CHITOSANS AS SOIL AMENDMENTS FOR THE REMEDIATION OF METAL CONTAMINATED SOIL

Azlan Kamari

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College of Science and Engineering
University of Glasgow

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Haji Kamari Haji Salehuddin
&
Hajjah Siti Janainah Haji Sidek

Love & Inspiration…

ABSTRACT

Research was conducted to evaluate the potential of chitosan, a fishery waste-based material, and its derivative cross-linked chitosans, as soil amendments for the remediation of metal contaminated land. This research comprised modification of chitosan followed by a characterisation study, a batch sorption study, two pot experiments and a biodegradation study. Chitosan was modified with three cross-linking reagents, namely glutaraldehyde (GLA), epichlorohydrin (ECH) and ethylene glycol diglycidyl ether (EGDE). The characterisation study used X-ray diffraction (XRD), scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) and Fourier transform infrared spectroscopy (FTIR) methods to investigate the effect of cross-linking treatment on the surface and physical properties of chitosan, the effect of metal interaction on the surface properties of chitosan and cross-linked chitosans, and the binding mechanism(s) of metal ions onto the chitosans. Cross-linking treatments on chitosan enhanced its chemical stability in acidic media and increased its BET surface area. Metal interaction reduced the crystallinity and changed the surface morphology of the chitosans. FTIR analysis revealed that the complexation of metal ions was through dative covalent interaction with the amino and hydroxyl groups of the chitosans. The batch sorption study evaluated the ability of chitosan and cross-linked chitosans to bind heavy metals. The effects of contact time, initial metal concentration and background electrolyte on metal binding were assessed. The binding behaviour was described by several kinetic and isotherm models. The maximum binding capacity (Q) values, estimated using the Langmuir isotherm model for the chitosans were comparable with other low-cost sorbents reported in the literature. The sorption-desorption study showed that the chitosans were able to retain metal ions on their surfaces, even at dilution factor of x11. The pot experiments evaluated the effectiveness of chitosan and chitosan-GLA in immobilising heavy metals in the contaminated soil. Their effects on plant growth and metal accumulation in plant tissue were determined using *Lolium perenne* (perennial ryegrass) and *Brassica napus* (rapeseed). For perennial ryegrass, the results were dependent on the rate of addition of the chitosans. Low application rates (up to 1% w/w) resulted in an increase in metal uptake, whereas 10% (w/w) addition decreased metal uptake. For rapeseed, metal uptake was decreased at all rates of application of chitosans. The ammonium acetate extractable metals in soil decreased following application of chitosan and plant growth. The biodegradation study measured microbial breakdown of the chitosans in both non-contaminated and contaminated soils. It was estimated that a longer period is required to complete the breakdown of the cross-linked chitosans (up to approximately 100 years) than unmodified chitosan (up to approximately 10 years). The influence of biodegradation on the bioavailable fraction of heavy metals in soil was studied concurrent with the biodegradation trial. It was found that the binding behaviour of chitosan for heavy metals in soils was not affected by the biodegradation process.

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AUTHOR'S DECLARATION

Except where specific reference is made to other sources, I certify that the work presented in this PhD thesis is entirely my own work. It has not been submitted, in part or in whole, for any other degree. Some of the results may have been published elsewhere.

Azlan Kamari

ABBREVIATIONS

AAS	Atomic Absorption Spectrometer
BCF	Bioconcentration factor
BET	Brunauer-Emmett-Teller
BJH	Barrett, Joyner and Halenda
CEC	Cation exchange capacity
CRM	Certified reference material
DOM	Dissolved organic matter
ECH	Epichlorohydrin
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy Dispersive X-ray
EEA	European Environment Agency
EGDE	Ethylene glycol diglycidyl ether
ESCA	Electron Spectroscopy for Chemical Analysis
FAO	Food and Agriculture Organization
FTIR	Fourier Transform Infrared
GLA	Glutaraldehyde
LSD	Least significant difference
MAC	Maximum Allowable Concentrations
MOSTE	Ministry of Science, Technology and the Environment Malaysia
NRSC	Natural Resources Conservation Service
SEM	Scanning Electron Microscopy
SGV	Soil Guideline Value
SRM	Standard reference material
TAV	Trigger Action Value
TF	Translocation factor
UKEA	UK Environment Agency
USDA	United States Department of Agriculture
USEPA	US Environmental Protection Agency
WHO	World Health Organization
XRD	X-ray Diffraction

1 INTRODUCTION

1.1 Rationale

This research was initiated as a direct response to the increase of soil contamination by heavy metals in Malaysia, and a number of cases related to this problem will be discussed in Sections 1.2.2 and 1.2.3. Solid waste disposal is becoming a major issue in Malaysia. For example, fishery industry in Malaysia generates approximately 70,000 tonnes of shellfish waste each year (see Section 1.5.2). The use of waste-based materials for environmental conservation has been stressed under Malaysia's Green Strategies of the National Policy on the Environment (MOSTE, 2002). Chitosan, a fishery waste-based material, has received much attention from local researchers in this context. To date, no specific study has been reported on the efficacy of chitosan and/or its derivatives as immobilising agents for heavy metals in contaminated soil. Therefore, this research was undertaken to evaluate the potential of chitosan and cross-linked chitosans as soil amendments for the remediation of metal contaminated land.

1.2 Soil contamination by heavy metals

1.2.1 Metal contaminated soil: A worldwide problem

Soil contamination by heavy metals is a worldwide environmental problem. For example, the UK Environment Agency (UKEA) reported that metals and metalloids were the main contaminants affecting 669 sites in England and Wales in 2007 (UKEA, 2008). Meanwhile, 6000 sites in Denmark were identified as being affected with metals in 2005 (Jensen *et al.*, 2009). Based on a survey involving 25 areas in 11 provinces in China, about 14,000,000 ha were identified to be contaminated by heavy metals, particularly As and Cd (Sun *et al.*, 2009).

A statistic published in 2007 by the European Environment Agency (EEA) revealed that soil and groundwater in Europe were mainly affected by heavy metals (> 37.3% of cases), with mineral oils, polycyclic aromatic hydrocarbons (PAH) and aromatic hydrocarbons (benzene, toluene, ethylbenzene and xylene) being responsible for 33.7%, 13.3% and 6.0% of cases respectively (EEA, 2007).

In the USA, of 1200 contaminated sites listed on the National Priority List (NPL) for immediate treatment, approximately 720 sites are contaminated by toxic heavy metals (USEPA, 1992). About 108 sites were affected by Pb, 79 sites were contaminated by Cr, while Cd and Cu were identified as the main contaminants at 58 and 50 sites (Evanko and Dzombak, 1997; Mulligan *et al.*, 2001).

1.2.2 Sources of soil contamination: Contribution vs. Challenge

In general, soil contamination can occur either by a point or a diffuse source (Pulford and Flowers, 2006; Vamerali *et al.*, 2010). A point source of contamination comes from a single, identifiable origin and affects a relatively restricted or localised area (Pulford and Flowers, 2006). Abandoned metal working facilities, accidental release or spillage of contaminants, and inappropriate municipal and industrial waste disposal are frequently associated with localised contaminated soils (French *et al.*, 2006; Vamerali *et al.*, 2010; Pichtel and Bradway, 2008).

In the metal manufacturing, refining and finishing industries, localised contamination may result from leaks or spillages of effluent drains and on-site disposal of sludges (DOEUK, 1995). For example, in primary Pb production, the collapse of a reverberatory furnace causes the spillage of slag and molten Pb onto the ground, therefore contaminating the operation site (DOEUK, 1995).

On the other hand, a diffuse source does not come from one specific source and its effects are usually seen over a large area (Pulford and Flowers, 2006). Atmospheric deposition and flowing water are the main pathways of diffuse soil contamination (Vamerali *et al.*, 2010). For example, Kayser *et al.* (2000) and Keller *et al.* (2003) noted that approximately 4.5 km^2 in Dornach, Switzerland have been contaminated with heavy metals through atmospheric emissions from nearby metal smelters. Kayser *et al.* (2000) measured 673 mg/kg Zn, 516 mg/kg Cu and 2.5 mg/kg Cd in soil, sampled at 300 m from a brass smelter in Dornach.

Besides their natural occurrence in the parent rocks, specific sources of heavy metals in soils are from anthropogenic activities such as mining, industry and agriculture. The concentration of heavy metals in soils has increased tremendously due to rapid global industrialisation. Nowadays, it is common that the concentrations of metals in soils, particularly in the vicinity of industrial areas, exceed the mean background concentrations of metals in surface soils, Maximum Allowable Concentrations (MAC) and

Trigger Action Value (TAV) for metals in agricultural soils, and, Soil Guideline Value (SGV) for residential and commercial areas. These values are listed in Table 1.1.

Table 1.1 Mean background concentrations of metals in surface soils, MAC and TAV values for metals in agricultural soils, and, UK SGV for residential and commercial areas (mg/kg).

Metal	Background[a]	MAC[a]	TAV[a]	SGV-Res.[b]	SGV-Com.[b]
As	4.7	15 – 20	10 – 65	20	500
Cd	1.1	1 – 5	2 – 10	2	1400
Cr	42	50 – 200	50 – 450	130	5000
Cu	14	60 – 150	60 – 500	-	-
Pb	25	20 – 300	50 – 300	450	750
Zn	62	100 – 300	200 – 1500	-	-

Note: [a] Kabata-Pendias and Mukherjee (2007); [b] UK SGV, SGV-Res.: SGV for residential area – with plant uptake, SGV-Com.: SGV for commercial area (Pulford and Flowers, 2006).

1.2.2.1 Mining

Mining is a major source of soil contamination with heavy metals (Kumpiene *et al.*, 2007; Fornes *et al.*, 2009). Metal mining not only extracts metals, but also generates immense quantities of tailings, a by-product of metal extraction. For example, Forsberg *et al.* (2009) mentioned that approximately a 10 km^2 area in Gällivare, Sweden was covered with tailing deposits as a result of copper mining at Aitik Cu mine. Mine tailings generally contain a considerable amount of metals. A study by Schwab *et al.* (2007) measured 17,850 mg/kg Zn and 125 mg/kg Cd in mine tailings sampled from a former mining area in Southeast Kansas, USA. Ye *et al.* (1999) analysed mine tailings at Lechang Pb/Zn mine, China and reported that the tailings contained 1042 mg/kg Pb and 2131 mg/kg Zn.

A study by Roongtanakiat *et al.* (2009) on mine tailings derived from a mining area in Tak, Thailand reported 5039 mg/kg Zn in the tailings. Meanwhile, Rotkittikhun *et al.* (2007) reported that mine tailings of Bo Ngam Pb mine in Kanchanaburi, Thailand contained 9020 mg/kg of Pb. Cd, Cu, Pb and Zn are often present together in ore minerals. Therefore, they can be found in the surrounding environment of mining sites. For example, a study carried out at Nenthead Pb/Zn mine, UK by Sneddon *et al.* (2008) found 32,300 mg/kg Zn, 8900 mg/kg Pb, 130 mg/kg Cu and 60 mg/kg Cd in the mine soils.

Tailings dam failure is one of the common mining accidents that contaminate soil significantly. On 25 April 1998, a tailings dam at Los Frailes pyrite mine in Aznalcóllar, Spain was breached and released about 6 million m^3 of tailings containing heavy metals into the Agrio and Guadiamar Rivers, and affected the Doñana National Park (Cabrera *et al.*, 1999). It was reported that approximately 4500 ha of agricultural land was contaminated by As, Cd, Cu, Pb and Zn (Madejón *et al.*, 2006; Cabrera *et al.*, 1999). Cabrera *et al.* (1999) analysed soil samples collected from the Guadiamar River bank and the mean concentrations of heavy metals were reported as 2878 mg/kg for As, 25.1 mg/kg for Cd, 1552 mg/kg for Cu, 7888 mg/kg for Pb and 7096 mg/kg for Zn. About 10 years after the incident, Fornes *et al.* (2009) measured 60 mg/kg As, 229 mg/kg Pb and 113 mg/kg Zn in the soil samples collected from the affected Guadiamar River bank.

As reported by the WHO (2001), the failure of a tailings dam at Nakhon Si Thammarat, Thailand in 1993 has caused serious As contamination of topsoil and groundwater over a 40 km^2 area, and the contamination was estimated to last for 40-50 years. Moreover, the As levels in the contaminated topsoil were reported 20-100 times higher than the guideline value set by local authorities, while the levels in the groundwater wells were about 50-100 times higher than the guideline value recommended by the WHO for drinking water (WHO, 2001). Truck spillage, is another kind of soil contamination deriving from mining activities. For example, on 2 June 2000 a truck from Yanacocha gold mine in Peru spilled more than 150 kg of Hg, a secondary product of the mine, onto a 43 km stretch of road (Earthworks and Oxfam America, 2004).

Mining waste can spread to the surrounding and lowland areas by erosion caused by rain and wind. Rahim *et al.* (2006; 2008) studied metal dispersion from a former Sn mine located at the base of Muntahak Mountain in Pelepah Kanan, Malaysia to the lowland areas. They noted that the lowland areas were significantly contaminated with heavy metals derived from the mine tailings. The highest soil metal concentrations were found to be 11,956 mg/kg for Mn, 4753 mg/kg for Cu and 699 mg/kg for Zn. A metal contamination study in an agricultural area, which was located about 2 km from an abandoned mining site in La Unión, Murcia, Spain by Clemente *et al.* (2007) found 3235 mg/kg Pb and 2706 mg/kg Zn in the soil samples. The high level of Pb and Zn was discussed due to the transport of fine particles by wind.

Mining operations can also contaminate nearby agricultural soils and food crops via fluvial transport. For example, Murtedza and Lee (1985) investigated metal concentrations in rice grown on a farmland located very close to Mamut River, which was contaminated by effluent from Mamut Cu mine in Sabah, Malaysia. They found that the levels of Cd, Cu, Pb and Zn exceeded the permissible limit of the Malaysian Food

Act 1983. Hao *et al*. (2010) reported that the spread of mining waste of Beishan Pb/Zn mine to a nearby farmland was through fluvial transport in the headwater of the Huanjiang River, China. They measured 817 mg/kg Pb and 614 mg/kg Zn in the topsoil samples of the farmland.

Mining is a long-term source of contamination. For example, about four years after the closure of the Mamut Cu mine, Ali *et al*. (2004; 2006) studied the levels of heavy metals in soil and herb plants found along the Mamut riverbank. They noted that the soil samples, which were collected at 0, 5 and 10 m from the edge of the river, remained highly contaminated with heavy metals, especially Pb (9180 mg/kg) at 0 m. They also reported that Cd was the main contaminant accumulated by three herb plant species, *Curculigo latifolia*, *Selaginella plana* and *Clidemia hirta*. Pulford *et al*. (2009) studied river sediment contamination as affected by metal dispersion from a disused Pb/Zn mine at Tyndrum, UK and measured 11,000 mg/kg Pb and 30,000 mg/kg Zn in the sediment sampled from the immediate vicinity of the mine. The production of Pb and Zn from the mine had stopped for over 80 years (MacKenzie and Pulford, 2002).

Statistical data on world metal mining in 1979, 2000 and 2003 are shown in Table 1.2, and revealed the great demand of metals in manufacturing and industrial sectors. Cr and Cu are the two metals most mined since 1979, followed by Zn and Pb. The production of high commercial value metals such as Cr, Cu, Zn and Pb rose consistently during the period of 1979 to 2003. Although there is no statistical data on world mining projection, it can be speculated that the production of these metals will continue to increase. Of course, their intensive production will pose a great impact to environmental quality and create a strenuous challenge to environmental scientists to overcome the environmental issues.

Table 1.2 World metal mining in 1979, 2000 and 2003.

Metal	1979[a]	2000[b]	2003[b]
As	36.2 kt	27.9 kt	38.8 kt
Cd	18.28 kt	19.21 kt	16.87 kt
Cr	10.50 Mt	13.91 Mt	15.83 Mt
Cu	7.61 Mt	13.23 Mt	13.68 Mt
Pb	3.5 Mt	3.1 Mt	6.8 Mt
Zn	5.98 Mt	8.73 Mt	9.17 Mt

Note: kt – thousand tonnes; Mt – million tonnes; [a] Adriano (1986); [b] Kabata-Pendias and Mukherjee (2007).

1.2.2.2 Industry

Smelting of metal ores has resulted in serious contamination of soils in the immediate vicinity of the smelter sites and the surrounding areas. The atmospheric metal emissions generated by smelters and metallurgical plants contaminated the surrounding soils through atmospheric deposition and sedimentation (Keller *et al.*, 2003; Sterckeman *et al.*, 2000). The airborne emissions from smelters normally contain heavy metals, as well as sulfur dioxide (SO_2), the prime source of acid rain. Therefore, such activities have significant contribution to the loss of environmental quality.

A study by Basta and McGowen (2004) found 69,200 mg/kg Zn, 5150 mg/kg Pb, 1090 mg/kg Cd and 152 mg/kg As in surface soil (0-20 cm) of an inoperative smelter site in Northeastern Oklahoma, USA. In a study evaluating metal contamination in the vicinity of a former Pb/Zn smelter in Arnoldstein, Austria, Puschenreiter *et al*. (2001) reported that the soil samples contained 12,300 mg/kg Pb, 2710 mg/kg Zn and 19.7 mg/kg Cd.

Sterckeman *et al*. (2000) noted that soils in Northern France were heavily contaminated with heavy metals due to smelting at a Pb/Zn smelter in Noyelles-Godault and at a Zn smelter in Auby. The concentrations of heavy metals measured in the soils were 31,175 mg/kg for Zn, 5410 mg/kg for Pb and 305 mg/kg for Cd. A study of soil contamination by Fernandez-Turiel *et al*. (2001) found maximum levels of Pb (8714 mg/kg), Zn (1637 mg/kg), Cr (94.1 mg/kg) and Cd (30.68 mg/kg) within the vicinity of a Pb smelter in Lastenia, Argentina.

Metal concentrations of three soils collected in the vicinity of a Cu smelter in Anaconda and Deer Lodge Valley in Montana, USA was analysed by Burt *et al*. (2003). They noted that the highest concentrations of Cu (1270 mg/kg), Zn (849 mg/kg), Pb (475 mg/kg) and Cd (9.9 mg/kg) were measured at the Beaverell site, which was located about 1 km from the smelter. Kachenko and Singh (2006) studied metal concentrations in two soils contaminated by metal smelting at Boolaroo and Port Kembla of New South Wales, Australia. At Boolaroo, the highest Cd, Cu, Pb and Zn concentrations were measured as 27.5, 223, 1626 and 4014 mg/kg, while 1.2 mg/kg Cd, 1032 mg/kg Cu, 430 mg/kg Pb and 1947 mg/kg Zn were analysed in Port Kembla soils.

Metal contamination in soils near the Daye Cu/Au smelter in China was assessed by Yan *et al*. (2007). The Cd concentrations in topsoil samples were reported in the range of 8.1 to 20.7 mg/kg, which far exceeded the Chinese limit level of 1.0 mg/kg. Karczewska *et al*. (2009) analysed soil samples within vicinities of smelters in Legnica and Głogów,

Poland, and measured 395 mg/kg of Cu and 212 mg/kg of Pb in soils. A study at a former agricultural field situated 500 m from a Zn smelter in Lommel, Belgium by Ruttens *et al*. (2010) found 240 mg/kg Pb, 226 mg/kg Zn and 6.3 mg/kg Cd in the topsoil (0-25 cm) samples. Earlier, Geebelen *et al*. (2006) measured 7693 mg/kg Zn, 1293 mg/kg Pb, 853 mg/kg Cu, 92 mg/kg As and 31 mg/kg Cd in soils sampled within the vicinity of the Lommel Zn smelter.

Sallaku *et al*. (2009) evaluated the degree of metal contamination around a metallurgical plant in Elbasan, Albania and found that metal concentrations in topsoil (0-20 cm) samples decreased with increasing distance from the metallurgical plant. They measured 159 mg/kg Cu, 147 mg/kg Zn and 2.3 mg/kg Cd at 1 km distance from the metallurgical plant, meanwhile at 14 km distance, 50 mg/kg Cu, 86 mg/kg Zn and 0.8 mg/kg Cd were analysed.

Contributions from battery recycling and metal-based industries are also important. An investigation by Brown and Elliott (1992) found 211,271 mg/kg Pb, 1383 mg/kg Cu, 655 mg/kg Zn and 332 mg/kg Cd in soil collected from a battery recycling plant in Pennsylvania, USA. Meanwhile, Cui *et al*. (2004) measured 31,000 mg/kg Pb and 480 mg/kg of Zn in surface soil near a battery recycling factory in Anhui, China. Jensen *et al*. (2009) reported that soil collected from Copenhagen Recycling Center, Denmark contained 3000 mg/kg Zn, 1000 mg/kg Cu, 500 mg/kg Pb and 15 mg/kg Cd.

Marchiol *et al*. (2007) measured 1527 mg/kg Cu, 980 mg/kg Zn, 309 mg/kg As, 50.9 mg/kg Co and 4.29 mg/kg Cd in soil sample of Caffaro chemical plant, Torviscosa, Italy. A study at an abandoned Pb-based explosives manufacturing and testing site in New Jersey, USA by Cooper *et al*. (1999) found 3212 mg/kg Pb in the soil. Blaylock *et al*. (1997) conducted a study at a former cable manufacturing site in Bayonne, New Jersey, USA and reported that the site was contaminated by Pb with a concentration of 1200 mg/kg.

1.2.2.3 Agriculture

Applications of fertilisers and pesticides, as well as irrigation of agricultural soils using wastewater have contributed to elevated levels of heavy metals in soil. Taylor (1997) assessed accumulation of Cd in New Zealand topsoils (0-15 cm) as affected by application of phosphate fertiliser. He compared the concentrations of Cd in 58 topsoil samples collected in 1940s-1960s to topsoil samples taken in 1997 from the same sites, and reported that the $CaCl_2$-extractable Cd increased by approximately 450%.

The extent of heavy metal contamination of soils and crops in Peninsular Malaysia was investigated by Zarcinas *et al*. (2004). They analysed 241 topsoils (0-15 cm) sampled from cultivated, forested and uncultivated regions, and 188 crop samples collected from agricultural regions. Due to positive correlation between heavy metals (As, Cd and Cu) and both total and bioavailable phosphorus in soil, they attributed the increase in As, Cd and Cu concentrations in the cultivated soils to addition of phosphate fertiliser to the agricultural soils. They also noted that the Cd concentration in cocoa was found to exceed the Malaysian Food Act 1983 permissible limit of 1.0 mg/kg, while the Cd level in groundnut and mustard seeds was above the Australian Maximum Levels of 0.1 mg/kg.

Over a century, copper-based fungicide such as Bordeaux mixture, a mixture of copper(II) sulfate and lime, has been used widely in fruit orchards, vegetable farms, gardens and nurseries to prevent infestations of fungi, primarily *Plasmopara viticola* (downy mildew) (Wightwick *et al*., 2008; Wang *et al*., 2009). After application on plants, the Cu residue will be bound to the organic matter, fine clay and minerals in soil (Epstein and Bassein, 2001), and will persist in the topsoil for hundreds of years. For example, Mirlean *et al*. (2007) studied metal concentration in vineyard soils in Rio Grande do Sul State, Brazil with 100 years history of Cu-based fungicide application and reported that the soils contained up to 3200 mg/kg of Cu.

In an earlier study, Flores-Vélez *et al*. (1996) reported approximately 100 to 1500 mg/kg of Cu accumulated in Beaujolais vineyard soils, France, of which Bordeaux mixture has been used for more than 100 years. Meanwhile, a study by Wightwick *et al*. (2008) on 98 vineyards across Australia, with 40 to 100 years of Cu fungicide usage record, demonstrated Cu concentrations ranged from 24 to 159 mg/kg. The lower Cu concentrations measured in Australian vineyard soils as compared to those reported in European vineyards, was ascribed to the lower usage of Cu fungicide.

An investigation by Nayek *et al*. (2010) found that metal concentrations in wastewater irrigated agricultural soil were much higher than those analysed in groundwater irrigated soil (control soil). The metal concentrations in wastewater irrigated soil were reported as 752 mg/kg Pb and 506 mg/kg Cr, whereas 29 mg/kg Pb and 109 mg/kg Cr contained in the control soil. The elevation of metal concentrations in agricultural soil was explained due to high metal concentrations in wastewater obtained from Mangalpur industrial complex in West Bengal, India. For example, they noted that the concentrations of Cr (0.69-0.83 mg/L) in wastewater exceeded the FAO irrigation standards of 0.1 mg/L for Cr.

Kisku *et al*. (2000) assessed the effect of industrial effluents irrigation to an agricultural land in Kalipur, India on heavy metal accumulation in soils. They reported that irrigation of industrial effluents increased metal concentrations in soils, especially for Cd and Pb. For example, it was reported that the Cd concentration in non-irrigated effluent soils ranged from 1.2 to 2.8 mg/kg, while in soils irrigated with industrial discharge ranged from 6.7 to 13.6 mg/kg. A similar observation was reported by Mapanda *et al*. (2007), who studied the levels of metal contamination in soils and vegetables at a vegetable farm with a history of 10 years irrigation using industrial effluents in Harare, Zimbabwe. In fact, the mean concentration of Pb in the leaf tissues of *Brassica juncea* (Indian mustard) was found to exceed the permissible limit recommended by FAO/WHO.

1.2.2.4 Other sources

Pb-based paint and leaded gasoline are two major sources of Pb in soil and dust. Parra *et al*. (2008) analysed metal concentrations in topsoil samples collected from a firing range in New Mexico and from a residence in Massachusetts, USA that had a history of Pb-based paint application. The Pb concentration in munition-derived soil was found to be 9600 mg/kg, while 1100 mg/kg Pb was analysed in paint-derived soil. An analysis on metal concentrations in firing range soil of the Small Arms Training Area, Aiken, USA by Wilde *et al*. (2005) found 3282 mg/kg Pb and 1762 mg/kg Cu.

According to Preciado and Li (2006), the highway system is a potential source of heavy metal contaminants to the environment through natural mechanisms such as atmospheric dust deposition and the hydrologic cycle. An investigation at two highway sites in Vancouver, Canada by Preciado and Li (2006) found that Pb was the metal most accumulated in the roadside soil with a maximum concentration of 1000 mg/kg. A similar observation was reported by Ramlan and Badri (1989). They measured 2466 mg/kg Pb, 344 mg/kg Zn and 2.96 mg/kg Cd in roadside soils of Kuala Lumpur, Malaysia.

An analysis of surface soil (0-5 cm depth) at a chemical waste dump at Merton Bank, Merseyside, UK by Clemente *et al*. (2010) found 975 mg/kg Pb and 456 mg/kg As. Kasassi *et al*. (2008) studied metal contamination at a closed unlined landfill in Thessaloniki, Greece by measuring metal concentrations at different soil depths (2.5-17.5 m). The concentrations of heavy metals measured were found to be 6.38-343.75 mg/kg for Zn, 8.13-356.25 mg/kg for Cu, 3.88-171.88 mg/kg for Cr and 0.50-18.75 mg/kg for Cd, with the highest metal concentrations being found at depths over 2.5 m. A study by Meers *et al*. (2005) at a disposal site at Meigem, Belgium found 907 mg/kg Zn, 178 mg/kg Pb and 8.8 mg/kg Cd in the surface soil samples.

Soil contamination by heavy metals at Dhapa landfill, India was studied by Bhattacharyya *et al*. (2008). They analysed topsoils (0-15 cm depth) collected from the landfill site and measured 389 mg/kg Pb, 261 mg/kg Zn and 7.1 mg/kg Cd in the soils. The concentration of heavy metals in topsoil (0-20 cm depth) samples of seven landfill sites in Kuala Lumpur, Malaysia was assessed by Rahman *et al*. (2000), who reported the Cd concentration ranged from 4.13 to 7.28 mg/kg, which was beyond the target level of 0.8 mg/kg. They reported that six sites were high in Ag with concentration ranged from 9.2 to 20.9 mg/kg, exceeded the common soil concentration of 0.1 mg/kg for Ag in Malaysia.

The level of heavy metals in soils may also increase by the disposal of ash residue from thermal power plants (Nriagu and Pacyna, 1988). For example, a study by Jambhulkar and Juwarkar (2009) at Khaperkheda thermal power station in Maharashtra, India measured 449 mg/kg Mn, 40.70 mg/kg Zn and 4.78 mg/kg Cd in fly ash. Meanwhile, Rau *et al*. (2009) reported that fly ash of Badarpur thermal power plant in Delhi, India, contained 102.68 mg/kg Zn, 77.26 mg/kg Pb and 56.61 mg/kg Cu. An analysis on coal fly ash produced by coal and wood combustion at Öresundskraft, Sweden by Kumpiene *et al*. (2007) found 425 mg/kg Zn, 71 mg/kg Cu, 32 mg/kg Pb and 2.4 mg/kg Cd.

1.2.3 The impact of soil contamination by heavy metals: A global concern

Soil contamination by heavy metals is of great concern with respect to human health risks, groundwater contamination, phytotoxicity to plants, adverse effects on microbial activity and diversity, long-term effects on soil fertility and depreciation of land (Huang and Cunningham, 1996; Kayser *et al*., 2000; Castaldi *et al*., 2009).

1.2.3.1 Health risks and food safety

Intake of heavy metals via the crop-soil system has been regarded as the predominant pathway of human exposure to toxic metals (Liu *et al*., 2007), and is normally chronic (USDA/NRCS, 2000). Wu *et al*. (2010) has estimated that over 8,000,000 ha of farmland in China were contaminated by heavy metals such as Cr, Pb, Sn and Zn. Li *et al*. (2006) conducted a field survey on heavy metals contamination in vegetables cultivated on an agricultural farm affected by mining and metal smelting, in Shuichuan, China. They reported that leafy vegetables (cabbage, turnip mustard, spinach and Chinese cabbage) accumulated much higher amounts of metals than non-leafy vegetables (cucumber, pumpkin, eggplant and capsicum). The highest concentrations of Pb (17.4 mg/kg), Cd

(1.3 mg/kg) and As (1.9 mg/kg) were analysed in Chinese cabbage, and were found to exceed the Chinese permissible limit of 0.2 mg/kg for Pb, 0.05 mg/kg for Cd and 0.5 mg/kg for As in vegetables.

Yan *et al*. (2007) studied metal concentrations in Chinese cabbage grown on a farmland located near Daye smelter in China and found mean concentrations of Pb (3.28 mg/kg) and Cd (0.21 mg/kg) exceeded the Chinese tolerance limit levels for vegetables. Liu *et al*. (2007) evaluated the uptake of heavy metals by rice grown on a contaminated farmland near Zhengzhou City, China, and the order of metal accumulation in rice plant was found to be root > straw > grain. For example, the mean concentrations of Pb were found to be 7.14 mg/kg (root), 2.05 mg/kg (straw) and 0.53 mg/kg (grain).

A study by Rahman *et al*. (2008) found that rice plants accumulated approximately 1.8 mg/kg As in straw, 1.6 mg/kg As in husk and 0.4 mg/kg As in grain when cultivated on a As contaminated paddy field (14.5 mg/kg) in Bangladesh. The daily intake of As was estimated to be 0.164-0.266 mg per kg body weight, about 3-5 times higher than the maximum tolerable value of 0.05 mg per kg body weight set by FAO/WHO. Moreover, the rice straw and husk produced from the paddy field were found to be not suitable for use as cattle feed because the As concentrations exceeded the guideline value of 0.2 mg/kg.

A study by Robinson *et al*. (2000) on Cd accumulation by Tangoio (*Salix matsudana x S. alba*), a willow clone that commonly used for supplementary stock fodder in New Zealand (Granel *et al*., 2002), found a shoot Cd concentration of 14 mg/kg in the dry leaves when grown on a soil containing just 0.6 mg Cd/kg. Therefore, they concluded that there was a potential adverse effect to the productivity of livestock such as sheep and cattle, and risk to human health.

A study on metal concentrations in crops sampled from agricultural fields of Mondragón, Tuero Chico, Tasapampa and Satomayor, along the Río Pilcomayo basin of Southern Bolivia by Miller *et al*. (2004) found that a sample of onion at Satomayor exceeded the Cd guideline for root vegetables, while the Cd, Pb and Zn contents in a lettuce sample at Tuero Chico were above the guideline values. High metal concentrations in soils and crops were explained as arising from contamination from both historic and modern mining operations within the Potosí mining district of Southern Bolivia. Ruttens *et al*. (2010) reported that in 2009 several vegetable crops such as carrot and black salsify were confiscated by Belgian Federal Agency for Food Safety due to high levels of Cd.

Nayek *et al*. (2010) analysed metal concentrations in tomato, pea, common bean and spinach sampled from an agricultural field irrigated with industrial wastewater. They reported that the concentrations of Cr (34-131 mg/kg), Cd (6-42 mg/kg) and Pb (6-36 mg/kg) in edible parts of the crops were much higher than the permissible limits of 5 mg/kg for Cr and Pb, and 0.3 mg/kg for Cd recommended by FAO/WHO for vegetable and fruit products. Kachenko and Singh (2006) investigated metal contamination in vegetable crops at two growing regions within close proximity to Boolaroo Pb/Zn smelter and Port Kembla Cu smelter of New South Wales, Australia. They reported that 95% of the vegetables collected from Boolaroo exceeded the Australian and New Zealand Food Authority maximum limit of 0.1 mg/kg fresh weight (FW) for Cd and Pb, and noted mint accumulated both metals with the highest mean concentrations of 1.89 mg/kg FW (Cd) and 43.0 mg/kg FW (Pb).

Ang and Ng (2000) studied metal contamination in mango, guava and papaya grown on ex-mining land in Bidor, Malaysia, and found 5.20-8.74 mg/kg Zn, 0.63-8.71 mg/kg Pb and 0.02-0.26 mg/kg Hg in the fruit samples. Both Pb and Zn concentrations in all fruit samples were reported to exceed the Malaysian Food Act 1983 permissible limits of 5.0 mg/kg for Zn and 0.5 mg/kg for Pb, while mango and papaya were found to accumulate Hg with concentrations greater than that permitted. The Pb level in guava was measured to be 17 fold greater than the permissible limit.

In Glasgow, UK the operation of chromium chemical works and disposal of chromite ore processing residue during the period of 1830 to 1968 has caused severe land and groundwater contamination by hexavalent chromium (Cr(VI)) (Farmer and Jarvis, 2009; Graham *et al*., 2006). An environmental assessment at four former landfill sites in the southeast of Glasgow by Farmer *et al*. (1999) measured 8830 to 25,000 mg/kg of Cr and 290 to 4670 mg/kg of Cr(VI) in the soil (0-50 cm depth) samples, and up to 33.3 mg/L of Cr(VI) in the groundwater. The highest Cr(VI) concentration analysed in the groundwater, exceeds the maximum allowable concentration of Cr(VI) in drinking water of 0.05 mg/L (WHO, 2003), by 666 times. It is apparent that soil contamination by such carcinogenic Cr(VI) can pose significant risks to human health.

1.2.3.2 Soil fertility, microbial activity and plant growth

Metal contaminated soils are commonly associated with high levels of heavy metals, low contents of nutrient and organic matter, low water retention capacity and low cation exchange capacity. Therefore, in practical terms, they are not suitable for cultivation. Ang (1994) reported that the available phosphorus (0.4 mg/L) and potassium (0.03

meq/100 g) contents in Sn mine soil were much lower than forested soil (4.3 mg/L of P and 0.27 meq/100 g of K). The average Zn, Co and Cd concentrations in Sn tailings at a former Sn mine were reported to be 1360, 193 and 2.4 mg/kg (Rahim *et al.*, 2008). Because of unfavourable soil quality, metal contaminated soils are generally low in plant diversity. In Malaysia, land contaminated with Sn tailings is normally colonised with creeper and shrub species such as *Melastoma malabathricum* and *Nepenthes gracilis* (Ang, 1994; Rahim *et al.*, 2006; 2008).

The potential of ex-mining sites and Sn tailings in Malaysia for cultivation of agricultural crops, forest trees and timber species was evaluated by Ang (1994), Majid *et al.* (1994), Hariandra and Amin (2008) and Ho *et al.* (2008). They observed symptoms of nutrient deficiency in plants and noted that plants survived only after fertilisation treatment. Majid *et al.* (1994) reported that 400 g NPK fertiliser per seedling was the optimum fertilisation rate for early growth of *Acacia mangium*, a timber species, on Sn tailings. A study by Ho *et al.* (2008) on chicken manure treatment on Sn tailings obtained a greater biomass yield of *Hibiscus cannabinus* (kenaf) at a higher rate of manure application. Overall, a high amount of fertiliser was required to improve the fertility of such contaminated soils for tree planting.

Soil microorganisms play a major role in organic matter decomposition, nutrient cycling and mineralisation in soils (Castaldi *et al.*, 2009; Liao and Xie, 2007). For example, urease is an enzyme that catalyses the hydrolysis of urea into carbon dioxide and ammonia, and is a key component in the nitrogen cycle in soils (Wang *et al.*, 2009). Meanwhile, hydrolase enzymes such as phosphatase and invertase are important in the soil phosphorus and carbon cycles due to their significant role in removing phosphate groups from substrates and in the hydrolysis of sucrose respectively (Wang *et al.*, 2009). Therefore, microbial and enzyme activities are often used as biochemical parameters to quantify the adverse effects of various contaminants on soil quality (Wang *et al.*, 2009; Cardelli *et al.*, 2009; Liao *et al.*, 2010).

Cardelli *et al.* (2009) studied the effect of Cd contamination on microbial and enzyme activities in six agricultural soils of Tuscany, Italy. They found that the amount of CO_2-C evolved during the incubation study decreased by up to 75%, while arylsulfatase and catalase activities were inhibited by 44% and 45%. A similar observation was reported by Brandt *et al.* (2010) on the microbial activity in a 5 year old agricultural soil exposed to Cu-based fungicides in Denmark. The soil respiration in the Cu contaminated soil was found to be 50% lower than in the non-contaminated soil.

The effect of long-term application of Cu-based fungicides on soil microbial and enzyme properties in 5, 15, 20, 30 and 45 year old apple orchards at Rizhao City, China was evaluated by Wang *et al*. (2009). They reported soil mean microbial biomass carbon (C_{mic}) values ranging from 44 to 116 mg/kg in the orchard soils, while 144 mg/kg was measured in the forest soil (reference soil). The ratio of soil C_{mic} to total organic carbon (C_{org}) was found to decrease with increasing orchard age. Based on significant correlation between total soil Cu and soil C_{mic}, as well as with acid phosphatase activity, they concluded that the Cu input from the fungicide application reduced microbial biomass carbon, carbon mineralisation, respiration rate, and suppressed phosphatase activity.

The presence of heavy metals in soil can inhibit the growth and reproduction of plants. For example, a 4 month field experiment by Santala and Ryser (2009) found that the addition of furnace slag collected from a Cu/Ni smelter in Sudbury, Canada affected the growth of *Betula papyrifera* (white birch). They reported that the 0.5% and 2.5% (w/w) slag treatments reduced biomass production by 14% and 74% as compared to control treatment (0% w/w). They also noted that the stem diameters were 2.4, 2.3 and 1.4 mm for 0%, 0.5% and 2.5% (w/w) slag treatments. Fedorkov (2007) reported the long-term (15 years) effects of metal contamination on the tree diameter growth of Scots pine grown on forest soil to which metallurgical dust derived from a Cu/Ni smelter in Monchegorsk, Russia containing 41 mg/kg Cu and 150 mg/kg Ni was added. He found that the radial growth was retarded by 7% with 2.5 kg application of metallurgical dust per 100 m^2 after 15 years of cultivation, while 20 kg of dust decreased Scots pine growth by 54% over the same period.

Probst *et al*. (2009) evaluated the response of *Vicia faba* (broad bean) to metal toxicity by cultivating plants on mine tailings collected from an abandoned Pb/Zn mine at the Pyrénées Mountains, France, containing 14,548 mg/kg Zn, 7772 mg/kg Pb, 166 mg/kg Cu and 43 mg/kg Cd. They reported that the plant growth was retarded by 38% when grown on mine tailings, as compared to plants grown on uncontaminated soil. The toxicity of Cu on rice growth was studied by Xu *et al*. (2006). The production of rice grain was found to decrease by 10% when cultivated on soil containing 100 mg Cu/kg, while about 50% reduction in rice grain yield was observed when the soil Cu concentration was 300-500 mg/kg. They noted that a soil Cu concentration of 1000 mg/kg hindered the growth of the whole rice plant severely, with a total yield loss of 90%.

Lal *et al*. (2008) evaluated the production potential of three annual flower crops, marigold, chrysanthemum and gladiolus grown on Cd contaminated soil in Karnal, India. Growth parameters such as plant height, flower yield and flower diameter were

reported to decrease as a direct response to the biotoxicity of Cd. They observed a significant reduction in the yield of fresh flower at high soil Cd concentrations. For example, at a soil Cd concentration of 0.6 mg/kg, 913 chrysanthemum flowers were produced, while they recorded only 549 chrysanthemum flowers when the Cd level was 68.4 mg/kg. The mean values of Cd accumulation in marigold, chrysanthemum and gladiolus flowers were reported as 6.5, 4.0 and 7.2 mg/kg, respectively.

1.3 Mobility of heavy metals in soils

The fate and mobility of heavy metals in soil are greatly influenced by the chemical behaviour of the metals (Evanko and Dzombak, 1997; Álvarez-Ayuso and García-Sánchez, 2003), as well as the chemical and physical properties of the soil (Alloway, 2001; Bradl, 2004). The solubility, availability and mobility of heavy metals in soils are normally governed by several chemical reactions, as discussed by McBride (1994) (Figure 1.1). However, adsorption reactions through the formation of complexes with the surfaces of organic matter, oxides and clays, and precipitation reactions leading to the formation of insoluble precipitates such as hydroxides, carbonates and phosphates, are the two major mechanisms involved in the retention of heavy metals by soils (Evans, 1989; Bradl, 2004). Both reactions will maintain the mobility of heavy metals at minimum and as a consequence, this will provide a long-term source of metal contaminants in soil environments.

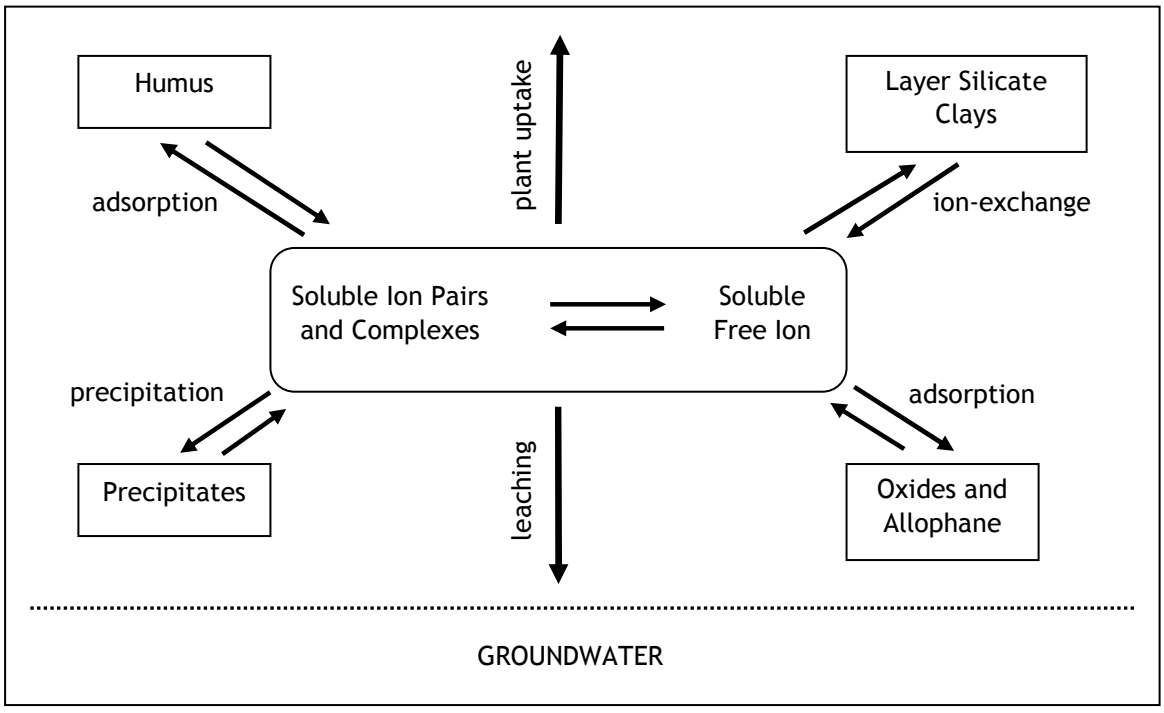


Figure 1.1 Dynamic interactive processes governing solubility, availability and mobility of heavy metals in soils (McBride, 1994).

1.3.1 Chemistry and behaviour of heavy metals in soil and soil solution

The term 'speciation' refers to the chemical forms or phases in which a heavy metal occurs in soils or soil solutions (McBride, 1994; Ure, 1990; Tack and Verloo, 1995). Sposito (1989) defined 'soil solution' as the aqueous liquid phase in soil, composed of a mixture of liquid water and dissolved substances such as sodium chloride and carbon dioxide. The amount and composition of metal species in soil solution are governed by several factors such as interaction with a highly diverse solid phase and uptake by plant roots (Kabata-Pendias and Mukherjee, 2007). In addition, soil pH, organic matter content, clay minerals, metal oxides, organic and inorganic ligands are also important in controlling the behaviour of metal contaminants in soil and soil solution.

Information on the speciation and the oxidation state of heavy metals in soil is vital in investigating the level of phytotoxicity as affected by metal contaminated soil, especially when different chemical forms of heavy metal differ in toxicity. For example, Chen *et al*. (2010) assessed the phytotoxicity of Cr to wheat seedlings grown on two Taiwanese acidic agricultural soils from Neipu and Pinchen, and reported that the oxidation state of Cr was influenced by the organic matter content of the soils. They observed no toxicity symptoms on wheat seedlings grown on the Neipu soil and this observation was explained as being due to the high content of organic matter, which was able to reduce hexavalent chromium (Cr(VI)) to trivalent chromium (Cr(III)). Pinchen soil was found to contain low organic matter, and was therefore unable to reduce Cr(VI) to Cr(III) completely. As a result, they observed a significant retardation of the wheat seedlings grown on the Pinchen soil.

Pb is found in ore minerals primarily as PbS (galena) and in smaller quantities as $PbCO_3$ (cerussite), $PbSO_4$ (anglesite) and $PbCrO_4$ (crocoite) (Fergusson, 1990; Mulligan *et al.*, 2001). The common ionic species of Pb in soil solutions are Pb^{2+}, $PbCl^{+}$, $PbOH^{+}$, $PbCl_3^{-}$ and $Pb(CO_3)_2^{2-}$ (Kabata-Pendias and Mukherjee, 2007). However, Pb^{2+} is the stable form of Pb in soil solutions (McBride, 1994), and mainly occurs between pH 2.0 and 6.0 (Adriano, 1986; Bunce, 1991). At pHs higher than 9.0, $Pb(OH)_2$ is the important species (Kobza *et al.*, 2001). Depending on the pH, the solubility of Pb in noncalcareous soils is controlled by $Pb(OH)_2$, $Pb_3(PO_4)_2$, $Pb_4O(PO_4)_2$ or $Pb_5(PO_4)_3OH$ species, while $PbCO_3$ affects Pb solubility in calcareous soils (Adriano, 1986).

Cu is a primary product of chalcopyrite ($CuFeS_2$), bornite (Cu_5FeS_4) and malachite ($Cu_2CO_3(OH)_2$) mineral ores (Adriano, 1986), and occurs principally in the +2 oxidation state in soil solutions (McBride, 1994). However, $CuOH^+$, $Cu_2(OH)_2^{2+}$, $Cu(OH)_3^-$ and $Cu(CO_3)_2^{2-}$ species are commonly found in soil solutions (Kabata-Pendias and Mukherjee, 2007), and are likely to occur at pH above 6.0 (McBride, 1994; Alvarez-Puebla *et al.*, 2004). Under reducing conditions, the presence of halide and sulfide ions facilitates reduction of Cu^{2+} (cupric) to Cu^+ (cuprous), or to Cu^0 (metallic copper) (McBride, 1994). The solubility of free Cu^{2+} in alkaline soils is extremely low (McBride, 1994).

Sphalerite (ZnS) is the most important mineral ore of Zn (Adriano, 1986) and is commonly found in association with galena (PbS) and pyrite (FeS_2) (Fergusson, 1990). Zn^{2+}, $ZnCl^+$, $ZnOH^+$, $ZnHCO_3^+$, ZnO_2^{2-}, $Zn(OH)_3^-$ and $ZnCl_3^-$ are the common ionic species of Zn in soil solutions (Kabata-Pendias and Mukherjee, 2007). However, Zn exists primarily in the +2 oxidation state in soil solutions (McBride, 1994; Adriano, 1986). Zn has moderate mobility and solubility in acidic soils because it is retained in exchangeable forms of clays and organic matter (McBride, 1994; Álvarez-Ayuso and García-Sánchez, 2003). Zn mobility at pHs higher than 6.0 is controlled mainly by the precipitation of oxides, hydroxides or hydroxycarbonates (McBride, 1994; Dimirkou, 2007).

As discussed by Evans (1989) and Sposito (1989), adsorption reactions between metal ions and the charged surfaces of soil particles may involve either the formation of outer- or inner-sphere complexes. An outer-sphere complex usually formed through cation exchange reactions (Evans, 1989), while an inner-sphere complex is considered more stable than an outer-sphere complex because the metal ion binds directly with the functional group of the surface (Sposito, 1989; Grossi and Sparks, 1994). A comprehensive sorption-desorption study by Grossi and Sparks (1994) found that Cu^{2+} sorption by goethite (α-FeOOH) was not influenced by background electrolyte concentrations and therefore, they postulated that Cu^{2+} bound on the surface of goethite through strong inner-sphere surface complexation. Dimirkou (2007) explained the decrease in the pH of solution after Zn^{2+} sorption onto clinoptilolite, a natural zeolite, was due to the release of protons (H^+) as products of the binding reaction.

Metal cations can form bonds with specific functional groups of organic matter such as carboxyl (-COOH), hydroxyl (-OH) and carbonyl (C=O) (Evans, 1989), and preferentially bind with ionised functional groups at high pH (Basta *et al.*, 2005). Alvarez-Puebla *et al.* (2004) found that the amount of Cu^{2+} sorbed onto humic substance increased with pH, and noted that the ionisation of carboxyl groups starting at about pH 4.0 favoured Cu^{2+} sorption. Based on a Fourier Transform Infrared (FTIR) study, they confirmed that Cu^{2+} forms stable complexes with the carboxyl groups of the humic substance at pH 6.0.

According to McBride (1994) and Hansen (2001), both cations and anions can form complexes with O^{2-} or OH^- surfaces, and pH greatly influences these complexation processes (Basta *et al.*, 2005). The chemical reactions of metal cation (M^{2+}) and phosphate anion (HPO_4^{2-}) are described below as examples (Hansen, 2001; McBride, 1994):

Cation: $S\text{-}OH + M^{2+} = S\text{-}OM^+ + H^+$

Anion: $S\text{-}OH + HPO_4^{2-} + 2H^+ = S\text{-}OPO_3H_2 + H_2O$

where S-OH represents an OH group at the surface of the solid.

Kanungo *et al*. (2004) studied the sorption behaviour of Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} onto amorphous hydrous manganese dioxide (δ-MnO_2) as a function of pH. The sorption of metal was reported to increase with pH and was found to be a maximum at pH 6.0 for Cu^{2+} and Zn^{2+}. Trivedi *et al*. (2004) studied Zn^{2+} sorption onto ferrihydrite and reported that the percentage of sorption increased with pH, and decreased when the $NaNO_3$ concentration was increased from 0.001 to 0.1 mol/L. Maliyekkal *et al*. (2010) reported that Pb^{2+} sorption by manganese oxide was maximum at pH 5.0 and decreased when the concentration of NaCl and $Ca(NO_3)_2$ electrolytes were increased from 0 to 1.0 mol/L. They attributed the decrease in the Pb^{2+} uptake by manganese oxide to competitive effects by Na^+ and Ca^{2+} for sorption sites.

A similar observation was reported by Wang *et al*. (2010) for Cu^{2+} sorption by birnessite, a crystalline manganese oxide. They observed a decrease in the amount of Cu^{2+} sorbed and an increase in desorption percentage when the concentrations of KCl and KNO_3 electrolytes were increased up to 1.0 mol/L at pH 4.5. They also noted that the decrease in the sorption percentage was more pronounced in KCl solution than in KNO_3 solution, and this was postulated as being due to the formation of $CuCl^+$ and $CuCl_2$ species, which reduced the sorption of Cu^{2+} on negatively charged birnessite. In contrast, Zhou *et al*. (1996) observed a decrease in the percentages of Cu^{2+} and Zn^{2+} desorption from iron and aluminium oxides when the concentration of KNO_3 electrolyte was increased from 0.001 to 1.0 mol/L at solution pH of 4.5.

From these observations, it is obvious that sorption-desorption behaviour of metal ions in soil solutions is very complex and does not depend on a single factor. Although the pH of the soil solution is an important factor, the point of zero charge (pzc) of the metal oxides is another influencing factor. According to Alloway (2001) and Evans (1989), the surfaces of oxides are (net) positively charged at pH below pzc, while at above pzc the surfaces become (net) negatively charged. As reported by Wang *et al*. (2010) and Zhou

et al. (1996), the pzc of manganese oxide was 2.5, while it was 8.2 and 8.8 for iron and aluminium oxides, respectively. Since desorption experiments were performed under the same conditons, using KNO_3 as background electrolyte and with the solution pH maintained at 4.5, it can be assumed that manganese oxide carried a net negative charge, while iron and aluminium oxides were positively charged. Therefore, each of the oxide surfaces had different modes of interaction with the cations and anions present in the solution.

The sorption of heavy metals onto three manganese oxide minerals, namely todorokite, birnessite and cryptomelane was studied by Feng *et al*. (2007). The order of maximum capacity for heavy metals was found to be birnessite ≥ cryptomelane > todorokite, while the binding affinity of heavy metals to manganese oxide minerals was reported as Pb^{2+} > Cu^{2+} > Zn^{2+}. They noted that the surface negative charge and sorption capacity of the minerals were closely related to the pzc value of the mineral. The pzc values for birnessite, cryptomelane and todorokite were reported as 1.75, 2.10 and 3.50.

A desorption study by Zaman *et al*. (2009) has shown that the presence of PO_4^{3-} and NO_3^- in the soil solution affected desorption of Pb^{2+} and Cu^{2+} from manganese dioxide surfaces. They observed that Pb^{2+} desorbed less as compared to Cu^{2+} in both KNO_3 and KH_2PO_4 electrolytes, and noted that more metal ions were desorbed in KNO_3 solution than in KH_2PO_4 solution. The low percentage of desorption in KH_2PO_4 background electrolyte was attributed to the formation of stable $Pb_3(PO_4)_2$ and $Cu_3(PO_4)_2$ complexes on manganese oxide surfaces, and they proved this speculation by using FTIR analysis.

In addition to metal oxides, clay minerals are also important for metal ion sorption. Gupta and Bhattacharyya (2005) studied interaction of Pb^{2+} with kaolinite and montmorillonite clay minerals, and noted that sorption of Pb^{2+} onto both clays increased with increasing pH. They noted that montmorillonite with cation exchange capacity (CEC) of 153.0 meq/100 g has a greater ability to sorb Pb^{2+} than kaolinite, which was determined to have a smaller CEC value of 11.3 meq/100 g. Mockovčiaková *et al*. (2010) reported that the optimum pH for Zn^{2+} uptake by bentonite was pH 5.0 and that precipitation of $Zn(OH)_2$ occurred at pH 7.0.

A study by Singh *et al*. (2006) found that the amount of Pb^{2+} sorbed by phosphatic clay increased with increasing pH and decreased with increasing in concentrations of KCl and KNO_3 background electrolytes. They found that NO_3^- caused a greater inhibition effect for Pb^{2+} sorption than for Cl^-. They also noted that the presence of organic ligands such as humic, oxalic and citric acids reduced Pb^{2+} uptake significantly by forming stable complexes and therefore inhibited the precipitation of Pb^{2+} to form pyromorphite.

Based on X-ray diffraction (XRD) and Energy Dispersive X-ray (EDX) analyses, they postulated that the immobilisation of Pb^{2+} by phosphatic clay was mainly controlled by precipitation of pyromorphite ($Pb_{10}(PO_4)_6(F,Cl,OH)_2$). However, Liu and Gonzalez (1999) noted that in the presence of humic acid, the binding efficiency of montmorillonite for Pb^{2+} increased with ionic strength.

Eren *et al.* (2010) reported that the uptake of Cu^{2+} onto sepiolite, a clay mineral with a chemical formula of $Mg_4Si_6O_{15}(OH)_2.6H_2O$, increased with increasing pH, but was found to decrease with an increase in the ionic strength and in the presence of inorganic ligands such as Cl^-, SO_4^{2-} and HPO_4^{2-}. They observed a marked decrease in the Cu^{2+} uptake by about 26% when the concentration of $NaNO_3$ was increased from 0.01 mol/L to 0.1 mol/L at pH 5.3, and the significant influence of inorganic ligands was reported to be in the order of $Cl^- > HPO_4^{2-} > SO_4^{2-}$. The formation of outer-sphere complexes between Na^+ and active surfaces of sepiolite, as well as the expansion of the electrical diffuse double layer following enhancement of ionic strength, were thought to be the two main mechanisms reducing Cu^{2+} sorption by sepiolite.

A study by Ma *et al.* (1994) has shown that the immobilisation of Pb^{2+} by hydroxyapatite ($Ca_5(PO_4)_3OH$) (HA) was increased in the presence of NO_3^-, Cl^-, F^-, SO_4^{2-} and CO_3^{2-}. They noted that the reaction of HA with Pb^{2+} in the presence of NO_3^-, SO_4^{2-} and CO_3^{2-} facilitated the formation of insoluble hydroxypyromorphite ($Pb_5(PO_4)_3OH$) precipitates, while chloropyromorphite ($Pb_5(PO_4)_3Cl$) and fluoropyromorphite ($Pb_5(PO_4)_3F$) precipitates were formed in the presence of Cl^- and F^-.

1.3.2 Chemical and physical properties of soil

The chemical and physical properties of soil such as pH, CEC, organic matter content, mineral phases, oxides and inorganic ligands play a major role in controlling the mobility of heavy metals in soil. Chemical processes that take place on the oxide surfaces, for example, not only influence the behaviour of heavy metals, but also the bioavailability of nutrients in soils (Ponthieu *et al.*, 2006). Organic matter has been regarded as the most important soil constituent in lowering the concentration of free metal ions in the soils and soil solutions (Covelo *et al.*, 2008; Bradl, 2004). However, the distribution of heavy metals in soil depends on the nature of their interaction with organic matter. In the case of Pb, specific adsorption reactions by soil organic matter reduce Pb mobility (Lund, 1990; Covelo *et al.*, 2008), whereas complexation with dissolved organic matter (DOM) such as humic and fulvic acids may facilitate Pb mobilisation (Pinheiro *et al.*, 1999; Liu and Gonzalez, 1999).

Wong *et al*. (2007) investigated the effects of DOM addition on Cd and Zn sorption by acidic sandy loam, calcareous clay loam and calcareous sandy loam soils, and observed a greater inhibitory effect on Zn sorption than on Cd. High mobility of metals in acidic sandy loam was attributed to the low pH, while low clay content was thought to be the main reason that fewer metals were retained by calcareous sandy loam. They reported that the application of DOM not only reduced metal uptake by soil, but also increased metal mobility in soil due to the formation of soluble DOM-metal complexes.

CEC refers to the capacity of soil material to exchange cations such as metals with the soil solution (Hansen, 2001; Alloway, 1992). In general, the higher the CEC of soil, the greater the amount of metal ion can be bound onto soil material (Adriano, 1986). A study by Appel *et al*. (2008) found that the retention capacity of Oxisol, Ultisol and Mollisol soils for Pb and Cd correlated very well with their CEC values.

Zhao and Selim (2010) studied Zn retention in three soils having different physical and chemical properties, and found that Zn was strongly retained in neutral soil with low percentages of Zn release, about 9% to 11%, but was retained less by acidic soils, with release percentages of 42% to 51%. Shaheen (2009) studied the mobility of Pb and Cd in surface soil samples, and found that that Cd retention was mainly influenced by the clay content, the CEC and the organic matter content, while Pb binding was reported to correlate well with clay content, total free and amorphous silicon oxides, and amorphous aluminium oxide content.

Sipos *et al*. (2008) investigated the effect of soil components such as carbonate and iron oxide on heavy metal retention by soil mineral particles. They found that Cu and Zn were retained strongly on swelling clay mineral particles such as smectites and vermiculites, while Pb was highly immobilised by organic matter and iron oxide phases. They also noted that the carbonate content increased precipitation of heavy metals in soils. Covelo *et al*. (2008) studied heavy metal retention in a Fibric Histosol and its organo-mineral fraction, and found that Pb and Cu were bound tightly onto organic matter, whereas Zn was preferentially sorbed by clays and oxides.

Mouni *et al*. (2009) analysed soil samples collected from an abandoned site at Amizour, Algeria using a sequential extraction procedure, and found that metals are mainly retained by carbonates and iron-manganese oxide phases. The relative distribution of Zn and Pb between different bonding modes was reported as (%Zn, %Pb): exchangeable (3.2, 2.8); carbonate bound (32.3, 10.8); iron-manganese oxide bound (51.8, 62.7); organic matter and sulfide bound (7.1, 9.6); and total extraction (5.6, 14.1). They

observed a strong correlation between soil pH and amount of metal retained, of which high soil pH was reported to favour metal retention by Amizour soil.

A sequential extraction analysis on Pb distribution in surface soil collected from an industrial region of Kattedan, India by Sekhar *et al*. (2005) found 2570 mg/kg in residual fraction, 1853 mg/kg in carbonate bound, 500 mg/kg in organic bound, 379 mg/kg in iron-manganese oxide bound and 342 mg/kg of Pb in exchangeable fraction.

Cu^{2+} binds strongly to colloidal surfaces such as oxides of iron, manganese and aluminium, humus and silicate clays (McBride, 1994; Covelo *et al.*, 2008), over a wide range of pH values (Ponthieu *et al.*, 2006; Kanungo *et al.*, 2004). This was confirmed by Peng *et al*. (2005) who studied the fractionation of Cu in an agricultural soil contaminated by Cu smelting in Fuyang, China, and found the binding capacity of Cu to different soil fractions to follow the order iron-manganese oxide > organic matter > carbonates > residual state > exchangeable state.

An investigation on binding and mobility of heavy metals in calcareous soils by Lafuente *et al*. (2008) found that Pb and Cr were the metals most retained, while Ni and Cd were the metals most mobile and easily transferred into the soil solution. Meanwhile, an intermediate behaviour for Cu and Zn was observed. The high retention capacity of calcareous soils was proposed to be due to the presence of calcite ($CaCO_3$) and dolomite ($CaMg(CO_3)_2$) minerals, which favoured the formation of insoluble compounds with heavy metals.

The degree and distribution of metal contamination are influenced by the particle size of soil constituents. In general, fine particles have a higher surface area and therefore are more reactive to retain heavy metals than coarser material (Evanko and Dzombak, 1997; Alloway, 1992). For example, Ramlan and Badri (1989) compared heavy metal concentrations in street dust and roadside soils, and found 2466 mg/kg Pb and 344 mg/kg Zn in the street dust, while 895 mg/kg Pb and 222 mg/kg Zn were measured in the roadside soils.

1.4 Remediation techniques for metal contaminated soil

Unlike organic contaminants that can be eliminated or reduced by microbial activity and chemical oxidation techniques (Cline and Reed, 1995; Kiikkilä *et al.*, 2001; Wu *et al.*, 2010), heavy metals cannot be degraded because they are elements (Holm, 2001; Parab *et al.*, 2006; Jiang *et al.*, 2010). Therefore, once metals are introduced into the soil, they will remain (USDA/NRCS, 2000). Soil is a biochemically and geochemically complex material and is highly heterogeneous in composition (Alloway, 2001), and therefore it retains heavy metals much longer than air and water (Lasat, 2002; Alkorta, 2004).

There are several techniques available for the remediation of soils contaminated by heavy metals, which can be applied both *in situ* (on-site) and *ex situ* (off-site). Remediation technologies generally involve physical, chemical or biological processes, or a combination of the processes (Holm, 2001; Mulligan *et al.*, 2001), as described in Table 1.3.

Table 1.3 Overview of remediation techniques for inorganic contaminants and the main processes involved, modified from Holm (2001) and Mulligan *et al.* (2001).

Technique	Description	Main process involved		
		Physical	Chemical	Biological
Flushing and extraction	Infiltration of water with extracting additives to leach contaminants	✓	✓	
Electrokinetics	Application of electrical current between anode and cathode electrodes to remove small charged particles and ionic contaminants	✓	✓	
Vitrification	Application of electrical energy to vitrify contaminants	✓		
Reactive barriers	Creation of permeable barrier to reduce the mobilisation of contaminants in groundwater at contaminated sites	✓	✓	
Solidification and stabilisation	Encapsulation of the contaminants in a solid matrix	✓	✓	
Bioremediation	Use of microorganisms or plants to sequester metal contaminants			✓
Phytoremediation	Use of plants for metal extraction and immobilisation			✓

The choice of remediation technique will depend upon some critical factors such as the nature of the contaminants, the geology and type of soil, the characteristics of the contaminated site, and, undoubtedly the relative cost and time frame of the remediation technique (Holm, 2001; Alloway, 1992; USDA/NRCS, 2000). Due to economic and logistical restrictions, many of these techniques are not realistic to implement (Illera *et al.*, 2004; Hao *et al.*, 2010). For example, the excavation and landfilling technique is effective at lowering risk (Basta and McGowen, 2004; Zhu *et al.*, 2004). However, due to the high cost of landfill, transportation and backfill of the original site with clean soil, this technique would be more ideal for relatively small contaminated sites (Alloway, 1992; Zhu *et al.*, 2004; Tandy *et al.*, 2009). Moreover, this remediation option tends to cause secondary environmental problems such as leachate leakage and land occupation (Long *et al.*, 2002; Hao *et al.*, 2010).

A number of *in situ* treatments have been tested and were found to have great potential to clean up metal contaminated sites and generally cost less, in comparison to excavation and *ex situ* treatments (USEPA, 2006). In recent years, phytoextraction and stabilisation techniques have received much attention as alternatives to traditional 'dig and dump', as well as high technology remediation techniques.

1.4.1 Biological treatment: Phytoextraction

Biological treatment, or also called bioremediation, refers to the use of living organisms to remediate contaminated soils (Wenzel, 2009). It exploits natural biological processes occurring in plants and microorganisms to accumulate and alter the valence of some hazardous metals, thereby making them less toxic and less mobile (USEPA, 2006; Evanko and Dzombak, 1997). Phytoremediation is a general term for the remediation technique that employs plants to extract, contain, immobilise or degrade contaminants in soil, sediment and groundwater (USEPA, 2006; Alloway, 2001). Phytoextraction specifically refers to the uptake and translocation of metal contaminants in the soil by plant roots into harvestable parts of the plants (Padmavathiamma and Li, 2007; Pulford and Watson, 2003), which may require that the above-ground biomass be harvested and properly disposed of in a landfill or chemically treated to recover the metals (USEPA, 2006; Cooper *et al.*, 1999; Cunningham *et al.*, 1995).

Prasad and Freitas (2003) criticised the incorrect use of the terms of phytoremediation and phytoextraction. In the literature, both terms are often used as synonyms. They stressed that phytoremediation is a concept of remediation technique, while phytoextraction is a specific clean up process.

1.4.1.1 Plant species studied for phytoextraction

A variety of plant species have been evaluated for their potential and effectiveness in cleaning up metal contaminated soil. Studies have demonstrated that plants have different natural abilities to accumulate metal from contaminated soils. For example, the ability of five common crop plants, namely *Zea mays* (maize), *Helianthus annuus* (sunflower), *Brassica napus* (rapeseed), *Hordeum vulgare* (barley) and *Lupinus albus* (white lupin) to extract Pb, Zn and Cu from tailings of San Quintín Pb/Zn mine, Spain was assessed by Ruiz *et al*. (2009). Zn accumulation (mg/kg) in plant shoots was found in the order of rapeseed (174.0) > lupin (94.7) > barley (78.2) > sunflower (62.5) > maize (44.5).

Franco-Hernández *et al*. (2010) analysed metal concentrations in *Viguiera dentata*, *Parthenium bipinnatifidum*, *Flaveria angustifolia* and *Flaveria trinervia* found at an ex-mine site in San Luis Potosí, Mexico. The concentrations of As, Cu, Pb and Zn in mine tailings were reported as 8420, 1154, 754 and 1386 mg/kg. They noted that *F. angustifolia* was more effective at accumulating As (199 mg/kg), Pb (21 mg/kg) and Zn (155 mg/kg) in its shoots than the other three plants studied, whereas *P. bipinnatifidum* contained the highest amount of Cu (70 mg/kg). A field investigation conducted by Li *et al*. (2008) at Yongzhou Pb/Zn/Cu mine in Hunan, China found that *Typha orientalis Presl* was capable of accumulating 619 mg/kg of Pb in its shoot tissues from the mine tailings with an average Pb concentration of 5798 mg/kg.

An investigation by Baker *et al*. (1994) on heavy metal concentrations in *Thlaspi caerulescens* sampled from five abandoned Pb mining sites in the Pennines, UK found mean shoot concentrations of up to 21,000 mg/kg for Zn, 660 mg/kg for Pb and 164 mg/kg for Cd. Puschenreiter *et al*. (2001) measured a shoot Pb concentration of 2840 mg/kg for *Thlaspi goesingense* when cultivated on soil contaminated by Pb/Zn smelting, containing 12,300 mg/kg Pb. In a field experiment at Palmerston North, New Zealand Robinson *et al*. (1997a) reported that *Berkheya coddii* accumulated 7500 mg/kg Ni in its shoots, yielding an off-take value of 100 kg/ha. In another remediation study carried out at a Ni-contaminated site in Murlo, Italy, Robinson *et al*. (1997b) reported that about 72 kg/ha of Ni was removed by *Alyssum bertolonii*.

A study of metal concentrations in *Pteris vittata* (brake fern) grown naturally on a site contaminated with chromated copper arsenate in Central Florida, USA by Ma *et al*. (2001) discovered that brake fern accumulated 3280-4980 mg/kg of As in its fronds. Due to its high tolerance of up to 1500 mg/kg of soil As concentration, they recommended

brake fern for use in the remediation of As-contaminated soils. Haque *et al*. (2008) found that *Baccharis sarothroides* (desert broom) grown on Cu mine tailings in Arizona, USA accumulated 1214 mg/kg Cu, 107 mg/kg Pb, 106 mg/kg Cr, 55 mg/kg Zn and 37 mg/kg As in its shoots.

Jordan *et al*. (2009) studied metal uptake by *Lolium perenne* (perennial ryegrass) grown on Pb/Zn tailings with metal concentrations of 11,424 mg/kg Zn, 1185 mg/kg Pb and 44 mg/kg Cd. They found that perennial ryegrass was able to accumulate 4193 mg/kg of Zn, 323 mg/kg of Pb and 17 mg/kg of Cd in its shoots. Bidar *et al*. (2007) assessed the potential of perennial ryegrass and *Trifolium repens* (white clover) to extract heavy metals from a site containing 1222 mg/kg Pb, 1301 mg/kg Zn and 26 mg/kg Cd, located near a closed Pb smelter in Northern France. They found that perennial ryegrass accumulated 218 mg/kg Zn, 46 mg/kg Pb and 12 mg/kg Cd in its shoots, while 97 mg/kg Zn, 36 mg/kg Pb and 9 mg/kg Cd were found in the shoots of white clover.

In a study evaluating the potential of *Sorghum bicolor* (sorghum) and sunflower to remediate the Caffaro chemical plant site in Torviscosa, Italy, Marchiol *et al*. (2007) reported the mean off-take value of As by sorghum was 7.48 g/ha, while 0.18 g/ha of As was successfully removed after harvesting the above-ground biomass of sunflower. Yoon *et al*. (2006) studied metal accumulation in the shoots of *Phyla nodiflora* and *Sonchus asper* collected from a former industrial site in Jacksonville, Florida, USA with Pb, Zn and Cu concentrations of 2405 mg/kg, 1000 mg/kg and 746 mg/kg. They reported that *P. nodiflora* was more effective in accumulating heavy metals than *S. asper*, with Pb, Zn and Cu concentrations of 83 mg/kg, 453 mg/kg and 47 mg/kg, as compared to 39 mg/kg Pb, 250 mg/kg Zn and 34 mg/kg Cu analysed in *S. asper*.

Wilde *et al*. (2005) found that *Vetiveria zizanioides* (vetiver grass) accumulated only 39.3 mg/kg of Cu in its shoots from the firing range soil of Small Arms Training Area, Aiken, USA, containing 1762 mg/kg Cu. Zhuang *et al*. (2007) studied the phytoextraction of heavy metals by several plant species from a contaminated farmland near Lechang Pb/Zn mine, China, containing 960 mg/kg Pb, 1050 mg/kg Zn and 7.2 mg/kg Cd. They reported that vetiver accumulated 19 mg/kg Pb, 144 mg/kg Zn and 3.7 mg/kg Cd in its shoots, while the shoot metal concentrations for *Sedum alfredii* were found to be 104 mg/kg Pb, 6279 mg/kg Zn and 9.2 mg/kg Cd.

