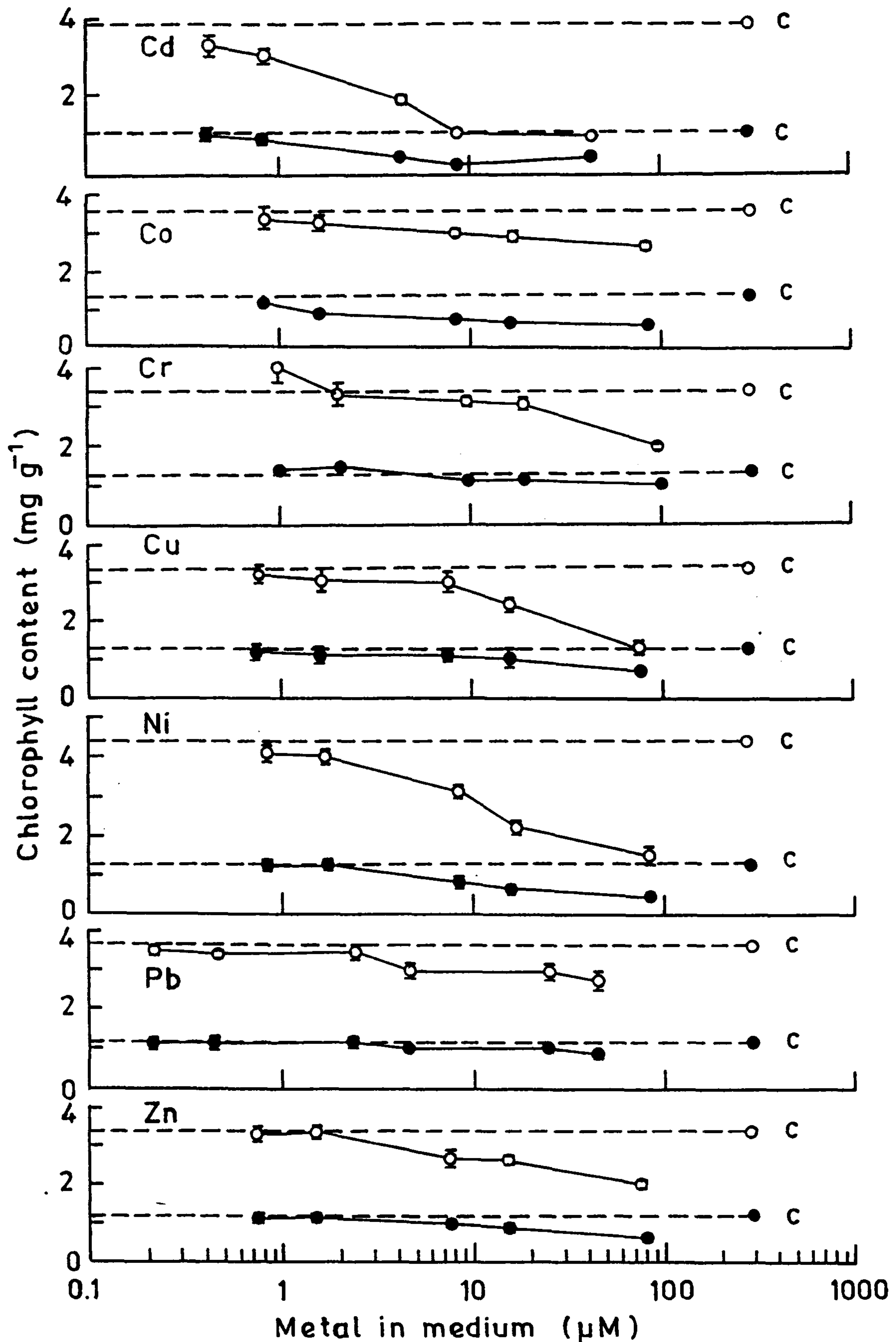


Fig. 2.3: Chlorophyll a and chlorophyll b content in *L. polyrrhiza* at different concentrations of heavy metals. Symbols: O Chlorophyll a; ● Chlorophyll b; broken lines show the controls. Error bars represent SD, n = 5.

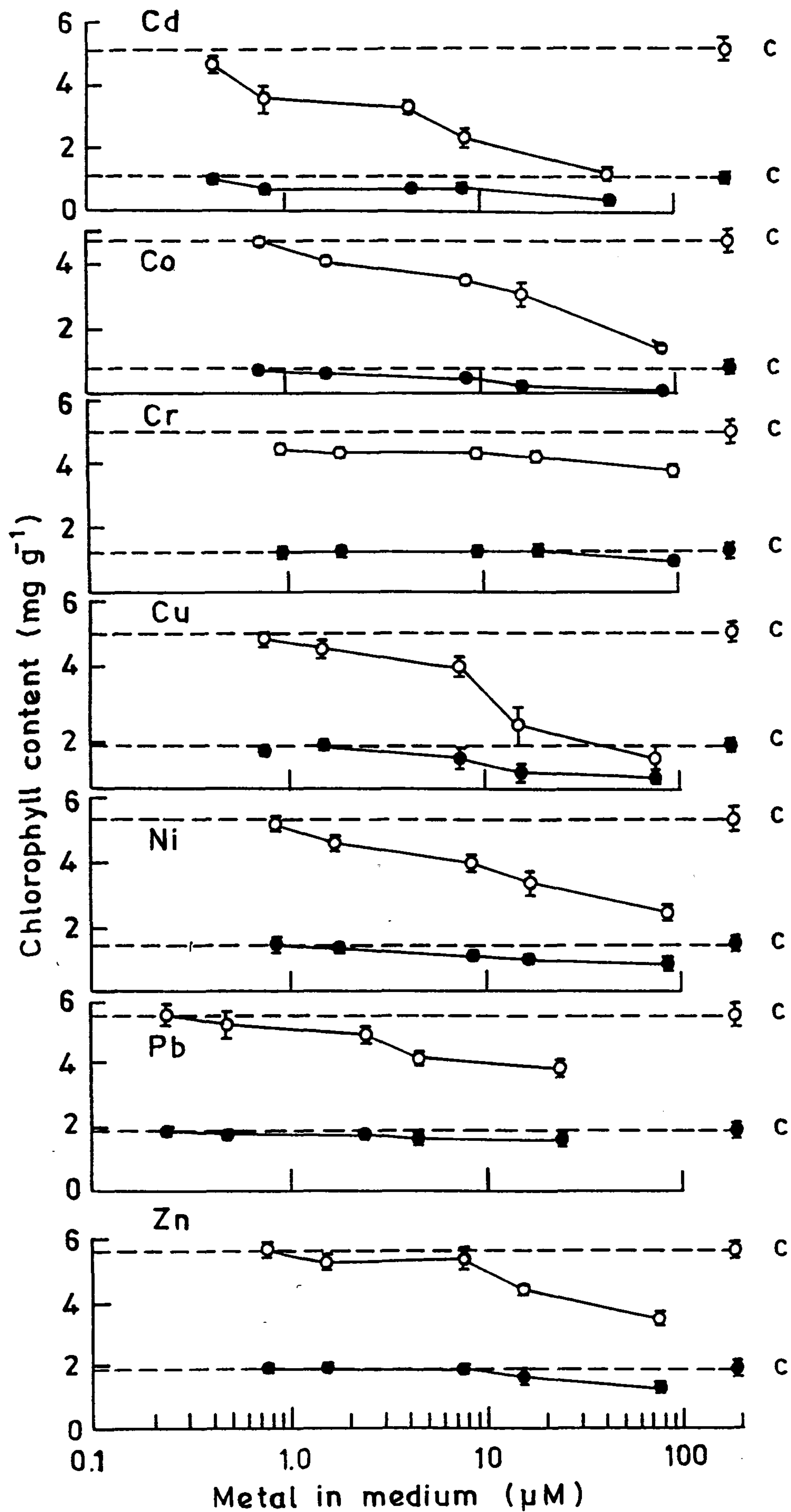


Fig. 2.4: Chlorophyll a and chlorophyll b content in *A. pinnata* at different concentrations of heavy metals. Symbols: O Chlorophyll a; ● Chlorophyll b, broken lines show the controls. Error bars represent SD, n= 5.

Table 2.6: *Regression equations, correlation coefficients and* EC_{50} for chlorophyll a and chlorophyll b of L. polyrrhiza treated with heavy metals.

Heavy metal	Regression equation	r	EC_{50} in µM (mean ± SD)
(a) Chlorophyll a			
Cd	$Y = -1.39 (\log_{10}x) +3.07$	-0.89	0.59±0.11
Co	$Y = -0.62 (\log_{10}x)+3.46$	-0.95	3.90±0.15
Cr	$Y = -0.75 (\log_{10}x)+3.92$	-0.88	11.90±0.10
Cu	$Y = -1.08 (\log_{10}x)+3.49$	-0.95	3.94±0.10
Ni	$Y = -1.23 (\log_{10}x)+4.19$	-0.97	3.26±0.10
Pb	$Y = -0.35 (\log_{10}x)+3.46$	-0.88	14.34±0.25
Zn	$Y = -0.67 (\log_{10}x)+3.39$	-0.97	10.14±0.20
(b) Chlorophyll b			
Cd	$Y = -0.36 (\log_{10}x)+0.92$	-0.82	0.57±0.05
Co	$Y = -0.33 (\log_{10}x)+1.30$	-0.94	6.97±0.12
Cr	$Y = -0.09 (\log_{10}x)+1.36$	-0.90	16.90±0.23
Cu	$Y = -0.11 (\log_{10}x)+1.17$	-0.84	10.70±0.20
Ni	$Y = -0.44 (\log_{10}x)+1.34$	-0.97	4.45±0.10
Pb	$Y = -0.09 (\log_{10}x)+1.15$	-0.76	20.00±0.21
Zn	$Y = -0.26 (\log_{10}x)+1.20$	-0.99	8.20±0.10

x = Concentration of metal in medium (µM); Y = concentration of metal in plants (µmol g^{-1}).

All values of r are significant ($p<0.001$).

Table 2.7: Regression equations, correlation coefficients and EC_{50} for chlorophyll a and chlorophyll b of *A. pinnata* treated with heavy metals.

Heavy metal	Regression equation	r	EC_{50} in µM (mean ± SD)
(a) Chlorophyll a			
Cd	$Y = -2.58\ (\log_{10}x) + 4.67$	-0.93	0.49±0.15
Co	$Y = -0.1.38\ (\log_{10}x) + 5.00$	-0.98	1.44±0.20
Cr	$Y = -0.56\ (\log_{10}x) + 4.94$	-0.92	9.08±0.22
Cu	$Y = -2.70\ (\log_{10}x) + 5.48$	-0.87	0.90±0.10
Ni	$Y = -1.67\ (\log_{10}x) + 5.57$	-0.98	1.75±0.12
Pb	$Y = -0.77\ (\log_{10}x) + 5.03$	-0.80	11.53±0.25
Zn	$Y = -1.13\ (\log_{10}) + 5.89$	-0.95	6.97±0.20
(b) Chlorophyll b			
Cd	$Y = -0.62\ (\log_{10}x) + 1.19$	-0.93	0.56±0.12
Co	$Y = -0.43\ (\log_{10}x) + 0.90$	-0.96	1.80±0.10
Cr	$Y = -0.07\ (\log_{10}x) + 1.34$	-0.88	8.56±0.10
Cu	$Y = -0.69\ (\log_{10}x) + 1.52$	-0.90	0.97±0.20
Ni	$Y = -0.41\ (\log_{10}x) + 1.59$	-0.89	1.90±0.15
Pb	$Y = -0.18\ (\log_{10}x) + 1.84$	-0.80	13.70±0.10
Zn	$Y = -0.32\ (\log_{10}x) + 2.00$	-0.87	8.65±0.10

x = Concentration of metal in medium (µM); Y = concentration of metal in plants (µmol g^{-1}).

All values of r are significant ($p<0.001$).

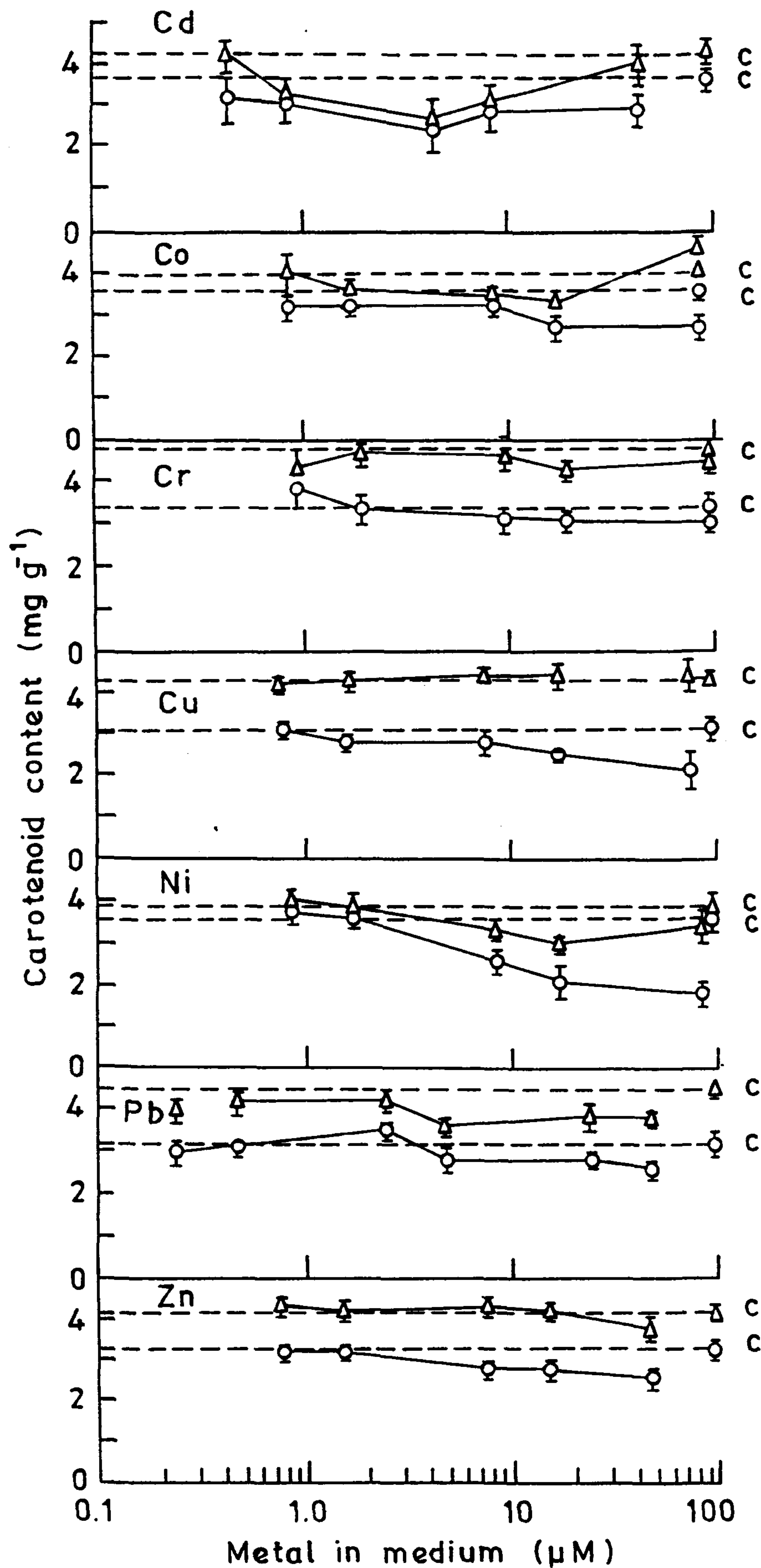


Fig. 2.5: Carotenoid content in L. polyrrhiza (o) and A. pinnata (Δ) at different concentrations of heavy metals. Controls for L. polyrrhiza and A. pinnata are shown by broken lines with circle and triangle respectively at one end. Error bars represent SD, n=5.

treatment lowered the level of carotenoid in L. polyrrhiza as well as in A. pinnata.

Essential cations (Ca, Mg, K) in test plants

Control plants were found to contain 135±7.5, 217±16, 550±60 µmol g^{-1} of Ca, Mg and K, respectively, in L. polyrrhiza, and 162±8, 183±4, 392±50 µmol g^{-1} of Ca, Mg and K, respectively, in A. pinnata. All metals caused lowering of Mg concentration in test plants (Fig. 2.6) and this effect depended on the concentration of metals in the external environment.

Of various metals, Cu was most inhibitory. Fig. 2.7 shows Ca content of test plants after a 4-day exposure to test metals. Whereas Ni, Cu and Cr caused concentration-dependent reduction of Ca level in test plants, Cd, Co, and Pb enhanced Ca level. Although the treatment with Cd caused increase in the concentration of K in A. pinnata, other heavy metals decreased the level of K in test plants in a concentration-dependent fashion (Fig. 2.8).

The exposure of test plants to heavy metals decreased water content of L. polyrrhiza (Fig. 2.9) and A. pinnata (Fig. 2.10). This effect was essentially dependent on the concentration of test metals in the medium. Nickel, Cu and Cr most drastically lowered water content of test plants, however, Zn and Pb displayed mild reduction.

Metal accumulation in test plants

The ability of L. polyrrhiza and A. pinnata to accumulate

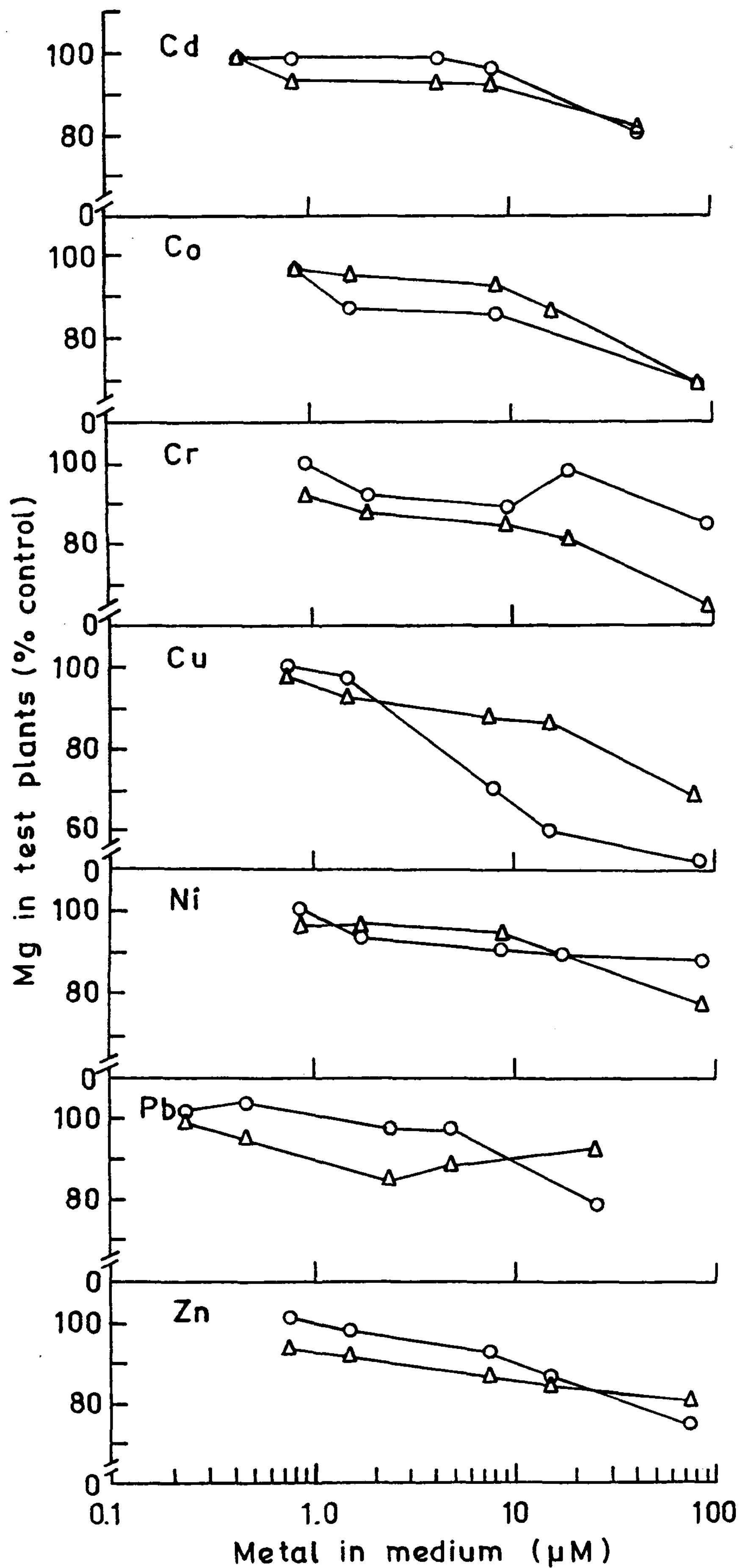


Fig. 2.6: Effects of heavy metals on Mg content (% control) in L. polyrrhiza and A. pinnata. Symbols: o L. polyrrhiza; Δ A. pinnata.

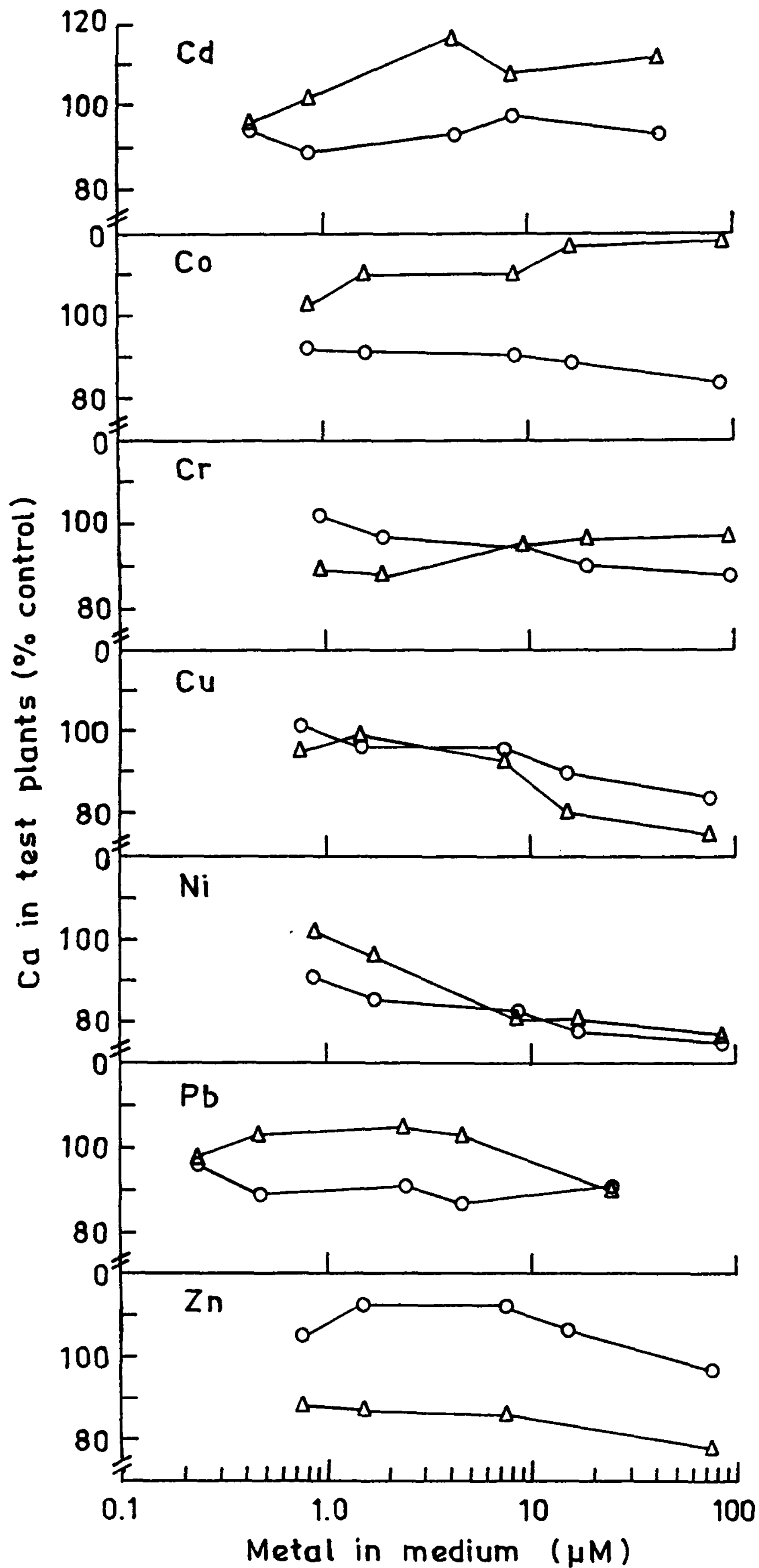


Fig. 2.7: Effects of heavy metals on Ca content (% control) in L. polyrrhiza and A. pinnata. Symbols: o L. polyrrhiza; Δ A. pinnata.

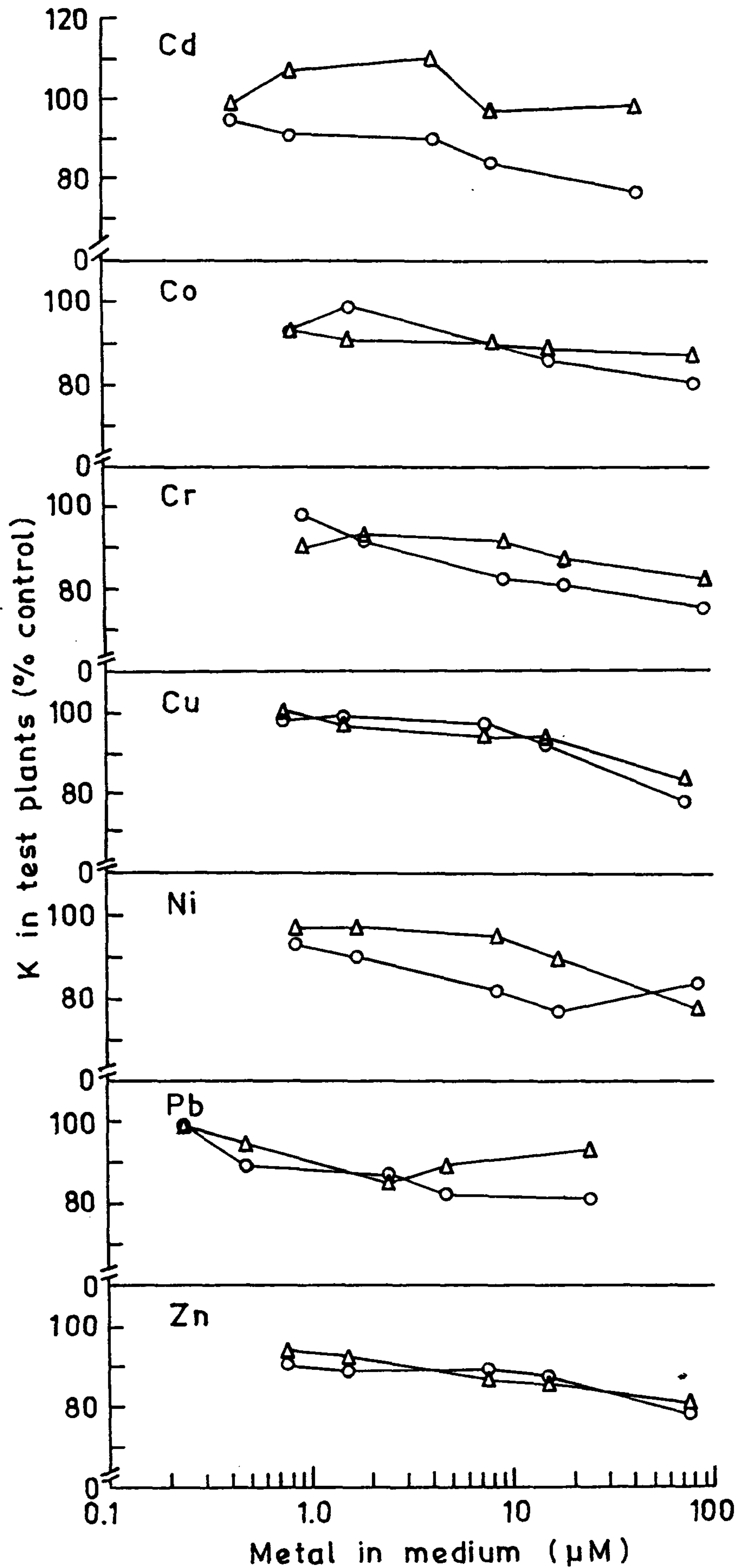


Fig. 2.8: Effects of heavy metals on K content (% control) in _L. polyrrhiza_ and _A. pinnata_. Symbols: o _L. polyrriza_; Δ _A. pinnata_.

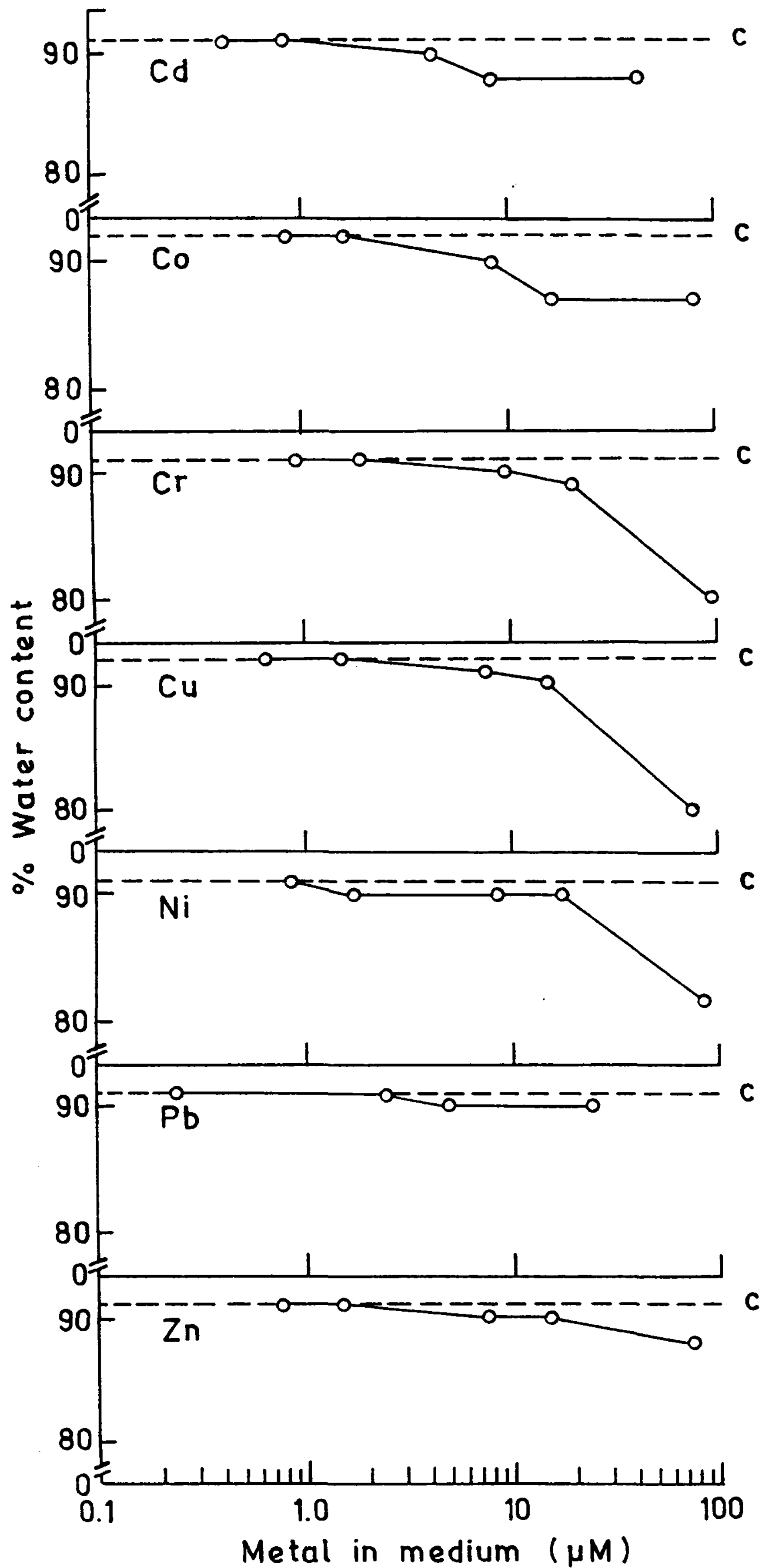


Fig. 2.9: Per cent water content in *L. polyrrhiza* at different concentrations of heavy metals. The broken line shows the control.

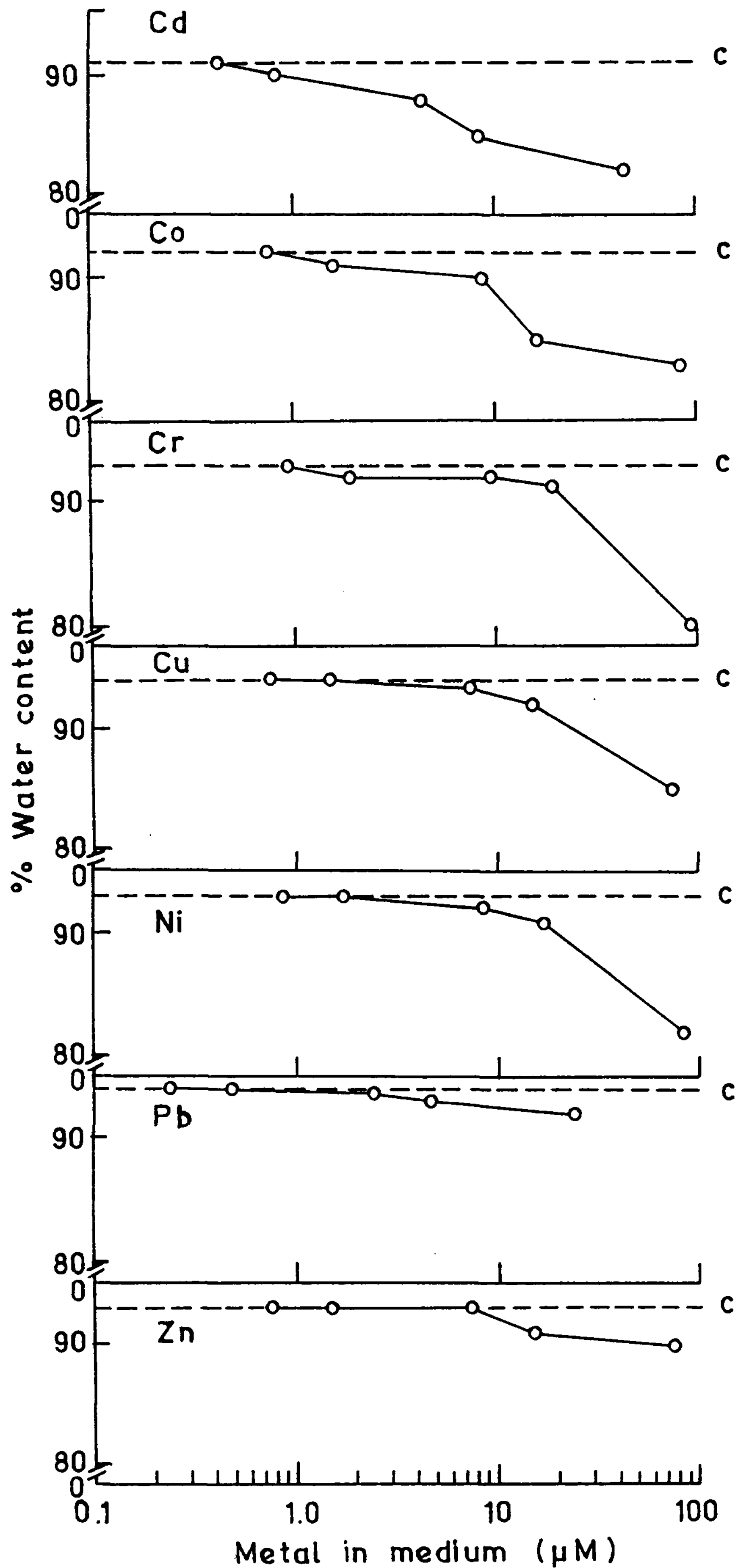


Fig. 2.10: Per cent water content in A. pinnata at different concentrations of heavy metals. The broken line shows the control.

Cd, Co, Cr, Cu, Ni, Pb and Zn was tested at different external concentrations of these metals after a 4-d exposure period. Metal accumulation data for L. polyrrhiza and A. pinnata are presented in Figs. 2.11 and 2.12, respectively. The accumulation of heavy metals by test plants was dependent on metal concentration in the medium. Both test plants accumulated Ni maximally, while Cr was accumulated least. A highly significant positive correlation was observed between metal concentration in plant and metal concentration in the medium for L. polyrrhiza (Table 2.8) and A. pinnata (Table 2.9). These tables also contain regression equations wherein Y is metal content (µmol g^{-1} dry weight) of test plants and X is metal concentration (µM) in the medium.

Discussion

All test metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) were found to reduce the growth of L. polyrrhiza and A. pinnata. Thus, present findings agree with previous works on metal toxicity to Lemna spp. (Hutchinson and Czyrska 1975, Nasu and Kugimoto 1981, Jain et al. 1990, Huebert and Shay 1991, 1992) and other aquatic plants (Hutchinson and Czyrska 1975, Sela et al. 1989). Charpentier et al. (1987) observed stimulation of growth of Lemna polyrrhiza at low concentrations (0.02-0.1 mg l^{-1}) of Cd, however, this phenomenon was not observed in the present study despite the fact that the same species was employed. Thus the isolates of L. polyrrhiza utilized in the present work and those by Charpentier et al. (1987) seem to have certain differences in their pysiological attributes.

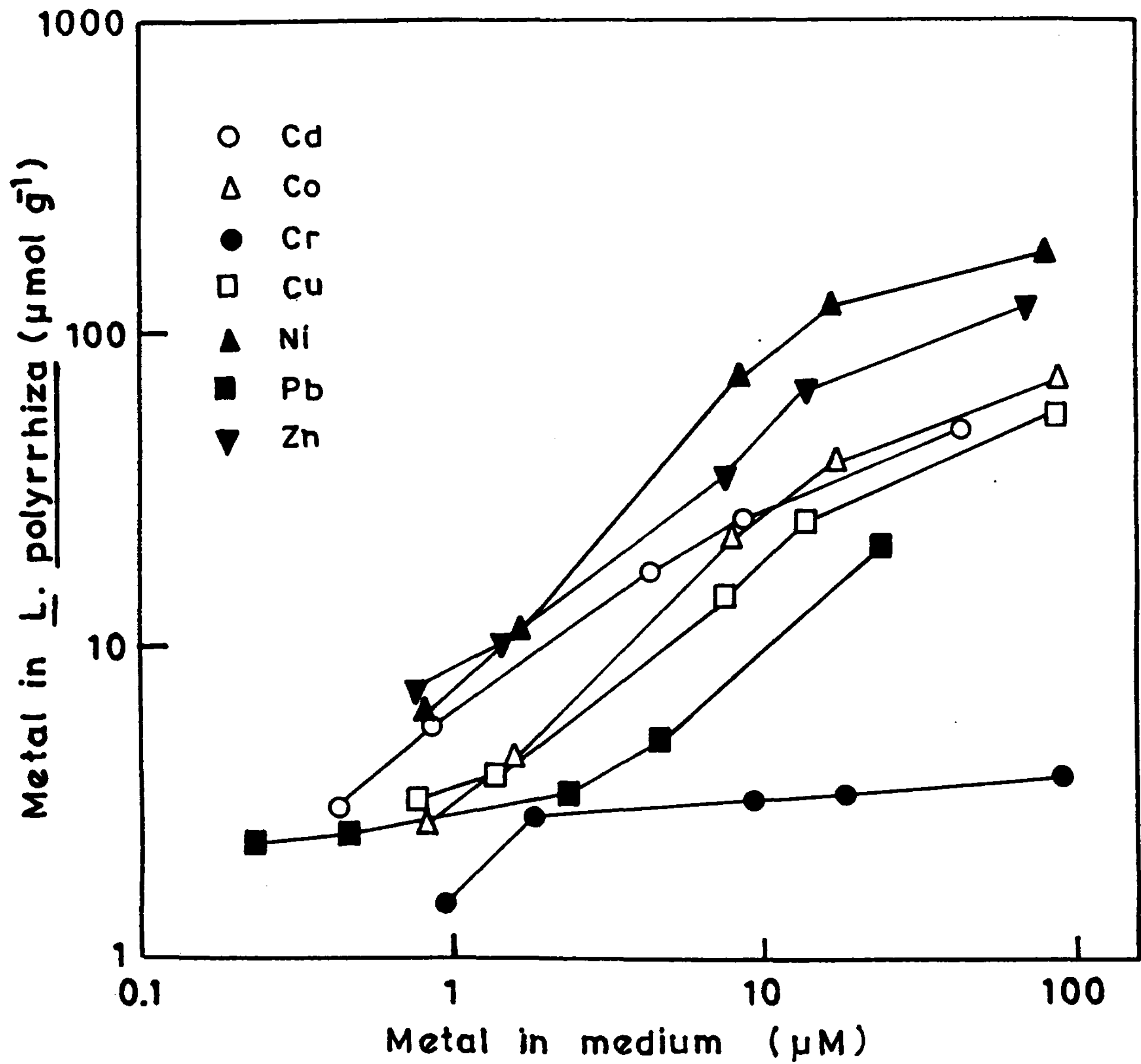


Fig. 2.11: Relationship between metal concentration in medium and metal concentration in L. polyrrhiza.

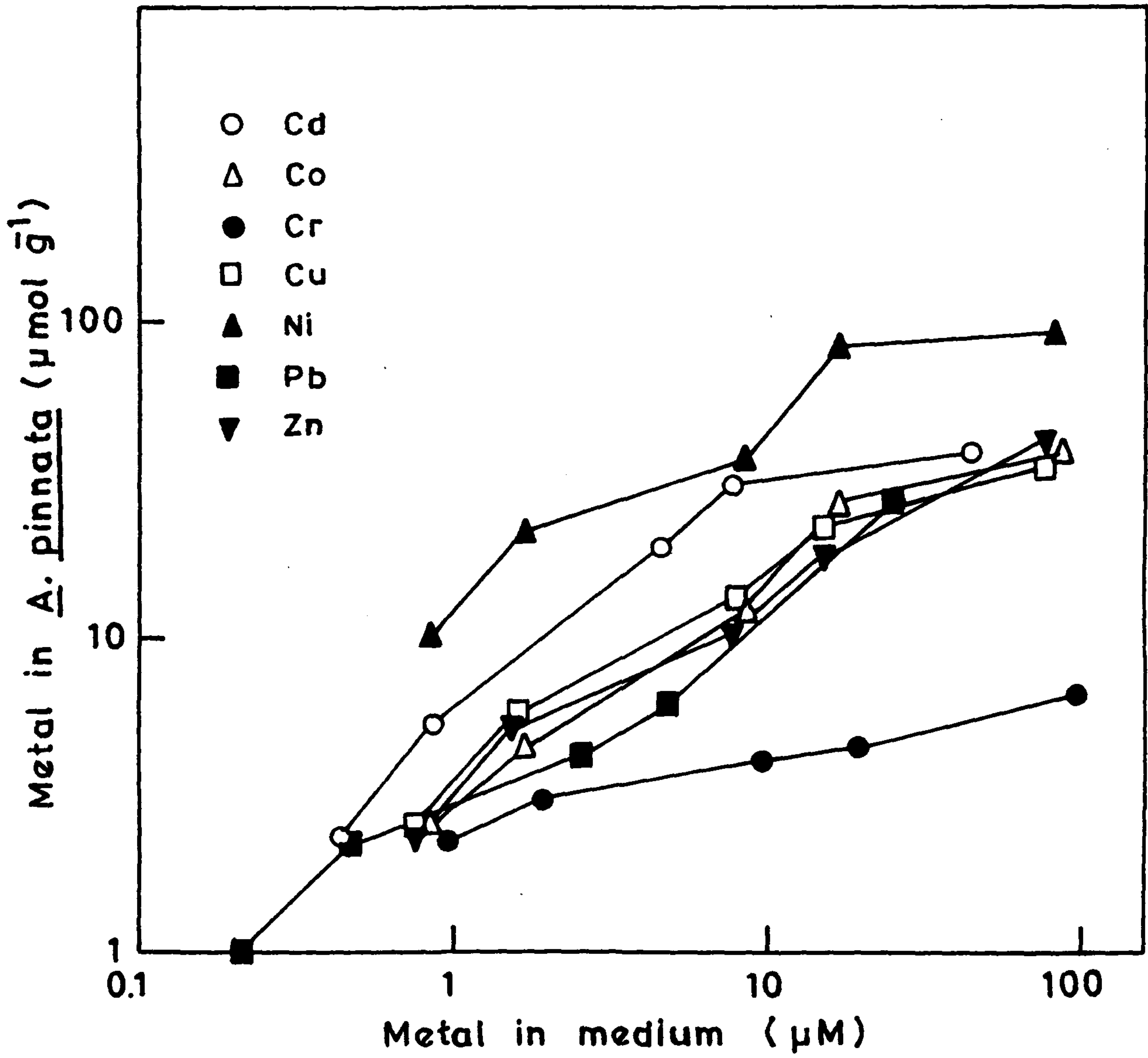


Fig. 2.12: Relationship between metal concentration in _A. pinnata_ and metal concentration in medium.

Table 2.8: Bivariate linear regression equations relating metals in L. polyrrhiza to metals in medium.

	Regression equation	r
Cd	$\log_{10} Y = 0.78 \log_{10}x + 0.72$	0.90
Co	$\log_{10} Y = 0.61 \log_{10}x + 0.50$	0.97
Cr	$\log_{10} Y = 0.20 \log_{10}x + 0.40$	0.89
Cu	$\log_{10} Y = 0.56 \log_{10}x + 0.58$	0.97
Ni	$\log_{10} Y = 0.47 \log_{10}x + 1.15$	0.95
Pb	$\log_{10} Y = 0.66 \log_{10}x + 0.44$	0.98
Zn	$\log_{10} Y = 0.59 \log_{10}x + 0.52$	0.98

x = Concentration of metal in the medium (µM); Y = concentration of metal in plants (µmol g^{-1}).

All values of r are significant ($p<0.001$).

Table 2.9: Bivariate linear regression equations relating metals in *A. pinnata* to metals in medium.

	Regression equation	r
Cd	$\log_{10} Y = 0.61 \log_{10}x + 0.76$	0.98
Co	$\log_{10} Y = 0.96 \log_{10}x + 0.24$	0.94
Cr	$\log_{10} Y = 0.17 \log_{10}x + 0.30$	0.83
Cu	$\log_{10} Y = 0.63 \log_{10}x + 0.57$	0.89
Ni	$\log_{10} Y = 0.75 \log_{10}x + 1.00$	0.96
Pb	$\log_{10} Y = 0.45 \log_{10}x + 0.52$	0.92
Zn	$\log_{10} Y = 0.64 \log_{10}x + 0.93$	0.79

x = Concentration of metal in medium (µM); Y = concentration of metal in plants (µmol g^{-1}).

All values of r are significant ($p<0.001$).

The present observations of metal-induced decline in water content of L. polyrrhiza and A. pinnata are in consonance with previous reports (Fuhrer 1982, Paivoke 1983, Barcelo et al. 1986, Sela et al. 1989). Hager et al. (1987) found that nano molar levels of triethyl lead (Et_3Pb^+) destroyed H^+ gradients in tonoplast vesicles of Zea mays coleoptiles. They indicated that the strong inhibitory effect of Et_3Pb^+ on the accumulation of ions within the vacuolar vesicles would result in an immediate collapse of turgor in intact cells. Another possible explanation for diminished water content in plants treated with heavy metals could be depression of cell enlargement and cell division by these pollutants (Greger et al. 1991). However, more efforts need to be made to precisely understand the effects of metals on cell water relations which are consequences of the primary metal effects on cell walls and plasmalemma (see Barcelo and Poschenrieder 1990).

Plant cell membranes are considered as primary sites of metal injury (Kennedy and Gonsalves 1987, Haug and Caldwell 1985, Barcelo and Poschenrieder 1990). Metal-induced alteration in the characteristics of plasma membrane should be expected to alter the cell's ability in regulating fluxes of various ions. L. polyrriza and A. pinnata showed considerable losses of essential cations (Ca, Mg, K) after exposure to test metals. The only exceptions were Cd- or Co-treated A. pinnata fronds which showed some increase in Ca content. Sela et al. (1989) have also observed enhanced Ca levels in A. filiculoides treated with Cd. Nevertheless, the general pattern of reduced

levels of Ca, Mg and K in plants treated with heavy metals is in agreement with previous reports (Fuchs and Garty 1983, Taylor and Foy 1985a, Sela et al. 1989, Barcelo and Poschenrieder 1990, Kaplan et al. 1990). Taylor and Foy (1985a) observed that ionic Cu caused lowering of Ca, Mg and Mn levels in wheat due to membrane failure. However, when Cu was supplied in a chelated form (Cu-EDTA), the leaves maintained fairly high levels of Ca, Mg and Mn. Taylor and Foy (1985a) suggested that sensitive membrane sites were preoccupied by Cu-EDTA, hence they became protected from Cu toxicity. It is worth mentioning here that EDTA was not employed in the present study, and consequently loss of cations was observed in metal-treated test plants. In essence, metal-induced element deficiencies are part of the overall metal toxicity syndrome, and may contribute to reduced yields of *L. polyrrhiza* and *A. pinnata* growing in metal-enriched waters.

Upon exposure to various test metals, *L. polyrrhiza* and *A. pinnata* showed reduced levels of chlorophyll *a* and *b*. Heavy metal-induced reduction in chlorophyll concentration has been found in several other cases as well (Filbin and Hough 1979, Rebhum and Ben-Amotz 1984, Sharma and Chopra 1987, Takamura et al. 1989, Wong and Chang 1991). In view of high redox potential of many heavy metals, the simplest explanation consistent with these observations is that metals inhibit the reductive steps in the biosynthetic pathway of these pigments (DeFilippis and Pallaghy 1976a and b). In addition, the key enzyme protochlorophyllide reductase, which is involved in the reduction of

protochlorophyll to chlorophyll, is well-known to be inhibited by heavy metals (Van Assche and Clijsters 1990). In comparison to chlorophyll b, chlorophyll a levels of L. polyrrhiza or A. pinnata were more severely lowered by various test metals. This trend became apparent when EC_{50} values for test metals derived from chlorophyll a and b data were compared. No explanation can be offered for this variability, however, increased chlorophyll a/chlorophyll b ratio has been found in certain cases after metal treatment (Wong and Chang 1991). The inhibition of chloropyll biosynthesis by heavy metals should be expected to decrease the photosynthetic efficiency of plants, although direct interferences of heavy metals with photosynthetic machinery have been observed (Cedeno-Maldonado et al. 1972, Bradeen et al. 1973, Rosko and Rachlin 1977, Stratton and Corke 1979, Stratton et al. 1979, Takamura et al. 1989, Wong and Chang 1991). Unlike chlorophylls, levels of carotenoid in L. polyrrhiza or A. pinnata did not change much after heavy metal treatment. Thus, present observations agree with other reports showing increased carotenoid/chlorophyll a ratio in plants exposed to heavy metals or other kinds of stresses (De Filippis and Pallaghy 1976, Rai et al. 1981b).

The results of the uptake studies performed while varying the initial concentration of heavy metals showed that L. polyrrhiza and A. pinnata have tremendous ability to remove metal ions from solution over a wide range of concentrations. The positive relationship between the degree of metal accumulation by test plants and external metal concentration was

statistically significant in all the treatments. Thus, present findings are in congruity with metal accumulation reports on macrophytes (Beckett and Brown 1984, Brown and Beckett 1985, Hagemeyer and Waisel 1989, Wells and Brown 1990, Kwan and Smith 1991) and algae (Bates et al. 1982, Skowronski 1984, 1986, Gadd 1988, Ting et al. 1991, Whorowski 1991). Nonetheless, Charpentier et al. (1987) could not find a significant relationship between Cd concentration in *Lemna polyrrhiza* and Cd concentration in the external environment. It has been often suggested that bioaccumulation of a particular metal is a function of its ionic level, and is not linearly related to total ambient metal concentrations in water where there is appreciable complexation of organic matter (Sunda and Guillard 1976, Darimont and Frenay 1990). The present work, however, shows that the accumulation of Cd, Cr, Co, Cu, Ni, Pb and Zn in test plants was directly related to total metal concentration in the culture medium. This relationship was obtained due perhaps to the reason that a chelator-free synthetic inorganic medium was used for cultivating *L. polyrrhiza* and *A. pinnata*. It should, however, be kept in mind that similar experiments with natural waters, which are known to be rich in natural chelators (Darimont and Frenay 1990), must define the ionic concentrations of test metals. *L. polyrrhiza* and *A. pinnata* accumulated Ni maximally and Cr minimally. No effort was made to differentiate metal accumulation in different plant parts, although Charpentier et al. (1987) and Sela et al. (1989) observed greater accumulation of metals in roots in comparison to other parts of floating macrophytes. This is perhaps the reason why

root senescence and shedding occurred in *L. polyrrhiza* and *A. pinnata* after exposure to Cd and Cu which were found to be most toxic to the growth of test plants.

Concentration factors (CF) of metals (metal in plant/metal in medium) obtained for *L. polyrrhiza* and *A. pinnata* have been compared with previous reports (see Table 2.10). The range for Cd in test plants falls within the range obtained by other workers. The CF for Cr was however greater than the values reported by Sela et al. (1989), and the same was the case for Ni. In comparison to *L. polyrrhiza*, *A. pinnata* was found to have higher CF for Cr. Consequently, the latter macrophyte showed a greater sensitivity to Cr. The values of CF for Cu, Pb and Zn in test plants lie within the range obtained by other workers. The ability of *L. polyrrhiza* and *A. pinnata* to concentrate heavy metals in their tissues bolster a strong case for using these plants for metal removal from wastewaters. This plea gets further support from the fact that these macrophytes occur abundantly in India and many other parts of the world.

The present study revealed that the order of metal toxicity to RGR of *L. polyrrhiza* was Cd > Cu = Ni > Co > Cr > Zn > Pb and the order of metal accumulation was Ni > Zn > Co > Cu > Cd > Pb > Cr. In *A. pinnata* however the hierarchies were Cd > Cr > Co > Cu > Ni > Pb > Zn for metal toxicity to RGR and Ni > Zn > Co = Cd > Cu > Pb > Cr for metal accumulation. Although not accumulated maximally in both test plants, Cd was the most toxic of all tested metals. On the other hand, Ni was accumulated

Table 2.10: Concentration factors (CF) of various heavy metals in aquatic plants as reported by others and observed in the present study.

Name of plant	Plant part	Metal	Concentration factor (CF)	Reference
Lemna valdiviana	Whole	Cd	6030	Hutchinson and Czyrska (1972)
Salvinia natans	Whole	Cd	9500	
Elodea	Roots	Cd	20000	Ravera et al. (1973)
Elodea	Shoots	Cd	21000	
Agrostis	Whole	Cd	583.3	Wu et al. (1975)
Equisetum		Cd	200	Ray and White (1979)
Callitriche platycarpa		Cd	14000	van der Werff and Pruyt (1982)
Azolla filiculoides	Whole	Cd	696	Sela et al. (1989)
	Roots	Cd	1582	
Lemna polyrrhiza	Roots	Cd	694	Charpentier et al. (1987)
	Fronds	Cd	222	
Lemna trisulca	Whole	Cd	24800	Huebert and Shay (1991)
Lemna polyrrhiza	Whole	Cd	1088-6716	Present study
Azolla pinnata	Whole	Cd	876-5222	
Lemna polyrrhiza	Whole	Co	822-3200	Present study
Azolla pinnata	Whole	Co	447-2941	
Azolla filiculoides	Whole	Cr	126.9	Sela et al. (1989)
	Root	Cr	687.3	
Lemna polyrrhiza	Whole	Cr	29-1520	Present study
Azolla pinnata	Whole	Cr	67-2600	
Lemna valdiviana	Whole	Cu	10000	Hutchinson and Czyrska (1975)
Aquatic macrophyte	Roots	Cu	2222	Hutchinson et al. (1975)
Equisetum		Cu	10000	Ray and White (1979)
Callitriche platycarpa	Root	Cu	8000	van der Werff and Pruyt (1982)
Azolla filiculoides	Whole	Cu	614.9	Sela et al. (1989)
	Roots	Cu	2918.6	
Lemna polyrrhiza	Whole	Cu	702-4769	Present study
Azolla pinnata	Whole	Cu	432-3164	

Contd. ...

Table 2.10 Contd.

Nuphar lutem		Ni	550	Dietz (1973)
Potamogeton		Ni	11429	
Sagittaria sagittifolia		Ni	120	
Azolla filiculoides	Whole	Ni	587.6	Sela *et al*. (1989)
	Roots	Ni	1355.9	
Lemna polyrrhiza	Whole	Ni	2115-7800	Present study
Azolla pinnata	Whole	Ni	1058-12117	
Mosses	Whole	Pb	3120-5300	Dietz (1973)
Submerged macrophytes	Whole	Pb	182-720	
Equisetum		Pb	9400	Ray and White (1979)
Callitriche	Root	Pb	6500	van der Werff and Pruyt (1982)
Lemna polyrrhiza		Pb	836-9600	Present study
Azolla pinnata		Pb	1112-4166	
Equisetum		Zn	34000	Ray and White (1979)
Callitriche platycarpa	Root	Zn	6000	van der Werff and Pruyt (1982)
Azolla filiculoides	Whole	Zn	427.2	Sela *et al*. (1989)
	Root	Zn	776.0	
Lemna trisulca	Whole	Zn	7100-16100	Huebert and Shay (1992)
Lemna polyrrhiza	Whole	Zn	1570-9260	Present study
Azolla pinnata	Whole	Zn	1056-6052	
Lemna minor	Whole	Ag	82.5	Hutchinson and Czyrska (1975)

maximally by test plants, and caused pronounced toxic effects on test plants. Chromium was not very toxic to L. polyrrhiza but ranked second in order of toxicity to A. pinnata. Lead was not accumulated much by both the plants and was found to be the least toxic.

Several studies have compared the toxic effects of different metals on algae and aquatic plants. Chromium has been found to be less toxic thanCu (Azeez and Banerjee 1988), and Cu, Zn, Hg, Cd and Pb (Thomas et al. 1980). Hollibaugh et al. (1980) compared the toxicity of ten metals on natural algal populations and gave Cr a low toxicity ranking. On the other hand, Lazinsky and Sicko-Goad (1990) found Cr more toxic than Cu, Zn and Cd to Cyclotella meneghiniana. The reason for the differences in toxic effects of a particular metal towards different test organisms are not clear. However, such differences can be expected to cause perceptible changes in community composition of metal-stressed waterbodies. The primary toxicity mechanisms of the different metal may be as different as their chemical properties, especially valence, ion radius and capacity to form organic complexes (Barcelo and Poschenrieder 1990). Fisher and Jones (1981) proposed that differences in metal toxicities are due to differential affinities of the cations for sulphur complexation. If this hypothesis was correct, metals having more affinity for sulphur (e.g., Cu) should be more toxic than the metals having lesser affinity for sulphur (e.g., Cd). The sulphur affinity hypothesis does not seem plausible as Cd was found more toxic than Cu in the present case.

Enough evidences have accumulated showing difficulties in assessing the effects likely to be caused by the presence of metals in waters simply on the basis of their concentrations, largely because the extent to whichthe metals are chelated is normally unknown. In order to overcome this problem, it has been suggested that the effects of metals on algae and plants should be studied, not in relation to the concentration in water, but to the amounts present in plant cells (Davies 1983). The present findings showed that metal concentration in test plants as well as their toxicities were functions of the metal concentration in the medium. Hence the best way of conducting metal toxicity bioassays of natural waters, which contain plenty of inorganic and organic chelators (Darimont and Frenay 1990), would be to measure metal concentration in test plants.

Chapter-III

EXTRACELLULAR AND INTRACELLULAR UPTAKE OF CADMIUM IN TEST PLANTS

Introduction

In order to utilize aquatic plants for stripping heavy metals from wastewaters, it is necessary to know the details of metal uptake process in these organisms. Reports are available showing that aquatic plants accumulate high amounts of heavy metals from ambient water (Chigbo et al. 1982, Wehr and Whitton 1983, Say and Whitton 1983, Sela et al. 1989, Turnquist et al. 1990, Kwan and Smith 1991). However, studies on the kinetics of metal accumulation in aquatic plants are extremely limited (Kwan and Smith 1991). Some studies have been carried out to elucidate the mechanisms of extracellular and intracellular uptake of metals in bryophytes and lichens (Wells and Brown 1990, Brown and Beckett 1985, Beckett and Brown 1984), and in algae (Bates et al. 1982). Similar investigations on other aquatic plants, which are also exposed to metal contaminants in aquatic environment, are virtually non-existent.

Different opinions have been expressed on the mode of metal uptake by higher plants. A non-metabolic uptake mechanism based on diffusion and sequestration of Cd was suggested by Cutler and Rain (1974), while a metabolic Cd uptake mechanism combined with the non-metabolic influx was suggested by Smeyers-Verbeke et al. (1978). Cataldo et al. (1983) found absorption of Cd a

metabolic process. Beckett and Brown (1984) found stimulation of intracellular Cd uptake by light in the lichen *Peltigera horizontalis*. Similar results have been found in some algae (Findenegg et al. 1971).

The efficiency of metal adsorption on plants can be expected to influence the rate of intracellular metal accumulation. Cell surfaces are composed of a mosaic of adsorption sites consisting of carboxylic, sulphydryl, phosphatidic, amino and other groups (Crist et al. 1981). Some of these sites are believed to be 'physiologically inert' while others are 'physiologically active' facilitating metal transport across the cell membrane (Rothstein 1959). The cell surface therefore acts as a barrier in regulating the transport of heavy metals. It is extremely important to distinguish extracellular and intracellular accumulation of metals in order to have more insights into metal uptake mechanism and tolerance than otherwise would be possible by measuring only total metal associated with the entire plant cells (Bates et al. 1982).

Dead plants are also known to accumulate high amounts of heavy metals. Dead biomass of *Azolla filiculoides* has a very high capacity to bind heavy metals (Sela et al. 1989). Kuyucak and Volesky (1988) found that non-living thallus of the brown alga *Sargassum* has an extraordinary capacity for Au uptake (420 mg g^{-1} dry weight). They also found that U, Zn and Cu were better adsorbed by the non-living yeast than the living cells. Similarly, dead *Chlorella* cells have been found to accumulate

more U than the living cells (Horikoshi et al. 1979). Similar observations have been made in case of fungi and bacteria (Gadd 1988, 1990).

An attempt has been made to study the kinetics of extracellular and intracellular Cd uptake by L. polyrrhiza and A. pinnata, and also to detrmine the possibilities of using dead biomass of these plants for stripping Cd from wastewaters.

Materials and Methods

Stock cultures of L. polyrrhiza and A. pinnata were maintained in laboratory as described in Chapter II. For each experiment, Cd solution was prepared afresh using cadmium acetate. As recommended (Wang 1990), experiments were carried out in Teflon beakers at 20°C. In each experiment about 10 mg (fresh weight) biomass of test plant was floated on the uptake medium. A minimum of five replicates were used for each treatment. In order to determine the effect of time on extracellular and intracellular uptake of Cd, a time-course study was carried out. Test plants were incubated in a medium containing 100 µM Cd at pH 7. Test plants were then harvested at different time intervals and analysed for extracellular and intracellular levels of Cd.

Several chemicals, such as EDTA and $NiCl_2$, have been recommended to displace heavy metals adsorbed onto plant surfaces (Bates et al. 1982, Beckett and Brown 1984). The use of these desorbing agents allows one to distinguish

extracellular and intracellular concentrations of heavy metals in plants. The estimation of metal content of plant after washing with a desorbing agent gives the intracellular metal concentration, whereas the amount of metal removed from plant biomass by a desorbant is the extracellular fraction. In the present work, the desorption of extracellular Cd was carried out by shaking and washing plant biomass with 20 ml of 10 mM EDTA for 10 min followed by rinsing with chilled double-distilled water. The analysis of this washing gave the extracellular metal level, and analysis of EDTA-washed plant material gave the intracellular metal level. All samples were dried at 60°C till constant weights were obtained.

Plant materials were digested following the method used by Bates et al. (1982). Weighed samples were taken in borosilicate test tubes alongwith 5 ml of digestion mixture consisting of concentrated nitric acid, hydrogen peroxide 30% (w/v) and double-distilled water in 1:1:3 ratio (v/v/v). Each sample was digested as already described in Chapter II. The digested samples were diluted with 2% (v/v) nitric acid to the desired volume, and were analysed at Regional Sophisticated Instrumentation Centre, NEHU, Shillong, using a Perkin Elmer atomic absorption spectrophotometer (model 2380). Metal content of test plant was calculated by the equation

$$M = \frac{N \times V}{1000 \times W}$$

where M is the metal content in test plant (mg g^{-1} dry weight), N is the average reading of atomic absorption spectrophotometer,

V is the final volume for estimation and W is the dry weight of digested samples.

Extracellular and intracellular Cd uptake as a function of Cd concentration in the medium were also measured for L. polyrrhiza and A. pinnata. Test plants were incubated in 10, 20, 40, 80 and 100 µM Cd for 2 h at 20°C and pH was adjusted to 7 at the beginning of the experiment. Extracellular Cd was displaced with 10 mM EDTA and measured. Intracellular level of Cd was measured after EDTA treatment. Similarly, experiments were carried out to evaluate the role of roots and light availability on extracellular and intracellular Cd uptake by test plants. A time-course study on Cd uptake by rootless and root-containing test plants was performed. Rootless plants were prepared just before the experiment by carefully removing roots from healthy plants. Plants without and with roots were incubated in the medium containing 5 µM Cd under conditions used in the previous experiment. The plants were harvested at different time intervals and analysed for Cd levels. In order to determine the effect of light on Cd uptake by L. polyrrhiza and A. pinnata a time-course experiment was carried out. Test plants were incubated in the light (PAR 45 µmol m^{-2} sec^{-1}) or in the dark in a medium containing 5 µM Cd and harvested 5, 10, 20 and 30 h after incubation.

Experiments were carried out to study Cd adsorption by dead biomass of test plants. Oven-dried plant biomass (10 mg) was taken in Teflon beakers (capacity 500 ml) and shaken with 200 ml

of 100 µM Cd. Plant samples were harvested at 5, 10, 30, 60 and 90 min, dried, weighed, digested and analysed for Cd concentration.

Efforts were made to find out how long dead plant material could be utilized for removing Cd from ambient medium. Ten mg dried biomass of test plants was kept in 200 ml of 100 µM Cd for 15 min. The biomass was harvested and Cd desorption was carried out with 50 ml of 10 mM EDTA for 15 min. After desorption, the biomass was washed in distilled water and once again kept in 100 µM Cd under similar conditions. This was again followed by desorption. The process was repeated as long as the biomass could be handled. The eluted samples were then analysed for Cd content.

Relative efficiencies of various chemical agents (5 mM HCl, 5 mM NaCl, 5 mM $CaCl_2$, 5 mM $CaCl_2$ + 5 mM HCl, and 5 mM EDTA) in desorbing Cd from dead biomass were evaluated. Dead biomass was exposed to 100 µM Cd for 48 h at room temperature. Subsequently, Cd-loaded biomass was treated with different chemical agents for 30 min. After desorption, all samples were oven dried at 60°C till constant weights were obtained. Each sample was digested and analyzed for Cd level following the method already described.

Results

The rate of extracellular, EDTA (Ethylenediaminetetraacetic acid)-displaceable, Cd uptake declined with time in both L.

polyrrhiza and A. pinnata. In L. polyrrhiza it occurred rapidly during the first 30 min of incubation but slowed down subsequently. No significant change in the level of extracellular Cd occurred after 120 min. Similarly, A. pinnata showed a rapid extracellular Cd uptake during the first 10 min of incubation followed by a slower uptake becoming saturated in 120 min. Cadmium remaining after EDTA treatment (i.e., intracellular) showed a constant uptake rate (Figs. 3.1 and 3.3) of 2.81±0.21 and 2.95±0.5 µmol g^{-1} h^{-1} for L. polyrriza and A. pinnata, respectively. Using double-reciprocal transformation (Figs. 3.2 and 3.4) it was possible to estimate the maximum extracellular Cd uptake (U_{max}) by the test plants; 35.3±5.2 and 33.79±6.6 µmol g^{-1} in L. polyrrhiza and A. pinnata, respectively, and the time required to reach half the maximum uptake as 38.8±4 min for L. polyrrhiza and 71.47±11 min for A. pinnata.

When measured over a range of Cd concentrations intra-cellular Cd uptake by both test plants displayed typical Michaelis-Menten kinetics (Figs. 3.5 and 3.7), and the data fitted straight lines using the Lineweaver-Burk double reciprocal plot (Figs. 3.6 and 3.8). K_m = 55.0±6.2 µM Cd and V_{max} = 2.93±2 µmol g^{-1} h^{-1} for L. polyrrhiza, and K_m = 93.7±21 µM Cd and V_{max} = 3.07±0.9 µmol g^{-1} $^{-1}$ A. pinnata were calculated (Table 3.1). Although extracellular uptake also showed saturation kinetics (Fig. 3.5 and Fig. 3.7), the kinetic constants similar to those for intracellular uptake were not calculated because the rate of extracellular uptake did not remain constant throughout the experimental period. However, Cd

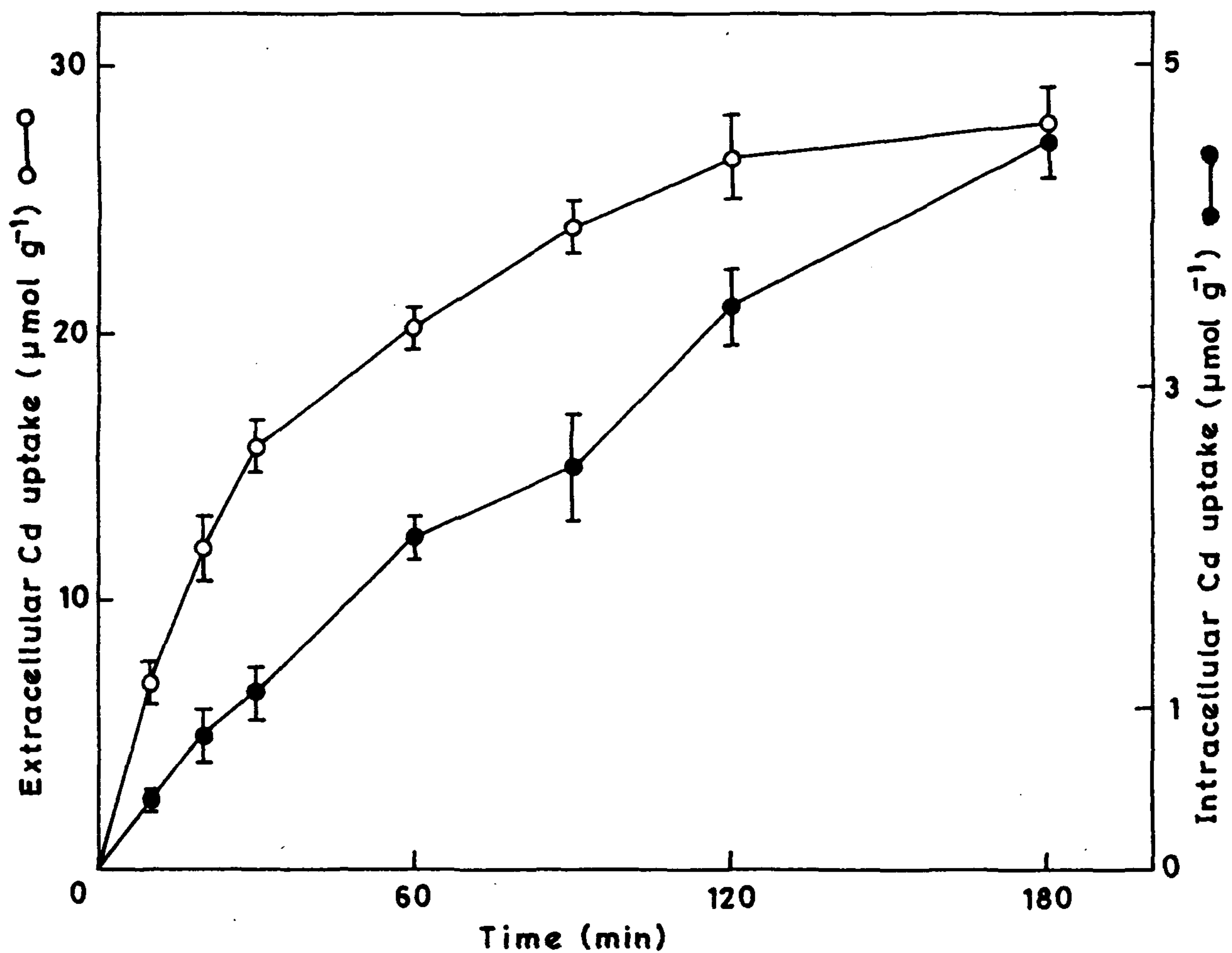


Fig. 3.1: Intracellular and extracellular Cd uptake by *L. polyrrhiza* as a function of time from a solution containing 100 μM Cd. Error bars represent SD, n=5.