Rotkittikhun *et al*. (2007) studied Pb accumulation by vetiver and *Thysanolaena maxima* grasses grown on mine tailings collected from Bo Ngam Pb mine, Thailand containing 9020 mg/kg of Pb. Shoot Pb concentrations were reported to be 128 mg/kg for vetiver and 20 mg/kg for *T. maxima*. Metal concentrations in the shoots of *Nepenthes*

hookeriana (pitcher plant) found at an ex-Sn mining site in Pelepah Kanan, Johor, Malaysia were analysed by Rahim *et al*. (2008). They reported that *N. hookeriana* accumulated 105.1 mg/kg Ni, 60.0 mg/kg Co, 5.4 mg/kg Cd, 3.1 mg/kg Pb and 0.03 mg/kg Zn. Due to low accumulation of metals in the shoot tissues, they did not recommend *N. hookeriana* for phytoextraction.

Some of the plants mentioned above, especially *T. caerulescens*, *T. goesingense*, *B. coddii* and *B. sarothroides* accumulated heavy metals much greater than the ranges of normal and toxic concentrations in plants, as shown in Table 1.4. This group of plants is known as hyperaccumulators (Baker and Brooks, 1989; McGrath and Zhao, 2003). Although they accumulated high metal concentrations, no symptom of toxicity was reported. According to Baker *et al*. (1994) and Salt *et al*. (1995) a potential plant species for phytoextraction, particularly hyperaccumulator plant, should have the critical characteristic of high tolerance to heavy metals. Metal concentrations in the dry biomass of hyperaccumulator plants should exceed the thresholds that have been set by Baker and Brooks (1989), given in Table 1.4.

Table 1.4 Common concentrations of heavy metals in shoots of various plant species and in shoots of hyperaccumulator plant species (mg/kg).

Metal	Normal[a]	Toxic[a,b]	Hyperaccumulator[c]
As	1.0 – 1.5	5 – 20	-
Cd	0.01 – 0.20	5 – 30	>100
Cr	0.1 – 0.5	5 – 30	-
Cu	5 – 30	20 -100	>1000
Ni	0.1 – 5.0	10 – 100	>1000
Pb	5 – 10	30 – 300	>1000
Zn	25 – 150	100 – 400	>10,000

Note: [a] Kabata-Pendias and Mukherjee (2007); [b] Values are given for moderate tolerant plant species; [c] Baker and Brooks (1989).

1.4.1.2 Factors affecting phytoextraction

The effectiveness of phytoextraction in remediating metal contaminated sites is inherently dependent on several plant and soil characteristics. The plant characteristics include fast growth and high above-ground biomass production, good tolerance to high concentrations of metals in plant tissues, and the ability to accumulate large amounts of metals in their shoots rapidly (Vamerali *et al.*, 2010; Padmavathiamma and Li, 2007). Meanwhile, soil pH, texture, salinity and extent of metal contamination are the main soil characteristics affecting phytoextraction (Cunningham *et al.*, 1995; Singh *et al.*, 2003; Prasad and Freitas, 2003).

Hammer *et al*. (2003) conducted long-term trials on the phytoextraction of Cd and Zn by *Salix viminalis* (willow) at two contaminated sites in Switzerland, namely Dornach and Caslano, which vary in soil properties such as pH, organic carbon content, calcium carbonate and clay contents, and metal concentrations. Dornach soil was reported to be calcareous and contaminated by a nearby brass smelter, while Caslano soil was acidic as a result of contamination by sludges from septic tanks. They reported that 0.17 kg/ha of Cd and 13.4 kg/ha of Zn were removed from the Dornach soil after 5 years, while 0.047 kg/ha of Cd and 14.5 kg/ha of Zn were extracted from Caslano soil after 2 years of trial. They explained that acidic soil (pH 5.2) at Caslano site favoured the growth of *S. viminalis* and increased the solubility and availability of Zn to plant roots for extraction. Therefore, phytoextraction of Zn at Caslano was found to be more effective than at Dornach.

On the same sites, Hammer and Keller (2003) assessed the influence of different methods of growing on the biomass yield and metal extraction efficiency by *T. caerulescens*. They reported that transplanted *T. caerulescens* showed higher biomass yield and annual metal off-take than sown *T. caerulescens* at both contaminated sites. For example, at Caslano, transplanted *T. caerulescens* with 2.1 tonne/ha of biomass yield was estimated to remove 20.0 kg/ha of Zn, while sown *T. caerulescens* with a lower biomass production of 1.0 tonne/ha was capable of extracting 7.8 kg/ha of Zn. They concluded that phytoextraction efficiency was dependent on both soil properties and the agricultural management.

Electrical conductivity of soil affects biomass production and phytoextraction efficiency of some plant species. For example, Rotkittikhun *et al*. (2007) studied the effect of pig manure application (20% w/w) to Pb mine tailings on growth performance and Pb extraction by vetiver and *T. maxima* grasses. The soil electrical conductivity was observed to increase from 0.23 to 3.13 dS/m. They noted that the survival of *T. maxima* decreased by 25% with pig manure addition, while vetiver was observed to survive well without any toxicity symptoms. Pb concentrations in shoots and roots of *T. maxima* grown on non-amended tailings were reported as 19.9 and 271 mg/kg, while 13.3 and 115 mg/kg were for *T. maxima* grown on amended tailings.

Wang *et al*. (2000) studied the effects of soil acidity on the uptake of Zn and Co by *Glycine max* (soybean) and *Lycopersicon esculentum* (tomato). They reported that metal uptake in the shoots and stems of soybean, cultivated on acidic soil (pH 4.2) was higher than soybean plants grown on normal soil (pH 6.4). Meanwhile, slow growth performance and low metal uptake were reported for tomato cultivated on acidic soil. The variety of a plant species may also influence the phytoextraction efficiency. The

efficiency of three varieties of *Sorghum bicolor*, namely *Keller*, *Mray* and *Rio*, for metal extraction from a contaminated farmland was evaluated by Zhuang *et al*. (2009). They observed a significant correlation between biomass yield and metal removals. The biomass production was found to be 25.8, 24.8 and 18.7 tonne/ha for *Keller*, *Mray* and *Rio*, while the removal of Zn by *Keller*, *Mray* and *Rio* sorghums were estimated as 1.44, 1.10 and 0.80 kg/ha.

However, a research finding on phytoextraction of heavy metals from a contaminated site by Indian mustard, *Nicotiana tabacum* (tobacco), willow, maize and *T. caerulescens* by Keller *et al*. (2003) has shown that biomass yield may not always be an indicator for removal efficiency. They reported that maize had the highest biomass production of 15.6 tonne/ha but could only remove 0.009 kg Cd per ha, as compared to *T. caerulescens* with a low biomass yield of 0.9 tonne/ha which gave the highest Cd off-take of 0.179 kg/ha. A 170 day field trial by Li *et al*. (2009) found that *Averrhoa carambola* (starfruit) attained 18.6 tonne/ha shoot biomass yield and extracted 0.213 kg Cd per ha when grown on a metal-contaminated site in Guangzhou, China.

According to Yoon *et al*. (2006), plant uptake of heavy metals from soil occurs either passively with the mass flow of water into the roots, or through active transport crosses the plasma membrane of root epidermal cells. Some plant species retain heavy metals in their root tissues, causing low metal transport to the above-ground parts and therefore reduce metal removal from contaminated soils. For example, a phytoextraction study by Peng *et al*. (2005) found that *Elsholtzia splendens* accumulated 50-70 mg/kg of Cu in shoots and 800-1200 mg/kg of Cu in roots. *V. zizanioides* and *Phragmites australis* were found to accumulate more Pb, Zn and Cu in roots than in shoots from Cu mine tailings (Chiu *et al*., 2006). They reported that 330 and 268 mg/kg Cu were retained in roots of *V. zizanioides* and *P. australis*, while 10 and 14 mg/kg Cu were contained in the plant shoots.

A similar trend of accumulation was observed by Marchiol *et al*. (2007) on As, Cd, Cu, Pb and Zn extraction by sorghum and sunflower from a former industrial site contaminated by chemical wastes in Torviscosa, Italy. They reported that all metals studied, especially Cu, were retained in roots of the plants. Cu concentrations in roots of sorghum and sunflower were 594 and 837 mg/kg, while 28.6 and 36.2 mg/kg Cu were accumulated in the shoots of the plants. However, a different observation was reported by Madejón *et al*. (2003) who studied the potential of sunflower to clean up soil affected by the Aznalcóllar mine spill. They found that Cd, Cu and Zn were accumulated more in the shoots than in the roots, while As and Pb were retained in roots. Cd was reported to be accumulated in the shoots most with a concentration of 500 mg/kg,

followed by Zn (98 mg/kg) and Cu (23 mg/kg). Pb and As concentrations in the roots were found to be 5210 and 2057 mg/kg, while 634 mg/kg of Pb and 21 mg/kg of As were found to be in the shoots.

Some of the reports on biomass production and removal of heavy metals by plants from contaminated soils, without any treatment, are summarised in Table 1.5.

Table 1.5 Biomass production (tonne/ha) and removal (kg/ha) of heavy metals by plants from contaminated soils.

Plant species	Soil type	Biomass	Removal			Reference
			Pb	Cu	Zn	
Sorghum bicolor var. *Keller* (sorghum)	Farmland affected by Pb/Zn mining	25.8	0.35	0.24	1.44	Zhuang *et al.* (2009)
Hordeum distichum (barley)	Soil affected by Aznalcóllar mine spill	5.8	0.035	0.057	1.07	Soriano and Fereres (2003)
Triticosecale (triticale)	Soil affected by Aznalcóllar mine spill	4.8	0.033	0.051	0.92	Soriano and Fereres (2003)
Brassica napus (rapeseed)	Soil affected by Aznalcóllar mine spill	4.9	0.025	0.036	0.90	Soriano and Fereres (2003)
Brassica carinata (Ethiopian mustard)	Soil affected by Aznalcóllar mine spill	5.2	0.037	0.037	0.80	Soriano and Fereres (2003)
Zea mays (maize)	Pb/Zn mine tailings	15.0	0.08	-	1.26	Ruiz *et al.* (2009)
Sedum alfredii	Farmland affected by Pb/Zn mining	5.5	0.55	-	32.7	Zhuang *et al.* (2007)
Rumex crispus	Farmland affected by Pb/Zn mining	20.0	1.0	-	27.0	Zhuang *et al.* (2007)
Helianthus annuus (sunflower)	Soil affected by Aznalcóllar mine spill	24.0	0.016	-	2.05	Madejón *et al.* (2003)
Brassica juncea (Indian mustard)	Soil contaminated by brass smelting	7.3	-	0.146	0.89	Keller *et al.* (2003)
Nicotiana tabacum (tobacco)	Soil contaminated by brass smelting	12.6	-	0.474	1.83	Keller *et al.* (2003)
Elsholtzia splendens	Farmland affected by Cu smelting	9.2	-	0.56	-	Peng *et al.* (2005)
Thlaspi caerulescens	Soil contaminated by brass smelting	0.9	-	0.076	3.7	Hammer and Keller (2003)
Thlaspi caerulescens	Soil contaminated by septic-tank wastes	2.1	-	0.065	20.0	Hammer and Keller (2003)

1.4.1.3 Amendments for enhancing phytoextraction

Although a number of plants have been identified as suitable for phytoextraction, slow growth rates and low annual production of harvestable biomass can limit their effectiveness to remove significant amounts of metals from contaminated soils (Alloway, 2001; Baker and Brooks, 1989; Zhuang *et al.*, 2007). Furthermore, elevated soil pH and high organic matter content can hinder the availability of metals to be

extracted by plants (Jung and Thornton, 1996; Rosselli *et al.*, 2003; Yoon *et al.*, 2006). Therefore, enhancing the production of above-ground biomass (Shtangeeva *et al.*, 2004) and accumulation of heavy metals in harvestable plant tissues is a prerequisite for such a technique to be practical (Meers *et al.*, 2005; Lin *et al.*, 2009).

Chemical amendments can be added to increase the solubility and availability of heavy metals to plant roots (Kayser *et al.*, 2000) and translocation of metals to aerial biomass (Johnson *et al.*, 2009). A number of amendments have been tested for enhancing phytoextraction. These include aminopolycarboxylic acids such as ethylenediaminetetraacetic acid (EDTA), ethylenediaminedisuccinic acid (EDDS) and nitrilotriacetic acid (NTA); organic acids such as acetic, citric, malic, oxalic, succinic and ascorbic acids; amino acid such as histidine; acid-producing fertilisers such as ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl) and ammonium sulfate ($(NH_4)_2SO_4$); and inorganic agent such as elemental sulfur (S_8).

The potential of NTA and S_8 to enhance phytoextraction of heavy metals from a contaminated site at Dornach, Switzerland was investigated by Kayser *et al.* (2000). They observed a slight increase in plant accumulation of Cd, Cu and Zn with application of NTA (4.2 and 8.4 mmol/kg) and S_8 (36 mol/m^2). NTA treatment with an application rate of 8.4 mmol/kg was found to be the most effective treatment. On the same site, Hammer *et al.* (2003) assessed long-term effects of S_8 treatment (36 mol/m^2) on phytoextraction of Cd and Zn by *S. viminalis*, and found that the application of S_8 did not significantly affect cumulative metal extraction over five years of a field trial. In addition, they observed a decrease in the biomass yield of *S. viminalis* following the treatment.

Cui *et al.* (2004) studied the influence of S_8 and EDTA application on Pb and Zn uptake by Indian mustard and *Triticum aestivum* (winter wheat) cultivated for 50 days in pots on a contaminated soil derived from a battery recycling factory. They noted that the combined application of S_8 (160 mmol/kg) and EDTA (8 mmol/kg) gave the best metal uptake. For example, shoot Pb uptake by Indian mustard was 4.122 mg/pot, as compared to 0.290 mg/pot for the control. Meanwhile, 0.723 and 0.023 mg/pot were estimated for shoot Pb uptake by winter wheat for treated and control samples respectively.

Zhuang *et al.* (2007) treated a contaminated agricultural soil with EDTA (Na_2-EDTA) at an application rate of 6 mmol/kg, one week before harvest. They reported that EDTA treatment increased the rate of Pb extraction by *Viola baoshanensis*, *Rumex K-1 (Rumex patientia* x *R. timschmicus)* and *Rumex crispus*, meanwhile they observed no significant

effect on Cd and Zn uptake by the plants. Wilde *et al*. (2005) applied 5 mmol/kg EDTA to a firing range soil one week prior to harvest, and found that Pb uptake by vetiver grass increased by 147% with EDTA treatment.

The influence of trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid (CDTA) (2 and 20 mmol/kg), diethylenetriaminepentaacetic acid (DTPA) (2 and 20 mmol/kg) and *N*-hydroxyethylethylenediaminetriacetic acid (HEDTA) (2 mmol/kg) on phytoextraction of Pb from a contaminated soil was evaluated by Cooper *et al*. (1999) in a 42 day greenhouse study. They reported that CDTA treatment at an application rate of 20 mmol/kg gave the highest Pb uptake of 1.6 mg/plant by ragweed, 3.0 mg/plant by redtop, 6.5 mg/plant by maize and 1.8 mg/plant by sunflower. The combination of CDTA (20 mmol/kg) with maize was found to be the most effective treatment, yielding a total removal of 0.4 kg Pb per ha. Huang and Cunningham (1996) observed a significant increase in Pb uptake by maize when grown on a Pb-contaminated soil treated with HEDTA at an application rate of 2 g/kg. One week after treatment, the shoot Pb concentration was found to be 10,600 mg/kg for plants cultivated on the HEDTA-treated contaminated soil, while only 40 mg/kg was found in control plants. However, this treatment caused a severe effect on the plant growth. They reported that maize plants died one week after HEDTA application.

A field study at a former cable manufacturing site containing 1200 mg/kg Pb by Blaylock *et al*. (1997) found that the combined application of EDTA (5 mmol/kg) and acetic acid (5 mmol/kg) resulted in a significant increase in Pb accumulation by Indian mustard. The shoot Pb uptake was estimated as 28, 785 and 1471 mg/kg for the control, EDTA and combined EDTA with acetic acid treatments, meanwhile, the root Pb concentrations were 101, 152 and 466 mg/kg for the same treatments, respectively. A different observation was reported by Miller *et al*. (2008). They analysed shoot Pb concentrations of *Sesbania exaltata* (coffeeweed) six days after application of EDTA (1 g/kg), acetic acid (1 g/kg) and combined EDTA with acetic acid to a soil contaminated with 1000 mg/kg Pb. They noted that EDTA addition gave the highest shoot Pb concentration. In fact, acetic acid and combined EDTA with acetic acid application have resulted in similar amount of Pb accumulated in shoots.

Zhuang *et al*. (2009) evaluated the effects of EDTA (6 mmol/kg), NH_4NO_3 (10 mmol/kg) and $(NH_4)_2SO_4$ (10 mmol/kg) treatments on removal of heavy metals by sorghum (var. Keller) from a contaminated farmland. They reported that EDTA, NH_4NO_3 and $(NH_4)_2SO_4$ treatments enhanced phytoextraction efficiency. For example, EDTA addition increased Pb removal from 0.35 kg/ha (control) to 0.64 kg/ha, while Zn removal was found to increase from 1.44 kg/ha (control) to 1.49 kg/ha with NH_4NO_3 application. They

observed a slight increase in Cd removal, 0.052 kg/ha (control), while 0.057 kg/ha following $(NH_4)_2SO_4$ treatment.

The application of such EDTA and $(NH_4)_2SO_4$ may not always increase plant metal uptake. For example, Puschenreiter *et al*. (2001) applied EDTA and $(NH_4)_2SO_4$ to a soil collected nearby a former Pb/Zn smelter and found that both treatments enhanced Cd uptake by *T. goesingense*. $(NH_4)_2SO_4$ treatment was reported to reduce Pb and Cu uptake by plants. In fact, a 60% decrease was observed for Pb uptake, as compared to untreated contaminated soil. Meanwhile, EDTA application was found to decrease Pb uptake by about 14%. The ability of EDTA and EDDS to enhance Cu, Ni and Zn uptake by sunflower from a contaminated soil derived from a disposal site was compared by Meers *et al*. (2005). They found that plants grown on EDDS-treated contaminated soil extracted higher amounts of metal than plants cultivated on EDTA-treated contaminated soil. For example, the shoot Zn concentration was found to be 96 mg/kg for untreated contaminated soil, 190 mg/kg for 2 + 2 mmol EDDS and 155 mg/kg for 2 + 2 mmol EDTA.

Karczewska *et al*. (2009) assessed the effects of EDTA (1 mmol/kg), EDDS (1 mmol/kg) and histidine (2 mmol/kg) treatments on heavy metal uptake by Indian mustard and maize cultivated in pots on a soil contaminated by Cu smelting. They found that EDDS treatment enhanced Cu extraction by both plants significantly, while histidine treatment had no obvious effect as compared to the control. For example, the shoot Cu concentrations for Indian mustard were reported as 18.6 mg/kg (control), 54.5 mg/kg (EDTA), 68.7 mg/kg (EDDS) and 18.0 mg/kg (histidine). Roongtanakiat *et al*. (2009) studied the influence of EDTA (1 g/kg), DTPA (1 g/kg) and compost (10 g/kg) application on heavy metal extraction by *Chrysopogon zizanioides* (vetiver grass) grown on Zn mine tailings. They reported that only EDTA and DTPA treatments were found to enhance Zn and Cu extraction significantly, while a very slight increase was observed for compost treatment as compared to untreated mine tailings.

A field study by Marchiol *et al*. (2007) found that application of mineral (NPK) and organic (mature cow manure) fertilisers enhanced the removal of heavy metals by sorghum and sunflower from a former chemical manufacturing site. For example, the removal of As by sorghum was found to be 7.48 g/ha (control), 158 g/ha (mineral) and 219 g/ha (organic). Meanwhile, the off-take values for As removal by sunflower were reported to be 0.18, 6.74 and 3.96 g/ha for the control, mineral and organic treated plants, respectively. A higher uptake was reported for Zn. They noted that sorghum removed 1223 and 1944 g/ha of Zn following mineral and organic fertilisation, while the

off-take values for Zn removal by sunflower were estimated to be 410 g/ha (mineral) and 804 g/ha (organic).

1.4.2 Stabilisation

Stabilisation refers to the technique that decreases the hazard potential of the contaminated soil by converting the contaminants into their least soluble, mobile or toxic form (Holm, 2001). Immobilising agents reduce the mobility and availability of heavy metals by enhancing geochemical processes such as precipitation, sorption, complexation and ion exchange (Ruttens *et al.*, 2010; Kumpiene *et al.*, 2008). This technique has been regarded as an 'environmentally friendly' technique because it causes less disruption to the environment (Aguilar-Carrillo *et al.*, 2009; Lee *et al.*, 2009). Soil stabilisation using waste-based materials has been considered as a cost-effective technique for soil remediation (Janoš *et al.*, 2010; Kumpiene *et al.*, 2007). In fact, the use of such materials for *in situ* metal immobilisation has received great attention in recent years due to their low-cost and ready availability in large quantities. Numerous agricultural and industrial by-products, as well as naturally occurring minerals have been tested for metal immobilisation in contaminated soils.

1.4.2.1 Agricultural by-products

The uptake of heavy metals by perennial ryegrass established on Pb/Zn tailings amended with spent mushroom compost (SMC) at application rates of 0, 50, 100, 200 and 400 tonne/ha was evaluated by Jordan *et al.* (2009) in a 6 month greenhouse study. The application of SMC was reported to decrease Cu, Pb and Zn accumulation in the shoot tissues of ryegrass. For example, at application rate of 200 tonne/ha the uptake of Cu, Pb and Zn was found to decline by 39%, 26% and 6%, as compared to unamended tailings. They attributed the decrease in metal uptake to the presence of functional groups such as hydroxyl, phosphoryl and phenolic groups on the surface of SMC, which were able to bind heavy metals from mine tailings.

Nwachukwu and Pulford (2009) conducted a 6 week greenhouse study in order to assess the potential of green waste compost, peat, coir and wood bark for heavy metal immobilisation in a soil contaminated with mine waste. The application of amendments at the rates of 1%, 10% and 20% (w/w) was found to reduce Pb concentration in the shoots of perennial ryegrass significantly. However, the shoot Cu concentration was reported to increase with amendment treatment. Fornes *et al.* (2009) applied an organic compost consisting of 80% (v/v) 'alperujo' (a by-product of olive oil extraction)

and 20% (v/v) sheep manure to a contaminated agricultural soil at the rate of 100 tonne/ha. The uptake of Pb and Zn in Indian mustard cultivated on organic compost amended soil was studied in a greenhouse trial for 16 weeks. The shoot Pb accumulation of Indian mustard was reported to decrease by 57%, while Zn uptake reduced by 85% as compared to untreated contaminated soil.

The performance of two strategies for immobilising heavy metals in contaminated soil, namely the co-composting of contaminated soil with organic wastes and the incorporation of mature compost into contaminated soil was compared by Tandy *et al*. (2009). After 28 days of a greenhouse study, the shoot Zn concentration of winter wheat was found to be 32 mg/kg for mine soil mixed with mature compost, while it was 28 mg/kg for plants grown on co-composted mine soil. Since there was no significant difference in plant metal uptake, as well as the added economic cost and logistics of co-composting, they did not recommend co-composting strategy for heavy metal immobilisation.

A field experiment at a contaminated farmland by Clemente *et al*. (2007) found that the application of olive husk at the rate of 53 tonne/ha favoured Cu, Mn and Zn uptake by *Beta vulgaris* and *Beta maritima*, as compared to the control treatment (200 kg/ha of NPK fertiliser). However, cow manure (51 tonne/ha) treatment was reported to reduce shoot Pb uptake of *B. vulgaris* by 29% and by 53% for *B. maritima*. Calderone and Frankenberger (1990) added orange peel and cattle manure at application rate of 3% (w/w) to a sediment sampled from the Kesterson Reservoir, California, USA contained 7.6 mg/kg Mo. After 56 days of incubation, the amount of deionised water soluble Mo leached from the soil columns was found to be 7 µg/kg for orange peel treated soil, 11 µg/kg for manure treated soil and 29 µg/kg for untreated soil.

In a field study, Sato *et al*. (2010) evaluated the effects of three types of animal waste compost (AWC) namely cattle, swine and poultry on Cd uptake by *Spinacia oleracea* (spinach), and the uptake by these plants was compared with plants cultivated on soil treated with chemical fertiliser (NPK). The application of cattle, swine and poultry amendments was reported to produce no significant difference in plant Cd accumulation. However, they noted that the AWC treatment resulted in 34-38% lower concentration of Cd as compared to plants grown on fertiliser amended soil.

Li *et al*. (2008) treated a contaminated paddy soil with pig manure and peat at application rate of 0.4% (w/w), and found that the calcium chloride (0.01 mol/L) extractable heavy metals in the amended contaminated soil decreased significantly after 3 months of the pot trial. For example, Cu and Cd concentrations were found to

be 339 and 2.39 mg/kg for unamended soil, 22 and 1.40 mg/kg for pig manure amended soil, and, 99 and 1.84 mg/kg for peat treated soil. Moreover, the Cu concentration in the rice grain was reported to decrease by 23% and 18% following application of pig manure and peat. Based on the reductions in heavy metal concentrations in both soil and rice grain, they concluded that pig manure was the most effective amendment.

A similar observation was reported by Rotkittikhun *et al*. (2007) when cultivated vetiver and *T. maxima* grasses on Pb mine tailings amended with pig manure (20% and 40% w/w) in pots for 60 days. They noted that the addition of pig manure reduced Pb accumulation in plant shoots and roots. For example, 20% (w/w) treatment was reported to decrease Pb concentration in vetiver by 91% (shoots) and 32% (roots), as compared to plants grown on mine tailings only. Meanwhile, shoot and root Pb concentrations of *T. maxima* were found to decrease by 33% and 58%. However, a 170 day field study by Peng *et al*. (2005) at a contaminated agricultural site found that the application of 1500 kg/ha organic manure has resulted in a slight decrease, about 4% in shoot Cu accumulation of *E. splendens*, as compared to plants grown on untreated soil.

Application of amendments to metal-contaminated soils may not always reduce the availability of heavy metals. Schwab *et al*. (2007) investigated heavy metal leaching from mine tailings amended with composted cattle manure (CCM), aged cattle manure (ACM) and composted yard waste (CYM) at an application rate of 90 tonne/ha. They found that all amendments increased the amount of heavy metal leached from the tailings, as compared to untreated mine tailings. For example, Zn concentration in the leachate (0.001 mol/L calcium chloride) on the sixth day of the column study was measured as 0.4 mg/L (untreated), 1.1 mg/L (CYM), 2.7 mg/L (ACM) and 3.7 mg/L (CCM). Of more concern, Cd concentration was found to exceed the guideline value of 0.003 mg/L for drinking water set by the WHO, following amendment treatment. Based on this observation, they noted that there was a possibility of groundwater contamination if these amendments were used for the remediation of mine tailings.

Chiu *et al*. (2006) studied the effects of sewage sludge and pig manure compost addition (5% w/w) to Cu mine tailings on metal uptake by vetiver and *P. australis*. After 3 months of the greenhouse study, they found that sewage sludge application increased the shoot Cu concentration of vetiver by 80%, while a 179% increase in Cu concentration was observed in the *P. australis* shoots as compared to unamended tailings. Pig manure compost treatment was reported to increase the shoot Zn concentration of *P. australis* from 19 mg/kg to 57 mg/kg, while no significant effect was observed for vetiver.

Hodson *et al*. (2000) evaluated the ability of bonemeal (2% w/w) to immobilise heavy metals in three UK mine soils, namely Parys Mountain, Leadhills and Wanlockhead. They noted that the addition of bone meal to mine soils reduced metal concentration in the leachate (0.01 mol/L calcium chloride). For example, in the first 500 hours of leaching, the Zn concentration in the leachate of Parys Mountain soil was found to be 173 mg/L for unamended soil and 12 mg/L for amended soil. Based on the results obtained from SEM and XRD analyses, they postulated that the formation of metal phosphates following bonemeal treatment reduced heavy metal availability in the mine soils.

The effectiveness of bone char to immobilise heavy metals in a contaminated agricultural soil was evaluated by Hao *et al*. (2010) in a batch column incubation study. After 3 months of bone char treatment at application rates of 1%, 2% and 5% (w/w), the acetic acid (0.11 mol/L) extractable soil Pb concentration was reported to reduce by 67%, 84% and 97%, as compared to unamended contaminated soil. Meanwhile, 2%, 20% and 41% reductions in Zn concentration were obtained following the treatments.

1.4.2.2 Industrial by-products

The immobilisation capacities of cyclonic ashes (CAH) and steel shots (SS) for heavy metals were evaluated by Ruttens *et al*. (2010) in a soil column study. They treated a contaminated agricultural soil with 5% (w/w) CAH, 1% (w/w) SS, and combined 5% (w/w) CAH with 1% (w/w) SS (CAH+SS). After 26 weeks of the soil column experiment, they found that CAH+SS treatment gave the lowest calcium nitrate (0.1 mol/L) extractable soil Zn and Cd concentrations, as compared to the other two treatments. The combined CAH+SS application was reported to reduce Zn concentration by 89%, while a 77% reduction was observed for Cd concentration, as compared to untreated contaminated soil.

Geebelen *et al*. (2006) conducted a greenhouse pot experiment in order to assess the ability of cyclonic ashes (CAH), Na-silicates (SIL) and lime (LM) to immobilise heavy metals in a smelter contaminated soil. The soil was treated with 5% (w/w) CAH, 1% (w/w) SIL, 0.5% (w/w) LM, and combined 5% (w/w) CAH with 1% (w/w) SIL (CAH+SIL). After 28 days of pot trial, shoot Zn concentrations of common bent grass were measured as 5330 mg/kg (unamended), 1041 mg/kg (SIL), 488 mg/kg (LM), 400 mg/kg (CAH) and 383 mg/kg (CAH+SIL).

In a 45 day greenhouse study, Lee *et al*. (2009) applied red mud and furnace slag to a metal-contaminated agricultural soil at the rates of 2% and 5% (w/w). The metal accumulation in plant tissue was reported to decrease significantly following amendments addition. For example, red mud treatment at 5% (w/w) reduced shoot Cd, Pb and Zn concentrations of lettuce by 86%, 58% and 73%, as compared to plants grown on unamended contaminated soil. The ability of red mud and furnace slag to reduce the availability of heavy metals in such contaminated soil was explained as being due to the presence of iron oxides, which act as binding sites for heavy metals.

However, in a 60 day greenhouse study assessing the potential of two steel processing wastes, namely furnace slag and baghouse dust as soil amendments for supporting plant growth, Pichtel and Bradway (2008) found that the application of both wastes incorporated with composted peat induced the uptake of heavy metals by spinach and cabbage. The high metal accumulation in plant tissue was discussed as being due to the high metal content in the wastes. The baghouse dust was reported to contain 169,760 mg/kg Zn, 4990 mg/kg Pb, 2880 mg/kg Ni and 266 mg/kg Cd, while 945 mg/kg Zn, 200 mg/kg Pb, 388 mg/kg Ni and 1.46 mg/kg Cd were measured in the furnace slag.

Castaldi *et al*. (2009) studied metal uptake of pea and wheat cultivated on a contaminated soil treated with red mud, zeolite and lime at an application rate of 10% (w/w) in a 7 week greenhouse study. The addition of amendments was found to reduce Cd, Pb and Zn accumulation in plant shoots and roots. For example, the shoot Zn concentration of pea was reported as 2568 mg/kg (untreated), 1757 mg/kg (zeolite), 99 mg/kg (lime) and 93 mg/kg (red mud). Meanwhile, the shoot Cd concentration of wheat was analysed as 11.7 mg/kg (untreated), 3.6 mg/kg (zeolite), and 0.7 mg/kg for both red mud and lime amended soils.

In a two week batch leaching study evaluating the potential of fly ash and peat for heavy metal immobilisation in a soil contaminated with ore deposits, Kumpiene *et al*. (2007) found that the application of both amendments at the rate of 5% (w/w) reduced the amount of metal leached from the soil significantly. The combined application of fly ash (5% w/w) with peat (5% w/w) was found to be the most effective treatment for immobilisation of Cu and Cd, with reductions of 98% and 99%, respectively.

Bertocchi *et al*. (2006) investigated the feasibility of immobilising heavy metals contained in mine tailings using fly ash and red mud, and found that the the addition of 15% (w/w) of amendments, separately, reduced metal concentrations in the leachate (distilled water). After 80 days of leaching column experiments, they noted that red mud was more effective than fly ash in reducing the mobilisation of As, Pb and Zn in the

mine tailings. The greater ability of red mud in binding heavy metals than fly ash was attributed to the higher amount of iron and aluminium oxides in the red mud.

A two year field trial at Avonmouth, UK by Gray *et al*. (2006) found that the application of red mud at the rates of 3% and 5% (w/w) to smelter contaminated soil reduced the uptake of heavy metals by red fescue, as compared to plants grown on untreated contaminated soil. For example, after 10 months of growth, Zn concentrations in plants were analysed as 1500 mg/kg for untreated soil, 700 mg/kg for 3% (w/w) red mud treatment and 300 mg/kg for 5% (w/w) red mud addition. Despite its ability to reduce the availability of heavy metals to plants, the addition of red mud, particularly at 5% (w/w) was found to increase the concentration of Cr in soil by about 143%. The red mud was reported to contain 1377 mg/kg Cr.

Garrido *et al*. (2005) tested the potential of four industrial by-products, namely red gypsum (RG), phosphogypsum (PG), sugar foam (SF) and dolomitic residue (DR) for reducing the availability of Cd in a contaminated soil. They treated the contaminated soil with amendments at an application rate of 3% (w/w) and incubated the mixtures for 12 weeks. They noted that both gypsum amendments were inefficient in reducing Cd availability, of which a 4-24% increase was observed following the treatments. In addition, DTPA extractable Cd was not affected by the SF treatment. However, they observed a significant decrease (65%) in Cd concentration with the DR amendment.

A different observation was reported by Aguilar-Carrillo *et al*. (2009), particularly on DTPA extractable of Cd in contaminated soil. They found that the addition of single sugar foam (SF) and combined SF with phosphogypsum (PG) (SF+PG), both at an application rate of 1% (w/w), reduced the DTPA extractable of Cd and As after 12 weeks of incubation period. The DTPA extractable Cd was reported to decrease from 7.76 mmol/kg (untreated contaminated soil) to 6.42 mmol/kg (SF) and 5.84 mmol/kg (SF+PG). Meanwhile, DTPA extractable As was found to decrease by 33% and 11% following SF and SF+PG treatments, as compared to untreated soil.

The effectiveness of sugar beet lime (SBL), biosolid compost (BC) and leonardite (LE) for *in situ* heavy metal immobilisation was assessed by Madejón *et al*. (2006) at the 'El Vicario' site, affected by the Aznalcóllar mine spill. The experimental site was treated with 30 tonne/ha SBL, 30 tonne/ha BC and combined 10 tonne/ha SBL with 25 tonne/ha LE (SBL+LE). They observed a slight reduction in soil metal concentration after one year of amendment application. For example, Pb concentration in soil was reported to decrease from 484 mg/kg (unamended) to 453 mg/kg (SBL+LE), 386 mg/kg (SBL) and 381 mg/kg (BC).

A leaching column study by Calace *et al*. (2005) found that the application of paper mill sludge to a mine soil at a weight ratio of 1:9 (sludge: soil) reduced Cd, Cu, Mn and Zn concentrations in the leachate (deionised water) significantly. For example, the amount of Zn released by leaching from untreated soil was found to be 596 mg/kg, while 281 mg/kg, 26 mg/kg and 4 mg/kg were measured after 0, 7 and 15 days of incubation with the sludge treatment. However, sludge amendment was reported to increase Pb concentration in the leachate by 63% (0 day) and 17% (7 and 15 days), as compared to untreated soil.

Metal accumulation in winter wheat cultivated on three contaminated agricultural soils treated with lignin, derived from paper mill sludge, at the rate of 5% (w/w) for 8 weeks in pots was studied by Zhang *et al*. (2004). They found that lignin application reduced shoot uptake of Cd, Cr, Cu, Ni, Pb and Zn significantly, as compared to plants grown on untreated soil. However, lignin amendment was found to increase Mo uptake from all soils by 30%-41%.

1.4.2.3 Natural occurring minerals

Kashem *et al*. (2010) added lherzolite at the rate of 5% (w/w) to a soil contaminated with effluents from industrial and mining operations in Tohoku, Japan. The effect of lherzolite amendment was investigated in a 35 day greenhouse study using radish and Japanese mustard spinach. They found that lherzolite application reduced Cd and Zn concentrations in the shoots of radish by 64% and 78%, while the shoot Cd and Zn concentrations of Japanese mustard spinach was reported to decrease by 74% and 90%, as compared to unamended contaminated soil.

Janoš *et al*. (2010) assessed the ability of zeolite to immobilise heavy metals in a smelter contaminated soil and found that the application of zeolite at the rates of 1%, 3% and 5% (w/w) reduced the acetic acid (0.11 mol/L) extractable metals in soil significantly. For example, after 3 months of incubation, the Pb concentration was reported to decrease by 30%, 54% and 64% following 1%, 3% and 5% (w/w) zeolite treatments, respectively.

Hartley *et al*. (2004) treated a contaminated soil derived from a former landfill site in Merton Bank, UK with goethite (α-FeOOH) at application rate of 1% (w/w). They found that the concentrations of Cd, Cu, Zn and Pb in the leachates (deionised water) decreased with the goethite application, whereas the amount of As leached from the goethite amended soil column was reported to increase by about 11%, as compared to

untreated contaminated soil. Zhu *et al*. (2004) amended a residential soil contaminated by smelting, with phosphate compound amendments namely hydroxyapatite (HA) and phosphate rock (PR) at application rates of 0.25% and 0.5% (w/w). After 2 months of a greenhouse study, the shoot Pb concentration of turnip mustard was found to be 66.3 mg/kg for unamended soil, 22.9 mg/kg for 0.5% (w/w) HA and 41.2 mg/kg for 0.5% (w/w) PR.

A 12 month incubation study by Madrid *et al*. (2006) found that the EDTA (0.05 mol/L, pH 7.0) extractable metal concentrations in four urban soils from Seville, Spain decreased with the addition of acid zeolite (AZ), sodium zeolite (SZ), Slovakite (SK) and apatite (AP) at application rate of 5% (w/w). AZ was found to be the most effective amendment in reducing soil metal concentrations. After 12 months of incubation, Zn concentrations in the El Valle soil were measured as 58 mg/kg (untreated), 53 mg/kg (SZ), 52 mg/kg (AP), 44 mg/kg (SL) and 40 mg/kg (AZ). However, a batch leaching study by van Herwijnen *et al*. (2007a) found that the incorporation of zeolite or bentonite at the rates of 7%, 14% and 20% (w/w) to green waste compost and sewage sludge compost caused no significant effects on the leaching of Cd and Zn from the amended contaminated soils.

The ability of phosphate rock (PR) and limestone (LM) to immobilise heavy metals in a smelter contaminated soil was evaluated by Basta and McGowen (2004) in a soil column study. They treated the contaminated soil with 6% and 18% (w/w) of PR, and 17% (w/w) of LM. The cumulative amount of Cd, Pb and Zn extracted with calcium nitrate (0.5 mol/L) through 60 pore volumes of elution was found to decrease with amendments application, as compared to untreated contaminated soil. For example, PR treatment at application rate of 18% (w/w) was reported to reduce metal elution by 27% for Cd, 18% for Pb and 27% for Zn.

The effect of palygorskite amendment at application rates of 2% and 4% (w/w) in a mine soil was studied by Álvarez-Ayuso and García-Sánchez (2003) in a leaching column study. They reported that the amount of metal leached from the palygorskite amended soil was lower than untreated soil, and a significant stabilisation effect was observed when palygorskite was used at 4% (w/w). The application of palygorskite at the rate of 4% (w/w) to the mine soil was found to reduce the leaching of Cd by 66%, Cu by 59%, Zn by 52% and Pb by 50%, as compared to untreated soil.

1.5 Chitosan

1.5.1 Background and properties

Chitin, poly(1→4)-2-acetamido-2-deoxy-β-D-glucan, is one of the most abundant biopolymers on earth (Carraher Jr., 2003), and was first discovered by French scientist Braconnot during his research on mushrooms in 1811 (Muzzarelli, 1977). The word 'chitin' is derived from the Greek word *chiton*, meaning 'envelope', and was first used by Odier in 1823 to describe the insect cuticle (Muzzarelli, 1977). Chitin occurs in nature as crystalline microfibrils forming structural components in the exoskeleton of anthropods or in the cell walls of fungi and yeast (Rinaudo, 2006). The structure of chitin is very similar to that of cellulose, but it has an acetamido group at the C-2 position in place of the hydroxyl group (Figure 1.2). Chitosan, poly(1→4)-2-amino-2-deoxy-β-D-glucan, was discovered by Rouget in 1859 when he boiled chitin in a concentrated potassium hydroxide solution (Muzzarelli, 1977). It is derived from chitin by partially deacetylating the acetamido groups with strong alkaline solution, and becomes chitosan when the degree of deacetylation reaches about 50% (Vachoud and Domard, 2001; Rinaudo, 2006). Chitosan has three reactive functional groups, an amino at the C-2, as well as both secondary and primary hydroxyl groups at the C-3 and C-6 positions (Figure 1.2).

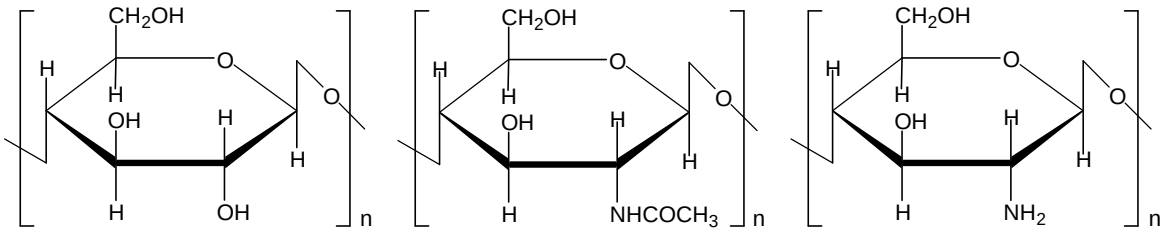


Figure 1.2 Molecular structure of cellulose, chitin and chitosan (n = repeating units).

Cellulose, chitin and chitosan are linear- and long-chain polysaccharides with high molecular weight. For example, cellulose from wood pulp has chain lengths ranging from 300 to 1700 units of D-glucose (Klemm *et al.*, 2005), while chitin from crab has typical chain lengths between 5000 and 8000 units of *N*-acetyl-D-glucosamine, a derivative of glucose (Synowiecki and Al-Khateeb, 2003). Depending on the processing conditions, the molecular weight of commercial chitosan is in the range between 10,000 and 1,000,000 Da (Gerente *et al.*, 2007). Cellulose and chitin have low chemical reactivity, and are highly hydrophobic and insoluble in most organic solvents (Rinaudo, 2006). The insolubility of natural cellulose and chitin in such media is attributed to the

high hydrogen bonding and dipole interactions (Kumar *et al.*, 2004), which enables them to serve as structural materials in plants and animals (Mathur and Narang, 1990; Kumar, 2000). Unlike cellulose and chitin, chitosan is soluble in dilute aqueous solutions of organic acids such as acetic acid, formic acid and oxalic acid, and inorganic acids such as hydrochloric acid and nitric acid (Rinaudo, 2006; Kumar, 2000). Therefore, chitosan can exist in three physical forms corresponding to solids, gels and solutions (Vachoud and Domard, 2001) and is more versatile than chitin and cellulose in terms of application. The appealing intrinsic properties of chitosan are given in Table 1.6.

Table 1.6 Intrinsic properties of chitosan (Crini and Badot, 2008).

Physical and chemical properties

- Linear aminopolysaccharide with high nitrogen content
- Rigid D-glucosamine structure; high crystallinity; hydrophilicity
- Capacity to form hydrogen bonds intermolecularly; high viscosity
- Weak base; the deprotonated amino group acts as a powerful nucleophile (pK_a 6.3)
- Insoluble in water; soluble in dilute aqueous acidic solutions
- Numerous reactive groups for chemical activation and cross-linking
- Forms salts with organic and inorganic acids
- Chelating and complexing properties
- Ionic conductivity

Polyelectrolytes (at acidic pH)

- Cationic biopolymer with high charge density (one positive charge per glucosamine residue)
- Flocculating agent; interacts with negatively charged molecules
- Entrapment and adsorption properties; filtration and separation
- Film-forming ability; adhesivity
- Materials for isolation of biomolecules

Biological properties

- Biocompability
    - Non-toxic
    - Biodegradable
    - Adsorbable
- Bioactivity
    - Antimicrobial activity (fungi, bacteria, viruses)
    - Antiacid, antiulcer, and antitumoral properties
    - Blood anticoagulants
    - Hypolipidemic activity
- Bioadhesivity

The presence of amino and hydroxyl groups in chitosan is highly advantageous, since modification can be performed by their reaction yielding numerous useful materials for different fields of application (Crini, 2005; Shahidi *et al.*, 1999). Chitosan can be modified by chemical reactions such as cross-linking, grafting, hydrolysis and oxidation, and physical treatments through preparation of membranes, gel beads and fibres (Guibal, 2004; Crini, 2005). These processes can be used to synthesise materials for specific applications, as well as to improve the chemical and physical characteristics of chitosan. For example, chitosan cross-linked with genipin, an active compound extracted from gardenia fruits, has better elastic properties for the use in cartilage regeneration, as compared to unmodified chitosan (Muzzarelli, 2009). Vallapa *et al.* (2011) reported that chemical treatment via a heterogeneous two-step process, namely reductive alkylation using aldehyde followed by methylation with methyl iodide improved the antibacterial activity of the chitosan surface. A study by Phompan and Hansupalak (2011) found that platinum-loaded carbon black entrapped in cross-linked chitosan had a great potential for enhancing the performance of proton-exchange membrane fuel cells.

Crini *et al.* (2008) reported that reacting chitosan with 4-formyl-1,3-benzene sodium disulfonate in the presence of sodium cyanoborohydride increased the BET surface area of chitosan by 39%. Meanwhile, modification with diacetylmonoxime was found to improve the porosity of chitosan (Monier *et al.*, 2010). A study by Ge and Huang (2010) found that the reaction between epichlorohydrin-cross-linked chitosan and EDTA dianhydride under microwave irradiation reduced the thermal stability of chitosan. Kyzas and Lazaridis (2009) grafted chitosan with poly(acrylamide) and poly(acrylic acid), and observed a significant decrease in the swelling degree of chitosan. Zuñiga *et al.* (2010) reported that the introduction of a propyl chain into a modified chitosan, namely *N*-methylene phosphonic chitosan, enhanced its water solubility and therefore, resulting in better application in the food and cosmetic industries.

1.5.2 Production

The worldwide annual production of chitin and chitosan is not exactly known. A survey in 1993 by Abdullah (1995) involving 30 marine food processing plants throughout Malaysia has estimated that at least 200 to 250 tonnes of chitin may be produced annually. Shells of shrimp, crab and lobster are the prime sources of chitin, and they are readily available in large quantities. For example, the frozen shrimps industry in Malaysia for the period 1991-1993 produced more than 9000 tonnes of waste per year (Abdullah, 1995). In Malaysia, the consumer demand for frozen and canned seafood

grows steadily, year by year. The FAO statistic data on shrimps, crabs and lobsters captured in Malaysia for three years period beginning 2006 (Table 1.7), confirms this scenario. From Table 1.7, the capture of three most popular crustaceans is found to increase by about 10,000 tonnes per year.

Table 1.7 Crustacean capture production (tonne) in Malaysia 2006-2008.

Fishing area	Crustaceans	2006	2007	2008
Inland waters	Freshwater shrimp, crab and lobster	291	299	408
Marine areas	Shrimp	61,898	71,729	80,417
	Crab	11,259	12,037	12,114
	Lobster	1,039	910	1,111
Total		74,487	84,975	94,050

Source: FAO – Fisheries and Aquaculture Information and Statistics Service.

According to Mathur and Narang (1990), about 80% of the total weight of crustaceans processed will be usually discarded as shell wastes. Based on this assumption, in 2008 a total of 75,240 tonnes (fresh weight) or 37,620 tonnes (dry weight) wastes have been generated in Malaysia. Although the content of chitin in the shell wastes varies widely depending on species and processing procedure (Synowiecki and Al-Khateeb, 2003; Kumar *et al.*, 2004), on a dry basis, crustacean shells normally contain 13-42% of chitin (Synowiecki and Al-Khateeb, 2003; Gerente *et al.*, 2007). In a pilot study evaluating the production of chitin and chitosan from shellfish waste, Abdullah (1995) reported that approximately 80% of chitin can be converted into chitosan through deacetylation process. Therefore, in 2008 over 5600 tonnes (dry weight) of chitin and 4500 tonnes (dry weight) of chitosan could be obtained from fishery wastes in Malaysia.

A method for the commercial production of chitin and chitosan is described by Mathur and Narang (1990) (Figure 1.3). As shown in Figure 1.3, the two main processes involved in the manufacturing of chitin are demineralisation and deproteination. Meanwhile, deacetylation has to be carried out to obtain chitosan. According to Guibal (2004) and Gerente *et al*. (2007), the fraction of deacetylation for commercial chitosan is usually from 70% to 90%. However, highly deacetylated chitosan is preferred for biomedical and pharmaceutical applications (Muzzarelli, 2009; Guibal, 2004). A study by Chatterjee *et al*. (2005) found that the cell wall of fungi could be an alternative source of chitosan. They noted that chitosan obtained by fermentation of *Mucor rouxii* had similar physical and chemical characteristics to that of derived from shellfish waste. However, due to the large amount of waste available, and the solid waste disposal problem, the use of shellfish waste as a main source of chitin and chitosan production is more practical.

Depending upon the quality and quantity, as well as transportation cost, labour and energy expenses, the production cost of chitin and chitosan varies significantly. However, based on a conservative estimation, 1 kg of chitosan can be produced at a cost of US$ 2.00-4.00 (Gupta and Ali, 2006).

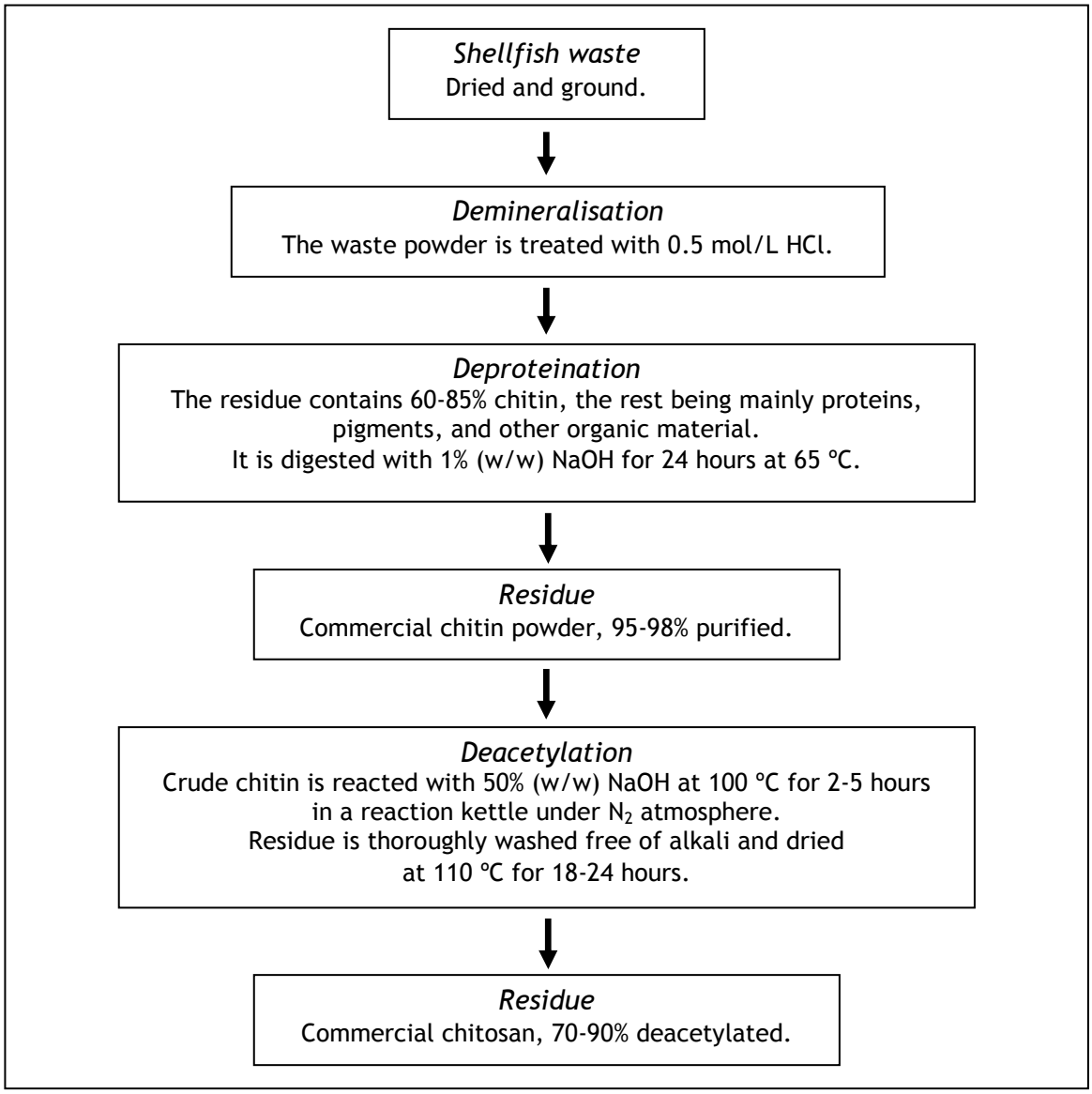


Figure 1.3 Flowsheet for preparation of chitin and chitosan from shellfish waste (Mathur and Narang, 1990).

1.5.3 Applications

As defined by Bailey *et al*. (1999), a low-cost material is one which is abundant in nature, requires little processing, or is a by-product from another industry. Chitin and chitosan are classified as low-cost materials because they can be obtained from the shellfish waste of the fishery industry, which is available in large amounts (Bailey *et al*., 1999; Babel and Kurniawan, 2003). Due to their excellent properties and affordable

cost, chitin and chitosan have been used in a variety of fields such as agriculture, food technology, pharmacy, medicine, cosmetics and toiletries, biotechnology, water treatment and the pulp industry (Rinaudo, 2006; Kumar, 2000).

In agriculture, studies have demonstrated that chitosan could be a promising soil amendment for plant growth. For instance, Ohta *et al*. (2004a) reported that 1% (w/w) chitosan treatment promoted seedling growth and hastened the flowering date of several ornamental plants. In another study, Ohta *et al*. (2004b) found that the addition of chitosan at the rate of 1% (w/w) to soil at sowing *Eustoma grandiflorum* (lisianthus) seeds resulted in remarkably greater shoot length, stem diameter, number and weight of the flowers. Uddin *et al*. (2004) observed a significant increase in the bud growth and petal pigmentation of lisianthus following chitosan application.

A study by Lafontaine and Benhamou (1996) found that chitosan treatment at concentrations of 12.5 and 37.5 mg/L reduced plant mortality and the root rot symptom attributed to *Fusarium oxysporum* f.sp. *radicis-lycopersici*. They also observed a significant increase in tomato yield at harvest for plants treated with chitosan. Li and Yu (2000) studied the effect of chitosan treatment at 5.0 and 10.0 mg/mL concentrations on the incidence of brown rot of peach fruit caused by *Monilinia fructicola*. They reported that both concentrations reduced the brown rot symptom significantly, as compared to untreated peaches. In fact, chitosan-treated peaches were found to contain more vitamin C and firmer than control peaches.

A field study in a commercial bell pepper farm at Boynton Beach, Florida, USA by Kim *et al*. (1997) found that chitosan application at 0.2% (w/v) reduced the crown root rot symptom caused by *Phytophthora capsici*, as compared to untreated plants. In fact, chitosan treatment was reported to reduce the disease incidence and severity better than other soil amendments tested such as humate, municipal solid waste, perennial peanuts, seed peanuts, sewage sludge-yard trimming and wood chips. Meanwhile, Bittelli *et al*. (2001) reported that foliar application of chitosan at a concentration of 1.0 g/L to pepper plants reduced the plant transpiration and the water used by 26-43% whilst maintaining biomass production and yield.

Hirano *et al*. (2000) noted that coating soybean seeds with a thin layer of chitin increased the bean harvest to approximately 118% of that of the uncoated seeds. A study by Wu and Liu (2008) found that coating NPK fertiliser with chitosan not only reduced the release of nutrient into soil, but also increased the water retention capacity of the soil.

1.5.4 Research and development on chitin and chitosan in Malaysia

1.5.4.1 Water treatment

The sorption efficiency of TiO_2 modified with chitin for Fast Green FCF (FG) dye was examined by Zainal *et al*. (2005). They noted that the presence of chitin in TiO_2 suspension increased FG removal by 72%, as compared to unmodified TiO_2. The greater sorption capacity of TiO_2-chitin was proposed to be due to the ability of functional groups in chitin to sorb FG through the formation of hydrogen bonds and van der Waals interactions, as well as ion exchange mechanisms. A similar observation was reported by Hasan *et al*. (2008), of which the modification of oil palm ash with chitosan increased the removal of Reactive Blue 19 dye from aqueous solution.

Saifuddin and Raziah (2007) studied the feasibility of Baker's yeast (*Saccharomyces cerevisiae*) immobilised in chitosan/lignosulfonate matrix for Cr(VI) uptake in aqueous solution. They noted that chitosan/lignosulfonate matrix had excellent immobilising support properties especially in maintaining high stability for bacterial cell for extended period of time and reusability. In fact, they achieved a 95% removal when the sorbent was applied under optimised conditions. The potential of chitosan coated oil palm shell charcoal composite for Cr(VI) sorption from a synthetic wastewater was studied by Nomanbhay and Palanisamy (2005). They found that the composite had a better sorption capacity than unmodified oil palm shell charcoal. A comparative sorption study by Zulkali *et al*. (2006) found that chitosan was more effective than rice husk in removing Pb^{2+} ions from aqueous solution.

The feasibility of two forms of chitosan, namely chitosan powder and chitosan flakes to sorb residue oil from palm oil mill effluent was investigated by Ahmad *et al*. (2005) using a batch sorption study. They found that chitosan in powder form exhibited a greater sorption capacity than the flake form. The higher sorption capacity of chitosan powder was explained as being due to its looser pore structure that facilitated the sorption of residue oil. Saifuddin and Chua (2006) treated wastewater containing fats and oils by a combination of microwave irradiation and lipase immobilisation on chitosan. Chitosan was found to be very effective in encapsulating lipase enzyme and had a high retention capability. After five cycles of treatment process, chitosan was reported to retain excellent activities of up to 70% of its initial activity. They noted that other immobilised lipases retained only 45% of their initial activity after five cycles of treatment.

Nawawi and Tram (2004) studied the effect of zeolite treatment at the rate of 0.25% (w/w) to chitosan membrane on separation of isopropanol from aqueous solution. They noted that the addition of zeolite improved the mechanical properties of chitosan membrane. In fact, the permeation flux and selectivity of chitosan-zeolite membrane was found better than unmodified chitosan membrane.

1.5.4.2 Polymer battery

Mohamed *et al*. (1995) fabricated a solid-state polymer battery using lithium nitrate and chitosan film. They found that chitosan was able to act as a host matrix for the conduction of ions, with a maximum electrical conductivity of 2.7×10^{-4} S/cm. A study by Subban and Arof (1996) found that the electrical conductivity of chitosan could be increased by the addition of sodium iodide to chitosan film. Meanwhile, Yahya *et al*. (2006) reported that the application of oleic and palmitic acids as plasticizers increased the electrical conductivity of chitosan electrolyte matrix. For example, the conductivity of chitosan matrix was found to increase from 2.2×10^{-7} S/cm to 1.1×10^{-5} S/cm and 5.5×10^{-6} S/cm at room temperature, when 10.0% (w/w) of oleic acid and 7.7% (w/w) of palmitic acid were added to the matrix.

1.5.4.3 Nanomaterials

Boey *et al*. (2007) studied the morphology of copper sulfides, CuS and Cu_2S, stabilised with chitosan and synthesised using thiourea as a reducing agent. The particle size of CuS and Cu_2S was found to decrease significantly from 257.0 to 76.6 nm in the presence of chitosan. Therefore, they recommended the use of chitosan as a stabilising agent in the preparation of advanced materials.

Adlim *et al*. (2004) synthesised colloidal Pt and Pd nanoparticles using chitosan as a stabiliser. The particle size of Pt and Pd was found to be in the range of 1.9-2.2 nm when methanol or sodium borohydride was used as reducing agent. They reported that chitosan-Pt prepared by methanol reduction showed 99% selectivity and converted octene to octane, while hydrogenation of octene catalysed by chitosan-Pd gave octane, 2-octene and 3-octene. They concluded that chitosan was not only able to reduce the size of colloidal particles, but also to produce nanoparticles with good catalytic properties.

1.5.4.4 Paper making

Ashori *et al*. (2006) studied the dry-strength properties of kenaf paper treated with chitosan. The dry-strength properties of paper such as tear index, burst index and tensile index were found to enhance significantly following chitosan treatment. However, they noted that the effectiveness of chitosan treatment was strongly dependent on the method of chitosan application and chitosan dosages. The spray application method was reported to yield superior strength properties as compared to equilibrium adsorption (pH 5.0) method and precipitation (pH 10.0) method. Meanwhile, a greater rate of improvement in the mechanical properties was observed when chitosan was applied at the rates of 0% to 0.5% (w/w) than 1.0% to 1.5% (w/w).

1.5.4.5 Ion selective electrode

Isa and Ghani (2007) developed a heterogeneous membrane of chitosan as ion selective electrode (ISE) for the determination of glutamate in food samples. The chitosan-based ISE was found to have a good selectivity and sensitivity, with a detection limit of 1.0 x 10^{-6} mol/L. Isa and Ghani (2009) analysed glutamate in food samples using chitosan-based ISE and High Performance Liquid Chromatography (HPLC). They found that the analysis of glutamate using chitosan-based ISE was comparable with the standard method of HPLC.

Yahaya and Ghani (2008) used chitosan as an active material on a heterogeneous membrane for the potentiometric determination of chloride ions. Based on the analysis of chloride in real samples such as sea water, mineral water and tap water, they found that the chitosan electrode membrane had a good selectivity and high recovery. In fact, the measurements obtained using the chitosan-based electrode was reported to be quite consistent with those measured using a commercial ISE electrode.

1.5.4.6 Cell and tissue engineering

Due to the high demand for cheaper implant materials, local resources such as chitosan, from fishery by-product and hydroxyapatite, from limestone were utilised in tissue engineering (Idrus, 2006). Norazril *et al*. (2004) cross-linked chitosan with collagen and the morphology of the cell was studied using SEM. They found that chitosan-collagen scaffold had a better cell morphology than chitosan scaffold. Therefore, they concluded that the incorporation of collagen to chitosan would enhance the cell attachment ability of chitosan and chitosan-collagen would be a potential scaffold in tissue engineering.

Poly(vinyl alcohol) (PVA) was modified with *N*,*O*-carboxymethylated chitosan and its potential as an extracellular matrix scaffold for cartilage engineering was studied by Abbas *et al*. (2008). PVA was found to have a non-porous structure with smooth and homogenous surface morphology, while PVA-chitosan had an interconnecting porous structure ranging between 1-200 μm. The porous structure of PVA-chitosan was thought to enhance chondrogenesis of implanted cells. Therefore, a PVA-chitosan based hydrogel scaffold was reported to have great potential as a cell carrier for cartilage tissue engineering. Lee *et al*. (2009) tested the mechanical behaviour of PVA-chitosan under unconfined compression and the results revealed that PVA-chitosan holds excellent potential as a scaffold for regenerating cartilage in a weight-bearing joint defect.

Zainol *et al*. (2008) modified nanoparticles of hydroxyapatite with chitosan and evaluated the potential of chitosan/nano hydroxyapatite composite for bone regeneration scaffolds. Chitosan addition was found to increase the osteoconductivity and biodegradability of hydroxyapatite. They reported that the composite exhibited higher tensile strength and was more stable when exposed to saline and blood, as compared to unmodified hydroxyapatite nanoparticles.

1.5.4.7 Biofilm

Khan *et al*. (2000) fabricated chitosan films using two different solvents, acetic acid (AA) and lactic acid (LA) for wound dressing. The mechanical and in-vitro bioadhesive strength properties, as well as the biological evaluation such as skin irritation of chitosan-AA and chitosan-LA were compared. They reported that chitosan-LA exhibited lower tensile strength, more flexible, pliable and bioadhesive than chitosan-AA. Chitosan-AA inflicted skin allergic reactions. In contrast, chitosan-LA was non-allergic and non-toxic. Therefore, they concluded that chitosan-LA is more suitable than chitosan-AA to be used in wound healing and skin burn treatments.

In another study, Khan and Peh (2003) compared the wound healing efficacy of two chitosan films, chitosan-AA and chitosan-LA, with Omiderm, a commercial wound dressing using punch biopsy wounds in rats. They found that both chitosan-AA and chitosan-LA were able to promote wound healing with a minimal scar formation. The cytotoxicity of three chitosan derivative films, namely oligo-chitosan (O-C), *N*-carboxymetyl-chitosan (N-CMC) and *N*,*O*-carboxymethyl-chitosan (NO-CMC) was assessed on primary human dermal fibroblasts by Asiah *et al*. (2006). They observed no toxicity

effect on fibroblast cells over the period of exposure to O-C and N-CMC. However, NO-CMC was found to cause a toxicity effect after 48 and 72 hours of exposure.

The effectiveness of chitosan-poly(ethylene oxide) blend membrane as a haemodialysis membrane was evaluated by Nasir *et al*. (2005). The permeability coefficients of urea and blood through chitosan-poly(ethylene oxide) blend membranes were found to be much higher than that of pure chitosan membrane and Cuprophan, a commercial haemodialysis membrane. In addition, they noted that chitosan-poly(ethylene oxide) blend membrane has low platelet adhesion and activation properties. Therefore, they concluded that chitosan-poly(ethylene oxide) blend membrane could be used as an alternative to commercial haemodialysis membranes.

1.6 Research aim and objectives

1.6.1 Aim

The preceding sections have given an overview of soil contamination by heavy metals and the use of waste-based materials to clean up contaminated soil. The work of this thesis was carried out in this context with the aim of evaluating the potential of chitosan and cross-linked chitosans as soil amendments for the remediation of metal contaminated soil.

1.6.2 Objectives

The objectives of this research were:

1. To modify chitosan using cross-linking treatment (Chapter 3).
2. To study the physical and surface properties of chitosan and cross-linked chitosans (Chapter 3).
3. To evaluate the ability of chitosan and cross-linked chitosans to bind metals in soil solution (Chapter 4).
4. To study the effect of chitosan and cross-linked chitosans on metal uptake by plants (Chapter 5).
5. To study the effect of metals on biodegradation of chitosan and cross-linked chitosans (Chapter 6).

2 MATERIALS AND METHODS

2.1 Chemicals and washing of glassware

All chemicals were of analytical grade, unless otherwise stated and were used with no further purification. All the glassware used in this research was first washed with tap water and soaked overnight in a 2% (v/v) solution of Decon 90 (Decon Laboratories Limited). It was then washed with tap water, rinsed twice with deionised water and dried in the drying cabinet.

2.2 Determination of degree of deacetylation

Chitosan, prepared from crab shells, was purchased from Sigma. The percentage of free amino groups in chitosan was determined as the degree of deacetylation by an infrared (IR) method outlined by Blair *et al*. (1987) and Baxter *et al*. (1992). This method is based on the relationship between absorbance values at 1655 cm^{-1}, which is attributed to amide, and the corresponding value of the hydroxyl band at 3450 cm^{-1}. The degree of deacetylation of the chitosan was then estimated using Equation 2.1 (Monteiro and Airoldi, 1999):

$$\text{Degree of deacetylation (\%)} = \left[1-\left(\frac{A_{1655}}{A_{3450}}\right)\left(\frac{1}{1.33}\right)\right] \times 100 \qquad (2.1)$$

The absorbance values recorded for amide at 1655 cm^{-1} and hydroxyl groups at 3450 cm^{-1}, were 0.090 and 0.525, respectively. Applying these figures to Equation 2.1, gave a value of 87% deacetylation for the chitosan used in this research.

2.3 Preparation of chitosan gel beads

Chitosan gel beads were prepared based on a similar procedure described previously (Kamari and Wan Ngah, 2009). 2.0 g of chitosan (Figure 2.1a) was dissolved into 60 mL of 5% (v/v) acetic acid solution and left overnight to form a yellow viscous chitosan gel solution (Figure 2.1b). The viscous solution was sprayed dropwise into a precipitation bath containing 500 mL of 0.5 mol/L NaOH, thus stimulating coagulation of the chitosan gel into spherical uniform beads through neutralisation. A magnetic stirrer was used to stir the NaOH solution to prevent the chitosan beads from sticking to glassware surfaces. The wet chitosan gel beads were filtered and rinsed thoroughly with deionised

water to remove any NaOH residue. The gel beads (Figure 2.1c) were then ready for cross-linking treatment.

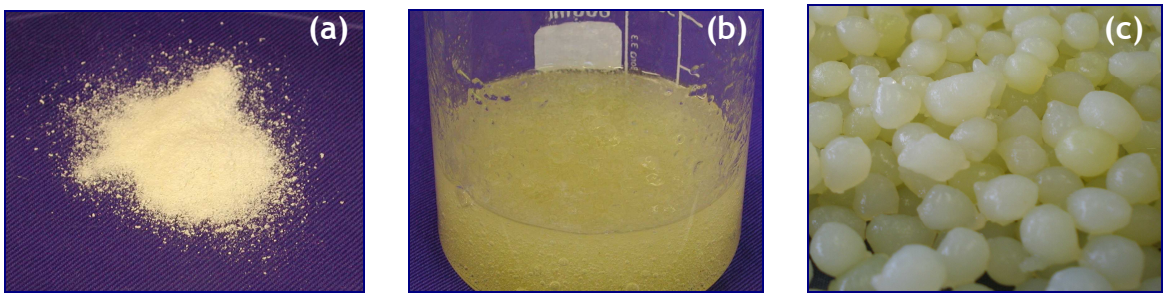
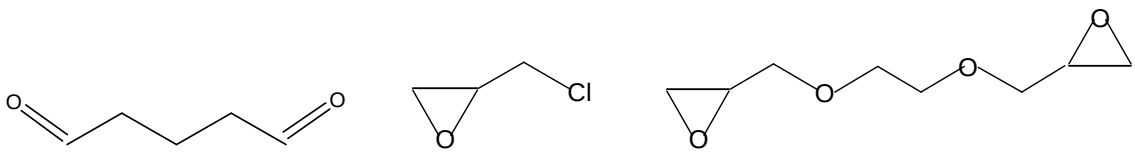


Figure 2.1 (a) Chitosan, (b) chitosan gel solution, and (c) chitosan gel beads (average bead diameter 5 mm).

5% (v/v) acetic acid solution was prepared by first adding 50 mL glacial acetic acid to approximately 400 mL of deionised water in a 1 L volumetric flask with a volumetric pipette. The solution was then made up to volume with deionised water. To prepare 1 L of 0.5 mol/L NaOH, 20 g of NaOH was dissolved in 500 mL of deionised water in a 1 L beaker and stirred using a magnetic stirrer. Then, the solution was transferred into a 1 L volumetric flask and made to the mark with deionised water.

2.4 Cross-linking treatment

The purpose of this treatment was to enhance the chemical stability of chitosan in acidic media, as well as to improve its physical properties. Glutaraldehyde (GLA) (50% solution in water), epichlorohydrin (ECH) (≥99% purity) and ethylene glycol diglycidyl ether (EGDE) (50% technical grade) were acquired from Sigma-Aldrich, and used as cross-linking reagents. The molecular structure of the cross-linking reagents is shown in Figure 2.2, while their physical and chemical properties are given in Table 2.1.



Glutaraldehyde Epichlorohydrin Ethylene glycol diglycidyl ether

Figure 2.2 Molecular structure of cross-linking reagents.

Table 2.1 Physical and chemical properties of cross-linking reagents.

Common name	Glutaraldehyde	Epichlorohydrin	Ethylene glycol diglycidyl ether
Chemical name	1,5-Pentanedial	1-Chloro-2,3-epoxypropane	1,2-Bis(glycidyloxy)ethane
Molecular formula	$C_5H_8O_2$	C_3H_5Cl	$C_8H_{14}O_4$
Molecular weight	100.12 g/mol	92.53 g/mol	174.12 g/mol
Physical state	Liquid	Liquid	Liquid
Colour	Colourless	Colourless	Faintly yellow-green
Boiling point	101 ºC	117 ºC	112 ºC
Density	1.11 g/cm^3	1.18 g/cm^3	1.118 g/cm^3
Solubility in water (at 20 ºC)	100%	6.5%	100%
Solubility in other solvents	Soluble in ether, alcohol and benzene	Soluble in ether and alcohol	Soluble in ether, alcohol and benzene

Source: Sigma-Aldrich Material Safety Data Sheet (MSDS), Weast and Astle (1979), Solvay Chemicals, Inc. (2003).