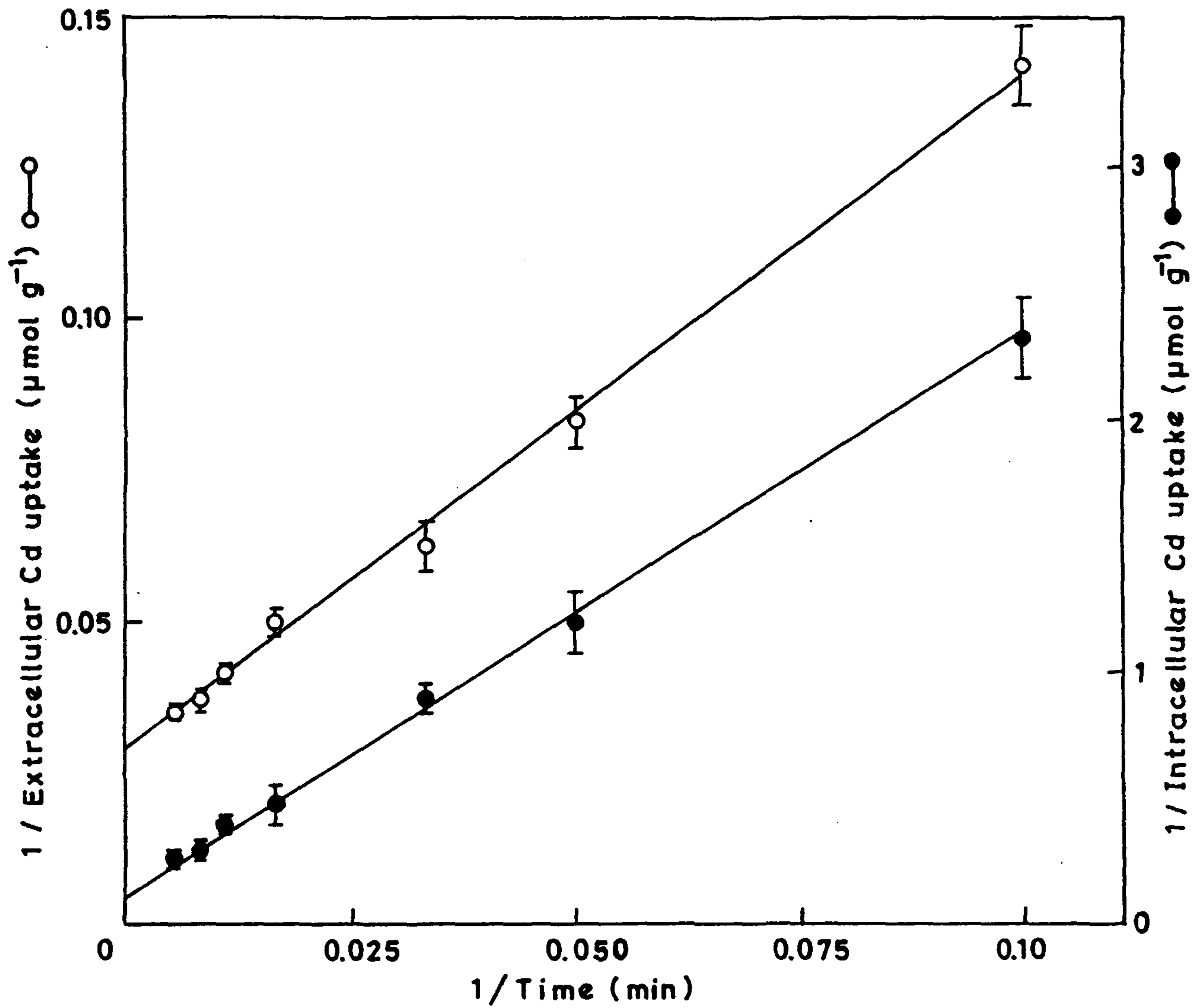


Fig. 3.2: Cadmium uptake by L. polyrrhiza as a function of time. Double reciprocal plot of intracellular and extracellular uptake from a medium containing 100 µM Cd. Error bars represent SD, n = 5.

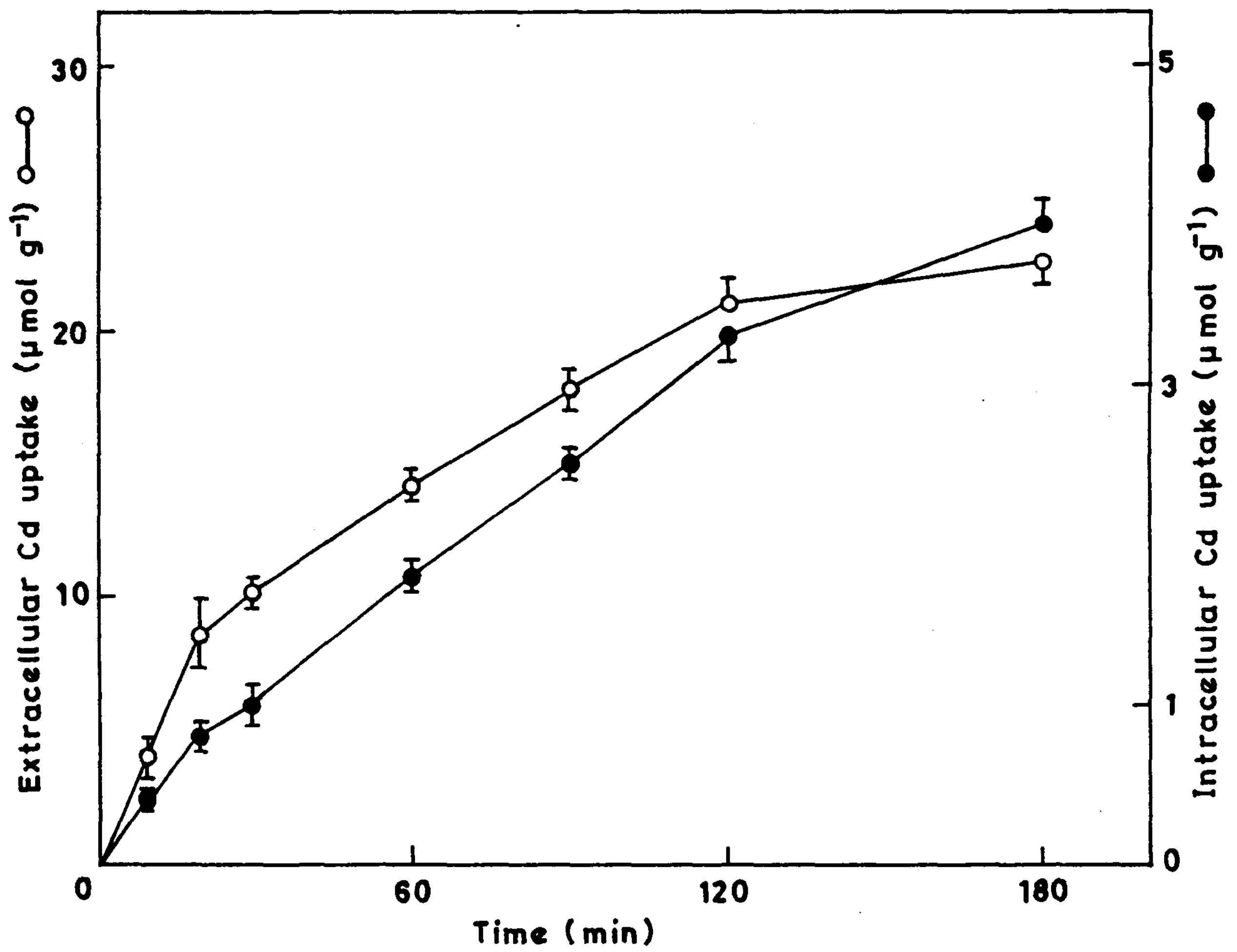


Fig. 3.3: Intracellular and extracellular Cd uptake by <u>A. pinnata</u> as a function of time from a solution containing 100 µM Cd. Error bars represent SD, n=5.

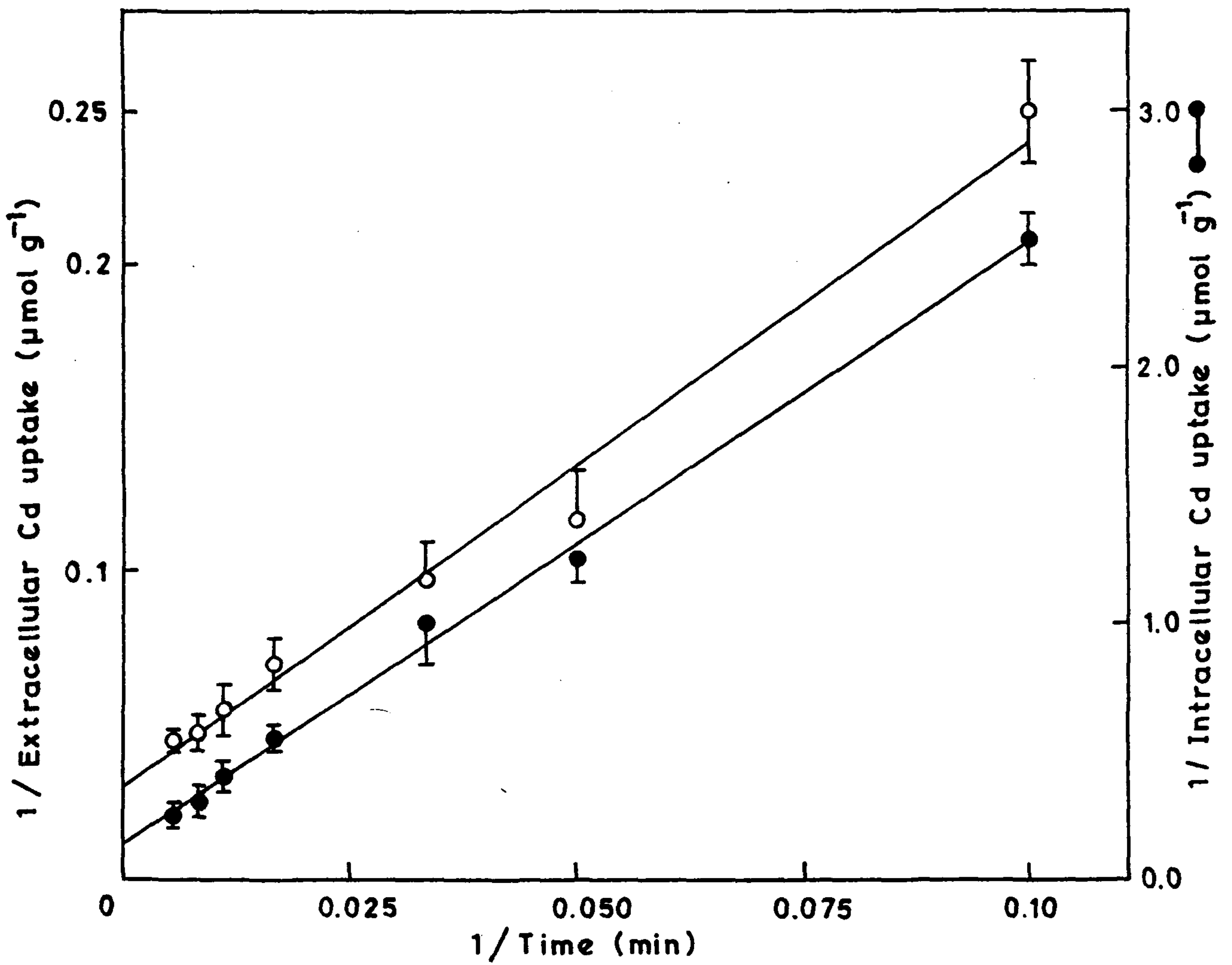


Fig. 3.4: Cadmium uptake by A. pinnata as a function of time. Double reciprocal plot of intracellular and extracellular uptake from a medium containing 100 µM Cd. Error bars represent SD, n=5.

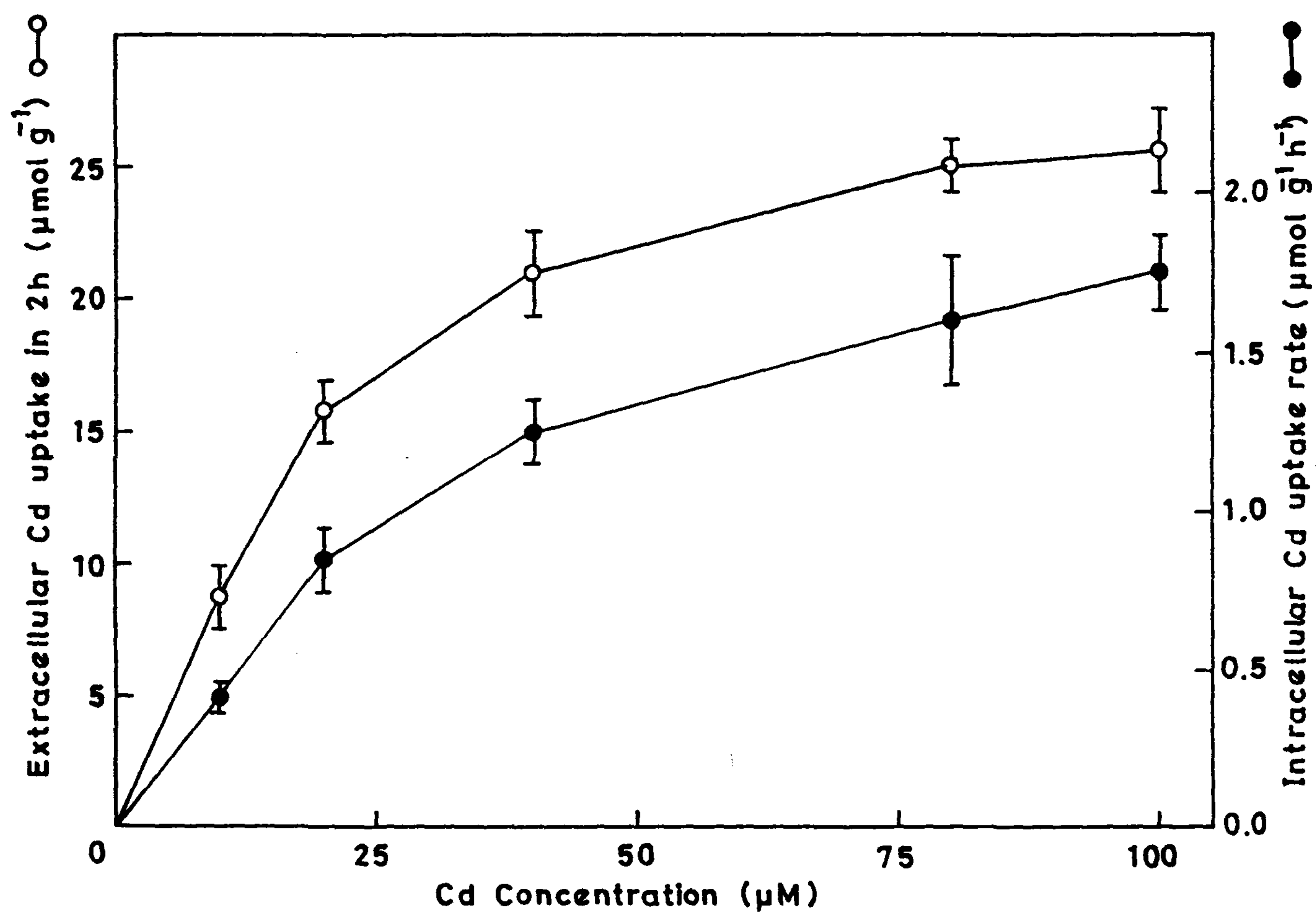


Fig. 3.5: Extracellular Cd uptake after 2h and intracellular uptake rate by L. polyrrhiza as a function of Cd concentration in the solution. Error bars represent SD, n = 5.

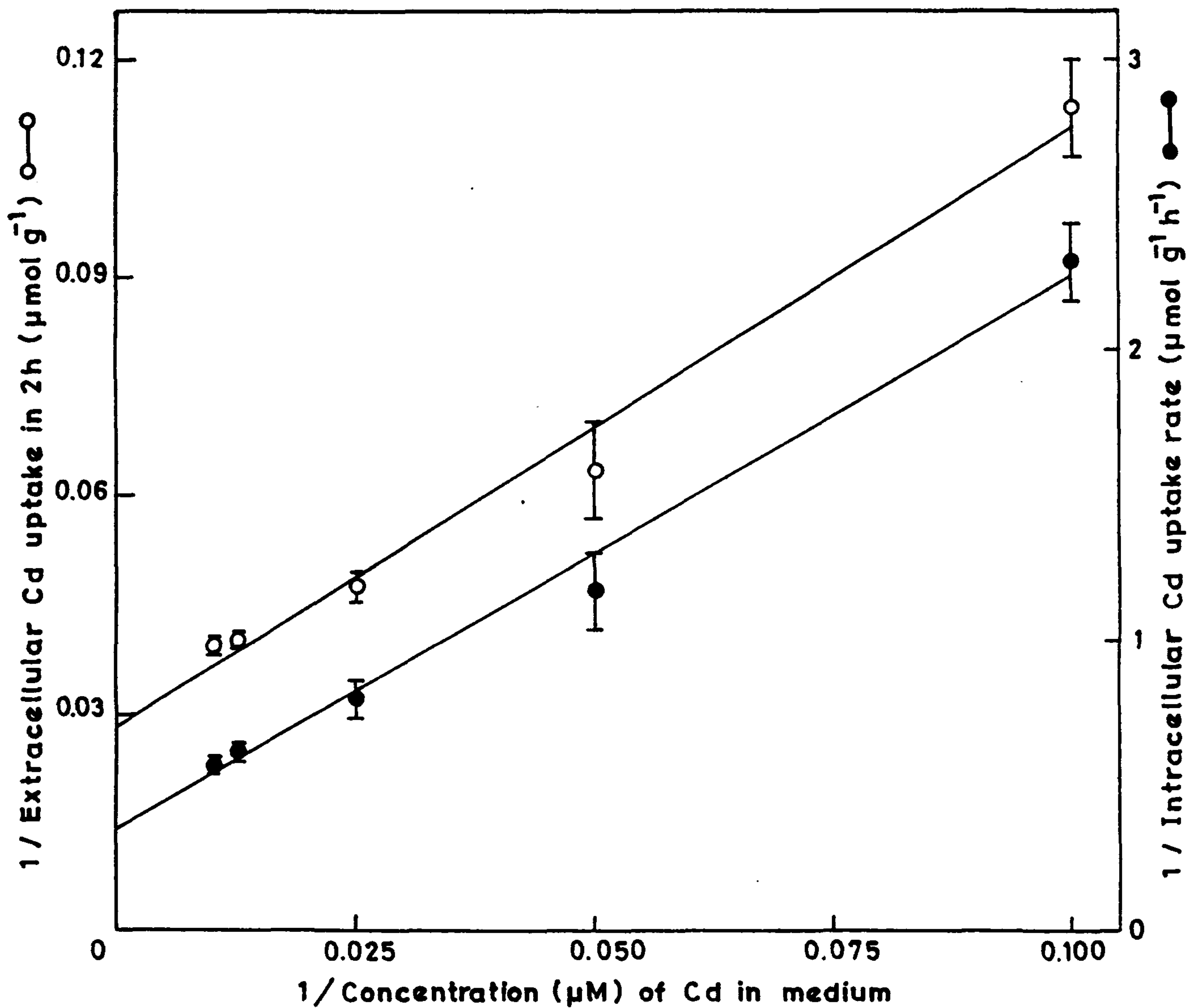


Fig. 3.6: Cadmium uptake by L. polyrrhiza as a function of Cd concentration in the medium after 2 h incubation. Double reciprocal plot of extracellular uptake in 2h (umol g^{-1}) and intracellular uptake rate (μmol g^{-1} h^{-1}). Error bars represent SD, n = 5.

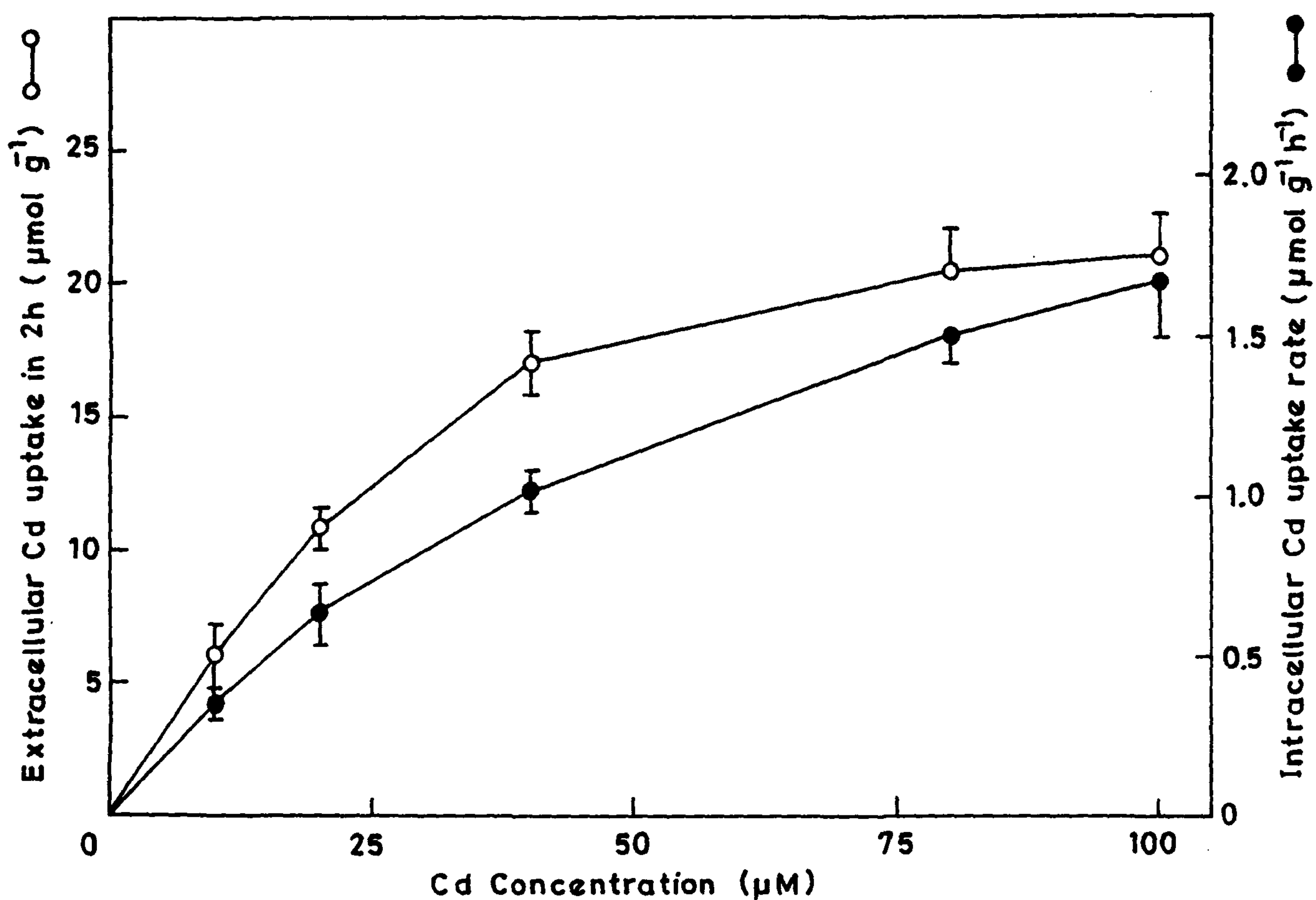


Fig. 3.7: Extracellular Cd uptake after 2h and intracellular Cd uptake rate of A. pinnata as a function of Cd concentration in the solution. Error bars represent SD, n = 5.

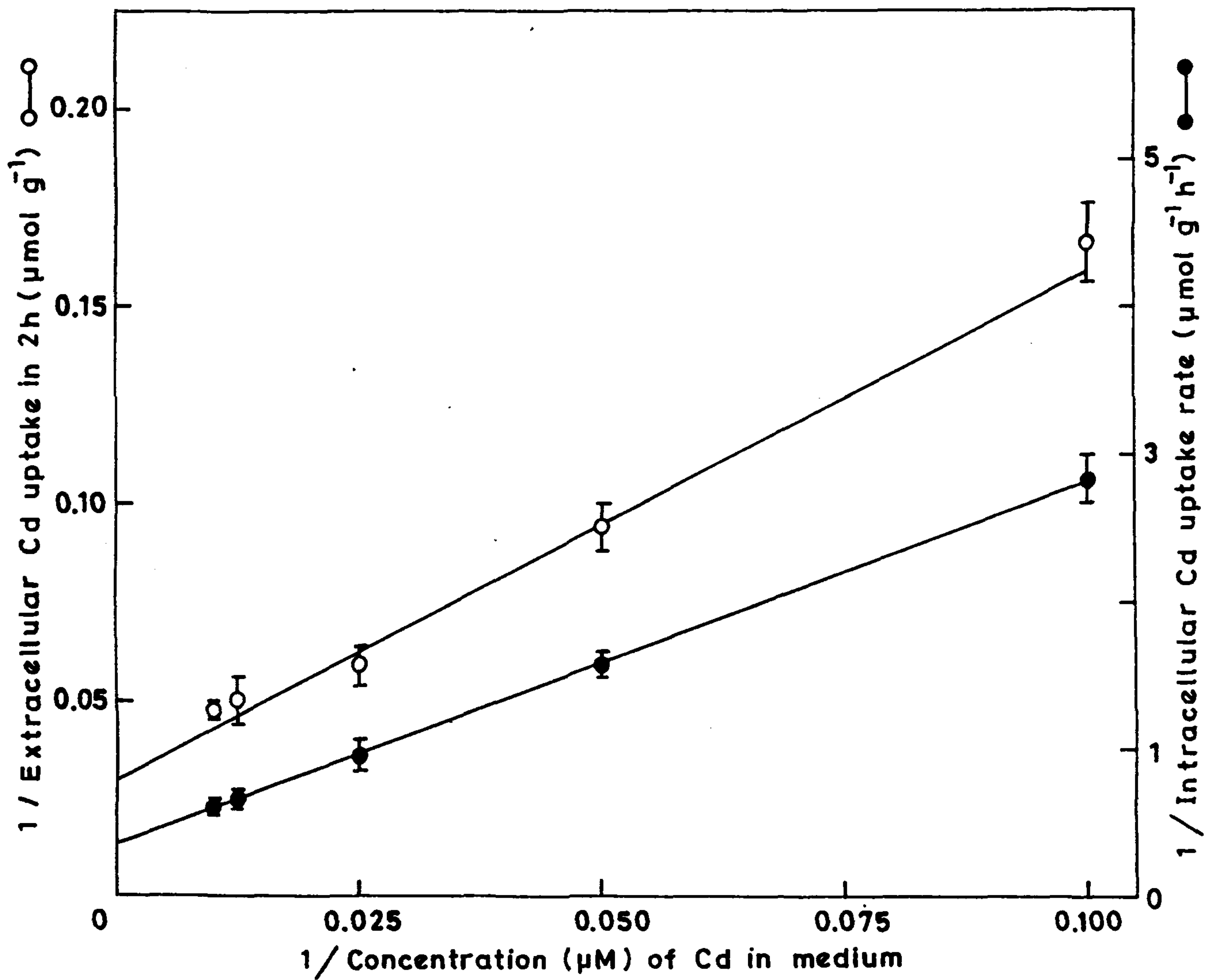


Fig. 3.8: Cadmium uptake by A. pinnata as a function of Cd concentration in the medium after 2 incubation. Double reciprocal plot of extracellular uptake after 2h (µmol g^{-1}) and intracellular uptake rate (µmol g^{-1} h^{-1}). Error bars represent SD, n = 5.

Table 3.1: Kinetic constants for Cd uptake in *L. polyrrhiza* and *A. pinnata*.

	A. Intracellular uptake	
	K_m (µM Cd)	V_{max} (µmol g^{-1} h^{-1})
L. polyrrhiza	55.0±6.2	2.93±0.2
A. pinnata	93.7±21.0	3.07±0.9
	B. Extracellular uptake	
	K_s (µM)	U_{max} (µmol g^{-1})
L. polyrrhiza	30.48±4.3	36.05±7.0
A. pinnata	45.45±8.0	33.48.11.0

All values are means ± SD.

K_m = Michaelis-Menten constant; µM Cd concentration required for half maximal uptake.

V_{max} = Maximal uptake rate.

K_s = Cd concentration (µM) required for half of maximum extracellular uptake possible after 2 h.

U_{max} = Maximum possible uptake (µmol g^{-1}) after 2 h.

concentrations required for half of the maximum possible extracellular uptake to occur after an exposure time of 2 h (K_s) were 29.5±4.5 and 46.5±7 µM for L. polyrrhiza and A. pinnata, respectively (Figs. 3.6 and 3.8). The maximum possible uptake recorded after 2 U_{max} in this experiment was 36.05±7 in L. polyrrhiza and 33.48±11 µmol g^{-1} in A. pinnata (Table 3.1). The Cd concentrations required for half maximum extracellular uptake (K_s) in both test plants were lower than the concentrations required for half maximum intracellular Cd uptake rates (K_m) (Table 3.1).

A time-course study of Cd uptake by test plants with and without roots indicated that both extracellular and intracellular Cd uptake were lower in plants without roots (Figs. 3.9 and 3.10). About 50% of the total Cd uptake could be accounted for by the roots in both test plants.

Data relating to light and dark effects on extracellular and intracellular Cd uptake by L. polyrrhiza and A. pinnata are given in Figs. 3.11 and 3.12, respectively. The intracellular Cd uptake by L. polyrrhiza and A. pinnata was enhanced by the presence of light, although extracellular Cd uptake was apparently not dependent on light. Thirty hours of incubation in light in 5 µM Cd solution resulted in intracellular Cd concentration of 2.4±0.14 and 1.94±0.2 µmol g^{-1} in L. polyrrhiza and A. pinnata, respectively; in the dark, Cd concentration in L. polyrrhiza and A. pinnata were respectively 1.72±0.14 and 1.3±0.2 µmol g^{-1}.

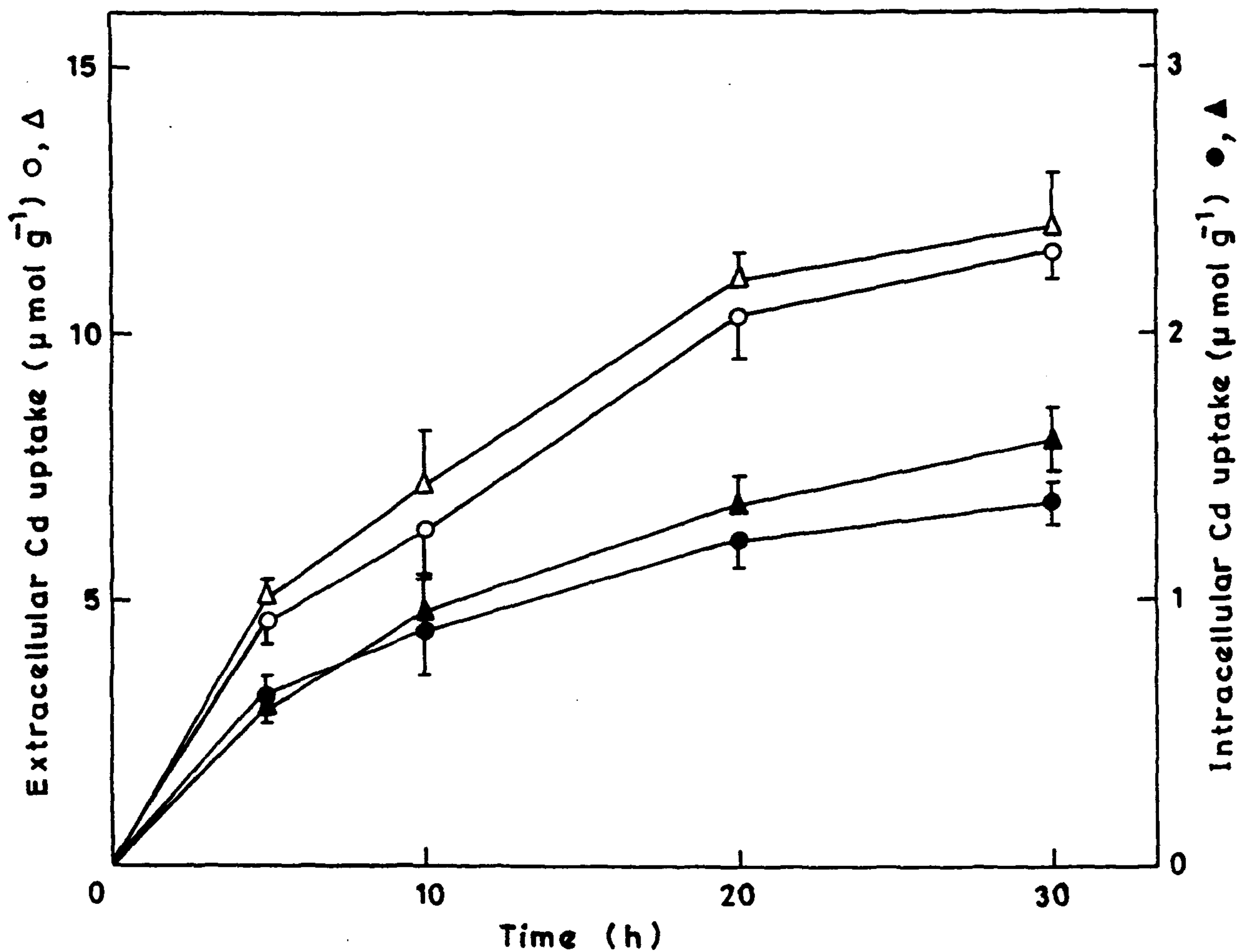


Fig. 3.9: Time-course study of extracellular and intracellular Cd uptake by L. polyrriza with root and without root. Symbols: ▲ Extracellular with root; ▲ Extra-cellular without root; o Intracellular with root; and ● Intracellular without root. Error bars represent SD, n = 5.