As shown in Figure 2.2, each cross-linking reagent molecule consists of two reactive groups. GLA contains two aldehyde CHO groups, while EGDE has two epoxy bridges with a CH_2O group in each bridge. Unlike GLA and EGDE, ECH has an epoxy bridge and a chlorine atom. Each chitosan monomer consists of an amino (NH_2) group, therefore two chitosan monomers and a cross-linking reagent molecule should react to form a cross-linking bridge. The concentration and volume of cross-linking reagents for cross-linking reaction depend upon the NH_2/CHO or NH_2/CH_2O ratio. In this research, chitosan was treated at a 1:1 mole ratio, according to a procedure outlined by Wan Ngah *et al*. (2005), but with some modification to the procedure of cross-linking reactions (will be described in Sections 2.4.1 and 2.4.2). The quantity of NH_2 was calculated using Equation 2.2.

$$\text{Mol of } NH_2 = \frac{\text{Weight of chitosan (g)}}{\text{Molecular weight of two units of chitosan monomer (g/mol)}} \times DD \qquad (2.2)$$

where, DD is the fractional degree of deacetylation.

Since the molecular weight for one unit of chitosan monomer ($C_6H_{11}NO_4$) is 161 g/mol and the deacetylation degree of chitosan is 87%, 2.0 g of chitosan contains 5.0×10^{-3} moles of NH_2. Therefore, to obtain a mole ratio of 1:1, 200 mL of 0.025 mol/L cross-linking reagent solution was required. This determination was discussed by Koyama and Taniguchi (1986), who modified chitosan using different NH_2/CHO ratios. Cross-linking reagent solutions were prepared as described by Wan Ngah *et al*. (2005).

2.4.1 GLA

The cross-linking treatment with GLA was performed by suspending chitosan gel beads in GLA solution for 24 hours at room temperature and stirring at 200 rpm using a magnetic stirrer. After 24 hours the cross-linked chitosan beads were thoroughly rinsed with deionised water, filtered and dried in air. After drying, the newly formed beads (hereafter called chitosan-GLA) were ground and sieved using a 500 µm size fraction British Standard 410 test sieve to remove large beads. Figure 2.3 represents the schematic diagram of cross-linking treatment with GLA.

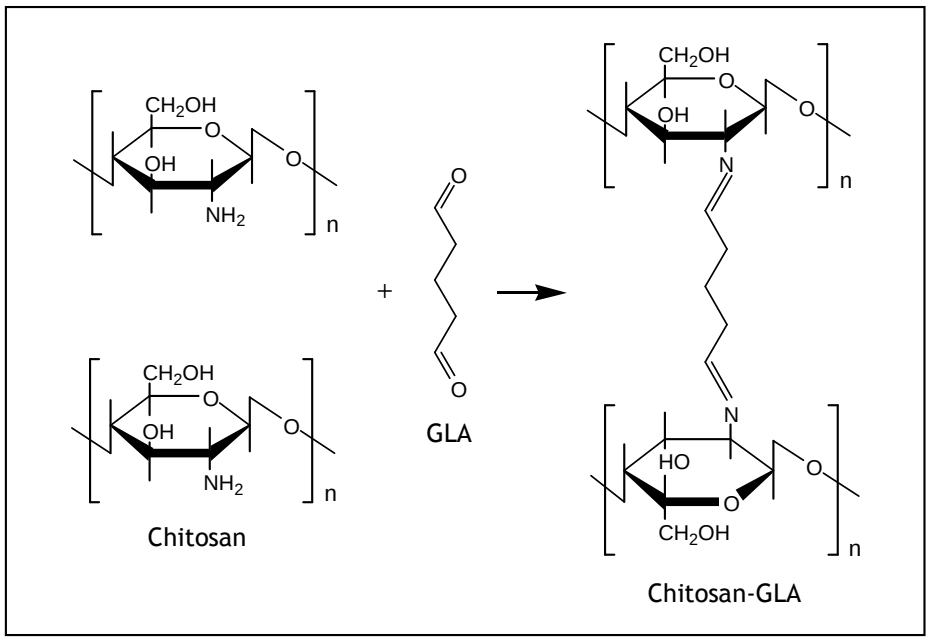


Figure 2.3 Schematic diagram of cross-linking treatment with GLA.

2.4.2 ECH and EGDE

ECH hydrolyses in an aqueous solution at ambient temperature (Solvay Chemicals, Inc., 2003; PerkinElmer, Inc., 2008) and this can be accelerated by heat (PerkinElmer, Inc., 2008; Laguerre *et al.*, 1989). For this reason, the chitosan gel beads in ECH solution were heated to a temperature between 40 °C and 50 °C for 3 hours and stirred continuously at 200 rpm using a magnetic stirrer. Since EGDE is a derivative of ECH and contains two epoxy bridges, the cross-linking reaction could also be driven by heat. After heating, the treated beads were filtered and rinsed thoroughly with deionised water, filtered and dried in air. The newly formed beads (hereafter called chitosan-ECH and chitosan-EGDE) were ground and sieved as described in Section 2.4.1. The schematic diagrams of the cross-linking treatment with ECH and EGDE are shown in Figures 2.4 and 2.5.

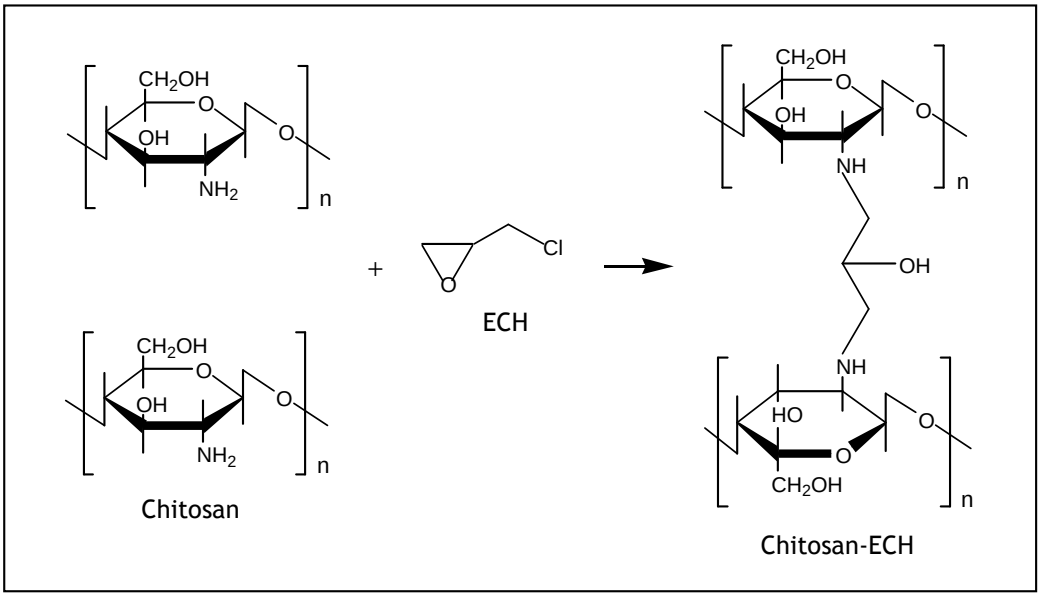


Figure 2.4 Schematic diagram of cross-linking treatment with ECH.

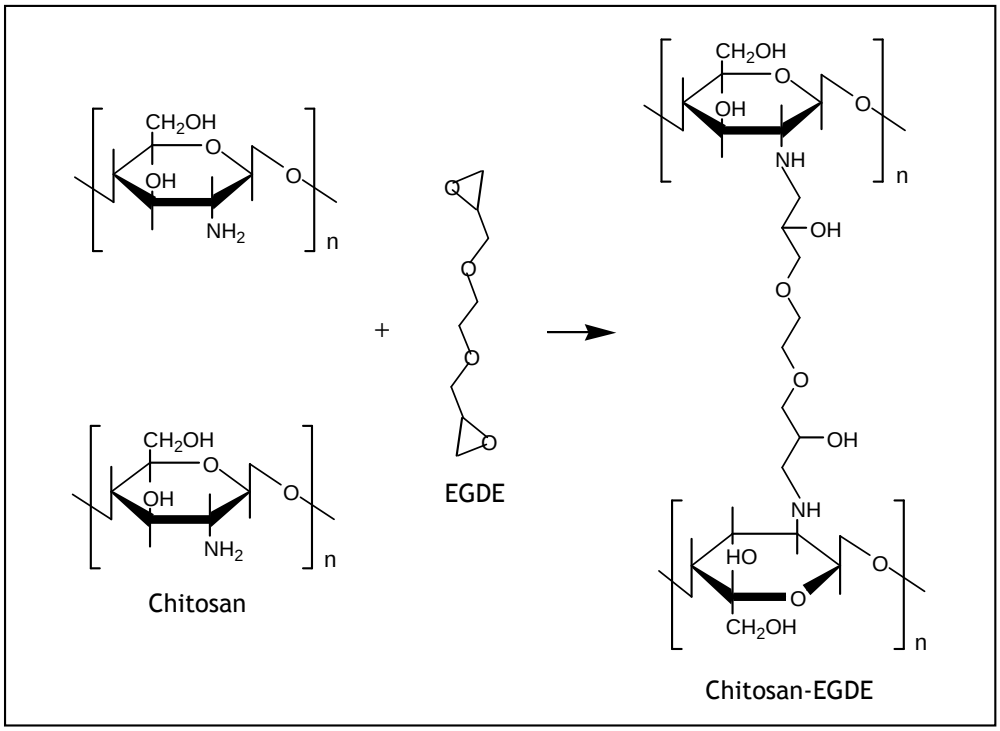


Figure 2.5 Schematic diagram of cross-linking treatment with EGDE.

2.5 Swelling percentage

In order to test the solubility and stability of chitosan and the treated chitosans, dissolution and swelling behaviour were tested in deionised water, 5% (v/v) acetic acid (pH 2.4), 0.1 mol/L HCl (pH 1.2), 0.1 mol/L KCl (pH 5.7) and 0.1 mol/L KNO_3 (pH 5.6) solutions. In triplicate, 2.5 g of each material was immersed in each of the five solutions (50 mL) at room temperature for a period of 5 days. Following this, the swollen chitosans were first blotted with filter paper and then weighed. The swelling percentage (SP) was calculated according to Equation 2.3 (Kamari and Wan Ngah, 2009):

$$SP\,(\%) = \frac{(W_s - W_d)}{W_d} \times 100 \qquad (2.3)$$

where, W_s and W_d (mg) are the weight of swollen and dry chitosans.

2.6 Characterisation study

2.6.1 Surface area and average pore diameter

Surface area and average pore diameter analyses were performed using a Gemini III 2375 Micromeritics Surface Analyser. For this purpose, 0.5 g of material was degassed under vacuum at elevated temperature for 24 hours prior to determination of N_2 physisorption isotherms at -196 ºC. There are three basic components of the surface analyser set-up, namely a gas reservoir, a twin-tube (a sample and a blank tube) and three transducers (Rouquerol *et al.*, 1999). The gas reservoir is filled with the adsorptive gas, usually nitrogen. Transducers monitor the pressure in the reservoir and detect any pressure difference between the two tubes.

The Brunauer-Emmett-Teller (BET) multipoint technique (Brunauer *et al.*, 1938; Rouquerol *et al.*, 1999) was applied for surface area determination and the Barrett, Joyner and Halenda (BJH) method (Barrett *et al.*, 1951; Rouquerol *et al.*, 1999) was used for pore diameter analysis. The amount of nitrogen that is adsorbed or desorbed by a sample is processed into an isotherm plot.

2.6.2 CHN elemental analysis

The percentage of C, H and N was determined using an Exeter CE-440 Elemental Analyser. About 2.0 mg of chitosan or treated chitosan was weighed into a tin capsule and then transferred into a combustion reactor for analysis. Acetanilide was run as standard, oxygen was used as oxidant and helium served as carrier gas. The main components of an elemental analyser instrument include a combustion reactor, a reduction tube and a series of thermal conductivity detectors (TCD) (Exeter Analytical, Inc., website).

Specialised reagents are required to eliminate the possibility of interference from sulfur, phosphorus and halogens, while copper is used to remove excess oxygen and to reduce any oxides of nitrogen to dinitrogen. Two pairs of TCD are used to trap water and carbon dioxide, which directly correlated with the concentration of hydrogen and carbon in the sample. Nitrogen measurement is carried out in the last two TCD cells by comparing the signal of nitrogen-helium mixture with a reference cell contains pure helium.

2.6.3 X-ray Diffraction (XRD) analysis

X-ray diffraction analysis was performed using a Siemens D5000 X-ray Diffractometer operated at 40 kV and 40 mA with CuK_α radiation (λ = 1.54056 Å). X-ray diffractograms were collected in the 2θ range from 5° to 90°, using a step size of 0.02° and a counting rate of 1s per step. In general, the pattern produced by the diffraction of X-rays through the closely spaced lattice of atoms in a crystal is analysed according to a mathematical relation known as Bragg's Law (Equation 2.4). Bragg's Law defines the relationship between the wavelength of an incoming ray and the *d*-spacings of the constituent lattice planes in a diffracting crystal (Schlögl, 2009).

$$n\lambda = 2d \sin \theta \qquad (2.4)$$

where, n is an integer, λ is the wavelength of incident ray, d is the interplanar spacing of the crystal, and θ is the angle of incidence and reflection of incident ray.

2.6.4 Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analyses

The surface morphology and elemental composition of the samples were examined using a Philips XL 30 Environmental Scanning Electron Microscope (ESEM). The surface morphology was observed at different magnifications. In order to remove complications due to charging, samples were gold-coated using a vacuum electric sputter coater (POLARON SC 7640) prior to analysis. SEM analysis was performed at 20.0 kV using a tungsten filament.

An SEM equipped with EDX generates a high energy beam of electrons to interact with the atoms of a sample. When the beam is focused on a sample, the electrons are absorbed or scattered by atoms of the sample. This interaction produces signals that contain surface morphology and elemental composition information (Goldstein *et al.*, 1992). Signals are electronically processed into an image and a spectrum.

2.6.5 Fourier Transform Infrared (FTIR) analysis

FTIR analysis was conducted on a Jasco 4100 FTIR Spectrometer using the KBr disc technique. KBr discs were prepared by mixing samples with IR-grade KBr in an agate mortar in the ratio 1 mg sample/100 mg KBr. The resulting mixture was milled to a fine powder and heated at 50 ºC for 3 hours. This heating procedure is important to remove any moisture which could interfere in the IR analysis. Finally, the mixture was pressed into a KBr disc in a die operating under vacuum. The IR spectrum was recorded in the wavenumber range from 4000 to 400 cm^{-1} with a resolution of 4 cm^{-1} over 30 cumulative scans.

Theoretically, when the infrared radiation is passed through a sample, an IR spectrometer measures the relative amount of energy as a function of the wavelength or frequency (Hollas, 2002). The frequency of the absorbed radiation represents the frequency of the bond or group that vibrates in the sample molecule. Since each bond or group absorbs only specific frequency, the chemical bond and molecular structure of a material can be identified. The interferometer in FTIR is able to collect the interferogram, the raw signal format, which can then be translated into an IR spectrum (Hollas, 2002).

2.7 Sorption study

2.7.1 Preparation of metal solutions

The Pb^{2+} and Ag^{+} solutions were made up with 0.1 mol/L KNO_3, while 0.1 mol/L KCl was used to prepare Cu^{2+}, Zn^{2+} and Cd^{2+} solutions. In this research, 0.1 mol/L KCl and KNO_3 were used as electrolytes to mimic the soil solution and to control the ionic strength. KCl and KNO_3 solutions were prepared in deionised water. The amount of salt required for the preparation of 1 L of metal (1000 mg/L) and electrolyte (0.1 mol/L) solutions is given in Table 2.2. To obtain lower metal concentrations, the metal solutions were diluted in the appropriate background electrolyte.

Table 2.2 Weight of salts required for the preparation of metal and electrolyte solutions.

Salt	Molecular formula	Molecular weight (g/mol)	Atomic weight (g/mol)	Weight of salt (g)
Lead(II) nitrate	$Pb(NO_3)_2$	331.20	Pb: 207.20	1.60
Silver nitrate	$AgNO_3$	169.87	Ag: 107.87	1.57
Copper(II) chloride dehydrate	$CuCl_2.2H_2O$	170.48	Cu: 63.55	2.68
Zinc chloride	$ZnCl_2$	136.28	Zn: 65.39	2.08
Cadmium chloride hemi(pentahydrate)	$CdCl_2.2.5H_2O$	228.34	Cd: 112.41	2.03
Potassium chloride	KCl	74.55	-	7.46
Potassium nitrate	KNO_3	101.11	-	10.11

2.7.2 Metal binding capacity

The kinetics study was conducted by shaking 0.1 g of each sorbent with 50 mL of a 500 mg/L metal ion solution in a 100 mL screw top glass jar. The suspension was agitated on an end-over-end shaker (30 rpm) at room temperature for 15, 30, 45, 60, 90, 120, 150, 180, 300, 600 and 1440 minutes. The equilibrium studies were accomplished by adding 0.1 g of each sorbent into 50 mL of the metal ion solutions with initial concentrations in the range of 5-500 mg/L and were then shaken for 90 minutes (chitosan) or 180 minutes (cross-linked chitosans).

After shaking, the sample was filtered through Whatman No. 2 filter paper (125 mm). The filtrate was collected in a plastic bottle and stored at 3-4 °C until analysis, while the sorbent loaded with metal was left to dry on the filter paper. Dried spent sorbent

was collected for analysis. Metal solution without sorbent was also shaken for 180 minutes, filtered and analysed to give an accurate measure of the initial metal concentration. The initial and final metal concentrations were determined by AAS, and the sorption capacity, q (mg/g) was calculated according to Equation 2.5.

$$q = \frac{(C_o - C_f)V}{W} \quad (2.5)$$

where, C_o and C_f are the initial and final metal concentrations, respectively. V is the volume of metal solution, and W is the weight of sorbent.

The pH of the solution was measured using a Mettler Delta 320 pH meter, fitted with a combined glass-reference electrode. The pH of the metal solutions was in the range of 4.7-5.7, which did not change much with dilution. Previous studies have reported that the optimum sorption of Ag^+, Pb^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} from aqueous solutions took place in the pH range 3.0-6.0 (Matos and Arruda, 2003; Ajmal *et al.*, 2006; Chen and Wang, 2008). In addition, the removal of metal ions by precipitation as the hydroxide occurs under alkaline conditions (Amarasinghe and Williams, 2007). Therefore, sorption experiments were carried out without adjusting the pH of the solution. Triplicate measurements were carried out and metal free blanks were used as controls.

2.7.3 Effect of background electrolytes

The influence of different background electrolytes on metal binding by the samples was investigated. For this experiment, stock solutions of 100 mg/L Zn^{2+} were prepared by dissolving $ZnCl_2$ and $Zn(NO_3)_2.6H_2O$ salts in either 0.1 mol/L KCl or KNO_3 electrolyte solution. The Zn^{2+} sorption trials were divided up as: Set A ($ZnCl_2$ + KCl), Set B ($Zn(NO_3)_2.6H_2O$ + KNO_3), Set C ($ZnCl_2$ + KNO_3) and Set D ($Zn(NO_3)_2.6H_2O$ + KCl). Zn^{2+} solution was added to 0.1 g of sorbent in a glass jar. The mixture was shaken and filtered as described in Section 2.7.2.

To prepare 500 mL of 100 mg/L Zn^{2+} solutions, 0.10 g $ZnCl_2$ and 0.23 g $Zn(NO_3)_2.6H_2O$ were required. The salts were then dissolved in about 200 mL of KCl or KNO_3 solution in a beaker. The solutions were transferred into 500 mL volumetric flasks and the volume was made up to 500 mL.

2.7.4 Sorption-desorption

Replicate samples of 0.02 g of each sorbent were treated separately with 10 mL of 100 and 500 mg/L metal solutions in 100 mL screw top glass jars and shaken for 24 hours on an end-over-end shaker at room temperature. After 24 hours, samples were diluted with either 0.1 mol/L KCl or KNO_3 at different dilution factors, namely 0, x1.5, x2.0, x3.5, x6.0, x8.5 and x11.0, as described in Table 2.3. An accurate volume of electrolyte solution was added into the glass jar with a volumetric pipette. Samples were then shaken for a further 24 hours for desorption to occur. The suspensions were filtered through Whatman No. 2 filter paper into plastic bottles and analysed by AAS. These experiments were conducted in triplicate and controls were run.

Table 2.3 Dilution factors for desorption study.

Volume (mL)		Dilution factor
Metal solution	Electrolyte added	
10.0	0	0
10.0	5.0	x1.5
10.0	10.0	x2.0
10.0	25.0	x3.5
10.0	50.0	x6.0
10.0	75.0	x8.5
10.0	100.0	x11.0

2.8 Atomic Absorption Spectrometry

2.8.1 Instrumentation and principle

The five primary components of an atomic absorption instrument are a light source, an atomiser, a monochromator, a detector and a display. The light source, normally a hollow cathode lamp, emits light energy of a specific wavelength corresponds to only one element. The atomiser, usually a flame, atomises the sample to produce ground-state atoms. The atoms will then absorb the light energy. The monochromator disperses and isolates the light to the detector. The amount of light absorbed is proportional to analyte concentration in the sample solution, as given by Beer's Law. To establish this relationship, calibration standard solutions are required. For quantitative measurements in atomic absorption, the Beer's Law can be written as Equation 2.6 (PerkinElmer, Inc., 1996).

$$C = kA \qquad (2.6)$$

where, *C* is the analyte concentration, *k* is the calibration curve coefficient, and *A* is the absorbance.

A Perkin-Elmer AAnalyst 400 Atomic Absorption Spectrometer (AAS) was used to measure the concentration of heavy metals in aqueous solutions, soil extracts and plant tissue digests, and the operating systems in which the metals were determined are shown in Table 2.4. For sorption studies, all AAS measurements were performed by angling the slot type burner out of the light beam path, decreasing the path length so that metal concentration can be measured up to 50 mg/L with a satisfactory precision. Hence, the top standard solution for the calibration was 50 mg/L. An air-acetylene flame with a temperature of about 2300 °C (PerkinElmer, Inc., 1996) was used for all measurements.

Table 2.4 Standard AAS conditions for the measurement of Ag, Cd, Cu, Pb and Zn.

Element	Wavelength (nm)	Energy	Linear range (mg/L)	Acetylene flow rate (L/min)	Air flow rate (L/min)
Ag	328.07	52-53	4.0	2.5	10.0
Cd	228.80	74-75	2.0	2.5	10.0
Cu	324.75	72-73	5.0	2.5	10.0
Pb	283.31	76-78	20.0	2.5	10.0
Zn	213.86	50-51	1.0	2.5	10.0

To measure each element, the hollow cathode lamp of a specific element was first inserted to the instrument, and both wavelength and energy were then set. The instrument was then programmed with information on the number of integrations and replications required per measurement, as well as the concentrations of the calibration standard solutions. A metal-free solution containing solvent only was used as a blank to 'zero' the instrument and one of the standard solutions was used to 're-slope' the instrument. Metal ion measurements were based upon calibration curves of the standard solutions. These curves were plotted several times during the period of analysis to ensure continued accuracy of the instrument readings. The drift in the readings was checked and minimised by re-sloping the calibration curve and 'zeroing' with the blank solution.

2.8.2 Preparation of AAS standard solutions

The 1000 mg/L calibration standard solutions were nitrates of Ag, Cd, Cu, Pb, and Zn in 0.5 mol/L nitric acid, as supplied. The Ag, Cu, Pb and Zn standard solutions were received from Fisher Scientific, while the Cd standard solution was from BDH Spectrosol.

Standard solutions for calibration were prepared by diluting an appropriate volume of 1000 mg/L calibration standard solutions with 0.1 mol/L of KCl or 0.1 mol/L KNO_3. For the soil extract measurements, standard solutions were made up with deionised water, EDTA (0.05 mol/L, pH 7.0), NH_4OAc (1.0 mol/L, pH 7.0) or aqua regia, whereas nitric acid was used to prepare standard solutions for analysis of plant tissue digests.

2.8.3 Determination of detection limit and quantification limit

Limit of detection and limit of quantification are terms used to describe the lowest concentration of an analyte that can be reliably determined by an analytical technique (Armbruster and Pry, 2008; PerkinElmer, Inc., 2004). In the case of atomic absorption, the instrument detection limit is defined as the concentration of analyte which equals three times the standard deviation of ten replicates of the calibration blank. Meanwhile, the practical quantification limit is the lowest level that can reliably measured within specified limits of precision and accuracy during routine operating conditions, often defined as ten times the standard deviation of ten replicates of the calibration blank (PerkinElmer, Inc., website; PerkinElmer, Inc., 2004).

The AAS was calibrated with standard solutions prior to determination of the detection and quantification limits. Then, 1.0 mg/L Ag, 0.5 mg/L Cd, 1.0 mg/L Cu, 5.0 mg/L Pb and 0.2 mg/L Zn standard solutions were selected separately, and the absorbance for each solution was measured ten times. Between each reading, the instrument was ‘zeroed’ with a blank and re-slope as described in Section 2.8.1. The estimated detection limits (mg/L) were: 0.13 for Ag, 0.22 for Cd, 0.11 for Cu, 0.57 for Pb and 0.17 for Zn, while the quantification limits (mg/L) were 0.45 (Ag), 0.74 (Cd), 0.35 (Cu), 1.92 (Pb) and 0.56 (Zn).

2.9 Soil and plant tissue analyses

2.9.1 Extraction of metal from soil

In this study, both bioavailable and total metal content in soil were measured. Deionised water, ethylenediaminetetraacetic acid (EDTA) (0.05 mol/L, pH 7.0) and ammonium acetate (NH_4OAc) (1.0 mol/L, pH 7.0) were used as extractants for the bioavailable metal content, while a combination of hydrochloric acid (6.0 mol/L) and nitric acid (69%) at a ratio of 3:1 (v/v) known as aqua regia was used to determine the total metal content in soil.

To prepare 1 L of 0.05 mol/L EDTA (pH 7.0) solution, 14.61 g EDTA was weighed and dissolved in approximately 900 mL of deionised water. Then, about 8 mL of ammonia solution was carefully added to the solution. The pH of the solution was adjusted to pH 7.0 by further adding a few drops of ammonia or nitric acid solution.

1 L of 1.0 mol/L NH_4OAc (pH 7.0) was prepared by adding 77.08 g NH_4OAc salt to about 900 mL of deionised water in a 2 L beaker. Acetic acid and ammonia solutions were used to bring the pH of the solution to pH 7.0.

To prepare 1 L of 6.0 mol/L HCl from concentrated HCl (12.0 mol/L), 500 mL of concentrated HCl was carefully added to approximately 400 mL of deionised water in a 1 L volumetric flask. The solution was mixed gently and made up to volume with deionised water.

2.9.1.1 Bioavailable metal content

The most readily available fraction of Cu, Pb and Zn in soil was determined by water extraction. For this experiment, water extraction was carried out on fresh and air-dried soil samples. In triplicate, 5.0 g of soil samples were weighed into 100 mL screw top glass jars and 50 mL of deionised water was added. The mixtures were then agitated on an end-over-end shaker for 1 hour at room temperature. Samples were filtered into plastic bottles and stored in the cold room until ready for analysis. EDTA and NH_4OAc extractions were performed by similar procedure to that described for metal extraction by deionised water. However, only air-dried soil samples were used in these experiments.

2.9.1.2 Total metal content

Using a four-figure balance, approximately 0.25 g of soil was weighed into a scoop, which had been lined with aluminium foil. With the aid of a long handle, the soil sample was then placed at the bottom of a block digestion tube. 9 mL of HCl (6.0 mol/L) and 3 mL of HNO_3 (69%) were added accurately to each soil sample. The digestion tubes were allowed to stand overnight at room temperature to allow the aqua regia to equilibrate with the soil. The digestion tubes containing soil samples, as well as aqua regia alone were placed in the digestion block. Ten replicates of each soil sample and blank giving a total of 40 samples on the digestion block. The temperature of digestion block was set at 125 ºC and the extraction unit of the fume cupboard was switched on to remove the NO_2 gas evolved during digestion process. After 3 hours of digestion, the digestion tubes were allowed to cool and 10 mL of deionised water was added to each tube. The digests were then filtered through Whatman No. 50 hardened filter paper into 50 mL volumetric flasks, made up to volume with deionised water and stored in the cold room at 3-4 ºC until ready for analysis.

2.9.2 Digestion of plant tissue

In triplicate, 0.25 g milled plant tissue (see Section 2.10.3) was weighed into a silica basin. The milled samples were ashed in a furnace at 450 ºC for 3 hours. In a 50 mL tall form beaker, 12 mL of HNO_3 (69%) was added to the ashed sample, and the beaker covered with a watch glass. The plant sample was left overnight in the fume cupboard for equilibration. Samples and blanks were then digested on a hot plate at a temperature of 110 ºC for 3 hours. The beakers were allowed to cool, and then about 8 mL of deionised water was added to each sample. The samples were filtered through Whatman No. 50 hardened filter paper into 25 mL volumetric flasks. Deionised water was added to make volume to the mark, transferred into plastic bottles and stored in cold room.

2.9.3 Standard/certified reference materials

Standard reference plant materials (SRM 1573a Tomato Leaves – National Institute of Standards & Technology, USA, and SRM 1575 Pine Needles – National Bureau of Standards, USA) and certified reference soil material (LGC 6135 Hackney Brick Works Soil – Laboratory of the Government Chemist, UK) were used to verify the accuracy of metal determination. Reference materials were treated and analysed using the same procedures applied for plant tissue and soil samples.

As shown in Table 2.5, the recovery rates were within 93-107% for soil and 87-92% for plant tissue, respectively. This suggests that the procedure used in this research for the determination of heavy metals in soil and plant tissues are feasible.

Table 2.5 Metal concentrations in reference materials.

Reference Material		Metal concentration (mg/kg)		
		Pb	Zn	Cu
LGC 6135	Certified	391 ± 16	316 ± 41	105 ± 5
	Measured	365 ± 9	298 ± 14	112 ± 1
	Recovery (%)	93.4	94.3	106.7
SRM 1573a	Certified	-	30.9 ± 0.7	4.7 ± 0.1
	Measured	-	28.5 ± 2.9	4.2 ± 0.5
	Recovery (%)	-	92.2	89.4
SRM 1575	Certified	10.8 ± 0.5	-	3.0 ± 0.3
	Measured	9.4 ± 0.9	-	2.6 ± 0.3
	Recovery (%)	87.0	-	86.7

Values represent mean of three replicates ± standard deviation.
LGC 6135: Hackney Brick Works Soil; SRM 1573a: Tomato Leaves; SRM 1575: Pine Needles.

2.10 Pot experiment (2009)

2.10.1 Preparation of soil

In this research, the source of heavy metals was an abandoned Pb/Zn mine at Tyndrum, Scotland (Figure 2.6). The mine began operation in 1741 and ceased operation in 1862, and reworking of the original waste dumps was carried out between 1916 and 1925 (MacKenzie and Pulford, 2002). The ore processing waste dumps contained high concentrations of Pb, Zn and Cu (Pulford *et al.*, 2009).

The mine waste used in this research was collected from a waste dump (Figure 2.6b) at the surface layer of up to 15 cm depth using a stainless steel trowel and was stored in the cold room at 3-4 ºC. Waste characteristics: pH 5.9; CEC 7.7 $cmol_{(+)}/kg$; 96% sand; 4% silt + clay; 65,600 mg Pb/kg, 25,100 mg Zn/kg and 2950 mg Cu/kg. The waste was mixed with sharp sand at a 1:1 (w/w) ratio to reduce metal toxicity (Nwachukwu and Pulford, 2009), hereafter referred to as bulk soil. The sharp sand used was a lime free horticultural grade and was supplied by William Sinclair Horticulture Ltd. Bulk soil samples were air-dried, sieved using a 2 mm sieve and stored in self-sealing plastic bags.

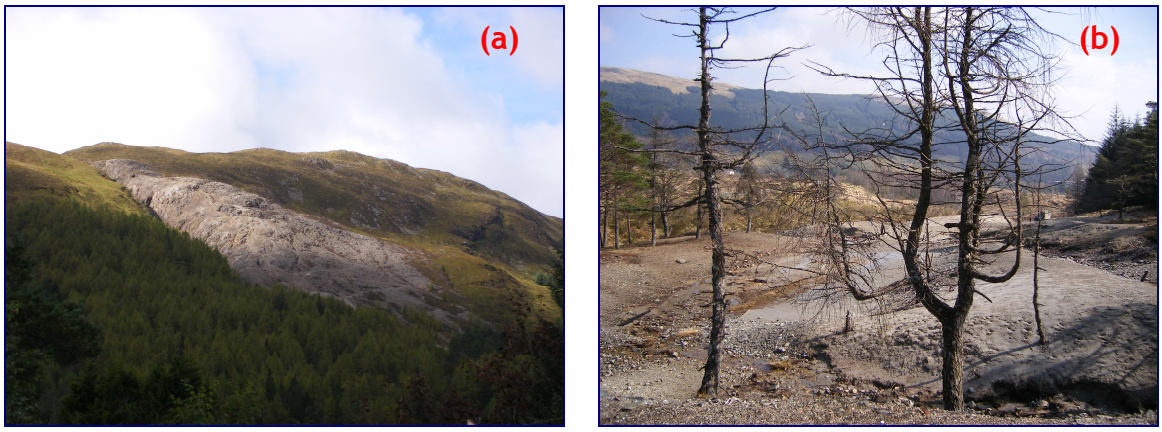


Figure 2.6 Abandoned Pb/Zn mine at Tyndrum, Scotland. Mining area (a) and waste dump (b). Photographs are courtesy of Dr Susie Fawley.

2.10.2 Pot experiment set-up

The first pot experiment was conducted from March 2009 to October 2009. Initially, five pots (9.5 cm in height and 10 cm diameter) containing 200 g of soil were saturated with deionised water and the water holding capacity was determined. For subsequent pot experiments, pots with a radius of 3.5 cm and a height of 5.5 cm were filled with 200 g of bulk soil. Chitosan and chitosan-GLA were added to the soil at three rates, 0%, 0.5% and 1.0% (w/w), in six replicates and mixed thoroughly. The soils were left to equilibrate for two weeks and watered thrice a week with deionised water to maintain the moisture. After two weeks, 1.0 g of *Lolium perenne* (perennial ryegrass) seed (Emorsgate Seeds, UK) was weighed on a two-figure balance, sprinkled evenly onto bulk soil and watered in. The pots were covered with a black plastic bag to prevent drying out until germination began.

After germination, plants were placed on a greenhouse bench and arranged in a randomised block design (Figure 2.7). The water content of the soil was adjusted to obtain 70% of the water holding capacity by adding deionised water daily, avoiding prolonged water logging.

T1R2	T5R3	T2R4	T5R2	T4R5	T2R2
T5R1	T4R4	T1R1	T3R4	T1R6	T3R5
T4R6	T1R5	T3R6	T2R1	T3R3	T5R4
T2R5	T3R1	T4R2	T1R4	T5R5	T1R3
T3R2	T2R3	T5R6	T4R3	T2R6	T4R1

Note: T: Treatment; R: Replicate; T1: 0% amendment; T2: Chitosan 0.5%; T3: Chitosan 1.0%; T4: Chitosan-GLA 0.5%; T5: Chitosan-GLA 1.0%

Figure 2.7 Layout of pot arrangement.

As the mine waste has a poor plant nutrient content, ¼ strength Hoagland's nutrient solution was added to each pot to ensure continued growth of the plants (Nwachukwu and Pulford, 2009). The nutrient solution was prepared according to a procedure described by Watson *et al*. (2003). The first addition of fertiliser was done 3 weeks after sowing and then once a week at application rate of 20 mL. Plants were grown for 24 weeks under natural light and both temperature and humidity were monitored with a 'Oakton' digital thermometer. Mean daily temperatures in the greenhouse were between 18 ºC and 43 ºC, while mean night temperatures ranged from 7 ºC to 14 ºC. Mean humidity was in the range of 45% to 78% for the duration of the experiment.

2.10.3 Harvest and biomass yield

The plants were harvested twice, at 12 and 24 weeks of growth. Only aerial parts were cut in the first harvest, while both shoots and roots were collected at the second harvest. Shoots were cut at 1.0 cm above the soil surface to avoid contamination by soil, and roots were washed thoroughly with deionised water to remove soil particles. The plant cuttings were put into envelopes and dried in an oven set at 70ºC for 48 hours. After two days, the dry weight of plant tissue was measured. The dried shoots and roots were milled separately using a grinder. A compressed air gun and a vacuum cleaner were used to clean the grinder after each grinding, to avoid contamination from previous samples. The ground tissue samples were stored in self-sealing bags until ready for digestion.

2.10.4 Soil analysis

At the end of the 24-week pot trial, EDTA and NH_4OAc extractable metal contents in the soil were determined at a soil:extractant ratio of 1:10, as described in Section 2.9.1.1. Soil pH was measured in deionised water with a soil:solution ratio of 1:2.5.

2.11 Pot experiment (2010)

2.11.1 Pot experiment set-up

The second pot trial was performed in March 2010 using two plant species, namely *Lolium perenne* (perennial ryegrass) and *Brassica napus* (rapeseed). Using a two-figure balance, about 200 g of bulk soil was added to a plastic pot with a radius of 3.5 cm and a height of 5.5 cm. The bulk soils were then amended separately with chitosan and chitosan-GLA at the rates of 0%, 1% and 10% (w/w) and mixed thoroughly using a glass

rod. Each treatment was replicated six times, making a total of 30 pots for each plant species. The pots were left to equilibrate before planting and deionised water was added where necessary to adjust the water content of the bulk soils. After two weeks of equilibration, each pot was sown with either 1.0 g of perennial ryegrass seed or 1.0 g of rapeseed seed (Cotswold Grass Seeds Direct, UK). The pots were covered with a black plastic bag which was removed when the seeds started to germinate.

Due to the similar number of treatment and replicate procedure (five treatments x 6 replicates), the pots for each plant species were arranged randomly in the greenhouse as described earlier in Figure 2.7 (Section 2.10.2). The pots were watered daily with deionised water and the growth was checked. In the third week of growth, plants were given 20 mL of ¼ strength Hoagland's nutrient solution and subsequently weekly until the end of the trial. Plants were allowed to grow under natural light and temperature conditions. The mean daily temperatures ranged from 16 °C to 40 °C, while mean night temperatures were between 8 °C and 13 °C. The humidity in the greenhouse was also recorded and the mean was in the range of 38% to 62%.

2.11.2 Harvest and biomass yield

The rapeseed was harvested once, after 8 weeks of growth. The ryegrass was harvested twice, the first harvest was after 9 weeks of growth, and the second was at 20 weeks. At 9 weeks, only shoot biomass was harvested, while both shoots and roots were collected at 20 weeks. Plants were cut with a pair of scissors, which were cleaned and wiped after each use to avoid contamination from previous plant samples. The plant tissues were rinsed in deionised water, dried, weighed and milled, as described in Section 2.10.3.

2.11.3 Soil analysis

At the end of the growth period, soils were analysed for the NH_4OAc extractable metal concentrations and pH values were determined. Procedures for these measurements were as outlined in Sections 2.9.1.1 and 2.10.4, respectively.

2.12 Biodegradation study

The first biodegradation study was carried out from June 2010 to September 2010. 100 g of garden sand and 5 g of chitosan were mixed thoroughly in a 500 mL beaker. The garden sand used in this study was a lime free horticultural grade (Bradstone, UK). The mixture was then transferred into a 1 L Kilner jar and 20 mL of ¼ strength Hoagland's nutrient solution was added using a volumetric pipette. The mixtures were then treated separately with Pb^{2+}, Zn^{2+} and Cu^{2+} solutions at concentrations of 5, 10 and 50 mg/kg by adding an appropriate amount of 5000 mg/L metal solution. Tables 2.6 and 2.7 summarise the weight of metal salt used in the preparation of 100 mL 5000 mg/L metal solutions (in deionised water) and the volume of metal solution required for the metal treatment. A mixture that received deionised water only was set as 0 mg/kg metal treatment. The mixtures were mixed gently using a glass rod. The jars were then sealed and left to incubate for two weeks. This procedure is important to allow microbial activity to stabilise before any readings were taken (Nwachukwu and Pulford, 2011). Since the measurement of CO_2 released was cumulative, as discussed by Nwachukwu and Pulford (2011), each metal treatment was set without replication.

After two weeks, 10 mL of 1.0 mol/L NaOH was transferred into a 30 mL glass jar using a volumetric pipette. In each Kilner jar, the NaOH solution was placed on the sand-chitosan mixture. The NaOH solution was also placed in three empty Kilner jars to serve as blanks. The jars were sealed carefully and left for one week, before the first titration for the determination of CO_2 evolved was conducted. After one week, the NaOH solution was taken out, 5 mL of 1.0 mol/L $BaCl_2$ and a few drops of 0.1% phenolphthalein indicator were added. The solution was then titrated immediately with 1.0 mol/L HCl. The colour of the solution changed from pink to colourless.

After titration, the jars were left open for 30 minutes to reaerate. Immediately after aeration, fresh NaOH solution was placed into each jar, and the jars were sealed. The weight of the jars was recorded once a week to monitor the moisture content of the sand-chitosan mixture, and deionised water was added where necessary. This experiment lasted for 12 weeks. The Kilner jars were left in a room at 20 °C. The amount of CO_2 released per week (mg C per kg per week) was calculated according to Equation 2.7 (Nwachukwu and Pulford, 2011).

$$CO_2\text{-C released per week} = \frac{\left[(\text{mean blank titre} - \text{treatment titre}) \times 6\right]}{\text{Weight of amendment (g)}} \times 1000 \qquad (2.7)$$

Table 2.6 Weight of metal salt used to prepare 100 mL of 5000 mg/L metal solution in deionised water.

Salt	Molecular formula	Molecular weight (g/mol)	Atomic weight (g/mol)	Weight of salt (g)
Lead(II) nitrate	$Pb(NO_3)_2$	331.20	Pb: 207.20	0.80
Copper(II) chloride dihydrate	$CuCl_2.2H_2O$	170.48	Cu: 63.55	1.34
Zinc chloride	$ZnCl_2$	136.28	Zn: 65.39	1.04

Table 2.7 Volume of 5000 mg/L metal solution required for the metal treatment.

Metal treatment (mg/kg)	Volume required (mL)	Treatment details
5	0.1	0.5 mg metal added to 0.1 kg sand = 5 mg/kg
10	0.2	1.0 mg metal added to 0.1 kg sand = 10 mg/kg
50	1.0	5.0 mg metal added to 0.1 kg sand = 50 mg/kg

To prepare 1 L of 1.0 mol/L $BaCl_2$ and NaOH solutions, 208.24 g of $BaCl_2$ and 40.00 g of NaOH were dissolved separately in approximately 500 mL of deionised water in a beaker and stirred using a magnetic stirrer. The solutions were transferred into 1 L volumetric flasks and made to the mark with deionised water. 1 L of 1.0 mol/L HCl solution was prepared by diluting approximately 83 mL concentrated HCl (12.0 mol/L) with deionised water in a 1 L volumetric flask.

The second biodegradation study was conducted on chitosan, chitosan-GLA, chitosan-ECH and chitosan-EGDE, according to a similar procedure as described earlier. The experiment commenced in September 2010 and was completed in December 2010, a total period of 12 weeks. The experiment was set-up as 4 materials x 3 metals x 3 concentrations, plus 1 treatment of 0 mg/kg metal for each material and 6 blanks, giving a total of 46 samples.

2.13 Metal bioavailability study

The metal bioavailability study was performed in September 2010 and was concurrent with the second biodegradation study. However, only chitosan was used for this study. Therefore, the set-up was 1 material x 3 metals x 3 concentrations, plus 1 treatment of 0 mg/kg metal, making a total of 10 samples. Chitosan and garden soil were mixed, treated with heavy metals and kept in 1 L Kilner jars, as outlined in Section 2.12. The NH_4OAc extractable metal concentrations in the mixture were determined at three periods, namely 1 day, 6 weeks and 12 weeks after the metal treatment. The jars were

left open for 30 minutes each week to allow replenishment of oxygen (Nwachukwu and Pulford, 2011). The jars were kept under the same experimental conditions as the second biodegradation study.

For metal extraction, about 5 g mixture (wet weight) was weighed into a 100 mL screw top glass jar and added with 50 mL of NH_4OAc solution. The mixtures were shaken, filtered and analysed using AAS, as described in Section 2.9.1.1. Analysis was run in duplicates. Since the extraction was on wet samples, a conversion factor was required for the calculation. For this purpose, 5 g of samples were weighed and dried in air. The weight of the dried sample was measured and the conversion factor was established.

2.14 Statistical analyses

Statistical analyses were carried out using Minitab 15 Statistical Software (Minitab Inc., PA, USA). Normality of data was verified using the Anderson-Darling test. The data were analysed using the general linear model of one-way analysis of variance (ANOVA), followed by Tukey's test at a significance level of $p = 0.05$ to determine least significant difference (LSD) for the comparison of means.

3 CHARACTERISATION STUDY

In this research, chitosan was treated using three cross-linking reagents, namely glutaraldehyde (GLA), epichlorohydrin (ECH) and ethylene glycol diglycidyl ether (EGDE) to enhance its chemical stability in acidic media and to improve its physical properties. The cross-linking treatments are fully discussed in Section 2.4. The effects of cross-linking treatment on the surface and physical properties of chitosan were determined.

As discussed in Section 1.4.2, the effectiveness of soil amendments for metal immobilisation is greatly influenced by their physical and chemical properties. Therefore, prior to application of chitosan and cross-linked chitosans to contaminated soil, it is necessary to understand their interaction with metals, and how the surface properties are altered by this interaction. Several techniques such as X-ray diffraction (XRD), scanning electron microscopy (SEM), energy dispersive X-ray analysis (EDX) and Fourier transform infrared spectroscopy (FTIR) were used in this study. The procedures are fully described in Sections 2.5 and 2.6.

There are two main sections in this chapter. The first section reports the surface and physical properties of chitosan and cross-linked chitosans. The surface properties of the chitosans following metal interaction, as well as the binding mechanism(s) of metal ions onto the chitosans are discussed in the second section.

3.1 Objectives of the study

1. To study the effect of cross-linking treatment on the surface and physical properties of chitosan.
2. To study the effect of metal interaction on the surface properties of chitosan and cross-linked chitosans.
3. To understand the binding mechanism(s) of metal ions onto the chitosan and cross-linked chitosans.

3.2 Dissolution and swelling behaviour

The swelling behaviour of chitosan and treated chitosans is presented in Table 3.1. Chitosan was completely dissolved in 5% (v/v) acetic acid (pH 2.4) and 0.1 mol/L HCl (pH 1.2) solutions due to the protonation of chitosan amino groups in low pH media. Chitosan was insoluble in deionised water, KCl and KNO_3 solutions because of the low

concentration of H^+ ions which protonate $-NH_2$ groups to yield $-NH_3^+$. Cross-linked treated chitosans did not dissolve in any of the media studied, but rather swelled to a lower degree than untreated chitosan. The reduction in the swelling percentage can be explained by the fact that during cross-linking, the reactive NH_2 groups of chitosan react with CHO or CH_2O groups of cross-linking reagents forming intermolecular bridges (Figures 2.3 to 2.5, Sections 2.4.1 and 2.4.2). These intermolecular bridges result in reduced mobility of the macromolecular chains. As a result, the swelling behaviour of chitosan is restricted and the rigidity is correspondingly improved.

Table 3.1 Swelling behaviour of chitosan and treated chitosans.

Adsorbent	Swelling percentage (%)				
	Deionised water	5% (v/v) acetic acid	0.1 mol/L HCl	0.1 mol/L KCl	0.1 mol/L KNO_3
Chitosan	23.6 ± 1.1	Soluble	Soluble	35.6 ± 0.2	33.8 ± 0.5
Chitosan-GLA	22.9 ± 0.9	86.4 ± 0.1	80.9 ± 0.7	30.4 ± 0.4	31.6 ± 0.4
Chitosan-ECH	19.1 ± 1.1	79.5 ± 0.2	77.2 ± 1.6	29.8 ± 0.9	28.2 ± 0.3
Chitosan-EGDE	20.7 ± 0.9	75.8 ± 0.2	72.1 ± 1.7	25.5 ± 0.3	26.4 ± 1.0

Values represent mean of three replicates ± standard deviation.

3.3 Characterisation: Cross-linked chitosans

3.3.1 Surface area and pore diameter

As can be seen in Table 3.2, the BET surface area of chitosan was increased by cross-linking. ECH and EGDE treatments increased the chitosan surface area by 142% and 108%, respectively. A slight increase, 17%, was observed for chitosan-GLA. However, the BET surface area obtained for chitosan-GLA (1.4 m^2/g) is higher than the value of 0.2 m^2/g reported by Cestari *et al*. (2004) using chitosan beads treated with 1% (m/v) GLA solution.

Table 3.2 BET surface area and average pore diameter of chitosan and treated chitosans.

Adsorbent	BET surface area (m^2/g)	Average pore diameter (nm)
Chitosan	1.2 ± 0.2	3.7 ± 0.6
Chitosan-GLA	1.4 ± 0.1	17.9 ± 0.2
Chitosan-ECH	2.9 ± 0.6	2.1 ± 0.4
Chitosan-EGDE	2.5 ± 0.3	2.2 ± 0.2

Values represent mean of three replicates ± standard deviation.

According to Zdravkov *et al*. (2007), the sorption performance of different sorbents is highly dependent on their internal pore structure. The pore size characteristics are important to identify pores that are accessible and can be occupied by contaminants. Treatment with ECH and EGDE decreased the average pore diameter of chitosan by 44% and 41%, respectively. Interestingly, GLA treatment resulted in a fourfold increase of chitosan average pore diameter. In fact, the average pore diameter obtained for chitosan-GLA (17.9 nm), is higher than the average pore diameter reported for ceramic α-alumina composite-chitosan (7.1 nm) (Boddu *et al*., 2008). By IUPAC convention, pore sizes are classified into three diameter groups (d), that is, micropores (d < 2 nm), mesopores (2 < d < 50 nm) and macropores (d > 50 nm) (Rouquerol *et al*., 1999). Based on this classification, chitosan and treated chitosans are mesoporous. The surface area and pore diameter of some low-cost sorbents, in both natural and treated forms are given in Table 3.3 for comparison.

The reported surface area of unmodified chitosan is found to be very close to the surface area determined for natural pine bark, meanwhile the pore size of untreated bagasse fly ash is similar to unmodified chitosan. Crab carapace and chitin have a greater surface area than chitosan and treated chitosans. Activated carbons show the best surface area property, whereby each of the activated carbons has a BET surface area of more than 1000 m^2/g. Despite their high surface areas, some activated carbons have a lower average pore diameter compared to unmodified chitosan and the treated chitosans, as well as some other sorbents. The activated carbon derived from coconut shell has an average pore diameter similar to chitosan-ECH and chitosan-EGDE, meanwhile activated carbon based on bamboo prepared by Hameed and El-Khaiary (2008) has an average pore diameter of 2.485 nm, about 1.2 nm lower than the value measured for unmodified chitosan. In fact, natural rubber leaves have an average pore diameter of approximately six times higher than bamboo derived activated carbon prepared by Hameed and El-Khaiary (2008). The pore diameter of chitosan-GLA is found to be quite similar to that of natural rubber leaves and lignin.

Depending on the chemical and physical nature of the sorbent, as well as the treatment procedure, chemical modification may not always increase the surface area and pore diameter of the sorbent. For example, Argun *et al*. (2009) reported that the surface area of pine bark increased from 1.41 to 3.15 m^2/g with NaOH treatment (Table 3.3). Meanwhile, a study by Wan Ngah and Hanafiah (2008) found that NaOH treatment did not affect the surface area of rubber leaves, whereas the average pore diameter decreased from 15.5 to 11.3 nm. A different observation was reported by Lin *et al*. (2008), whereby H_2SO_4 treatment on thermal power plant fly ash was found to increase

the surface area from 1.786 to 6.236 m^2/g and decrease the average pore diameter from 12.12 to 8.075 nm.

Table 3.3 Comparison of surface area and pore diameter of some low-cost sorbents, natural and treated form.

Sorbent	Surface area (m^2/g)[a]	Pore diameter (nm)[b]	Reference
Chitin, natural	6.5	N/A	Barriada *et al*. (2008)
Crab carapace, natural	33.4	N/A	Cochrane *et al*. (2006)
Lignin, natural	21.7	14.8	Guo *et al*. (2008)
Bone char, natural	107	9.43	Ip *et al*. (2009)
Sphagnum peat, natural	0.7	5.52	Ip *et al*. (2009)
Brown algae (*Fucus vesiculosus*), natural	0.22	N/A	Cochrane *et al*. (2006)
Pine bark, natural	1.41	N/A	Argun *et al*. (2009)
Pine bark, treated with NaOH	3.15	N/A	Argun *et al*. (2009)
Rubber leaves, natural	0.478	15.5	Wan Ngah and Hanafiah (2008)
Rubber leaves, treated with NaOH	0.436	11.3	Wan Ngah and Hanafiah (2008)
Tea leaves, natural	0.79	1.92	Amarasinghe and Williams (2007)
Yellow passion fruit peel, natural	30.0	3.0	Pavan *et al*. (2008)
Coir pith, natural	7.42	7.68	Suksabye *et al*. (2007)
Rice husk ash, natural	36.44	4.26	Srivastava *et al*. (2009)
Bagasse fly ash, natural	168.8	3.39	Srivastava *et al*. (2006)
Thermal power plant fly ash, natural	1.786	12.12	Lin *et al*. (2008)
Thermal power plant fly ash, treated with H_2SO_4	6.236	8.075	Lin *et al*. (2008)
Fertiliser plant waste carbon, natural	357	6.841	Mall *et al*. (2006)
Coconut shell derived activated carbon	1113	2.24	Suksabye *et al*. (2007)
Bamboo derived activated carbon, raw material was treated with H_3PO_4 at impregnation ratio of 2:1	1400	5.32	Ip *et al*. (2009)
Bamboo derived activated carbon, raw material was treated with K_2CO_3 at impregnation ratio of 1:1	1724	2.485	Hameed and El-Khaiary (2008)
Red mud, natural	108	N/A	Gupta and Sharma (2002)
Furnace sludge, natural	78.54	N/A	Naiya *et al*. (2009)
Furnace slag, natural	4	N/A	Jain *et al*. (2003)

[a] BET surface area, [b] Average pore diameter (BJH), N/A - Data not available.

A study by Rao *et al*. (2007) has demonstrated that even a small increase in the surface area can cause a significant increase in metal uptake. They modified neem sawdust with HCl and observed a 20% increase in the BET surface area of the sawdust whereas the sorption capacity of Cu^{2+} increased by 492%.

3.3.2 CHN elemental analysis

Alterations in the elemental composition of chitosan induced by chemical reagents can be examined using CHN elemental analysis (Muzzarelli, 1977; Yang and Yuan, 2001; Dal Pozzo *et al.*, 2000). In this study, CHN elemental analysis was performed to ascertain the empirical formula of treated chitosans. The theoretical and experimental CHN composition of chitosan and treated chitosans are presented in Table 3.4.

As expected, cross-linking treatment reduced the N/C ratio (weight basis) of chitosan. The N/C ratios for chitosan-GLA and chitosan-ECH were 0.146 and 0.153, respectively. EGDE, with a molecular weight (MW) of 174.12 g/mol, reduced the N/C ratio from 0.198 to 0.107, which is more than for the GLA (MW 100.12 g/mol) and ECH (MW 92.53 g/mol) treatments. The reduction in the N/C ratio can be attributed to incorporation of cross-linking reagents into the structure of the D-glucosamine repeat unit of the chitosan. In general, the experimental values estimated from CHN elemental analysis were in line with the theoretical values, which assumed total consumption of NH_2 groups of chitosan.

Table 3.4 Composition of chitosan and treated chitosans.

Adsorbent	Monomer (g/mol)	Weight (%)			
		C	H	N	N/C
Chitosan ($C_6H_{11}NO_4$)	161	42.4 ± 0.5 (44.7)	6.3 ± 1.2 (6.8)	8.4 ± 0.1 (8.7)	0.198 (0.195)
Chitosan-GLA ($C_{17}H_{26}N_2O_8$)	386	50.7 ± 0.9 (52.8)	6.0 ± 0.3 (6.7)	7.4 ± 0.5 (7.3)	0.146 (0.138)
Chitosan-ECH ($C_{15}H_{25}N_2O_9$)	377	46.4 ± 0.8 (47.7)	5.9 ± 0.9 (6.6)	7.1 ± 0.8 (7.4)	0.153 (0.155)
Chitosan-EGDE ($C_{20}H_{34}N_2O_{12}$)	494	49.7 ± 1.6 (48.6)	6.5 ± 0.6 (6.9)	5.3 ± 0.1 (5.7)	0.107 (0.117)

Theoretical data are in brackets. Values represent mean of three replicates ± standard deviation.

3.3.3 XRD analysis

It is known that the nature of the crystal structure of a sorbent plays an important role in its performance. Onsøyen and Skaugrud (1990), Tang *et al.* (2007), and Wang and Kuo (2008) showed that decreasing the crystallinity of chitosan improves metal uptake, mainly by enhancing the diffusivity of metal ions into the centre of chitosan particles (Guibal *et al.*, 1999; Jeon and Höll, 2003). Koyama and Taniguchi (1986), Milot *et al.* (1998), and Monteiro and Airoldi (1999) reduced the crystallinity of chitosan through cross-linking treatment.

X-ray diffractograms of chitosan and treated chitosans are shown in Figure 3.1. Chitosan shows a prominent broad reflection at 20.42° 2θ and a very broad reflection at 41.88° 2θ. Both peaks are attributed to the allomorphic tendon form of chitosan (Saitô *et al.*, 1987). However, it is apparent that cross-linking treatments applied to natural chitosan have reduced these reflections, in particular that at 20.42°. Cross-linking treatment with ECH and EGDE were pronounced in their effects in this respect. The decrease in crystallinity can be attributed to the deformation of hydrogen bonds due to the substitution of amino groups by the cross-linking reagents (Tang *et al.*, 2007), leading to the formation of amorphous structure in cross-linked treated chitosans (Wang and Kuo, 2008).

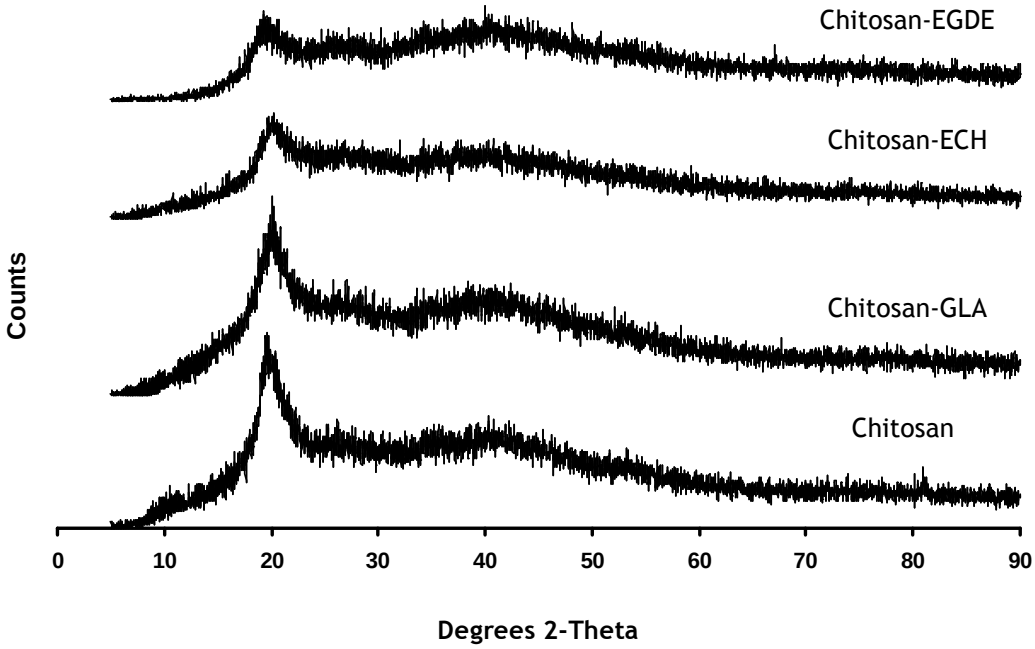


Figure 3.1 X-ray diffractograms of chitosan and treated chitosans.

Beppu *et al.* (2007) cross-linked chitosan membranes with 0.25% (w/w) GLA solution at 25 ºC for 14 hours and observed a significant reduction in the 20.42° reflection. In contrast, Cestari *et al.* (2008) reported that chitosan retained its structure after cross-linking treatment with a 2% (m/v) GLA solution. Lee *et al.* (2004) added 0.01 mol/L ECH solution to chitosan fibres and stirred the mixture for 60 seconds. They observed no significant change in the 20.42° reflection. A possible explanation for different effects reported in the literature may be the different methods used for cross-linking treatment. The present results indicate that treated chitosans had less crystalline structures compared to their chitosan precursor.

In order to study the effect of heat on the crystallinity, an XRD comparison was made between a chitosan sample heated in air at 100 ºC for two hours and one heated similarly at 400 ºC. The results are presented in Figure 3.2. As can be seen, the chitosan remained crystalline following 100 ºC treatment. Meanwhile, 400 ºC treatment shifted the reflection at 20.42° 2θ to 26.82° 2θ and decreased the intensity of this reflection from 250 counts to 180 counts, implying a significant reduction in crystalline structure of chitosan.

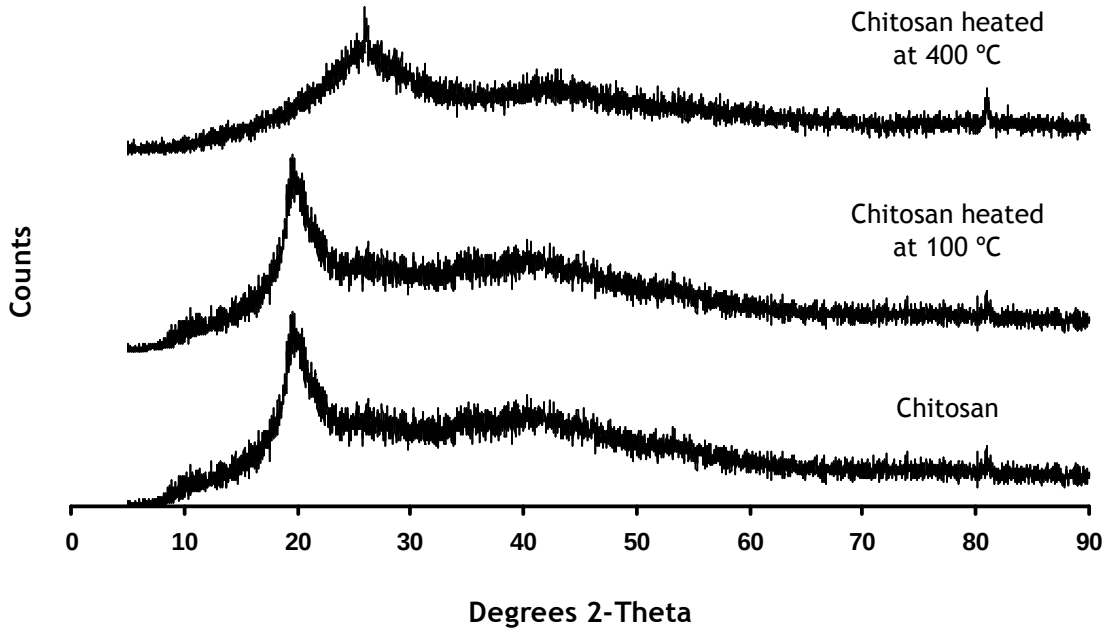


Figure 3.2 X-ray diffractograms of chitosan and heated chitosans.

Further, the melting point of chitosan was also studied. This was performed using the electrothermal capillary technique. It was observed that the white colour of chitosan changed into light brown at 220 ºC. This indicates that decomposition of chitosan starts at this temperature. Chitosan was not melt, but instead caramelizes at 250 ºC and turns dark brown. Therefore, chitosan does not have a true melting point. Since the cross-linking treatments were carried out below 100 ºC (Sections 2.4.1 and 2.4.2), it can be speculated that the change in the crystallinity observed (Figure 3.1) was due to chemical reaction between chitosan and the cross-linking reagents.

3.3.4 SEM analysis

SEM images of chitosan and treated chitosans are shown in Figure 3.3, which shows that there is a significant change in the surface texture of the treated chitosans. Unmodified chitosan displayed a dense surface, while treated chitosans have a rougher surface texture. Wang and Kuo (2008) observed a rough surface morphology on chitosan-poly(acrylic acid) nanoparticles. Chitin was reported to have a smooth surface texture (Barriada *et al.*, 2008). Meanwhile, Chen *et al.* (2002) observed a rugged surface morphology on calcium alginate-based ion-exchange resin, a polysaccharide-like polymer.

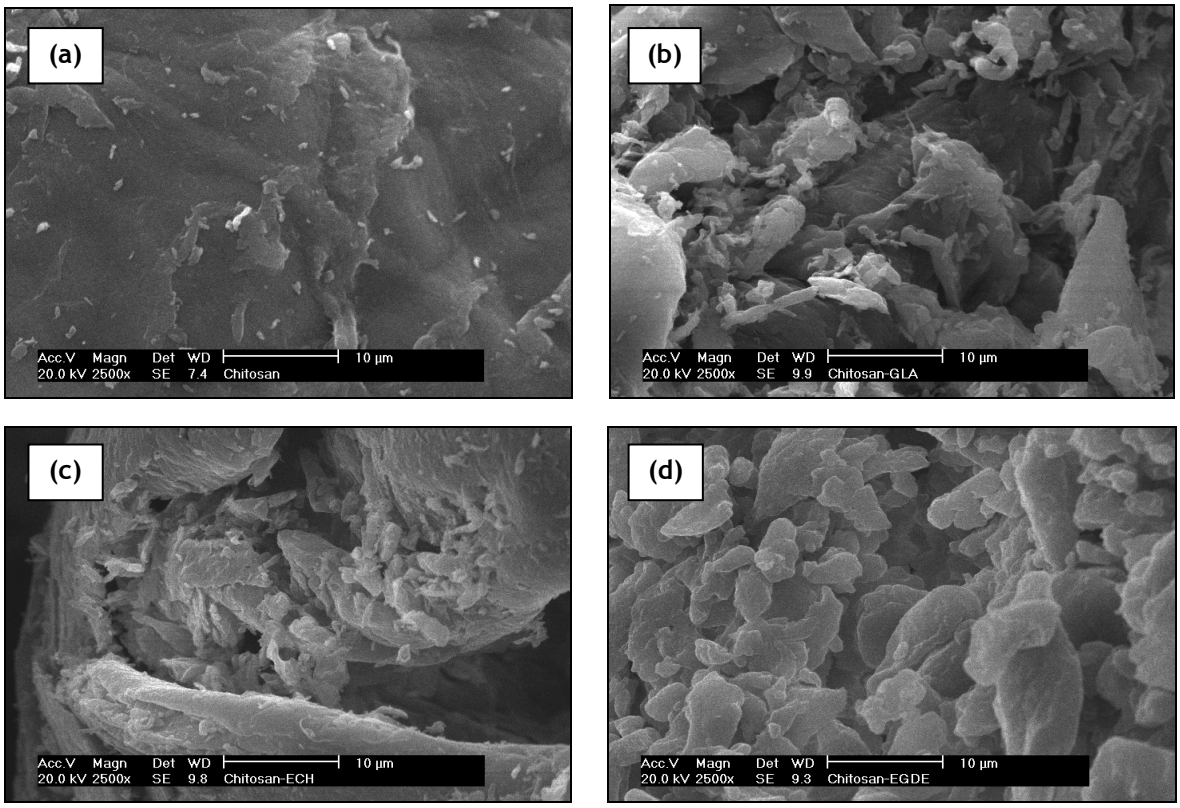


Figure 3.3 SEM images of (a) chitosan, (b) chitosan-GLA, (c) chitosan-ECH and (d) chitosan-EGDE at 2500x magnification.

A study by Vieira and Beppu (2005) has shown that physical modification may affect the surface properties of natural chitosan. They reported that chitosan formed into a membrane had a microporous structure, while a dense surface texture was observed on unmodified chitosan. A porous structure was also observed on chitosan-epichlorohydrin hydrogel films (Seki and Yurdakoc, 2008). However, chitosan granules were reported to have similar surface characteristics to unmodified chitosan (Rumyantseva *et al.*, 2008). Denkbaş and Odabaşi (2000) studied the effects of their cross-linking procedures on the

surface morphology of chitosan sponges. They observed a fibrillar structure when the cross-linker was applied during the formation of the chitosan sponges, while a leaflet structure was obtained when the cross-linker added after the formation of chitosan sponges.

Since treated chitosans were ground during the preparation in this study, the surface morphology of ground chitosan was also analysed. From Figure 3.4, it can be seen that both chitosans, with and without grinding have a similar morphology. Although grinding could affect the surface morphology of a material, no effects were apparent in this study.

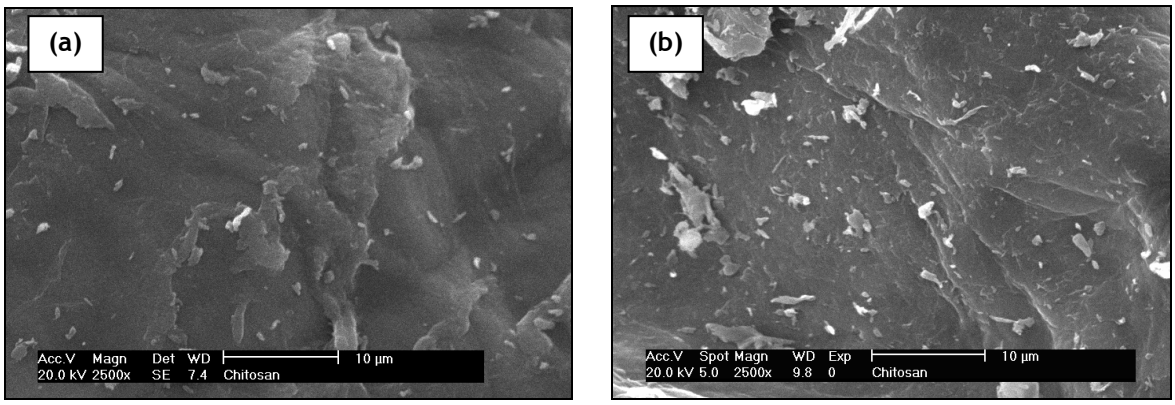


Figure 3.4 SEM images of (a) chitosan and (b) ground chitosan at 2500x magnification.

Changes in the surface morphologies of materials are dependent upon several factors such as the modifiers used, the method of modification and the nature of the material. For example, although H_2SO_4 treatment on thermal power plant fly ash was found to increase the surface area significantly, Lin *et al*. (2008) reported that the dense surface texture of the fly ash remained after the treatment. In contrast, whilst NaOH treatment reduced the surface area and average pore diameter of rubber leaves, Wan Ngah and Hanafiah (2008) observed a significant change in the surface morphology of the leaves, whereby the dense texture of the leaves became irregular following the treatment. Modification of pine bark with different chemicals such as NaOH, Fenton's reagent and poly-acrylamide was reported to cause a similar effect on the surface structure, whereby the morphology of the bark became porous after the treatment (Argun *et al.*, 2009).

3.3.5 FTIR analysis

FTIR spectroscopy has been used widely as a tool to identify the presence of certain functional groups or chemical bonds in a material because each specific chemical bond often has a unique energy absorption band (Li and Bai, 2005). It was expected that the cross-linking reaction involved the amino groups of chitosan (Muzzarelli, 2009). To prove this FTIR analysis was carried out. The FTIR spectra of chitosan and treated chitosans are shown in Figure 3.5.

Based on Figure 3.5a, a broad and strong band is present at 3433 cm^{-1} (1), corresponding to the stretching vibration of –OH groups, the extension vibration of N-H and intermolecular hydrogen bonds of polysaccharides. The absorption bands at 2920 and 2875 cm^{-1} (2) can be assigned to asymmetric and symmetric $-CH_2$ stretches. The FTIR spectrum of chitosan exhibits characteristic frequencies for –NH bending vibrations for primary amine groups at 1650 cm^{-1} (3) and 1595 cm^{-1} (4). The bands observed at 1419, 1378 and 1320 cm^{-1} (5) can be attributed to the C-N stretching vibration. According to Crews *et al*. (1998), the band at 1260 cm^{-1} (6) corresponds to the –OH bending vibration. The C-O stretching vibration associated with secondary alcohol groups was observed at 1155 cm^{-1} (7), while the C-O stretching vibration for primary alcohol groups appeared at 1075 and 1029 cm^{-1} (8) (Williams and Fleming, 1995). The band at 895 cm^{-1} (9) can be attributed to a C-H out-of-plane bending vibration, while the absorption band observed at 664 cm^{-1} (10) represents a $-NH_2$ wagging vibration.

Some changes in the FTIR spectrum of chitosan were observed after cross-linking. For example, bands representing amine deformation at 1650 and 1595 cm^{-1} shifted, suggesting an interaction of chitosan-NH_2 groups with CHO or CH_2O groups of the cross-linking reagents. Further evidence of the involvement of $-NH_2$ groups in the cross-linking mechanism was given by the disappearance of the NH_2 wagging vibration band at 664 cm^{-1}. This can be observed clearly in the FTIR spectra of cross-linked treated chitosans.