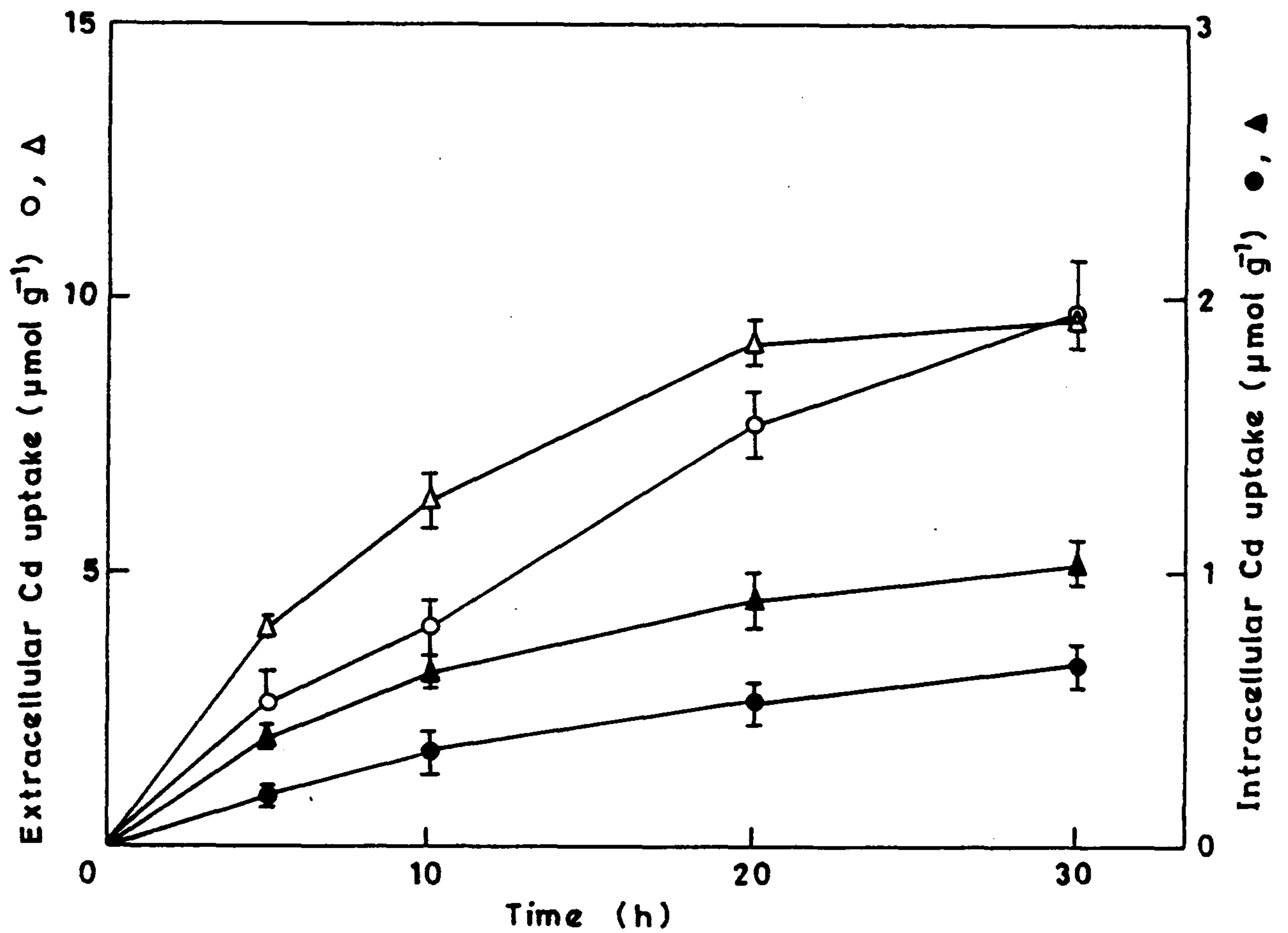


Fig. 3.10: Time-course study of extracellular and intracellular Cd uptake by A. pinnata with root and without root. Symbols: Δ Extracellular with root; ▲ Extracellular without root; o Intracellular with root; and ● Intracellular without root. Error bars represent SD, n = 5.

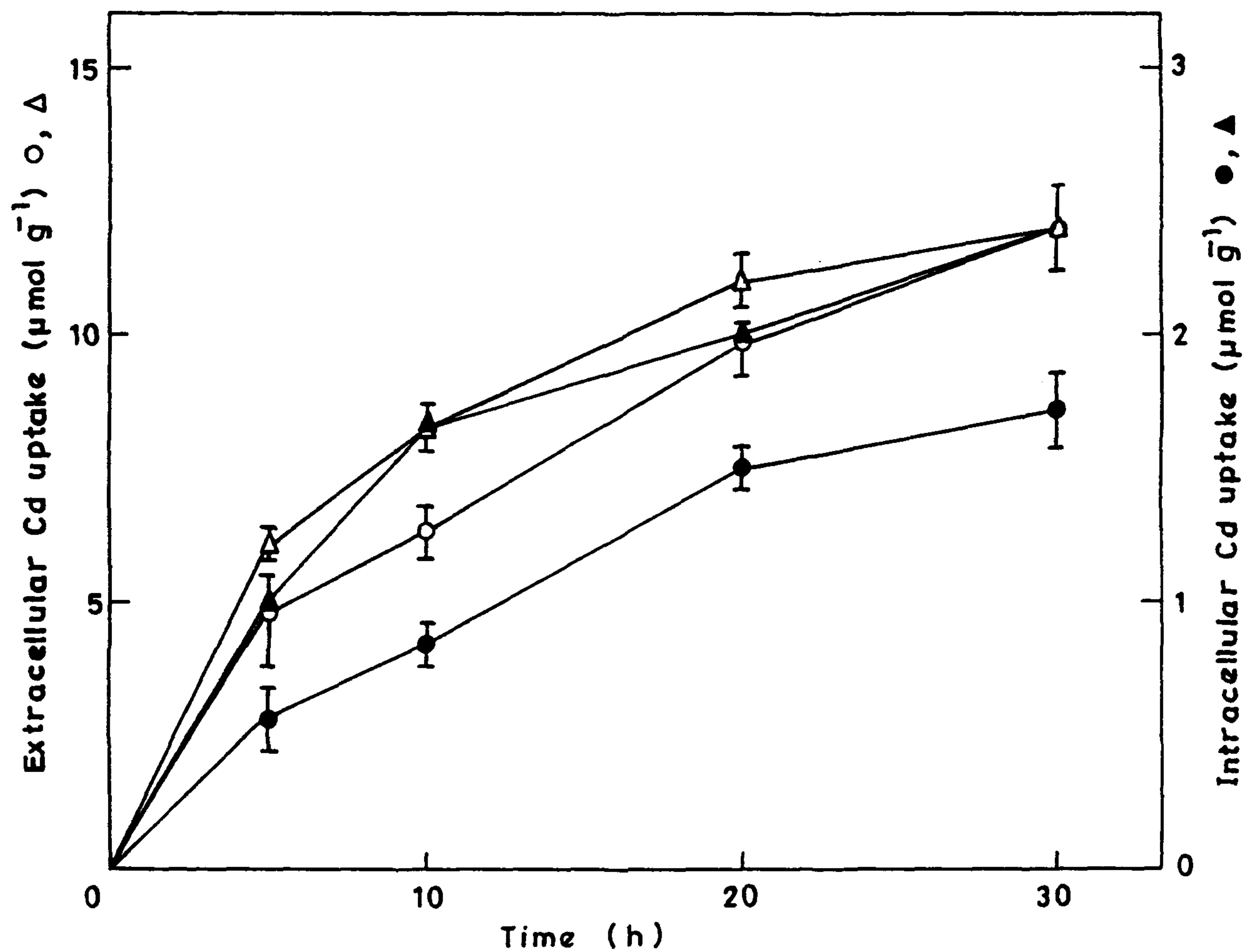


Fig. 3.11: Time-course study of extracellular and intracellular Cd uptake by L. polyrrhiza in light (PAR 45 µmol m^2 sec^{-1}) and in dark. Symbols: Δ Extracellular in light; ▲ Extracellular in dark; o Intracellular in light; and ● Intracellular in dark. Error bars represent SD, n = 5.

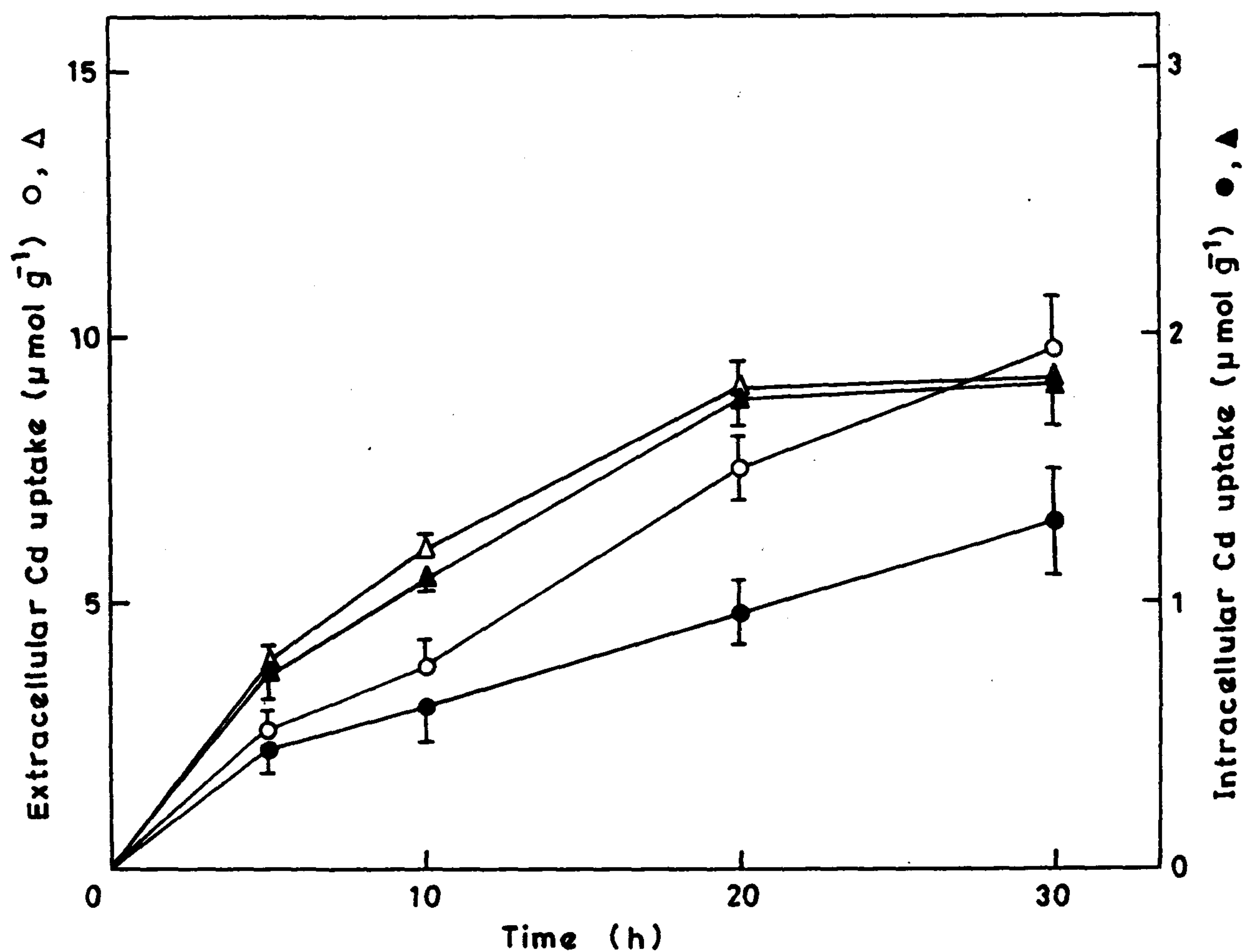


Fig. 3.12: Time-course study of extracellular and intracellular Cd uptake by A. pinnata in light (PAR 45 µmol m^2 sec^{-1}) and in dark. Symbols: Δ Extracellular in light; ▲ Extracellular in dark; o Intracellular in light; and ● Intracellular in dark. Error bars represent SD, n = 5.

The efficiency of dead biomass of test plants in adsorbing Cd at different time intervals could be found in Table 3.2. It is apparent that increased incubation period caused increased Cd level in the biomass. Raid sorption took place within 5 min of incubation. Adsorption within 10 min was more than 75% of the total Cd adsorbed in 90 min. Further increase in Cd content were observed with increase in incubation period, although values were much lower as compared to those obtained during the first 10 min.

Efficiency of Cd adsorption by dead biomass of test plants through successive cycles of adsorption and desorption was determined (Table 3.3). Dead biomass of both test plants retained their efficiencies of adsorption for several cycles. However, with repeated adsorption and desorption, the biomass of both test plants became so fragmented after the 10th cycle that its handling became virtually impossible.

Various chemical agents were also tested for their efficacies in desorbing Cd adsorbed by dead biomass of L. polyrrhiza and A. pinnata. Results of the experiment are given in Table 3.4. Among the chemical agents, 5 mM HCl was the least and 5 mM EDTA was most efficient in desorbing Cd from dead biomass. However, NaCl (5 mM), $CaCl_2$ (5 mM) and $CaCl_2$ (5 mM) + HCl (5 mM) could successfully desorb more than 75% of Cd from the Cd-laden dead biomass.

Table 3.5 shows Cd desorption by dead biomass of test

Table 3.2: Cadmium adsorption by dead biomass of test plants incubated in 100 µM Cd solution for different time intervals.

Test plants	Cd adsorption (µmol g^{-1}) at different time intervals				
	5 min	10 min	30 min	60 min	90 min
L. polyrrhiza	25±4*	34±4	35±3	38±2	40±4
A. pinnata	20±3	30±3	35±4	37±5	41±5

*Mean ± SD.

Table 3.3: Efficiency of Cd adsorption by dead biomass of test plants through successive cycles.

No. of cycle	Cd adsorbed (µmol g^{-1})	
	L. polyrrhiza	*A. pinnata*
1	20±3*	21±2*
2	19±2	19±2
3	21±3	20±3
4	17±1	19±3
5	22±2	19±3
6	24±2	15±2
7	26±3	19±1
8	22±2	10±1
9	23±1	21±3
0	18±3	20±2

*Mean + SD.

The dead biomass was kept in 100 µM Cd for 15 min, adsorbed-Cd was desorbed after 10 min treatment with 10 mM EDTA.

The biomass became so fragmented after the 10th cycle that it could not be used further.

Table 3.4: Relative efficiencies of various chemical agents in desorbing Cd from dead biomass of test plants.

Chemical agent	*L. polyrrhiza* Cd remaining in biomass ($\mu mol\ g^{-1}$)	*L. polyrrhiza* %desorption	*A. pinnata* Cd remaining in biomass ($\mu mol\ g^{-1}$)	*A. pinnata* %desorption
5 mM HCl	25±3*	37.5	30±2*	27.0
5 mM NaCl	8±1	80.0	2±2	70.7
5 mM $CaCl_2$	6±2	85.0	8±2	80.5
5 mM $CaCl_2$ + 5 mM HCl	6±2	85.0	6±1	85.4
5 mM EDTA	5±1	87.5	5±2	87.8

Desorption was carried out for 30 min. Before desorption, *L. polyrrhiza* and *A. pinnata* respectively contained 40±2 and 41±2 $\mu mol\ g^{-1}$ of Cd.

*Mean ± SD.

Table 3.5: Cd desorption from dead biomass of *L. polyrrhiza* and *A. pinnata* by 10 mM EDTA.

	Cd desorption ($\mu mol\ g^{-1}$) at different time intervals				
	5 min	10 min	30 min	60 min	90 min
L. polyrrhiza	28±4* (62.5%)	34±3 (75.5%)	39±5 (87.6%)	40±3 (88.4%)	42±2 (93.3%)
A. pinnata	22±3 (55%)	26±3 (65%)	33±3 (83%)	35±2 (87.5%)	37±4 (92.5%)

Dead biomass was exposed to 100 uM Cd for 48 h at room temperature. Before desorption Cd levels in *L. polyrrhiza* and *A. pinnata* were 45±3 and 40±2 $\mu mol\ g^{-1}$, respectively. Values for % desorption are given in the parentheses.

*Mean ± SD.

plants after treatment with 10 mM EDTA. Within 5 min of treatment more than 50% of Cd was desorbed from the dead biomass of L. polyrrhiza and A. pinnata, and less than 20% Cd remained in the biomass after 30 min of treatment.

Table 3.1 compares the relative efficiencies of Cd uptake in two test plants. Apparently, L. polyrrhiza could accumulate more of extracellular and intracellular Cd than A. pinnata. The rate of intracellular Cd uptake was slightly higher in L. polyrrhiza than in A. pinnata. However, the kinetic constants for intracellular uptake and the concentration required for half maximum uptake possible in extracellular Cd uptake in A. pinnata were higher than those of L. polyrrhiza.

Discussion

Both L. polyrrhiza and A. pinnata were found to accumulate high levels of Cd and it seemed worthwhile to find out the relative contributions of adsorbed and intracellular Cd in test plants for two main reasons: (i) Intracellular metal causes toxicity, and (ii) metal adsorbed by plants could be desorbed and plant biomass could be repeatedly utilized for gleaning heavy metals from effluents. This would nevertheless require understanding the mechanisms of extracellular uptake of metals in these plants. In the present study the extracellular Cd was efficiently extracted with 10 mM EDTA from the test plants. Davies (1970, 1973), Schulz-Baldes and Lewin (1976), Sakaguchi et al. (1979), Leita et al. (1991), respectively, used 3×10^{-3} M, 1×10^{-2} M, 1×10^{-2} M and 5×10^{-4} M EDTA to extract

surface-bound metals. It seems likely that such an extraction removes some of the intracellular metal (Leita et al. 1991). Hence, the amount of intracellular metal obtained in this way may be less than the actual intracellular metal content in the plant. It is assumed that the intracellular Cd values reported here are for Cd firmly bound either on the cell surface (i.e., wall and membrane) or in the interior of the cell (symplasm). The change in amount of extracellular Cd with incubation time in the presence of Cd for both test plants suggests a complex adsorption mechanism. Given a fixed number of adsorption sites, one would expect, if an adsorption equilibrium were attained rapidly, an initial rapid rise in extracellular Cd followed immediately by a plateau, corresponding to the attainment of the equilibrium. This idealized pattern was not present in test plants. The initial rapid adsorption of Cd could be seen, but a constant value was not immediately attained. Instead, there was a gradual increase of extracellular Cd before an apparent plateau was reached. These findings suggest that there are two types of sites on the surfaces of both plants: (i) sites which readily adsorb Cd, and (ii) sites which react more slowly with Cd. Bates et al. (1982) have also observed two kinds of sites for Zn adsorption in two green algae. Present work also shows that L. polyrrhiza has more of type (i) sites in comparison to A. pinnata. The variability in behaviour of two kinds of adsorption sites warrants further research efforts.

In some cases losses of adsorbed metal have been reported after prolonged incubation. This kind of reports have been

published on algae (see Bates et al. 1982) and some aquatic plants (Lee et al. 1991). No such loss occurred in the test plants. Of the various explanations for the loss of cellular metal, the most common involves the excretion of organic matter either already bound to the metal, or able to complex and remove the adsorbed metal metal once the organic matter is in solution (Butler et al. 1980). No such mechanism seems to be present in both the test plants.

L. polyrrhiza and A. pinnata differed greatly in their abilities to accumulate Cd in extracellular and intracellular compartments. L. polyrrhiza adsorbed more Cd than A. pinnata, hence the former should have a greater number of negatively charged surface sites per unit biomass for metal adsorption. Both plants differ morphologically and may be the surface area of L. polyrrhiza directly in contact with growth medium is greater than that of A. pinnata. It was, however, not possible to measure surface areas of test plants.

Root-containing plants of L. polyrrhiza and A. pinnata took up more Cd than rootless test plants. These observations are in congruity with the results obtained by Charpentier et al. (1987), Jana (1988), Sela et al. (1989), and Leita et al. (1991). Root-containing plants had more extracellular as well as intracellular metal levels than the rootless plants. It has been suggested that after uptake by roots Cd is easily transported within the plants (Peel et al. 1978, Adriano 1986). Leita et al. (1991) found progressive Cd accumulation in stem as

a function of Cd concentration in the roots. This trend of saturation was also revealed by Petit and Van de Geijn (1978). In the present case most parts of the fronds of L. polyrrhiza and A. pinnata were in direct contact with the medium, and it was expected that plant parts other than roots may also contribute to the total Cd uptake by test plants. However, from the results it is clear that the contribution of roots to Cd uptake by L. polyrrhiza and A. pinnata was extremely large.

Dead biomass accumulated more of Cd in both L. polyrrhiza and A. pinnata than live test plants. Enhanced Cd accumulation by dead biomass probably means that Cd was adsorbed not only on the surface of the biomass but also on the inner phase of the cell wall of of test plants. The absence of a permeability barrier and creation of more sites for metal accumulation could be the possible explanations for greater metal accumulation by dead biomass. Present observations agree with previous reports showing greater efficiency of metal removal by dead biomass in contrast to live biomass (Sakaguchi et al. 1979, Sela et al. 1989, Maeda et al. 1990a).

The relative contribution of intracellular Cd to the total Cd uptake was between 14 to 19% and 15 to 17% for L. polyrrhiza and A. pinnata, respectively. Similar results were obtained by Brown and Beckett (1985) in the moss Rhytidiadelphus squarrosus, but in the lichen Peltigera horizontalis intracellular Cd was merely 10% of the total Cd uptake (Beckett and Brown 1984).

Intracellular Cd uptake in relation to time occurred at almost a constant rate, whereas extracellular accumulation reached a plateau within 120 min in both test plants due obviously to the reason that all available negatively charged adsorption sites became occupied with Cd. The constant rate of intracellular uptake indicated slow transport or permeation across the membrane and a carrier-mediated mechanism. In their study Rebhum and Ben-Amotz (1984) observed that intracellular accumulation of Cd increased in proportion to the exposure time. The increased intracellular accumulation by test plants when incubation period is increased would also explain incorporation of Cd ions into cell proteins and possibly other cellular components.

Extracellular uptake was not affected by light in both _L. polyrrhiza_ and _A. pinnata_. Hence it can be reasoned that this process is not under metabolic control, and is a purely physico-chemical mechanism. On the other hand, intracellular accumulation was stimulated by light thereby revealing its dependence on cell energetics and metabolism. These results are in consonance with the observations of Cataldo et al. (1983), Findenegg et al. (1971), Beckett and Brown (1984) and Bariaud et al. (1985).

The pattern of intracellular heavy metal localization has been studied by various workers (Sela et al. 1988, Taylor 1987, Leita et al. 1991). High quantities of Cd were localized within the cell wall in the root and shoot of _Azolla filiculoides_ (Sela

et al. 1988). Leita et al. (1991) found that 27.2% of total Cd in the roots of Phaseolus vulgaris was associated with proteins and the presence of Cd in the growing medium induced the synthesis of water-soluble proteins in plant tissues. Similar observations have been made by Grill et al. (1985). However, in the present study no attempt was made to determine the cellular location of Cd in the test plants.

The ability of L. polyrrhiza and A. pinnata to accumulate large concentrations of extracellular Cd may be exploited for the treatment of Cd-rich effluents. Prospects seem promising as these plants occur widely and abundantly in water bodies, and also because the conventional methods for Cd stripping from effluents (such as precipitation, ion exchange, electrochemical treatment and evaporative recovery) are generally very expensive.

Chapter-IV

INFLUENCE OF CATIONS AND OTHER HEAVY METALS ON CADMIUM UPTAKE BY TEST PLANTS

Introduction

While a great deal of information has accumulated on the toxicity and uptake of single species of heavy metals in aquatic plants and algae, relatively little is known about the combined effects of two or more metals (Braek et al. 1980, Wong et al. 1983). This ignorance is concerning as natural waters and effluents always contain a mixture of metals. There is a pressing need to understand how uptake and toxicity of a metal is influenced by the presence of other metals and cations. This exigency gets support from Wong et al. (1978) who reported the results of a series of tests on a mixture of metals whose individual concentrations each satisfied the recommended discharge levels. However, when present in combination, the metals proved to be extremely toxic to algae.

In general, a mixture of heavy metals can produce three possible types of interactions: synergistic, antagonistic and non-interaction (Ting et al. 1991). Most studies on combined effects reveal simple cases of either synergism or antagonism. Several explanations have been proposed for these synergistic and antagonistic actions. For instance, when the combined effect of copper and nickel on the cell population of *Chlorella vulgaris* was found to be synergistic, it was postulated that

This response resulted from an increase in membrane permeability (Hutchinson and Stokes 1975). Passow et al. (1961) have shown that the cell membrane is the target site of activity for cupric ions and mercuric ions. In contrast, antagonistic interaction was reported in the case of cadmium and selenium for the same alga and it was suggested that screening or competition for the binding sites on the cellular surfaces had resulted in the metal ions mutually ameliorating their individual toxic effects.

An extensive literature survey by Ting et al. (1991) reveals that few researchers have employed metal uptake as the test criterion in algae-metal interaction. Stokes (1975) found that Cu and Ni enhanced each others uptake in *Scenedesmus acutiformis*, and thus acted synergistically. Mierle (1982) observed inhibition of Cu uptake by a number of metals. However, Braek et al. (1980) reported both antagonistic and synergistic cations for Zn and Cd uptake by different algae. Similar investigations are also fewer in case of lichens (Beckett and Brown 1984), bryopyte (Brown and Beckett 1985, Wells and Brown 1990) and higher plants including aquatic macrophytes (Huebert and Shay 1991, Turnquist et al. 1990).

This chapter deals with the influence of certain metals (Cu, Ni, Fe and Zn) and cations (Ca, Mg, K, Na) on the extra-cellular and intracellular uptake of Cd in *L. polyrrhiza* and *A. pinnata*. Attempts were also made to see if these metals and cations could change the kinetic parameters for Cd uptake in test plants.

Materials and Methods

Healthy growing plants of L. polyrrhiza and A. pinnata were selected for setting up of experiments. Prior to incubation in test solutions, selected test plants were gently washed in double distilled water for 10 min. For each experiment freshly prepared metal solutions were used, and the following salts of analytical grade were used for interaction experiments: Cd as cadmium acetate, Ca as calcium chloride, Mg as magnesium chloride, K as potassium carbonate, Na as sodium chloride, Cu as cupric chloride, Fe as ferric chloride, Ni as nickel chloride and Zn as zinc sulphate.

The purpose of washing test plants before setting up the experiment was to remove nutrient solution generally present on the surface of the roots and to avoid interference of nutrient cations to test cations in the kinetic studies. However, washing test plants with double distilled water will not completely remove cations adhering to the walls of root cells and other parts of test plant, but this provided uniformity in the experiments. The importance of naturally-occurring cell wall ions has been pointed out by Wells and Brown (1987), who showed that the cell wall ions particularly Ca, could significantly alter the kinetics of intracellular Cd uptake in two populations of Rhytidiadelphus squarossus.

Unless stated otherwise, the experiments were performed at 20°C and the pH was adjusted to 7. All the test solutions were

unbuffered. Experiments were carried out in double distilled water by adding the desired doses of Cd or the cation. For control experiment, five concentrations of Cd (i.e., 10, 20, 40, 80 and 100 µM) were taken. In each of these solutions about 5 fronds of test plants were floated and incubated for 2 h. Each treatment had a minimum of 5 replicates. In order to determine the kinetics of inhibition of Cd uptake in test plants by different competing cations and metals (Ca, Mg, K, Na, Cu, Fe, Ni and Zn), L. polyrrhiza and A. pinnata were incubated in test solutions containing 100 µM of interacting cation and 10, 20, 40, 80 and 100 µM Cd, in a manner similar to that described by Brown and Beckett (1985). Test plants were then harvested 2h after incubation. Similar to the methods described in Chapter III, extracellular and intracellular Cd contents were determined.

Kinetic parameters (K_m= Michaelis-Menten constant; the ion concentration required for half maximal uptake, and V_{max} = maximal uptake rate) for intracellular uptake, and (K_s = dissociation constant, the ion concentration required for half maximal uptake and U_{max} = maximal uptake capacity) for extracellular Cd uptake were determined using procedures outlined by Wells and Brown (1987, 1990). The inhibition kinetics were calculated from the slopes and intercepts of double reciprocal plots of Cd uptake against supplied concentration in the presence and absence of an interacting ion as described by Royer (1982).

Results and Discussion

When *L. polyrrhiza* and *A. pinnata* were incubated at different concentrations of Cd (10, 20, 40, 80 and 100 µM) for 2h at 20°C and pH adjusted to 7, both extracellular and intracellular uptake showed saturation kinetics. The kinetic parameters for *L. polyrrhiza* for intracellular uptake, K_m and V_{max} were respectively 55.6±6.2 µM Cd and 2.93±0.2 µmol g^{-1} h^{-1} (Table 4.1). For extracellular uptake the K_s was 30.48±4.3 µM Cd, and U_{max} 36.05±7 µmol g^{-1}. In *A. pinnata* the K_m and V_{max} for intracellular Cd uptake were 93.7±17 µM and 3.07±0.9 µmol g^{-1} h^{-1}, respectively (Table 4.2). The K_s was 45.5±6 µM Cd, whereas U_{max} was 33.48±11 µmol g^{-1}.

Figures 4.1 to 4.4 exhibit changes in extracellular uptake of Cd as elicited by Ca, Mg, K, Na, Cu, Fe, Ni and Zn. All these cations and metals inhibited extracellular uptake of Cd. Changes in kinetic parameters for extracellular Cd uptake, i.e., K_s and U_{max} in presence of inhibiting cations could be found in Tables 4.1 and 4.2 for *L. polyrrhiza* and *A. pinnata*, respectively. Figs. 4.1 and 4.2 and Tables 4.1 and 4.2 make it amply clear that Ca, Mg and K did not change K_s whereas U_{max} for extracellular Cd uptake was significantly decreased in both test plants. Hence, these cations are causing non-competitive inhibition of extracellular uptake. The present findings are contrary to the observations of Wells and Brown (1990) who found Ca and Mg to be competitive inhibitors for Cd adsorption. According to Brown and Beckett (1985) the inhibition of

Table 4.1: Kinetic constants for the inibition of intracellular and extracellular Cd uptake by Ca, Mg, K, Na, Cu, Fe, Ni and Zn in *L. polyrrhiza*.

A. Intracellular uptake

Inhibiting cation	K_m (µM)	V_{max} (µmol g^{-1} h^{-1})
None (control)	56.6±6.2	2.93±0.20
Ca	99.0±11.0****	2.80±0.05
Mg	90.0±14.0****	2.80±0.60
K	56.0±6.6	1.80±0.30**
Na	58.0±8.0	2.00±0.50*
Cu	62.0±7.0***	1.10±0.50***
Fe	77.0±13.0****	2.80±0.30
Ni	78.0±4.0****	2.70±0.20
Zn	111.0±18.0****	3.10±0.30

B. Extracellular uptake

Inhibiting cation	K_s (µM)	U_{max} (µmol g^{-1})
None (control)	30.48±4.3	36.05±7
Ca	30.40±0.9	24.50±6****
Mg	32.20±6	28.01±3****
K	33.40±8	29.70±5**
Na	35.51±4**	24.01±6****
Cu	40.16±6****	30.70±4
Fe	37.70±6***	27.50±3****
Ni	51.00±11****	34.30±5
Zn	45.40±8****	32.70±5

All data denote mean ± SD.

Data marked with asterisks are significantly different from control according to Student's 't' test. ****$p<0.001$, ***$p<0.005$, **$p<0.01$, *$p<0.05$.

Table 4.2: Kinetic constants for the inhibition of intracellular and extracellular Cd uptake by Ca, Mg, K, Na, Cu, Fe, Ni and Zn in *A. pinnata*.

A. Intracellular uptake

Inhibiting cation	K_m (µM)	V_{max} (µmol g^{-1} h^{-1})
None (control)	93.7±17	3.07±0.8
Ca	126.0±7****	2.70±0.3
Mg	125.0±17****	3.00±0.4
K	100.0±10	2.50±0.2*
Na	100.0±5	2.50±0.4*
Cu	111.0±14***	2.50±0.5*
Fe	142.0±25****	3.00±0.4
Ni	97.0±4	2.10±0.3****
Zn	95.0±10	2.10±0.2****

B. Extracellular uptake

Inhibiting cation	K_s (µM)	U_{max} (µmol g^{-1})
None (control)	45.5±6	33.5±4
Ca	47.6±5	22.9±9****
Mg	46.2±7	23.9±7****
K	47.6±10	26.1±7***
Na	47.8±7	23.9±5****
Cu	57.3±6***	28.9±7
Fe	56.5±4****	32.7±6
Ni	46.7±6	25.8±6****
Zn	59.9±5	30.0±2**

All data denote mean ± SD.

Data marked with asterisks are significantly different from control according to Student's 't' test (****$p<0.001$, ***$p<0.005$, **$p<0.01$, *$p<0.05$).

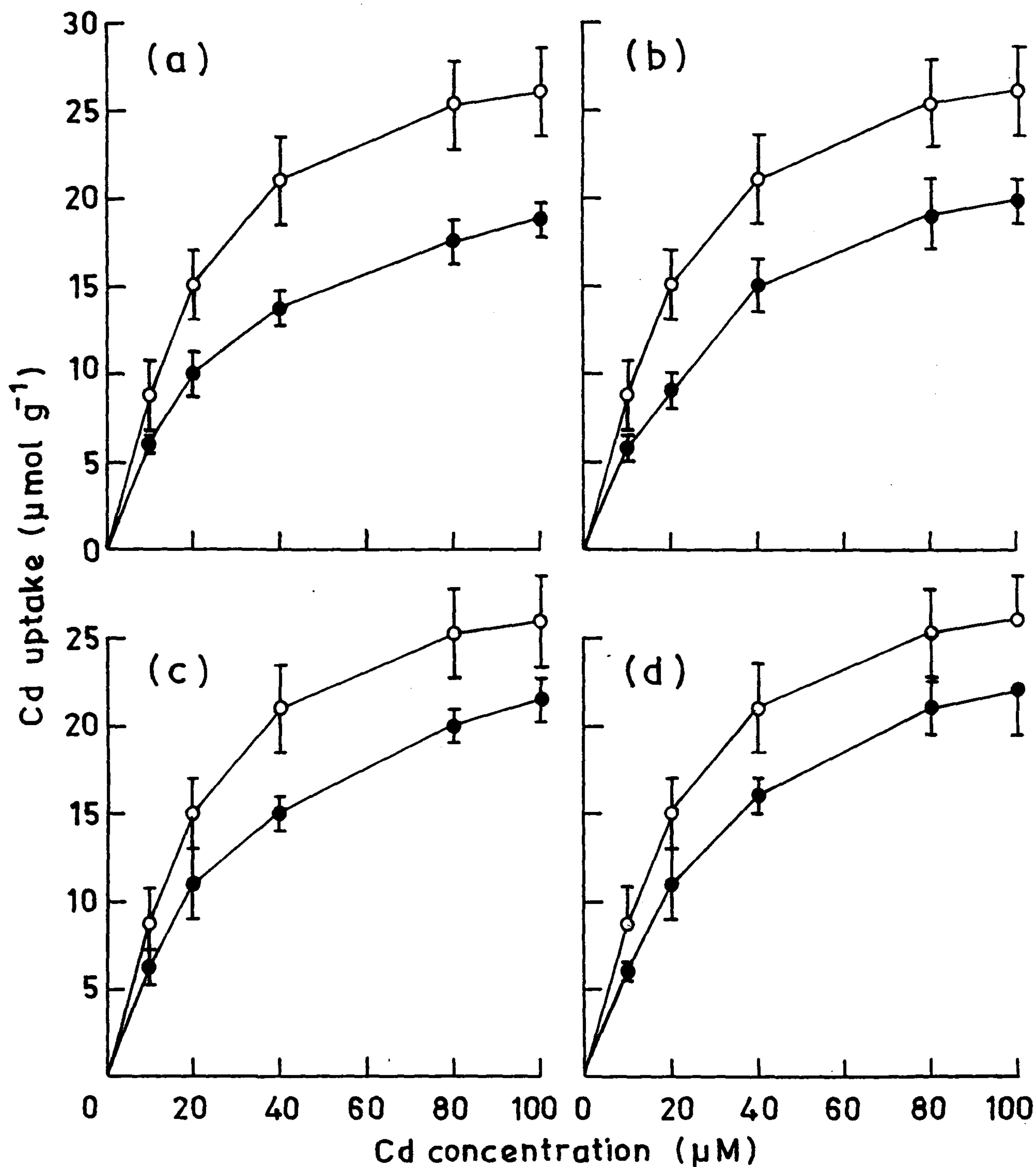


Fig. 4.1: Extracellular Cd uptake by *L. polyrrhiza* after an incubation period of 2h in the presence (●) or absence (o) of 100 µM Ca, Mg, K or Na from a range of solutions containing 10 to 100 µM Cd. (a) Ca, (b) Mg, (c) K, (d) Na. Error bars represent SD, n=5.