Treatment with ECH results in the appearance of new absorption bands at 1725 and 743 cm^{-1} (Figure 3.5b). The band at 1725 cm^{-1} (11) is related to the –CO stretching vibration in –C-OH, while the absorption band found at 743 cm^{-1} (12) is assigned to the CH_2 rocking vibration within the $-NHCH_2CH(OH)CH_2NH-$ bridge of chitosan-ECH (Guibal *et al.*, 1999). The –NH bending vibration bands were shifted to 1642 and 1587 cm^{-1}, with a marked increase in absorption intensity of the primary amine band at 1587 cm^{-1}. Meanwhile, the absorption intensity of C-N stretching bands decreased and the bands were shifted to 1410, 1382 and 1316 cm^{-1}.

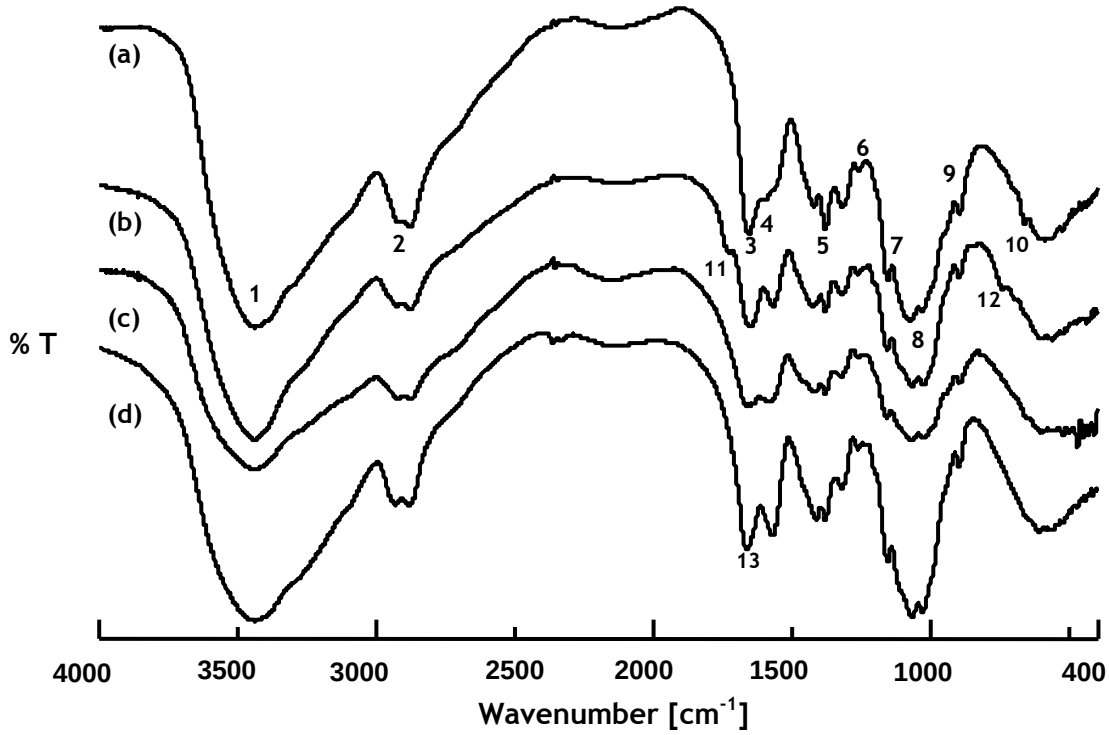


Figure 3.5 FTIR spectra of (a) chitosan, (b) chitosan-ECH, (c) chitosan-EGDE and (d) chitosan-GLA.

The relative intensity of the –NH bending vibration band at 1650 cm^{-1} decreased significantly after EGDE treatment, while the second primary amine band was shifted from 1595 to 1590 cm^{-1} (Figure 3.5c). Treatment with EGDE decreased the intensity of the C-N vibration, particularly for the C-N vibration at 1378 cm^{-1} of natural chitosan. The C-N vibration bands were shifted to 1408, 1383 and 1315 cm^{-1}.

As shown in Figure 3.5d, the prominent band observed at 1662 cm^{-1} (13) corresponds to the imine bond (–C=N–) (Beppu *et al.*, 2007; Monteiro and Airoldi, 1999). The imine bond formed during cross-linking reaction, which involves the $–NH_2$ groups of chitosan and the –CHO groups of GLA, according to Equation 3.1 (Guibal *et al.*, 1999):

$$—NH_2 + O{=}CH— \quad .... \rightleftharpoons —N{=}CH— \quad .... + H_2O \qquad (3.1)$$

It can be seen that the absorption intensity of the second primary amine band increased and shifted from 1595 to 1568 cm^{-1}. The absorption intensity of C-N stretching vibration bands at 1378 and 1320 cm^{-1} were decreased significantly and shifted to 1380 and 1318 cm^{-1}. FTIR analysis confirmed that cross-linking reaction between chitosan and cross-linking reagents involved the amino groups of chitosan.

3.4 Characterisation: Metal interaction

3.4.1 XRD analysis

The crystallinity of chitosan and treated chitosans before and after metal uptake (see Section 2.7.2) were examined, and are shown in Figures 3.6 to 3.9. Interaction of Ag^{+}, Pb^{2+} and Cu^{2+} with the materials led to a significant change in the intensity of the crystalline reflection at 20.42° 2θ. There was a minor change in the X-ray diffractograms after Zn^{2+} uptake, suggesting a small extent of complexation. The high extent of Ag^{+}, Pb^{2+} and Cu^{2+} complexation with chitosan and treated chitosans is evident from the change in intensity of the reflection at 20.42°. These profiles were consistent with the amount of metal ions bound at equilibrium (as will be discussed in Section 4.6).

Pb-treated chitosan, chitosan-GLA and chitosan-EGDE showed distinct and complex peaks in their X-ray diffractograms. These can be attributed to the deposition of crystalline lead nitrate ($Pb(NO_3)_2$). A similar observation for Pb-treated cellulose/chitin blend beads was reported by Zhou *et al*. (2005). However, the X-ray diffractogram of Pb^{2+} complexation with chitosan obtained in this study contradicts the result reported by Trimukhe and Varma (2008). They noted a negligible change in the X-ray diffractogram of chitosan after Pb^{2+} adsorption, and therefore they inferred that the interaction of Pb^{2+} with the chitosan adsorption sites was a weak complexation. The different effects in the X-ray diffractogram of Pb-treated chitosan apparent in the present study might be due to differences in the deacetylation degree. For Ag-treated chitosan-ECH, crystalline AgCl was evident (Figure 3.7).

XRD analysis indicates that metal uptake decreased the crystallinity of chitosan and treated chitosans. According to Trimukhe and Varma (2008), significant reduction in the intensity of the crystalline peak might be due to the complete disruption of the interpolymer bonds. Complexation of metal with chitin-based materials is expected to involve the formation of co-ordination bonds, which may change the arrangement of the polymer and increase the disorder of the polymer network (Milot *et al.*, 1998). This rearrangement can be expected to decrease the crystalline structure of chitosan and treated chitosans.

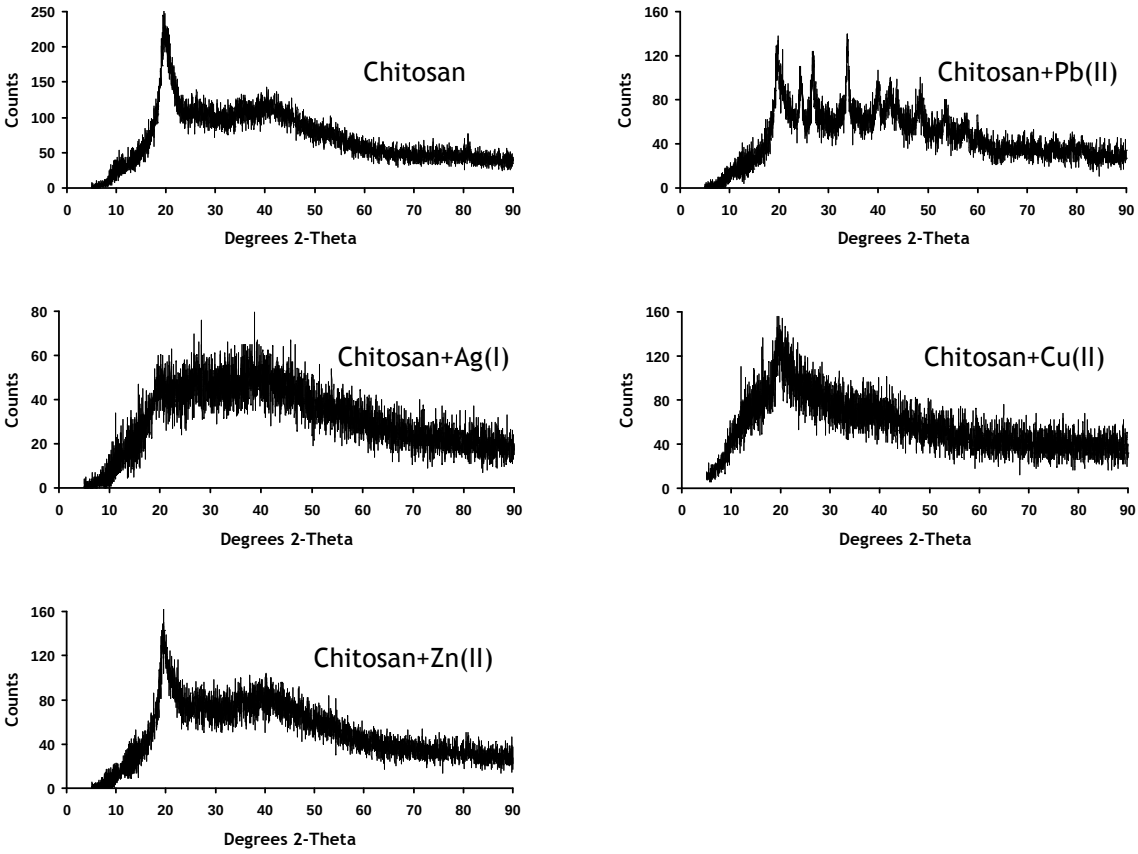


Figure 3.6 X-ray diffractograms of chitosan before and after metal sorption.

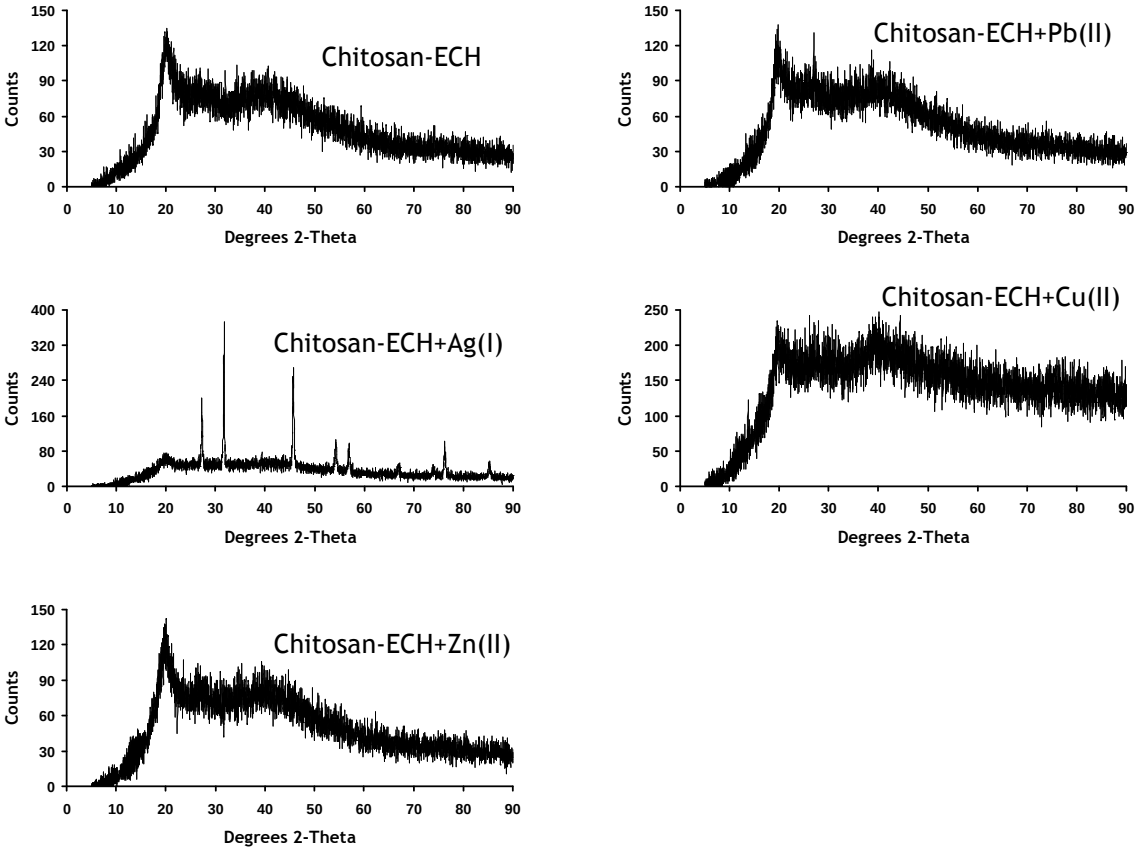


Figure 3.7 X-ray diffractograms of chitosan-ECH before and after metal sorption.

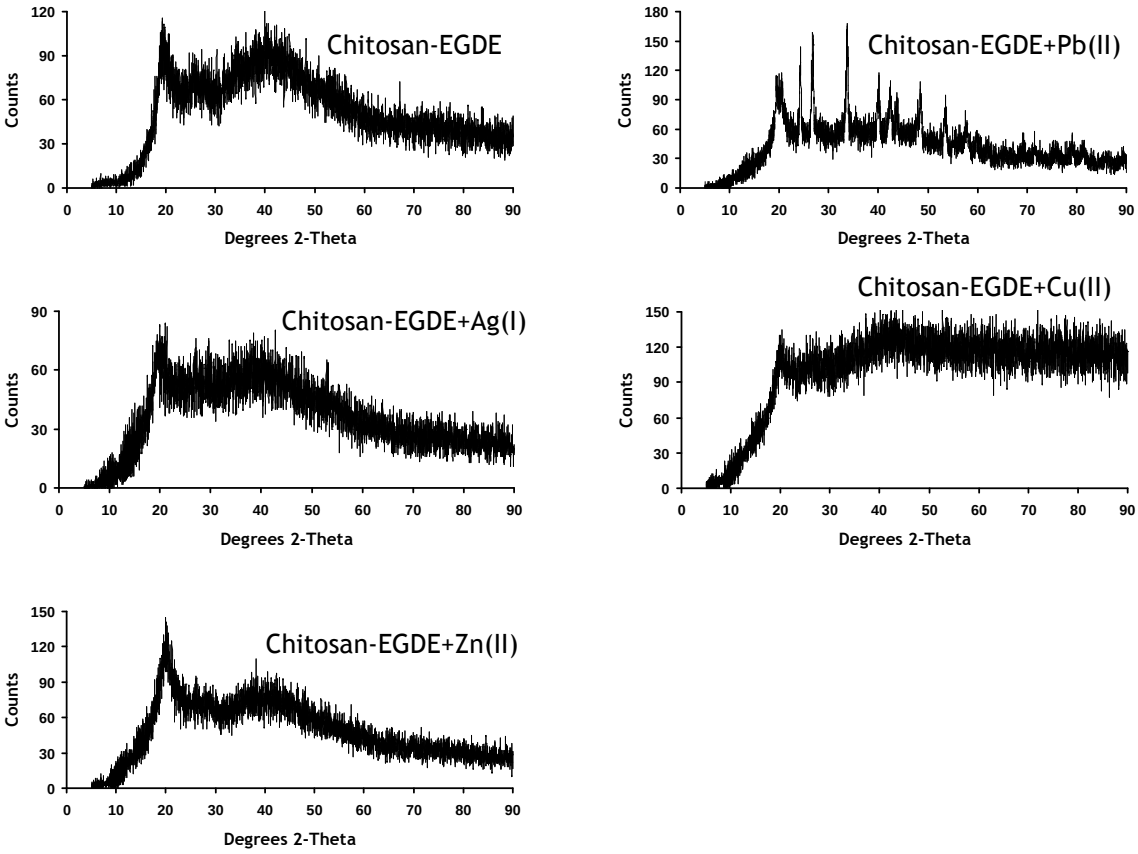


Figure 3.8 X-ray diffractograms of chitosan-EGDE before and after metal sorption.

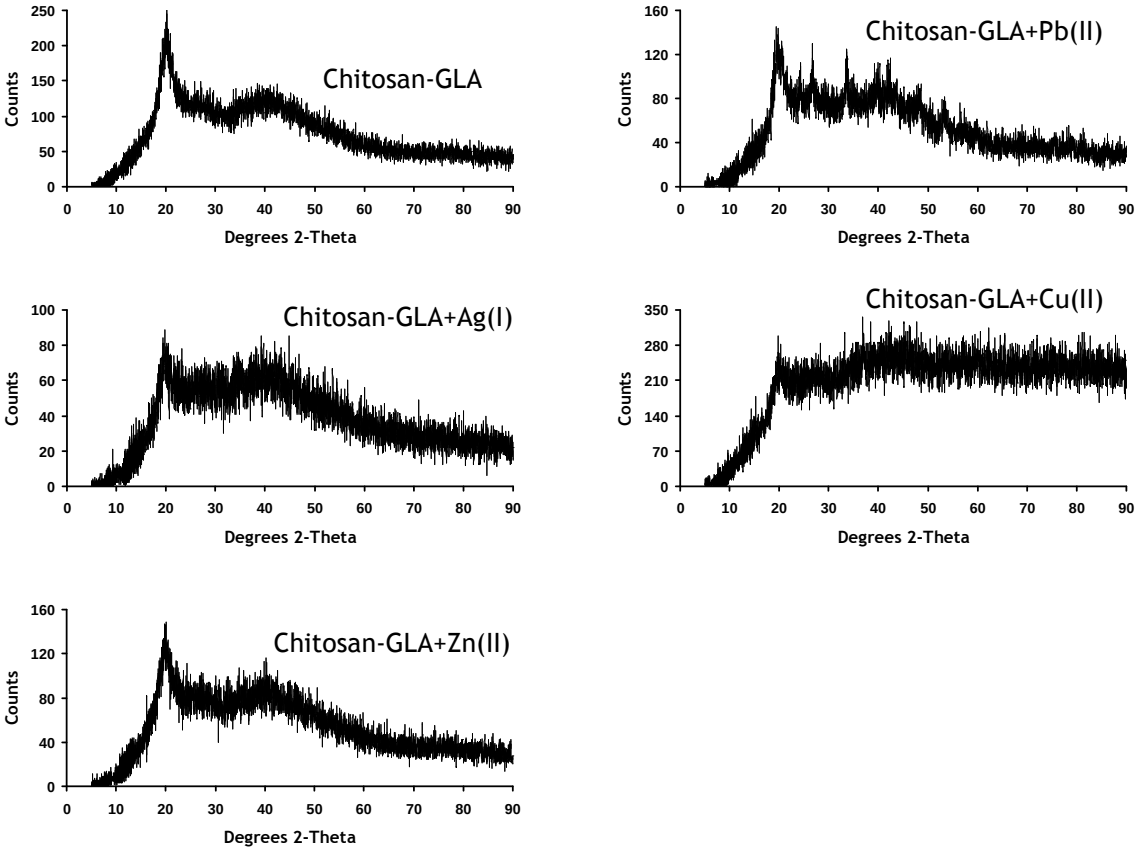


Figure 3.9 X-ray diffractograms of chitosan-GLA before and after metal sorption.

3.4.2 SEM analysis

Figures 3.10 to 3.13 present the SEM images at 2500x magnification of chitosan and treated chitosans, before and after metal uptake. There are significant changes to the surface morphology of the chitosans, as well as the formation of discrete aggregates on their surfaces after metal uptake. After Ag^{+} and Cu^{2+} uptake, the surface of unmodified chitosan became more dense (Figures 3.10a and 3.10b), while grooves were observed on the surface of chitosan after Pb^{2+} and Zn^{2+} uptake (Figures 3.10c and 3.10d). A similar observation was reported by Chen *et al*. (2002), whereby the rugged surface morphology of calcium alginate-based ion-exchange resin became smooth after interaction with Cu^{2+}. Interaction of chitosan with Cu^{2+} and Pb^{2+} has also resulted in the deposition of tiny nodules and sphere-like deposits on its surface (Figures 3.10b and 3.10c). Rumyantseva *et al*. (2008) observed irregular-shape deposits on the surface of chitosan granules after Cu^{2+} sorption, meanwhile Eiden *et al*. (1980) observed flake-like deposits on chitosan after equilibration with a Pb^{2+} solution. Analysis using Electron Spectroscopy for Chemical Analysis (ESCA) by Eiden *et al*. (1980) and Rumyantseva *et al*. (2008) confirmed the deposits observed on chitosan after interaction with Pb^{2+} and Cu^{2+} solutions were aggregates of $Pb(OH)_2$ and $Cu(OH)_2$.

The surface of chitosan-ECH appeared to be rather swollen after contact with Zn^{2+} (Figure 3.11d). Sphere-like deposits were observed on the surface of chitosan-ECH after exposure to Cu^{2+} (Figure 3.11b), while the chitosan-ECH surface appears littered with dust-like material after uptake of Ag^{+} (Figure 3.11a). Another striking feature is the appearance of needle-edge crystal-like deposits obtained on the chitosan-ECH surface after treatment with Pb^{2+} (Figure 3.11c).

Uptake of Ag^{+} and Zn^{2+} completely altered the surface morphology of chitosan-GLA from a rough to a dense surface texture (Figures 3.12a and 3.12d), while interaction with Cu^{2+} resulted in a cracked structure (Figure 3.12b). The effect of Cu^{2+} interaction is consistent with that reported by Du *et al*. (2009), who observed a wrinkled surface morphology on formaldehyde cross-linked chitosan microspheres after Cu^{2+} sorption. Modification of the lacunose surface texture of *N,O*-carboxymethyl-chitosan resin after Zn^{2+} uptake was observed by Sun and Wang (2006). Sphere-like deposits were also observed on the chitosan-GLA surface after Cu^{2+} uptake (Figure 3.12b), while there were sphere and crystal-like deposits spread on its surface after Pb^{2+} interaction (Figure 3.12c).

In contrast all the other materials tested, chitosan-EGDE displayed no significant change to its surface morphology after Ag^{+} uptake (Figure 3.13a). Meanwhile, interaction of chitosan-EGDE with Cu^{2+} led to the formation of a network structure morphology (Figure 3.13b). Pb^{2+} and Zn^{2+} changed its surface morphology significantly, whereby the surface morphology became flake-like and exhibited a swollen network structure after Pb^{2+} and Zn^{2+} uptake (Figures 3.13c and 3.13d), respectively.

SEM analysis revealed that the rough texture of treated chitosans was lost upon metal uptake. These observations indicate that the mechanism between binding sites of the materials and metal ions is very complex and may affect the original polymer chain structure of chitosan and treated chitosans.

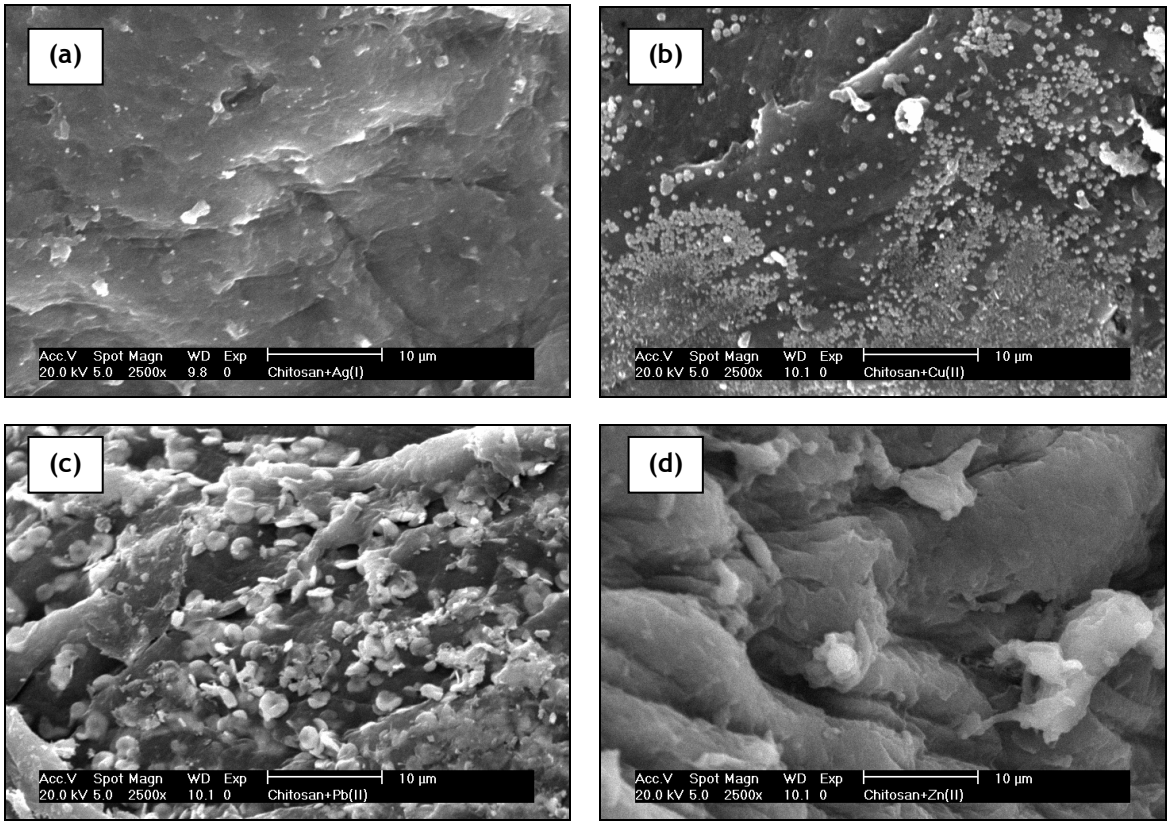


Figure 3.10 SEM images of chitosan at 2500x magnification after sorption of (a) Ag^{+}, (b) Cu^{2+}, (c) Pb^{2+} and (d) Zn^{2+}.

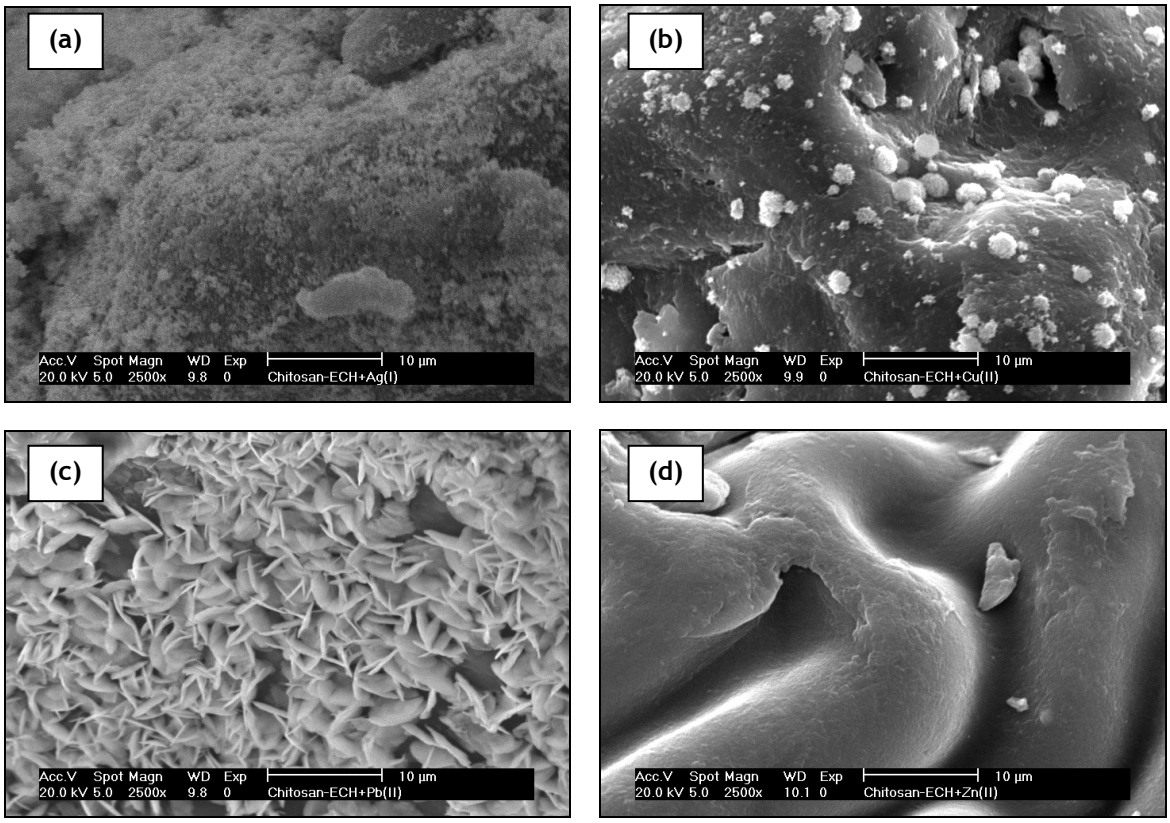


Figure 3.11 SEM images of chitosan-ECH at 2500x magnification after sorption of (a) Ag^{+}, (b) Cu^{2+}, (c) Pb^{2+} and (d) Zn^{2+}.

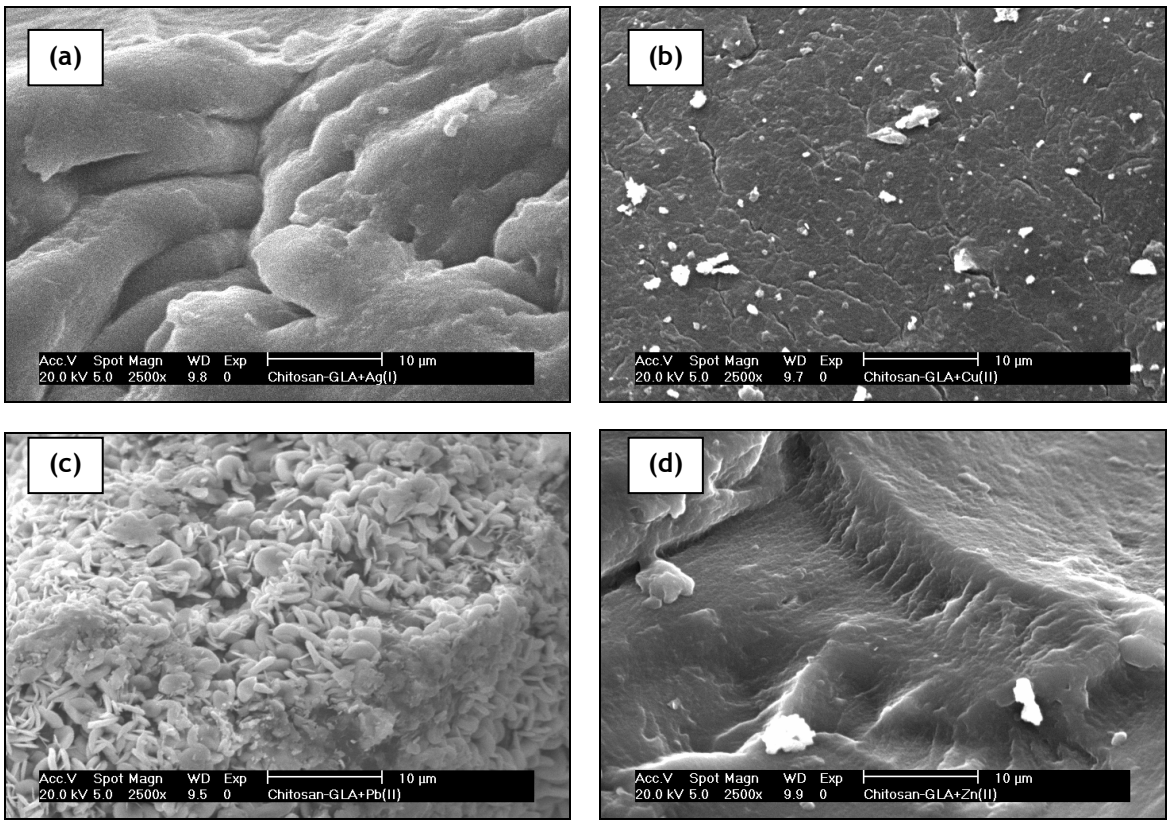


Figure 3.12 SEM images of chitosan-GLA at 2500x magnification after sorption of (a) Ag^{+}, (b) Cu^{2+}, (c) Pb^{2+} and (d) Zn^{2+}.

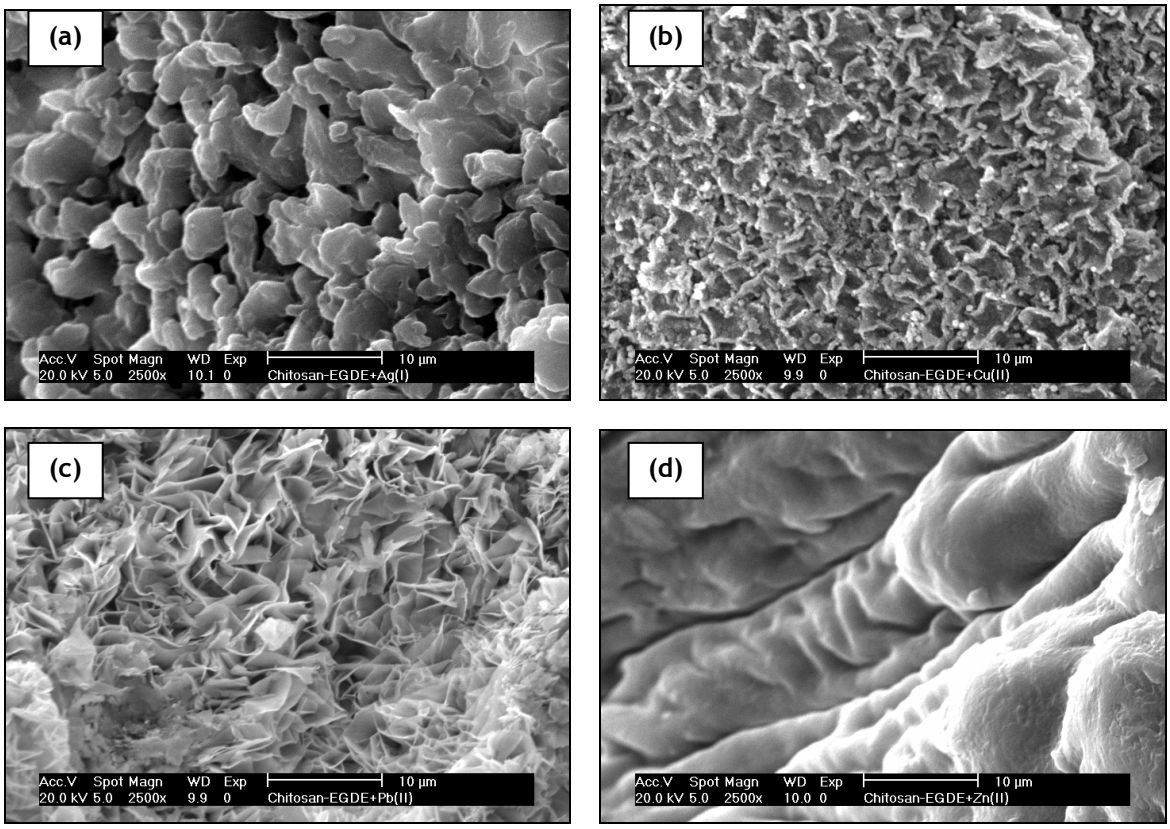


Figure 3.13 SEM images of chitosan-EGDE at 2500x magnification after sorption of (a) Ag^{+}, (b) Cu^{2+}, (c) Pb^{2+} and (d) Zn^{2+}.

3.4.3 EDX analysis

The EDX spectra of sorbents before and after heavy metal sorption are presented in Figures 3.14 to 3.17. After metal sorption, new peaks representing Ag, Pb, Cu and Zn were observed. The EDX spectrum of chitosan shows three prominent peaks at the energy values of 0.277, 0.392 and 0.525 KeV, which can be assigned to CK_{α}, NK_{α}, and OK_{α}, respectively (Figure 3.14). Carbon, nitrogen and oxygen are the three major constituents in chitosan and treated chitosans. Features for Au, AuM_{α} and AuL_{α} detected at the energy values of 2.121 and 9.712 KeV, originate from the gold film used to coat the samples prior to analysis, in order to prevent charging. The NaK_{α} feature observed at 1.041 KeV (Figures 3.15 to 3.17), suggests the presence of sodium in the treated chitosans, which arises as a result of cross-linking treatment which involves NaOH solution to neutralise the sorbents.

Exposure of the chitosan samples to silver solution results in the appearance of four silver features, namely AgL_{l}, AgL_{α}, AgL_{β} and AgL_{γ} at 2.692, 2.984, 3.150 and 3.347 KeV, respectively. For Pb^{2+} interaction, only the PbM_{α} feature was detected for chitosan and chitosan-ECH (Figures 3.14 and 3.15), while PbM_{α}, PbL_{α} and PbL_{β} observed at 2.342,

10.550 and 12.612 KeV were apparent for chitosan-EGDE and chitosan-GLA (Figures 3.16 and 3.17). Interaction with copper and zinc solutions led to the presence of ClK_{α} and ClK_{β} at 2.621 and 2.815 KeV. Three peaks related to CuL_{α}, CuK_{α} and CuK_{β} were detected at 0.930, 8.040 and 8.904 KeV after Cu^{2+} sorption. Meanwhile, ZnL_{α}, ZnL_{l}, ZnK_{α} and ZnK_{β} were observed at the energy values of 1.012, 1.022, 8.630 and 9.579 KeV. The NaK_{α} feature for treated chitosans disappeared after metal interaction suggesting the possible involvement of ion exchange in metal sorption.

For the EDX spectral features, K, L and M represent the energy lines associated with the principal quantum shells, while α and β represent principal emission lines associated with the electronic transitions. In principle, when a high energy beam of electrons is focused on a sample, the incident beam may excite an electron in an atomic inner shell, creating a hole that can be occupied by an electron from an outer shell (Goldstein *et al.*, 1992). This interaction will produce X-ray emission of characteristic energy. The signal produced is normally processed into an EDX spectrum which contains information on number and energy of the X-rays emitted.

EDX is a semi-qualitative technique (Goldstein *et al.*, 1992). Therefore, information on percentage of metal determined on the surface of materials does not represent the total amount of metal sorbed by materials. It is evident from EDX analysis that chitosan and treated chitosans are capable of binding metal ions, even in the presence of K^{+}, Cl^{-} and NO_3^{-}, which are soil dominant cation and anions, and which are present in the model soil solutions applied.

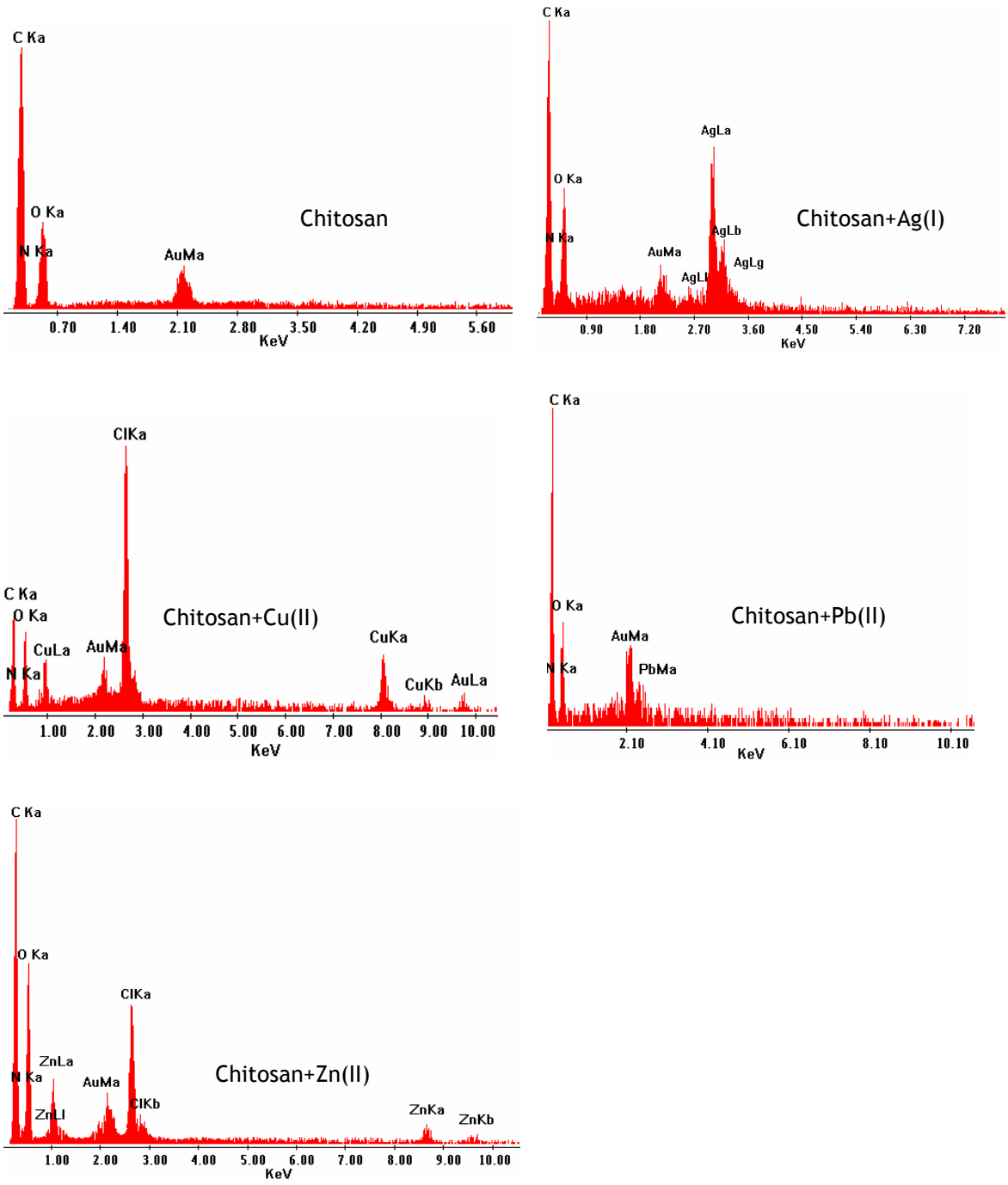


Figure 3.14 EDX spectra of chitosan before and after metal sorption.

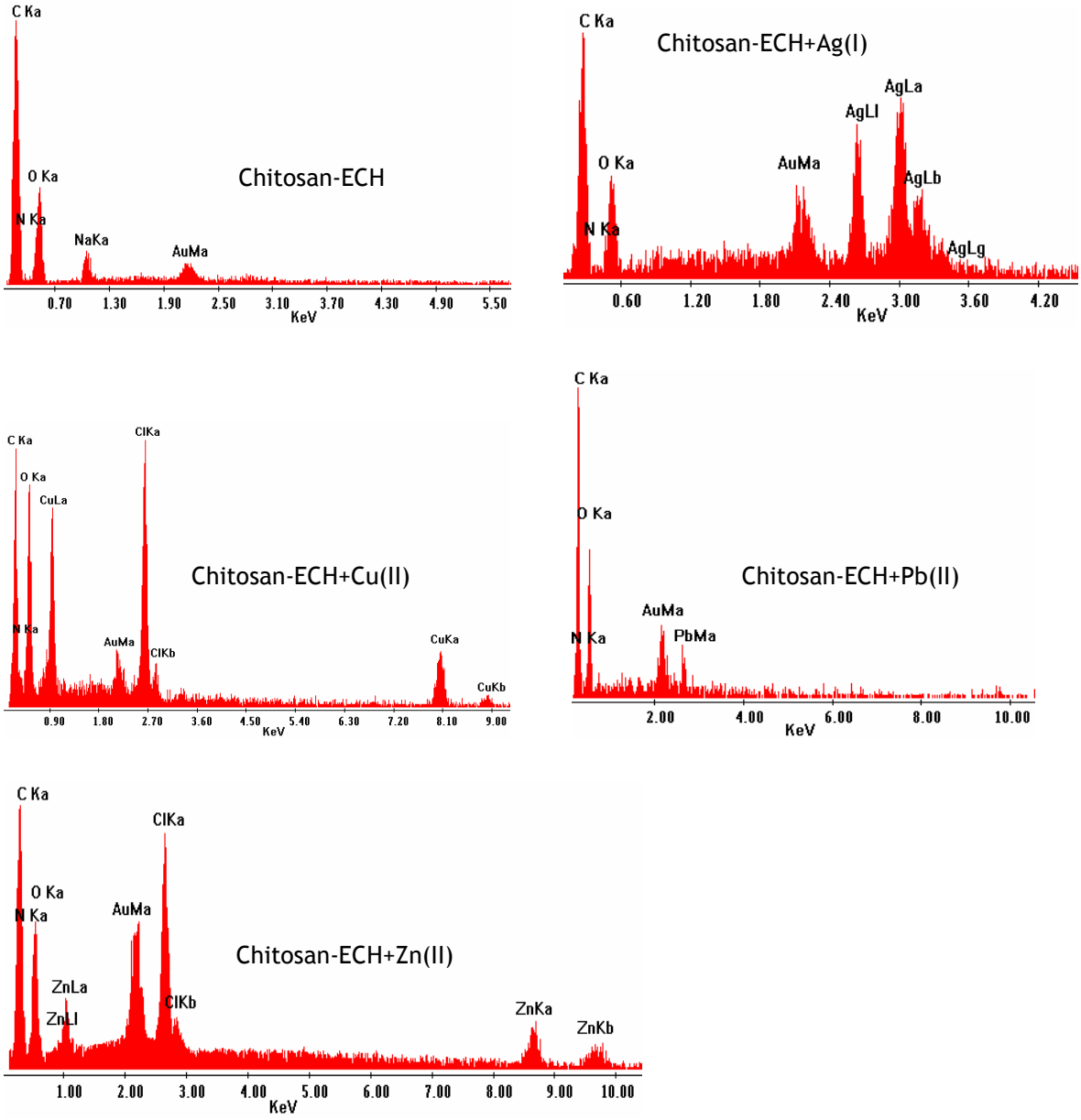


Figure 3.15 EDX spectra of chitosan-ECH before and after metal sorption.

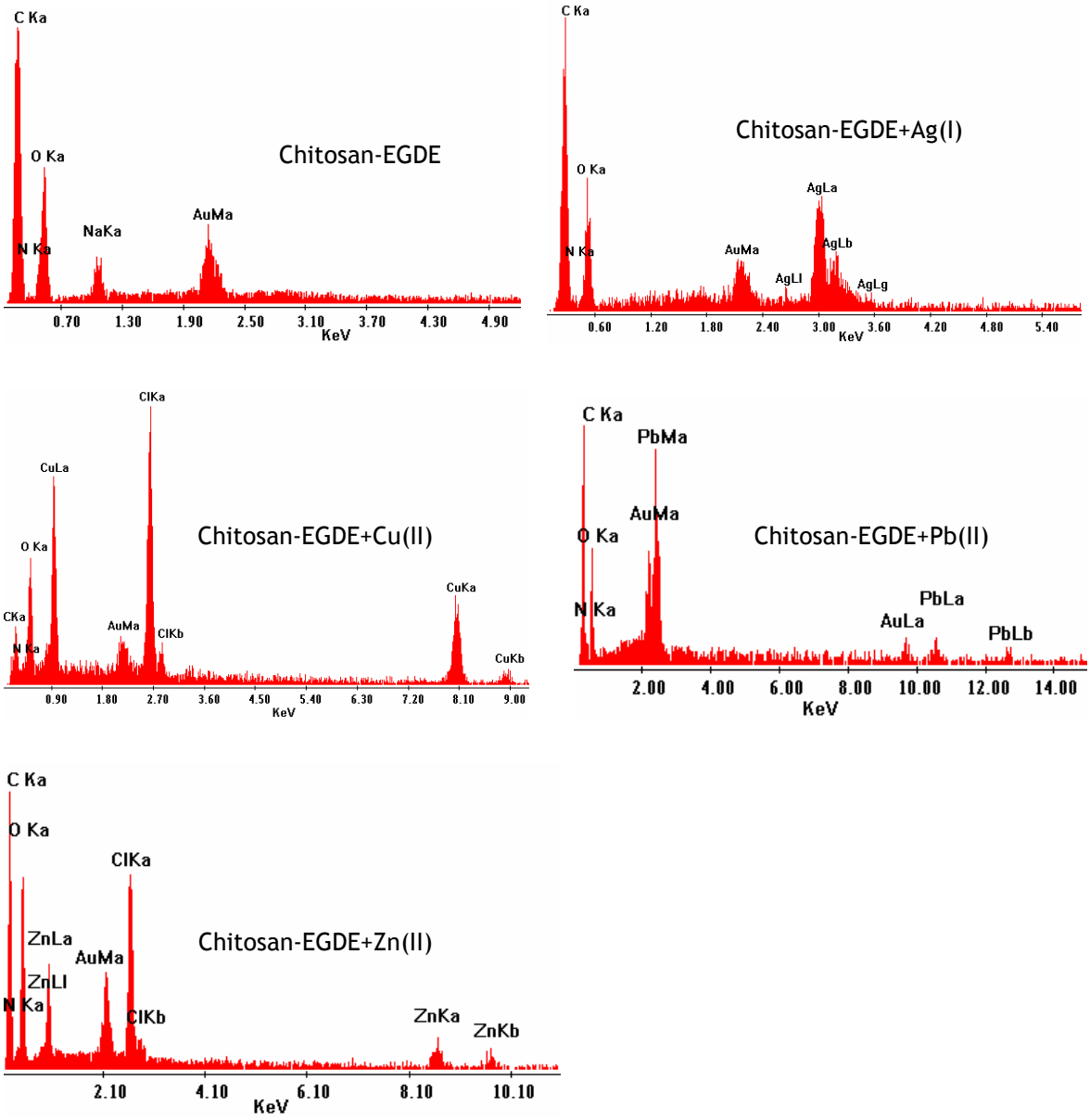


Figure 3.16 EDX spectra of chitosan-EGDE before and after metal sorption.

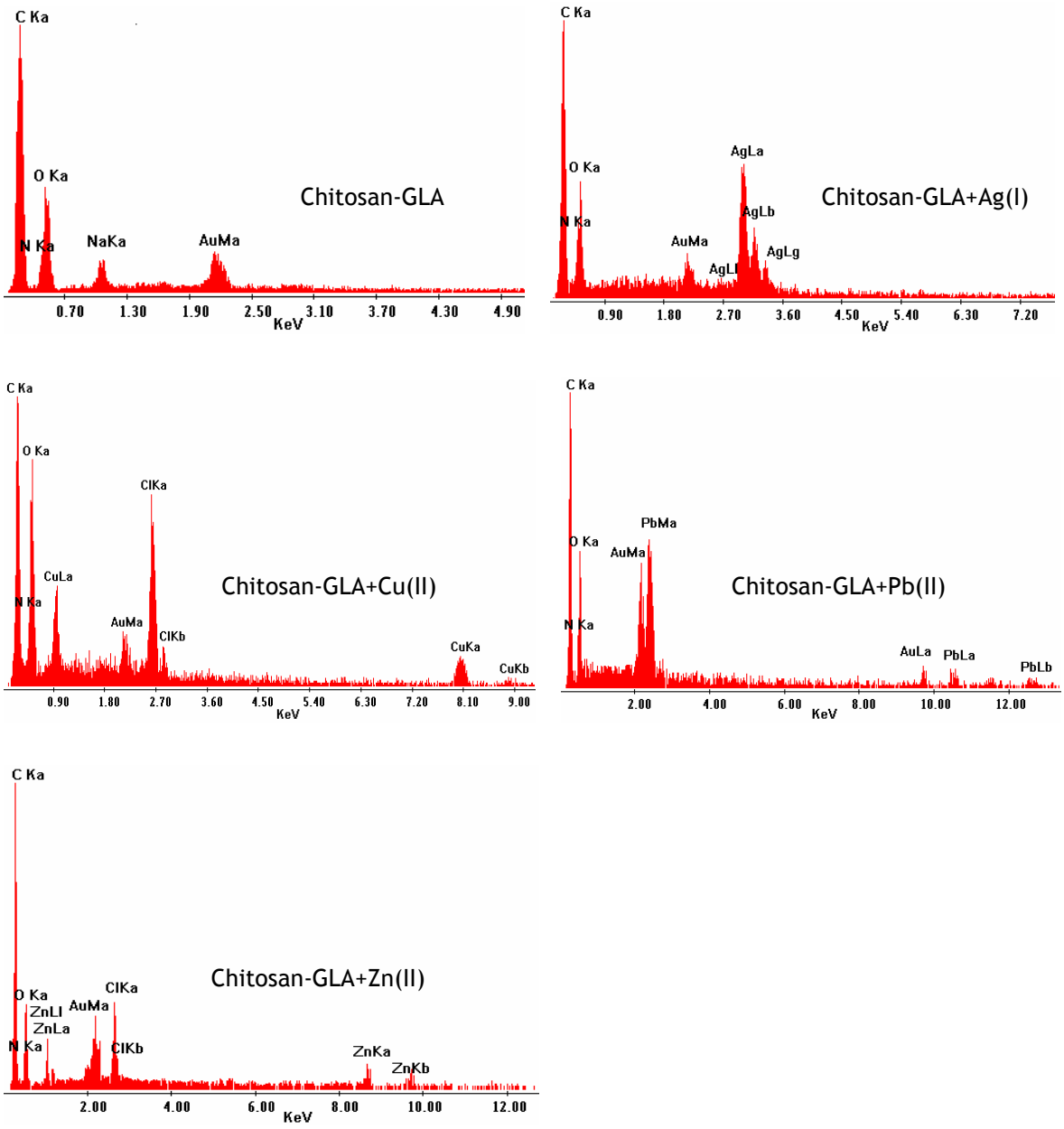


Figure 3.17 EDX spectra of chitosan-GLA before and after metal sorption.

3.4.4 FTIR analysis

Whilst EDX investigation of the metal-treated materials confirmed the presence of the various metals on the chitosan surfaces as anticipated, it does not yield specific information on the nature of the binding interactions involved. Accordingly, FTIR investigations were undertaken to elucidate these.

FTIR spectra were obtained for each material before and after metal uptake, in order to understand the nature of metal uptake and identify possible sites for metal binding (Figures 3.18 to 3.25). In general, the major changes observed in the FTIR spectra of materials after metal uptake seem to be rather similar to each other, suggesting similar

mechanisms. It was evident that the C-N stretching vibration bands at 1419, 1378 and 1320 cm^{-1} for chitosan and treated chitosans changed significantly from sharp and small bands to broad and prominent bands, particularly after Ag^{+} and Pb^{2+} uptake (Figures 3.18, 3.20, 3.22 and 3.24). Meanwhile, the absorption intensity of the N-H bending vibration for the primary amine group at 1595 cm^{-1} decreased after metal ion uptake. These suggest a strong complexation between metal ions and binding sites. This had been substantiated previously, based on the results obtained from XRD analysis.

After Ag^{+} and Pb^{2+} uptake, a new absorption band was observed at 826 cm^{-1}. The wavenumber region of 900 to 650 cm^{-1} can be assigned to the N-H bending vibration (Crews *et al.*, 1998). Hence, the new band apparent at 826 cm^{-1} can be attributed to the interaction between amino groups and metal ions. This interaction was further evident by the appearance of new band observed at 679 cm^{-1} of chitosan-EGDE after Pb^{2+} uptake (Figure 3.24b).

Treating chitosan and cross-linked chitosans with Cu^{2+}, results in the appearance of discernible bands in the O-H stretching vibration region. The new absorption bands observed at the wavenumbers of 3515, 3513, 3440 and 3380 cm^{-1} for chitosan (Figure 3.19a), chitosan-GLA (Figure 3.21a), chitosan-ECH (Figure 3.23a) and chitosan-EGDE (Figure 3.25a) indicate that O atoms also played a role in binding Cu^{2+} ions on the surfaces. In addition, four new absorption bands were observed at wavenumbers of 858, 818, 786 and 705 cm^{-1} in the chitosan-GLA FTIR spectrum, confirming that N atoms served as binding sites for Cu^{2+} ions (Figure 3.21a). Unlike Ag^{+}, Pb^{2+} and Cu^{2+}, no new band was observed in the FTIR spectra after Zn^{2+} uptake. However, there were changes in the absorption intensity and wavenumber for C-N stretching vibrations after Zn^{2+} complexation. The C-N stretching vibration bands were shifted from 1419, 1378 and 1320 cm^{-1} to 1411, 1370 and 1312 cm^{-1}.

FTIR analysis showed that metal uptake affected all chemical bonds associated with N atoms. Hence, it is reasonable to speculate that N atoms are indeed the main binding sites for metal on chitosan and treated chitosans. However, the presence of O atoms in the hydroxyl groups of the polymer chain should not be neglected because results suggest the involvement of O atoms in binding metals from the soil solutions. It is known that both N and O atoms can bind metal ions through complexation. However, due to the stronger attraction of the lone pair of electrons to the nucleus in an O atom than in a N atom, O atoms can be considered less favourable for metal ion uptake (Jin and Bai, 2002).

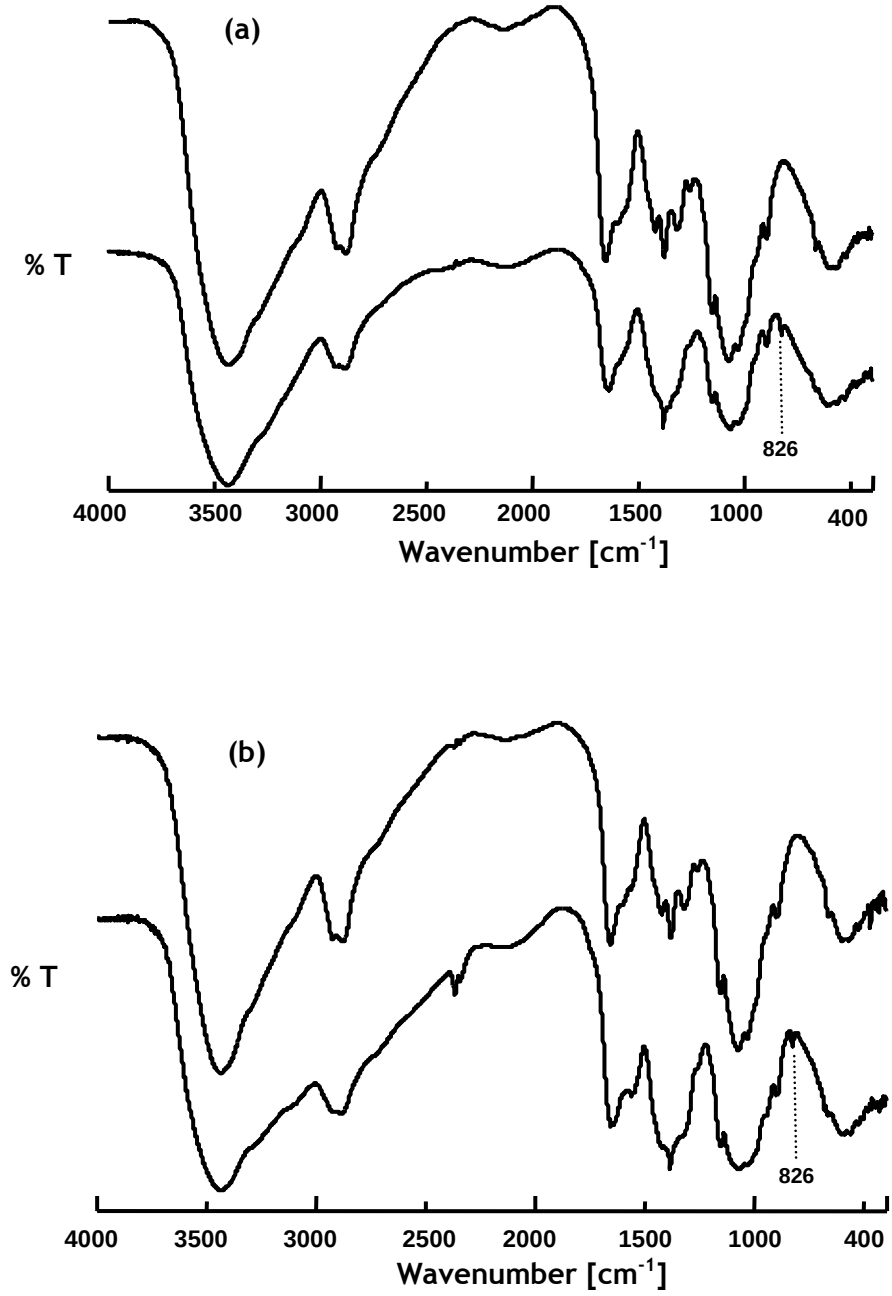


Figure 3.18 FTIR spectra of chitosan before (upper line) and after sorption (bottom line) of (a) Ag^{+} and (b) Pb^{2+}.

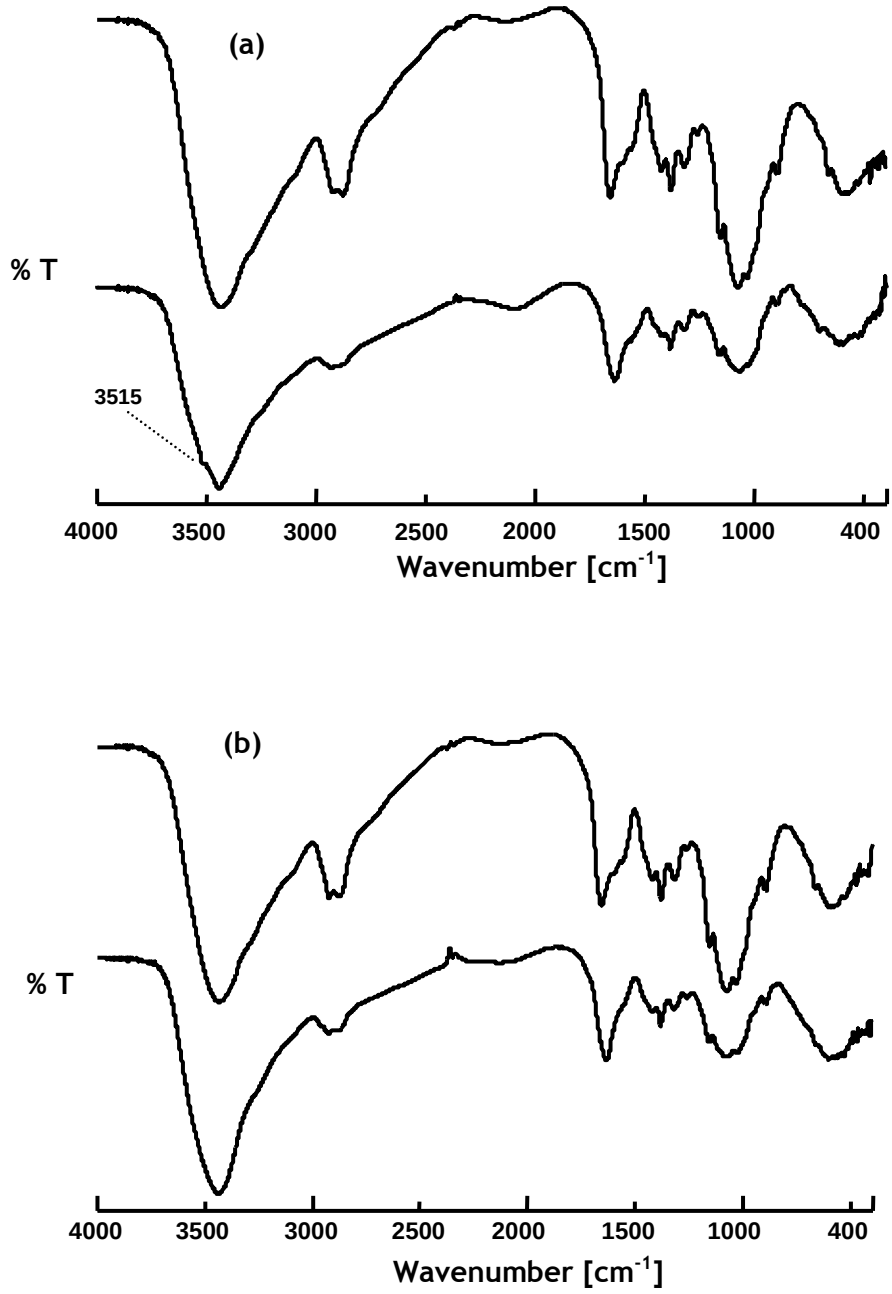


Figure 3.19 FTIR spectra of chitosan before (upper line) and after sorption (bottom line) of (a) Cu^{2+} and (b) Zn^{2+}.

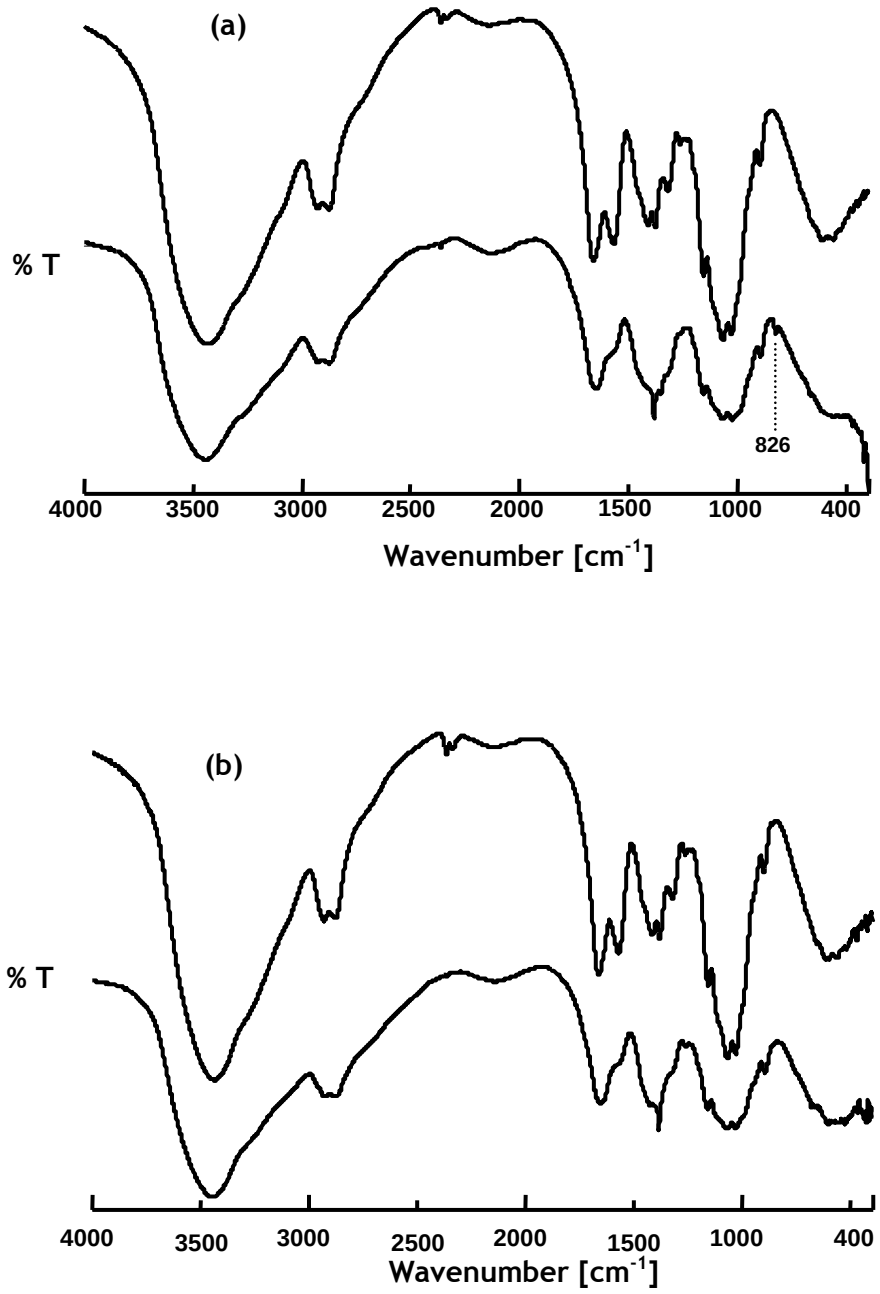


Figure 3.20 FTIR spectra of chitosan-GLA before (upper line) and after sorption (bottom line) of (a) Ag^{+} and (b) Pb^{2+}.

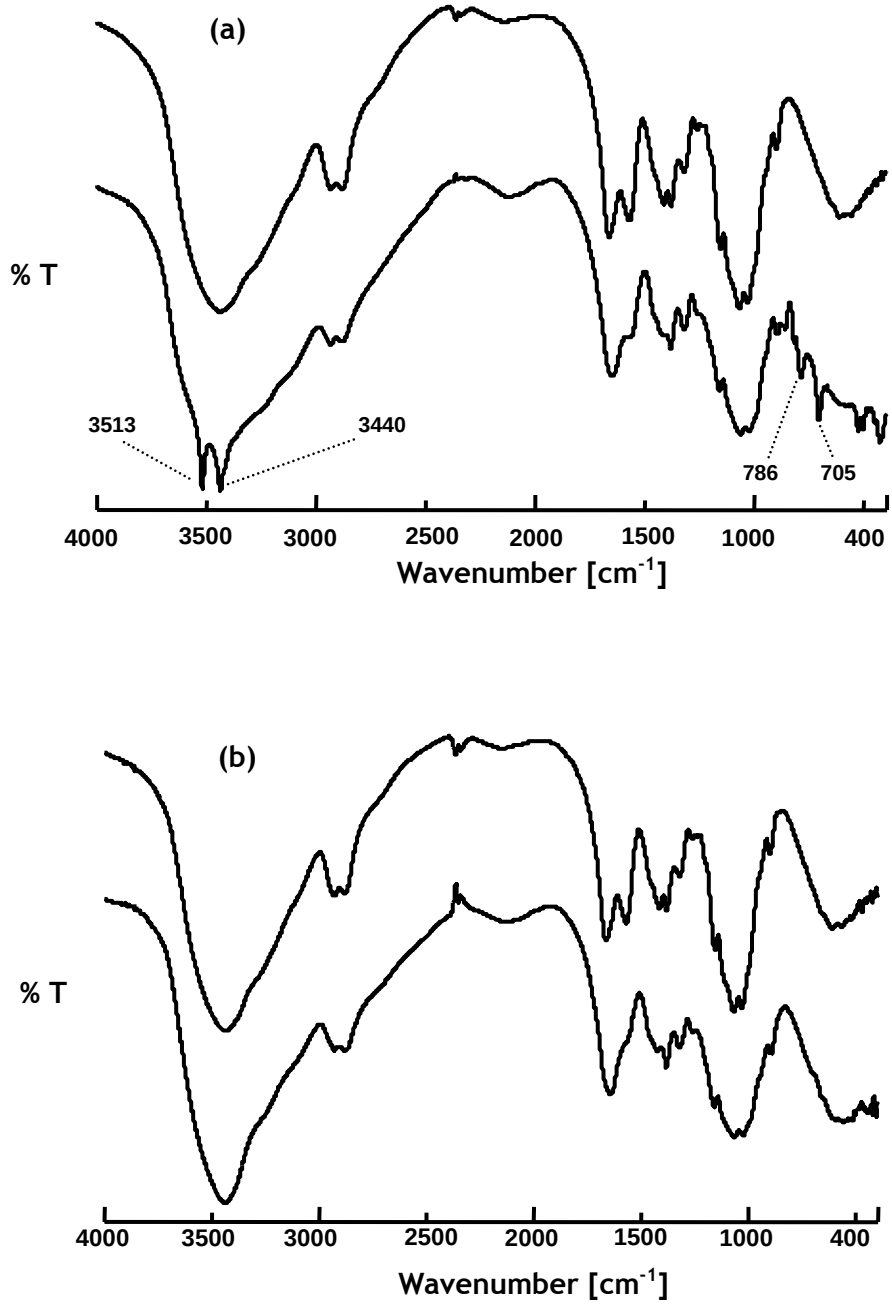


Figure 3.21 FTIR spectra of chitosan-GLA before (upper line) and after sorption (bottom line) of (a) Cu^{2+} and (b) Zn^{2+}.