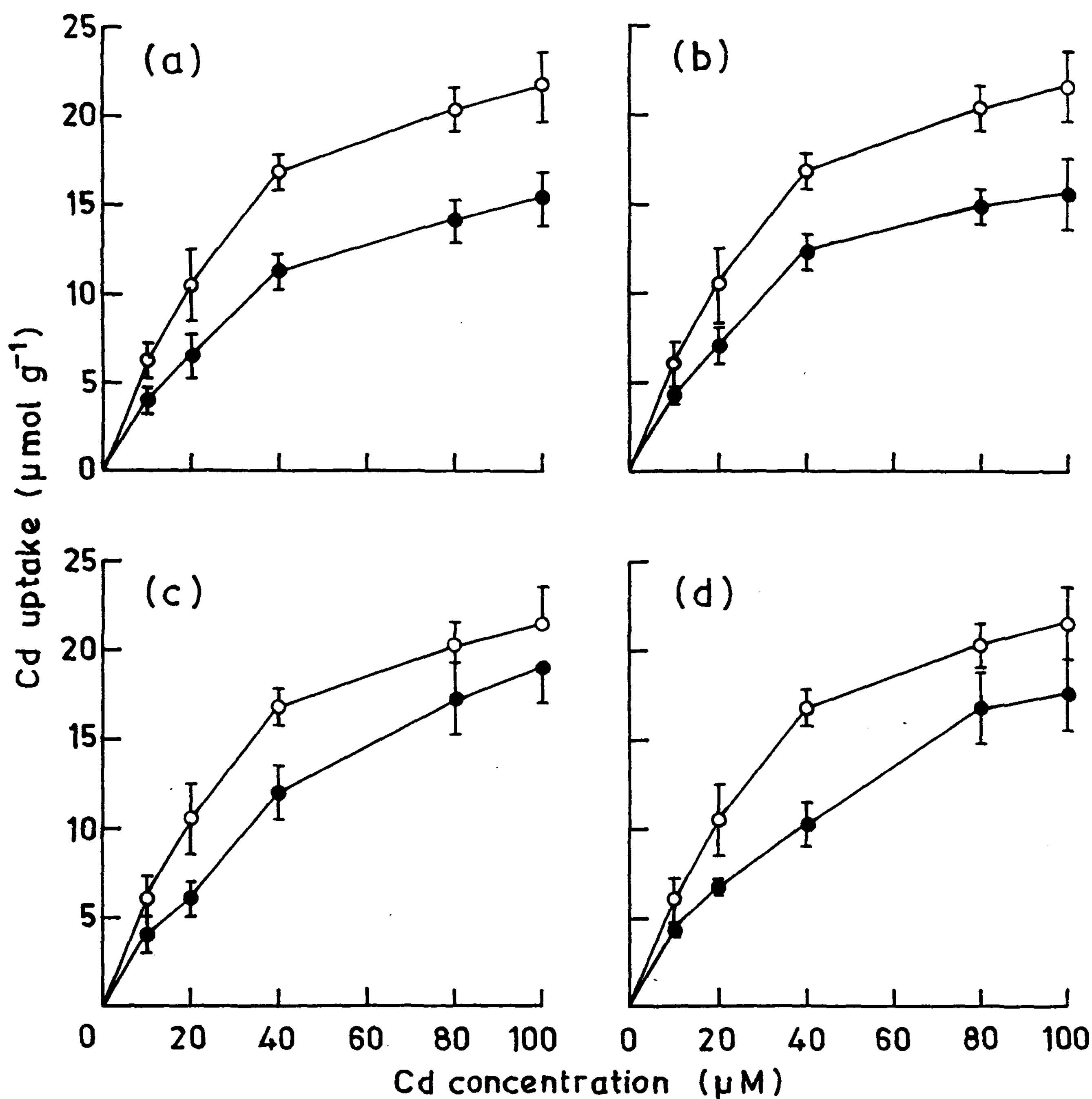


Fig. 4.2: Extracellular Cd uptake by A. pinnata after an incubation period of 2h in the presence (●) or absence (o) of 100 μM Ca, Mg, K or Na from a range of solutions containing 10 to 100 μM Cd. (a) Ca, (b) Mg, (c) K, (d) Na. Error bars represent SD, n=5.

extracellular Cd uptake by Ca and Mg was not wholly competitive in a moss studied by them. The non-competitive inhibition of extracellular uptake of Cd by K has been observed by Wells and Brown (1990) as well. A suggestion has been made that the surface binding capacity for divalent cations could be increased by increasing the concentration of a monovalent cation (De-marty et al. 1978, Wolterbeek 1987). It is believed that the stimulation of divalent cation binding is due to increase of electrostatic attraction (rather than chemical bindings) by anionic cell wall groups depleted of protons by the monovalent cation. This was definitely not the case in the present study, as K and Na inhibited Cd adsorption.

Copper caused competitive inhibition of extracellular uptake of Cd as it increased K_s in both test plants. Nickel and Zn also inhibited Cd adsorption, which was competitive in L. polyrrhiza and non-competitive in A. pinnata (Figs. 4.3 and 4.4). The nature of inhibition of extracellular Cd uptake by Fe was mixed in L. polyrrhiza but competitive in A. pinnata (Figs. 4.3 and 4.4). Sodium caused mixed inhibition of extracellular Cd uptake in L. polyrrhiza, and non-competitive in A. pinnata (Figs. 4.1 and 4.2).

The data relating to inhibitory effects of various cations and metals on extracellular Cd uptake by test plants reveal that the mode of inhibition of Cd uptake by a particular cation differ greatly in two test plants. This disparity was particularly evident in case of inhibiting cation viz. Na, Fe,

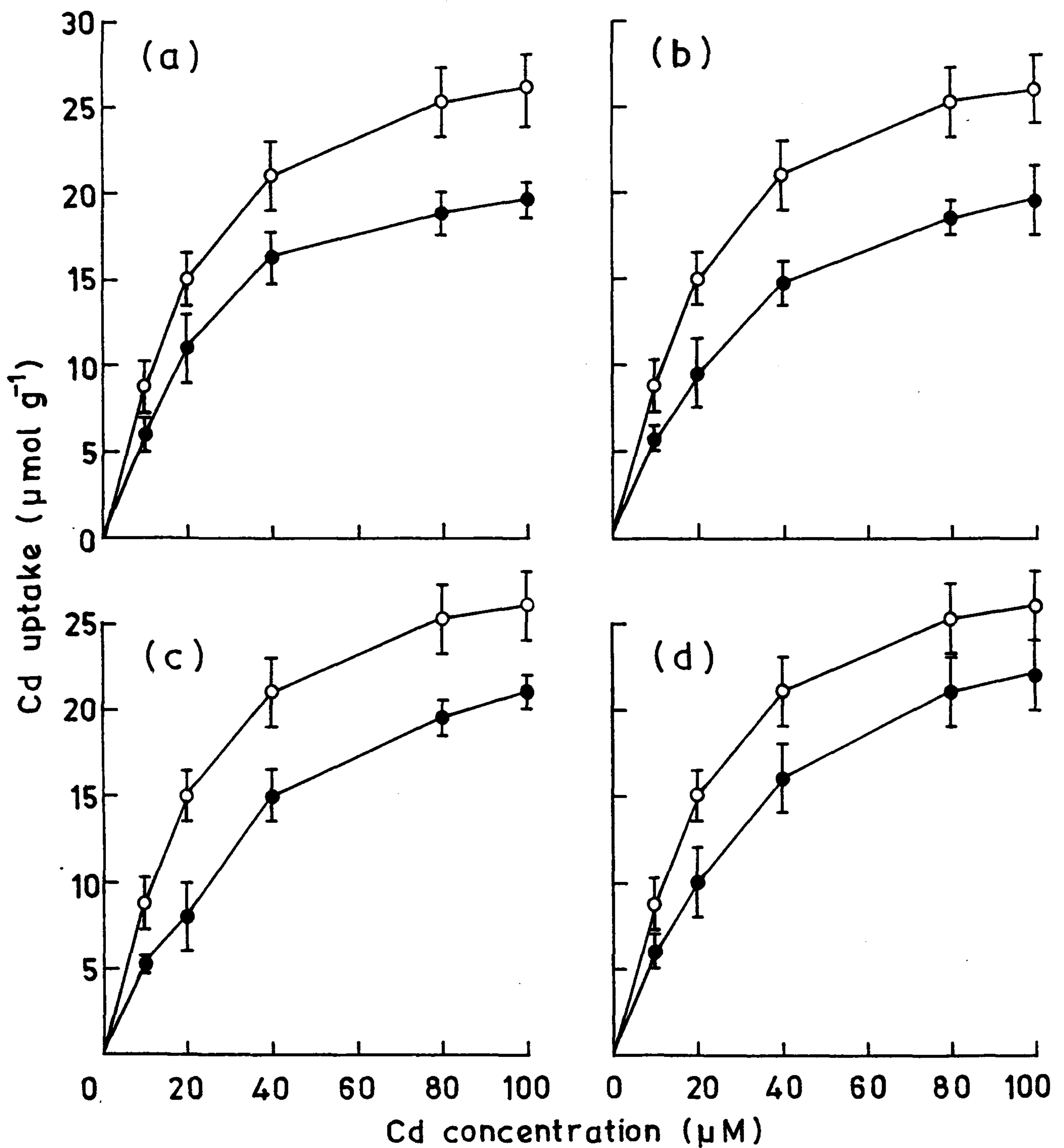


Fig. 4.3: Extracellular Cd uptake by L. polyrrhiza after an incubation period of 2h in the presence (•) or absence (o) of 100 μM Cu, Fe, Ni, or Zn from a range of solutions containing 10 to 100 μM Cd. (a) Cu, (b) Fe, (c) Ni, (d) Zn. Error bars represent SD, n=5.

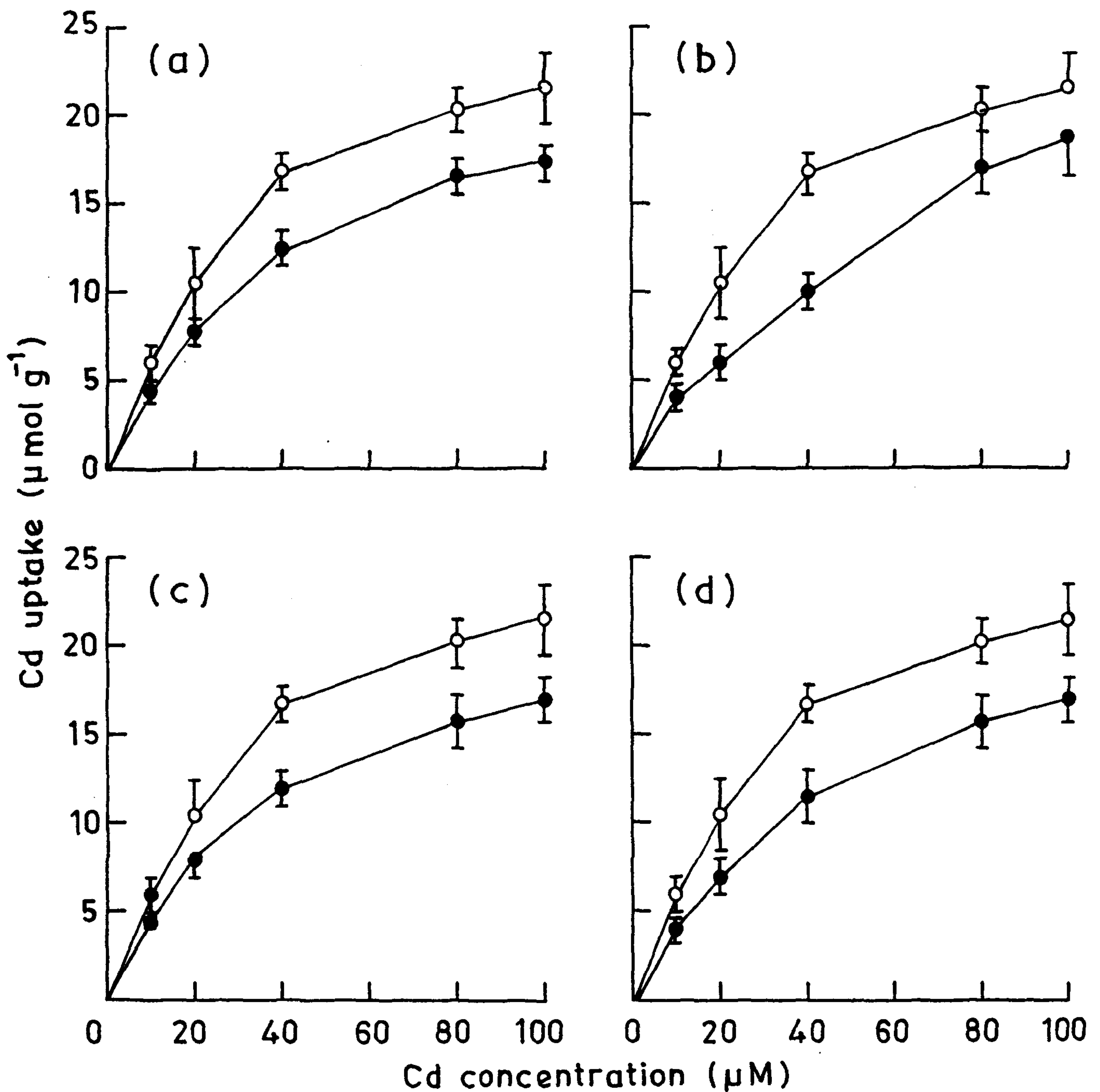


Fig. 4.4: Extracellular Cd uptake by A. pinnata after an incubation period of 2h in the presence (●) or absence (o) of 100 μM Cu, Fe, Ni or Zn from a range of solutions containing 10 to 100 μM Cd. (a) Cu, (b) Fe, (c) Ni, (d) Zn. Error bars represent SD, n=5.

Ni and Zn. It may be related to differences in characteristics of anionic sites on the surfaces of test plants. The cations causing competitive inhibition of Cd uptake are obviously competing for the same binding sites. The ability of certain cations (e.g., Ca, Mg, K) to noncompetitively inhibit extracellular Cd binding implies that binding of cations affected the surface charged density of anionic sites available for Cd binding (see Wells and Brown 1990). It has been suggested that Cd^{2+} and K^{+} bind to different sites and K^{+} binding reduces the number of Cd^{2+} binding sites (Wells and Brown 1990). The ionic radius of K^{+} is 40% greater than that of Cd^{2+} (Weast and Astle 1983). It seems probable that K^{+} binding to one site reduces the space available in the extracellular matrix, excluding Cd^{2+} binding.

Similar to the extracellular Cd uptake, Ca, Mg, K, Na, Cu, Fe, Ni and Zn caused reduction in intracellular Cd uptake in both _L. polyrrhiza_ (Figs. 4.5 and 4.7; Table 4.1) and _A. pinnata_ (Figs. 4.6 and 4.8; Table 4.2). Calcium and Mg significantly increased the apparent K_m for Cd uptake whereas V_{max} remained unchanged in both test plants (Figs. 4.5 and 4.6; Tables 4.1 and 4.2). Thus Ca and Mg competitively inhibited intracellular Cd uptake in test plants. John (1976) and Wells and Brown (1990) also found competitive inhibition of Cd uptake by Ca. Magnesium has, however, been found to be a non-competitive inhibitor of Cd uptake in a moss (Wells and Brown 1990). Beckett and Brown (1984) also found competitive inhibition of Cd uptake by Mg and postulated that intracellular

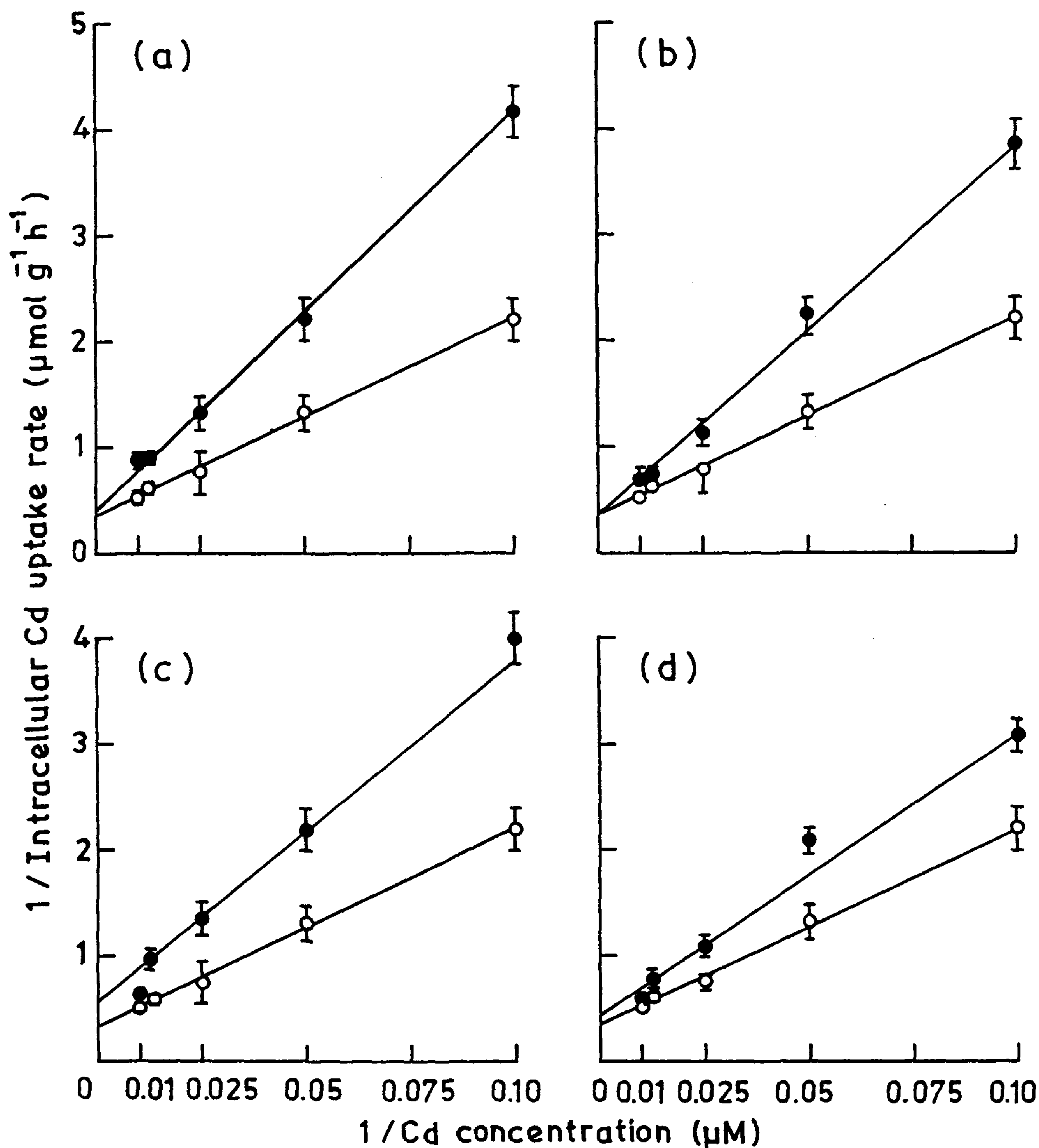


Fig. 4.5: Intracellular Cd uptake by *L. polyrrhiza* after an incubation period of 2 h in the presence (●) or absence (o) of 100 μM Ca, Mg, K or Na from a range of solutions containing 10 to 100 μM Cd. Double reciprocal plots (a) Ca, (b) Mg, (c) K, (d) Na. Error bars represent SD, n = 5.

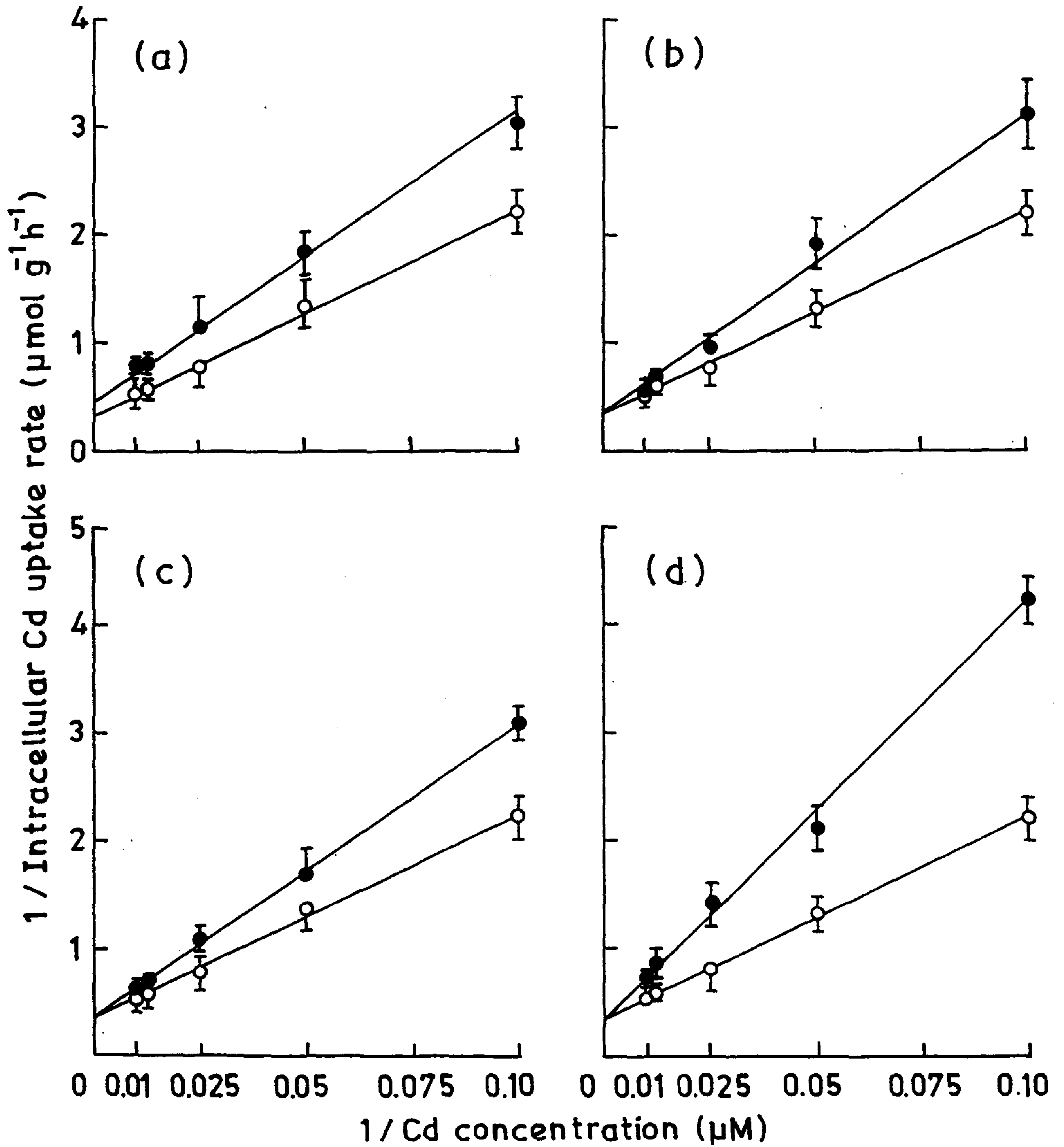


Fig. 4.6: Intracellular Cd uptake by L. polyrrhiza after an incubation period of 2h in the presence (●) or absence (o) of 100 μM Cu, Fe, Ni or Zn from a range of solutions containing 10 to 100 μM Cd. Double reciprocal plots (a) Cu, (b) Fe, (c) Ni, (d) Zn. Error bars represent SD, n = 5.

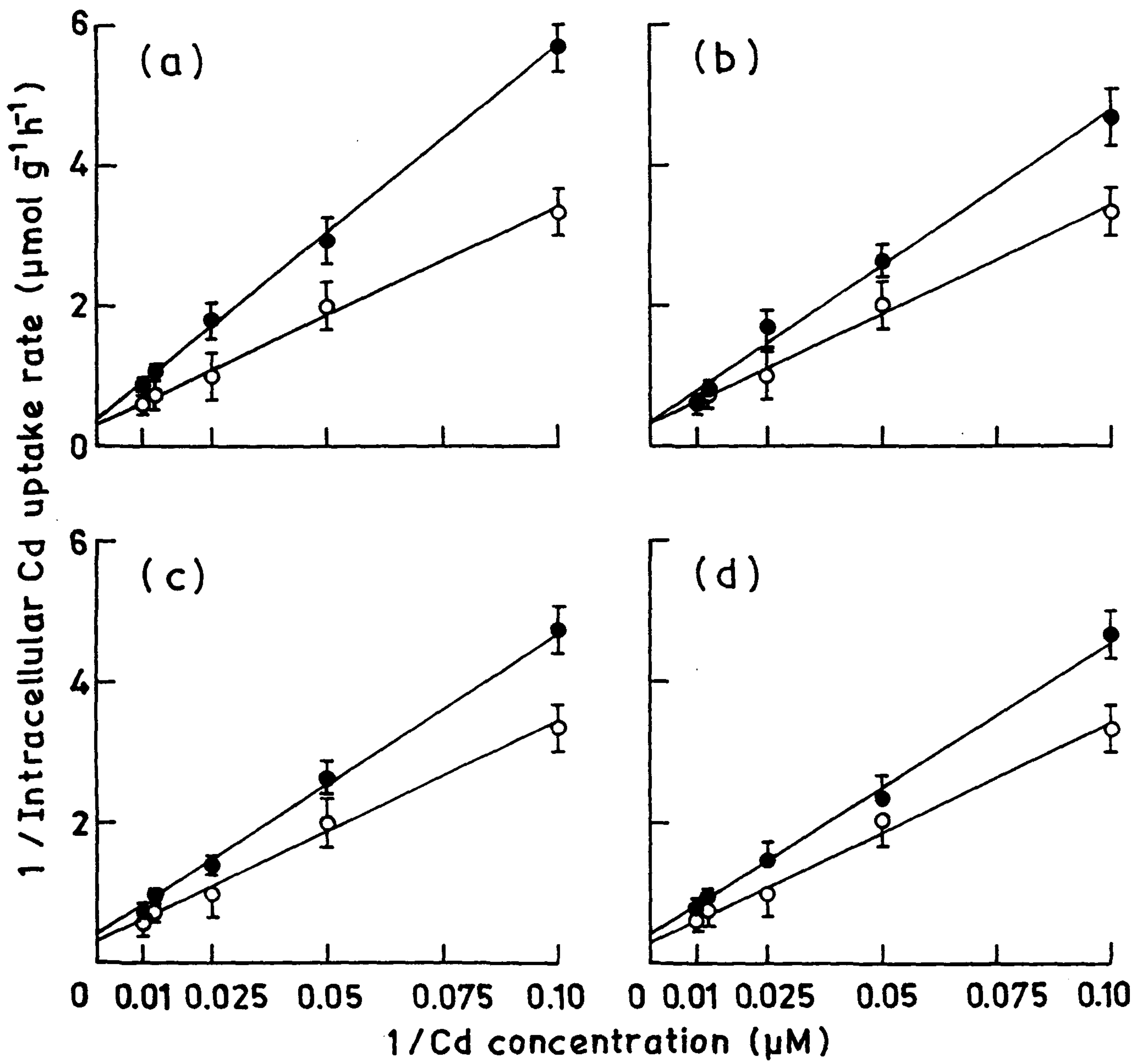


Fig. 4.7: Intracellular Cd uptake by A. pinnata after an incubation period of 2h in the presence (●) or absence (o) of 100 μM, Ca, Mg, K or Na from a range of solutions containing 10 to 100 μM Cd. Double reciprocal plots (a) Ca, (b) Mg, (c) K, (d) Na. Error bars represent SD, n = 5.

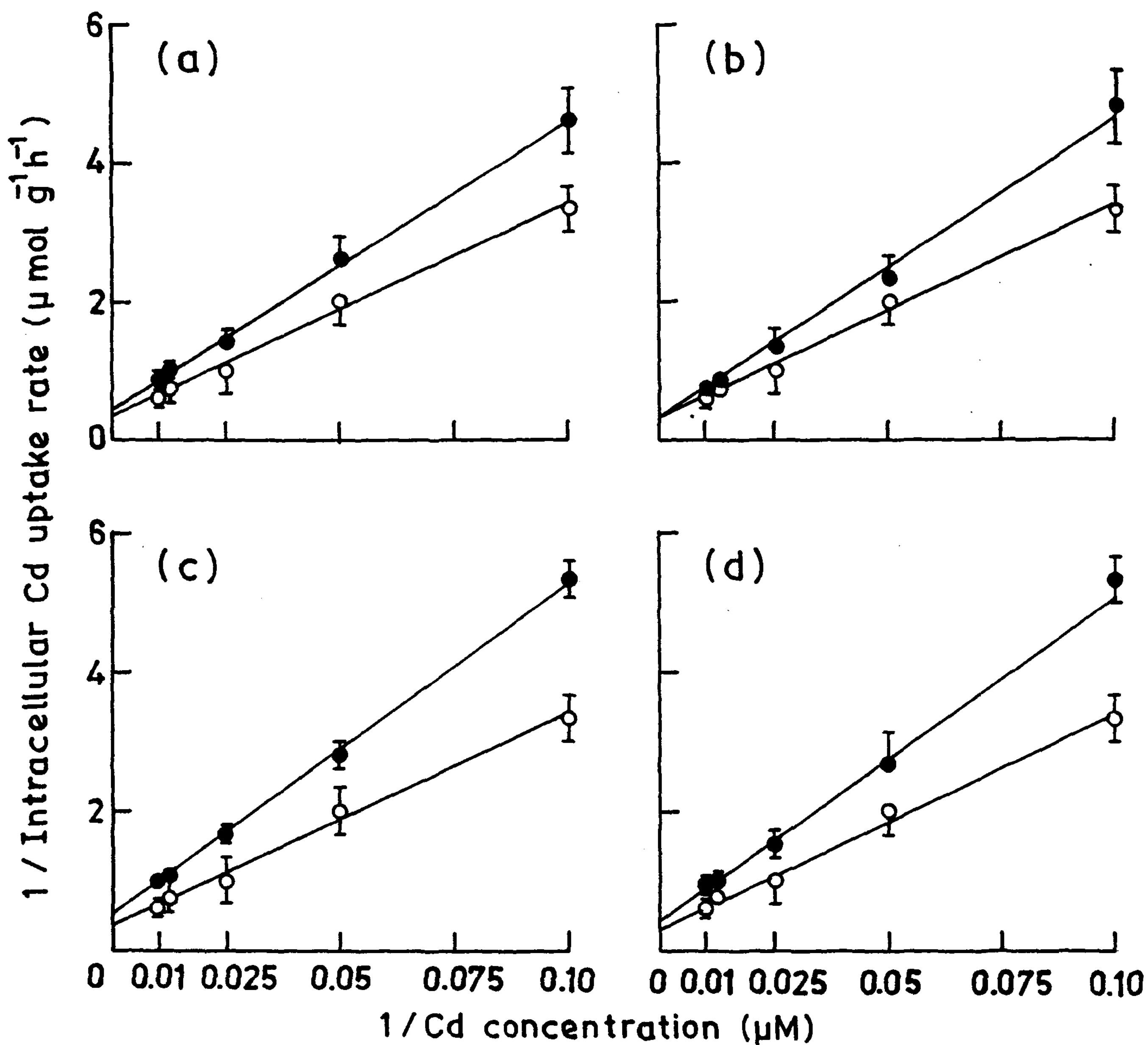


Fig. 4.8: Intracellular Cd uptake by A. pinnata after an incubation period of 2 h in the presence (●) or absence (o) of 100 µM Cu, Fe, Ni or Zn from a range of solutions containing 10 to 100 µM Cd. Double reciprocal plots (a) Cu, (b) Fe, (c) Ni, (d) Zn. Error bars represent SD, n = 5.

Cd uptake occur by the system normally transporting Mg. A possible explanation for competitive inhibition of Cd uptake by Ca could be their ionic similarities, the ionic radius is 99 pm for Ca^{2+} and 97 pm for Cd^{2+}. Iron also caused competitive inhibition of Cd uptake (Figs. 4.7 and 4.8; Tables 4.1 and 4.2). Gipps and Coller (1982) also reported antagonistic action of Fe on Cd toxicity which obviously resulted through decreased intracellular transport of Cd. These authors suggested that at higher pH and Fe level co-precipitation of Cd with Fe may reduce the concentration of Cd available to organisms.

Potassium and Na did not bring about any change in apparent K_m for intracellular Cd uptake, however, V_{max} was significantly decreased. This suggests non-competitive inhibition of Cd uptake. Both of these inhibiting cations are monovalent, that is why they showed similar effect. Potassium has been found to be competitive inhibitor of Cd uptake in oats and lettuce seedlings (John 1976). Wells and Brown (1990) found that concentration of KNO_3 greater than 1 mM caused both stimulation in transport site activity (V_{max}) and reduced affinity for Cd (increased K_m). The mode of inhibition of intracellular uptake by Ni and Zn differed in test organisms (Figs. 4.6 and 4.8, Tables 4.1 and 4.2). These heavy metals inhibited Cd uptake competitively in *L. polyrrhiza*, and non-competitively in *A. pinnata*. It seems that test plants differ greatly with regard to inhibitiory action of Ni and Zn. This aspect warrants detailed researches, however, it should become clear that broad generalizations should not be made

regarding the inhibitory effects of metals and cations on Cd uptake. Ting et al. (1991) could not find any effect of Zn on intracellular transport of Cd in a green alga. Others however report competitive interaction with Zn and Cd (Gipps and Coller 1982, Huebert and Shay 1992).

In presence of Cu the apparent K_m for intracellular uptake of Cd was increased, V_{max} was decreased concomitantly (Figs. 4.6 and 4.8; Tables 4.1 and 4.2). Therefore Cu caused 'mixed inhibition' of Cd uptake in both test plants. Others have observed competitive action of Cu on Cd uptake (Bowen 1969, Schmid et al. 1965, Brown and Beckett 1985). Beckett and Brown (1984) interestingly found stimulation of Cd uptake by copper. They suggested that Cu perhaps temporarily damaged plasma-membrane making them more freely permeable to Cd.

Chapter-V

INFLUENCE OF CERTAIN PHYSICO-CHEMICAL FACTORS ON Cd TOXICITY AND UPTAKE IN TEST PLANTS

Introduction

Metal availability and toxicity to aquatic organisms are influenced by physico-chemical factors, such as, pH, temperature, light, levels of various ions, and organic and inorganic complexing agents (Wong et al. 1980, Gipps and Coller 1982, Martell et al. 1988). Excepting light, all other factors determine speciation of any metal in solution as a free ion, bound to ligand in a complex, adsorbed on a solid surface or as a distinct precipitate (Darimont and Frenay 1990). Of the various species of a metal occurring in water, free ions are the only forms which can easily enter into living organisms and cause toxicity at higher concentrations (Börgmann 1983). Interestingly, there are some reports showing toxicity of metal chelaters to various kinds of living organisms (Gadd and Griffiths 1978).