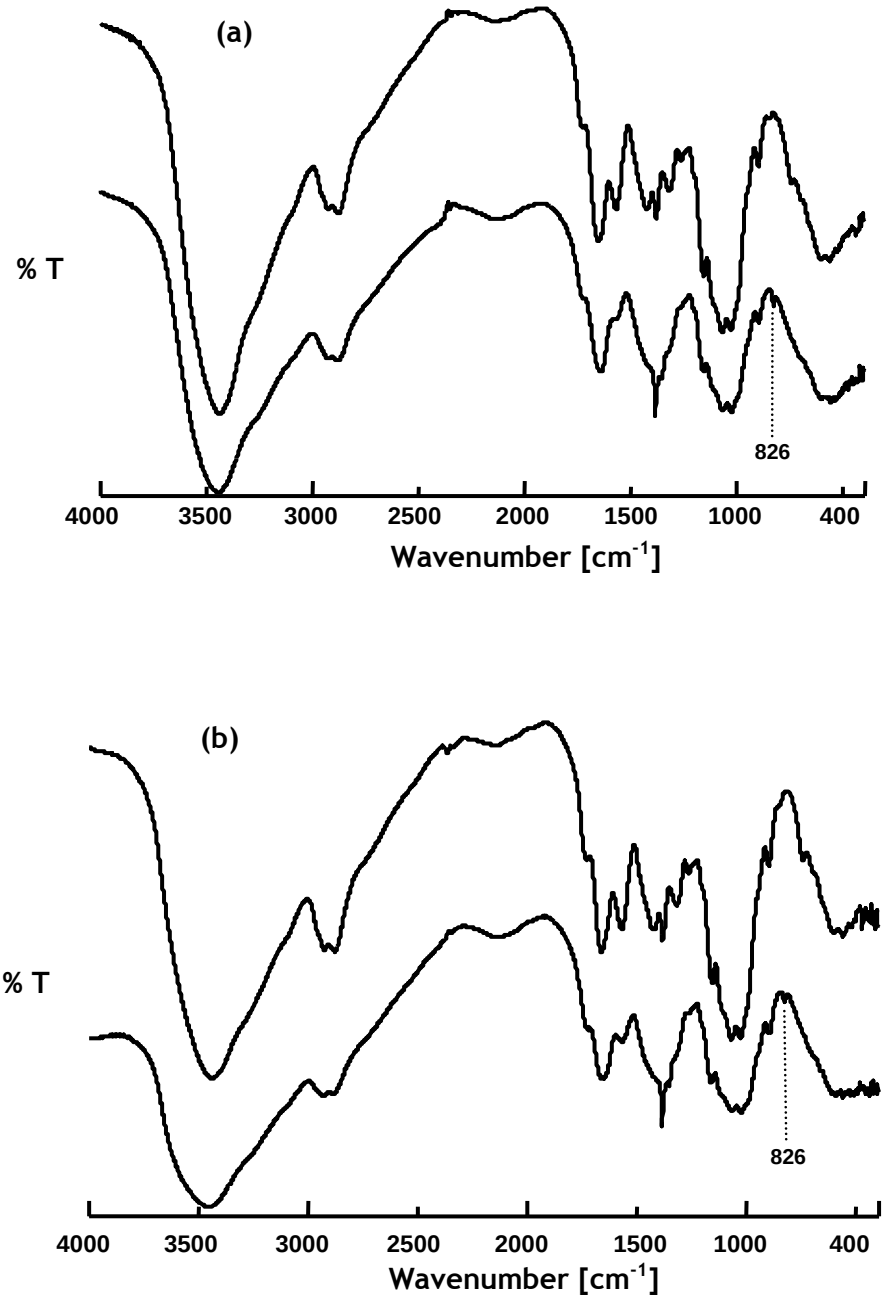


Figure 3.22 FTIR spectra of chitosan-ECH before (upper line) and after sorption (bottom line) of (a) Ag^{+} and (b) Pb^{2+}.

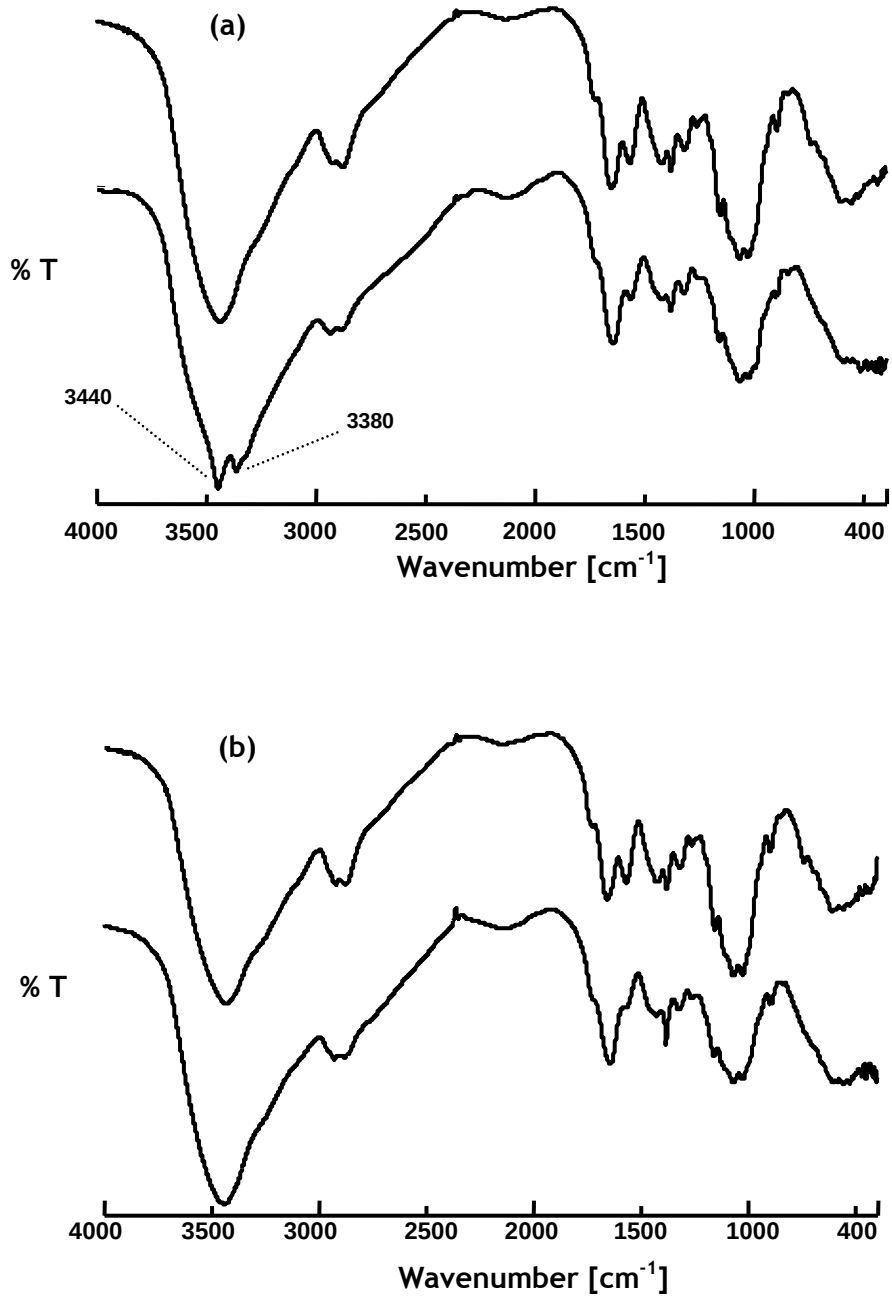


Figure 3.23 FTIR spectra of chitosan-ECH before (upper line) and after sorption (bottom line) of (a) Cu^{2+} and (b) Zn^{2+}.

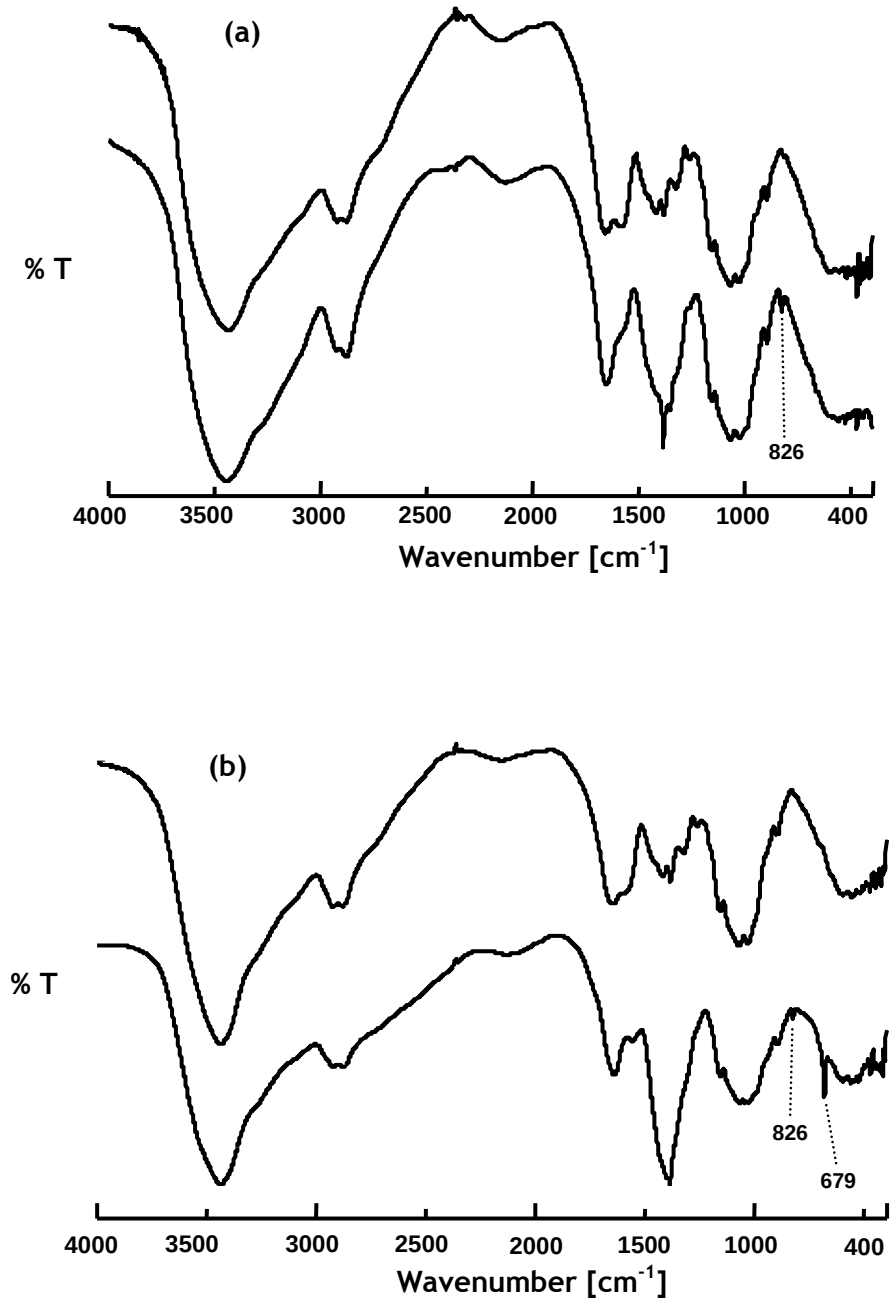


Figure 3.24 FTIR spectra of chitosan-EGDE before (upper line) and after sorption (bottom line) of (a) Ag^{+} and (b) Pb^{2+}.

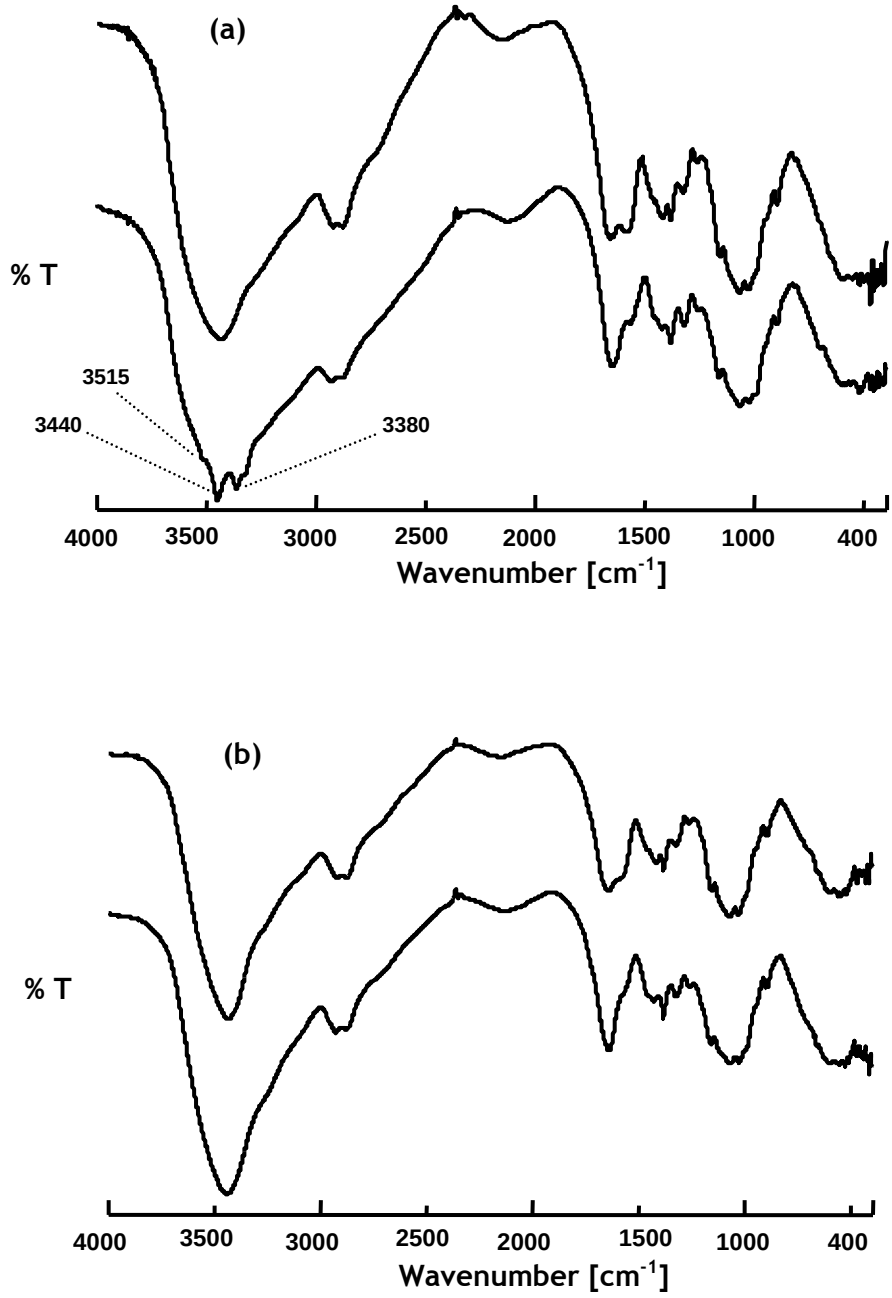


Figure 3.25 FTIR spectra of chitosan-EGDE before (upper line) and after sorption (bottom line) of (a) Cu^{2+} and (b) Zn^{2+}.

3.5 Summary

The formation of intermolecular bridges as a result of cross-linking enhanced the chemical stability of chitosan in acidic media (Table 3.1). Cross-linking treatments increased the BET surface area of chitosan. The average pore diameter of chitosan decreased after the ECH and EGDE cross-linking treatments, whereas a fourfold increase was achieved with the GLA treatment (Table 3.2). The crystallinity of chitosan was reduced following cross-linking treatment (Figure 3.1) and such reduction is important for enhancing the intraparticle diffusion of metal ions. Cross-linked chitosans appeared to have a rougher surface texture than unmodified chitosan (Figure 3.3). It was evident by FTIR analysis that the cross-linking reaction between chitosan and cross-linking reagents involved the amino groups of chitosan (Figure 3.5).

Metal complexation increased the disorder of the polymer network, led to a significant reduction in crystallinity of the chitosans (Figures 3.6 to 3.9). In addition, their surface morphology was affected by this interaction, particularly after contact with Ag^{+}, Pb^{2+} and Cu^{2+} (Figures 3.10 to 3.13). The presence of metals on the surface of the chitosans was confirmed using EDX analysis (Figures 3.14 to 3.17). FTIR spectra revealed that Ag^{+}, Pb^{2+} and Cu^{2+} had a strong interaction with binding sites of the chitosans, whereas a small extent of complexation was obtained for Zn^{2+} (Figures 3.18 to 3.25). Overall, FTIR analysis suggests that dative covalent interaction is a major binding mechanism for metal ion complexation by the chitosans.

4 SORPTION STUDY

Immobilisation of heavy metals in contaminated soil is highly dependent on the ability of amendments to bind metals, and therefore it is necessary to evaluate their capacity for metal uptake, and to understand the binding behaviour of metals onto amendments (Hodson *et al.*, 2000; Meunier *et al.*, 2004; Nwachukwu and Pulford, 2008). Batch sorption studies are generally effective in assessing metal binding at the laboratory level, for which the sorption kinetics and isotherms can be determined (Illera *et al.*, 2002; Zhao and Selim, 2010). In addition, the effects of several factors such as the reaction time, the initial metal concentration and the background electrolytes on metal binding can also be evaluated (Moreira *et al.*, 2008; Soares *et al.*, 2009).

In Chapter 3 (Characterisation Study), the surface properties of chitosan and cross-linked chitosans as affected by the interaction with Ag^{+}, Cu^{2+}, Pb^{2+} and Zn^{2+} were characterised. Although the effect of Cd^{2+} uptake on the surface properties of the chitosans was not examined in the characterisation study, the range of metals was extended to include Cd, which is a soft metal.

There are three main sections in this chapter. The first section reports the effects of contact time and initial metal concentration on metal binding. For the kinetics study, amendments were exposed to metal ion solutions at different contact times, ranging from 15 to 1440 minutes, using only one metal concentration of 500 mg/L (Section 2.7.2). Following on from this, sorption experiments were carried out at the optimum contact times determined and with initial metal concentrations ranging from 5 to 500 mg/L (Section 2.7.2). The effect of background electrolytes on Zn^{2+} binding was also studied (Section 2.7.3). The kinetics and equilibrium data were fitted to several kinetic and isotherm models and this is discussed in the second section. Finally, the third section discusses the ability of the amendments to retain metal ions on their surfaces assessed in a sorption-desorption study (Section 2.7.4).

4.1 Objectives of the study

1. To evaluate the binding capacity of chitosan and cross-linked chitosans for metal ions.
2. To assess the ability of chitosan and cross-linked chitosans to retain metal ions on their surfaces.

4.2 Effect of contact time

The time profiles for the sorption of metal ions by chitosan and cross-linked chitosans are presented in Figures 4.1 to 4.4. The amount of metal ions sorbed increased with contact time before levelling out, implying equilibrium has been reached. The initial high amount of metal ions sorbed indicates instantaneous sorption, which can be attributed to the availability of binding sites of the sorbents. However, as these sites progressively react, the sorption of metal ions slowed before attaining equilibrium at 90 minutes for chitosan and 180 minutes for cross-linked chitosans. Cross-linked chitosans reached equilibrium later than natural chitosan due to their rigid structure, which may have made some binding sites less accessible.

Qi and Aldrich (2008) reported that the sorption of Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} by tobacco dust attained equilibrium at 90 minutes at room temperature. A study by Çoruh *et al*. (2010) found that the Ag^{+} sorption onto clinoptilolites, a natural zeolite, was optimum at 120 minutes. The kinetics experiment was carried out at 23 ºC with initial Ag^{+} concentration of 100 mg/L and 10 g/L of clinoptilolites. Benguella and Benaissa (2002) performed a kinetics study of Cd^{2+} uptake by chitin at 25 ºC and with an initial Cd^{2+} concentration of 100 mg/L, and noted that 400 minutes was the optimum contact time for the sorption. A much longer period of contact time was reported for lignin, whereby the Cd^{2+} and Cu^{2+} sorption reached equilibrium at 1440 minutes (Mohan *et al.*, 2006). The kinetics of Cd^{2+} and Cu^{2+} sorption onto lignin was examined at 25 ºC and initial metal concentration of 100 mg/L.

Based on the results shown in Figures 4.1 to 4.4, the subsequent batch sorption studies are carried out at 90 minutes for chitosan and 180 minutes for cross-linked chitosans.

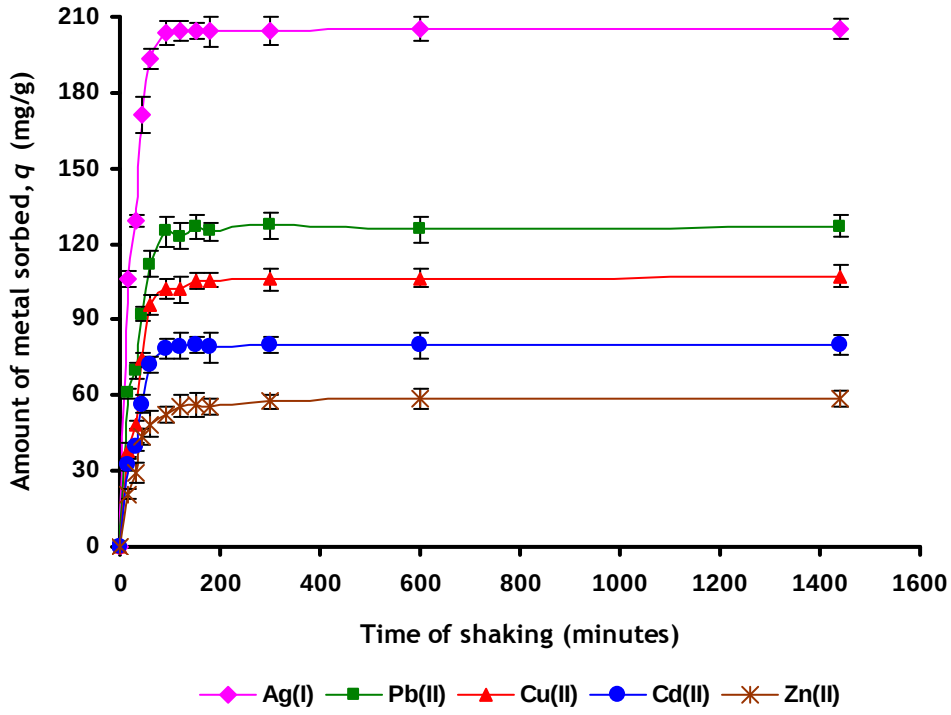


Figure 4.1 Effect of contact time on the sorption of metal ions by chitosan (Error bars are ± standard deviation of three replicates).

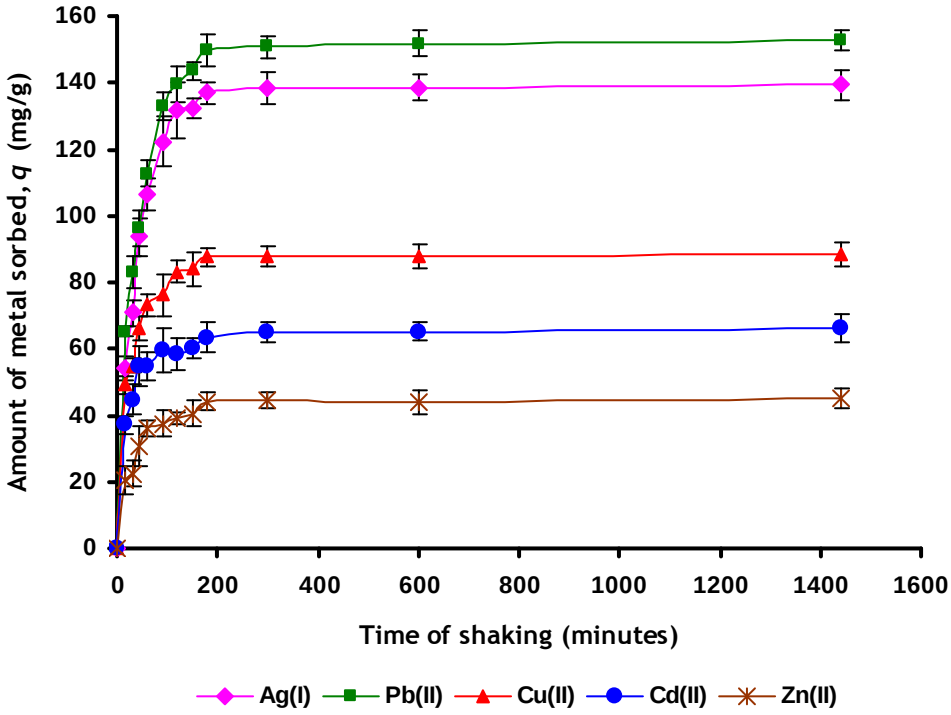


Figure 4.2 Effect of contact time on the sorption of metal ions by chitosan-EGDE (Error bars are ± standard deviation of three replicates).

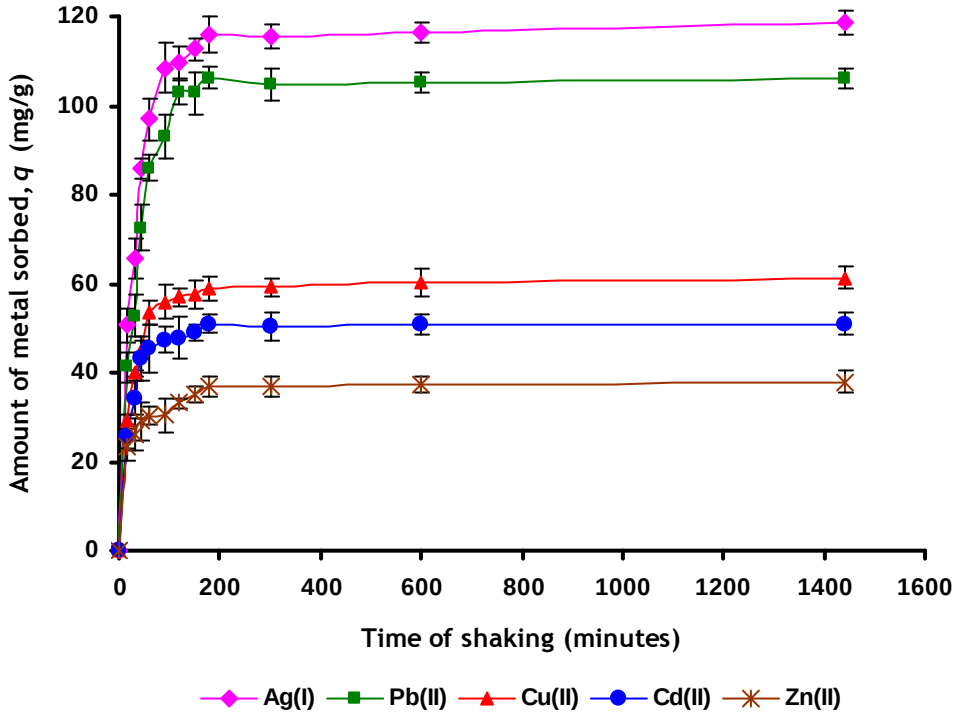


Figure 4.3 Effect of contact time on the sorption of metal ions by chitosan-GLA (Error bars are ± standard deviation of three replicates).

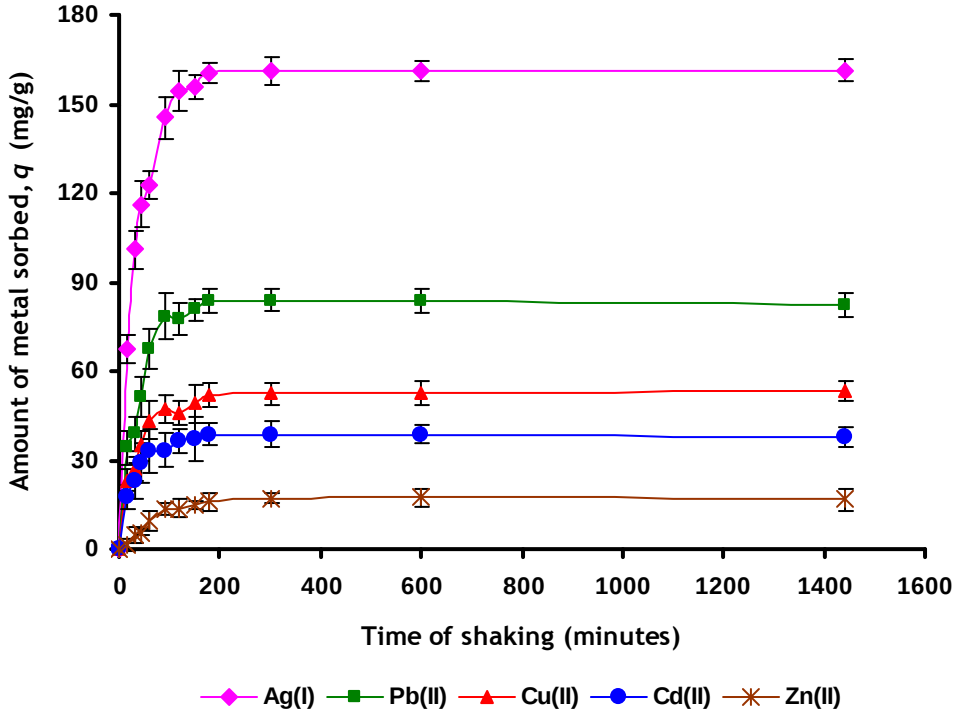


Figure 4.4 Effect of contact time on the sorption of metal ions by chitosan-ECH (Error bars are ± standard deviation of three replicates).

4.3 Effect of initial metal concentration

Table 4.1 presents the effect of initial metal concentration on uptake by chitosan and cross-linked chitosans at equilibrium, q_e. It is apparent that they have similar capacity to bind metal ions at initial metal concentrations of 5 and 10 mg/L. However, when the initial metal concentration was increased from 100 to 500 mg/L, a great difference was observed. A similar observation was reported by Dahiya *et al*. (2008). They observed no significant difference in the amount of Cu^{2+} and Pb^{2+} sorbed onto crab shells for initial metal concentrations between 10 and 100 mg/L.

Table 4.1 Effect of initial metal concentration (C_o) on amount of metal ions sorbed by chitosan and cross-linked chitosans.

Metal ion	C_o (mg/L)	Amount of metal ion sorbed, q_e (mg/g)			
		Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Ag^{+}	5	2.44	2.41	2.38	2.42
	10	4.85	4.83	4.80	4.76
	50	24.3	23.9	24.2	24.4
	100	46.6	41.9	41.3	42.5
	200	95.8	74.0	90.8	87.1
	300	141	93.7	111	123
	500	205	119	162	147
Pb^{2+}	5	2.30	2.06	1.95	2.26
	10	4.43	4.57	4.13	4.51
	50	21.6	23.6	17.2	23.7
	100	32.8	46.5	32.4	44.9
	200	65.5	67.9	64.7	87.7
	300	92.9	79.9	75.0	116
	500	127	105	83.5	153
Cu^{2+}	5	2.39	2.24	2.14	2.44
	10	4.78	4.75	4.11	4.77
	50	23.7	20.1	13.9	20.8
	100	42.7	28.3	20.2	31.7
	200	66.6	40.9	30.3	49.6
	300	82.6	46.7	38.9	68.5
	500	107	60.0	52.9	90.3
Cd^{2+}	5	1.97	2.35	1.26	1.49
	10	4.40	4.12	3.37	3.80
	50	22.3	14.2	15.3	16.8
	100	36.0	17.6	23.3	25.3
	200	47.3	28.7	27.5	35.6
	300	57.9	36.9	31.2	48.0
	500	81.0	49.7	38.7	66.4
Zn^{2+}	5	2.18	1.99	1.83	1.97
	10	4.00	3.66	3.39	3.74
	50	15.5	11.5	8.20	10.6
	100	22.1	16.6	10.4	15.5
	200	38.9	23.2	12.3	25.8
	300	44.2	27.0	13.2	36.3
	500	58.2	36.7	16.3	45.8

Values represent mean of three replicates.

From Table 4.1, it can be seen that the amount of metal ions bound per unit weight of sorbent is higher at high initial metal concentrations. However, the sorption percentage decreased with increasing initial metal concentration (Figures 4.5 to 4.8). This can be explained by the fact that the ratio of available binding sites to the total metal ions is high at low initial concentration, and all metal ions may be bound to the sites. At high concentrations, however, this ratio is lower and consequently the binding of metal depends on the initial concentrations (Li *et al.*, 2008; Naiya *et al.*, 2009). For example, when the Ag^+ concentration was increased from 5 to 50 mg/L, the Ag^+ sorption by chitosan-ECH (Figure 4.8) remained at its maximum (98% to 99%), and decreased to 94% with 200 mg/L Ag^+ solution. A further reduction was observed (78%), when 300 mg/L was used as the initial concentration.

In the case of Cd^{2+} sorption by chitosan and chitosan-EGDE (Figures 4.5 and 4.6), there was a continuous increase in the sorption percentage up to an initial concentration of 50 mg/L for chitosan and 25 mg/L for chitosan-EGDE. For chitosan-ECH, a 16% increase in the Cd^{2+} sorption was obtained when the initial concentration was increased from 5 to 10 mg/L (Figure 4.8). At further higher concentrations, however, the uptake of Cd^{2+} by the sorbents decreased consistently. There is no obvious reason for this behaviour. It could be proposed that a competition binding process involving K^+ and Cd^{2+} ions is operative. However, this should be investigated further.

Of metal ions studied, Ag^+ was the most effectively sorbed ion by chitosan and cross-linked chitosans, whereas Zn^{2+} was the least bound for the initial concentrations of 50 mg/L to 500 mg/L. At lower initial metal concentrations, particularly at 5 mg/L, Cd^{2+} had a lower affinity than Zn^{2+} for chitosan, chitosan-EGDE and chitosan-ECH. A 20% difference in the percent of Zn^{2+} and Cd^{2+} removed was obtained for chitosan-EGDE and chitosan-ECH at initial concentration of 5 mg/L (Figures 4.6 and 4.8). For example, 82% of Zn^{2+} and 61% of Cd^{2+} were sorbed onto chitosan-EGDE at this concentration (Figure 4.6). However, chitosan-GLA sorbed 95% of Cd^{2+} and 86% of Zn^{2+} at the same concentration (Figure 4.7). In most cases, Pb^{2+} showed a higher affinity to the chitosans than Cu^{2+}. In fact, its behaviour was similar to Ag^+ for chitosan-EGDE.

Results obtained from this study suggest that chitosan and cross-linked chitosans are able to bind metal ions over a wide range of initial metal concentration.

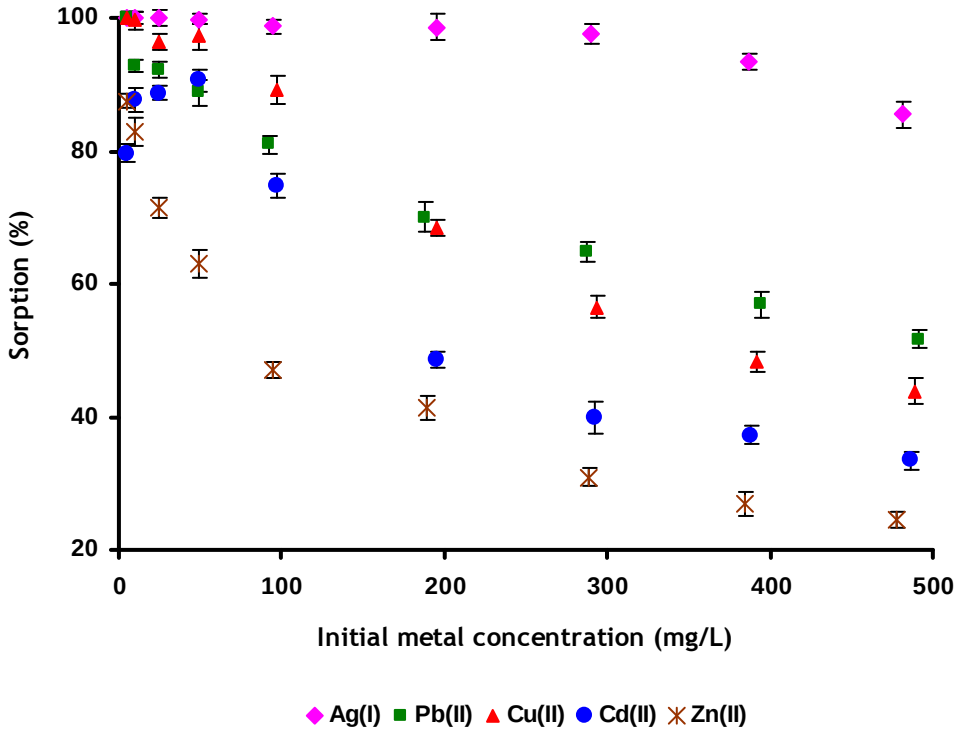


Figure 4.5 Effect of initial metal concentration on the sorption percentage of metal ions by chitosan (Error bars are ± standard deviation of three replicates).

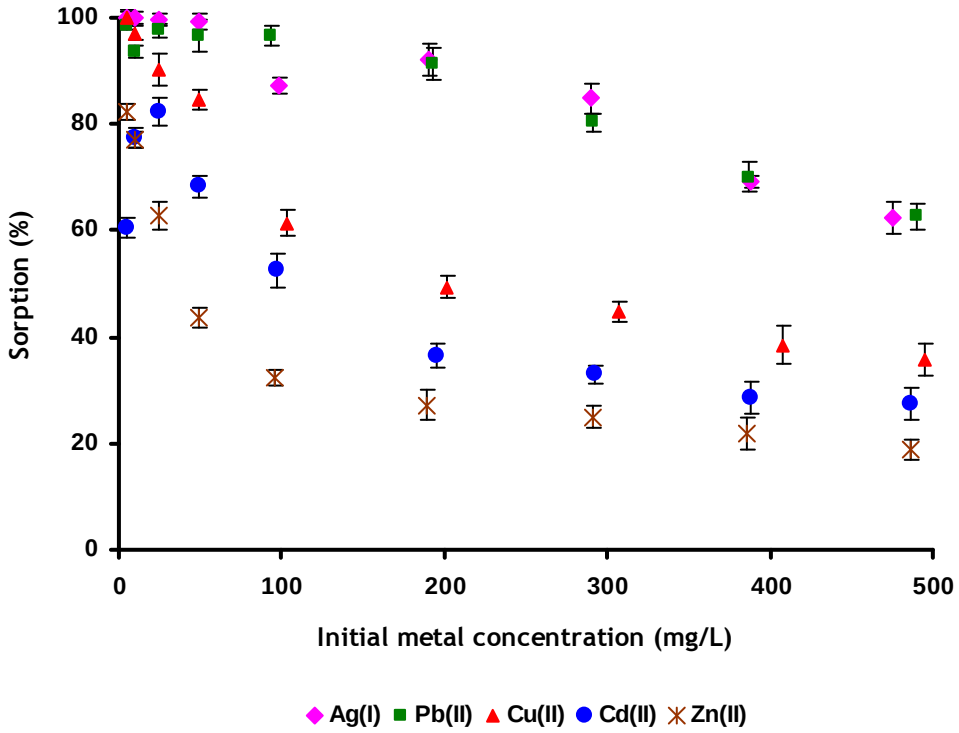


Figure 4.6 Effect of initial metal concentration on the sorption percentage of metal ions by chitosan-EGDE (Error bars are ± standard deviation of three replicates).

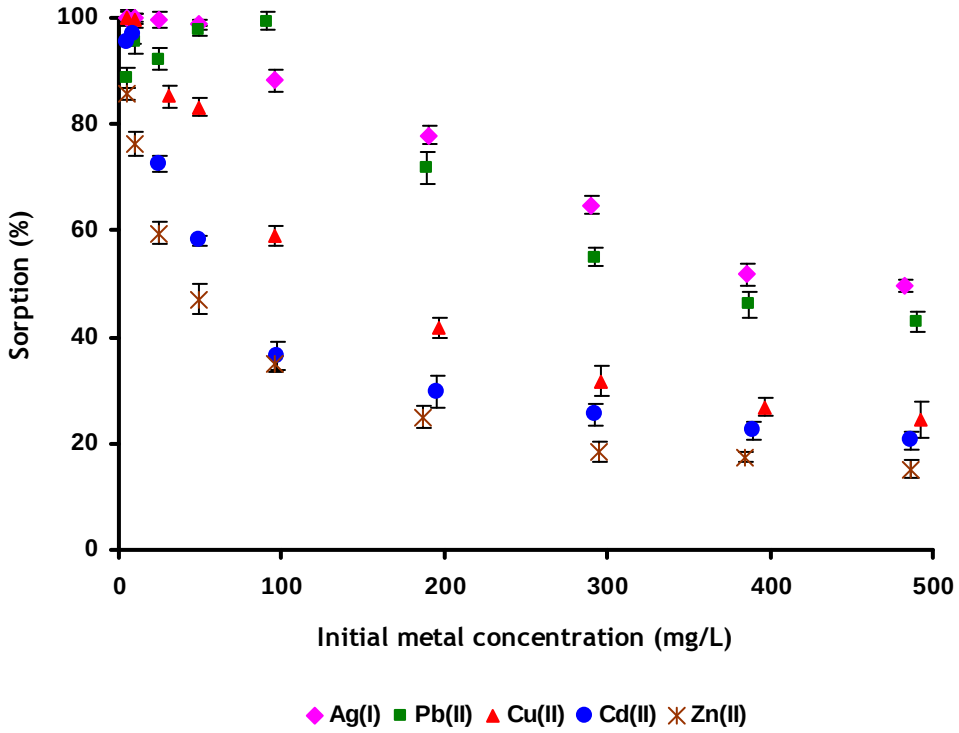


Figure 4.7 Effect of initial metal concentration on the sorption percentage of metal ions by chitosan-GLA (Error bars are ± standard deviation of three replicates).

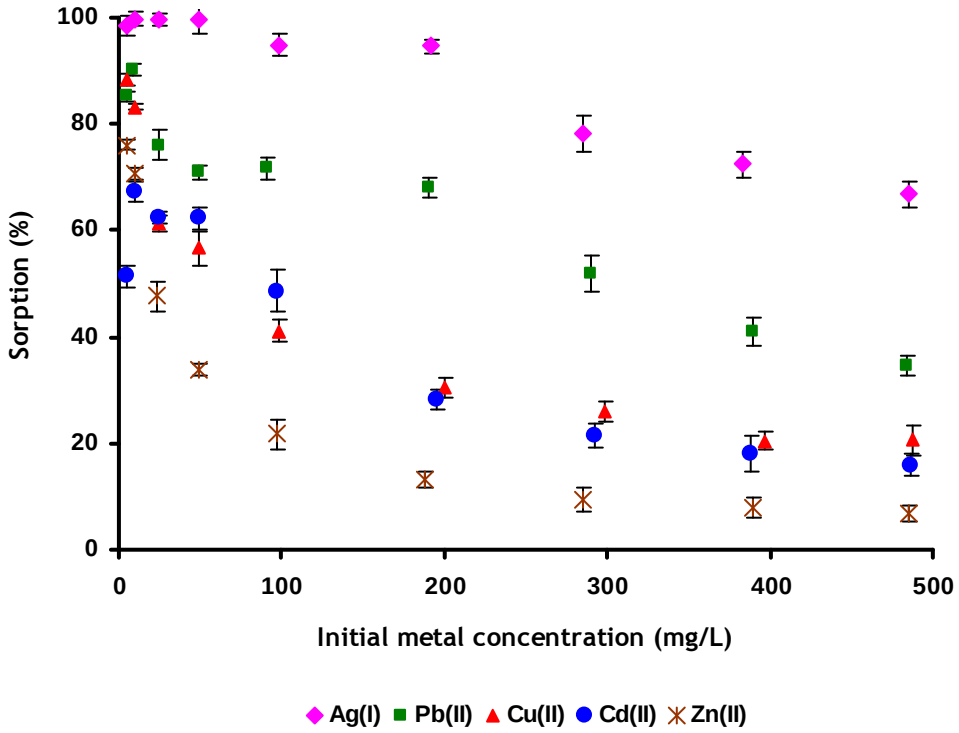


Figure 4.8 Effect of initial metal concentration on the sorption percentage of metal ions by chitosan-ECH (Error bars are ± standard deviation of three replicates).

4.4 Effect of background electrolytes

Soil normally contains a number of different cations and anions. In general, K^+, Na^+, Ca^{2+}, Cl^- and NO_3^- are commonly found in soil solution. Therefore, it is important to examine the ability of sorbents to bind one specific metal ion from mixed ion solutions.

In this research, the influence of Cl^- and NO_3^- ions on Zn^{2+} binding by chitosan and cross-linked chitosans was investigated using KCl and KNO_3 (0.1 mol/L) background electrolytes in four sorption systems, as described in Section 2.7.3. Kinetic parameters for Zn^{2+} sorption for each system are given in Table 4.2. Based on the R^2 values, the sorption data were best fitted with the pseudo-second-order kinetic model, while the pseudo-first-order and intraparticle diffusion models described the data poorly, as discussed in Section 4.5. Therefore, the amount of Zn^{2+} sorbed onto chitosans at equilibrium, q_e, was obtained from the pseudo-second-order equation. From Table 4.2, the estimated q_e values for each system showed no significant difference at $p < 0.05$ (Tukey's Least Significant Difference). This suggests that the nature of Cl^- and NO_3^- ions did not affect Zn^{2+} binding by the chitosans.

Results obtained from this study are in agreement with the research findings discussed by Benguella and Benaissa (2002). They noted that the nature of cadmium salts, namely cadmium sulfate, cadmium fluoride, cadmium acetate and cadmium nitrate did not influence the kinetics behaviour of Cd^{2+} removal by chitin, in which Cd^{2+} binding was reported to follow the pseudo-second-order kinetic model. In addition, the estimated amount of Cd^{2+} sorbed at equilibrium was found to be similar for all cadmium salts studied.

Studies have shown that the presence of cations and anions inhibited metal ion binding by sorbents. For example, Huang *et al*. (2009) reported that Cu^{2+} sorption onto aspen wood fibres was influenced by Na^+, Ca^{2+} and Al^{3+}. Moreira *et al*. (2008) noted that Ca^{2+} caused a greater inhibition effect than Na^+ upon Ni^{2+} sorption by two Oxisols and an Alfisol. Guo *et al*. (2008) observed a significant decrease in Cu^{2+} sorption onto lignin when $NaNO_3$ was used as a background electrolyte. The presence of Cl^- was found to reduce As(V) sorption by crab shells (Niu *et al*., 2007), Cr(III) removal by kaolinite (Turan *et al*., 2007) and Cd(II) binding to *Eleocharis acicularis* biomass (Miretzky *et al*., 2010). A study by Singh *et al*. (2008) has demonstrated that the removal efficiency of Cr(VI) by *Cassia marginata* seed gum was affected by the presence of SO_4^{2-} and Cl^-, whereby the latter was reported to have a lower effect with respect to the competition for binding sites.

Table 4.2 Kinetic parameters for Zn^{2+} sorption in different background electrolytes.

Sorbent	Set	q_e	Pseudo-first-order		Pseudo-second-order		Intraparticle diffusion	
			k_1	R^2	k_2	R^2	k_{id}	R^2
Chitosan	A	23.2	2.93×10^{-3}	0.3429	3.93×10^{-3}	0.9988	3.00×10^{-1}	0.2366
	B	28.4	2.56×10^{-3}	0.4301	6.99×10^{-3}	0.9999	4.48×10^{-1}	0.3210
	C	28.6	2.52×10^{-3}	0.7543	4.37×10^{-3}	0.9999	4.34×10^{-1}	0.3332
	D	25.1	2.76×10^{-3}	0.8194	3.82×10^{-3}	0.9999	3.91×10^{-1}	0.3557
LSD		8.27						
Chitosan-GLA	A	15.8	2.04×10^{-3}	0.2824	1.60×10^{-3}	0.9996	2.03×10^{-1}	0.3659
	B	19.9	2.12×10^{-3}	0.4331	2.97×10^{-3}	0.9992	4.33×10^{-1}	0.4840
	C	19.6	2.30×10^{-3}	0.5399	2.63×10^{-3}	0.9995	3.99×10^{-1}	0.5518
	D	16.1	2.07×10^{-3}	0.3748	8.77×10^{-3}	0.9998	2.64×10^{-1}	0.3720
LSD		7.84						
Chitosan-ECH	A	8.69	1.92×10^{-3}	0.1530	8.04×10^{-3}	0.9989	1.81×10^{-1}	0.4424
	B	8.85	1.84×10^{-3}	0.1634	6.33×10^{-3}	0.9982	2.05×10^{-1}	0.5019
	C	7.60	2.78×10^{-3}	0.6846	9.49×10^{-3}	0.9998	1.46×10^{-1}	0.4739
	D	6.53	2.53×10^{-3}	0.7800	1.02×10^{-3}	0.9998	1.22×10^{-1}	0.4764
LSD		5.35						
Chitosan-EGDE	A	17.4	2.61×10^{-3}	0.5098	9.19×10^{-3}	0.9999	2.80×10^{-1}	0.3461
	B	20.8	2.38×10^{-3}	0.5877	5.68×10^{-3}	0.9999	3.58×10^{-1}	0.3923
	C	20.2	2.34×10^{-3}	0.5316	3.12×10^{-3}	0.9996	4.01×10^{-1}	0.5089
	D	18.5	2.21×10^{-3}	0.6768	6.78×10^{-3}	0.9999	2.94×10^{-1}	0.3539
LSD		4.91						

Set A ($ZnCl_2$ + KCl), Set B ($Zn(NO_3)_2.6H_2O$ + KNO_3), Set C ($ZnCl_2$ + KNO_3) and Set D ($Zn(NO_3)_2.6H_2O$ + KCl).
q_e (mg/g), k_1 (1/min), k_2 (g/mg/min) and k_{id} (mg/g/$min^{0.5}$).

The SEM images at 2500x magnification of chitosan and cross-linked chitosans after Zn^{2+} sorption in different background electrolytes are presented in Figures 4.9 to 4.12. SEM images reveal that the irregular surface of chitosan (Figure 3.3a, Section 3.3.4) became grooved in all systems tested (Figure 4.9), whereas a dense and less rugged texture was observed on chitosan-GLA (Figure 4.10). Meanwhile, the rough surface texture of chitosan-ECH and chitosan-EGDE (Figures 3.3c and 3.3d, Section 3.3.4) changed into a swollen network structure (Figures 4.11 and 4.12). From the study of surface morphology, it can be postulated that Zn^{2+} sorption in different background electrolytes involves a similar binding mechanism, producing similar morphological effects.

Following on the results obtained from this study, the subsequent Zn^{2+} sorption studies are carried out with Zn^{2+} solution prepared using $ZnCl_2$ salt in 0.1 mol/L KCl background electrolyte as described in Section 2.7.1.

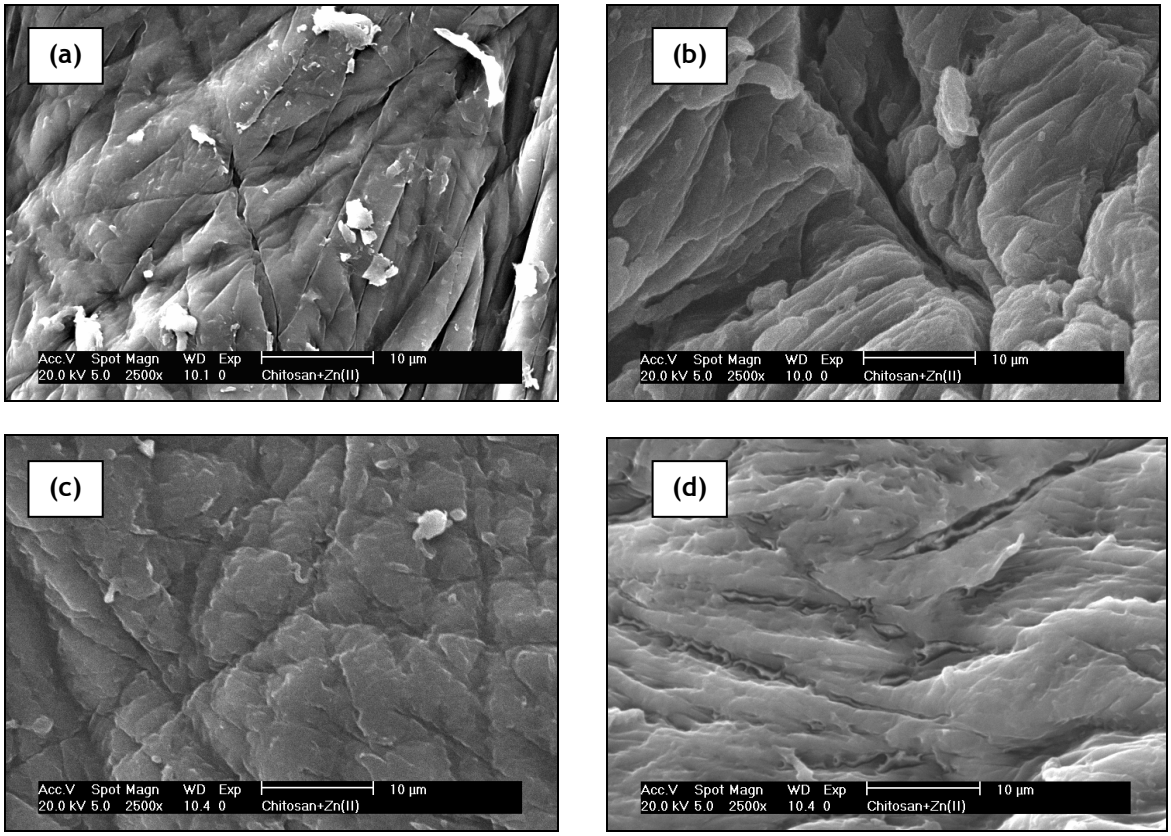


Figure 4.9 SEM images of chitosan at 2500x magnification after Zn^{2+} sorption in different background electrolytes: (a) $ZnCl_2$ in KCl, (b) $Zn(NO_3)_2$ in KNO_3, (c) $ZnCl_2$ in KNO_3, and (d) $Zn(NO_3)_2$ in KCl.

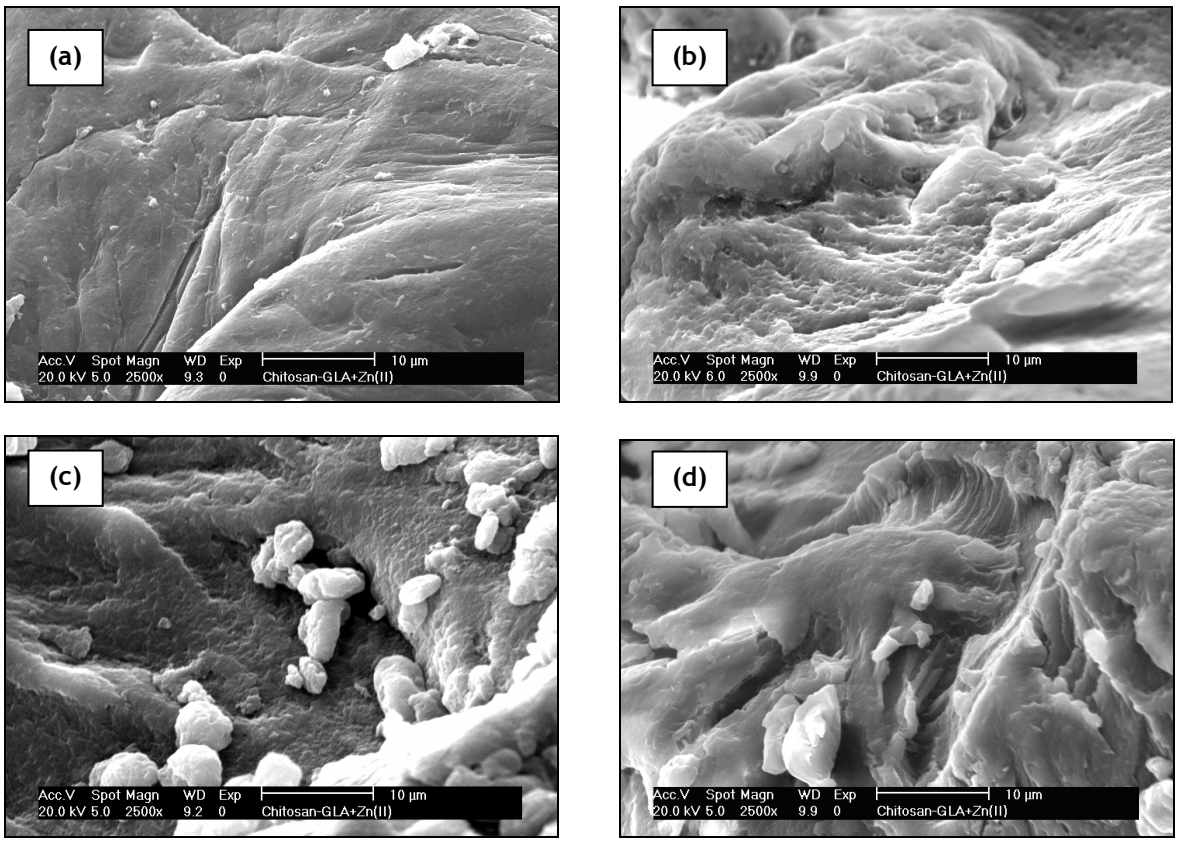


Figure 4.10 SEM images of chitosan-GLA at 2500x magnification after Zn^{2+} sorption in different background electrolytes: (a) $ZnCl_2$ in KCl, (b) $Zn(NO_3)_2$ in KNO_3, (c) $ZnCl_2$ in KNO_3, and (d) $Zn(NO_3)_2$ in KCl.

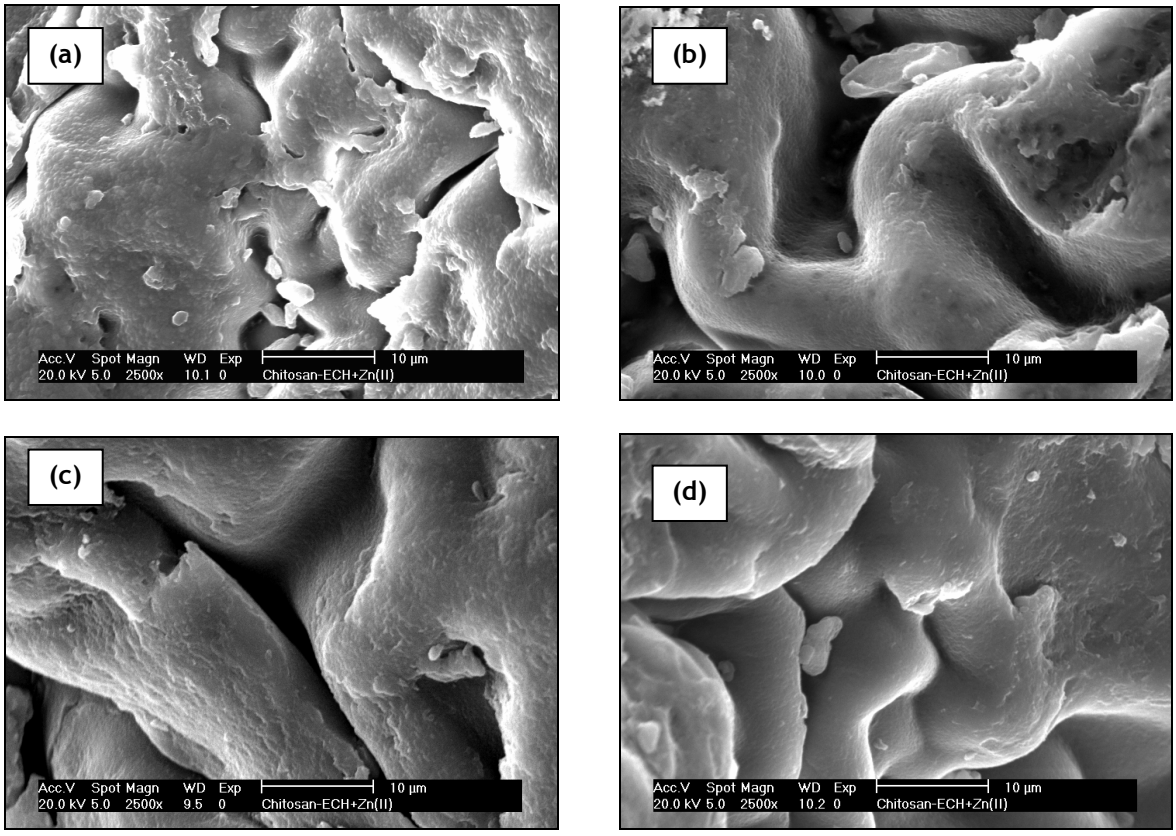


Figure 4.11 SEM images of chitosan-ECH at 2500x magnification after Zn^{2+} sorption in different background electrolytes: (a) $ZnCl_2$ in KCl, (b) $Zn(NO_3)_2$ in KNO_3, (c) $ZnCl_2$ in KNO_3, and (d) $Zn(NO_3)_2$ in KCl.

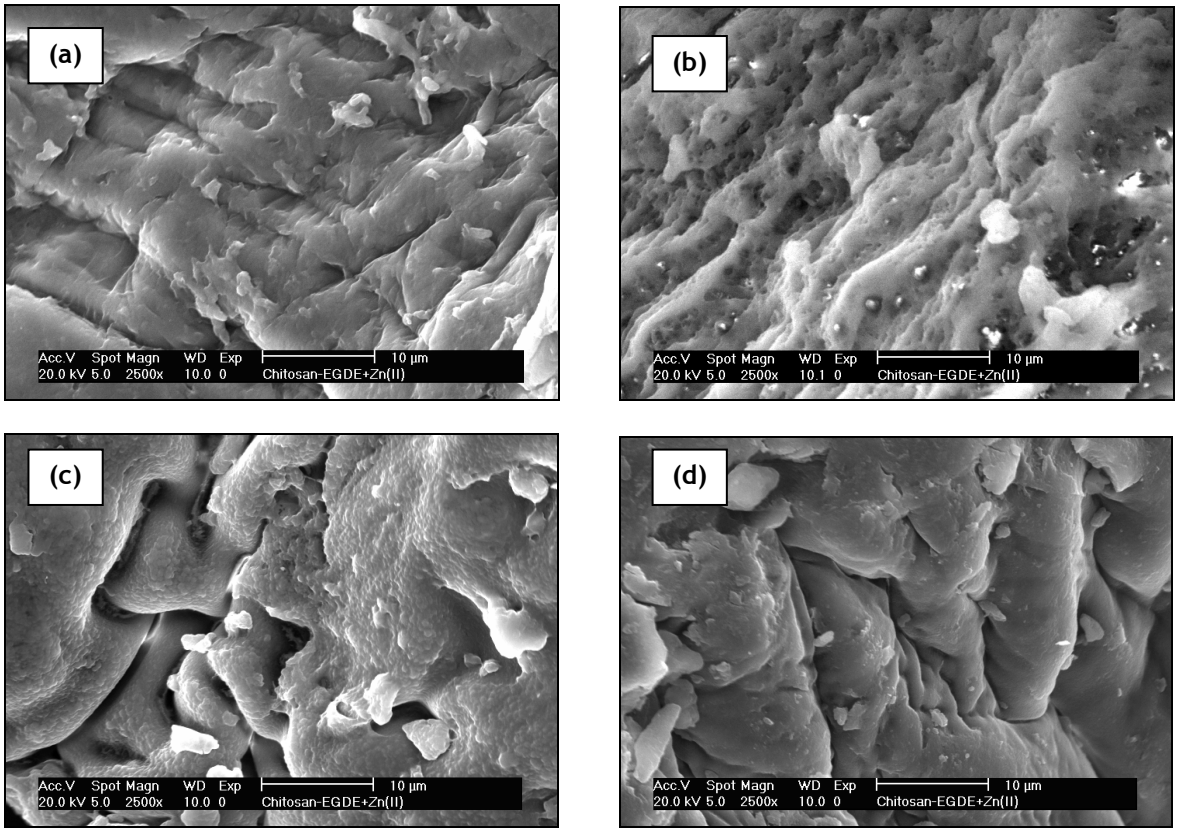


Figure 4.12 SEM images of chitosan-EGDE at 2500x magnification after Zn^{2+} sorption in different background electrolytes: (a) $ZnCl_2$ in KCl, (b) $Zn(NO_3)_2$ in KNO_3, (c) $ZnCl_2$ in KNO_3, and (d) $Zn(NO_3)_2$ in KCl.

4.5 Sorption kinetics

An ideal sorbent for metal decontamination should not only have a large sorbate capacity but also a fast sorption rate (Dąbrowski, 2001; Kurniawan *et al.*, 2006; Crini and Badot, 2008). According to Demirbas (2008) and Sud *et al.* (2008), predicting the rate at which sorption takes place and knowing the binding mechanism are both vital to determine the efficiency of a sorption process.

Unlike the extent of sorption, which depends only on the initial and final equilibrium state, the rate of sorption depends on the whole process (Lodeiro *et al.*, 2006; Gerente *et al.*, 2007). There are four independent processes that may control the sorption kinetics in a solid-liquid system (Lodeiro *et al.*, 2006; Ho *et al.*, 1996): (i) solute transfer from the bulk solution to the boundary film that surrounds the sorbent's surface, (ii) solute transport from the boundary film to the sorbent's surface, (iii) solute transfer from the sorbent's surface to the active intraparticle sites, and (iv) interaction(s) between solute and binding sites of the sorbent. The first and second steps are generally considered as rapid processes (Miretzky *et al.*, 2010; Dąbrowski, 2001). Therefore, intraparticle diffusion or interaction(s) between solute and binding sites (chemical binding reaction) may potentially control the sorption kinetics (Crini and Badot, 2008).

In this research, the pseudo-first-order (Lagergren, 1898), pseudo-second-order (Ho and McKay, 1998) and intraparticle diffusion (Weber and Morris, 1963) kinetic models were employed to determine the rate constant and the controlling mechanisms of the sorption processes.

The pseudo-first-order kinetic model was originally developed by Lagergren in 1898 to describe the sorption of oxalic and malonic acids onto charcoal (Lagergren, 1898; Ho, 2004), and is generally expressed as Equation 4.1 (Lagergren, 1898; Gerente *et al.*, 2007).

$$\frac{dq_t}{dt} = k_1(q_e - q_t) \qquad (4.1)$$

Integration of Equation 4.1 with applying boundary conditions, $t = 0$ to $t = t$ and $q_t = 0$ to $q_t = q_t$, gives Equation 4.2 (Lagergren, 1898; Gerente *et al.*, 2007; Febrianto *et al.*, 2009).

$$q_t = q_e(1 - e^{-k_1 t}) \qquad (4.2)$$

The linear form of pseudo-first-order equation is written as Equation 4.3 (Lagergren, 1898; Gerente *et al.*, 2007).

$$\log(q_e - q_t) = \log(q_e) - \frac{k_1}{2.303} t \quad (4.3)$$

where, q_e and q_t (mg/g) are the amount of metal ions sorbed at equilibrium and at time t (min), respectively and k_1 (1/min) is the rate constant of pseudo-first-order equation. The plot $\log(q_e - q_t)$ against t should give a straight line if this kinetic model is applicable (Febrianto *et al.*, 2009). The q_e and k_1 values can be determined from the intercept and slope of the plot.

Ho and McKay (1998) derived a pseudo-second-order relationship based on the assumption that chemical sorption is the rate-limiting step. Chemical sorption was assumed to involve valency forces through exchange or sharing of electrons between sorbent and sorbate (Ho and McKay, 1998). The kinetic equation for this model can be written as Equation 4.4 (Ho and McKay, 1998; Ho, 2006).

$$\frac{dq_t}{dt} = k_2(q_e - q_t)^2 \quad (4.4)$$

Integration of Equation 4.4 for the boundary conditions $t = 0$ to $t = t$ and $q_t = 0$ to $q_t = q_t$, gives Equation 4.5 (Ho and McKay, 1998; Ho, 2006).

$$q_t = \frac{q_e^2 k_2 t}{1 + q_e k_2 t} \quad (4.5)$$

Equation 4.5 can be rearranged to obtain Equation 4.6 (Ho and McKay, 1998; Ho, 2006).

$$q_t = \frac{t}{\frac{1}{k_2 q_e^2} + \frac{t}{q_e}} \quad (4.6)$$

The linear form of pseudo-second-order equation is rendered as Equation 4.7 (Ho and McKay, 1998; Ho, 2006).

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (4.7)$$

where, k_2 (g/mg/min) is the rate constant of pseudo-second-order equation. A straight line of t/q_t against t suggests the applicability of this kinetic model (Ho, 2006).

In 1963, Weber and Morris proposed intraparticle diffusion equation based on the assumption that the sorption process involves simple diffusion in the sorbent particles (Weber and Morris, 1963). The intraparticle diffusion equation is described as Equation 4.8 (Weber and Morris, 1963).

$$q_t = k_{id} t^{0.5} \quad (4.8)$$

where, k_{id} (mg/g/min$^{0.5}$) is the rate constant of intraparticle diffusion equation and can be determined from the slope of a plot q_t against $t^{0.5}$, which would result in a linear relationship if intraparticle diffusion controls the sorption process (Weber and Morris, 1963).

The kinetic plots for metal ion binding by chitosan and cross-linked chitosans are shown in Figures 4.13 to 4.24. From these figures, it can be seen that only the pseudo-second-order model gives a linear relationship to the kinetic data (Figures 4.17 to 4.20). The kinetic parameters estimated for the sorption along with their corresponding R^2 values are given in Table 4.3. The R^2 values obtained for the pseudo-second-order plots ranged from 0.9842 to 0.9999, whereas 0.1199 to 0.5484 and 0.3485 to 0.4974 were obtained for pseudo-first-order and intraparticle diffusion plots, respectively. The high R^2 values indicate the best fit of the pseudo-second-order kinetic equation to the sorption data. Moreover, the experimental equilibrium sorption capacities ($q_{e\ experimental}$) determined from the contact time study were in good agreement with the theoretical equilibrium sorption capacities ($q_{e\ theoretical}$) calculated using the pseudo-second-order kinetic model as shown in Table 4.3. This suggests the applicability of this model in describing the kinetic behaviour of metal ions binding to the chitosans over the period of contact times.

The rate of the chemical binding reaction depends on the nature of the specific interaction(s) that occur between metal ion and the functional groups of the sorbent (Weber *et al.*, 1991; McCoy and Liapis, 1991; Lodeiro *et al.*, 2006). The k_2 values obtained from this study (range from 0.50 x 10^{-3} to 4.33 x 10^{-3} g/mg/min) are comparable with the k_2 values reported for Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} sorption by lignin (1.3 x 10^{-2} to 4.5 x 10^{-2} g/mg/min) (Guo *et al.*, 2008); Cd^{2+}, Cu^{2+} and Pb^{2+} binding to wood bark (1.0 x 10^{-4} to 5.0 x 10^{-4} g/mg/min) (Munagapati *et al.*, 2010); and Ag^{+} sorption onto clinoptilolites (2.02 x 10^{-1} g/mg/min) (Çoruh *et al.*, 2010).

Interestingly, the k_2 values determined for Cd^{2+} binding to the chitosans (1.22 x 10^{-3} to 4.17 x 10^{-3} g/mg/min) are consistent with the k_2 value reported by Benguella and Benaissa (2002) for Cd^{2+} sorption onto chitin (1.29 x 10^{-3} g/mg/min). This perhaps suggests a similar binding mechanism for Cd^{2+} uptake by chitin and chitosans, leading to

a similar kinetic rate constant. It is known that the nitrogen atom of the chitin acetamido ($NHCOCH_3$) group is able to donate and share the lone electron pair with empty orbital of metal ions (Niu *et al.*, 2007; Barriada *et al.*, 2008). It would be expected that the nitrogen atom of the chitosan amino (NH_2) group would behave in a similar manner.

Several other metal sorption studies using sorbents of biological origin, such as swine bone char (Pan *et al.*, 2009), crab shells (Cochrane *et al.*, 2006), *Saccharomyces cerevisiae* (budding yeast) (Chen and Wang, 2008), tobacco stems (Li *et al.*, 2008) and tea leaves (Amarasinghe and Williams, 2007), showed that sorption kinetics followed the pseudo-second-order kinetic model.

In this study, the pseudo-first-order and intraparticle diffusion equations did not fit well throughout the whole range of contact time (Figures 4.13 to 4.16 and Figures 4.21 to 4.24). A similar trend was observed by Mohan *et al.* (2006). They noted that the plots of Lagergren's pseudo-first-order model for Cd^{2+} and Cu^{2+} sorption by *Eucalyptus* black liquor lignin deviated considerably after 40 minutes. Meanwhile, Miretzky *et al.* (2010) reported that the binding of Cd^{2+} onto *Eleocharis acicularis* biomass followed the intraparticle diffusion kinetic model for the first 5 minutes only. However, Liu *et al.* (2009) reported that the pseudo-first-order equation fitted Cd^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} binding to *Laminaria japonica* (brown alga) very well. A possible explanation for different kinetic behaviour relates to the different physical and chemical properties of the sorbents, as well as the different in experimental conditions applied.

The results presented in this chapter suggest that the binding of metal ions onto the various chitosans studied can be best described by the pseudo-second-order kinetic model implying that chemical binding was the rate-limiting step, as discussed by Ho and McKay (1998; 2000).

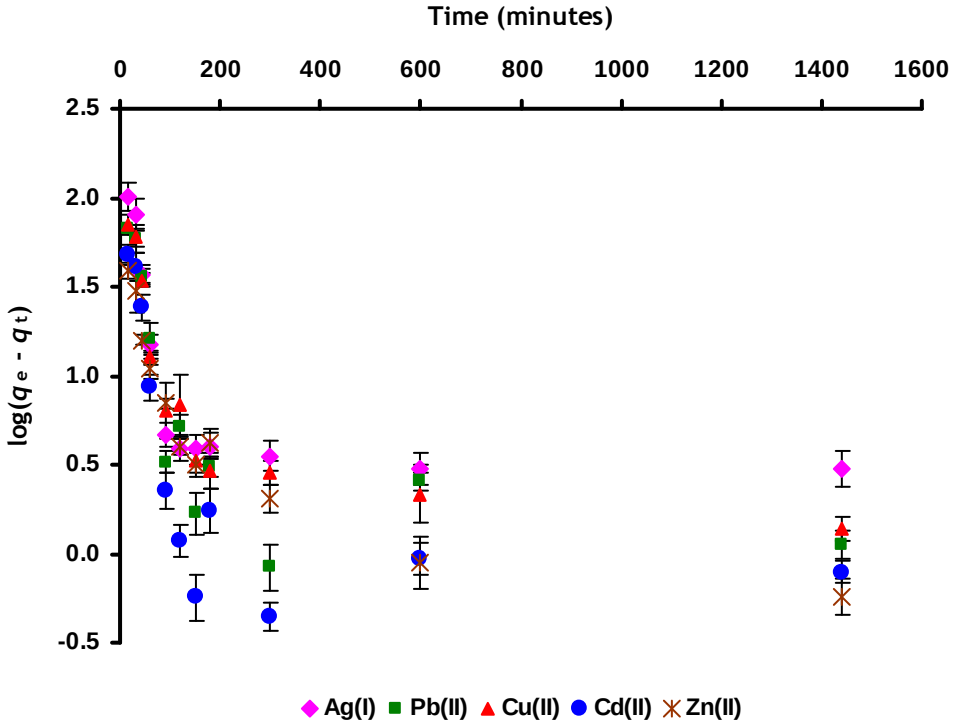


Figure 4.13 Pseudo-first-order kinetics of metal ions binding by chitosan (Error bars are ± standard deviation of three replicates).