Among various physico-chemical factors, pH has been considered to be the most important determinant of metal speciation in natural waters. A great deal of efforts have been made to understand this (Börgmann 1983, Campbell and Stokes 1985), and the intricate relationship between metal speciation and its toxicity and accumulation in living organisms (Babich and Stotzky 1983a). Ecotoxicologists have

recently shown much inclination for studying the effect of pH on toxicity or accumulation of metals in living organisms due to two reasons: (i) the pH of many natural waters is declining due to acidic precipitation or due to disposal of acidic wastes, and (ii) the pH of some natural waters is increasing owing to discharge of alkaline wastes (e.g., soda ash discharge). It is generally believed that metal toxicity to plants and metal uptake rates generally increase with a decrease in pH (Gächter 1976, Monahan 1976, Harding and Whitton 1977, Müller and Payer 1979, Rai et al. 1981a and b, Starodub et al. 1987), but several workers have reported the opposite (Peterson et al. 1984, Skowronski 1986a and b, Skowronski et al. 1991, Crist et al. 1988), and conclusive metal pH effects have not been established. Several inorganic ligands (e.g., Cl^-, HS^-, O^-, F^-, S^{2-}, SO^{2-}, CN^-, PO_4^{3-}) are able to interact with metals to form complex compounds (Darimont and Frenay 1990). The ability of a ligand to form a metallic complex is determined mainly by its activity in solution. This activity depends on several parameters: concentration, temperature, ionic strength, acidity (pH), and potential of the solution. Inorganic ligands also alleviate metal accumulation and toxicity due to formation of complex inorganic compounds (Darimont and Frenay 1990,Honeyman and Santschi 1988). It is concerning to note that studies on pH effects on Cd toxicity or accumulation in living organisms are very few, and those on aquatic plants are even more meagre. All these studies have been carried out under extremely narrow range of pH, taking

different test organisms and experimental conditions; hence, they are not comparable.

Organic complexing agents in natural waters, although generally not as abundant as inorganic ligands, possess a strong affinity for heavy metals (Börgmann 1983). Several studies have revealed that both synthetic and natural chelators or metal-organic complexes alter the toxicity of metal ions to aquatic organisms (Khoboty'ger et al. 1975, Bitten and Freihofer 1978, Gnassiabarelli et al. 1978, Tanaka et al. 1982, Nor and Cheng 1986, Van den Berg et al. 1979, Huebert and Shay 1992). Metal-organic complexes reduce the bioavailability (Martell et al. 1988) and toxicity of heavy metals, probably by decreasing the attraction between the chelated metals and the surface of the cells (Babich and Stotzky 1983). Although binding of metals to organics or solids usually reduces metal toxicity, toxicity has been known to persist even when no free metallic ions are present (Gadd and Griffiths 1978). Aquatic plants have been sporadically studied with regard to effect of organic chelators on metal toxicity (Huebert and Shay 1992). Some studies describe that the synthetic chelator EDTA antagonizes uptake and toxicity of heavy metals (Tanaka et al. 1982, Schreinemakers and Dorhont 1985, Nor and Cheng 1986). While others reveal that EDTA is ineffective in alleviating metal toxicity (Nasu et al. 1983, Polar and Kücükcezzar 1986).

A few efforts have been made to study the effect of anions on metal toxicity or accumulation in living organisms. Nitrate

and PO_4^{3-} have been found to ameliorate the toxicity of metals to certain algae (Li 1978, Rai et al. 1981a, b, Babich and Stotzky 1983a). A similar effort on aquatic vascular plants does not seem to have been made before. Likewise, effects of light and temperature variations on metal accumulation and toxicity have not been studied in case of aquatic vascular plants.

This chapter describes the effects of pH, EDTA, NO_3^-, PO_4^{3-}, light and temperature on the accumulation and toxicity of Cd in L. polyrrhiza and A. pinnata.

Materials and Methods

Test plants, L. polyrrhiza and A. pinnata were obtained from stock cultures described in Chapter II. After careful selection, healthy growing plants were gently washed in distilled water prior to incubation in test medium. Unless otherwise stated, all experiments were carried out under similar conditions: temperature 25±1°C, a 14 h light (45 µmol m^{-2} sec^{-1}) and 10 h dark cycle, and 7 pH.

Effect of pH

Growth medium with 4 µM Cd concentration was freshly prepared before experiment and six different pHs (pH 4, 5, 6, 7, 8 and 9) were selected. The adjustment of pH was done by either 0.1N KOH or 0.1N HCl. Each treatment had a minimum of 5 replicates. About 5 to 6 fronds were floated in each of the replicate after their fresh weights were estimated. A separate

control without Cd was run side by side under similar conditions. Test plants were harvested after 4-d incubation and placed on blotting papers to remove liquid medium attached to test plants. Fresh weights of test plants were taken again. Test samples were then treated with 20 ml of 10 mM EDTA for 15 min to displace extracellular Cd. Eluted samples were then analysed for extracellular Cd content. The treated samples were dried at 60°C till constant weights were obtained. Dried samples were then weighed and digested following the procedure described in Chapters II and III. The digested samples were analysed for intracellular metal content by atomic absorption spectrophotometry.

Effect of temperature

Similar to the experiment described above, test plants were incubated in freshly-prepared medium containing 4 µM Cd at 5, 15, 25 and 35°C for 4 days. Controls without Cd were run under similar conditions. Fresh weight of each replicate sample (n = 5) was taken prior to incubation. At the termination of the experiment fresh weights were measured again. Estimations of extracellular and intracellular Cd contents were carried out as detailed above.

Effect of light intensity

Experiments were set at three different light intensities (PAR 30, 45 and 60 µmol m^{-2} sec^{-1}). Cadmium concentration in the medium was 4 µM Cd. Fresh weights were recorded at the

beginning and end of the experiment. Controls were kept for all light intensities.

Effect of EDTA

In order to study the effect of EDTA on Cd toxicity, test plants were incubated in growth medium containing 5, 10 and 20 µM EDTA spiked with 4 µM Cd. Controls were run without Cd in the medium. Fresh weights at the beginning, and at the end of experiment were also recorded. A short term experiment was conducted to determine the effect of EDTA on extracellular and intracellular Cd uptake. Test plants were incubated in medium containing 100 µM Cd for 2h. At the close of the experiment, test plants were harvested, and extracellular and intracellular Cd contents were determined.

Effects of anions on Cd uptake

The effects of PO_4^{3-} and NO_3^- concentration in medium on Cd uptake in _L. polyrrhiza_ and _A. pinnata_ were evaluated at 5 different concentrations of each anion (½x, x, 5x, 10x and 20x), where x = 6 mM for PO_4^{3-} and x = 300 mM for NO_3^-. Healthy test plants were incubated in growth medium containing 4 µM Cd and desired PO_4^{3-} or NO_3^- concentration. After 4-d incubation test plants were harvested, dried at 60°C, and analysed for Cd content.

Results

At lower pH values the intracellular Cd uptake by _L. polyrrhiza_ and _A. pinnata_ was higher as compared to uptake at

higher pHs. As the pH increased from 4 to 9, there was a gradual, but mild, decrease in intracellular Cd uptake. The extracellular uptake was reduced at pH 8 and 9 in both test plants (Figs. 5.1 and 5.2). Table 5.1 presents the RGR of test plants in relation to Cd toxicity at different pHs (4, 5, 6, 7, 8 and 9). The results presented here indicate higher toxicity at lower pH than at higher pH. At pH 4, there was a 90% reduction of RGR, whereas at pH 9 it was only 62% in L. polyrrhiza. A. pinnata showed a similar trend.

Effects of temperature on the extracellular and intracellular Cd uptake in L. polyrrhiza and A. pinnata were investigated. Fig. 5.3 shows that while rise in temperature significantly increased the intracellular Cd uptake, there was no significant change in extracellular Cd uptake in L. polyrrhiza. Table 5.2 indicates a relationship between temperature and relative growth rate (RGR) of L. polyrrhiza. At low temperature (5°C) the toxicity of Cd was lower than at high temperature (35°C). As the temperature increased the toxicity also increased proportionately from 20% inhibition at 5°C to 66% inhibition at 35°C. Similar results were obtained in A. pinnata. Fig. 5.4 shows that temperature had no significant influence on extracellular Cd uptake but there was marked increase in intracellular Cd uptake with increased temperature. Elevation of temperature increased RGR of control plants, but decreased RGR of plants treated with Cd.

Values of RGR of L. polyrrhiza and A. pinnata incubated for 4-d in a medium containing 4 µM Cd under different light

Table 5.1: Effect of pH on RGR of _L. polyrrhiza_ and _A. pinnata_ in presence or absence of 4 μM Cd.

pH	_L. polyrrhiza_		_A. pinnata_	
	-Cd	+Cd	-Cd	+Cd
4	0.14±0.02*	0.015±0.002 (10)	0.15±0.01	0.02±0.01 (12)
5	0.16±0.02	0.018±0.002 (11)	0.18±0.02	0.02±0.01 (11)
6	0.22±0.04	0.070±0.010 (32)	0.25±0.01	0.06±0.01 (24)
7	0.29±0.02	0.100±0.020 (34)	0.33±0.01	0.08±0.01 (24)
8	0.28±0.02	0.108±0.010 (36)	0.34±0.02	0.11±0.02 (32)
9	0.17±0.02	0.060±0.010 (38)	0.20±0.02	0.08±0.01 (40)

*RGR d^{-1} (mean ± SD).

% control responses in parentheses.

Table 5.2: Effect of temperature on the RGR of test plants in presence or absence of 4 µM Cd.

Temperature (°C)	*L. polyrrhiza*		*A. pinnata*	
	-Cd	+Cd	-Cd	+Cd
5	0.05±0.01*	0.04±0.01(80)	0.01±0.005	0.009±0.001(90)
15	0.12±0.03	0.06±0.02(50)	0.23±0.01	0.070±0.010(30)
25	0.31±0.03	0.12±0.01(38)	0.33±0.01	0.070±0.020(21)
35	0.32±0.01	0.11±0.01(34)	0.34±0.02	0.040±0.010(12)

*RGR d^{-1} (mean ± SD).

Values in parentheses denote % control response.

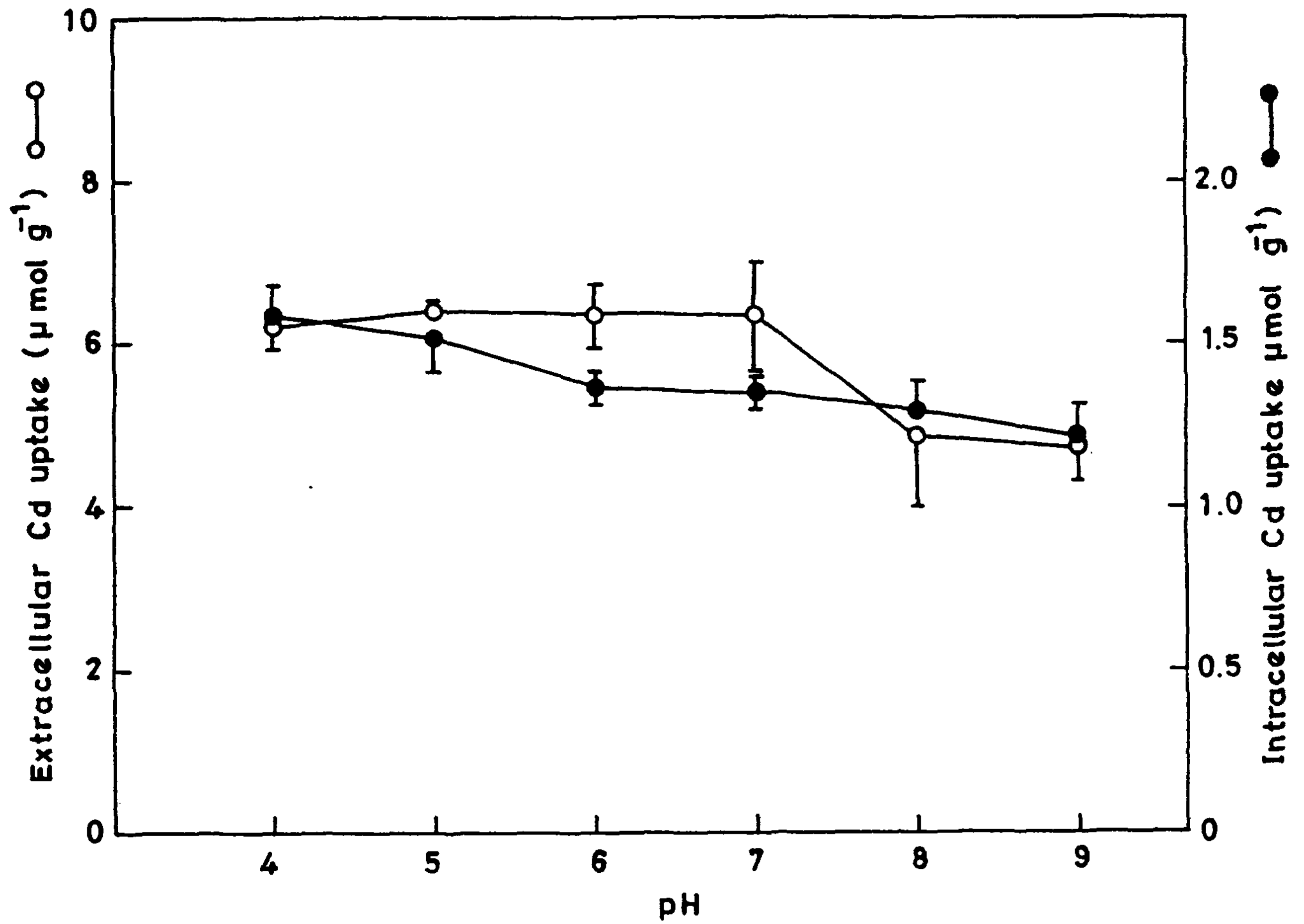


Fig. 5.1: Effect of pH on intracellular and extracellular Cd uptake in L. polyrrhiza incubated for 4 days in a medium containing 4 μM Cd. Error bars represent SD, n = 5.

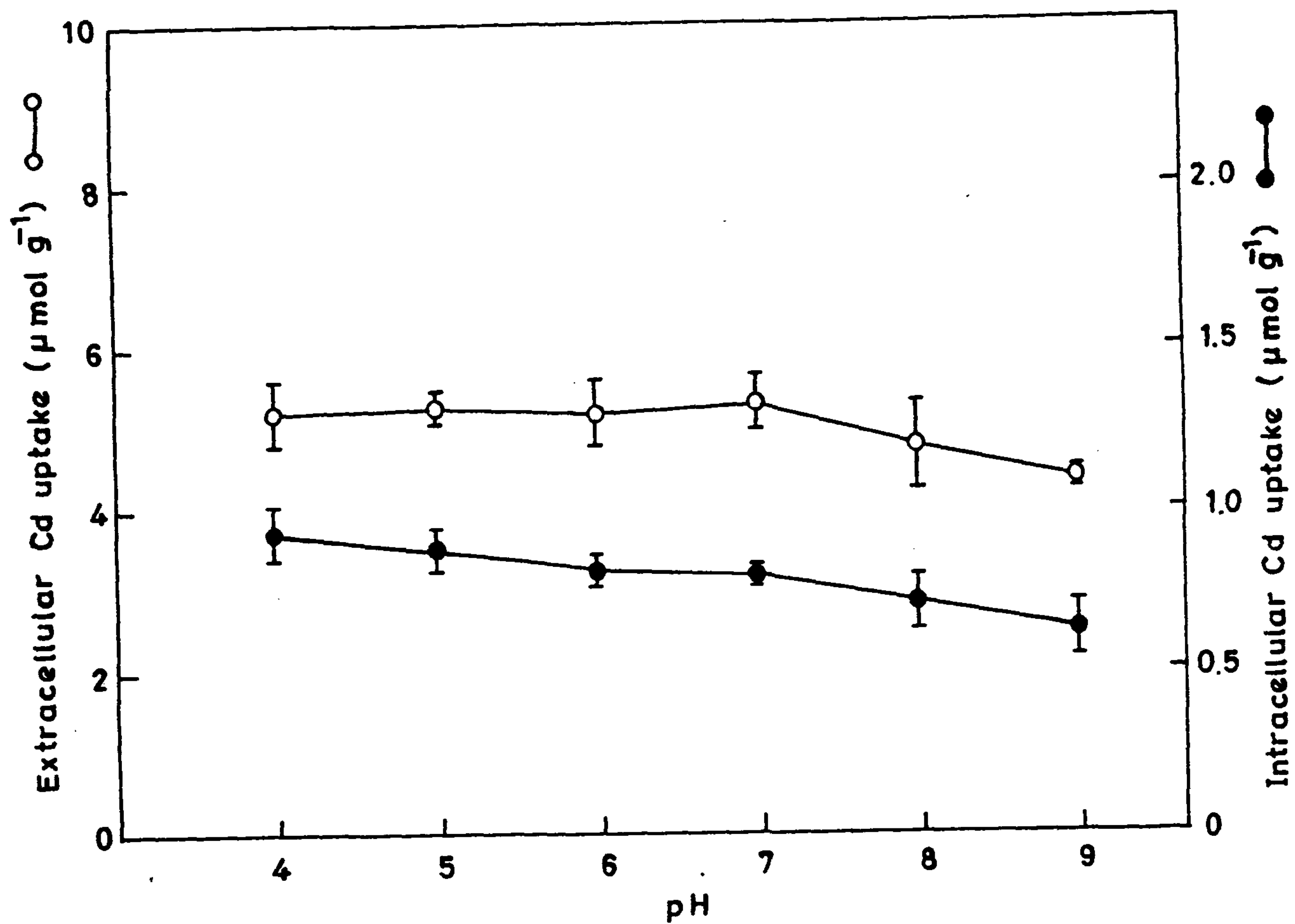


Fig. 5.2: Effect of pH on intracellular and extracellular Cd uptake in A. pinnata incubated for 4 days in a medium containing 4 µM Cd. Error bars represent SD, n = 5.

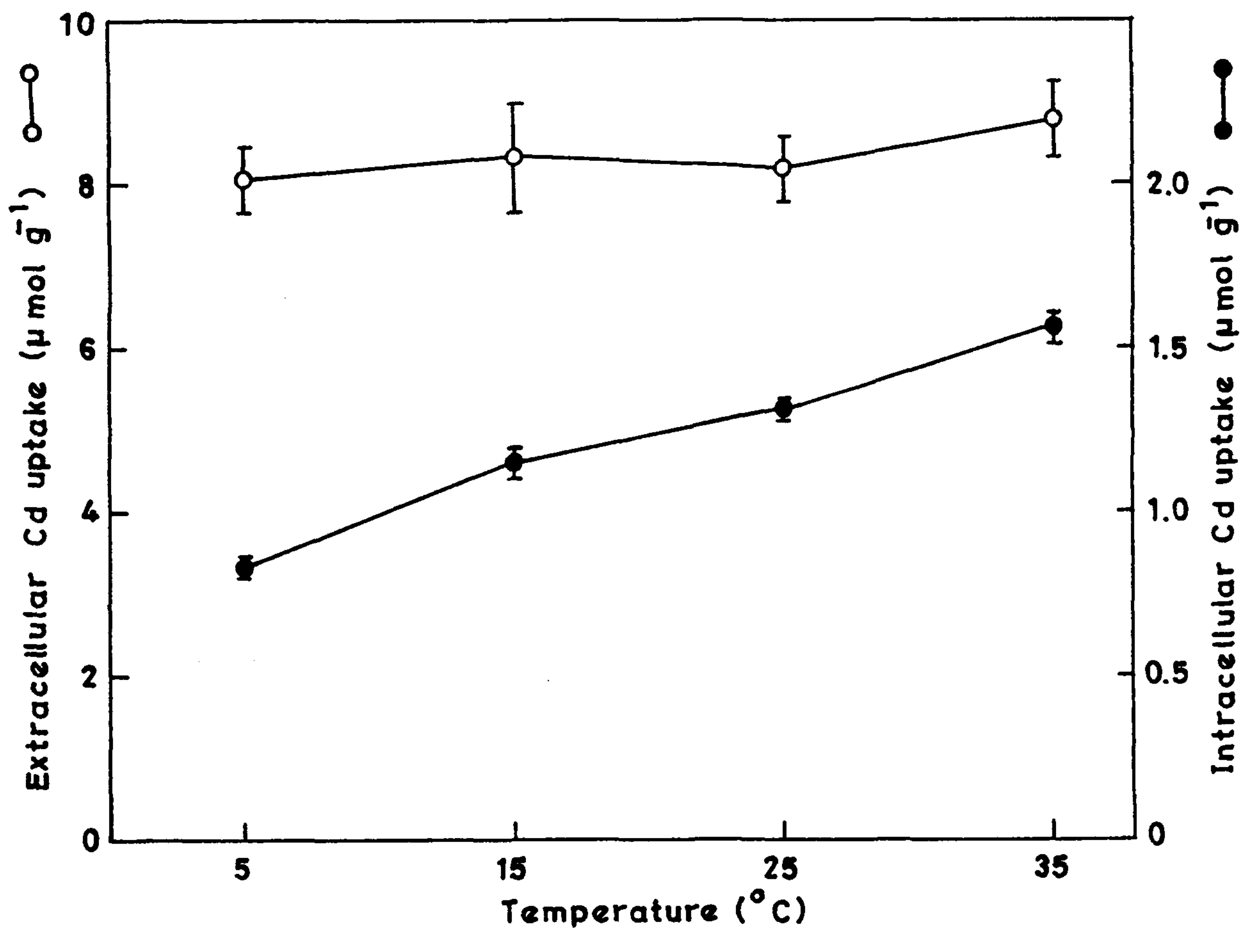


Fig. 5.3: Effect of temperature on intracellular and extracellular Cd uptake in L. polyrrhiza incubated for 4 days in a medium containing 4 µM Cd. Error bars represent SD, n = 5.

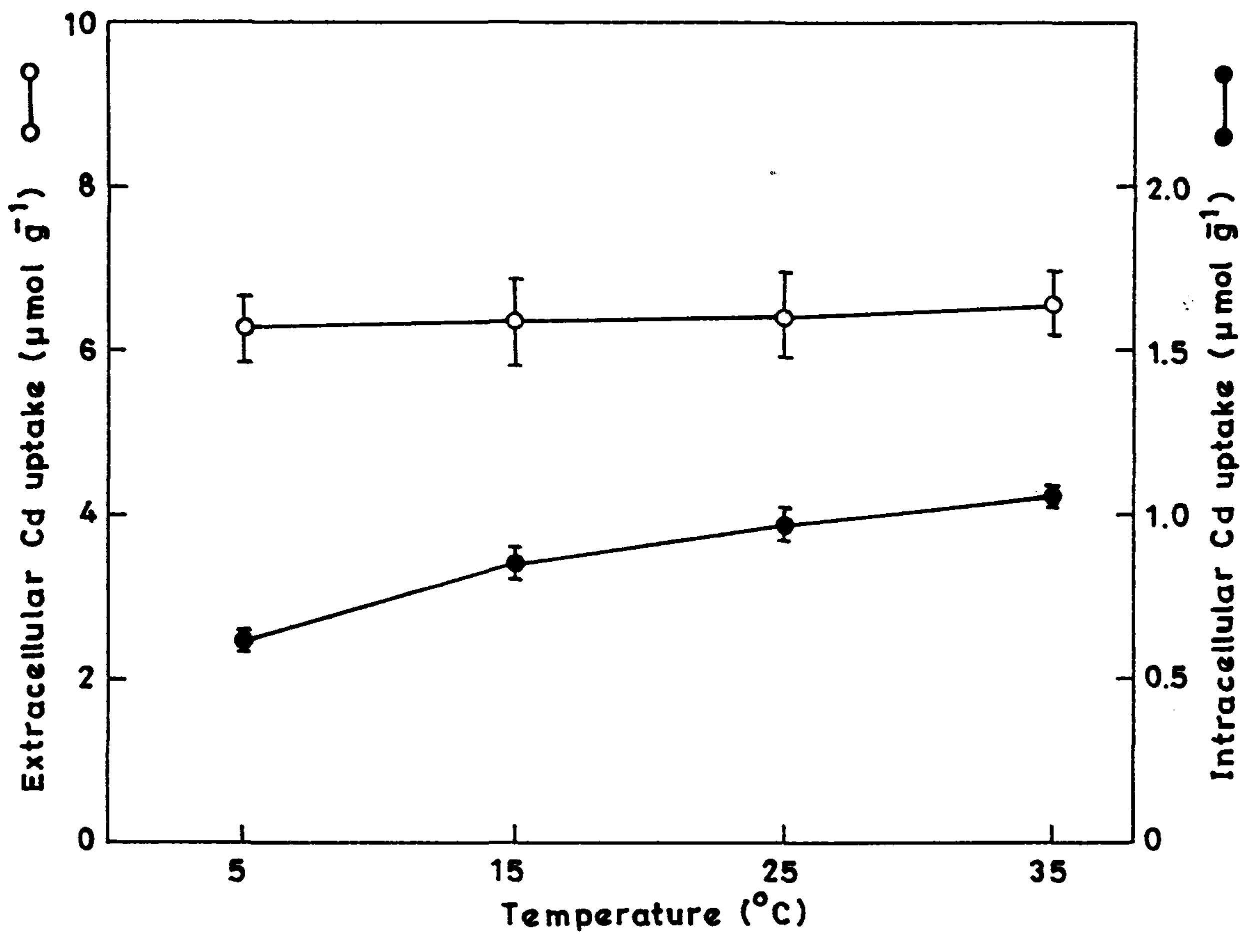


Fig. 5.4: Effect of temperature on intracellular and extracellular Cd uptake in A. pinnata incubated for 4 days in a medium containing 4 μM Cd. Error bars represent SD, n = 5.

intensities are given in Table 5.3. It can be seen that at lower light intensity, per cent inhibition of RGR by Cd uptake was less than higher light intensities. The data relating to extracellular Cd uptake under different light intensities were not significantly different from each other (Table 5.4). However, the intracellular uptake was significantly affected by light intensity. With increase in light intensity there was an increase in the intracellular Cd uptake in both test plants.

The presence of EDTA in the growth medium decreased extracellular and intracellular Cd uptake, of which intracellulr uptake was more severely affected at higher EDTA concentrations in the growth medium (Figs. 5.5 and 5.6). Table 5.5 presents the RGR of both test plants at different EDTA concentrations. With increase in EDTA concentration in the medium the toxicity of Cd decreased in L. polyrrhiza as well as A. pinnata. From the same table it can also be seen that EDTA concentration in the medium did not affect the RGR of A. pinnata, however at 20 µM EDTA, the RGR of L. polyrrhiza was decreased even in the absence of Cd in the growth medium.

Both phosphate and nitrate significantly lowered Cd uptake in L. polyrrhiza and A. pinnata. The amount of Cd uptake by test plants at different concentrations of phosphate and nitrate could be found in Table 5.6.

Discussion

The present work showed an inverse relationship between

Table 5.3: Effect of variation in PAR on the relative growth rate of test plants in presence or absence of 4 μM Cd.

PAR (μmol m^2 sec^{-1})	*L. polyrrhiza*		*A. pinnata*	
	-Cd	+Cd	-Cd	+Cd
30	0.28±0.02*	0.10±0.02 (36)	0.28±0.02	0.06±0.01 (22)
45	0.31±0.03	0.11±0.01 (35)	0.33±0.01	0.07±0.01 (22)
60	0.33±0.02	0.09±0.01 (26)	0.35±0.02	0.06±0.03 (18)

*RGR d^{-1} (mean ± SD).

Values in parentheses denote % control response.

Table 5.4: Effect of PAR levels on Cd uptake in *L. polyrrhiza* and *A. pinnata* after 4-d incubation in a medium containing 4 µM Cd.

PAR (µmol m^{-2} sec^{-1})	*L. polyrrhiza*		*A. pinnata*	
	Extra-cellular Cd (µmol g^{-1})	Intra-cellular Cd (µmol g^{-1})	Extra-cellular Cd (µmol g^{-1})	Intra-cellular Cd (µmol g^{-1})
30	6.5±0.3	1.22±0.04	5.1±0.05	0.62±0.01
45	6.5±0.53	1.29±0.02	5.2±0.3	0.63±0.01
60	6.53±0.05	1.32±0.04	5.3±0.2	0.65±0.02

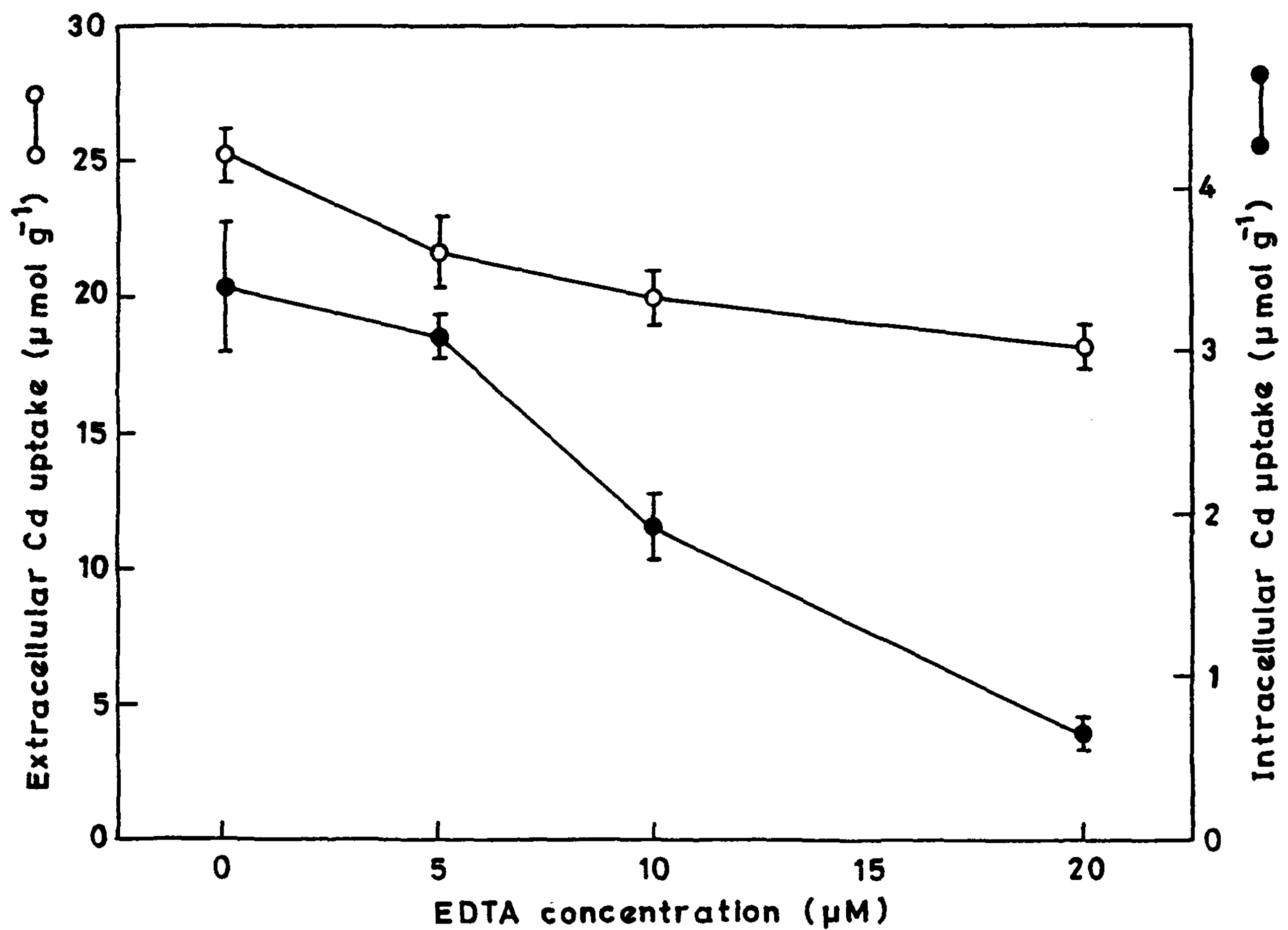


Fig. 5.5: Effect of EDTA on intracellular and extracellular Cd uptake in L. polyrrhiza incubated for 2h in a solution containing 100 μM Cd. Error bars represent SD, n = 5.

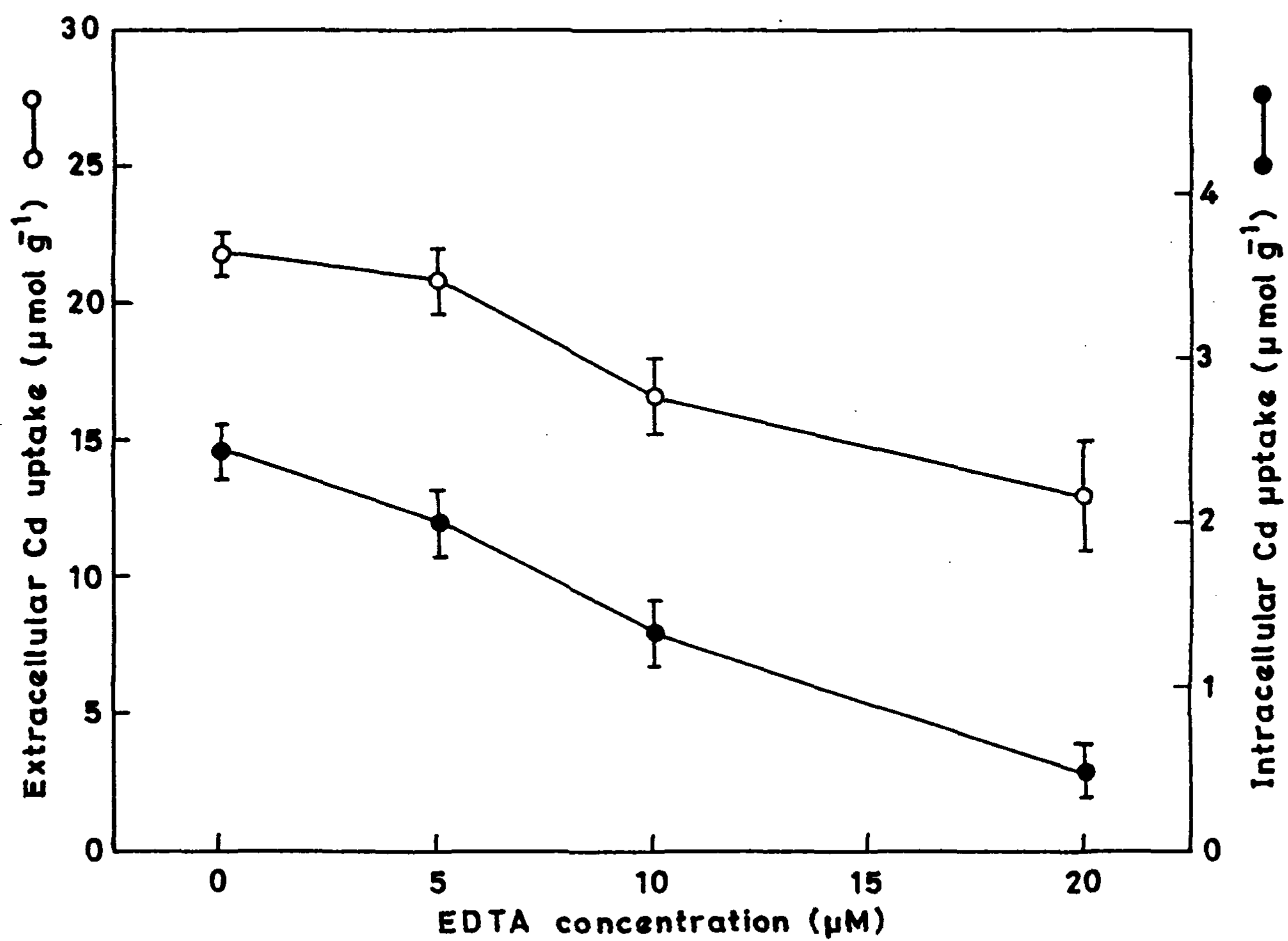


Fig. 5.6: Effect of EDTA on intracellular and extracellular Cd uptake in A. pinnata incubated for 2h in a solution containing 100 μM Cd. Error bars represent SD, n=5.