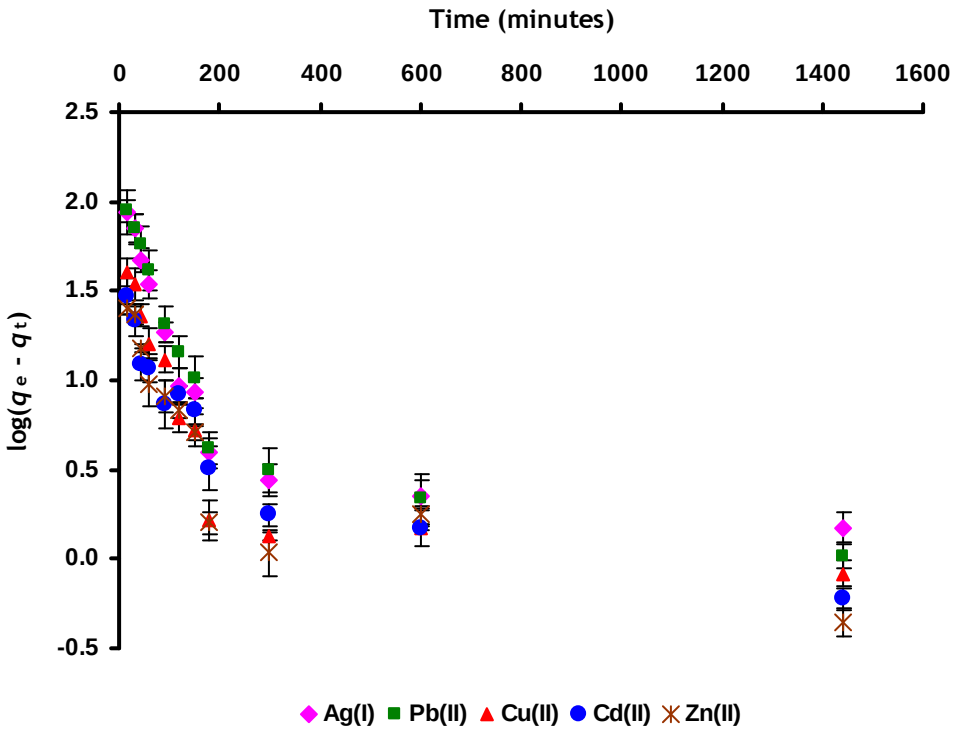


Figure 4.14 Pseudo-first-order kinetics of metal ions binding by chitosan-EGDE (Error bars are ± standard deviation of three replicates).

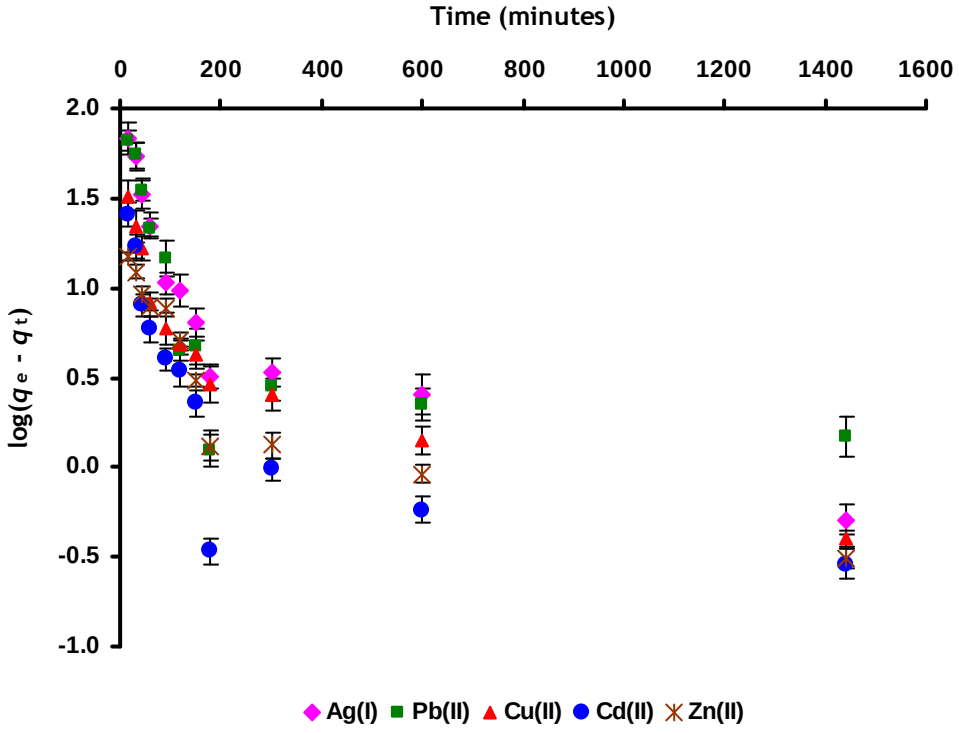


Figure 4.15 Pseudo-first-order kinetics of metal ions binding by chitosan-GLA (Error bars are ± standard deviation of three replicates).

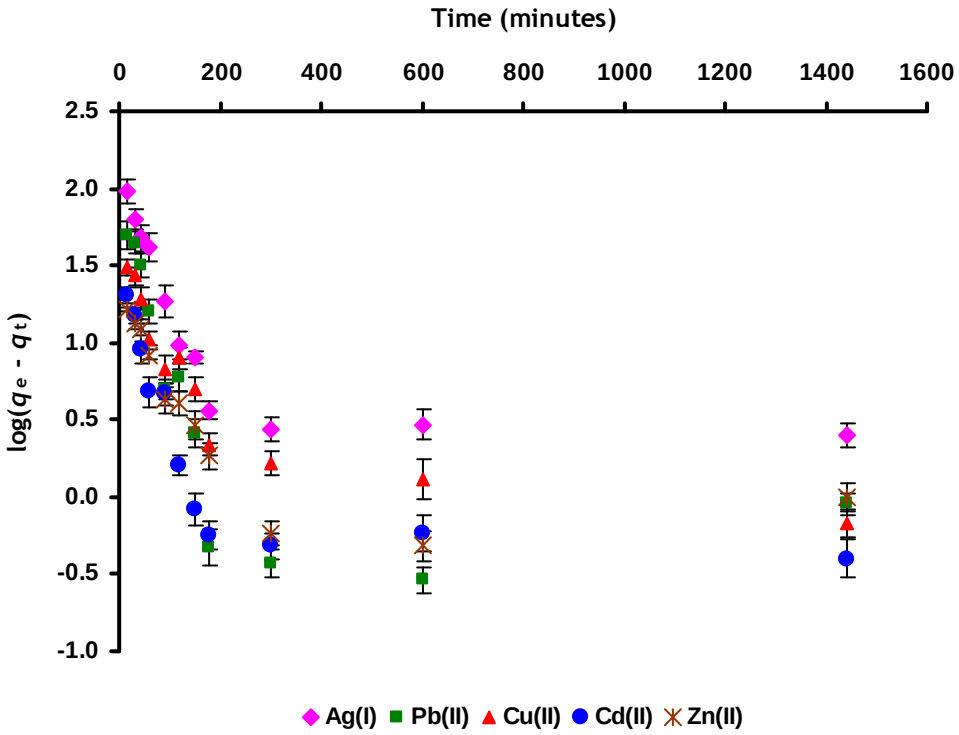


Figure 4.16 Pseudo-first-order kinetics of metal ions binding by chitosan-ECH (Error bars are ± standard deviation of three replicates).

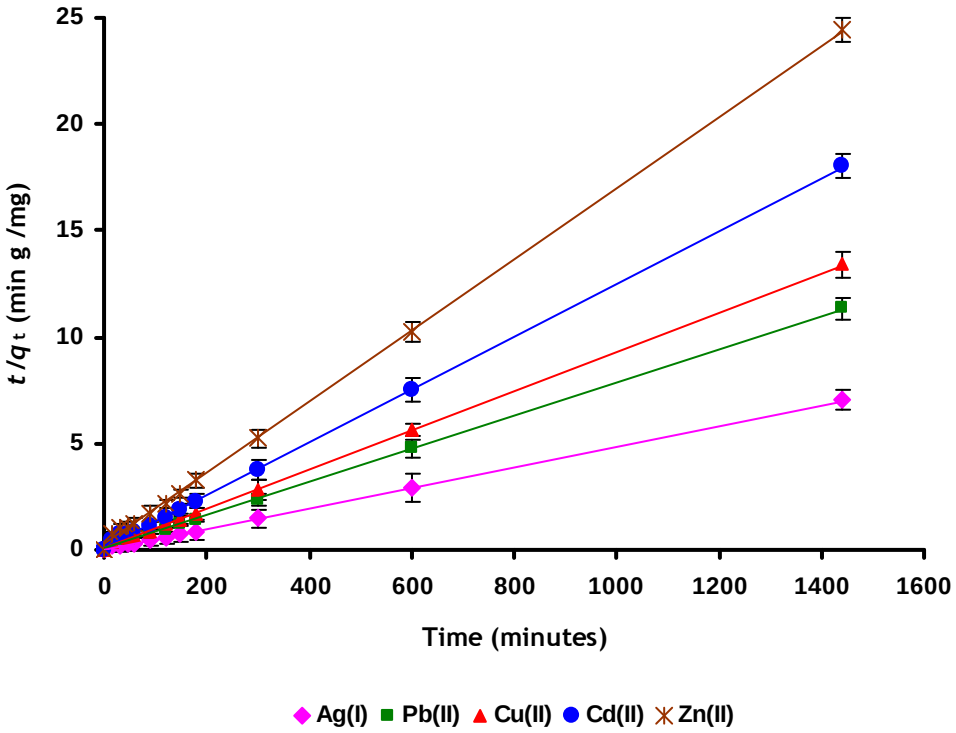


Figure 4.17 Pseudo-second-order kinetics of metal ions binding by chitosan (Error bars are ± standard deviation of three replicates).

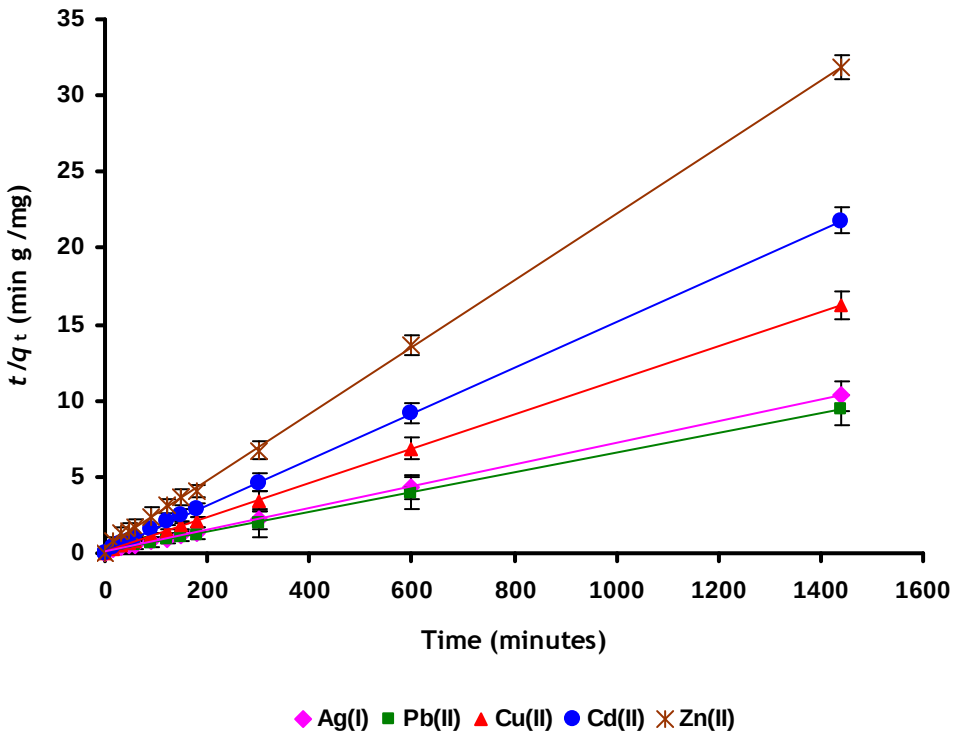


Figure 4.18 Pseudo-second-order kinetics of metal ions binding by chitosan-EGDE (Error bars are ± standard deviation of three replicates).

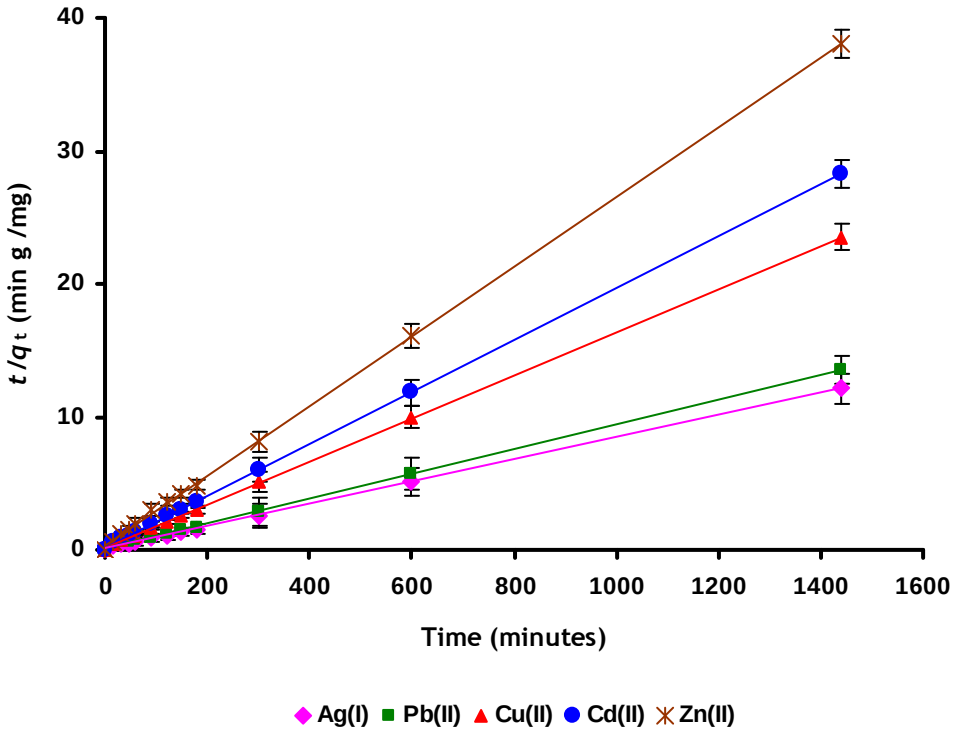


Figure 4.19 Pseudo-second-order kinetics of metal ions binding by chitosan-GLA (Error bars are ± standard deviation of three replicates).

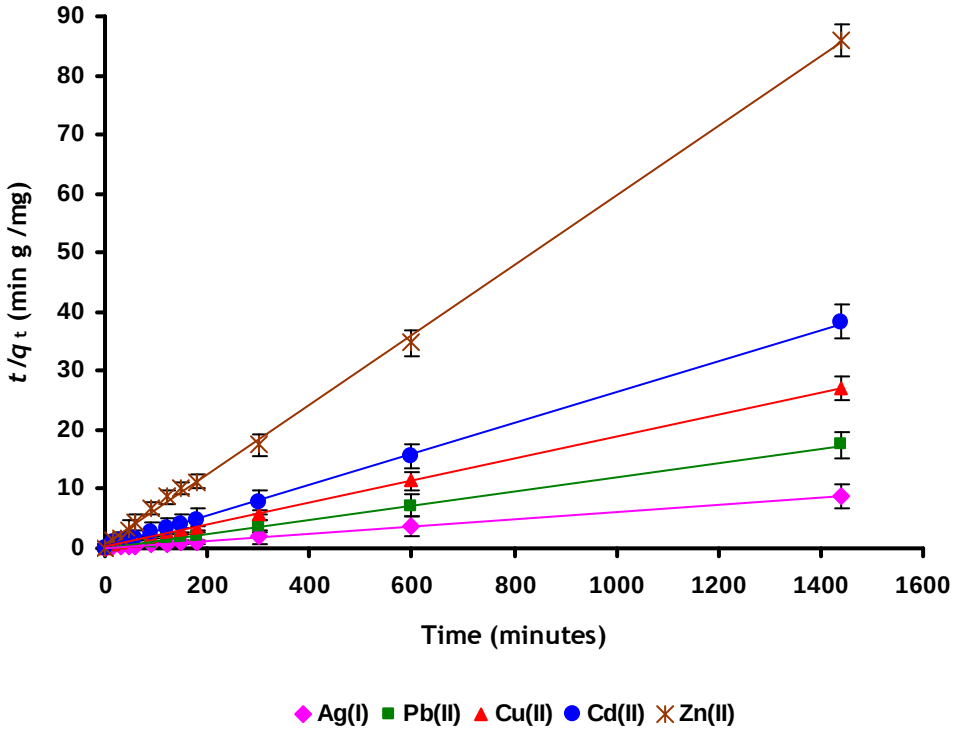


Figure 4.20 Pseudo-second-order kinetics of metal ions binding by chitosan-ECH (Error bars are ± standard deviation of three replicates).

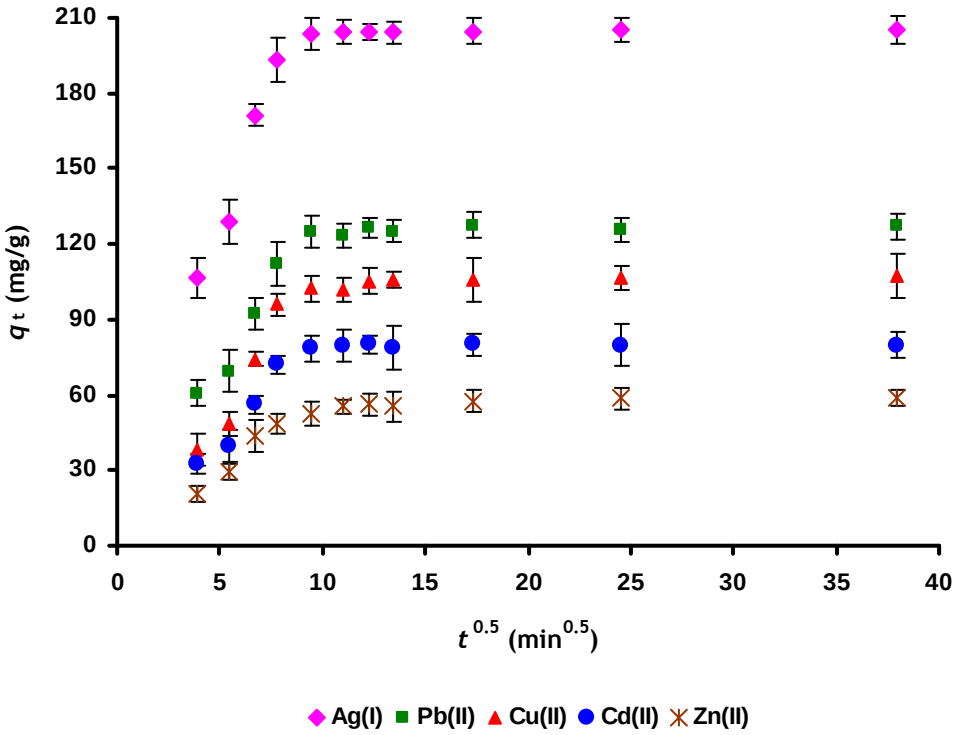


Figure 4.21 Intraparticle diffusion kinetics of metal ions binding by chitosan (Error bars are ± standard deviation of three replicates).

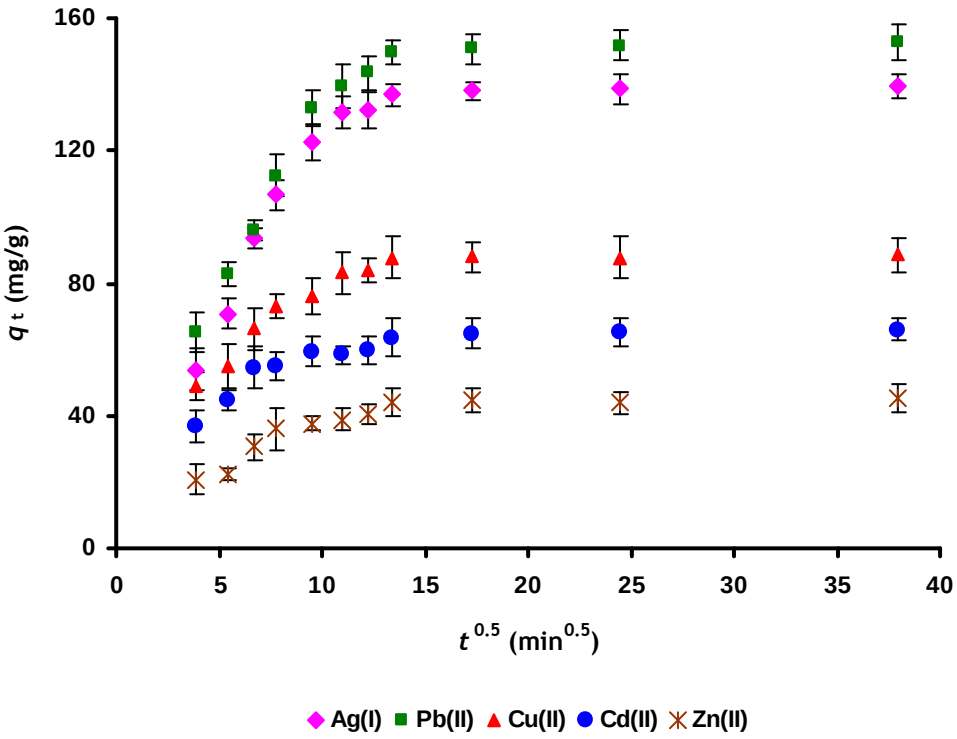


Figure 4.22 Intraparticle diffusion kinetics of metal ions binding by chitosan-EGDE (Error bars are ± standard deviation of three replicates).

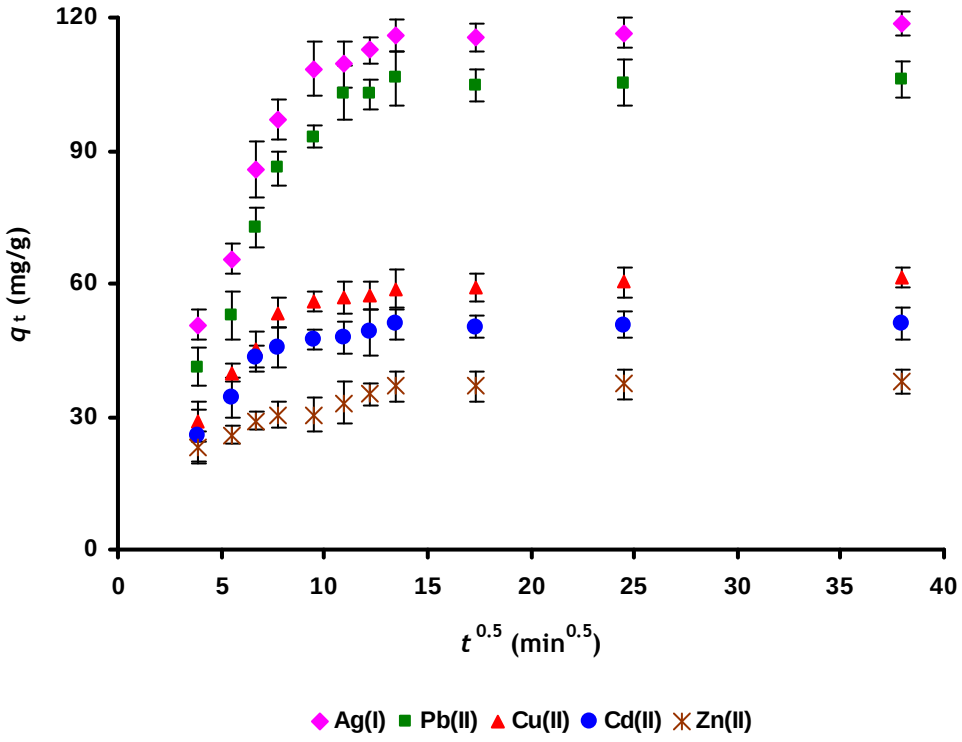


Figure 4.23 Intraparticle diffusion kinetics of metal ions binding by chitosan-GLA (Error bars are ± standard deviation of three replicates).

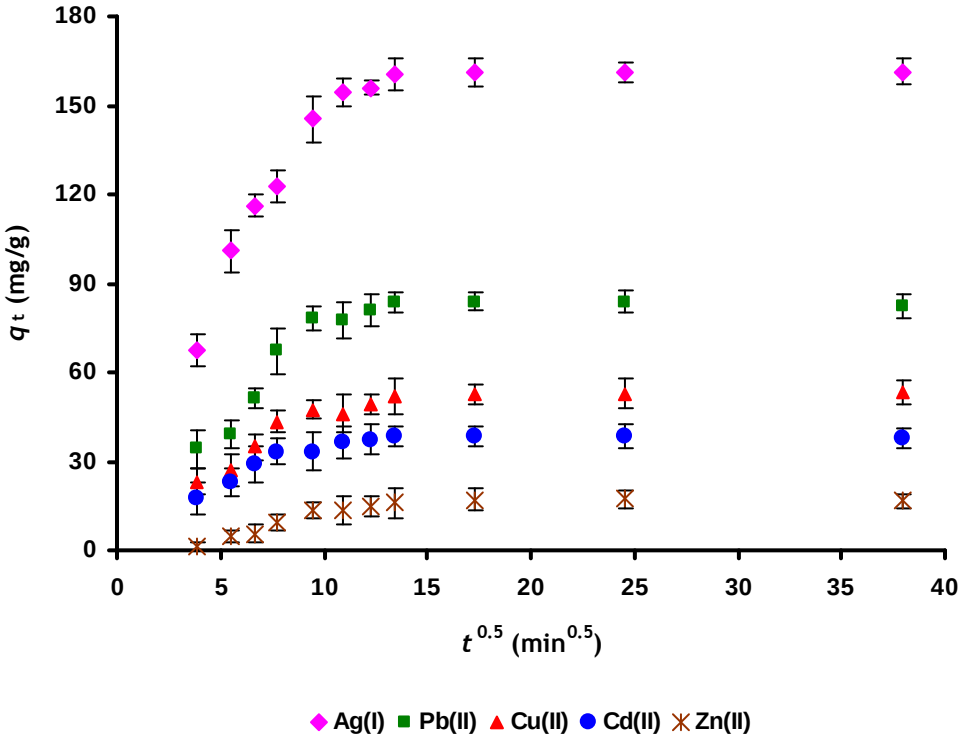


Figure 4.24 Intraparticle diffusion kinetics of metal ions binding by chitosan-ECH (Error bars are ± standard deviation of three replicates).

Table 4.3 Kinetic parameters for metal ions sorption onto chitosan and cross-linked chitosans.

Metal ion	Sorbent	Pseudo-first-order		Pseudo-second-order				Intraparticle diffusion	
		k_1 (1/min)	R^2	k_2 (g/mg/min)	q_e (mg/g)		R^2	K_{id} (mg/g/min$^{0.5}$)	R^2
					Expt.	Theot.			
Ag^+	Chitosan	1.15×10^{-3}	0.1199	0.87×10^{-3}	206	208	0.9998	3.61	0.3485
	Chitosan-GLA	2.53×10^{-3}	0.4369	0.67×10^{-3}	118	119	0.9997	2.35	0.4458
	Chitosan-ECH	1.61×10^{-3}	0.2102	0.55×10^{-3}	159	163	0.9997	3.22	0.4456
	Chitosan-EGDE	2.07×10^{-3}	0.2904	0.50×10^{-3}	143	140	0.9995	2.94	0.4754
Pb^{2+}	Chitosan	1.84×10^{-3}	0.2072	0.90×10^{-3}	125	128	0.9996	2.43	0.3970
	Chitosan-GLA	1.72×10^{-3}	0.2235	0.73×10^{-3}	106	107	0.9995	2.21	0.4518
	Chitosan-ECH	2.26×10^{-3}	0.2364	1.04×10^{-3}	82.4	83.3	0.9991	1.75	0.4498
	Chitosan-EGDE	2.30×10^{-3}	0.3595	4.33×10^{-3}	151	154	0.9996	3.20	0.4938
Cu^{2+}	Chitosan	1.67×10^{-3}	0.2448	0.73×10^{-3}	107	109	0.9993	2.23	0.4208
	Chitosan-GLA	2.39×10^{-3}	0.5036	1.42×10^{-3}	60.2	61.7	0.9999	1.14	0.4216
	Chitosan-ECH	2.18×10^{-3}	0.4046	1.18×10^{-3}	53.3	54.0	0.9996	1.10	0.4868
	Chitosan-EGDE	2.05×10^{-3}	0.3390	1.10×10^{-3}	88.4	89.3	0.9998	1.64	0.4309
Cd^{2+}	Chitosan	1.62×10^{-3}	0.1842	1.35×10^{-3}	79.9	80.7	0.9994	1.60	0.3975
	Chitosan-GLA	2.36×10^{-3}	0.4051	4.17×10^{-3}	50.3	51.3	0.9999	0.90	0.3782
	Chitosan-ECH	2.11×10^{-3}	0.3228	3.79×10^{-3}	38.9	38.0	0.9995	0.73	0.4121
	Chitosan-EGDE	2.28×10^{-3}	0.4582	1.22×10^{-3}	66.1	66.7	0.9999	1.17	0.4141
Zn^{2+}	Chitosan	2.29×10^{-3}	0.4303	1.17×10^{-3}	58.6	59.5	0.9996	1.22	0.4540
	Chitosan-GLA	2.53×10^{-3}	0.5484	2.07×10^{-3}	36.4	38.2	0.9998	0.68	0.4399
	Chitosan-ECH	1.21×10^{-3}	0.2934	1.05×10^{-3}	16.8	17.8	0.9842	0.54	0.4857
	Chitosan-EGDE	2.47×10^{-3}	0.4448	1.53×10^{-3}	43.9	45.7	0.9996	0.93	0.4974

Expt. means Experimental; Theot. means Theoretical.

4.6 Sorption isotherms

A sorption isotherm correlates solute concentration in bulk solution and in the sorbed state at a given temperature under equilibrium conditions (Limousin *et al.*, 2007; Li *et al.*, 2008; Febrianto *et al.*, 2009). The sorption isotherms of metal ions by chitosan and cross-linked chitosans are shown in Figures 4.25 to 4.29.

Based on the classification of solid solute adsorption systems as discussed by Giles *et al.* (1974a; 1974b), the isotherms for Ag^{+} sorption by the chitosans can be classified as H-curve type (Figure 4.25), whereas the isotherms for Cd^{2+} and Zn^{2+} sorption can be described as L-curve (Figures 4.28 and 4.29). In the case of Pb^{2+}, the sorption isotherms for chitosan and chitosan-ECH are L-curve, whereas the isotherms for chitosan-GLA and chitosan-EGDE are H-curve (Figure 4.26). The isotherms for Cu^{2+} sorption by chitosan is of H-curve shape, whereas isotherms for Cu^{2+} sorption onto chitosan-GLA, chitosan-ECH and chitosan-EGDE are of L-curve type (Figure 4.27).

The H-curve isotherm occurs when the solute exhibits a high affinity for the solid (Giles *et al.*, 1974a; Limousin *et al.*, 2007), and normally has a very steep initial slope (Giles *et al.*, 1974b). The L-curve isotherm suggests a progressive saturation of the solid, whereby the ratio between the concentrations of the solute remaining in solution and adsorbed on the solid decreases when the concentration increases (Limousin *et al.*, 2007; Giles *et al.*, 1974a; 1974b). From Figures 4.25 to 4.29, it is apparent that each metal ion studied has different affinity for the chitosans, and this can be described as below.

Ag^{+}: chitosan > chitosan-ECH ≈ chitosan-EGDE > chitosan-GLA

Pb^{2+}: chitosan-EGDE > chitosan > chitosan-GLA > chitosan-ECH

Cd^{2+}, Cu^{2+} and Zn^{2+}: chitosan > chitosan-EGDE > chitosan-GLA > chitosan-ECH

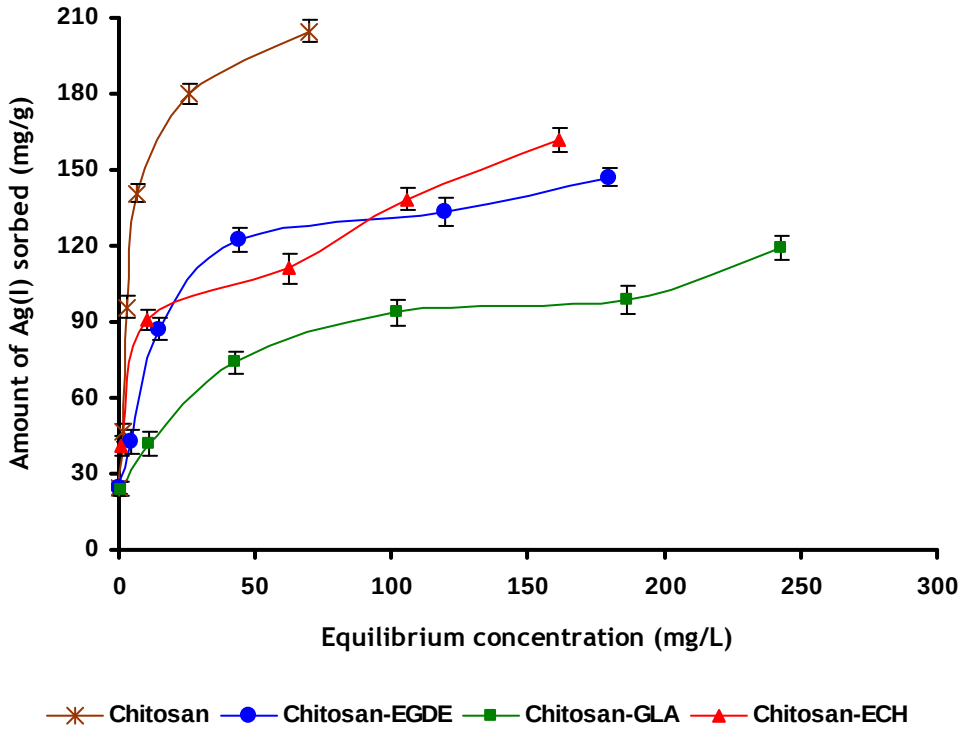


Figure 4.25 Sorption isotherms of Ag^{+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

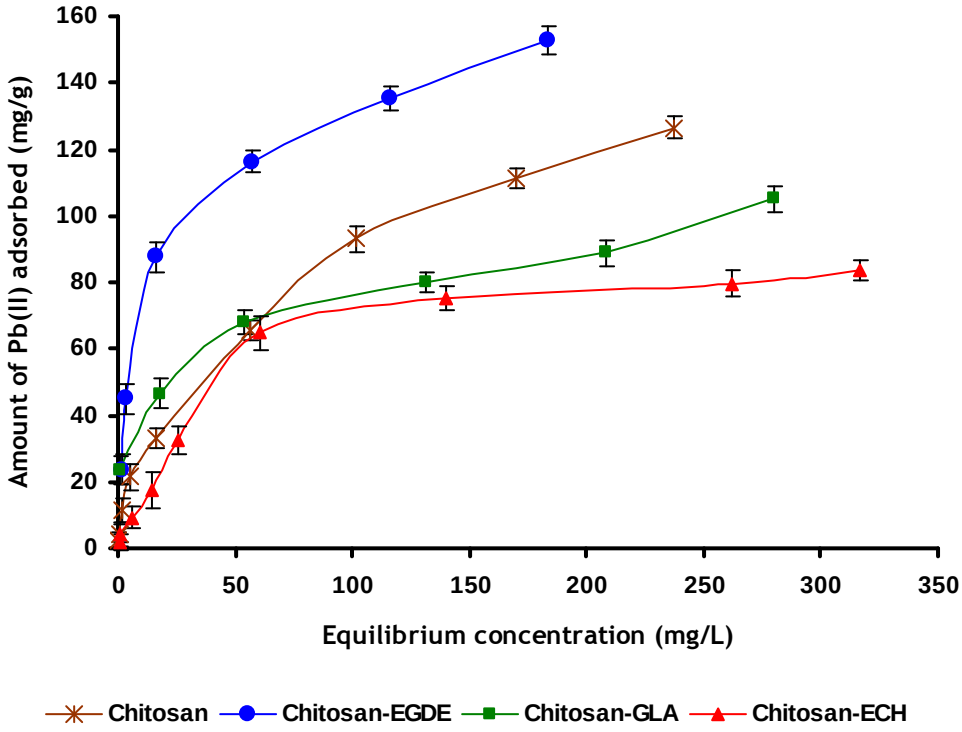


Figure 4.26 Sorption isotherms of Pb^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

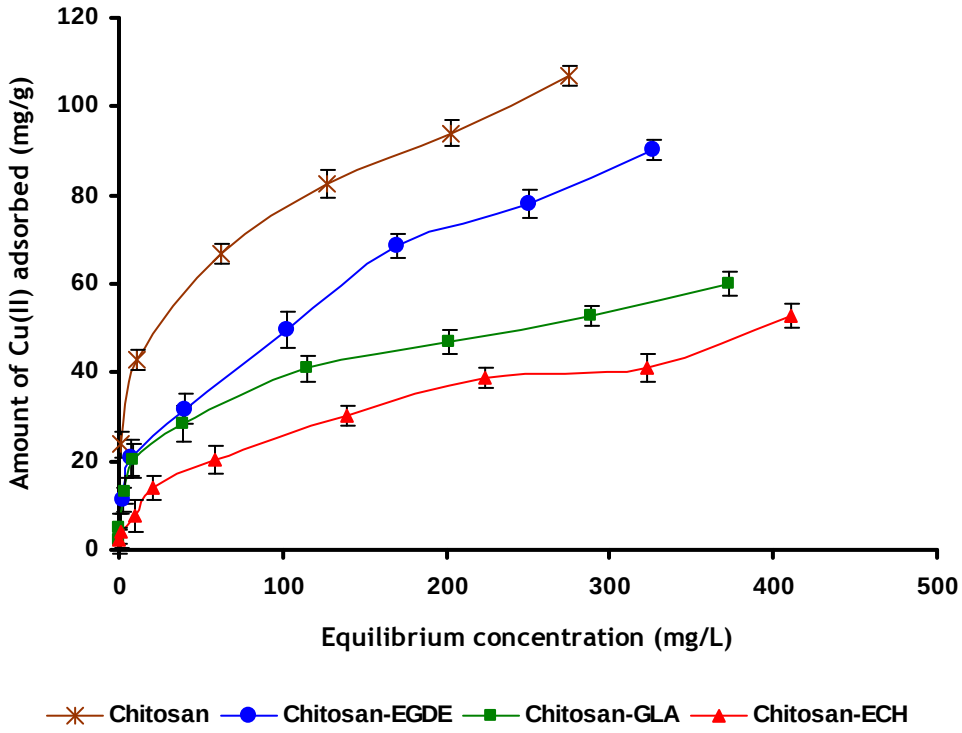


Figure 4.27 Sorption isotherms of Cu^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

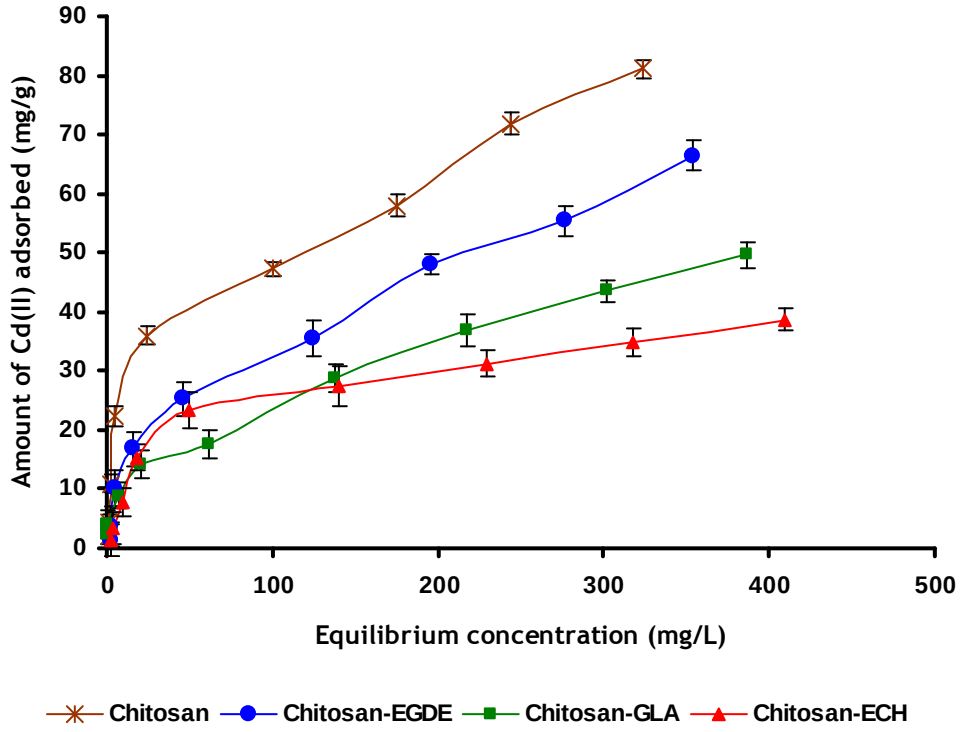


Figure 4.28 Sorption isotherms of Cd^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

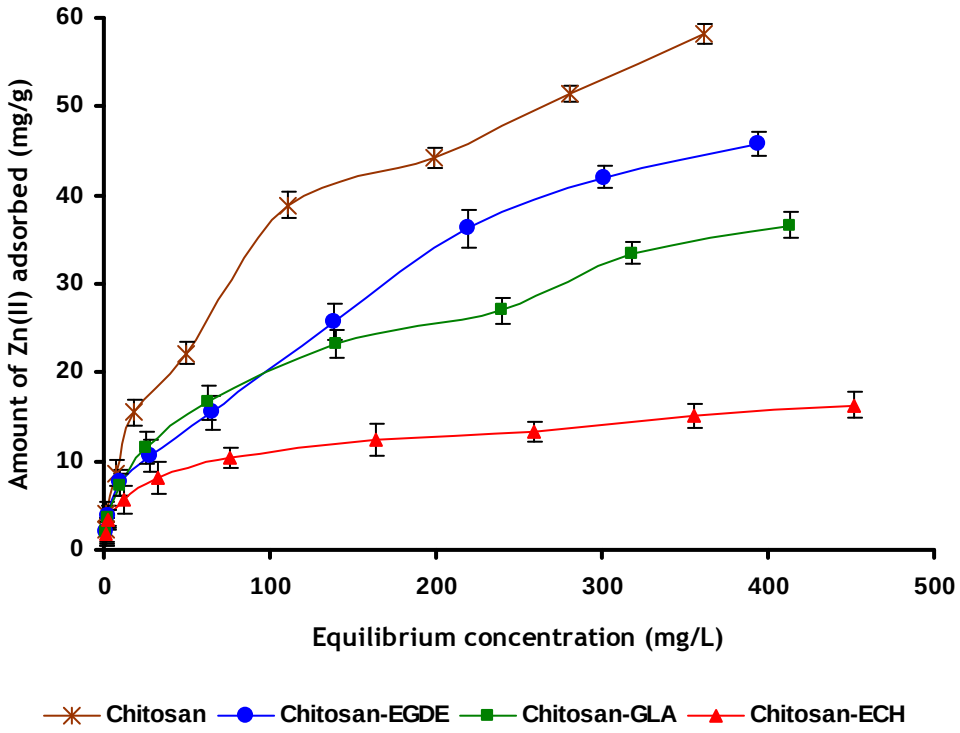


Figure 4.29 Sorption isotherms of Zn^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

Several isotherm models originally used to describe gas phase sorption can be readily adapted to explain solution equilibria (Febrianto *et al.*, 2009; Limousin *et al.*, 2007). The most widely used are the Freundlich (1906) and Langmuir (1916) isotherms (Lodeiro *et al.*, 2006; Sud *et al.*, 2008). The equation parameters of these equilibrium isotherm models provide information on the surface properties and affinity of the sorbent (Gerente *et al.*, 2007). In this research, the Freundlich and Langmuir models were applied to describe the isotherms and determine the isotherm constants of the metal ion sorption by the chitosans.

The Freundlich isotherm is the earliest known sorption isotherm equation (Gerente *et al.*, 2007; Limousin *et al.*, 2007). It was developed by Freundlich in 1906. This empirical equation applies to sorption on heterogeneous surfaces, and is generally expressed as Equation 4.9 (Freundlich, 1906; Febrianto *et al.*, 2009).

$$q_e = K_F C_e^{1/n} \qquad (4.9)$$

The linear form of the Freundlich isotherm is expressed as Equation 4.10 (Freundlich, 1906; Febrianto *et al.*, 2009; Demirbas, 2008).

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \quad (4.10)$$

where, C_e (mg/L) is the equilibrium concentration of metal ion, K_F (mg/g) is the sorption capacity and n is the sorption affinity.

The Langmuir isotherm assumes that the sorbent is structurally homogeneous with all adsorption sites being identical and energetically equivalent (Langmuir, 1916; Crini and Badot, 2008). The Langmuir isotherm further assumes that the sorption takes place at specific sites (Langmuir, 1916; Febrianto *et al.*, 2009). The general Langmuir equation is defined by Equation 4.11 (Langmuir, 1916; Gerente *et al.*, 2007).

$$q_e = \frac{QbC_e}{1 + bC_e} \quad (4.11)$$

The Langmuir equation can be written in the linear form, as described by Equation 4.12 (Langmuir, 1916; Crini and Badot, 2008).

$$\frac{C_e}{q_e} = \frac{C_e}{Q} + \frac{1}{Qb} \quad (4.12)$$

where, b (L/mg) is the Langmuir constant related to the affinity of binding sites, and Q (mg/g) is the maximum sorption capacity. A linear plot of C_e/q_e against C_e gives Q and b.

The Freundlich and Langmuir sorption isotherm models for metal ion binding to the chitosans are shown in Figures 4.30 to 4.39, and the isotherm constants obtained are summarised in Table 4.4. From Table 4.4, it can be observed that the Freundlich and Langmuir models fitted the isotherm equilibrium data reasonably well with R^2 values of 0.9604 to 0.9981 for the Freundlich isotherm plots and 0.9639 to 0.9982 for the Langmuir isotherm plots. The values of maximum sorption capacity (Q) obtained from the Langmuir equation were close to the $q_{e\ theoretical}$ and $q_{e\ experimental}$ values determined from the kinetic study (Table 4.3). Therefore, the equilibrium data were more satisfactorily fitted to the Langmuir isotherm than the Freundlich isotherm model. Studies on Cu^{2+} and Pb^{2+} sorption by crab shells (Dahiya *et al.*, 2008); Cd^{2+}, Cu^{2+} and Pb^{2+} removal by peat (Qin *et al.*, 2006); and Cd^{2+}, Cu^{2+} and Pb^{2+} sorption onto activated carbon-zeolite composite (Jha *et al.*, 2008) have demonstrated that the Langmuir equation gave a better fit to the equilibrium data than the Freundlich model.

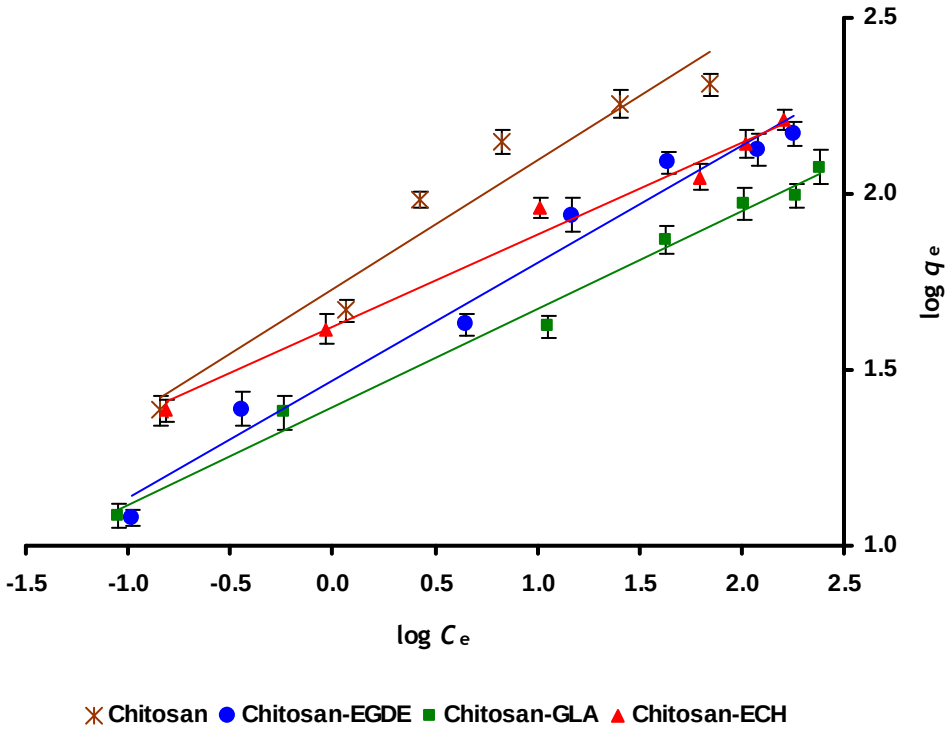


Figure 4.30 Freundlich sorption isotherms of Ag^+ by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

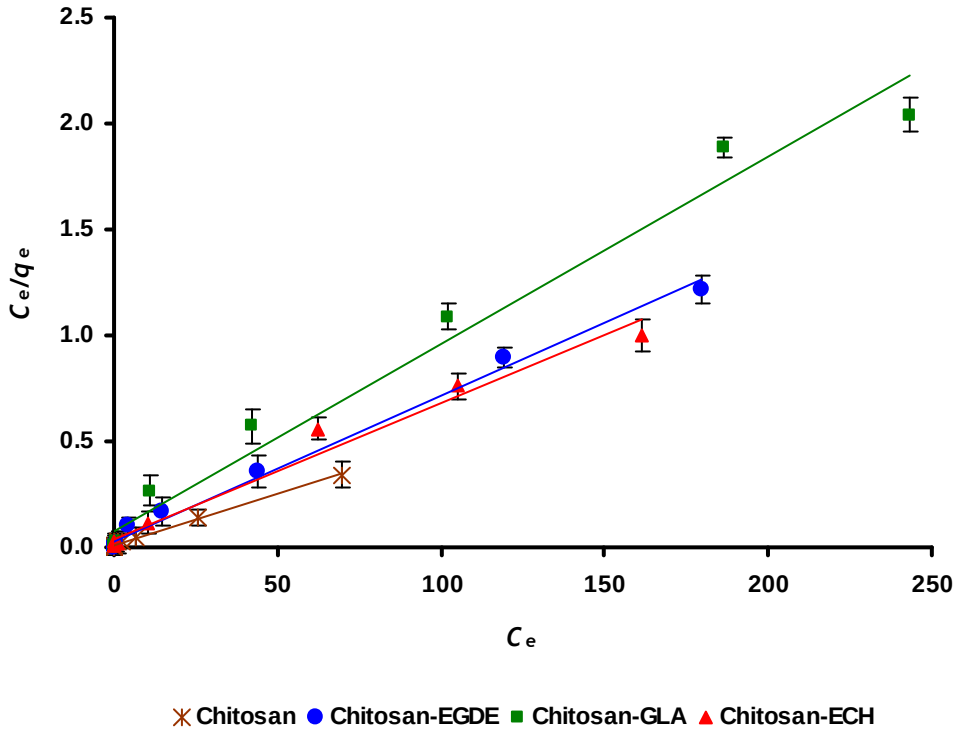


Figure 4.31 Langmuir sorption isotherms of Ag^+ by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

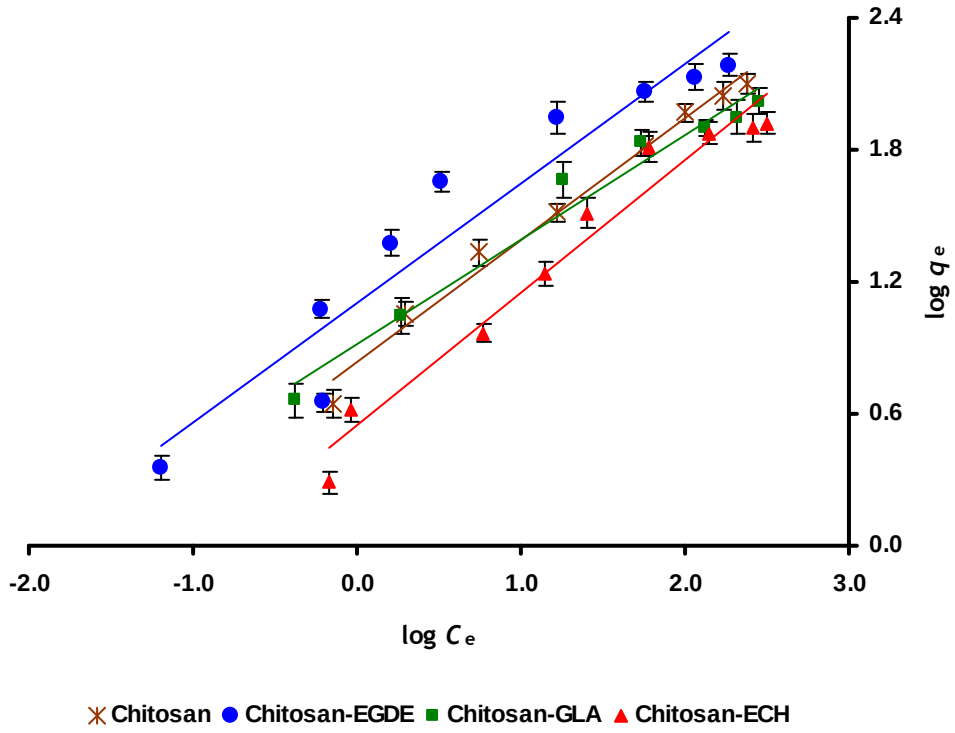


Figure 4.32 Freundlich sorption isotherms of Pb^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

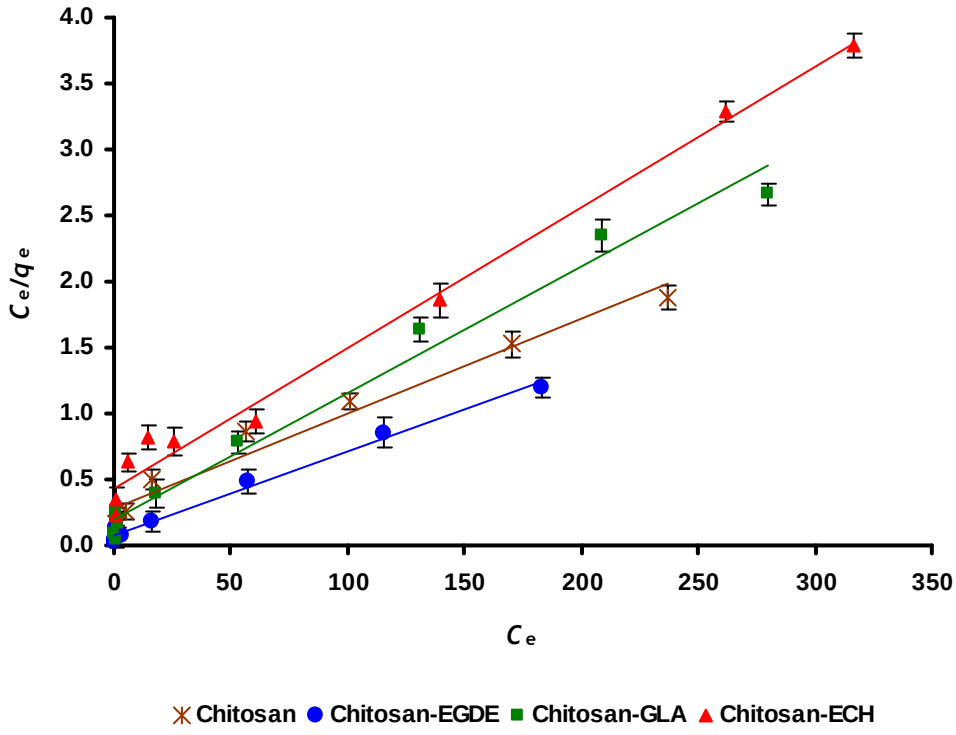


Figure 4.33 Langmuir sorption isotherms of Pb^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

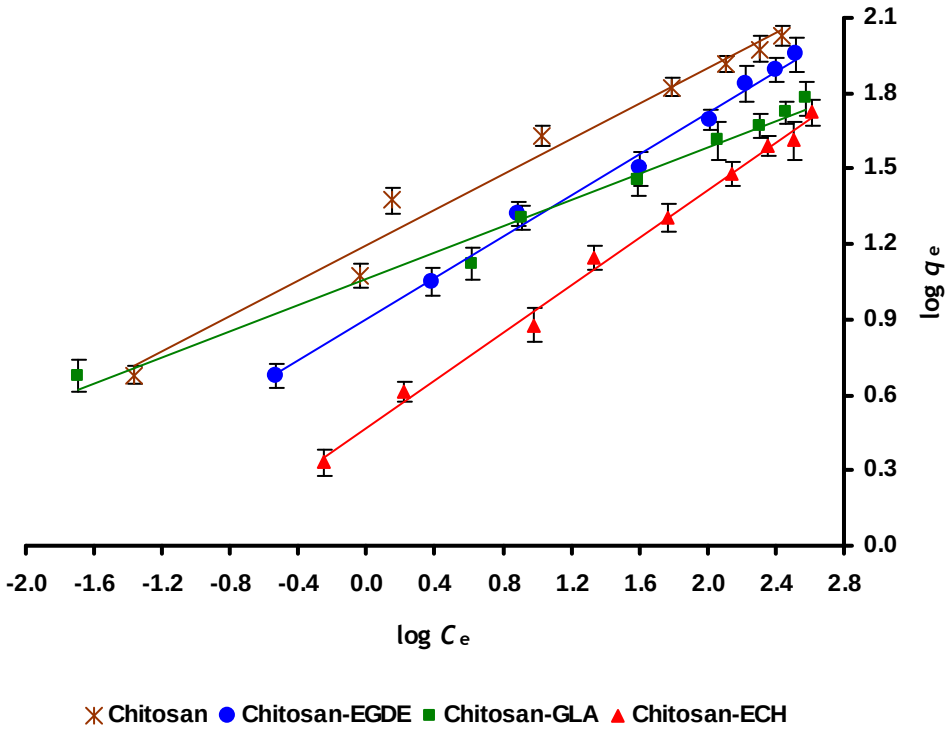


Figure 4.34 Freundlich sorption isotherms of Cu^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

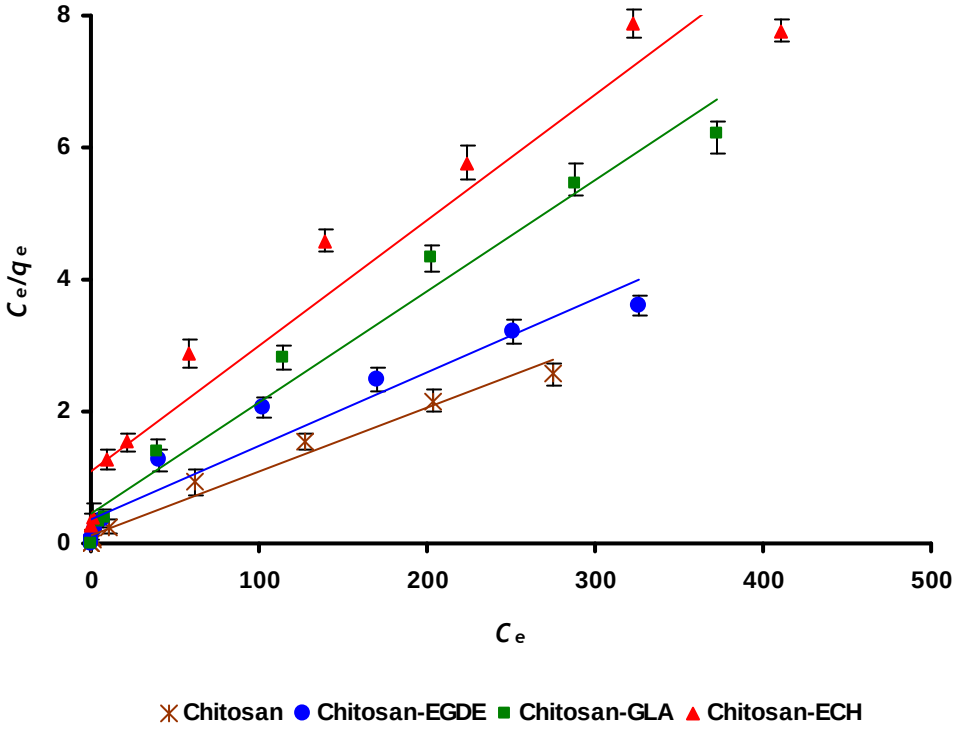


Figure 4.35 Langmuir sorption isotherms of Cu^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

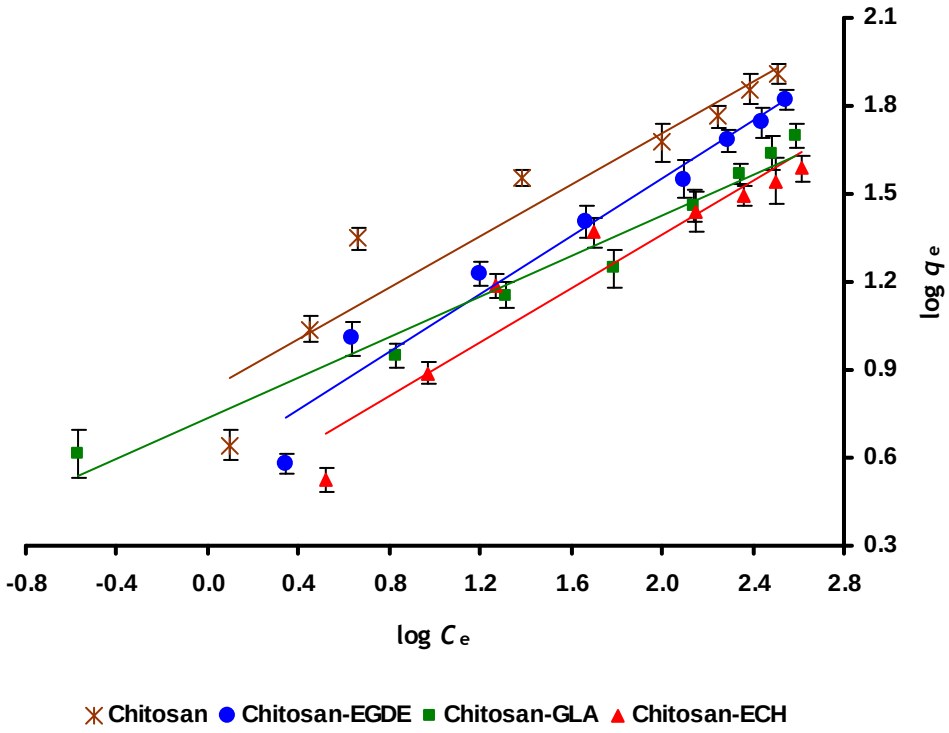


Figure 4.36 Freundlich sorption isotherms of Cd^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

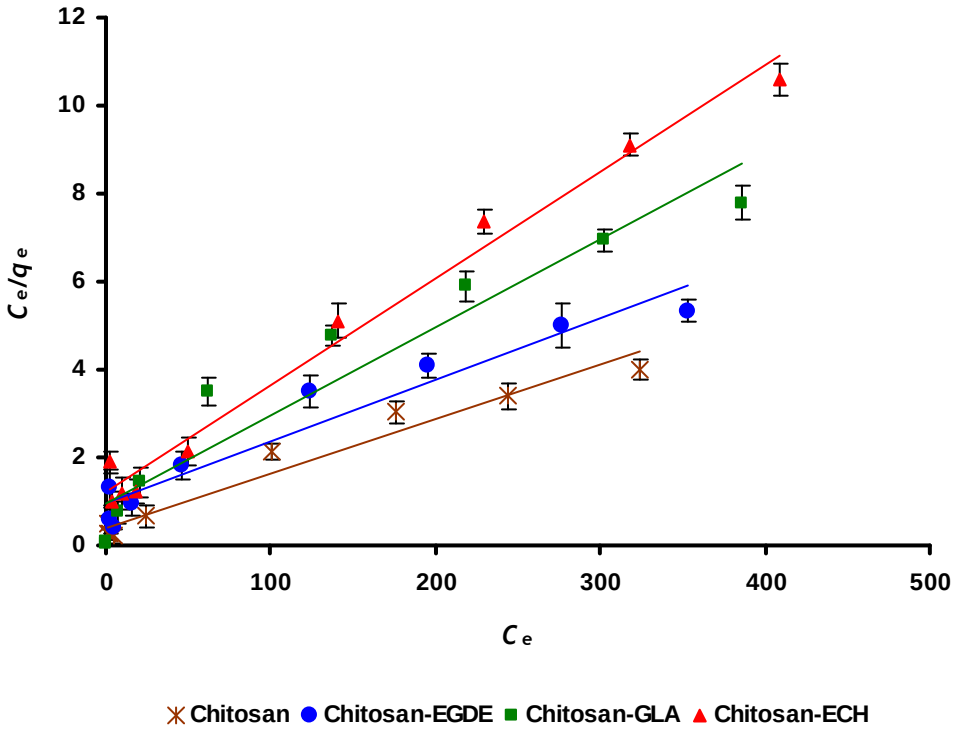


Figure 4.37 Langmuir sorption isotherms of Cd^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

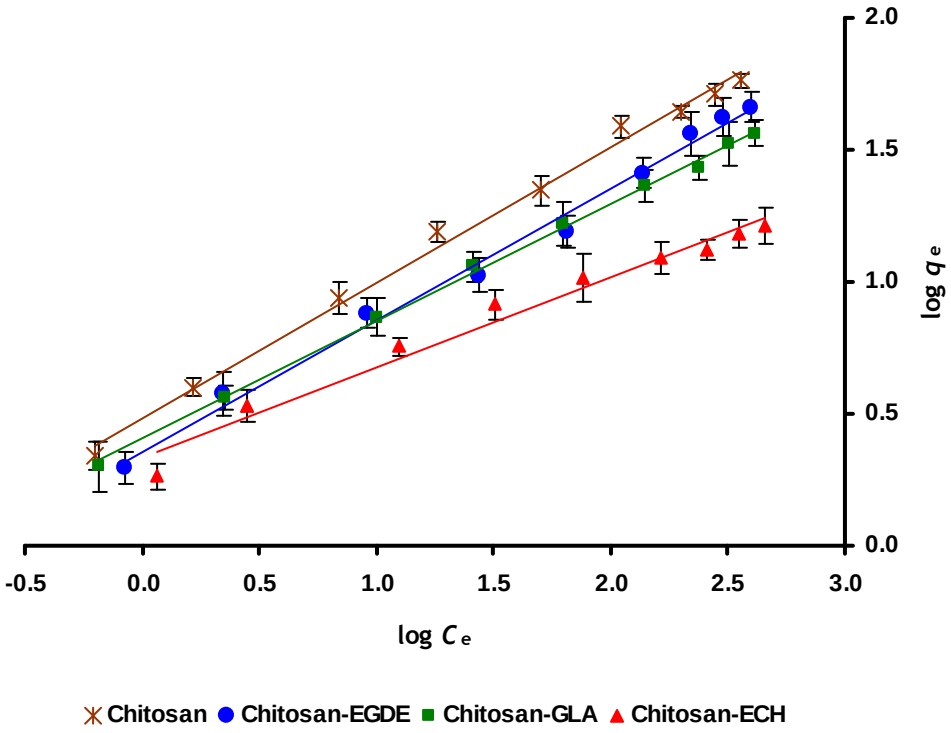


Figure 4.38 Freundlich sorption isotherms of Zn^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

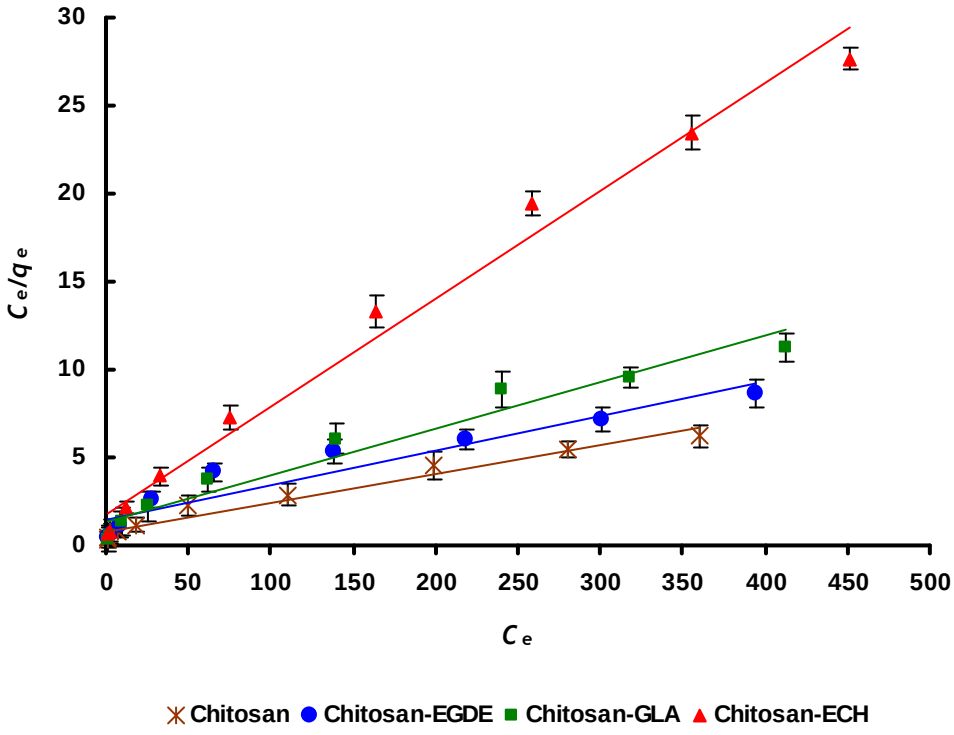


Figure 4.39 Langmuir sorption isotherms of Zn^{2+} by chitosan and cross-linked chitosans (Error bars are ± standard deviation of three replicates).

Table 4.4 Freundlich and Langmuir isotherm constants for metal ions binding to chitosan and cross-linked chitosans.

Metal ion	Sorbent	Freundlich			Langmuir		
		K_F (mg/g)	1/*n*	R^2	*Q* (mg/g)	*b* (L/mg)	R^2
Ag^+	Chitosan	53.9	0.3656	0.9789	208	0.5581	0.9945
	Chitosan-GLA	24.8	0.2773	0.9889	112	0.1147	0.9787
	Chitosan-ECH	41.8	0.2628	0.9851	154	0.1934	0.9780
	Chitosan-EGDE	29.5	0.3335	0.9746	147	0.2092	0.9933
Pb^{2+}	Chitosan	6.89	0.5525	0.9864	133	0.0333	0.9656
	Chitosan-GLA	10.9	0.4192	0.9659	104	0.0505	0.9816
	Chitosan-ECH	3.54	0.6010	0.9619	93.5	0.0249	0.9875
	Chitosan-EGDE	12.7	0.5452	0.9803	156	0.0849	0.9903
Cu^{2+}	Chitosan	15.6	0.3509	0.9774	103	0.0865	0.9800
	Chitosan-GLA	11.6	0.2597	0.9818	58.1	0.0483	0.9753
	Chitosan-ECH	2.95	0.4718	0.9950	52.6	0.0176	0.9911
	Chitosan-EGDE	7.95	0.4111	0.9942	89.3	0.0312	0.9788
Cd^{2+}	Chitosan	6.73	0.4400	0.9899	81.3	0.0297	0.9639
	Chitosan-GLA	5.42	0.3450	0.9673	50.3	0.0206	0.9824
	Chitosan-ECH	2.78	0.4600	0.9706	41.2	0.0200	0.9846
	Chitosan-EGDE	3.67	0.4916	0.9604	70.9	0.0147	0.9978
Zn^{2+}	Chitosan	3.07	0.5117	0.9948	61.4	0.0205	0.9657
	Chitosan-GLA	2.54	0.4463	0.9981	37.7	0.0199	0.9982
	Chitosan-ECH	2.17	0.3412	0.9751	16.4	0.0344	0.9856
	Chitosan-EGDE	2.27	0.4996	0.9921	51.0	0.0131	0.9895

From the Freundlich isotherm model viewpoint, favourable sorption should have a 1/*n* value between 0 and 1 (Freundlich, 1906; Febrianto *et al.*, 2009). As presented in Table 4.4, the 1/*n* values lie within the range indicating the metal ions are favourably sorbed by the chitosans. The 1/*n* value can be used to predict binding affinity of the sorbents toward metal ions, whereby a smaller value of 1/*n* implies stronger interaction between sorbent and metal ion (Freundlich, 1906; Febrianto *et al.*, 2009; Limousin *et al.*, 2007). From Table 4.4, the 1/*n* values for Ag^+ were found to be lower than for Pb^{2+} suggesting stronger binding interaction between Ag^+ and active sites of the chitosans. This was further evident by the greater values of *b* for Ag^+ obtained from the Langmuir model.