Table 5.5: Effect of EDTA on RGR of test plants in presence or absence of 4 µM Cd.

EDTA concentration (µM)	*L. polyrrhiza*		*A. pinnata*	
	-Cd	+Cd*[2]	-Cd	+Cd
5	0.31±0.01*	0.11±0.01 (35)	0.33±0.01	0.08±0.01 (24)
10	0.31±0.01	0.16±0.02 (52)	0.33±0.02	0.13±0.01 (39)
20	0.29±0.02	0.18±0.02 (62)	0.33±0.02	0.20±0.02 (60)

*RGR d^{-1} (mean ± SD).

% control responses in parentheses.

Table 5.6: Effect of anions on Cd uptake in *L. polyrrhiza* and *A. pinnata* after 4-d incubation in a medium containing 4 µM Cd.

Concentration of anion	*L. polyrrhiza*		*A. pinnata*	
	NO_3^-	PO_4^{3-}	NO_3	PO_4^{3-}
½x	8.15±0.3	8.22±0.3	6.23±0.8	6.25±0.8
x	8.12±0.3	8.20±0.09	6.07±0.2	6.20±0.3
5x	8.50±0.5	8.20±0.2	5.11±0.2	5.10±0.2
10x	7.56±0.5	7.05±0.2	5.00±0.3	4.67±0.4
20x	6.40±0.2	5.80±0.2	4.95±0.4	3.68±0.2

Data denote means of Cd uptake (µmol g^{-1}) ± SD.
The values for x are 300 and 6 mM for NO_3^- and PO_4^{3-}, respectively.

rise in pH and the extent of metal accumulation. Build up of Cd concentration in test plants at lower pH led to accentuation of Cd toxicity. These results are in agreement with observations of some earlier workers. Hart and Scaife (1977) found lower Cd toxicity to Chlorella pyrenoidosa at pH 8 than at pH 7. Almost similar observations were made by Müller and Payer (1979). The interaction of Cd with pH may be interpreted in terms of the availability of Cd at different pHs. It is now well-known that the availability of heavy metals in an aquatic environment depends mainly on the pH, and that heavy metals in general exists as free ions at acidic pH. At alkaline pHs, metals and other cations tend to precipitate as insoluble salts (Darimont and Frenay 1990). Thus the availability of metal ions is generally increased at low pH (Gadd and Griffiths 1978). The present findings do not agree with many earlier reports showing higher Cd uptake and toxicity at higher pH regimes (Sakaguchi et al. 1979, Riisgard et al. 1980, Geisweid and Urbach 1983, Peterson et al. 1984, Campbell and Stokes 1985, Skowronski et al. 1991). This kind of response has been linked to rise in level of protons (H^+) with rise in pH. Peterson et al. (1984) suggested that competition between heavy metals and H^+ for the same cellular binding sites results in a decrease in cellular heavy metal uptake and toxicity. However, in the present study competition between proton and Cd for the binding sites does not seem to have played a major role as Cd accumulation and toxicity were more at lower pH values. This fact becomes abundantly clear when the result of intracellular Cd uptake at lower and higher pHs are compared.

Synthetic organic ligands such as EDTA are added to defined inorganic culture media to ensure the availability of trace elements (principally Fe and Mn) for algal growth, and it is generally assumed that EDTA chelates iron exclusively as Fe-EDTA. However, the formation of this complex depends on pH, and other trace metals and cation (such as, Cu, Zn, Pb and Cd) can compete with Fe for EDTA. It has been reported that EDTA reduces the toxicity of heavy metals (Tanaka et al. 1982, Nor and Cheng 1986, Huebert and Shay 1992). This happens probably because chelated form of metals are less toxic than their free, non-complexed forms. Chelation has been suggested to be the single most important abiotic factor for the reduction of Cu toxicity in aquatic ecosystems (Hodson et al. 1979). The reduction of intracellular Cd uptake in L. polyrrhiza and A. pinnata by EDTA treatment also indicates that chelated species of Cd does not easily transport across the cell membrane. Hence high concentration of EDTA in the medium possibly complexed more Cd, resulting in its less uptake by the test plants. These results are in consonance with the results of Kwan and Smith (1991), and Huebert and Shay (1992), but at variance with the observations of Nasu et al. (1983) who found EDTA ineffictive in antagonizing the toxic effect of Cd towards Lemna paucicostata. Polar and Kücükcezzar (1986) also found chelators ineffective in ameliorating Cd toxicity to L. gibba. Huebert and Shay (1992) found that decreasing the Fe concentration from 9 to 2.25 µM in the presence of 9 µM EDTA caused a 97% reduction in Cd uptake and antagonized the toxicity of 1.28 µM Cd. This suggests that the amount of EDTA available for

chelating Cd is controlled by the Fe concentration, which is not unexpected since since Fe has the highest EDTA binding capacity (Skoog and West 1969). In contrast, Foster and Morel (1982) found that only when Fe was increased, was excess EDTA effective in ameliorating Cd capacity in the alga _T. weissflogii_. They concluded that there was a physiological interaction between Fe and Cd, and in a later study showed that Cd inhibited Fe uptake (Harisson and Morel 1983).

The reduction of Cd uptake in _L. polyrrhiza_ and _A. pinnata_ was observed with increase in concentration of PO_4^{3-} and NO_3^- in the medium. Earlier workers have shown that anions are able to reduce metal toxicity by precipitation. Phosphate ions can form precipitated with heavy metals, depending on their concentrations and the pH of the solution/medium (Gadd and Griffiths 1978). The addition of phosphate, thiosulphate or carbonate to the growth media often reduces metal toxicity (Sadler and Trudinger 1987). It is possible that the uptake of Cd in high phosphate media is limited by formation of cadmium phosphate in colloid form. According to Martell and Smith (1976)

$$\text{Log } K_{sp} [Cd_3 (PO_4)_2] = -32.60$$

and $$pK_a (HPO_4^-) = 7.207$$

on a simple basis it can be calculated that the saturation concentration of free Cd^{2+} should be about 4.8×10^{-1} M or 54 $\mu g\ l^{-1}$. With activity coefficients and complexing by chloride,

sulfate, etc., the concentration of soluble cadmium may be higher, but it is clear that the level of Cd in a given medium should be limited by the concentration of phosphates (Gipps and Coller 1980). Another possible role of PO_4^{3-} may be alleviation of Cd toxicity by protecting the susceptible sites within the cell. It is known that Cd ions generally bind to polyphosphate bodies in the cell (Rachlin et al. 1984). With increased PO_4^{3-} concentration the synthesis of polyphosphate bodies could be enhanced as a result of which more Cd ions are intracellularly immobilized. This mechanism was obviously not important in the present case as test plants accumulated lower levels of Cd at higher PO_4^{3-} concentrations. Nitrate ions are also known to mitigate the toxicity of Cd to microorganisms. The degree of inhibition by Cd of the growth of _Thalassiosira fluviatilis_ is more severe at low than high NO_3^- levels but the difference severely diminishes as Cd concentration increases, indicating competitive interaction (Li 1978).

Temperature and light intensity are also known to influence metal uptake. Intracellular Cd uptake in test plants was significantly increased with rise in temperature or irradiance. Similar results were reported by Skowronski et al. (1986b). They found that Cd transport into _Stichococcus bacillaris_ was completely inhibited at 4°C, but increased with temperature increase in the studied temperature range (4°C to 35°C). Gjengedal and Steinnes (1990) also observed an increase in metal uptake with increasing temperature in a moss.

Obviously, the rise in light or temperature has increased metabolic activity of test plants thereby leading to increased accumulation and toxicity of Cd. Elevation of temperature may increase metal adsorption by an organism (Rai et al. 1981a, Beckett and Brown 1984). It seems likely that increased Cd adsorption at higher temperature may also have contributed to enhanced intracellular level of Cd.

GENERAL DISCUSSION

Ever-increasing contamination of aquatic environment with heavy metals is posing a serious threat to diverse aquatic biota, including floating macrophytes. These macrophytes are, besides algae, the main primary producers in ponds and lakes. Hence, it is pertinent to assess the effects of metals in them. These plants can be potentially utilized in toxicity bioassays. The available reports suggest that these plants can accumulate extremely high levels of heavy metals (Sela et al. 1989, Turnquist et al. 1990, Wang 1990). It should, therefore, be possible to use them for gleaning heavy metals from wastewaters. The present study was aimed at investigating heavy metal toxicity and accumulation in *L. polyrrhiza* and *A. pinnata*. Greater emphasis was placed on elucidating the mechanism of extracellular and intracellular uptake of Cd. In addition, modification of Cd uptake and toxicity by various environmental factors was also investigated in test plants. A major part of the present work deals with Cd toxicity and accumulation due mainly to the reasons that it is regarded as one of the most toxic heavy metals, and in certain wastes (e.g., mine waters) its concentration may be more than 300 µM (Fleischeret et al. 1974, Wong et al. 1980).

All test metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) were accumulated by test plants in a concentration-dependent manner. The elevation of metal concentration in the medium led to

increase in metal accumulation. Heavy metals have strong affinity for acidic and thiol groups of proteins and nucleotides, and therefore they interfere with the functions of these biologically-important macromolecules (Sandman and Böger 1983). Hence, increased metal uptake by test plants, at higher concentrations in the external environment, has enhanced metal interaction with susceptible and sensitive sites within the cell, eventually causing higher toxicity to test plants.

One of the inhibitory effects of heavy metals, including Cd, was reduction in concentrations of biologically-important cations (Ca, Mg and K) in test plants. Previous reports give enough indications of loss of permeability and membrane damage by metals (Taylor and Foy 1985, Kennedy and Gonsalves 1987, Barcelo and Poschenrieder 1990). Metal-induced reduction in levels of cations has been observed in some other cases also (Fuch and Gartz 1983, Sela et al. 1989, Barcelo and Poschenrieder 1990). Once the level of essential cations in a metal-treated plant declines, almost the entire metabolic machinery of the cell will be affected. The reduction in levels of Ca and Mg by Cd treatment could be explained in terms of competitive interaction between Cd and the nutrient cations. Indeed, Ca and Mg caused competitive inhibition of intracellular Cd uptake in test plants. Test metals also caused lowering of water content in _L. polyrrhiza_ and _A. pinnata_. This was perhaps a consequence of the primary metal effects on cell wall and plasmamembrane (Barcelo and Poschenrieder 1990).

Test plants showed declination in the level of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoid) after exposure to heavy metals. Of the various pigments, the level of chlorophyll a was most severely inhibited by metals followed in decreasing order by chlorophyll b and carotenoid. The inhibition of pigment biosynthesis has been related to metal-induced inhibition of reductive steps in the biosynthesis of pigments (DeFillippis and Pallaghy 1976a and b). Photochlorophyllide reductase, the key enzyme needed for reducing protochlorophyll to chlorophyll, is inhibited by heavy metals (Van Assche and Clijsters 1990). It is logical to assume that lowering of pigment levels by heavy metals will reduce photosynthetic efficiency of test plants. Nonetheless, direct inhibition of photosynthetic process has been observed in several studies (Shioi et al. 1978, Bohner et al. 1980, Silverberg 1975). Higher concentrations of test metals also caused degradation of chlorophyll because the test plants showed definite symptoms of chlorosis.

The relative growth rate (RGR) of test plants declined in presence of high concentrations of heavy metals. Heavy metals are known to inhibit a multitude of metabolic processes including chlorophyll biosynthesis as observed in the present study. All such inhibitory effects culminate in inhibition of RGR. RGR was found to be a more sensitive parameter of metal toxicity in comparison to chlorophyll and carotenoid concentrations. This fact became apparent when concentrations

of various metals causing 50% inhibition, i.e., EC_{50}, of various parameters were compared. EC_{50} values derived from RGR data were much lower than those obtained from chlorophyll or carotenoid data. Therefore, RGR can be routinely used in metal toxicity bioassays.

Of the various metals considered in the present study, Cd was the most toxic whereas Pb was the least toxic. The variability of toxicity of heavy metals has been related with their chemical properties, especially valence, ionic radius and ability to form organic complexes (Barcelo and Poschenreider 1990). It has been suggested that metals having greater affinity for sulfur are more toxic than those having lower affinity for sulfur (Fisher and Jones 1981). This suggestion does not seem to be applicable in the present case as Cd, well known to have lower affinity for sulfur in comparison to Cu, was found to be more toxic than Cu.

L. polyrrhiza and *A. pinnata* have tremendous ability to accumulate metal ions from solutions over a wide range of metal concentrations. The positive relationship between the degree of metal accumulation by test plants and external metal concentration was statistically significant in all the treatments. The present results agree with metal accumulation reports on macrophytes (Hagemeyer and Waisel 1989, Wells and Brown 1990, Kwan and Smith 1991). The existence of such a significant relationship was perhaps due to the reason that a chelator-free inorganic medium was used for performing these

experiments. In case natural water samples are to be utilized, it is necessary to measure the concentration of free ions using a computer programme (Martell et al. 1988) or analytical methods like differential pulse anodic stripping voltammetry (DPASV) (Starodub et al. 1987) or ion specific electrodes. The concentration factors of various metals for test plants were by and large within the limit obtained by previous workers. However, the concentration factors for certain metals (e.g., Cr and Ni) were much higher than any of the previous report. The order of metal toxicity and the order of metal accumulation did not match for many metals. This was because of the fact that total metal content of test plants were measured instead of intracellular concentration, whereas toxicity is a manifestation of intracellular metal concentration. In fact, it was subsequently found that extracellular Cd binding accounted for greater than 80% of total Cd uptake by test plants.

An attempt was made to study intracellular and extracellular uptake of Cd in test plants. EDTA was used to displace extracellular (adsorbed) metal from cell surfaces. Extracellular uptake was found to be a purely physico-chemical process as it was not affected by temperature and light. Plant surfaces have different kinds of anionic sites at which metals are adsorbed (Davies 1978, Crist et al. 1981). The rate of extracellular uptake of Cd was high in the beginning, but slowed down with prolonged exposure reaching a plateau within 120 min. This would mean that there are two kinds of anionic

sites: the sites reacting rapidly with Cd and the sites reacting slowly. Further researches may throw some light on the nature of these sites. Another interesting observation was that _L. polyrrhiza_ has more of sites which rapidly reacted with Cd. Root-containing plants took up more of extracellular Cd on a per gram dry weight basis, in comparison to rootless plants. That means root surfaces have a high metal adsorbing capacity. The ability of Cd adsorption was found in dead biomass of test plants as well. Actually, dead biomass accumulated much more EDTA-displaceable Cd in comparison to live biomass. Enhanced efficiency of dead plants in adsorbing high levels of Cd could be explained in terms of increased availability of binding sites in the intracellular compartment due to the breakdown of permeability barrier.

A number of factors affected the extracellular uptake of Cd in test plants. It has been often suggested that metal adsorption occurs most efficiently at higher pHs due to their reduced competition with H^+ for the surface binding sites (Peterson et al. 1984). The present study, however, showed just the opposite trend. Extracellular Cd uptake was more at lower than at higher pH. The reduced extracellular uptake at higher pH seems to be due to formation of insoluble salts of Cd. EDTA, known for its ability to chelate metals, reduced extracellular uptake. It should however, be kept in mind that EDTA is a synthetic chelator, whereas natural waters contain chelators like humic acid, fulvic acid, etc. (Wilson 1978, Babich and Stotzky 1983, MacCarthy 1989). Extracellular uptake

of Cd was inhibited by monovalent and divalent cations used in the present study. Calcium, Mg and K caused non-competitive inhibition of Cd adsorption, whereas Wells and Brown (1990) found Ca and Mg to be competitive inhibitors of Cd uptake. Other cations caused competitive or non-competitive inhibition of Cd adsorption.

Whereas extracellular uptake became saturated within 120 min exposure of test plants to Cd, intracellular uptake continued at almost a constant rate. The constant rate of intracellular uptake is suggestive of a carrier-mediated mechanism for Cd uptake. Intracellular accumulation was enhanced by light thereby showing its dependence on metabolism of the cell. Although intracellular uptake was a mere 14 to 19% of total Cd uptake, it was responsible for toxicity in test plants. All factors which contributed to enhanced intracellular Cd uptake caused severe toxic effects; for example, intracellular accumulation was more at lower than higher pH due to increased availability of ionic forms of Cd. Consequently, Cd toxicity was more at lower pH. Increase in light intensity and temperature, known to increase cellular metabolism in plants, enhanced intracellular uptake, thereby increasing the toxic effects of Cd. Conversely, the presence of EDTA in the external environment reduced intracellular uptake resulting in mitigated toxicity of Cd to test plant.

Intracellular uptake of Cd in test plants was inhibited by Ca, Mg, K, Na, Cu, Fe, Ni and Zn. Calcium and Mg competitively

inhibited intracellular uptake. Similar observations have been made by Wells and Brown (1990). This was perhaps the reason why Ca and Mg levels in test plants declined after 4-d incubation in Cd solution. Potassium and Na caused non-competitive inhibition of Cd uptake, whereas Fe caused competitive and Cu caused mixed inhibition of Cd uptake. The mode of intracellular uptake of Ni and Zn varied in test plants. These elements inhibited intracellular uptake competitively in L. polyrrhiza, but non-competitively in A. pinnata.

In conclusion, the present work clearly shows the prospects of using L. polyrrhiza and A. pinnata as test plants for metal toxicity bioassays. It is concerning to know that metal toxicity bioassays have been generally carried out with algae. In comparison to algae, floating macrophytes, like the test plants, can be easily cultivated in the laboratory without sophisticated infrastructure (Wang 1990). Two species of Lemna, namely L. minor and L. gibba, have been recommended as test organisms by American Public Health Association and American Society for Testing and Materials (see Wang 1990). The present work demonstrates that L. polyrrhiza as well as A. pinnata can serve this purpose very well. Among the various parameters employed for evaluating Cd toxicity, RGR yielded the best results. The present work shows that several environmental factors affect Cd uptake and toxicity in test plants. Therefore, these factors must be given due

consideration in all future studies on metal accumulation and toxicity. It should become possible to use these plants for alleviating heavy metal burden of wastewaters. Even if live plants are not available, dead biomass can be repeatedly utilized for stripping metals from wastewaters.

SUMMARY

Accumulation and toxicity of heavy metals was studied in two floating macrophytes, namely, *L. polyrrhiza* and *A. pinnata*. Heavy metals (Cd, Co, Cr, Cu, Ni, Pb and Zn) selected for the study reduced the relative growth rate (RGR), and contents of chlorophyll *a* and *b* in a concentration-dependent manner. However, carotenoid content was not significantly reduced. EC_{50} values derived from chlorophyll data suggest the following hierarchies of metal toxicity:

Cd > Ni > Co > Cu > Zn > Cr > Pb for chlorophyll *a* in *L. polyrrhiza*,

Cd > Ni > Co > Zn > Cu > Cr > Pb for chlorophyll *b* in *L. polyrrhiza*,

Cd > Cu > Co > Ni > Zn > Cr > Pb for chlorophyll *a* in *A. pinnata*, and

Cd > Cu > Co > Ni > Cr > Zn > Pb for chlorophyll *b* in *A. pinnata*.

Test metals generally caused lowering of Ca, Mg and K levels in test plants. This was in all probabilities a result of metal-induced alteration in the characteristics of plasma membrane and subsequent changes in fluxes of various ions, and membrane damage.

The accumulation of heavy metals by test plants was dependent on metal concentration in the medium. Both test

plants accumulated Ni maximally, while Cr was accumulated the least. The linear relationship between uptake and metal concentration in the medium could be due to the reason that a chelator-free synthetic inorganic medium was used for cultivating test plants.

The present study revealed that the order of metal toxicity to L. polyrrhiza was Cd > Cu = Ni > Co > Cr > Zn > Pb and the order of metal accumulation was Ni > Zn > Co > Cu > Cd > Pb > Cr. In A. pinnata, however, the hierarchies were Cd > Cr > Co > Cu > Ni > Pb > Zn for metal toxicity to RGr and Ni > Zn > Co = Cd > Cu > Pb > Cr for metal accumulation. These results show that metal concentration in test plants as well as their toxicities were functions of metal concentration in the medium. At the same time the reasons for the differences in toxic effects of a particular metal towards different test organisms are not clear.

The intracellular Cd uptake by both test plants displayed typical Michaelis-Menten kinetics (K_m = 55.0±6 µM Cd and V_{max} = 2.92±2 µmol g^{-1} h^{-1} for L. polyrrhiza and K_m = 93.7±2 µM Cd and V_{max} = 3.07±0.9 µmol g^{-1} h^{-1} for A. pinnata). Rate of extracellular Cd uptake in L. polyrrhiza and A. pinnata did not remain constant throughout the experimental period. The values for concentration required for half maximum possible uptake (K_s) and maximum possible uptake after an exposure time of 2 h (U_{max}) were 29.5±4.5 µM Cd and 36.05±7 µmol g^{-1}, respectively for L. polyrrhiza, whereas for A. pinnata the K_s was 46.5±7 and U_{max} after 2 h was 33.48±11 µmol g^{-1}.

The results of extracellular uptake experiments showed the existence of two types of anionic sites on plant surfaces: sites reacting rapidly with Cd and the sites reacting slowly. Results obtained from time-course study indicate that extracellular Cd uptake reaches plateau much earlier than the intracellular uptake. This could be due to difference in uptake mechanism. While extracellular Cd uptake was a purely physico-chemical mechanism as it was independent of light and temperature, intracellular uptake indicated slow transport and a carrier-mediated mechanism. Intracellular accumulation was stimulated by light, revealing its dependence on cell energetics and metabolism. Root containing test plants accumulated more Cd than rootless test plants. Nearly 50% of the total Cd uptake could be accounted for by the roots in both test plants.

Dead biomass of test plants showed enhanced accumulation of Cd which probably means that Cd was adsorbed not only on the surface of the biomass but also on the inner phase of the cell walls of test plants due to the absence of permeability barrier in dead plants.

All cations and metals (Ca, Mg, K, Na, Cu, Fe, Ni and Zn) inhibited extracellular and intracellular Cd uptake in both test plants. Calcium, Mg and K caused non-competitive inhibition of extracellular uptake. Copper caused competitive *inhibition of extracellular uptake of Cd in both test plants.* Nickel and Zn were competitive inhibitors of Cd uptake in L.

polyrrhiza but non-competitive in A. pinnata. The nature of inhibition of extracellular Cd uptake by Fe was mixed in L. polyrrhiza but non-competitive in A. pinnata. These variations reveal that the mode of inhibition of Cd uptake by a particular cation differ in the two test plants. This could be due to differences in characteristics of anionic sites on the surfaces of test plants.

Calcium and Mg competitively inhibited intracellular Cd uptake in test plants. Competitive inhibition of Cd uptake by Ca could possibly be due to similarities in their ions, and competitive inhibition by Mg could be due to intracellular Cd uptake by the system normally transporting Mg. Potassium and Na exhibited non-competitive inhibition. While Ni and Zn were competitive in L. polyrrhiza, they were non-competitive in A. pinnata. Copper caused mixed inhibition in both test plants. This difference in inhibition among species of cations and metals as well as between the two test plants warrants further research.

At lower pH values the intracellular Cd uptake by test plants was higher as compared to uptake at higher pHs; correspondingly, Cd was more toxic at lower pH than at higher pH. This was obviously due to the predominance of Cd^{2+} ions at lower pH.

EDTA decreased extracellular and intracellular uptake of Cd by test plants. This was due to remarkable ability of EDTA to chelate Cd.

Elevation of temperature increased intracellular Cd uptake and increased Cd toxicity. Obviously, the rise in temperature has increased metabolic activity of test plants leading to increased Cd accumulation and toxicity. Similarly light also affected the uptake and toxicity of Cd.

Both PO_4^{3-} and NO_3^- inhibited the uptake of Cd in *L. polyrrhiza* and *A. pinnata*. Inhibitory effect of PO_4^{3-} was probably due to formation of insoluble cadmium phosphate.

LITERATURE CITED

Adriano DC (1986) Trace Elements in the Terrestrial Environment. Springer-Verlag, New York.

Aulio K, Saling M (1982) Enrichment of copper, zinc, manganese and iron in five species of pond weed (Potamogeton spp.). Bull. Env. Contam. Toxicol. 29: 320-325.

Azeez PA, Banerjee DK (1988) Effect of chromium on cyanobacteria and its accumulation. Toxicol. Environ. 16: 229-240.

Babich H, Stotzky G (1983a) Influence of chemical speciation on the toxicity of heavy metals. In: Nriagu JO (ed.) Aquatic Toxicology, Advances in Environmental Science and Technology, John Wiley & Sons, New York, 1-46.

Babich H, Stotzky G (1983b) Nickel toxicity to estuarine/marine fungi and its amelioration by magnesium in sea water. Water, Air Soil Pollut. 19: 193-202.

Babich H, Stotzky G (1985) Heavy metal toxicity to microbe mediated ecological processes: A review and potential application to regulatory policies. Env. Res. 36: 111-137.

Barcelo J, Poschenrieder Ch (1990) Plant water relations as affected by heavy metal stress: A review. J. Plant Nutr. 13: 1-37.

Barcelo J, Poschenrieder Ch, Andreu I, Gunse B (1986) Cadmium-induced decrease of water stress resistance in bush bean plants (Phaseolus vulgaris L. cv Contender). 1. Effects of Cd on water potential, relative water content and cell wall elasticity. J. Plant Physiol. 125: 17-25.

Bariaud A, Bury M, Mestre JC (1985) Mechanism of Cd^{2+} resistance in Euglena gracilis. Physiol. Plant. 63: 382-386.

Bates SS, Tessier A, Campbell PGC, Buffle J (1982) Zinc adsorption and transport by Chlamydomonas variabilis and Scenedesmus subspicatus (Chlorophyceae) grown in semi-continuous culture. J. Phycol. 18: 521-529.

Bauda P and Block JC (1990) Role of envelopes of gram-negative bacteria in cadmium binding and toxicity. Toxicity Asses. 5: 47-60.

Beckett RP, Brown DH (1984) The control of cadmium uptake in the lichen genus Peltigera. J. Exp. Bot. 35: 1071-1082.

Behemann JR, Weisman JC, Koopman BL, Oswald WJ (1977) Energy production by microbial photosynthesis. Nature 268: 19-23.

Bitten G, Freihofer N (1978) Influence of extracellular polysaccharides on the toxicity of copper and cadmium to Klebsiella aerogenes. Microb. Ecol. 4: 119-125.

Bohner H, Böhme H, Böger P (1980) Reciprocal formation of cytochrome C-553 and the influence of cupric ions on photosynthetic electron transport. Biochim. Biophys. Acta 592: 103-112.

Bórgmann W (1983) Metal speciation and toxicity of free metal ions to aquatic biota. In: Nriagu JO (ed.) Aquatic Toxicology, John Wiley & Sons, London, pp. 47-72.

Bowen JE (1969) Absorption of Cu, Zn and Mn by sugarcane leaf tissue. Plant Physiol. 44: 255-261.

Bradeen DA, Wingei GD, Gould JM, Ort DR (1973) Site-specific inhibition of photophosphorylation in isolated spinach chloroplasts by mercury chloride. Plant Physiol. 52: 680-682.

Braek GS, Malnes D, Jensen A (1980) Heavy metal tolerance of marine phytoplankton. IV. Combined effect of zinc and cadmium on growth and uptake in some marine diatoms. J. Exp. Mar. Biol. Ecol. 42: 39-54.

Brand LE, Sunda WG, Guillard RRL (1986) Reduction of marine phytoplankton reproduction rates by copper and cadmium. J. Exp. Mar. Biol. Ecol. 96: 22-250.

Breckle SW (1989) Growth under stress: Heavy metals. In: Waisel Y, Kafkafi U, Eshel A (eds.) The Root System: The Hidden Half, Marcel Dekker Inc., New York.

Brown DH, Beckett RP (1985) Intracellular and extracellular uptake of cadmium by the moss Rhytidiadelphus squarrosus. Ann. Bot. 55: 179-188.

Bryan GW, Langston WJ, Hummerstone LG, Butt GR (1985) A guide to the assessment of heavy metal contamination in estuaries using biological indicators. Occasional publication No. 4, Marine Biological Association of the United Kingdom, Plymouth, Devon.

Butler M, Haskew AEJ, Young MM (1980) Copper tolerance in the green alga, Chlorella vulgaris. Plant Cell Env. 3: 119-126.

Button DK, Dunker SS, Morse ML (1973) Continuous culture of Rhodotorula rubra: Kinetics of phosphate-arsenate uptake, inhibition, and phosphate limited-growth. J. Bacteriol. 113: 599-611.

Campbell PGC, Stokes PM (1985) Acidification and toxicity of metals to aquatic biota. Can. J. Fish. Aquat. Sci. 42: 2034-2049.

Carty AJ, Taylor NJ, Wong YS (1976) The binding of heavy metals (mercury and cadmium) at biologically important sites. Univ. Waterloo Res. Inst. Proj. No. 3082-2, Final Report.

Cataldo DA, Garland TR, Wildung RE (1983) Cadmium uptake kinetics in intact soybean plants. Plant Physiol. 73: 844-848.

Cedeno-Maldonado A, Swader JA, Heath RL (1972) The cupric ion as an inhibitor of photosynthetic electron transport in isolated chloroplasts. Plant Physiol. 50: 698-701.

Chaney RL, White MC, Simon PW (1975) Plant uptake of heavy metals from sewage sludge applied to land. In: Proc. 2nd Natl. Conf. Munic. Sludge Manage., Rockville, MD, pp 167-178.

Charpentier S, Garnier J, Flaugnatti R (1987) Toxicity and bioaccumulation of cadmium in experimental cultures of duckweed, Lemna polyrrhiza L. Bull. Env. Contam. Toxicol. 38: 1055-1061.

Chigbo FE, Smith RW, Shore FL (1982) Uptake of arsenic, cadmium, lead and mercury from polluted waters by the water hyacinth Eichhornia crassipes. Env. Pollut. 27: 31-36.

Colwell FS, Hornor SG, Cherry DS (1989) Evidence of structural and functional adaptation in epilithon exposed to zinc. Hydrobiologia 171: 79-90.

Coombes AJ, Lepp NW (1974) The effect of Cu and Zn on the growth of Marchantia polymorpha and Funaria hydrometrica. Bryologist 77: 447-452.

Creed IF, Havas M, Trick CG (1990) Effects of arsenate on growth of nitrogen- and phosphorus-limited Chlorella vulgaris (Chlorophyceae) isolates. J. Phycol. 26: 641-650.

Crist RH, Oberholser K, Shank H, Nguzen M (1981) Nature of bonding between metallic ions and algal cell walls. Env. Sci. Technol. 15: 1212-1217.

Crist RH, Oberholser K, Schwartz D, Marzoff J, Ryder D, Crist DR (1988) Interactions of metals and protons with algae. Env. Sci. Technol. 22: 755-760.

Cutler JM, Rains DW (1974) Characterization of cadmium uptake by plant tissue. Plant Physiol. 54: 67-71.

Darimont A, Frenay J (1990) Metals in aqueous solutions. In: Volesky B (ed.) Biosorption of Heavy Metals, CRC Press Inc., Boca Raton, pp. 66-79.

Davies AG (1970) Iron chelation and the growth of marine phytoplankton. 1. Growth kinetics and chlorophyll production in cultures of the euryhaline flagellate Dunaliella tertiolecta under iron limiting conditions. J. Mar. Biol. Assoc. UK 50: 65-86.

Davies AG (1973) The kinetics of and preliminary model for the uptake of radio-zinc by Phacodactylum tricornutum in culture. In: Radioactive Contamination of the Marine Environment, IAFA-SM-158/25, International Atomic Energy Agency, Vienna, pp. 403-420.

Davies AG (1978) Pollution studies with marine plankton. Part II. Heavy metals. Adv. Mar. Biol. 15: 381-508.

Davies AG (1983) The effects of heavy metals upon natural marine phytoplankton populations. In: Round FE, Chapman DJ (eds.) Progress in Phycological Research, Elsevier, Amsterdam, pp. 113-145.

De Filippis LF, Hampp R, Ziegler H (1981) The effects of sublethal concentrations of zinc, cadmium and mercury on Euglena. Growth and pigments. Z. Pflanzenphysiol. 101: 37-47.

De Filippis LF, Pallaghy CK (1976a) The effect of sub-lethal concentrations of mercury and zinc on Chlorella. 1. Growth characteristics and uptake of metals. Z. Pflanzenphysiol. 78: 197-207.

De Filippis LF, Pallaghy CK (1976b) The effects of sub-lethal concentrations of mercury and zinc on Chlorella. II. Photosynthesis and pigment composition. Z. Pflanzenphysiol. 78: 314-322.

Delhaize E, Jackson PJ, Lujan ID, Robinson NJ (1989) Poly (-glutamylcysteinyl) glycine synthesis in Datura innoxia and binding with cadmium. Plant Physiol. 89: 700-706.

Demarty M, Morvan C, Thellier M (1978) Exchange properties of isolated cell walls of Lemna minor L. Plant Physiol. 62: 477-481.

Devlin RM (1979) Plant Physiology, 3rd ed. Affiliated East West Press, New Delhi.

Dietz F (1973) The enrichment of heavy metals in submerged plants. In: Jenkins SH (ed.) Advances in Water Pollution Research, Proc. Sixth Int. Conf., Jerusalem, pp. 53-62.

Dixon NE, Gazzola C, Blakely RL, Zarer B (1975) Jack bean urease (E.C. 3.5 1.5) a metalloenzyme. A simple biological role of nickel. J. Am. Chem. Soc. 97: 4131-4153.

Doyle RJ, Mathews TH, Streips VN (1980) Chemical basis for selectivity of metal ions by Bacillus subtilis cell wall. J. Bacteriol. 143: 471-480.

Farnsworth M and Kline CH (1973) Zinc chemicals. Zinc Development Association, London, UK and Zinc Institute Inc., New York.

Filbin GJ, Hough RA (1979) The effects of excess copper sulfate on the metabolism of the duckweed Lemna minor. Aquat. Bot. 7: 79-86.

Findenegg GR, Paschinger H, Broda E (1971) Untersuchung der lichtabhangigkeit der Aufnahme von Rubidium, Zink, Kobalt, Blei und Cer durch Chlorella nach einer Flussmethode. Planta 99: 163-173.

Fisher NS, Bohe M, Teyssie J-L (1984) Accumulation and toxicity of Cd, Zn, Ag, and Hg in four marine phytoplankters. Mar. Ecol. Prog. Ser. 18: 201-213.

Fisher NS, Jones GJ (1981) Heavy metals and marine phytoplankton: Correlation of toxicity and sulfhydryl-binding. J. Phycol. 17: 108-111.

Fleischer M, Sarofim AF, Fasett DW, Hammond P, Shacklett HT, Nisbet ICT, Epstein S (1974) Environmental Impact of Cadmium - A review by the Panel on Hazardous Trace Substances. Env. Health Perspect. 7: 253-323.

Förstner U, Wittmann GTW (1981) Metal Pollution in the Aquatic Environment. Springer-Verlag, Berlin.

Foster PI, Morel FMM (1982) Reversal of cadmium toxicity in a diatom: An interaction between cadmium activity and iron. Limnol. Oceanogr. 27: 745-752.

Fuchs C, Garty J (1983) Element content in the lichen Ramalina duriaci (DeNot.) Jatta at air quality biomonitoring stations. Env. Exp. Bot. 23: 29-43.

Fuhrer J (1982) Early effects of excess cadmium uptake in Phaseolus vulgaris. Plant Cell Env. 5: 263-270.

Gächter R (1976) Untersuchungen uber die Beeinflussung der Planktischen Photosynthese durch anorganische metallsalze im eutrophen Alpanachersee und der mesotrophen Horwer Bucht. Schweiz. Z. Hydrol. 35: 252-261.

Gächter R, Mares A (1979) MELIMEX, An experimental heavy metal pollution study: Effects of increased heavy metal loads on phytoplankton communities. Schweiz. Z. Hydrol. 41: 228-246.

Gadd GM (1988) Accumulation of metals by microorganisms and algae. In: Rehm HJ (ed.) Biotechnology Vol. 6b, Special Microbial Processes, VCH Verlagsgesellschaft, Weinheim, pp. 401-433.

Gadd GM (1990) Heavy metal accumulation by bacteria and other microorganisms. Experientia 46: 834-840.

Gadd GM, Griffiths AJ (1978) Microorganisms and heavy metal toxicity. Microb. Ecol. 4: 303-317.

Geisweid HJ, Urbach W (1983) Sorption of cadmium by the green microalgae Chlorella vulgaris, Ankistrodesmus braunii and Eremosphaera viridis. Z. Pflanzenphysiol. 109: 127-141.

Gekeler W, Grill E, Winnacker EL, Zenk MH (1988) Algae sequester heavy metals via synthesis of phytochelatin complexes. Arch. Microbiol. 150: 197-202.

Gipps JF, Coller BAW (1980a) Effect of physical and culture conditions on uptake of cadmium by Chlorella pyrenoidosa. Aust. J. Mar. Freshwater Res. 31: 747-755.

Gipps JF, Coller BAW (1982) Effect of some nutrient cations on uptake of cadmium by Chlorella pyrenoidosa. Aust. J. Mar. Freshwater Res. 33: 979-987.

Gjengedal E, Steinnes E (1990) Uptake of metal ions in moss from artificial precipitation. Env. Monit. Assess. 14: 77-37.

Gnassia-Barelli M, Romeo M, Laumond F, Pesando D (1978) Experimental studies on the relationship between natural copper complexes and their toxicity to phytoplankton. Mar. Biol. 47: 15-19.

Greger M, Brammer E, Lindberg S, Larsson G, Iderotamalmquist J (1991) Uptake and physiological effects of Cd in sugar beet (Beta vulgaris) related to mineral provision. J. Exp. Bot. 42: 729-738.

Grill E, Thumann J, Winnacker EL, Zenk MH (1988) Induction of heavy-metal binding phytochelatins by inoculation of cell cultures in standard media. Plant Cell Rep. 7: 375-378.

Grill E, Winnacker EL, Zenk MH (1985) Phytochelatins: The principal heavy metal complexing peptides of higher plants. Science 23: 676-682.

Grill E, Winnacker EL, Zenk MH (1986) Synthesis of seven different homologous phytochelatins in metal-exposed Schizosaccharomyces pombe cells. FEBS Lett. 197: 115-120.

Grill E, Winnacker EL, Zenk MH (1987) Phytochelatins, a class of heavy-metal-binding peptides from plants, are functionally analogous to metallothioneins. Proc. Natl. Acad. Sci., USA 84: 439-443.

Hagemeyer J, Waisel Y (1989) Uptake of Cd^{2+} and Fe^{2+} by excised roots of Tamarix aphylla. Physiol. Plant. 77: 247-253.

Hager A, Moser I, Berthold W (1987) Organolead toxicity in plants: Triethyl lead (Et_3Pb^+) acts as a powerful transmembrane Cl/OH exchange dissipating H gradients at nano molar levels. Z. Naturforsch. 42C: 1116-1120.

Harding JPC, Whitton BA (1977) Environmental factors reducing the toxicity of zinc to Stigeoclonium tenue. Br. Phycol. J. 12: 17-21.

Harding JPC, Whitton BA (1981) Accumulation of zinc, cadmium and lead by field populations of Lemanea. Water Res. 15: 301-319.

Harrison GI, Morel FMM (1983) Antagonism between cadmium and iron in the marine diatom Thalassiosira weissflogii. J. Phycol. 19: 495-507.

Hart BA, Scaife BD (1977) toxicity and bioaccumulation of cadmium in Chlorella pyrenoidosa. Env. Res. 14: 401.

Haug A, Caldwell CR (1985) Aluminium toxicity in plants: Role of the root plasmamembrane and calmodulin. In: John JB St, Berlin E, Jackson PC (eds.) Frontiers of Membrane Research, Beltsville Symposium 9, Rowman & Allanheld, Totwa, pp. 359-381.

Hillman WS (1976) Calibrating duckweeds: Light, clocks, metabolism, flowering. Science 193: 453-458.

Hillman WS, Culley DD (1978) The uses of duckweed. Am. Sci. 66: 442-451.

Hirt H, Casari G, Barta A (1989) Cadmium enhanced gene expression in suspension-culture cells of tobacco. Planta 179: 414-420.

Hodson PV, Borgmann U, Shear H (1979) Toxicity of copper to aquatic biota. In: Nriagu JO (ed.) Copper in the Environment. Part II. Health Effects, John Wiley & Sons, New York, pp. 307-372.

Hollibaugh JT, Seibert DLR, Thomas WH (1980) A comparison of the acute toxicities of ten metals to phytoplankton from Saanich Inlet, B.C., Canada. Estuar. Coast Shelf Sci. 10: 93-105.

Honeyman BD, Santschi PH (1988) Metals in aquatic systems. Predicting their scavenging residence times from laboratory data remains a challenge. Env. Sci. Technol. 22: 962-871.

Horikoshi T, Nakajima A, Sakaguchi T (1979) Uptake of uranium by Chlorella regularis. Agr. Biol. Chem. 43: 617-623.

Huebert DB, Shay JM (1991) The effect of Cd and its interaction with external calcium in the submerged aquatic macrophyte Lemna trisulca L. Aquat. Toxicol. 20: 57-72.

Huebert DB, Shay JM (1992) zinc toxicity and its interaction with cadmium in the submerged aquatic macrophyte Lemna trisulca L. Env. Toxicol. Chem. 11: 715-720.

Hutchinson TC, Czyrska H (1972) Cadmium and zinc toxicity and synergism to floating aquatic plants. Water Pollut. Res. Can. 7: 59-65.

Hutchinson TC (1973) Comparative studies of the phytotoxicity of heavy metals to phytoplankton and their synergistic interactions. Water Pollut. Res. Can. 8: 68-89.

Hutchinson TC and Czyrska H (1975) Heavy metal toxicity and synergism to floating aquatic weeds. Verh Internat. Verein. Limnol. 19: 2102-2111.

Hutchinson TC, Stokes P (1975) Heavy metal toxicity and algal bioassays. In: Water quality Parameters, American Society for Testing of Materials, Special Technical Publication, no. 573, Philadelphia, pp. 320-343.

Hutton M (rapporteur) (1987) Group report: Cadmium. In: Hutchinson TC, Meema KM (eds.) Lead, Mercury, Cadmium and Arsenic in the Environment, Scope 31, John Wiley, Chichester, pp. 35-41.

Jain SK, Vasudevan P, Jha NK (1990) Azolla pinnata R. Br. and Lemna minor L. for removal of lead and zinc from polluted water. Water Res. 24: 177-183.

Jamir S (1982) Study on the Pteridophytic flora of Nagaland. Ph.D. Thesis, North-Eastern Hill University, Shillong.

Jana S (1988) Accumulation of Hg and Cr by three aquatic species and subsequent changes in several physiological and biochemical parameters. Water, Air Soil Pollut. 38: 105-109.

Jaworski (Rapporteur) (1987) Group report: Lead. In: Hutchinson TC, Meema KM (eds.) Lead, Mercury, Cadmium and Arsenic in the Environment, SCOPE 31. John Wiley, Chichester, pp. 53-68.

Jensen TE, Baxter M, Rachlin JW, Jani V (1982) Uptake of heavy metals by Plectonema boryanum (Cyanophyceae) into cellular components, especially polyphosphate bodies, an X-ray energy dispersive study. Env. Pollut. 27: 119-127.

Jennet JC, Hassett JM, Smith JE (1977) Removal of heavy metal trace elements from water by algae: Quantitative screening methods for selecting efficient organisms. In: Hemphill DD (ed.) Trace Substances in Environmental Health XI, University of Missouri, pp. 1-6.

John MK (1976) Interrelationships between plant cadmium and uptake of some other elements from culture solutions by oats and lettuce. Env. Pollut. 11: 85-95.

Kaplan DI, Adriano DC, Carlson CL, Sajwan KS (1990) Vanadium: Toxicity and accumulation by beans. Water, Air and Soil Pollut. 49: 81-91.

Kapur A, Chopra RN (1989) Effects of some metal ions on protonemal growth and bud formation in the moss Timmiella anomala grown in aseptic cultures. J. Hattori Bot. Lab. 66: 293-298.

Kay SH, Haller WT, Garrard LA (1984) Effects of heavy metals on water hyacinth (Eichhornia crassipes (Mart.) Solms. Aquat. Toxicol. 5: 117-128.

Kelly M (1988) Mining and the Freshwater Environment. Elsevier Applied Science Publishers, London.

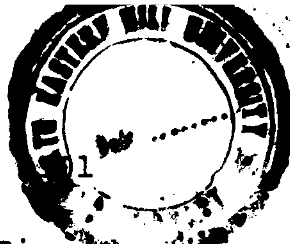
Kennedy CD, Gonsalves FAN (1987) The action of divalent zinc, cadmium, mercury, copper and lead on the trans-root potential and H^+ efflux of excised roots. J. Exp. Bot. 38: 800-817.

Khobot'yer VG, Kapkor VI, Rukhadze YG, Turunina NV, Shidlovskaya NA (1975) The toxic effect of copper complexes on algae. Hydrobiol. J. 11: 33-38.

Kneip TJ, Laver GJ (1973) Trace metal concentration factors in aquatic ecosystems. Prog. Anal. Chem. 5: 43-62.

Koeppe DE (1977) The uptake, distribution and effect of Cd and Pb in plants. Sci. Tot Environ. 7: 197-206.

Kumari JN, Venkateswarlu V, Rajkumar B (1991) Heavy metal pollution and phytoplankton in the river Moosi (Hyderabad), India. Int. J. Env. Studies 38: 157-164.



Kuyucak N, Volesky B (1988) Biosorbent for recovery of metals from industrial solutions. Biotechnol. Lett. 10: 137-142.

Kwan KHM, Smith S (1991) Some aspects of the kinetics of cadmium and thallium uptake by fronds of Lemna minor L. New Phytol. 117: 91-102.

Lane B, Kajioka R, Kennedy T (1987) The wheat-germ E_c protein is a zinc-containing metallothionein. Biochem. Cell Biol. 65: 1001-1005.

Langston WJ (1990) Toxic effects of metals and the incidence of *metal pollution in marine ecosystems. In: Furness RW,* Rainbow PS (eds.) Heavy Metals in the Marine Environment, CRC Press, Boca Raton, Florida, pp. 101-122.

Langston WJ, Bryan GW (1984) The relationship between metal speciation in the environment and bioaccumulation in aquatic organisms. In: Kramer CJM, Duinher JC (eds.) Complexation of Trace Metals in Natural Waters, Martinus Nijhoff/W. Junk Publ., The Hague, pp. 375-392.

Lazinsky D, Sicko-Goad L (1990) Morphometric analyses of phosphate and chromium interactions in Cyclotella meneghiniana. Aquat. Toxicol. 16: 127-140.

Lee CK, Low KS, Hew NS (1991) Accumulation of arsenic by aquatic plants. Sci. Tot. Env. 103: 215-228.

Leita L, Contin M, Maggioni A (1991) Distribution of Cd and induced Cd-binding proteins in roots, stems and leaves of Phaseolus vulgaris. Plant Sci. 77: 139-148.

Lerch K (1980) Copper metallothionein, a copper-binding protein from Neurospora crassa. Nature 284: 368-370.

Li WKW (1978) Kinetic analysis of interactive effects of cadmium and nitrate on growth of Thalassiosira fluviatilis (Bacillariophyceae). J. Phycol. 14: 454-460.

Lindsay WL (1972) Zinc in soils and plant nutrition. In: Brady NC (ed.) Advances in Agronomy, Academic Press, Vol. 24, pp. 147-186.

MacCarthy P (1989) Aquatic humic substances and their influence on the fate and treatment of pollutants. In: Suffet IH, MacCarthy P (eds.) Aquatic Humic Substances: Influence of Fate and Treatment of Pollutants, Amer. Chem. Soc., Washington, D.C., pp. xvii-xxx.

Maeda S, Mizoguchi M, Ohki A, Takeshita T (1990) Bioaccumulation of zinc and cadmium in freshwater alga, Chlorella vulgaris. 1. Toxicity and accumulation. Chemosphere 21: 953-963.

102789

Martell AE, Motekaitis RJ, Smith RM (1988) Structure-stability relationships of metal complexes and metal speciation in environmental aqueous solutions. Environ. Toxicol. Chem. 7: 417.

Martell AE, Smith RM (1976) Critical Stabiliity Constants, Vol. 4, Plenum Press, New York.

Martin JM, Whitfield M (1983) The significance of the river input of chemical elements to the ocean. In: Wong CS, Boyle EA, Burland KW, Button J, Goldberg ED (eds.) Trace Metals in Sea Water, Plenum Press, New York, pp. 265-296.

Maybeck M, Chapman D, Helmer R (eds.) (1989) Global Fresh Water Quality: A First Assessment. Blackwell Scientific, Oxford.

Mierle G (1982) Studies on the uptake and toxicity of copper and other transition metal ions with a green alga, Scenedesmus acuminatus. Ph.D. dissertation, Department of Botany, University of Toronto, Toronto, Ont.

Mildvan AS (1970) Metals in enzyme catalysis: In: Boyer PD (ed.) The Enzymes, Vol. 11, Academic Press, London & New York, pp. 445-536.

Miramand P, Bentley D (1992) Heavy metal concentrations in 2 biological indicators (Patella vulgata and Fucus serratus) collected near the French nuclear fuel reprocessing plant of La-Hague. Sci. Total Env. 111: 135-150.

Monahan TH (1976) Lead inhibition of chlorophycean microalgae. J. Phycol. 12: 358-362.

Moore JW, Ramamoorthy S (1984) Heavy Metals in Natural Waters: Applied Monitoring and Impact Assessment. Springer-Verlag, New York.

Müller KW, Payer HD (1979) The influence of pH, zinc, and light on the cadmium-repressed growth of the green alga Coelastrum proboscideum. In: Proceedings, Int. Conf. Management Control Heavy Metals in the Environment, London, CEP Consultants, Edinburg, U.K., pp. 296-299.

Münger K, Lerch K (1985) Copper metallothionein from the fungus Agaricus bisporus: Chemical and spectroscopic properties. Biochemistry 24: 6751-6756.

Nakada M, Fukaya K, Takeshita S, Wada Y (1979) The accumulation of heavy metals in the submerged plants (Elodea nuttali), Env. Contam. Toxicol. 22: 21-27.

Nakamo Y, Okamoto K, Toda S, Fuwa K (1978) Toxic effects of cadmium on Euglena gracilis grown in zinc deficient and zinc sufficient media. Biol. Chem. 42: 901-907.

NAS (1977) Coper. Medical and biological effects of environmental pollutants. Division of Medical Sciences, Nat. Acad. Sci., Washington, DC.

Nasu Y, Kugimoto M (1981) Lemna (duckweed) as an indicator of water pollution. 1. The sensitivity of Lemna paucicostata to heavy metals. Arch. Env. Contam. Toxicol. 10: 159-169.

Nasu Y, Kugimoto M, Tanaka O, Takimoto A (1983) Comparative studies on the adsorption of cadmium and copper in Lemna paucicostata. Env. Pollut. 32: 201-209.

Nasu Y, Kugimoto M, Tanaka O, Yanase D, Takimoto A (1984) Effects of cadmium and copper co-existing in the medium on the growth and flowering of Lemna paucicostata in relation to their absorption. Env. Pollut. 33: 267-274.

Nieboer E, Richardson DHS (1980) The replacement of nondescript term 'heavy metals' by a biologically and chemically significant classification of metal ions. Env. Pollut. 1: 3-26.

Nor YM, Cheng HH (1986) Chemical speciation and bioavailability of copper: Uptake and accumulation by Eichhornia. Env. Toxicol. Chem. 5: 941-947.

NRC (1976) Effects of chromium in the Canadian environment. Associate Committee on Scientific Criteria for Environmental Quality, National Research Council, Canada, NRCC No. 15017.

Nriagu JO, Kemp ALW, Wong HKT, Harper N (1979) Sedimentary record of heavy metal pollution in Lake Erie. Geochim. Cosmochim. Acta 43: 247-258.

Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature 333: 134-139.

Oron G, Wildschut LR, Porath D (1984) Waste water recycling by duckweed for protein production and effluent renovation. Water Sci. Technol. 17: 803-817.

Outridge PM, Hutchinson TC (1990) Effects of cadmium on integration and resource allocation in the clonal fern Salvinia molesta. Oecologia 84: 215-223.

Outridge PM and Hutchinson TC (1991) Induction of Cd tolerance by acclimation transferred between ramets of the clonal fern Salvinia minima Baker. New Phytol. 117: 597-605.

Paivoke A (1983) The long-term effects of zinc on the growth and development, chlorophyll content and nitrogen fixation of the garden pea. Ann. Bot. Fenn. 20: 205-213.

Paliouris G, Hutchinson TC (1991) Arsenic, cobalt and nickel tolerance in 2 populations of Silene vulgaris (Moench) Garcke from Ontario, Canada. New Phytol. 117: 449-460.

Parry GDR, Hayward J (1973) The uptake of Zn by Dunaliella tertiolecta Butcher. J. Mar. Biol. Assoc. UK 53: 915-922.

Passow H, Rothstein A, Clarkson TW (1961) The general pharmacology of heavy metals. Pharmacol. Rev. 13: 185-223.

Peel JW, Vetter RJ, Christian JE, Kessler WV, McFee WW (1978) In: Adriano DC, Brisbin IL (eds.) Environmental Chemistry and Cycling Processes, Springfield, VA, pp. 628-636.

Peterson HG, Healey FP, Wagemann R (1984) Metal toxicity to algae: A highly pH-dependent phenomenon. Can. J. Fish. Aquat. Sci. 41: 974-979.

Petit CM, Van de Geijn SC (1978) In vivo measurement of Cd transport and accumulation in the stems of intact tomato plants. Planta 138: 137-143.

Planas D, Healey FP (1978) Effects of arsenate on growth and phosphorus metabolism of phytoplankton. J. Phycol. 14: 337-341.

Polacco JC (1977) Nitrogen metabolism in soyabean tissue culture. II. Urea utilization and urease synthesis require Ni. Plant Physiol. 59: 827-830.

Polar E, Kücükcezzar R (1986) Influence of some metal chelators and light regimes on bioaccumulation and toxicity of Cd^{2+} in duckweek (Lemna gibba). Physiol. Plant. 66: 87-93.

Porter MR, Francko DA (1991) Effect of heavy metals on short-term photosynthetic rates in Potamogeton amplifolius. J. Aquat. Plant Manage. 29: 51-52.

Prahalad AK, Seenayya G (1986) In situ compartmentation and biomagnification of copper and cadmium in industrially polluted Husainsagar Lake, Hyderabad, India. Arch. Environ. Contam. Toxicol. 16: 417-425.

Prahalad AK, Seenayya G (1988) In situ partitioning and biomagnification of mercury in industrially polluted Husainsagar Lake, Hyderabad, India. Water, Air Soil Pollut. 39: 81-87.

Price NM, Morel FMM (1988) Cadmium and cobalt substitution for zinc in marine diatom. Nature 344: 658-660.

Rachlin JW, Jensen TE, Warkentine B (1984) The toxicological response of the alga Anabaena flos-aquae (Cyanophyceae) to cadmium. Arch. Env. Contam. Toxicol. 13: 143-151.

Rai LC, Gaur JP, Kumar HD (1981a) Phycology and heavy metal pollution. Biol. Rev. 56: 99-151.

Rai LC, Gaur JP, Kumar HD (1981b) Protective effects of certain environmental factors on the toxicity of zinc, mercury and methyl mercury to Chlorella vulgaris Beij. Env. Res. 25: 250-259.

Rauser WE (1990) Phytochelatins. Ann. Rev. Biochem. 59: 61-86.

Ravera O, Gommes R, Muntau H (1973) Cadmium distribution in aquatic environment and its effects on aquatic organisms. Problems of the contamination of man and his enviroment by mercury and cadmium. Colloquium, Commission of the European Communities, Luxembourg, pp. 317-331.

Ray SN, White WJ (1979) Equisetum arvense an aquatic vascular plant as a biological monitor for heavy metal pollution. Chemosphere 3: 125-128.

Rebhum S, Ben-Amotz A (1984) The distribution of cadmium between marine alga Chlorella stigmatophora and sea water medium. Wat. Res. 18: 173-178.

Reeder SW, Demayo A, Taylor MC (1979) Guidelines for Water Quality, Vol. I. Inorganic Chemical Substances. Cadmium, pp. 1-19. Inland Water Directorate,Water Qual.Br.,Canada.

Reese RN, Wagner GJ (1987) Properties of tobacco (Nicotiana tabacum) cadmium-binding peptide(s). Biochem. J. 241: 641-647.

Riisgard HU, Nielson KN, Sogaard-Jensen B (1980) Futher studies on volume regulation and effects of copper in relation to pH and EDTA in the naked marine flagellate Dunaliella marina. Mar. Biol. 56: 267-276.

Robinson NJ (1989) Algal metallothioneins: Secondary metabolites and proteins. J. Appl. Phycol. 1: 5-18.

Robinson NJ, Jackson PJ (1986) 'Metallothionein-Like' metal complexes in angiosperms; their structure and function. Physiol. Plant. 67: 499-506.

Robinson NJ, Ratliff RL, Anderson PJ, Delhaize E, Berger JM, Jackson PJ (1988) Biosynthesis of poly (-glutamyl cysteiny) glycines in cadmium-tolerant Datura innoxia (Mill.) cells. Plant Sci. 56: 197-204.

Romeo M, Nicolas E (1986) Cadmium, copper, lead and zinc in three species of planktonic crustaceans from the east coast of Corsica. Mar. Chem. 18: 359-367.

Rosko JJ, Rachlin JW (1975) The effects of copper, zinc, cobalt and manganese on the growth of the marine diatom *Nitzschia closterium*. Bull. Torr. Bot. Club 102: 100-106.

Rosko JJ, Rachlin JW (1977) The effect of cadmium, copper, mercury, zinc and lead on cell division, growth, and chlorophyll-*a* content of the chlorophyte *Chlorella vulgaris*. Bull. Torr. Bot. Club 104: 226-233.

Rothstein A (1959) Cell membrane as site of action of heavy metals. Fed. Proc. 18: 1026-1035.

Royer GP (1982) Fundamentals of Enzymology, Rate Enhancement, Specificity, Control and Applications. John Wiley & Sons, New York.

Rueter JG (1983) Alkaline phosphate inhibition by copper: Implications to phosphorus nutrition and use as a biochemical marker of toxicity. Limnol. Oceanogr. 28: 743-748.

Sadler WR, Trudinger PA (1967) The inhibition of microorganisms by heavy metals. Mineral Dep. 2: 158-168.

Sakaguchi T, Tsuji T, Nakajima A, Horikoshi T (1979) Accumulation of cadmium by green microalgae. Eur. J. Appl. Microbiol. Biotechnol. 8: 207-215.

Salt DE, Thurman DA, Tomsett AB, Sewell AK (1989) Copper phytochelatins of *Mimulus guttalus*. Proc. Royal Soc. Lond. Ser. B 236: 79-89.

Sanders JG, Osman RW, Riedel GF (1989) Pathways of arsenic and incorporation in estuarine phytoplankton and the filter feeding invertebrates *Eurytemora affinis*, *Balanus improvisus* and *Crassostrea virginica*. Mar. Biol. 103: 319-325.

Sandmann G, Böger P (1983) The enzymological function of heavy metals and their role in electron transfer processes of plants. In: Lauchli A, Bieleski RL (eds.) Encyclopedia of Plant Physiology, New Series, Vol. 15, Inorganic Plant Nutrition, Springer, Berlin-Heidelberg, pp. 563-596.

Say PJ, Whitton BA (1983) Accumulation of heavy metals by aquatic mosses. 1. *Fontinalis antipyretica* Hedw. Hydrobiologia 100: 245-260.

Schmid WE, Haag HP, Epstein E (1965) Absorption of zinc by excised barley roots. Physiol. Plant. 18: 860-869.

Schreinemakers WAC, Dorhout R (1985) Effects of copper ions on growth and ion absorption by *Spirodela polyrrhiza*. J. Plant Physiol. 121: 343-351.

Schulz Baldes M, Lewin RA (1976) Lead uptake in two marine phytoplankton organisms. Biol. Bull. 150: 118-127.

Sculthorpe CD (1967) The Biology of Aquatic Vascular Plants. St. Martins Press, New York.

Seenayya G, Prahalad AK (1987) In situ compartmentation and biomagnification of chromium and manganese in industrially polluted Husainsagar Lake, Hyderabad, India. Water, Air Soil Pollut. 35: 233-239.

Sela M, Tel-or E, Frizt E, Huttermann A (1988) Localization and toxic effects of cadmium, copper and uranium in Azolla. Plant Physiol. 88: 30-36.

Sela M, Gaity J, Tel-or E (1989) The accumulation and the effect of heavy metals on the water fern Azolla filiculoides. New Phytol. 112: 7-12.

Seto M, Tadahoshi Y, Ushijima T, Tazaki T (1979) Chlorotic death of Lemna gibba by cadmium in different concentration of nutritional minerals. Jap. J. Limnol. 40: 61-65.

Sharma SD, Chopra RN (1987) Effect of lead nitrate and lead acetate on growth of the moss Semibarbula orientalis (Web.) Wijk. et Marg. Grown in vitro. J. Plant Physiol. 129: 242-249.

Shimwell DW, Laurie AE (1972) Lead and zinc contamination of vegetation in the southern Pennies. Env. Pollut. 3: 291-301.

Shioi Y, Tamai H, Sasa T (1978) Effects of copper on photosynthetic electron transport systems in spinach chloroplast. Plant Cell Physiol. 19: 203-209.

Silverberg BA (1975) Ultrastructural localization of lead in Stigeoclonium tenue (chlorophyceae, Ulotricales) as demonstrated by cytochemical and X-ray microanalysis. Phycologia 14: 265-274.

Simpson WR (1981) A critical review of cadmium in the marine environment. Prog. Oceanogr. 10: 1-70.

Skipnes O, Roald T, Haug A (1975) Uptake of zinc and strontium by brown algae. Physiol. Plant. 34: 314-320.

Skoog DA, West DM (1969) Fundamentals of Analytical Chemistry. Holt, Rinehart and Winston, Toronto.

Skowronski T (1984a) Energy-dependent transport of cadmium by Stichococcus bacillaris. Chemosphere 13: 1379-1384.

Skowronski T (1984b) Uptake of Cd by Stichococcus bacillaris. Chemosphere 13: 1385-1389.

Skowronski T (1986a) Adsorption of Cd on green microalga Stichococcus bacillaris. Chemosphere 15: 69-76.

Skowronski T (1986b) Influence of some physico-chemical factors on Cd uptake by the green alga Stichococcus bacillaris. Appl. Microbiol. Biotechnol. 24: 423-425.

Skowronski T, Szubinska S, Pawlik B, Jakubowski M, Bilewicz R, Cukrowska E (1991) The influence of pH on Cd toxicity to the green alga Stichococcus bacillaris and on the Cd forms present in the culture medium. Env. Pollut. 74: 89-100.

Smeyers-Verbeke J, De Graeve M, Francois M, De Jaegere R, Massart DL (1978) Cd uptake by intact wheat plants. Plant Cell Env. 1: 291-296.

Smith DG (1986) Heavy metals in the New Zealand aquatic environment: A review. Water and Soil Miscellaneous Publication No. 100, National Water and Soil Conservation Authority, Wellington.

Soeder CJ, Hans DP, Payer HD, Runkel KH, Bein J, Briele E (1978) Sorption and concentration of toxic minerals by mass cultures of Chlorococcales. Mitt. Int. Ver. Limnol. 21: 575-584.

Srivastava A, Jaiswal VS (1989) Biochemical changes in duckweed after cadmium treatment. Enhancement of senescence. Water, Air Soil Pollut. 50: 163-170.

Stacey NH, Klassen CD (1980) Cd uptake by isolated rat hepatocytes. Toxicol. Appl. Pharmacol. 55: 448-455.

Starodub ME, Wong PTS, Mayfield CI, Chau YK (1987) Influence of complexation and pH on individual and combined heavy metal toxicity to a freshwater alga. Can. J. Fish. Aquat. Sci. 44: 1173-1180.

Steffens JC (1990) The heavy metal-binding peptides of plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 41: 553-575.

Stokes PM(1975) Uptake and accumulation of copper and nickel by metal tolerant strains of Scenedesmus. Ver. Int. Verein. Limnol. 19: 2128-2137.

Stokes PM (1983) Response of freshwater algae to heavy metals. In: Round FE, Chapman DJ (eds.) Progress in Phycological Research, Vol. 2, Elsevier, Amsterdam, pp. 87-112.

Stratton GW, Corke CT (1979) The effect of cadmium ion on the growth, photosynthesis and nitrogenase activity of Anabaena inaequalis. Chemosphere 5: 277-282.

Stratton GW, Huber AL, Corke CT (1979) Effect of mercuric ion on the growth, photosynthesis and nitrogenase activity of Anabaena inaequalis. Appl. Env. Microbiol. 38: 537-543.

Strickland JDH, Parsons TR (1968) A Practical Handbook of Sea Water Analysis, 2nd ed. Bull. Fish. Res. Board Can., 167.

Sunda WG, Barber RT, Huntsman SA (1981) Phytoplankton growth in nutrient rich seawater: Importance of copper-manganese interactions. J. Mar. Res. 39: 567-586.

Sunda WG, Guillard RRL (1976) The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J. Mar. Res. 34: 511-529.

Takamura N, Kasai F, Watanabe MM (1989) Effects of Cu, Cd and Zn on photosynthesis of freshwater benthic algae. J. Appl. Phycol. 1: 39-52.

Tanaka O, Nasu Y, Takimoto A, Kugimoto M (1982) Absorption of copper by Lemna as influenced by some factors which nullify the copper effect on flowering and growth. Plant Cell Physiol. 23: 1291-1296.

Tateda Y, Hirano S, Koyanagi T (1985) Accumulation of iron-59 by black-fish Girella punctata from food organisms. Bull. Jap. Soc. Sci. Fish/Nissuishi 51: 5067-5072.

Taylor GJ (1987) Exclusion of metals from the symplasm: A possible mechanism of metal tolerance in higher plants. J. Plant Nutr. 10: 1213-1222.

Taylor GJ, Foy CD (1985) Differential uptake and toxicity of ionic and chelated copper in Triticum aestivum. Can. J. Bot. 63: 1271-1275.

Thomas WH, Hollibaugh JT, Seibert DLR (1980) Effects of heavy metals on the morphology of some marine phytoplankton. Phycologia 19: 202-209.

Tiller KG (1989) Heavy metals in soils and their enviromnental significance. In: Steward BA (ed.) Advances in Soil Science, Vol. 9, Springer Verlag, New York, pp. 113-142.

Ting YP, Lawson F, Prince IG (1989) Uptake of cadmium and zinc by the alga Chlorella vulgaris 1. Individual ion species. Biotechnol. Bioengg. 34: 990-999.

Ting YP, Lawson F, Prince IG (1991) Uptake of cadmium and zinc by the alga Chlorella vulgaris. 2. Multi-ion situation. Biotechnol. Bioengg. 37: 445-455.

Turnquist TD, Urig BM, Hardy JK (1990) Nickel uptake by the water hyacinth. J. Env. Sci. Health Part A 25: 897-912.

Tyler G (1981) Heavy metals in soil biology and biochemistry. In: Paul EA, Ladd JN (eds.) Soil Biochemistry, Marcel Dekker, New York, pp. 371-414.

Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Env. 13: 195-206.

Van den Berg CMC, Wong PTS, Chau YK (1979) Measurement of complexing materials exerted from algae and their ability to ameliorate copper toxicity. J. Fish. Res. Board Can. 36: 901-905.

Van der Werff M, Pruyt MJ (1982) Long-term effects of heavy metals on aquatic plants. Chemosphere 2: 727-739.

Vocke RW, Sears KL, O'Toole JJ, Wildman RB (1980) Growth responses of selected freshwater algae to trace elements and scrubber ash slurry generated by coal-fired power plants. Water Res. 14: 141-150.

Vymazal JC (1987) Toxicity and accumulation of cadmium with respect to algae and cyanobacteria: A review. Toxicity Assess. 2: 387-415.

Walker WM, Miller JE, Hassett JJ (1977) Effect of lead and cadmium upon the calcium, magnesium, potassium and phosphorus concentration in young corn plants. Soil Sci. 124: 145-151.

Wang W (1986a) Toxicity tests of aquatic pollutants by using common duckweed. Environ. Pollut. 11: 1-14.

Wang W (1986b) Site specific phytotoxicity of heavy metals. Report to U.S. Environmental Protection Agency, R8 108 34, Illinois State Water Survey Contract Report 403.

Wang W (1990) Review: Literature review on duckweed toxicity testing. Env. Res. 52: 7-22.

Weast RC, Astle MJ (eds.) (1983) CRC Handbook of Physics and Chemistry. CRC Press, Boca Raton.

Webb M (1975) Metallothionein and the toxicity of cadmium. Env. Sci. Res. 7: 177-186.

Wehr JD, Whitton BA (1983) Accumulation of heavy metals by aquatic mosses. 2: _Rhynchostegium riparioides_. Hydrobiologia 100: 261-284.

Weigel HJ, Jager (1980) Subcellular distribution and chemical form of cadmium in bean plants. Plant Physiol. 65: 480-482.

Wells JM, Brown DH (1987) Factors affecting the kinetics of intra- and extracellular cadmium uptake by the moss Rhytidiadephus squarrosus. New Phytol. 105: 123-137.

Wells JM, Brown DH (1990) Ionic control of intracellular and extracellular Cd uptake by the moss Rhytidiadelphus squarrosus. New Phytol. 116: 541-553.

Whitton BA (1970) Toxicity of heavy metals to algae: A review. Phykos 9: 116-126.

Whitton BA (1984) Algae as monitors of heavy metals in freshwaters. In: Shubert LE (ed.) Algae as Ecological Indicators, Academic Press, London, pp. 257-280.

Williams LC, Mount DI (1965) Influence of zinc on periphytic communities. Am. J. Bot. 52: 26-34.

Wilson DE (1978) An equilibrium model describing the influence of humic materials on the speciation of Cu^{2+}, Znl2+, and Mn^{2+} in freshwaters. Limnol. Oceanogr. 23: 499-507.

Wolterbeck H Th (1987) Cation exchange in isolated xylem cell walls of tomato. 1. Cd^{2+} and Rb^{2+} exchange in adsorption experiments. Plant Cell Env. 10: 39-44.

Wnoroski AU (1991) Selection of bacterial and fungal strains for bioaccumulation of heavy metals from aqueous solutions. Water Sci. Technol. 23: 309-318.

Wong PK, Chang L (1991) Effects of copper, chromium and nickel on growth, photosynthesis and chlorophyll a synthesis of Chlorella pyrenoidosa 251. Env. Pollut. 72: 127-140.

Wong PTS, Chau YK, Luxon PL (1978) Toxicity of a mixture of metals on freshwater algae. J. Fish. Res. Board Can. 35: 479-481.

Wong PTS, Chau YK, Patel D (1983) Physiological and biochemical responses of several freshwater algae to a mixture of metals. Chemosphere 11: 367-376.

Wong PTS, Mayfield CI, Chau YK (1980) Cadmium toxicity to phytoplankton and microorganisms. In: Nriagu JO (ed.) Cadmium in the environment, Part I, John Wiley & Sons Inc., New York, pp. 572-585.

Wu L, Thurman DH, Bradshaw AD (1975) The uptake of copper and its effect upon respiratory processes of roots of copper-tolerant clones of Agrostis stolonifera. New Phytol. 75: 225-229.

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