Meanwhile, the higher value of *b* (from the Langmuir model) for chitosan-EGDE (0.0849) than chitosan (0.0333) suggests that it has better affinity to sorb Pb^{2+} ions. This might be attributed to greater surface area, less crystalline structure and better surface morphology, which favour metal binding on chitosan-EGDE. The change in the crystallinity and surface properties of chitosan following cross-linking treatment has

been discussed in Sections 3.3.1, 3.3.3 and 3.3.4. Except for Pb^{2+} sorption by chitosan-EGDE, cross-linked chitosans had lower binding capacity than chitosan for the metal ions studied. A possible reason for this is that the cross-linking treatment restricts the diffusion of metal ion to the internal sites of the sorbents by establishing insoluble cross-linked networks. As a consequence, fewer metal ions are bound to cross-linked chitosans.

The maximum binding capacity (Q) values, estimated using the Langmuir isotherm model for various low-cost sorbents are listed in Table 4.5. It is observed that the Q values obtained from this research are comparable with other low-cost sorbents reported in the literature. In fact, chitosan and cross-linked chitosans have excellent binding capacities for Pb^{2+} and Cu^{2+} as compared to crab shells. For example, the maximum binding capacities of chitosan and chitosan-EGDE for Pb^{2+} are 6 to 7 times higher than crab shells. In addition, the uptake values of Cu^{2+} and Zn^{2+} by chitosan-GLA obtained from this work are comparable with brown alga-GLA, reported by Liu *et al*. (2009).

It was found that cross-linked chitosans had higher binding capacity for Ag^{+} and Pb^{2+} as compared to hydroxyl azacrown ether-grafted chitosan and chitosan/TiO_2 film, respectively. In the case of Cu^{2+}, chitosan-EGDE had slightly higher sorption capacity to that of chitosan coated alumina. The uptake capacity of unmodified chitosan for Cd^{2+} is 5 times higher than the maximum binding capacity estimated for chitin. It was also observed that chitosan had a higher binding affinity for Zn^{2+} than alginate/phosphorylated chitin.

The essential feature of the Langmuir isotherm can be expressed in terms of a dimensionless constant separation factor or equilibrium parameter, R_L, which was defined by Hall *et al*. (1966), and is given by Equation 4.13.

$$R_L = \frac{1}{1 + bC_o} \tag{4.13}$$

where, C_o is the initial concentration of metal ion. R_L values indicate the shape of the isotherm (Hall *et al*., 1966): (i) irreversible ($R_L = 0$), (ii) favourable ($0 < R_L < 1$), (iii) linear ($R_L = 1$), and (iv) unfavourable ($R_L > 1$). The calculated R_L values are presented in Table 4.6. It was found that the R_L values are in the range of $0 < R_L < 1$, which suggests that the sorption of metal ions on the chitosans is favourable.

Table 4.5 Binding capacities of various low-cost sorbents for metal ions.

Metal ion	Sorbent	*Q* (mg/g)	Reference
Ag^{+}	Clinoptilolites	31.44	Çoruh *et al*. (2010)
	Budding yeast	42.72	Chen and Wang (2008)
	Bread mold	48.75	Kogej and Pavko (2001)
	Hydroxyl azacrown ether-grafted chitosan	55.01	Yang and Yuan (2001)
	Distillery-derived biomass	58.7	Bustard and McHale (1998)
	Melamine-formaldehyde-thiourea chelating resin	60.05	Yirikoglu and Gülfen (2008)
Pb^{2+}	Almond shells	5.43	Bulut and Tez (2007)
	Crab shells	19.83	Dahiya *et al*. (2008)
	Chitosan/TiO_2 film	36.8	Tao *et al*. (2009)
	Tea leaves	65	Amarasinghe and Williams (2007)
	Budding yeast	85.57	Chen and Wang (2008)
	Furnace sludge	92.51	Naiya *et al*. (2009)
Cu^{2+}	Rubber leaves treated with NaOH	14.97	Wan Ngah and Hanafiah (2008)
	Brown alga treated with GLA	26.06	Liu *et al*. (2009)
	Crab shells	38.62	Dahiya *et al*. (2008)
	Tea leaves	48	Amarasinghe and Williams (2007)
	Chitosan coated alumina	86.2	Boddu *et al*. (2008)
	Eucalyptus black liquor lignin	137.03	Mohan *et al*. (2006)
Cd^{2+}	Almond shells	3.18	Bulut and Tez (2007)
	Bagasse fly ash	6.194	Srivastava *et al*. (2006)
	Chitin	14.71	Benguella and Benaissa (2002)
	Parthenium hysterophorous weed	27.00	Ajmal *et al*. (2006)
	Bread mold	34.02	Kogej and Pavko (2001)
	Eucalyptus black liquor lignin	87.06	Mohan *et al*. (2006)
Zn^{2+}	Alginate/phosphorylated chitin	2.85	Jayakumar *et al*. (2009)
	Bonemeal	8.4	Nwachukwu and Pulford (2008)
	Oil palm ash	10.66	Chu and Hashim (2002)
	Bread mold	15.37	Kogej and Pavko (2001)
	Tobacco dust	25.1	Qi and Aldrich (2008)
	Brown alga treated with GLA	40.54	Liu *et al*. (2009)

Table 4.6 R_L values for metal ions binding to chitosan and cross-linked chitosans at different initial metal concentration (C_o).

Metal ion	C_o (mg/L)	R_L values			
		Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Ag^+	5	0.2638	0.6355	0.5084	0.4888
	25	0.0669	0.2586	0.1714	0.1605
	50	0.0346	0.1485	0.0937	0.0873
	100	0.0176	0.0802	0.0492	0.0456
	300	0.0059	0.0282	0.0169	0.0157
	500	0.0036	0.0171	0.0102	0.0095
Pb^{2+}	5	0.8573	0.7984	0.8893	0.7020
	25	0.5457	0.4420	0.6163	0.3203
	50	0.3752	0.2837	0.4454	0.1907
	100	0.2309	0.1653	0.2865	0.1054
	300	0.0910	0.0619	0.1181	0.0378
	500	0.0567	0.0381	0.0743	0.0230
Cu^{2+}	5	0.6981	0.8055	0.9191	0.8651
	25	0.3162	0.4530	0.6944	0.5618
	50	0.1878	0.2928	0.5319	0.3906
	100	0.1036	0.1715	0.3623	0.2427
	300	0.0371	0.0646	0.1592	0.0965
	500	0.0226	0.0398	0.1020	0.0602
Cd^{2+}	5	0.8707	0.9066	0.9091	0.9315
	25	0.5739	0.6601	0.6667	0.7313
	50	0.4024	0.4926	0.5000	0.5764
	100	0.2519	0.3268	0.3333	0.4049
	300	0.1009	0.1393	0.1429	0.1848
	500	0.0631	0.0885	0.0909	0.1198
Zn^{2+}	5	0.9070	0.9095	0.8532	0.9385
	25	0.6612	0.6678	0.5376	0.7533
	50	0.4938	0.5013	0.3676	0.6042
	100	0.3279	0.3344	0.2252	0.4329
	300	0.1399	0.1435	0.0883	0.2028
	500	0.0889	0.0913	0.0549	0.1325

4.7 Sorption-desorption study

Sorption experiments have shown that chitosan and cross-linked chitosans are capable of binding Ag^{+}, Cd^{2+}, Cu^{2+}, Pb^{2+} and Zn^{2+} in KCl and KNO_3 background electrolyte solutions. However, such information alone is not sufficient to elucidate the feasibility of the chitosans to immobilise metal ions in real contaminated soil. Therefore, it is necessary to study the reversibility of the process. This was undertaken by subjecting the metal-saturated chitosans to desorption using 0.1 mol/L KCl and KNO_3 as eluents at different dilution factors. In order to understand the nature of bonding between sorbents and sorbates, desorption studies were carried out using low and high initial metal concentrations (100 and 500 mg/L).

Table 4.7 presents the percentage of metal retained on the surface of the sorbents following desorption (see Appendix A, Tables A1 and A2 for full data set). Overall, Ag^{+} ions were most effectively retained on the chitosans, and Zn^{2+} the least retained. In all cases, more than 90% of the Ag^{+} ions were held following dilution, implying a strong bond between the Ag^{+} and the functional groups on the chitosans. For Zn^{2+}, shaking for an additional 24 hours with no dilution resulted in loss of between 20-35% of the sorbed Zn^{2+}, while an 11-fold dilution caused a loss of between 70-97% at the lower concentration, and about 60-70% at the higher.

The behaviour shown by Pb^{2+}, Cu^{2+} and Cd^{2+} on all of the chitosans lay between these two extremes, with the exception of that on chitosan-ECH at an initial concentration of 100 mg/L. For Pb^{2+} and Cu^{2+} with all samples, and for Cd^{2+} at the lower dilutions, there was an apparent enhancement of sorption above that expected due to experimental error, and despite apparent equilibrium having been obtained during the sorption phase. For Pb^{2+}, there was an apparent increase in the amount sorbed by a factor of 2.5. A possible reason for this effect, which was reproducible, might be due to a change in the morphology on prolonged shaking. Chitosan-ECH has a rigid structure, and it may be that the additional shaking results in physical breakdown, or even chemical modification, allowing access to more binding sites. The ionic size and strength of binding may also be important. Based on the values of effective radius for hydrated ions as discussed by Kielland (1937), Ag^{+} (470 pm) is the smallest of the ions tested, and may be able to penetrate the structure, so no additional sorption occurs during the desorption phase. Zn^{2+} (680 pm) however is roughly the same size as Pb^{2+} (590 pm), Cu^{2+} (680 pm), and Cd^{2+} (640 pm), but is the most weakly sorbed ion. In this case desorption may be a preferable process to further sorption.

Table 4.7 Percentage of metal ions retained on the surface of sorbents (C_o = 100 and 500 mg/L).

Metal ion	Dilution factor	Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Ag^{+}	0	99 (102)	99 (106)	100 (103)	99 (102)
	1.5	99 (101)	99 (99)	99 (101)	99 (99)
	2.0	98 (100)	99 (97)	99 (99)	99 (99)
	3.5	98 (97)	98 (98)	99 (97)	98 (97)
	6.0	98 (97)	97 (96)	97 (97)	97 (95)
	8.5	97 (96)	95 (96)	96 (97)	96 (95)
	11.0	97 (95)	95 (94)	94 (97)	96 (95)
Pb^{2+}	0	98 (116)	97 (106)	249 (98)	98 (112)
	1.5	94 (116)	97 (104)	216 (86)	97 (111)
	2.0	93 (114)	95 (102)	210 (85)	97 (107)
	3.5	88 (111)	95 (102)	200 (81)	97 (99)
	6.0	79 (107)	94 (91)	162 (63)	96 (93)
	8.5	73 (102)	94 (85)	155 (50)	96 (85)
	11.0	65 (100)	94 (78)	119 (46)	95 (80)
Cu^{2+}	0	117 (103)	104 (125)	158 (106)	104 (105)
	1.5	114 (98)	100 (119)	146 (85)	102 (104)
	2.0	111 (98)	99 (118)	131 (69)	101 (102)
	3.5	108 (96)	97 (114)	130 (63)	99 (99)
	6.0	101 (94)	92 (111)	129 (60)	97 (98)
	8.5	99 (92)	91 (104)	125 (43)	93 (94)
	11.0	99 (90)	87 (103)	115 (43)	92 (88)
Cd^{2+}	0	90 (92)	87 (85)	163 (95)	92 (92)
	1.5	86 (95)	77 (97)	150 (96)	86 (100)
	2.0	83 (95)	75 (94)	119 (93)	83 (94)
	3.5	77 (93)	68 (89)	114 (87)	69 (92)
	6.0	72 (91)	60 (79)	85 (80)	58 (86)
	8.5	66 (77)	54 (76)	81 (72)	56 (82)
	11.0	60 (74)	51 (70)	60 (68)	47 (72)
Zn^{2+}	0	72 (79)	65 (81)	70 (70)	65 (80)
	1.5	70 (76)	63 (74)	69 (66)	60 (73)
	2.0	61 (65)	58 (69)	63 (64)	52 (68)
	3.5	51 (62)	52 (64)	54 (52)	47 (64)
	6.0	42 (56)	45 (57)	25 (46)	34 (43)
	8.5	36 (53)	50 (50)	17 (38)	31 (41)
	11.0	20 (43)	31 (40)	3 (33)	27 (35)

Values represent mean of three replicates. Values within parentheses are for C_o = 500 mg/L.

The reversibility of the process is not only dependent upon the properties of the metal ions and the background solutions, but also physical characteristics of the sorbents (Ip *et al.*, 2009). From sorption studies (Tables 4.3 and 4.4), it was found that chitosan has greater affinity to bind metal ions than cross-linked chitosans. However, results from the desorption study suggest that in some cases cross-linked chitosans have better ability to retain metal ions than chitosan. This observation may relate to the dissolution of chitosan. Cross-linked chitosans are rigid and chemically stable as compared to chitosan (Section 3.2). Therefore, they are able to withstand such changes in the sorption system.

During desorption, metal ions can be replaced by H^+ or K^+ ions from the eluents, which becomes significant if the interaction between the metal ions and the functional groups of the chitosans involves weak binding forces. With the exception of Zn^{2+}, addition of eluents yielded low desorption percentage. This implies that binding of metal ions onto chitosans involves chemical binding, as opposed to ion exchange. From sorption-desorption studies, it is apparent that chitosan and cross-linked chitosans are able to retain metal ions on their surfaces. This highlights their potential use as immobilising agents for heavy metals in contaminated soil.

4.8 Factors affecting metal binding capacity

Based on the equilibrium sorption capacity (q_e) (Table 4.3) and Langmuir sorption capacity (Q) (Table 4.4), metal ion affinity was in the order of $Ag^+ > Pb^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$. This sequence was in accordance with the findings of Kogej and Pavko (2001) for metal ions binding to bread mold. However, Argun *et al*. (2009) noted that the amount of metal ions sorbed onto Fenton modified *Pinus nigra* (pine) bark at equilibrium (mg/g) decreased in the order of Cu^{2+} (55.3) > Cd^{2+} (35.3) > Pb^{2+} (30.9), which was different to the results of many studies. These effects can be explained by the fact that sorbents have different abilities to bind heavy metals, resulting in different orders of maximum sorption capacities. Metal binding capacity is mainly affected by two important factors, namely the properties of the metal ions and the characteristics of the sorbents (Chen and Wang, 2008; Hawari and Mulligan, 2007).

4.8.1 Properties of metal ions

Metal ion properties such as electronegativity, ionic radius and hydration control the binding of a metal ion to a sorbent (Chen and Wang, 2008; Hawari and Mulligan, 2007; Amarasinghe and Williams, 2007; Hanzlík *et al.*, 2004).

Chen and Wang (2008) studied the potential of budding yeast to bind metal ions from aqueous solution. They reported that the uptake values were in the order Pb^{2+} > Ag^{+} > Sr^{2+} > Cs^{+}, which correlated with their electronegativity (Pauling) values (2.33 for Pb^{2+}, 1.93 for Ag^{+}, 0.95 for Sr^{2+} and 0.79 for Cs^{+}). Tarley and Arruda (2004) observed a similar trend in metal binding capacities on rice husks, of which Pb^{2+} was preferentially bound with respect to Cd^{2+}. The suggested effect was stronger attraction due to the higher electronegativity of Pb^{2+} (2.33) compared to Cd^{2+} (1.69). The same suggestion was made by Chen *et al*. (2005), who noted Pb^{2+} was sorbed to a greater extent than Cd^{2+} onto spent mushroom compost.

Srivastava *et al*. (2009) investigated the binding behaviour of Cd^{2+} and Ni^{2+} onto rice husk ash, and found that the binding capacity of Ni^{2+} was greater than Cd^{2+}. They proposed that Ni^{2+} with a smaller ionic radius of 1.62 Å than the 1.71 Å for Cd^{2+} had more accessibility to the surface and pores of the sorbent. Hawari and Mulligan (2007) reported the affinity order of anaerobic sludge as Pb^{2+} > Cu^{2+} > Cd^{2+} and mentioned that metal ions of low hydrated radius are preferentially bound to sorbents. The hydrated ionic radii values of Pb^{2+}, Cu^{2+} and Cd^{2+} were reported as 4.01 Å, 4.19 Å and 4.26 Å, respectively. They reported that Pb^{2+} which has the lowest hydrated radius had the highest sorption affinity by the anaerobic sludge. Meanwhile, Mohan *et al*. (2006) proposed that due to the larger hydrated radius of Cd^{2+} than Cu^{2+}, Cd^{2+} induces a quick saturation of binding sites or known as the steric crowding effect. This effect was suggested to cause a physical blockage for further Cd^{2+} attachment to the binding sites, resulting in lower amount of Cd^{2+} sorbed onto lignin.

Amarasinghe and Williams (2007) attributed the higher binding capacity and affinity of Pb^{2+} as compared to Cu^{2+} to hydration enthalpy effect, which was defined as the energy that permits the detachment of H_2O molecules from cations. They argued that the more a cation is hydrated the greater its hydration enthalpy and then less it can interact with the sorbent. The hydration enthalpies of Pb^{2+} and Cu^{2+} were reported as -1481 and -2100 kJ/kg, respectively. Pb^{2+} was explained to be less hydrated than Cu^{2+}, leading to a higher affinity for tea leaves.

The selectivity of activated carbon-zeolite composite prepared from coal fly ash was established as Pb^{2+} > Cd^{2+} > Ni^{2+} by Jha *et al*. (2008). The higher binding capacity of Pb^{2+} and Cd^{2+} as compared to Ni^{2+} was proposed to be due to the effect of zero-ligand-field stabilisation in octahedral aqua complexes. Pb^{2+} and Cd^{2+} have a d^{10} outer electronic configuration. Therefore, it was thought that both metal ions interact strongly with the binding sites of the sorbent by comparison with Ni^{2+}, which has a d^{8} outer electronic configuration.

As several factors or parameters may contribute to metal ion binding, Nieboer and McBryde (1973) proposed the concept of the covalent index for metal ions, which is a complex parameter expressed as $X_m^2 r$, where X_m is electronegativity and r is ionic radius. This has been used to describe metal uptake capacity of some sorbents. For example, Brady and Tobin (1995) studied the binding of metal ions to *Rhizopus arrhizus* biomass and reported that the equilibrium metal uptake decreased in the order Pb^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}, consistent with the covalent index values. From the sorption studies (Tables 4.3 and 4.4), with the exception of chitosan-EGDE, the bonding affinity of metal ions was in the order Ag^{+} > Pb^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}. This is in agreement with the covalent index values of 4.25 (Ag^{+}), 3.29 (Pb^{2+}), 2.98 (Cu^{2+}), 2.71 (Cd^{2+}) and 2.13 (Zn^{2+}), as estimated by Nieboer and Richardson (1980), Brady and Tobin (1995) and Chen and Wang (2008).

Desorption studies revealed that Ag^{+} formed the most stable complex and strong bonding with the chitosans, while Zn^{2+} had the least potential to be retained on the sorbent surfaces. According to the hard and soft metal ion principle (Nieboer and Richardson, 1980), metal ions can be classified into three groups (Class A, Class B and borderline) based on the magnitude of the complex formation constants. Class A ions have a donor atom preference sequence for metal-binding ligands of O > N > S, while Class B have the opposite preference. Ag^{+} is a Class B metal ion, while Pb^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} are borderline. Since the chitosans contain nitrogen atoms, Ag^{+} preferentially binds to form a stable complex, while the uptake and stability of complexes of Pb^{2+}, Cu^{2+}, Cd^{2+} and Zn^{2+} are expected to be lower. As discussed in Chapter 3 (Characterisation Study), the FTIR spectra of the chitosans showed a significant change in the absorption intensity of the N-H bending and the C-N stretching vibration bands after Ag^{+} uptake suggesting a great extent of complexation (Figures 3.18 to 3.25, Section 3.4.4).

4.8.2 Properties of sorbents

The physical and chemical properties of the sorbent such as surface area, pore size, surface morphology, crystallinity and surface functional groups determine the binding characteristics of a sorbent (Amarasinghe and Williams, 2007; Crini and Badot, 2008; Kurniawan *et al.*, 2006; Demirbas, 2008).

Amarasinghe and Williams (2007) studied the sorption capacity of tea leaves and coconut shells based granulated activated carbon (GAC) for Pb^{2+} and Cu^{2+} ions. They reported that the BET surface area and pore size of GAC (683.1 m^2/g and 2.3 nm) were

higher than those measured for tea leaves (0.8 m^2/g and 1.9 nm, respectively). However, the percentage removal for tea leaves was found to be higher than GAC for most of the experiments performed under the same conditions. A reason suggested for the lower binding capacity of GAC was a hindrance effect due to the sphericity of GAC, of which impeded the diffusion of metal ions to the interior of the GAC.

Bulut and Tez (2007) observed no significant difference in the binding capacities of hazelnut shells and almond shells for Pb^{2+} and Cd^{2+}, and this was explained as being due to the similarity in chemical and physical properties of the sorbents. For example, the BET surface area for the hazelnut shells was reported as 4.3 m^2/g, while 4.1 m^2/g was measured for the almond shells.

Romera *et al*. (2007) assessed the metal binding capacity of different types of algae, namely *Codium vermilara* (green alga), *Chondrus crispus* (red alga) and *Fucus spiralis* (brown alga), and the order of binding capacity was reported as *F. spiralis* > *C. crispus* > *C. vermilara*. The high binding capacity of *F. spiralis* was explained as being due to the presence of alginates in its cell wall that served as binding sites for metal ions. They noted that the presence of carragenates, which behaved similarly to the alginates in *F. spiralis*, favoured metal uptake by *C. crispus*. Meanwhile, the low sorptive capacity of *C. vermilara* was ascribed as being due to low content of carboxylic groups in its composition.

Studies have shown that chemical modifications can enhance the binding capacity of sorbents. For example, Argun *et al*. (2009) modified pine bark using several reagents such as HCl, NaOH, Fenton's reagent, poly-acrylamide and ethanol. Chemical modifications were reported to improve the porosity and surface morphology of the bark, and increased the binding capacity for Cd^{2+}, Cu^{2+}, Ni^{2+} and Pb^{2+}. Liu *et al*. (2009) modified brown alga with epichlorohydrin (ECH) and glutaraldehyde (GLA) as cross-linking reagents, and potassium permanganate (PP) as oxidising reagent. The binding capacity of modified algae for Cd^{2+}, Cu^{2+} and Zn^{2+} was compared with alga washed with deionised water (DW) only. The order of binding efficiency was reported as ECH > PP > DW > GLA. The higher uptake capacity of modified algae was discussed due to their greater surface properties as compared to unmodified alga.

In the case of chitosan, Vieira and Beppu (2005) noted that cross-linking using GLA increased Hg(II) uptake by 198%, as compared to unmodified chitosan. The higher capacity of a chitosan-GLA membrane was proposed to be due to its less crystalline structure which facilitated the accessibility of Hg(II) to the internal sites. A different observation was obtained from this study. As shown in Tables 4.3 and 4.4, chitosan-GLA

showed a lower sorption capacity for heavy metals than unmodified chitosan and as discussed earlier, the rigid structure of chitosan-GLA, may have made some binding sites less accessible. An earlier study by Chiou and Li (2003) found that chitosan-ECH had better binding capacity for Reactive Red 189 dye than unmodified chitosan, and this was explained to be due to its rigid structure that favoured dye uptake at low pH.

4.9 Summary

With an initial metal concentration of 500 mg/L, 0.1 g of sorbent and at room temperature, the binding of metal ions onto chitosan and cross-linked chitosans attained equilibrium at 90 and 180 minutes, respectively (Figures 4.1 to 4.4). The chitosans were capable of binding metal ions over a wide range of initial metal concentration (Table 4.1). Zn^{2+} binding by the chitosans was not affected by the presence of Cl^- and NO_3^- in the model soil solution studied (Table 4.2). The sorption kinetics was best described by the pseudo-second-order kinetic model (Figures 4.17 to 4.20, Table 4.3). The Langmuir isotherm model fitted the equilibrium data more satisfactorily than the Freundlich model (Tables 4.4 and 4.3). Based on the equilibrium parameter (R_L) values, the binding of metal ions by the chitosans was favourable (Table 4.6).

The formation of insoluble cross-linked networks following the cross-linking treatments restricts the diffusion of metal ion to the internal sites of the sorbents and therefore reducing the binding capacity of cross-linked chitosans. However, results obtained from the desorption study suggest that in some cases cross-linked chitosans retain metal ions better than unmodified chitosan (Table 4.7) and this can be attributed to their rigid and chemically stable properties (Section 3.2). Based on the equilibrium sorption capacity ($q_{e\ theoretical}$ and $q_{e\ experimental}$) estimated from the kinetics study (Table 4.3) and Langmuir sorption capacity (Q) (Table 4.4), with the exception of chitosan-EGDE, the bonding affinity of metal ions to the chitosans was in the order $Ag^+ > Pb^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+}$. Overall, results from sorption study highlight the potential of chitosan and cross-linked chitosans for immobilising metal in contaminated soil.

5 POT EXPERIMENTS

Following on the results obtained from the sorption study (Chapter 4), which confirmed the applicability of chitosan and cross-linked chitosans for metal binding, pot experiments were conducted to evaluate the ability of the chitosans to immobilise heavy metals in a contaminated soil.

As discussed by van Herwijnen *et al*. (2007b), Nwachukwu and Pulford (2009) and Lee *et al*. (2009), it is necessary to assess the potential of amendments for soil remediation in pot experiments, prior to full scale field studies. They further discussed that pot experiments allow a realistic evaluation of the ability of amendments to immobilise metal and reduce the risk of metal toxicity to plants. Such information should be considered when applying amendments on a real contaminated site, and therefore, costly future mistakes can be avoided (Song *et al*., 2004; van Herwijnen *et al*., 2007b).

There are two main sections in this chapter. The first section reports the results obtained from the first pot trial, which was carried out in 2009. The results obtained from the second pot trial, performed in 2010, are discussed in the second section. In each section, the results are separated into three topics, namely plant growth, metal uptake by plants and metal concentration in soil.

5.1 Objectives of the study

1. To determine the effects of amendments on plant growth and metal concentration in plant tissue.
2. To evaluate the ability of amendments to immobilise heavy metals in a contaminated soil.

5.2 Pot experiment 2009

Prior to undertaking the pot experiments, the feasibility of chitosan and cross-linked chitosans to support plant growth was assessed in a plant growth screening test. For this purpose, perennial ryegrass seeds were sown on compost treated with chitosans at different weight ratios, namely 0%, 0.5%, 1.0% and 5.0% (w/w), and plant growth was monitored for 60 days. Plants were observed to grow well on chitosan and chitosan-GLA treated compost, at all weight ratios tested, whereas plants grown on compost treated with chitosan-ECH and chitosan-EGDE at application rate of 5.0% (w/w) died after 2

weeks of germination. In the case of 0.5% and 1.0% (w/w) of chitosan-ECH and chitosan-EGDE treatments, plant growth was stunted after four weeks of the trial. These results indicate that chitosan-ECH and chitosan-EGDE are not efficient in promoting plant growth. Therefore, the first pot experiment was carried out using chitosan and chitosan-GLA.

The first pot experiment was carried out from March 2009 to October 2009, for a total of 24 weeks. Chitosan and chitosan-GLA were applied at three rates of application, namely 0%, 0.5% and 1.0% (w/w). The influence of amendments on the growth and metal accumulation in shoot and root tissue of perennial ryegrass was studied. The metal concentration in soil after the pot experiment was determined. The procedure is fully discussed in Section 2.10.

5.2.1 Plant growth

The perennial ryegrass seeds germinated five days after sowing and no obvious difference in plant growth was observed up to five weeks of the pot experiment. Figures 5.1 to 5.3 show the plant growth observed after 3, 9 and 20 weeks of the pot experiment. In this preliminary study, ryegrass seeds were also planted on compost (John Innes No. 2 Compost, Homebase Ltd., UK) and received no amendment, in six replicates. This enabled a direct comparison in terms of growth performance of perennial ryegrass grown on untreated contaminated soil (zero treatment) and uncontaminated soil (compost). From these figures, it can be seen that plants grown on compost are healthier than plants cultivated on untreated contaminated soil. This difference was observed as early as at the third week of the pot experiment (Figure 5.1).

Chitosan and chitosan-GLA treatments gave rise to a healthy appearance on the ryegrass leaves, whereby the leaves were both longer and greener as compared to plants grown on zero treatment soil. Although the growth progress of plants grown on untreated soil was found to be slower than plants grown on treated soil, no toxicity symptom such as a reddish or burnt appearance was apparent on leaves of untreated perennial ryegrass. The bulk soil used in the first pot experiment contained 3923 mg/kg Pb, 1686 mg/kg Zn and 172 mg/kg Cu.

A similar observation was reported by Hooda and Alloway (1994), who studied the effects of sewage sludge amendment on growth and metal uptake by perennial ryegrass, in a one year pot experiment. Renoux *et al*. (2007) exposed perennial ryegrass to a soil-

sludge mixture containing 5000 mg/kg Zn, 3000 mg/kg Cu and 100 mg/kg Cd, and they observed no toxicity symptoms on the plant leaves. They also noted that plants survived with a low percentage of growth inhibition. This suggests that perennial ryegrass is a robust plant species and has great tolerance to high metal concentrations.

At week 9 (day 67) of the pot experiment, some leaves were observed to have a curled and dried appearance. This was also observed on plants grown on compost (Figure 5.2). In the case of perennial ryegrass, such observations may be due to stress symptoms related to high temperature, nutrient deficiency and metal toxicity (University of Rhode Island GreenShare Factsheets, website; Ren *et al.*, 2006). The Oakton digital thermometer recorded two rare events of high temperature, 45 ºC on 3 June 2009 and 40 ºC on 4 June 2009 (days 65 and 66). These observations were no longer apparent the following week. Therefore, it can be speculated that such curled and dried appearance were due to the high temperatures experienced on these dates.

The first harvest was performed after 12 weeks of the pot experiment and only shoots were sampled. The re-growth of the plants was quite rapid, new shoots with a length of 1.0 cm were developed within two days. However, slower re-growth was observed for the zero treatment plants, for which a 1.0 cm length for the new shoots was only obtained after three days. No obvious difference in plant growth was observed up to four weeks after the harvest. The second harvest was carried out after 24 weeks of the pot experiment. Both shoots and roots were harvested, and soils were collected for analysis.

Table 5.1 presents the dry biomass yield of perennial ryegrass after the total of 24 weeks of growth. As expected, addition of chitosan and chitosan-GLA to contaminated soil increased biomass yield. Shoot dry matter yield was pronounced for this effect. The highest percentage of increment in shoot yield was achieved with chitosan-GLA 1.0% (w/w) treatment, followed by application of chitosan at 1.0% (w/w). Chitosan-GLA 1.0% (w/w) treatment gave a 57% increase; meanwhile a 46% increase was obtained for chitosan 1.0% (w/w). Although there was an increase in the root yield following application of the chitosans, statistical analysis revealed no significant difference was obtained between the treatments. From Table 5.1, the total shoot yield obtained for plants grown on compost is found to be higher than plants cultivated on untreated contaminated soil by a factor of 2.7. Meanwhile, the root yield for perennial ryegrass grown on compost was greater than plants grown on untreated contaminated soil by a factor of 2.5. However, when adding chitosan-GLA at the rate of 1.0% (w/w) the increase was only a factor of 1.8. Lower yields of shoot and root obtained for zero treatment plants can be attributed to metal toxicity.

Elemental analysis was performed to investigate the effect of chitosan and chitosan-GLA amendment on composition of perennial ryegrass shoots. From Table 5.2, there was no clear effect in the weight percentage of carbon following application of both amendments. A clear trend was obtained for the weight percentage of nitrogen, whereby adding both amendments to contaminated soil increased the nitrogen content measured in plant shoots. Perennial ryegrass grown on untreated contaminated soil had the lowest nitrogen content (2.1%), whereas the highest was achieved following chitosan-GLA 1.0% (w/w) treatment (3.0%). This coincides with the results obtained for biomass production (Table 5.1), whereby chitosan-GLA 1.0% (w/w) gave the highest shoot yield of perennial ryegrass. It is observed that the biomass production of perennial ryegrass increased with the rates of chitosans application. Increasing the rates of application also caused an increase in nitrogen content of plant shoots (Table 5.2). This can be related to nitrogen content in chitosan (8.4%) and chitosan-GLA (7.4%) (Section 3.3.2), which provides plant nitrogen nutrition for ryegrass growth.

A significant linear response for dry matter production of perennial ryegrass to incorporation rates of spent mushroom compost, added to Pb/Zn mine tailings was obtained by Jordan *et al*. (2009). Nwachukwu and Pulford (2009) reported a greater biomass yield of perennial ryegrass when application rate of compost, coir and peat was increased from 1% to 20% (w/w). Amending a metal contaminated paddy soil with a higher application rate of pig manure and peat resulted in a greater yield of rice grain (Li *et al*., 2008).

A different observation was reported by Chiu *et al*. (2006), who studied the effect of pig manure compost and sewage sludge amendment on the growth of common reed cultivated on Pb/Zn mine tailings. They noted that application of manure compost at 5% and 10% (w/w) did not significantly affect the shoot yield of common reed, whereby the dry matter production was found to be rather similar to that of plants grown on untreated mine tailings. Although sewage sludge amendment at 5% and 10% (w/w) was found to increase shoot production significantly as compared to untreated mine tailings, no significant difference in shoot yield was reported for the two treatments. Lee *et al*. (2009) obtained an 80% increase in the shoot yield of lettuce when grown on a contaminated agricultural soil amended with red mud at incorporation rate of 2% (w/w), as compared to lettuce planted on unamended contaminated soil. However, an increase in application rate from 2% to 5% (w/w) was reported to reduce shoot production by 15%.

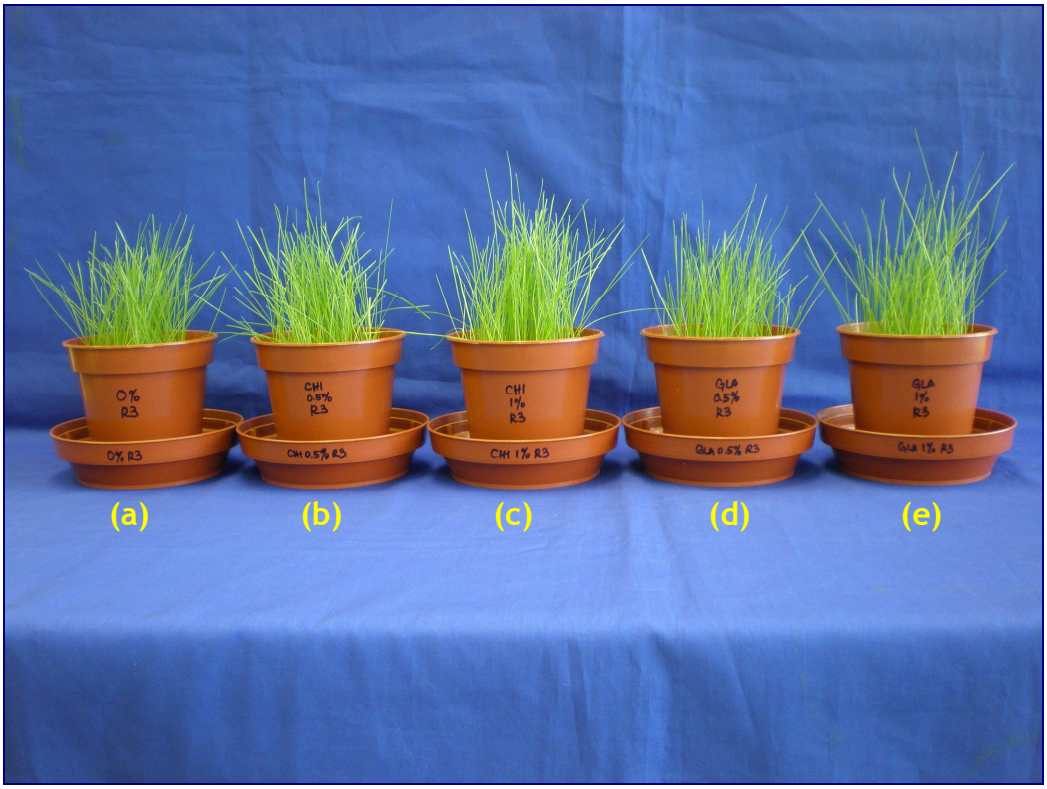


Figure 5.1 Perennial ryegrass after 3 weeks of growth. (a) Zero treatment, (b) Chitosan 0.5% (w/w), (c) Chitosan 1.0% (w/w), (d) Chitosan-GLA 0.5% (w/w), (e) Chitosan-GLA 1.0% (w/w) and (f) Compost.

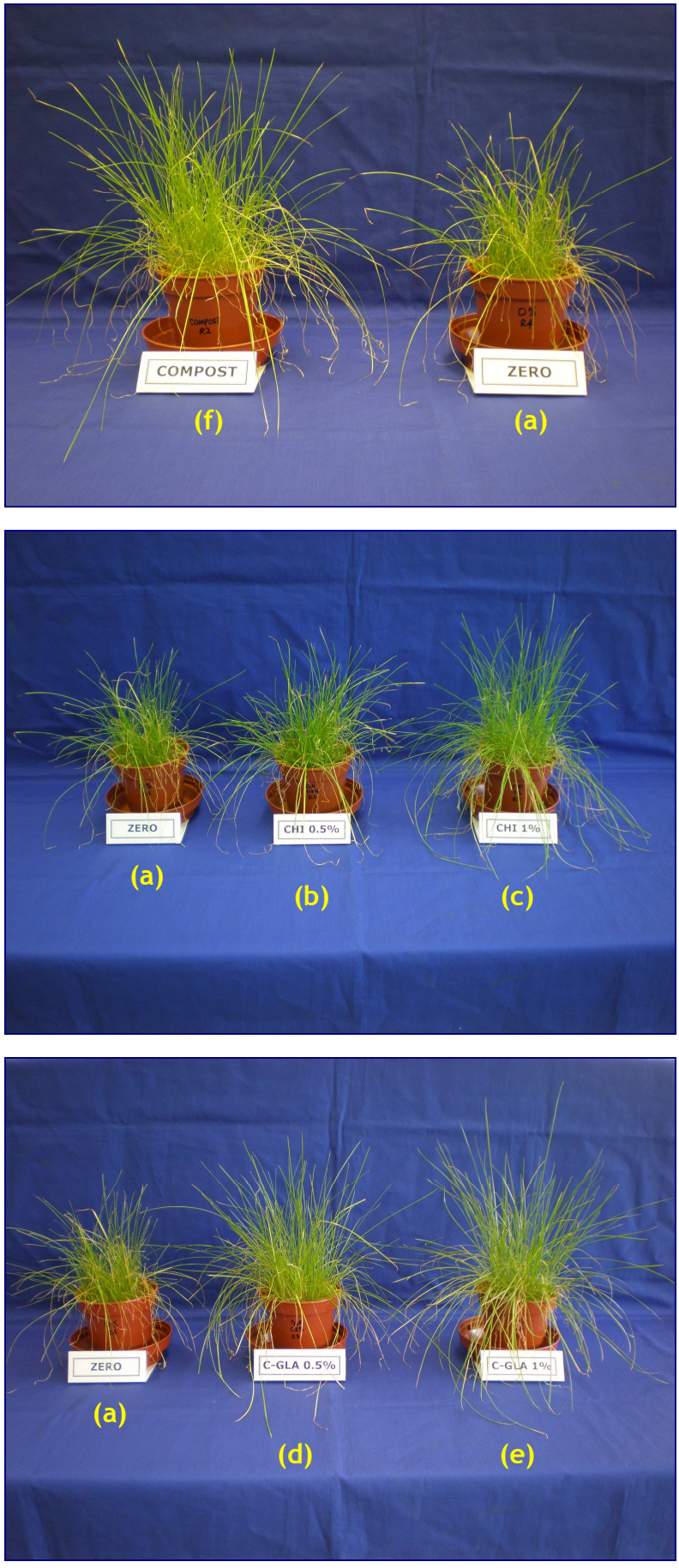


Figure 5.2 Perennial ryegrass after 9 weeks of growth. (a) Zero treatment, (b) Chitosan 0.5% (w/w), (c) Chitosan 1.0% (w/w), (d) Chitosan-GLA 0.5% (w/w), (e) Chitosan-GLA 1.0% (w/w) and (f) Compost.

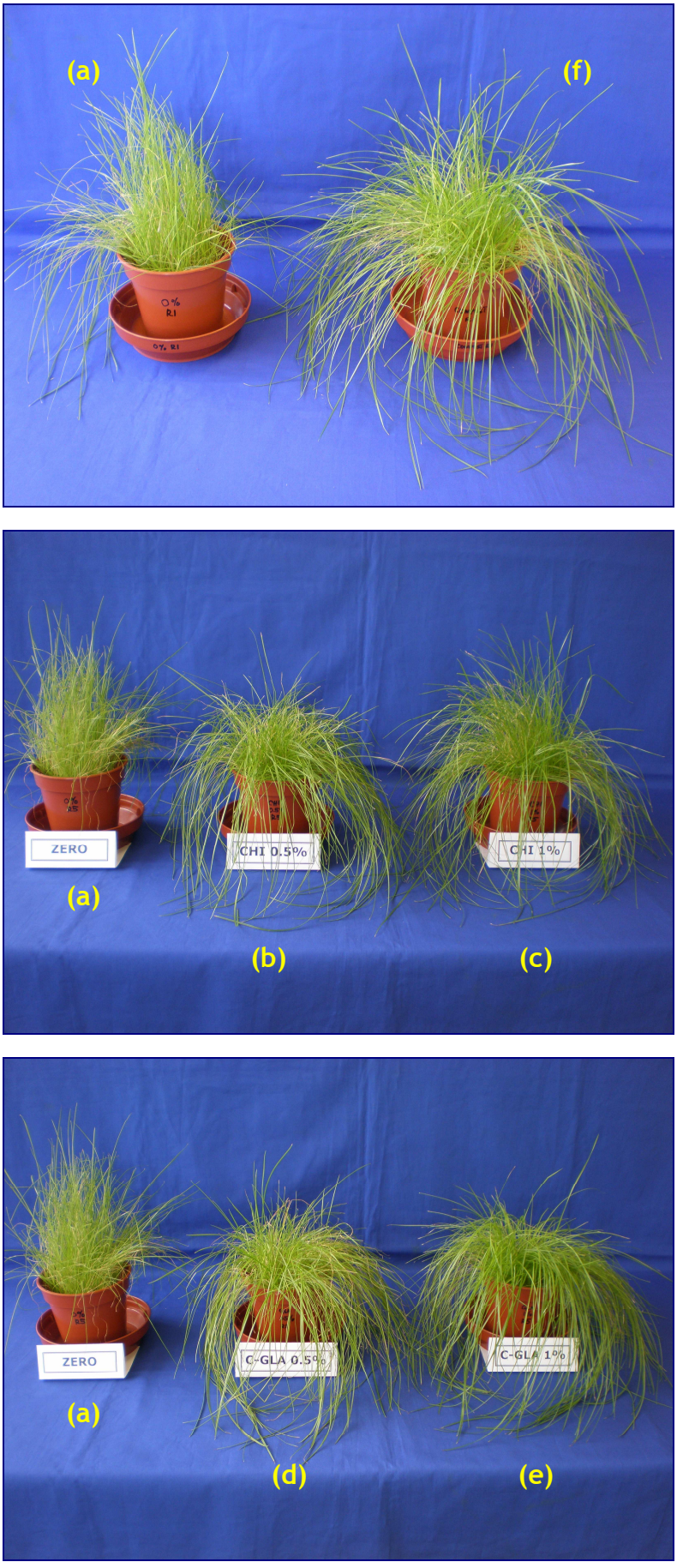


Figure 5.3 Perennial ryegrass after 20 weeks of growth. (a) Zero treatment, (b) Chitosan 0.5% (w/w), (c) Chitosan 1.0% (w/w), (d) Chitosan-GLA 0.5% (w/w), (e) Chitosan-GLA 1.0% (w/w) and (f) Compost.

Table 5.1 Biomass yield of perennial ryegrass.

Treatment	Dry weight (g/pot)			
	Shoot yield			Root yield
	First harvest	Second harvest	Total shoot yield	Second harvest
Compost*	2.62	2.47	5.09	3.37
Zero	0.79 a	1.11 a	1.90 a	1.35 a
Chitosan 0.5%	1.06 b	1.39 b	2.45 b	1.75 b
Chitosan 1.0%	1.23 c	1.54 bc	2.77 bc	1.72 b
Chitosan-GLA 0.5%	1.13 bc	1.48 bc	2.61 b	1.81 b
Chitosan-GLA 1.0%	1.41 d	1.57 c	2.98 c	1.89 b
LSD	0.16	0.15	0.31	0.23

* Plants grown on compost only (uncontaminated soil). Values represent mean of 6 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table 5.2 Elemental composition of plant shoots.

Treatment	Weight (%)		
	C	N	C/N
Zero	41.2 ± 0.5	2.1 ± 0.2	19.6
Chitosan 0.5%	40.6 ± 0.2	2.5 ± 0.1	16.2
Chitosan 1.0%	40.9 ± 0.6	2.7 ± 0.3	15.1
Chitosan-GLA 0.5%	41.1 ± 0.8	2.6 ± 0.2	15.8
Chitosan-GLA 1.0%	41.1 ± 0.4	3.0 ± 0.4	13.7

Values represent mean of 3 replicates ± standard deviation.

5.2.2 Metal uptake by perennial ryegrass

5.2.2.1 Metal concentration in plant tissues

The concentrations of Cu, Pb and Zn in plant tissues after 12 and 24 weeks of growth are shown in Figures 5.4 and 5.5 (see Appendix B, Table B1 for full data set). At first harvest, addition of chitosan and chitosan-GLA increased Pb shoot concentration (Figure 5.4). When comparing to the zero treatment, application of amendments at the rate of 0.5% (w/w) did not significantly affect the concentration of Zn in the plant shoots at first harvest. However, a different effect in Zn uptake was obtained when amendments were applied at 1.0% (w/w). From Figure 5.4, it is observed that the Zn shoot concentration reduced with chitosan 1.0% (w/w) treatment, whereas addition of chitosan-GLA at the same rate enhanced Zn concentration in the shoot tissue. A slight increase in Cu uptake was observed when amendments were added at application rate of 1.0% (w/w).

At the second harvest, Pb shoot concentration was highly influenced by the chitosan 0.5% and 1.0% (w/w) treatments. Meanwhile, chitosan-GLA at both rates of application caused no great effect in the uptake of Pb. In the case of Zn, a great increase in Zn shoot concentration was obtained for plants grown on soils treated with chitosan-GLA at 1.0% (w/w). A similar trend was observed for Cu, whereby chitosan-GLA 1.0% (w/w) caused a great effect in enhancing Cu shoot concentration.

From Figure 5.5, it is obvious that amending contaminated soil with chitosan and chitosan-GLA increased Pb and Cu concentrations in the root tissue of perennial ryegrass. This effect was less apparent for Zn root concentration. The enhanced metal concentration in the shoot tissue may be related to an increase in metal concentration in the root tissue following the treatments. Kalis *et al*. (2007) studied the relationship between metal concentration in the shoots and roots of perennial ryegrass. They noted that the metal content of the shoots depends on the metal content of the root. They obtained a linear relationship for Zn, while a less linear relationship was reported for Cd, Cu and Pb.

A shown in Figure 5.4, the Pb shoot concentration determined at the second harvest was found to be higher than the concentration measured at the first harvest. In contrast, a lower concentration was obtained for Cu at the second harvest. Amendments caused a consistent effect on Zn uptake, whereby no great difference in Zn shoot concentration was observed at both harvests.

Higher concentration of Pb measured in the plant shoots at second harvest confirms the findings of Jordan *et al*. (2009). They reported a significant increase in Pb concentration of perennial ryegrass shoots determined at the second harvest, after 84 days of growth. The first harvest was carried out at day 42 of the pot experiment. They attributed the higher Pb concentration obtained at the second harvest to the natural decay of the spent mushroom compost, amendment used to reduce plant metal uptake. They explained that the natural decay caused the remobilisation of Pb that was initially complexed with the compost.

The enhanced uptake of metal, particularly Pb, at the second harvest may be the result of cutting. As discussed in Section 2.10.2, soils received ¼ strength Hoagland's nutrient solution to ensure continued growth of the plants. The nutrient solution contained trace elements such as Ca, Fe, K, Mg, Mn and Mo, as well as Cu and Zn (Watson *et al*., 2003). Following the first harvest, plants seek nutrients for re-growth. The uptake of such nutrients may have also contributed to the increase in Cu and Zn shoot concentrations. In addition, it can be speculated that Pb, Zn and Cu were also extracted from the soils

into the plant tissues during the uptake of nutrients, leading to an increase in the shoot concentration.

In this research, the increase in metal uptake may be also related to the enzyme chitinase, which may be present in the soil-plant environment, and may have an effect on the plant's behaviour, enhancing the plant's tolerance to metal toxicity and therefore inducing metal accumulation in plant tissues.

Chitinase is present in various organisms such as animals, plants, fungi and yeasts (Kasprzewska, 2003; Matsumoto, 2006). In plants, this enzyme is involved in the defence mechanism against stress and toxicity, and plays an important role in delivering carbon and nitrogen to the cells (Patil *et al.*, 2000; Kasprzewska, 2003). However, its role in enhancing metal accumulation is not exactly known (Békésiová *et al.*, 2008; van Keulen *et al.*, 2008). The influence of metal toxicity on accumulation of chitinase in the roots of five plant species, namely barley, bean, maize, soybean and pea was studied by Békésiová *et al.* (2008). They measured higher amount of chitinase when the roots were exposed to higher concentrations of As, Cd and Pb. A similar observation was reported by van Keulen *et al.* (2008), who studied the effect of metal stress on accumulation of chitinase in dwarf sunflower. Although they obtained an unclear pattern in the relationship between chitinase content and metal uptake, they postulated that chitinase influenced metal uptake by plants, particularly metal accumulation in the root tissue.

As discussed by Vamerali *et al.* (2010), Wenzel (2009) and Prasad and Freitas (2003), a number of genes and enzymes have been identified to cause overproduction of metal chelating molecules such as phytochelatins and phytosiderophores, or overexpression of metal transporter proteins in plants. For example, Idris *et al.* (2004) reported that the presence of 1-aminocyclopropane-1-carboxylic acid deaminase, an enzyme produced by bacteria found in the rhizosphere of *Thlaspi goesingense*, not only stimulated the plant's resistance to metal toxicity but also increased its ability to accumulate metals. Meanwhile, Indian mustard treated with Gsh2, a glutathione (GSH) synthase gene produced by *Escherichia coli*, was found to have great tolerance to Cd toxicity and high capacity for Cd uptake (Singh *et al.*, 2003).

Overall, results obtained from the first pot experiment showed an increase in metal concentration in the plant tissues following the application of amendments. This may have been due to low application rates of amendments and high metal concentrations in soil, which masked the effect of amendments. The bulk soil used in the first pot trial contained 3923 mg/kg Pb, 1686 mg/kg Zn and 172 mg/kg Cu.

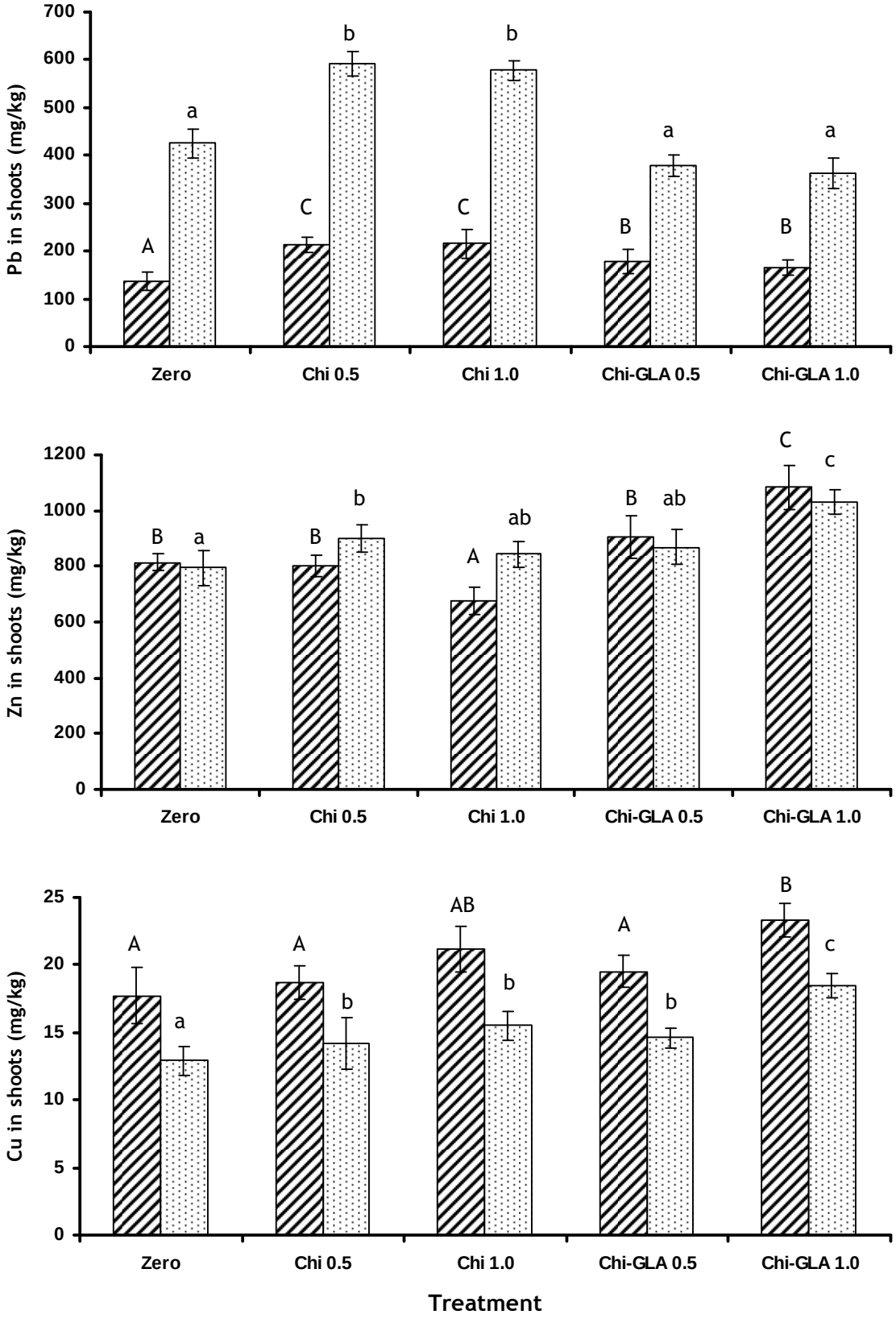


Figure 5.4 Metal concentrations in plant shoots after 12 and 24 weeks of growth. Error bars are ± standard deviation of 18 replicates. Letters represent statistical differences (Tukey's test at $p < 0.05$) for the first harvest (upper case) and second harvest (lower case). Chi 0.5: Chitosan 0.5% (w/w), Chi 1.0: Chitosan 1.0% (w/w), Chi-GLA 0.5: Chitosan-GLA 0.5% (w/w), and Chi-GLA 1.0: Chitosan-GLA 1.0% (w/w).

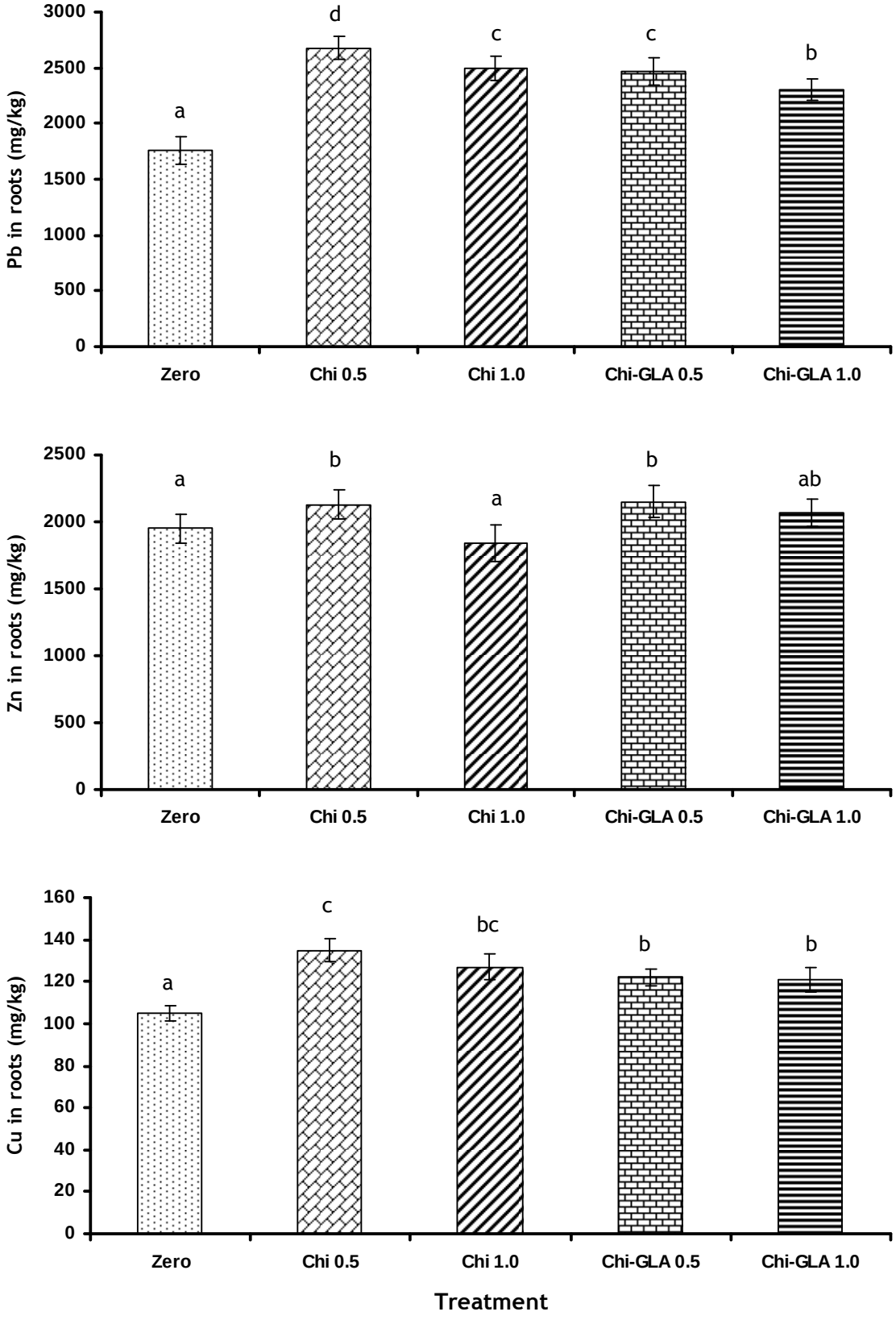


Figure 5.5 Metal concentrations in plant roots after 24 weeks of growth. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 0.5: Chitosan 0.5% (w/w), Chi 1.0: Chitosan 1.0% (w/w), Chi-GLA 0.5: Chitosan-GLA 0.5% (w/w), and Chi-GLA 1.0: Chitosan-GLA 1.0% (w/w).

5.2.2.2 Bioconcentration Factor

The bioconcentration factor (BCF) is defined as the ratio of metal concentration in plant shoots to metal concentration in soil (McGrath and Zhao, 2003), and is a measure of the ability of a plant to accumulate metals from soils (Yoon *et al.*, 2006). According to Hooda and Alloway (1994), the BCF can also be used to determine the availability of an element in the soil-plant system. In this research, the influence of chitosan and chitosan-GLA amendment on BCF values of the metals was determined after each harvest, and the values are given in Table 5.3.

The BCF values estimated at the first harvest suggest that the ability of perennial ryegrass to take up Pb from soils increased slightly with the addition of amendments. Application of amendments at the rate of 0.5% (w/w) did not affect the ability of plants to accumulate Zn in their shoots. However, addition of amendments at 1.0% (w/w) caused a different effect in Zn accumulation. The Zn shoot accumulation was found to reduce with chitosan 1.0% (w/w) treatment, while chitosan-GLA 1.0% (w/w) increased the BCF value of Zn from 0.483 (zero treatment) to 0.642 at the first harvest, suggesting a great enhancement in the plant's ability to accumulate Zn. The decrease in BCF value of Zn following chitosan addition can be attributed to Zn sorption onto chitosan. In the case of Cu, only chitosan-GLA 1.0% (w/w) had an effect on the BCF value, whereby the value increased from 0.103 to 0.136.

The BCF values of Pb estimated at the second harvest were found to be greater than the values obtained at the first harvest. This effect can be directly linked to the higher concentration of Pb measured in the plant shoots at the second harvest (Figure 5.4, Section 5.2.2.1) and as previously discussed, the accumulation of Pb was induced by harvesting. In the case of Zn, there was no great difference between the BCF values estimated at both harvests. In fact, the BCF values of Zn estimated for the treatments at the second harvest showed similar trend to that of values obtained at the first harvest. In the case of Cu, a lower BCF value was estimated at the second harvest and this effect can be related to the lower concentration of Cu determined in the plant shoots at the second harvest (Figure 5.4, Section 5.2.2.1).

In general, Zn had a greater BCF value than Pb and Cu. The BCF value is highly dependent on metal concentration in plant shoots and soil. When comparing to Pb, more Zn was found in the plant shoots (Figure 5.4, Section 5.2.2.1), and the total concentration of Zn in the bulk soil (1686 mg/kg) was lower than the 3923 mg/kg measured for Pb. Therefore, Zn had a greater BCF value than Pb.

Several studies have also reported an unclear effect on BCF values of heavy metals resulting from the application of amendments. For example, Padmavathiamma and Li (2010) observed a different trend in metal uptake by three plant species, namely perennial ryegrass, red fescue and Kentucky bluegrass grown on a contaminated soil amended with phosphate and compost for 90 days. They reported that perennial ryegrass and red fescue had a similar behaviour in accumulating Mn and Pb, whereby application of both amendments was found to increase the BCF value of Mn, and decrease the BCF value of Pb. In the case of Kentucky bluegrass, amending contaminated soil with both amendments was reported to reduce the BCF values of Mn and Pb. They attributed the increase in the BCF value of Mn to the increase in the exchangeable Mn fraction in the soil following the addition of phosphate and compost. However, this effect was not relevant to Kentucky bluegrass.

Hooda and Alloway (1994) applied sewage sludge at 50 tonne/ha and reported an increase in the ability of perennial ryegrass to take up Zn from soils. However, the BCF value of Zn was found to decrease when the sewage sludge was applied at the rate of 150 tonne/ha. The decrease in the BCF value was explained to be due to toxicity effect of Zn to plants, which reduced Zn uptake by plants. They mentioned that sewage sludge can be used as both a source and an adsorptive medium for heavy metals.

Fornes *et al*. (2009) studied the effect of organic compost amendment at the rate of 100 tonne/ha on the uptake of Pb and Zn by *Brassica oleracea* and *Brassica rapa* from a contaminated agricultural soil. After 16 weeks of a greenhouse study, they observed no significant difference in the BCF value of Pb estimated for both plants when compared to plants grown on untreated contaminated soil. However, the addition of compost was reported to reduce the BCF value of Zn by *B. oleracea*, whereas a significant increase was obtained for the BCF value of Zn by *B. rapa*.

A study on metal accumulation in 17 plant species by Yoon *et al*. (2006) has shown that plants have different preference in taking up heavy metals. For example, the BCF values estimated for *Gentiana pennelliana* was in the order of Pb > Cu > Zn, while Zn > Pb > Cu was obtained for *Sesbania herbacea*.

It is clear that application of amendments to contaminated soils may influence the behaviour of plants in accumulating metals. This behaviour is mainly affected by several factors such as the nature of the amendment, application rate of amendment, the nature of the metal contaminant, plant species and soil pH (Audet and Charest, 2007). Some amendments such as sewage sludge, furnace slag and red mud contain a considerable amount of heavy metals (Hooda and Alloway, 1994; Pichtel and Bradway,

2008; Gray *et al.*, 2006). The addition of these materials may increase metal concentrations in the contaminated soil. This will probably enhance metal uptake by plants, and as a consequence, the BCF values of the metals will be increased.

Table 5.3 BCF values for Pb, Zn and Cu.

Treatment	First harvest			Second harvest		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.035 a	0.483 b	0.103 a	0.108 a	0.471 a	0.075 a
Chitosan 0.5%	0.055 c	0.475 b	0.109 a	0.151 b	0.534 b	0.083 b
Chitosan 1.0%	0.055 c	0.400 a	0.123 ab	0.147 b	0.500 ab	0.090 b
Chitosan-GLA 0.5%	0.046 b	0.537 b	0.113 a	0.097 a	0.516 ab	0.085 b
Chitosan-GLA 1.0%	0.042 b	0.642 c	0.136 b	0.092 a	0.610 c	0.107 c
LSD	0.005	0.065	0.022	0.018	0.059	0.007

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

5.2.2.3 Translocation Factor

The translocation factor (TF) is defined as the ratio of metal concentration in plant shoots to metal concentration in roots (Ruiz *et al.*, 2009), and is a measure of the ability of a plant to translocate metals from the roots to the shoots (Yoon *et al.*, 2006). The effect of chitosan and chitosan-GLA amendment on TF values of metals was determined after each harvest, and the values are given in Table 5.4.

Based on the estimation of TF values at the first harvest, it was found that application of amendments did not significantly affect the translocation of heavy metals in the plant tissue. A slight reduction in the TF value of Pb was obtained following chitosan-GLA 0.5% and 1.0% (w/w) treatments. Meanwhile, amending contaminated soil with chitosan-GLA at 1.0% (w/w) led to a slight increase in translocation of Zn and Cu.

The TF values of Zn and Cu obtained at the second harvest showed no great difference to that of values estimated at the first harvest. This indicates that there was no significant change in the ability and behaviour of plant to translocate both metals over the period of pot experiment. In contrast to Zn and Cu, the TF values of Pb estimated at the second harvest were found to be greater than the values obtained at the first harvest. Although the trend of the TF values remained the same, all treatments showed a greater value at the second harvest. This suggests that the translocation of Pb in the plant tissue became efficient after the first harvest. This observation can be related to the higher concentration of Pb in the plant shoots determined at the second harvest

(Figure 5.4, Section 5.2.2.1). Harvesting might have a great effect in inducing the translocation of Pb from the roots to the shoots, leading to a significant increase in Pb accumulation in the plant shoots.

From Table 5.4, the order of TF values estimated for the treatments at the first harvest was found to be Zn > Cu > Pb, while Zn > Pb > Cu was obtained at the second harvest. Based on these values, it was determined that Zn was the metal most transported from the root to the shoot. Cu and Zn are known to be essential for the growth of plants. Therefore, plants tend to translocate both micronutrients from the roots to the shoots. Low translocation of Pb might be due to its toxic effect to plants (Yoon *et al.*, 2006). Kim *et al.* (2003) discussed the toxic effect of Pb on chlorophyll synthesis, photosynthetic activity and antioxidant enzymes. According to Jarvis and Leung (2002), two main mechanisms holding Pb in plant roots are binding of Pb to ion exchange sites on the root cell walls and extracellular precipitation. Studies of metal accumulation in perennial ryegrass tissue by Kalis *et al.* (2008; 2007) found that Pb was retained at the root surface of the plant better than Cd, Cu, Ni and Zn. The order of the TF values established at first harvest was in agreement with these discussions.

Plants have different abilities to translocate heavy metals. For example, sunflower and maize were found to translocate metals in the order of Zn > Cu > Pb (Ruiz *et al.*, 2009). Meanwhile, Yoon *et al.* (2006) reported the order of Pb > Cu > Zn for *Cyperus esculentus* and Cu > Zn > Pb for *Rubus fruticosus*. Padmavathiamma and Li (2010) reported an increase in the TF values of Mn and Pb for perennial ryegrass following compost treatment. In the case of red fescue, the same treatment was found to reduce the TF value of Mn, while the TF value of Pb was reported to increase.

Table 5.4 TF values for Pb, Zn and Cu.

Treatment	First harvest			Second harvest		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.080 a	0.428 a	0.173 a	0.243 b	0.414 a	0.124 b
Chitosan 0.5%	0.081 b	0.379 a	0.142 a	0.221 b	0.422 a	0.107 a
Chitosan 1.0%	0.088 b	0.378 a	0.169 a	0.235 b	0.468 b	0.124 b
Chitosan-GLA 0.5%	0.073 a	0.426 a	0.160 a	0.153 a	0.406 a	0.120 b
Chitosan-GLA 1.0%	0.071 a	0.529 b	0.194 b	0.156 a	0.501 b	0.154 c
LSD	0.010	0.069	0.034	0.029	0.051	0.013

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

5.2.2.4 Off-take values

The effect of amendments application on plant metal uptake was further evaluated in terms of off-take value. Although information on metal concentration in plant tissues is important in evaluating the effectiveness of soil amendments for metal immobilisation, the off-take value is a better parameter for such evaluation because it considers both metal concentration in plant tissues and biomass yield. The amount of Pb, Zn and Cu removed by perennial ryegrass from contaminated soil is given in Table 5.5. The off-take value (mg/pot) was estimated using Equation 5.1.

Total metal concentration in shoot (mg/kg) x Total shoot yield (dry weight, kg) (5.1)

It is also important to estimate the off-take value in kg/ha unit as this will provide an insight into the real effect of soil amendments if applied on a contaminated site. The off-take value (kg/ha) was based on conversion factor of pot area to hectare. As described in Section 2.10.2, the pot experiments were carried out using pots with a radius of 3.5 cm. Since 1 hectare equals to 10,000 m^2, the conversion factor of pot to hectare was determined as Equation 5.2. And, since 1.0 mg equals to 1.0 x 10^{-6} kg, the conversion from mg/pot to kg/ha was estimated by multiplying the off-take value (mg/pot) by 2.6.

$$\frac{10\,000}{\pi r^2} = 2.6 \times 10^6 \qquad (5.2)$$

From Table 5.5, it is observed that application of chitosan and chitosan-GLA enhanced the removal of Pb and Zn by perennial ryegrass. In fact, the off-take values of Pb and Zn increased with increasing the rate of amendments. Of the metals studied, Zn was removed at a higher amount as compared to Pb and Cu. Cu was the metal least extracted by perennial ryegrass. In fact, the removal of Cu was not affected by the treatments. At the end of the pot experiment, it is estimated that 1.53 mg/pot of Zn, 0.58 mg/pot of Pb and 0.03 mg/pot of Cu were removed from the untreated contaminated soil. Chitosan 1.0% (w/w) had a great effect in enhancing Pb removal by perennial ryegrass. Following this treatment, the off-take value of Pb increased from 0.58 mg/pot (1.51 kg/ha) to 1.15 mg/pot (2.99 kg/ha). Chitosan-GLA 1.0% (w/w) treatment could assist perennial ryegrass to extract 3.14 mg/pot (8.16 kg/ha) of Zn and 0.06 mg/pot (0.16 kg/ha) of Cu, respectively.

The results reported for this study contradict the results obtained from the sorption study (Chapter 4), which confirmed the ability of chitosan and chitosan-GLA to bind Cu, Pb and Zn (Section 4.6). In fact, in Chapter 3 (characterisation study) it was evident by

FTIR analysis that the metals were bound to the amendments through dative covalent interaction with amino and hydroxyl groups (Section 3.4.4).

As discussed in Section 1.4.2, application of amendments to metal contaminated soils may not always reduce metal uptake by plants. The effectiveness of soil amendments in immobilising metal is influenced by several factors such as soil properties, the nature of the amendments and the nature of the metal contaminants. Whilst the main aim of this research was to apply chitosan and cross-linked chitosans for metal immobilisation, results from the first pot experiment highlight their potential for enhancing phytoextraction. As discussed in Section 1.4, phytoextraction is also an important technique for the remediation of metal contaminated soils.

Due to the formation of stable complexes with soil organic matter, as well as the formation of insoluble precipitates, Pb is highly retained in soil (Saifullah *et al.*, 2009; Basta *et al.*, 2005; Evans, 1989). Several amendments such as EDTA, acetic acid, cow manure and olive husk have been tested and reported to increase the phytoextraction of Pb, mainly by enhancing the availability of Pb in the rhizosphere (Section 1.4.1.3). In this study, phytoextraction of Pb by perennial ryegrass increased with chitosan and chitosan-GLA treatments.

Kayser *et al*. (2000) and Johnson *et al*. (2009) discussed that amendments for enhancing phytoextraction should have the ability to increase both the solubility and availability of heavy metals to plant roots, and metal accumulation in aerial biomass. In Section 5.2.2.1, it was speculated that the chitinase enzyme enhanced plant's tolerance to metal toxicity, and consequently induced metal accumulation in the plant shoots. Overall, it is apparent that application of chitosan and chitosan-GLA up to 1.0% (w/w) enhanced the removal of Pb and Zn from contaminated soil and therefore, their application for enhancing phytoextraction is feasible.

Table 5.5 Removal of heavy metals using perennial ryegrass (sum of the first and second harvests).

Treatment	Off-take (mg/pot)			Off-take (kg/ha)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.58	1.53	0.03	1.51	3.98	0.08
Chitosan 0.5%	1.05	2.10	0.04	2.73	5.46	0.10
Chitosan 1.0%	1.15	2.12	0.05	2.99	5.51	0.13
Chitosan-GLA 0.5%	0.76	2.31	0.04	1.98	6.01	0.10
Chitosan-GLA 1.0%	0.80	3.14	0.06	2.08	8.16	0.16

5.2.3 Metal concentration in soil

According to Vamerali *et al*. (2010) and Kabata-Pendias and Mukherjee (2007), uptake of heavy metals by plants is mainly influenced by their bioavailable fraction rather than by the total amount in soil. Therefore, in addition to the total fraction, the bioavailable fraction of metals in bulk soil was also determined using deionised water, ammonium acetate (1.0 mol/L, pH 7.0) and ammonium EDTA (0.05 mol/L, pH 7.0) as extractants. The procedure is fully described in Sections 2.9.1.1 and 2.9.1.2. Table 5.6 presents bioavailable and total metal content in the bulk soil used in the first pot experiment.

Table 5.6 Bioavailable and total metal content in bulk soil.

Extractant	Metal concentration (mg/kg)					
	Pb	RSD (%)	Zn	RSD (%)	Cu	RSD (%)
Deionised water[a]	1.5	51.4	4.5	22.2	0.6	5.6
Deionised water[b]	1.2	25.9	4.4	24.3	0.3	17.0
Ammonium acetate	800	8.1	256	9.4	4	16.2
EDTA	1555	5.6	578	4.4	13	3.7
Aqua regia	3923	75.9	1686	17.1	172	52.1

Values represent mean of ten replicates. [a] Fresh soil; [b] Air-dried soil.

Extraction of metals from fresh and air-dried bulk soils using deionised water produced similar results, which suggests that the most bioavailable fraction of metals can be determined in both soil conditions. In general, ammonium acetate gave a lower concentration of metals, as compared to EDTA. According to Saifullah *et al*. (2009) and Alkorta *et al*. (2004), EDTA is an efficient extraction agent because it can form a stable complex with a variety of metals in soils.

The ammonium acetate and EDTA extractable metals in soil after 24 weeks of the pot experiment are shown in Figures 5.6 and 5.7 (see Appendix B, Tables B2 and B3 for full data set). From these figures, it is apparent that there was no great difference in amount of metal extracted from the untreated and treated soils. However, when comparing to metal concentrations in bulk soil before pot experiment (Table 5.6), the ammonium acetate extractable metals were found to reduce after the experiment (Figure 5.6). The EDTA extraction also showed a decrease in metal concentrations (Figure 5.7), but the percentage of reduction was less apparent as compared to ammonium acetate extraction. For example, the EDTA extractable Pb was found to decrease from 1555 mg/kg to 1282-1378 mg/kg, while the ammonium acetate extractable Pb reduced from 800 mg/kg to 362-402 mg/kg following the treatments.

Whilst the reduction in metal concentration of soil can be related to immobilisation effect of the amendments and uptake by plants, leaching may have also contributed to the decrease. During the pot experiment, plants were watered with deionised water daily (Section 2.10.2). Therefore, metals may have leached from the soil to the pot saucer. In addition, plants were also received ¼ strength Hoagland's nutrient solution once a week (Section 2.10.2). The nutrient solution contained a number of cations and anions that could interact with metals, and might have also favoured metal leaching from the soil. Although every effort was made to make sure soil samples contain no root tissue, there were fine root tissues remained in the soil samples. In Section 5.2.2.1, it was evident that root tissue contained high concentration of metals. It can be speculated that the fine root tissues also contain a considerable amount of metals. During soil extraction, metals in the fine root tissues may have also been extracted. As a consequence, no obvious effect of amendments on metal immobilisation was observed.

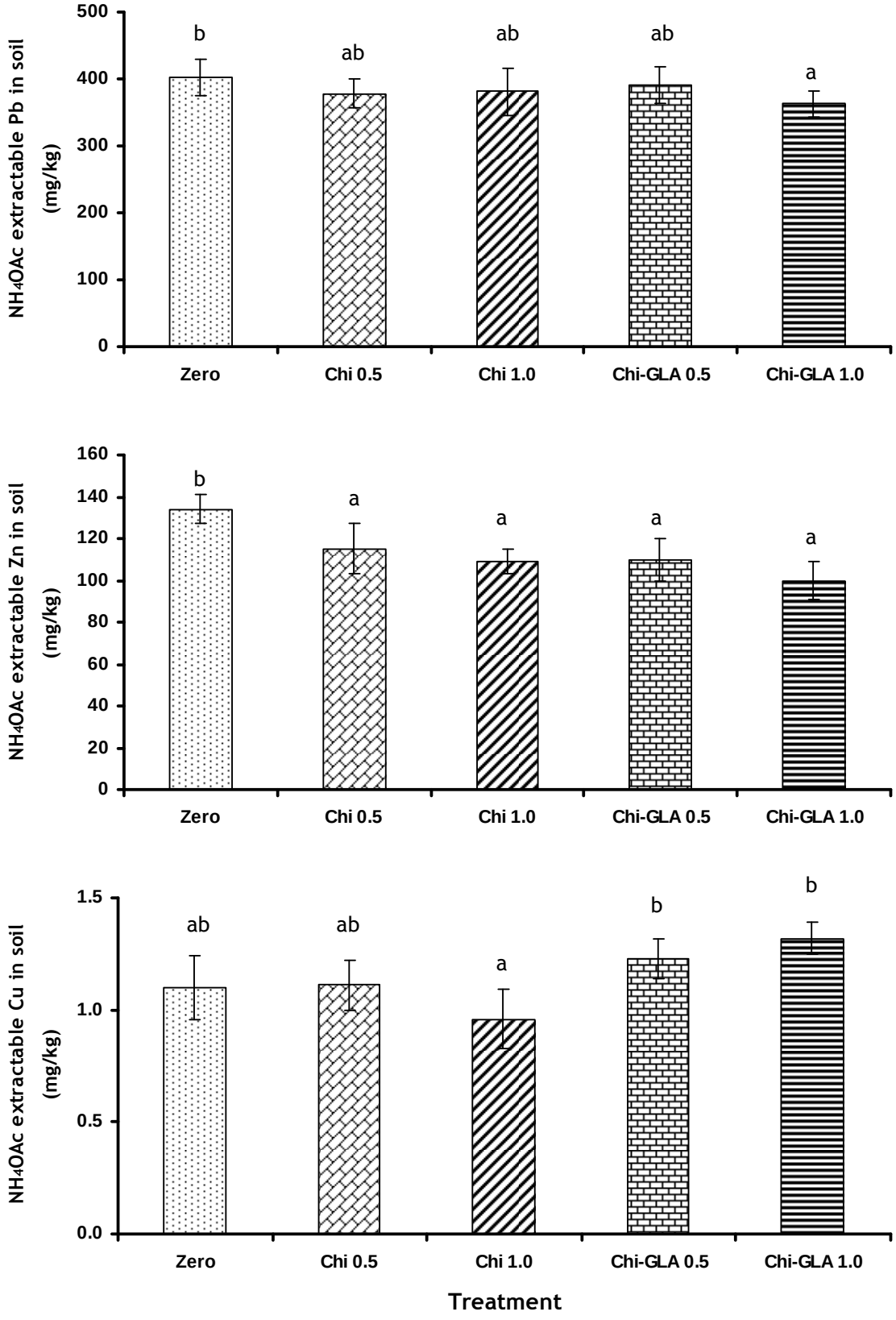


Figure 5.6 Ammonium acetate extractable metals in soil after 24 weeks of pot trial. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 0.5: Chitosan 0.5% (w/w), Chi 1.0: Chitosan 1.0% (w/w), Chi-GLA 0.5: Chitosan-GLA 0.5% (w/w), and Chi-GLA 1.0: Chitosan-GLA 1.0% (w/w).

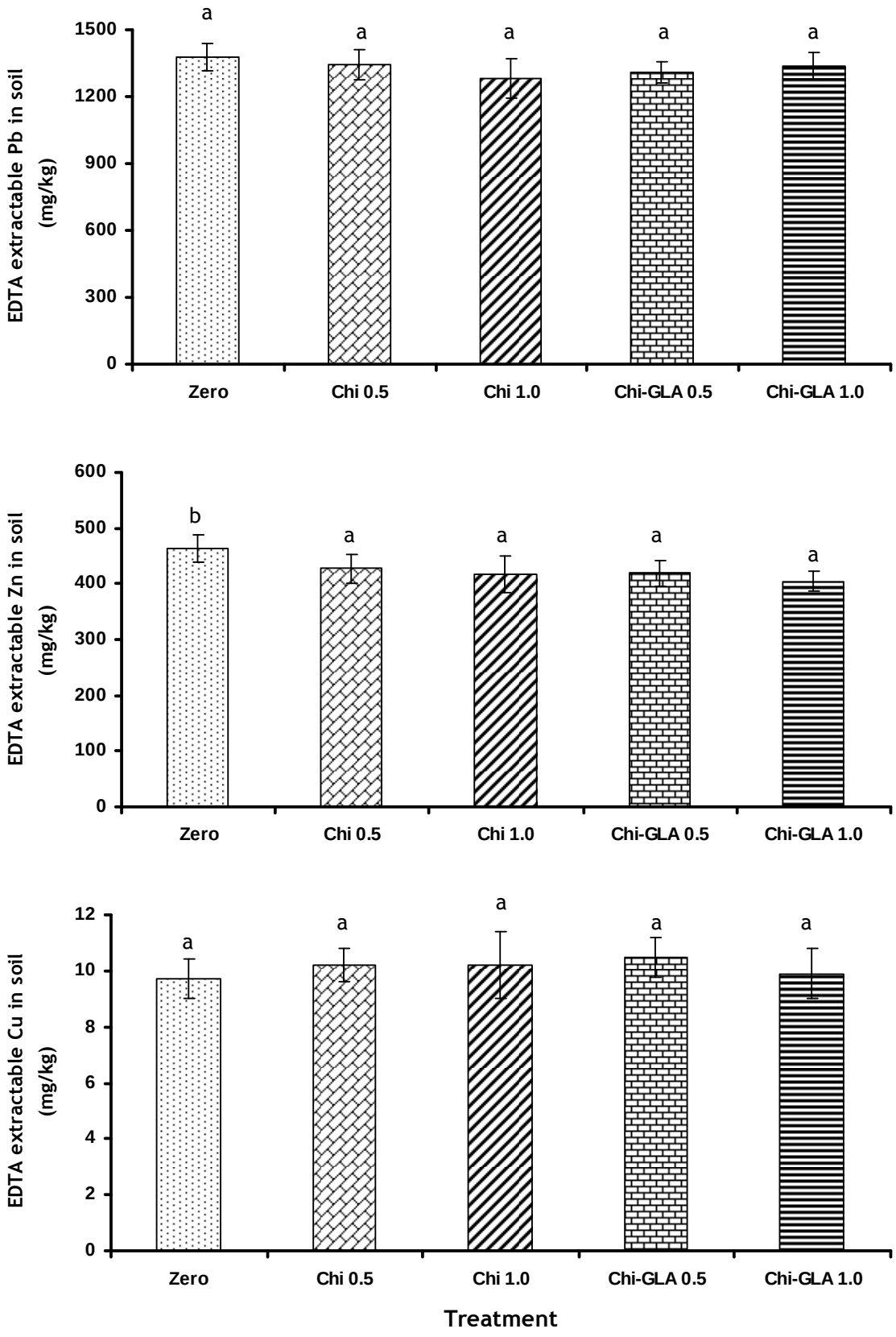


Figure 5.7 EDTA extractable metals in soil after 24 weeks of pot experiment. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 0.5: Chitosan 0.5% (w/w), Chi 1.0: Chitosan 1.0% (w/w), Chi-GLA 0.5: Chitosan-GLA 0.5% (w/w), and Chi-GLA 1.0: Chitosan-GLA 1.0% (w/w).

5.2.4 Soil pH

Table 5.7 presents the effect of chitosan and chitosan-GLA application on soil pH. The pH values of the soils and amendments were measured in deionised water with a solid:solution ratio of 1:2.5 (Section 2.10.4). It is observed that application of both amendments caused a slight increase in the soil pH, whereby the change in the pH was within 0.3 to 0.7 units. The pH values of chitosan and chitosan-GLA were determined as 7.0 and 7.2, respectively.

The change in the soil pH is influenced by the pH of the amendment and the nature of the soil. For example, Li *et al*. (2008) added pig manure (pH 7.7) and peat (pH 4.0) at 0.4% (w/w) to a metal contaminated soil. After 3 months of a greenhouse study, the pH of the soil (pH 4.0) was reported to increase by 1.7 and 0.5 units, respectively. A greater increase in the soil pH after pig manure application observed by Li *et al*. (2008), maybe due to the higher pH value of pig manure than peat. Clemente *et al*. (2007) treated a contaminated soil with cow manure (pH 8.8) and reported that the soil pH increased from 7.7 to 7.9 after 9 months of the treatment.

Table 5.7 Effect of chitosan and chitosan-GLA amendment on soil pH.

Treatment	pH
Bulk soil (before pot experiment)	4.7 ± 0.3
Zero	5.0 ± 0.7
Chitosan 0.5%	5.2 ± 1.2
Chitosan 1.0%	5.4 ± 0.4
Chitosan-GLA 0.5%	5.3 ± 0.8
Chitosan-GLA 1.0%	5.4 ± 1.5

Values represent mean of three replicates ± standard deviation.

5.3 Summary of pot experiment 2009

Addition of chitosan and chitosan-GLA to contaminated soil increased the biomass yield of perennial ryegrass. An increase in application rate caused an increase in nitrogen content of plant shoots. Both amendments contain 7-8% of nitrogen that can provide nitrogen nutrition for plant growth. No toxicity symptoms were observed over the pot experiment. This suggests that perennial ryegrass is a robust plant species and has great tolerance to high metal concentrations.

Amending contaminated soil with both amendments increased metal concentration in plant tissues. Harvesting may have an effect in enhancing metal uptake by plants. It was speculated that the chitinase enzyme increased plant's tolerance to metal toxicity and therefore induced metal accumulation in plant tissues. When the results were expressed as off-take values, it was apparent that application of amendments enhanced the removal of heavy metals from contaminated soil. Zn was the metal most removed, while Cu was the least.

The ammonium acetate and EDTA extractable metals were found to decrease after the pot experiment, with the latter showing less of a decrease. No great difference was obtained between untreated and treated soils. The decrease in metal concentration of soil can be related to the effect of metal immobilisation by the amendments, plant uptake and leaching. The determination of metal concentration in soil after the pot experiment was influenced by the presence of fine root tissues.

The ultimate aim of this research was to investigate the potential of chitosan and cross-linked chitosans for metal immobilisation. However, results from the first pot experiment highlight the potential of chitosan and chitosan-GLA for enhancing metal uptake by plants. Since phytoextraction has also received much attention for the remediation of metal contaminated soils, the use of chitosan and chitosan-GLA at 0.5% and 1.0% (w/w) can be promising in enhancing the efficiency of phytoextraction. Overall, unclear effect on metal immobilisation may have been due to low rate of application and high metal concentration in soil, which masked the effect of the amendments.

5.4 Pot experiment 2010

The first pot experiment yielded unexpected results, in which application of chitosan and chitosan-GLA at 0.5% and 1.0% (w/w) increased the uptake of Pb and Zn. At the same time, the effect of both amendments on the bioavailability of heavy metals in soil was not very clear. To understand the influence of both amendments better, a second pot experiment was carried out. Amendments were applied at 1% and 10% (w/w), and their effects on plant growth and metal accumulation in plant tissue were determined using perennial ryegrass and rapeseed. The procedure is fully discussed in Section 2.11.

5.4.1 Plant growth

The perennial ryegrass seeds germinated five days after sowing. A more rapid germination was observed for rapeseed, whereby the seeds germinated two days after sowing. The plant growth observation for both plants is shown in Figures 5.8 to 5.12. Slow growth performance was observed for plants grown on chitosan-GLA 10% (w/w) treatment (Figures 5.8 and 5.10). Following this treatment, the growth of perennial ryegrass was stunted after two weeks of the pot experiment, while rapeseed showed no progress in growth after one week of the experiment. Plants grown on chitosan-GLA 10% (w/w) died after three weeks of growth. It can be speculated that it might be due to the effect of glutaraldehyde (GLA), the chemical used in cross-linking treatment of chitosan (Section 2.4).

No obvious difference in growth of the perennial ryegrass was observed up to five weeks of the pot experiment. In the case of rapeseed, the effect of both amendments on biomass production was observed after three weeks of growth (Figure 5.11). An obvious difference in physical appearance of the plant leaves was observed. Soils receiving amendments resulted in plants with healthy leaves, as compared to plants grown on untreated contaminated soil (Figures 5.9 and 5.12). Plants grown on chitosan 10% (w/w) treated soil particularly exhibited this effect. No toxicity symptoms were observed on plants grown on untreated contaminated soil.

The rapeseed was harvested once, after 8 weeks of growth. Meanwhile, the perennial ryegrass was harvested twice, after 9 and 20 weeks of growth. Plants grown on treated soils showed better re-growth performance, as compared to plants cultivated on untreated soil. The best re-growth was observed for chitosan 10% (w/w) treatment, whereby new shoots of 1.0 cm length developed one day after harvest. Chitosan and

chitosan-GLA 1% (w/w) treatments had less of an effect, in which the development of 1.0 cm new shoots was only achieved after two days.

Tables 5.8 and 5.9 present the dry biomass yield of perennial ryegrass and rapeseed after the pot experiment. Application of amendments increased the biomass yield. In fact, the biomass production increased with the rates of application. The highest percentage increment in the shoot and root yield was achieved with chitosan 10% (w/w) treatment, while amendments at 1% (w/w) showed a similar effect in improving biomass production. Although a higher total biomass yield was obtained for rapeseed following the treatments, statistical data suggest that the effects of chitosan and chitosan-GLA on biomass production of both plants were similar. The order of treatment efficacy on biomass production (both shoot and root) was chitosan 10% (w/w) > chitosan and chitosan-GLA 1% (w/w) > zero. The total shoot yield for perennial ryegrass grown on soils treated with chitosan and chitosan-GLA at 1% (w/w) (Table 5.8) was found to be lower than the shoot yield obtained with the same treatments in the first pot experiment (Table 5.1, Section 5.2.1). However, there was no great difference for the root yield obtained at the end of both pot experiments for chitosan and chitosan-GLA 1% (w/w) treatments (Tables 5.8 and 5.1). Pot experiments were carried out in a greenhouse between March and October of 2009 and 2010. Plants were allowed to grow under natural lighting, ambient temperature and humidity (Sections 2.10.2 and 2.11.1). Therefore, the weather and more important, the sunlight contributed to the difference in biomass production.

The elemental composition of perennial ryegrass and rapeseed shoots are given in Tables 5.10 and 5.11. It is observed that the addition of both amendments did not affect the weight percentage of carbon. Chitosan 10% (w/w) treatment resulted in a great increase of the nitrogen content in plant shoots. Following this treatment, the nitrogen content in perennial ryegrass shoots increased from 2.6 (zero treatment) to 6.6. In case of the rapeseed shoots, the nitrogen content increased from 2.4 to 6.9. It is found that shoot with a higher nitrogen content gave a lower C/N ratio. Due to its low nitrogen content, shoots for the zero treatment had the highest C/N ratio. The C/N ratio for shoots of perennial ryegrass grown on soils treated with chitosan and chitosan-GLA at 1% (w/w) (Table 5.10) was found to be lower than the C/N ratio estimated for the same treatments in the first pot experiment (Table 5.2, Section 5.2.1). This can be attributed to the difference in amount of nitrogen available in soil and the distribution of nitrogen in the plant tissue. The availability of nitrogen in soil depends on the breakdown of the chitosans. It is known that nitrogen is a primary macronutrient and the increase in nitrogen content implies the importance of nitrogen for plant growth and survival.

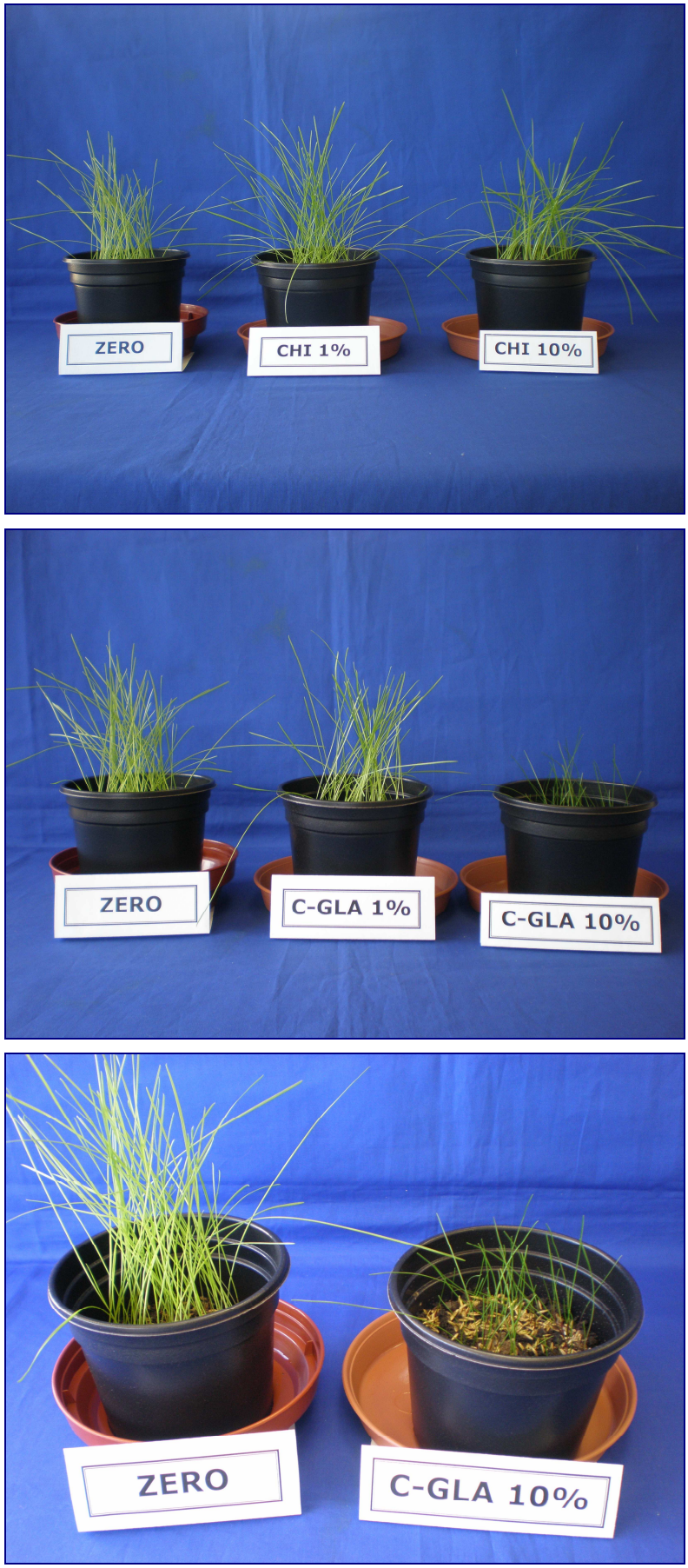


Figure 5.8 Perennial ryegrass after 2 weeks of growth. Zero: untreated contaminated soil, CHI 1%: Chitosan 1% (w/w), CHI 10%: Chitosan 10% (w/w), C-GLA 1%: Chitosan-GLA 1% (w/w), and C-GLA 10%: Chitosan-GLA 10% (w/w).

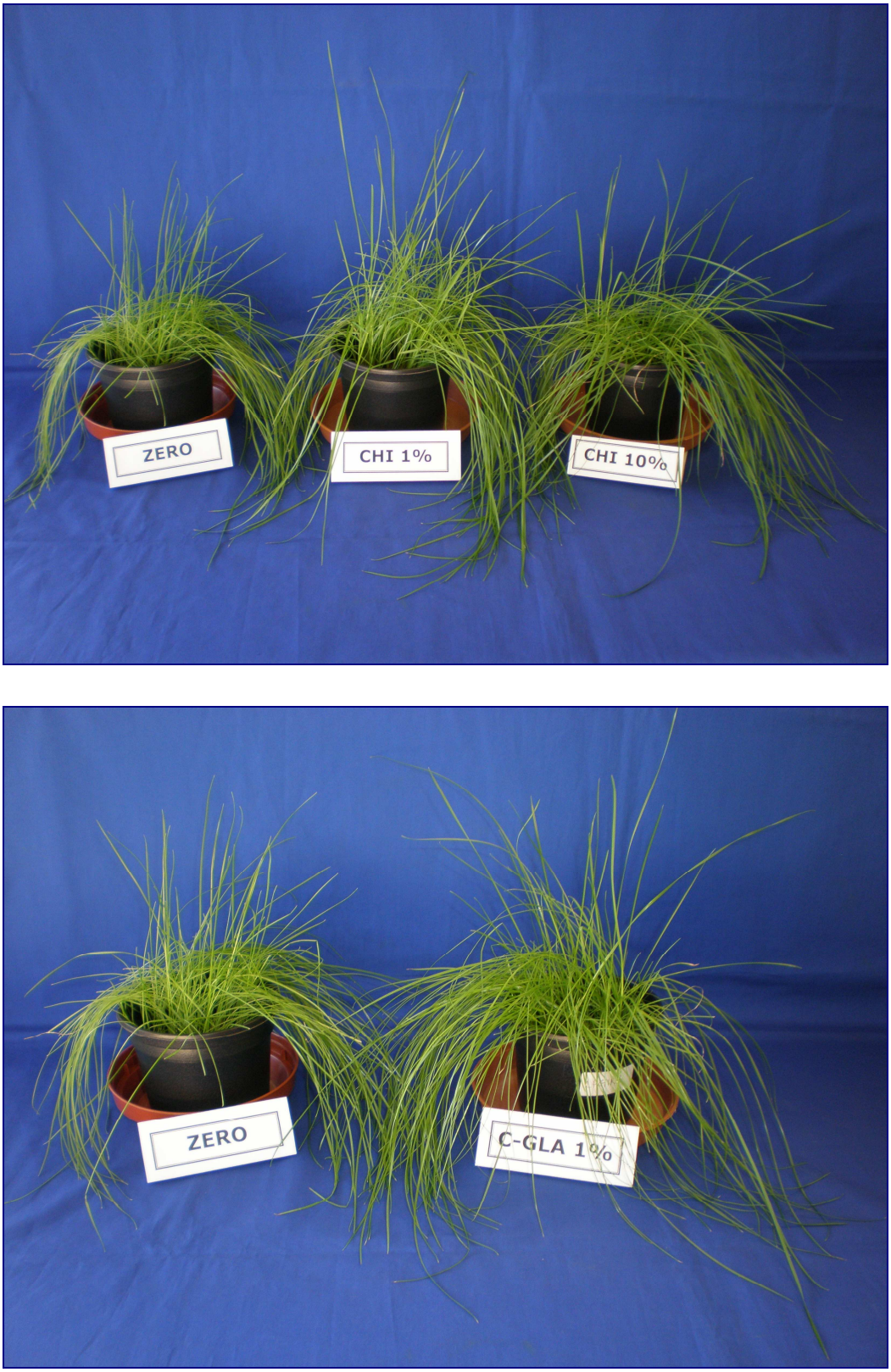


Figure 5.9 Perennial ryegrass after 8 weeks of growth. Zero: untreated contaminated soil, CHI 1%: Chitosan 1% (w/w), CHI 10%: Chitosan 10% (w/w), and C-GLA 1%: Chitosan-GLA 1% (w/w).

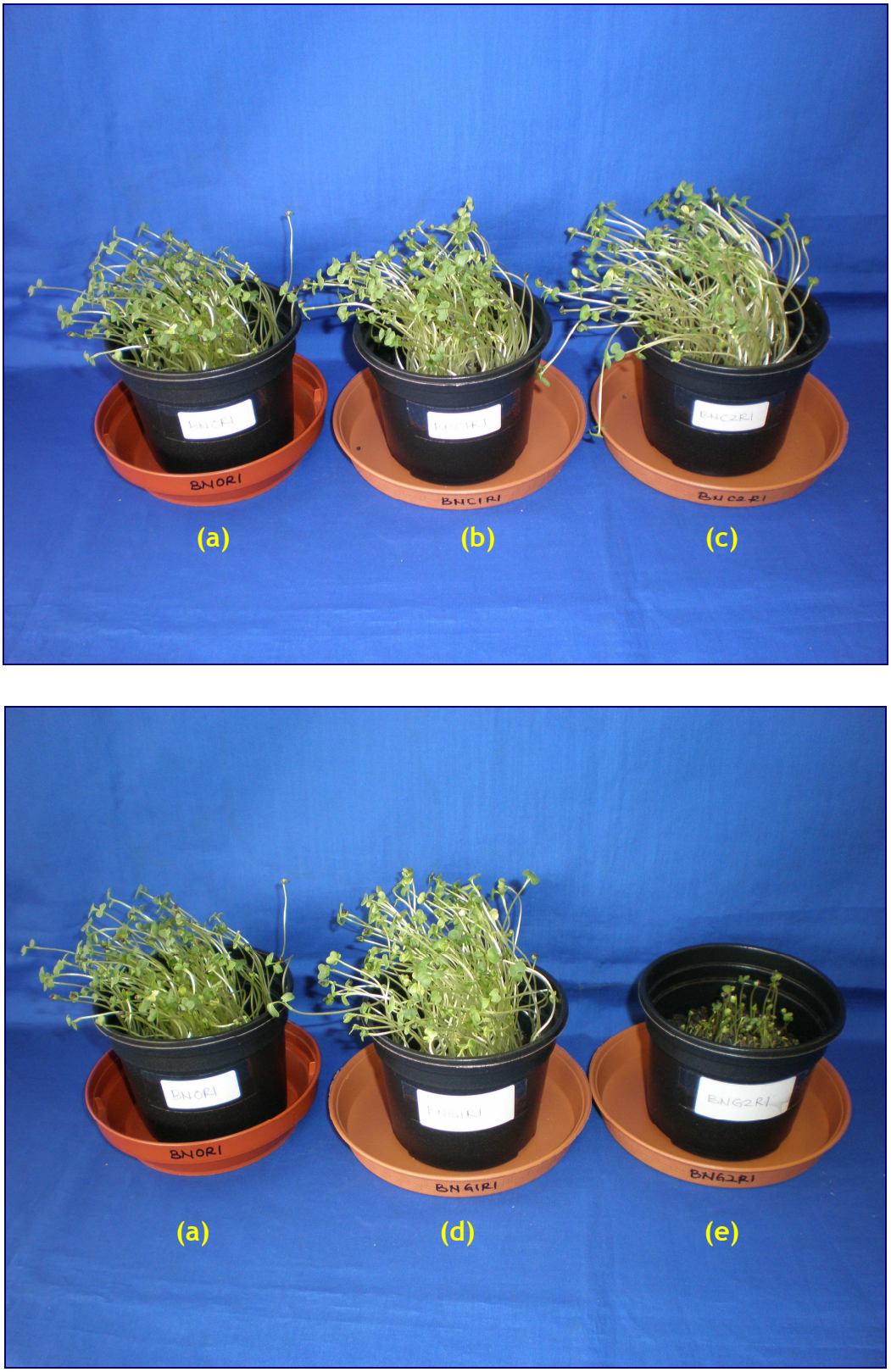


Figure 5.10 Rapeseed after one week of growth. (a) Zero treatment, (b) Chitosan 1% (w/w), (c) Chitosan 10% (w/w), (d) Chitosan-GLA 1% (w/w), and (e) Chitosan-GLA 10% (w/w).

Figure 5.11 Rapeseed after 3 weeks of growth. Zero: untreated contaminated soil, CHI 1%: Chitosan 1% (w/w), CHI 10%: Chitosan 10% (w/w), and C-GLA 1%: Chitosan-GLA 1% (w/w).

Figure 5.12 Rapeseed after 7 weeks of growth. Zero: untreated contaminated soil, CHI 1%: Chitosan 1% (w/w), CHI 10%: Chitosan 10% (w/w), and C-GLA 1%: Chitosan-GLA 1% (w/w).

Table 5.8 Biomass yield of perennial ryegrass.

Treatment	Dry weight (g/pot)			
	Shoot yield			Root yield
	First harvest	Second harvest	Total shoot yield	Second harvest
Zero	0.58 a	0.61 a	1.19 a	1.18 a
Chitosan 1%	0.83 b	0.79 b	1.62 b	1.68 b
Chitosan 10%	0.84 b	1.02 c	1.86 c	2.05 c
Chitosan-GLA 1%	0.74 b	0.81 b	1.55 b	1.79 b
LSD	0.13	0.10	0.23	0.15

Values represent mean of 6 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table 5.9 Biomass yield of rapeseed.

Treatment	Dry weight (g/pot)	
	Shoot yield	Root yield
Zero	1.99 a	1.49 a
Chitosan 1%	2.55 b	1.82 b
Chitosan 10%	2.95 c	2.16 c
Chitosan-GLA 1%	2.54 b	1.70 b
LSD	0.25	0.13

Values represent mean of 6 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table 5.10 Elemental composition of perennial ryegrass shoots.

Treatment	Weight (%)		
	C	N	C/N
Zero	40.1 ± 0.6	2.6 ± 0.2	15.4
Chitosan 1%	38.4 ± 0.4	4.4 ± 0.1	8.7
Chitosan 10%	39.1 ± 0.2	6.6 ± 0.4	5.9
Chitosan-GLA 1%	39.8 ± 0.7	3.1 ± 0.2	12.8

Values represent mean of three replicates ± standard deviation.

Table 5.11 Elemental composition of rapeseed shoots.

Treatment	Weight (%)		
	C	N	C/N
Zero	37.4 ± 0.3	2.4 ± 0.6	15.6
Chitosan 1%	38.9 ± 0.4	3.0 ± 0.2	13.0
Chitosan 10%	38.8 ± 0.6	6.9 ± 0.5	5.6
Chitosan-GLA 1%	38.1 ± 0.2	2.9 ± 0.1	13.1

Values represent mean of three replicates ± standard deviation.

5.4.2 Metal uptake by perennial ryegrass and rapeseed

5.4.2.1 Metal concentration in plant tissues

The concentrations of Cu, Pb and Zn in plant tissues after the pot experiment are shown in Figures 5.13 to 5.16 (see Appendix C, Tables C1 and C2 for full data set). From these figures, it is apparent that chitosan 10% (w/w) treatment reduced Pb and Zn concentrations in plant tissues of both plants. Marked reductions in Pb and Zn concentrations were obtained for the shoot tissues (Figures 5.13 and 5.15). Addition of chitosan and chitosan-GLA at 1% (w/w) increased the uptake of both metals by perennial ryegrass (Figures 5.13 and 5.14). This observation is consistent with the trend obtained in the first pot experiment (Figures 5.4 and 5.5, Section 5.2.2.1). The Pb and Zn root concentrations of rapeseed were also found to increase with chitosan and chitosan-GLA 1% (w/w) treatments (Figure 5.16). However, these treatments reduced the concentrations of Pb and Zn in the shoot tissue of rapeseed (Figure 5.15). This can be explained by the fact that rapeseed is known as a high biomass plant species (Fornes *et al.*, 2009) and that the addition of amendments increased its shoot yield (Table 5.9). This may have diluted the concentrations of Pb and Zn in its shoots.

The effect of amendments on Cu uptake was not very clear. There was no great difference in Cu shoot concentration for perennial ryegrass grown on untreated and chitosan 10% (w/w) treated soils (Figure 5.13). Meanwhile, perennial ryegrass cultivated on soils receiving amendments at 1% (w/w) showed a greater Cu shoot concentration than zero and 10% (w/w) chitosan treatments. In the case of rapeseed shoots, there was a reduction in Cu concentration following chitosan 1% (w/w) treatment (Figure 5.15). Rapeseed grown on untreated soil and soils treated with chitosan 10% and chitosan-GLA 1% (w/w) showed no difference in Cu shoot concentration. As shown in Figures 5.14 and 5.16, application of amendments at 1% (w/w) caused no great effect in reducing Cu root concentration of both plants. However, a slight decrease in Cu root concentration was obtained when chitosan was applied at 10% (w/w). The unclear effect on Cu uptake as affected by application of amendments confirms the findings of Nwachukwu and Pulford (2009). They reported that the addition of compost at 20% (w/w) did not affect the uptake of Cu by perennial ryegrass, in which they obtained similar Cu shoot concentration for plants grown on untreated and compost treated soils.

The concentrations of Pb and Zn in the shoots of perennial ryegrass (Figure 5.13), particularly for plants grown on untreated soil (zero treatment), were found to be lower than the concentrations determined in the first pot experiment (Figure 5.4, Section

5.2.2.1). However, no great difference was observed for Cu shoot concentration determined at both pot experiments. In addition, the root tissue of perennial ryegrass grown on the zero treatment seemed to accumulate similar amounts of metals (Figures 5.14 and 5.5). In Section 5.4.1, the difference in total shoot yield of perennial ryegrass for the zero and 1% (w/w) treatments, obtained at the end of both pot experiments was discussed in relation to several factors such as temperature, humidity and sunlight. It can be speculated that these factors may have also contributed to the difference in Pb and Zn uptake by perennial ryegrass grown on untreated soil and soils treated with 1% (w/w) amendments, determined at both experiments.

Hooda and Alloway (1994) studied the effect of temperature on metal accumulation in perennial ryegrass. They reported that the uptake of Cu was not influenced by the temperature, whereas the accumulation of Pb and Zn in plant shoots was highly affected by this factor. They found that plants grown in the warm environment (25 °C) accumulated higher concentrations of Pb and Zn than plants grown under cooler condition (15 °C). However, this effect was not reported for metal accumulation in the root tissue. The enhanced accumulation in the shoots of perennial ryegrass was thought to be due to the increase in transpiration rate of the plants.

Overall, results obtained from the second pot experiment highlight the potential of chitosan for Pb and Zn immobilisation, if applied at 10% (w/w). This observation is in agreement with the results obtained from the sorption study (Chapter 4), which confirmed the ability of chitosan to bind heavy metals. Depending on plant species, addition of chitosan and chitosan-GLA at 1% (w/w) may either enhance or reduce metal accumulation in plant tissues.

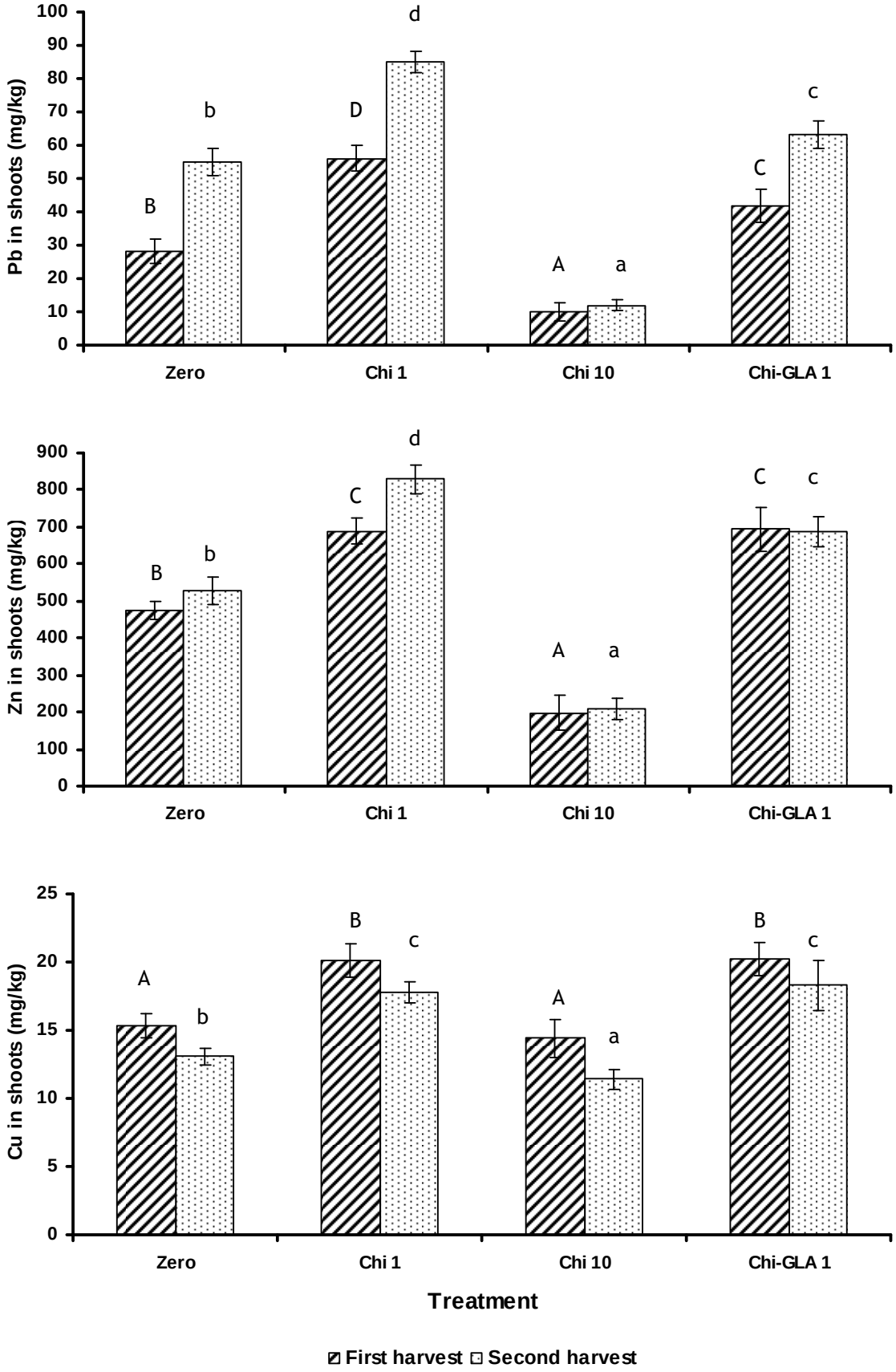


Figure 5.13 Metal concentrations in ryegrass shoots after 9 and 20 weeks of growth. Error bars are ± standard deviation of 18 replicates. Letters represent statistical differences (Tukey's test at $p < 0.05$) for the first harvest (upper case) and second harvest (lower case). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

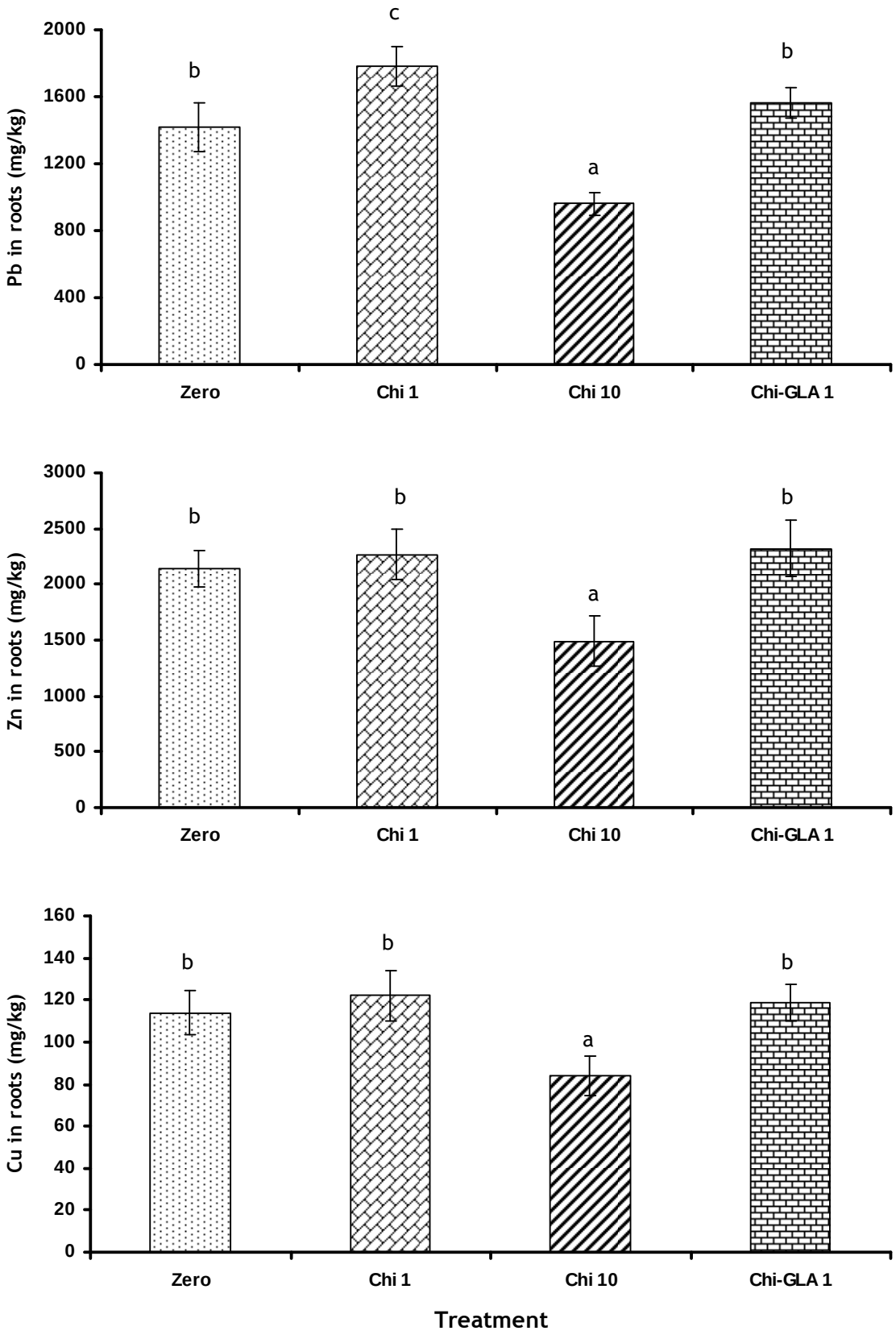


Figure 5.14 Metal concentrations in ryegrass roots after 20 weeks of growth. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

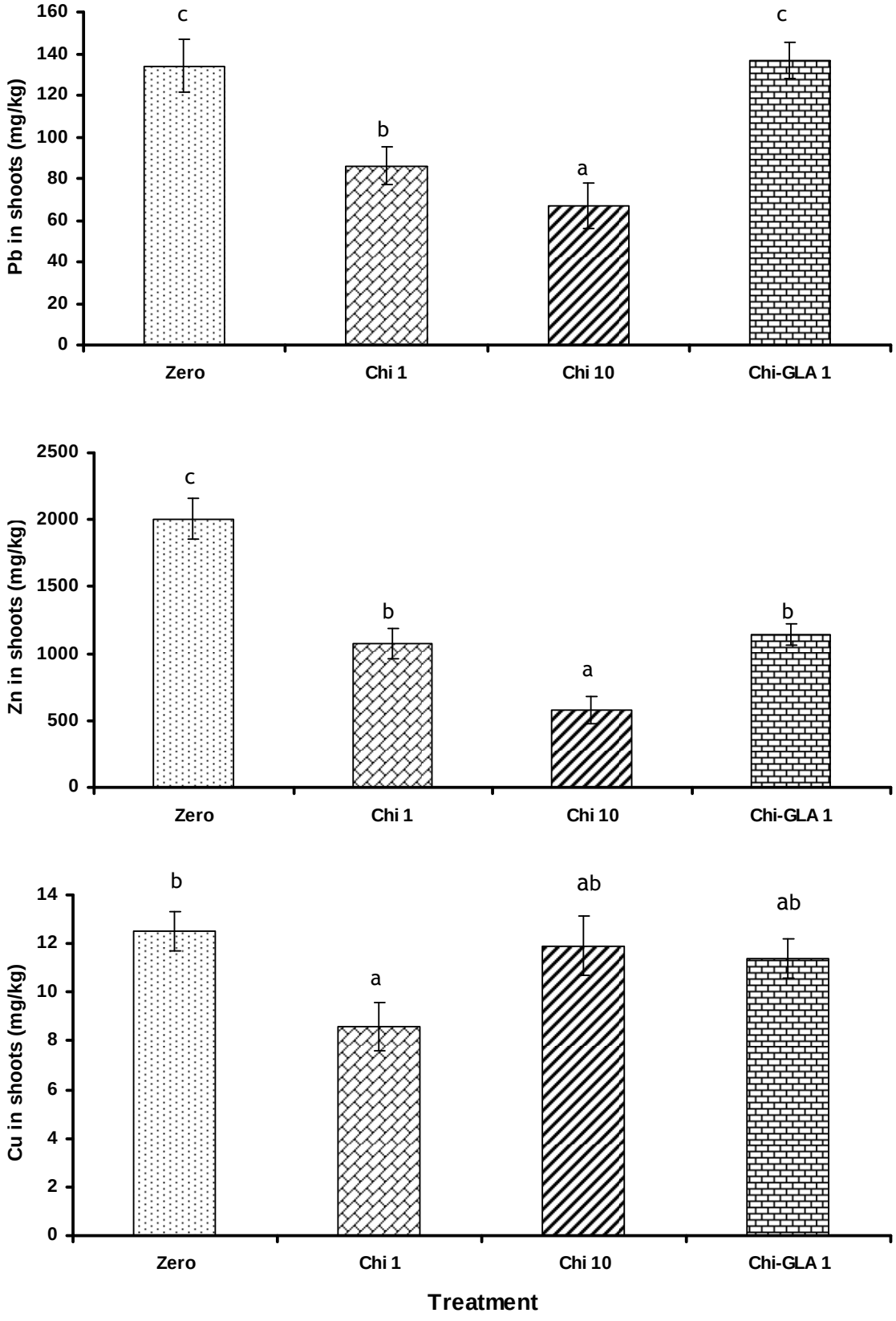


Figure 5.15 Metal concentrations in rapeseed shoots after 8 weeks of growth. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

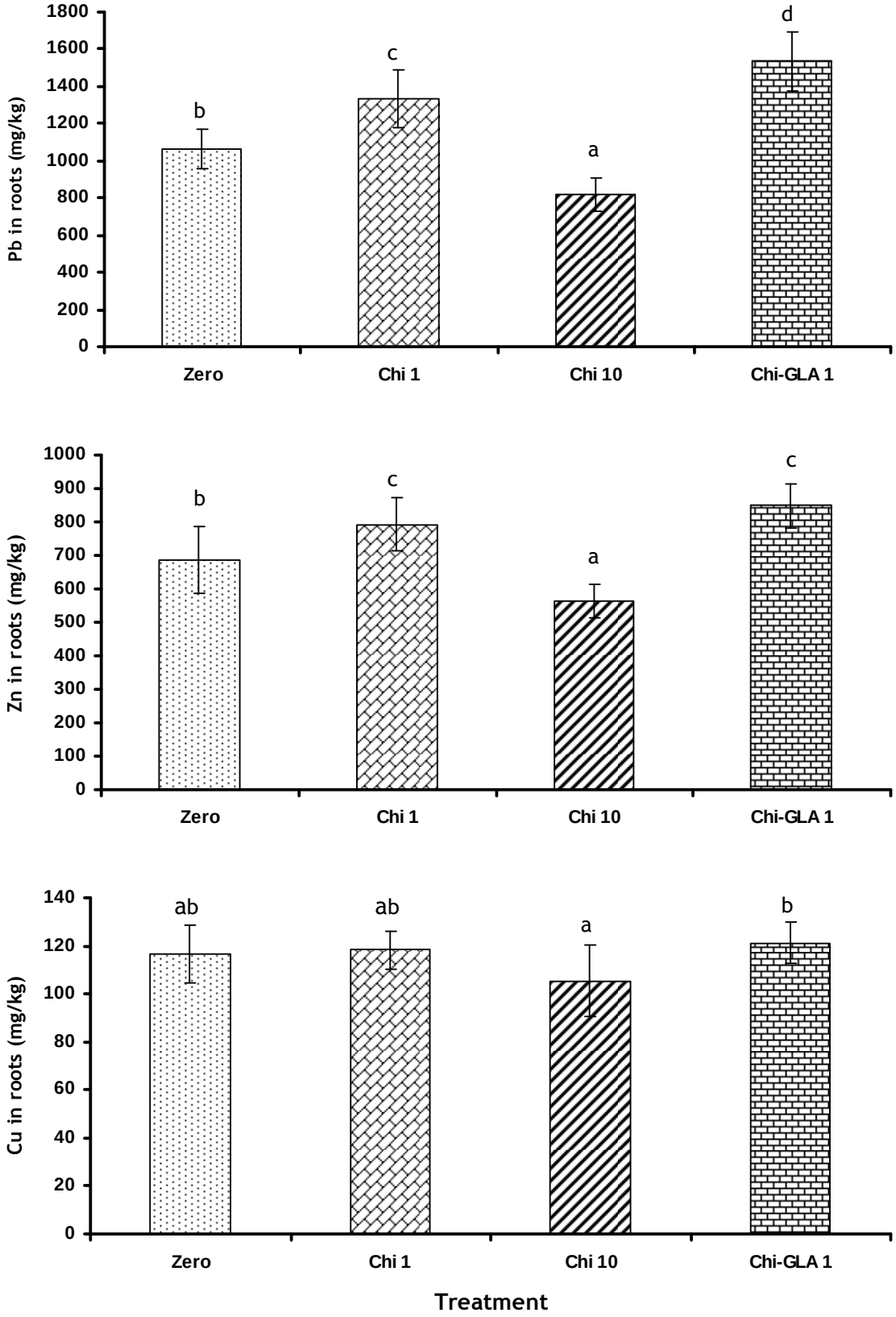


Figure 5.16 Metal concentrations in rapeseed roots after 8 weeks of growth. Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

5.4.2.2 Bioconcentration Factor

Tables 5.12 and 5.13 present the BCF values of metals for perennial ryegrass and rapeseed. The BCF values of Zn determined for zero treatment were found to be greater than 0.2. As discussed by McGrath and Zhao (2003), plants grown on contaminated soils often have a BCF value for heavy metals of less than 0.2. However, some plant species are able to take up and distribute large amounts of metals to their shoots (Vamerali *et al.*, 2010; Wenzel, 2009). In fact, the BCF value of Zn by rapeseed (1.211) is greater than 1. Plants with a BCF value of higher than 1 are suitable for phytoextraction (McGrath and Zhao, 2003; Yoon *et al.*, 2006; Ruiz *et al.*, 2009). Therefore, phytoextraction of Zn using rapeseed is feasible. Results suggest that both plants have great natural ability to accumulate Zn in their shoots.

For perennial ryegrass, addition of both amendments at the rate of 1% (w/w) increased the BCF values of metals at both harvests (Table 5.12). The ability of perennial ryegrass to accumulate Pb and Zn in its shoots was found to decrease with chitosan 10% (w/w) treatment. The BCF values of Pb and Zn estimated at the second harvest were found to be greater than the values obtained at the first harvest, suggesting the plants accumulated more Pb and Zn in their shoots after the first harvest. As discussed in Section 5.2.2.1, harvesting may have an effect in inducing the uptake of metal by perennial ryegrass. In contrast to Pb and Zn, a lower BCF value was obtained for Cu at the second harvest and this effect can be related to the lower concentration of Cu determined in plant shoots (Figure 5.13, Section 5.4.2.1). A similar trend was observed in the first pot experiment, for which lower BCF values of Cu were estimated at the second harvest as compared the BCF values obtained at the first harvest (Table 5.3).

In the case of rapeseed, in most cases application of amendments reduced the BCF values of metals (Table 5.13). However, the effect of amendments was less apparent for Cu, particularly for chitosan 10% and chitosan-GLA 1% (w/w) treatments. The accumulation of Pb in the rapeseed shoots was not affected by the chitosan-GLA 1% (w/w) treatment. Chitosan 10% (w/w) treatment gave the best effect in reducing Pb and Zn accumulation in the rapeseed shoots. The BCF value of Pb decreased from 0.064 (zero treatment) to 0.032, while the BCF value of Zn reduced from 1.211 to 0.349.

From Tables 5.12 and 5.13, the BCF values estimated for the treatments were in the order Zn > Cu > Pb, for both plants. Estimation of the BCF values of Pb and Zn for rapeseed grown on untreated and compost-treated contaminated soil by Fornes *et al.* (2009) yielded a greater BCF value for Zn than for Pb.

Table 5.12 BCF values for Pb, Zn and Cu (perennial ryegrass shoots).

Treatment	First harvest			Second harvest		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.013 b	0.286 b	0.116 a	0.026 b	0.319 b	0.099 b
Chitosan 1%	0.027 d	0.416 c	0.153 b	0.040 d	0.502 d	0.135 c
Chitosan 10%	0.005 a	0.120 a	0.109 a	0.006 a	0.126 a	0.086 a
Chitosan-GLA 1%	0.020 c	0.420 c	0.153 b	0.030 c	0.416 c	0.139 c
LSD	0.005	0.029	0.008	0.002	0.028	0.008

Values represent mean of 18 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table 5.13 BCF values for Pb, Zn and Cu (rapeseed shoots).

Treatment	Pb	Zn	Cu
Zero	0.064 c	1.211 c	0.095 b
Chitosan 1%	0.041 b	0.650 b	0.065 a
Chitosan 10%	0.032 a	0.349 a	0.090 ab
Chitosan-GLA 1%	0.066 c	0.689 b	0.087 ab
LSD	0.006	0.065	0.027

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

5.4.2.3 Translocation Factor

The effects of chitosan and chitosan-GLA amendment on the TF values of the metals are given in Tables 5.14 and 5.15. For perennial ryegrass, amending contaminated soil with amendments at 1% (w/w) increased the TF values of Pb and Zn at both harvests (Table 5.14). Meanwhile, application of chitosan at 10% (w/w) reduced the TF values of Pb and Zn at both harvests. Great reductions were obtained at the second harvest, whereby the TF value of Pb reduced from 0.039 to 0.013 and the TF value of Zn decreased from 0.253 to 0.143. Cu behaved differently, of which the translocation of Cu from the root to the shoot of perennial ryegrass enhanced with the application of amendments. Generally, the TF and BCF values of Pb and Zn showed a similar trend following the treatments (Tables 5.14 and 5.12), suggesting amendments had a similar effect on metal transport and accumulation in perennial ryegrass shoots. For example, chitosan 10% (w/w) treatment showed a consistent effect in lowering the TF and BCF values of Pb and Zn.

There was no great difference in the TF values of the metals estimated at both harvests, particularly for Pb and Zn following chitosan 10% (w/w). This treatment resulted in similar TF values of Pb and Zn at both harvests (Table 5.14). This observation

can be directly linked to the concentrations of both metals in the perennial ryegrass shoots determined at the first and second harvests (Figure 5.13). As shown in Figure 5.13, it is apparent that chitosan 10% (w/w) treatment had a consistent effect in reducing the accumulation of Pb and Zn, as compared to plants grown on untreated contaminated soils. The TF values of Pb and Zn for perennial ryegrass obtained in the second pot experiment were found to be lower than the values estimated in the first pot experiment, particularly for the zero treatment (Table 5.4). This can be related to the lower concentrations of Pb and Zn in the perennial ryegrass shoots (Figure 5.13), as compared to the concentrations determined in the first pot experiment (Figure 5.4).

From Table 5.15, rapeseed grown on zero treatment has a TF value of 2.983 for Zn. Plants with a TF value of greater than 1 are efficient in transporting metals from the root tissue to the shoot tissue and this characteristic is important for phytoextraction of heavy metals from contaminated soils (Cunningham *et al.*, 1995; Ruiz *et al.*, 2009; Kidd *et al.*, 2007). However, application of amendments decreased the TF value of Zn by rapeseed. A significant reduction in the TF value of Zn was achieved with chitosan 10% (w/w) treatment, whereby the TF value decreased from 2.983 to 1.036. Amendments applied at 1% (w/w) showed a similar effect in lowering the TF value of Zn.

The TF value of Pb by rapeseed reduced following the treatments. For example, chitosan 1% (w/w) decreased the TF value of Pb from 0.130 to 0.069. However, when comparing the TF value of Pb following chitosan 1% (w/w) treatment with the other two treatments, no great difference between the treatments was observed. In the case of Cu, the TF value was found to decrease with 1% (w/w) treatments, whereas chitosan 10% (w/w) treatment did not affect the transport of Cu from the root to shoot of rapeseed. In Section 5.4.2.2, it was observed that the addition of amendments, particularly chitosan at 10% (w/w), caused no great effect in reducing the accumulation of Cu in the rapeseed shoots (Table 5.13).

Table 5.14 TF values for Pb, Zn and Cu (perennial ryegrass).

Treatment	First harvest			Second harvest		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.019 b	0.225 b	0.141 a	0.039 b	0.253 b	0.122 a
Chitosan 1%	0.032 c	0.309 c	0.169 b	0.048 c	0.373 d	0.149 b
Chitosan 10%	0.011 a	0.134 a	0.173 b	0.013 a	0.143 a	0.138 ab
Chitosan-GLA 1%	0.027 c	0.306 c	0.174 b	0.041 b	0.302 c	0.158 b
LSD	0.007	0.038	0.024	0.005	0.041	0.022

Values represent mean of 18 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table 5.15 TF values for Pb, Zn and Cu (rapeseed).

Treatment	Pb	Zn	Cu
Zero	0.130 c	2.983 c	0.116 b
Chitosan 1%	0.069 a	1.375 b	0.077 a
Chitosan 10%	0.088 b	1.036 a	0.120 b
Chitosan-GLA 1%	0.093 b	1.352 b	0.081 a
LSD	0.016	0.292	0.017

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

5.4.2.4 Off-take values

Tables 5.16 and 5.17 present the off-take values of metals estimated for perennial ryegrass and rapeseed. The off-take value (mg/pot) was estimated according to Equation 5.1 (Section 5.2.2.4), while the estimation of off-take value (kg/ha) was based on conversion factor of pot area to hectare (Section 5.2.2.4). Amending contaminated soil with amendments at 1% (w/w) enhanced the removal of Pb and Zn by perennial ryegrass (Table 5.16). In general, application of amendments at the rate of 1% (w/w) showed a similar trend to that of results obtained in the first pot experiment (Table 5.5, Section 5.2.2.4). Following chitosan 10% (w/w) treatment, the removal of Pb by perennial ryegrass reduced from 0.05 mg/pot (0.13 kg/ha) to 0.02 mg/pot (0.05 kg/ha), while the off-take value of Zn decreased from 0.60 mg/pot (1.56 kg/ha) to 0.38 mg/pot (0.99 kg/ha).

When comparing to perennial ryegrass grown on untreated contaminated soil, it is obvious that the removal of Cu was not affected by the application of amendments. The off-take values of metals estimated in the second pot experiment were found to be lower than the values obtained in the first pot experiment. For example, for the zero treatment, the off-take values of Pb and Zn were found to be 0.58 and 1.53 mg/pot in the first pot experiment (Table 5.5), whereas 0.05 and 0.60 mg/pot were estimated in the second pot experiment (Table 5.16). As discussed in Section 5.4.2.1, experimental parameters such as temperature and humidity may have influenced the accumulation of heavy metals in the shoot and root tissues of perennial ryegrass.

In the case of rapeseed, addition of amendments reduced Zn removal (Table 5.17). A great reduction in the Zn removal was achieved with chitosan 10% (w/w) treatment, whereby the off-take value of Zn reduced from 3.99 mg/pot (10.37 kg/ha) to 1.70 mg/pot (4.42 kg/ha). Amendments at 1% (w/w) showed a similar effect in reducing the Zn removal. Application of chitosan decreased the off-take value of Pb, while chitosan-

GLA 1% (w/w) treatment enhanced the removal of Pb by rapeseed. In contrast to Pb and Zn, the removal of Cu was not affected by the treatments.

Overall, Zn was the metal most extracted by both plants, whereas Cu was the least. Rapeseed showed greater ability to extract Pb and Zn than perennial ryegrass, and therefore had higher off-take values of the metals. The removal of Pb and Zn by both plants was affected by the application of amendments, whereas the treatments did not influence the removal of Cu. The reduction in the off-take values of metals can be attributed to the immobilisation effect of the amendments. It is clear that the evaluation on metal immobilisation using plants as a receptor is greatly influenced by the ability of the plant to accumulate metal in its tissues. In this study, both plants showed low uptake of Cu. Therefore, the effect of amendments on Cu immobilisation in the contaminated soil and Cu uptake by plants was not very clear.

Table 5.16 Removal of heavy metals using perennial ryegrass (sum of the first and second harvests).

Treatment	Off-take (mg/pot)			Off-take (kg/ha)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.05	0.60	0.02	0.13	1.56	0.05
Chitosan 1%	0.11	1.23	0.03	0.29	3.20	0.08
Chitosan 10%	0.02	0.38	0.02	0.05	0.99	0.05
Chitosan-GLA 1%	0.08	1.07	0.03	0.21	2.78	0.08

Table 5.17 Removal of heavy metals using rapeseed.

Treatment	Off-take (mg/pot)			Off-take (kg/ha)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	0.27	3.99	0.03	0.70	10.37	0.08
Chitosan 1%	0.22	2.74	0.02	0.57	7.12	0.05
Chitosan 10%	0.19	1.70	0.04	0.49	4.42	0.10
Chitosan-GLA 1%	0.35	2.89	0.03	0.91	7.51	0.08

5.4.3 Metal concentration in soil

The concentrations of heavy metals in bulk soil used in the second pot experiment are given in Table 5.18. The bulk soil used for the second pot experiment had slightly higher content of ammonium acetate extractable Pb (945 mg/kg) as compared to 800 mg/kg Pb extracted using the same extractant in the first pot experiment (Table 5.6, Section 5.2.3). However, the total content of Pb measured in the bulk soil used for the second pot experiment (2088 mg/kg) was lower than total concentration of Pb obtained in the first pot experiment (3923 mg/kg, Table 5.6). In fact, the RSD (%) value estimated for total concentration of Pb in bulk soil for the first pot experiment (75.9%, Table 5.6) was greater than the RSD value obtained in the second pot experiment (24.5%). This suggests that the nature of distribution of Pb in the bulk soil used for the first pot experiment was less homogeneous.

Table 5.18 Bioavailable and total metal content in bulk soil samples.

Extractant	Metal concentration (mg/kg)					
	Pb	RSD (%)	Zn	RSD (%)	Cu	RSD (%)
Deionised water[a]	2.5	41.9	4.9	37.3	0.2	19.1
Deionised water[b]	3.7	57.0	6.1	50.5	0.2	45.7
Ammonium acetate	945	8.5	277	3.6	5	4.6
EDTA	1572	3.3	601	5.1	14	3.2
Aqua regia	2088	24.5	1654	33.6	132	44.0

Values represent mean of ten replicates. [a] Fresh soil; [b] Air-dried soil.

In the first pot experiment, untreated and treated contaminated soils produced no significant difference in the EDTA extractable Pb and Cu in the soils, after 24 weeks of the trial (Figure 5.7). In addition, the results obtained for EDTA extraction suggest that there were slight reductions in amount of metals following the application of amendments, as compared to bulk soil (before the pot experiment). In contrast, ammonium acetate extraction revealed the differences in metal bioavailability following the treatments (Figure 5.6). Therefore, only ammonium acetate extractable metals were determined after the plant growth of the second pot experiment.

The ammonium acetate extractable metals in soil after 20 and 8 weeks of the pot experiment are shown in Figures 5.17 and 5.18 (see Appendix C, Tables C3 and C4 for full data set). From these figures, it is apparent that chitosan 10% (w/w) treatment decreased the bioavailability of heavy metals in soil. In fact, this treatment caused a consistent effect in reducing metal bioavailability. For example, the ammonium acetate extractable Pb was found to decrease from 945 mg/kg (Table 5.18) to 625 mg/kg

(Figure 5.17) and 633 mg/kg (Figure 5.18), while the concentration of Zn reduced from 277 mg/kg (Table 5.18) to 176 mg/kg (Figure 5.17) and 185 mg/kg (Figure 5.18).

A lower effect in reducing the metal bioavailability was observed when amendments were applied at 1% (w/w). For example, the ammonium acetate extractable Zn in bulk soil, 277 mg/kg (Table 5.18), decreased to 270 mg/kg (zero treatment), 185 mg/kg (chitosan 10% w/w), 246 mg/kg (chitosan 1% w/w) and 258 mg/kg (chitosan-GLA 1% w/w) after 8 weeks of pot trial for rapeseed (Figure 5.18). In the same experiment, the concentration of Cu in bulk soil, 5.0 mg/kg (Table 5.18), was found to reduce to 2.1 mg/kg (zero treatment), 0.8 mg/kg (chitosan 10% w/w), 1.2 mg/kg (chitosan 1% w/w) and 1.4 mg/kg (chitosan-GLA 1% w/w) (Figure 5.18). In the first pot experiment, chitosan and chitosan-GLA 1% (w/w) treatments also resulted in low percentage of reductions, as compared to the zero treatment (Figure 5.6).

As discussed in Section 5.2.3, the reduction in the amount of metal extracted after the pot experiment can be related to immobilisation effect of the amendments and uptake by plants. Since plants were watered with deionised water daily and received nutrient solution once a week, metals may have leached from the soil to the pot saucer. Therefore, leaching may have also contributed to the decrease in metal concentration of soil.

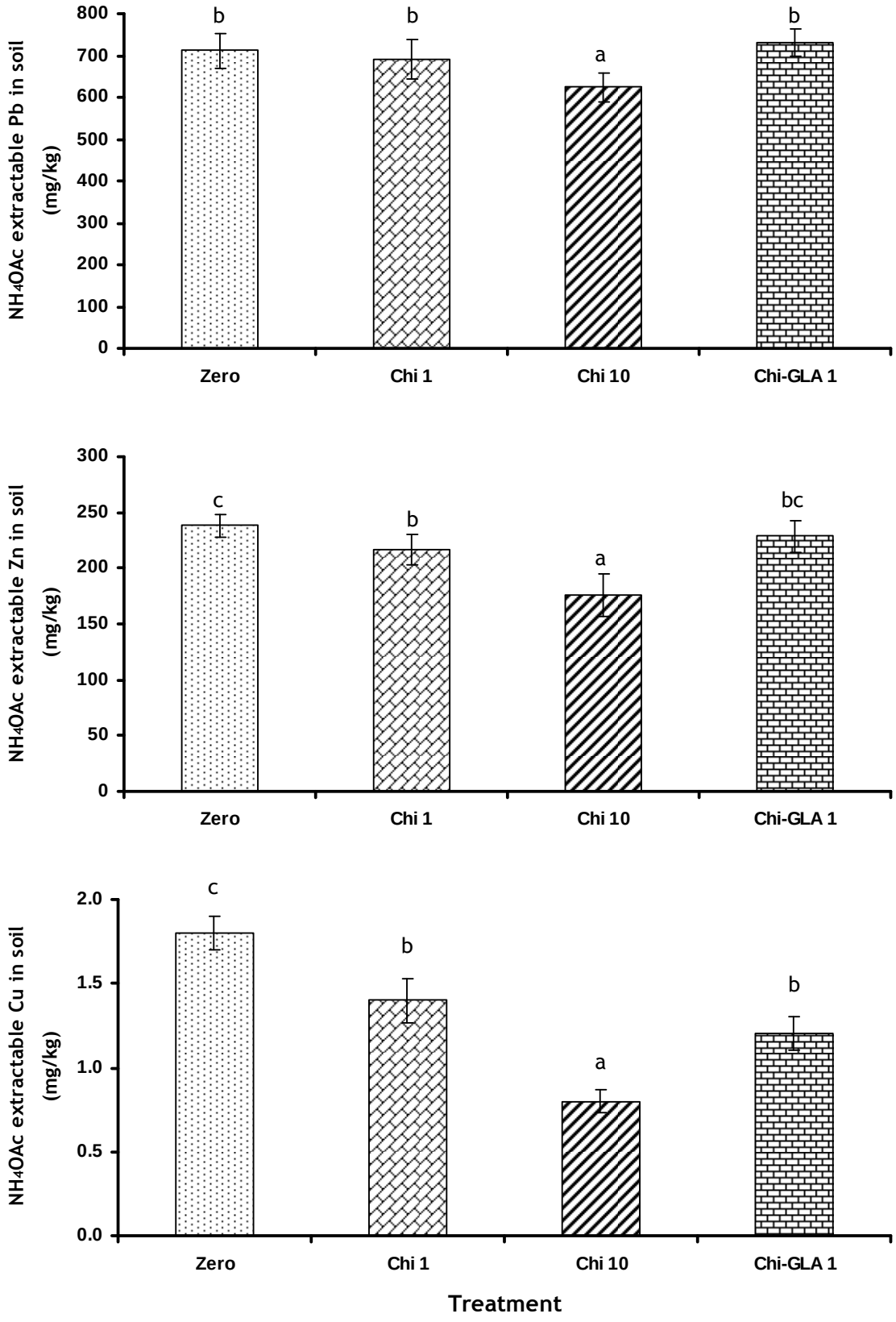


Figure 5.17 Ammonium acetate extractable metals in soil after 20 weeks of pot trial (perennial ryegrass). Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

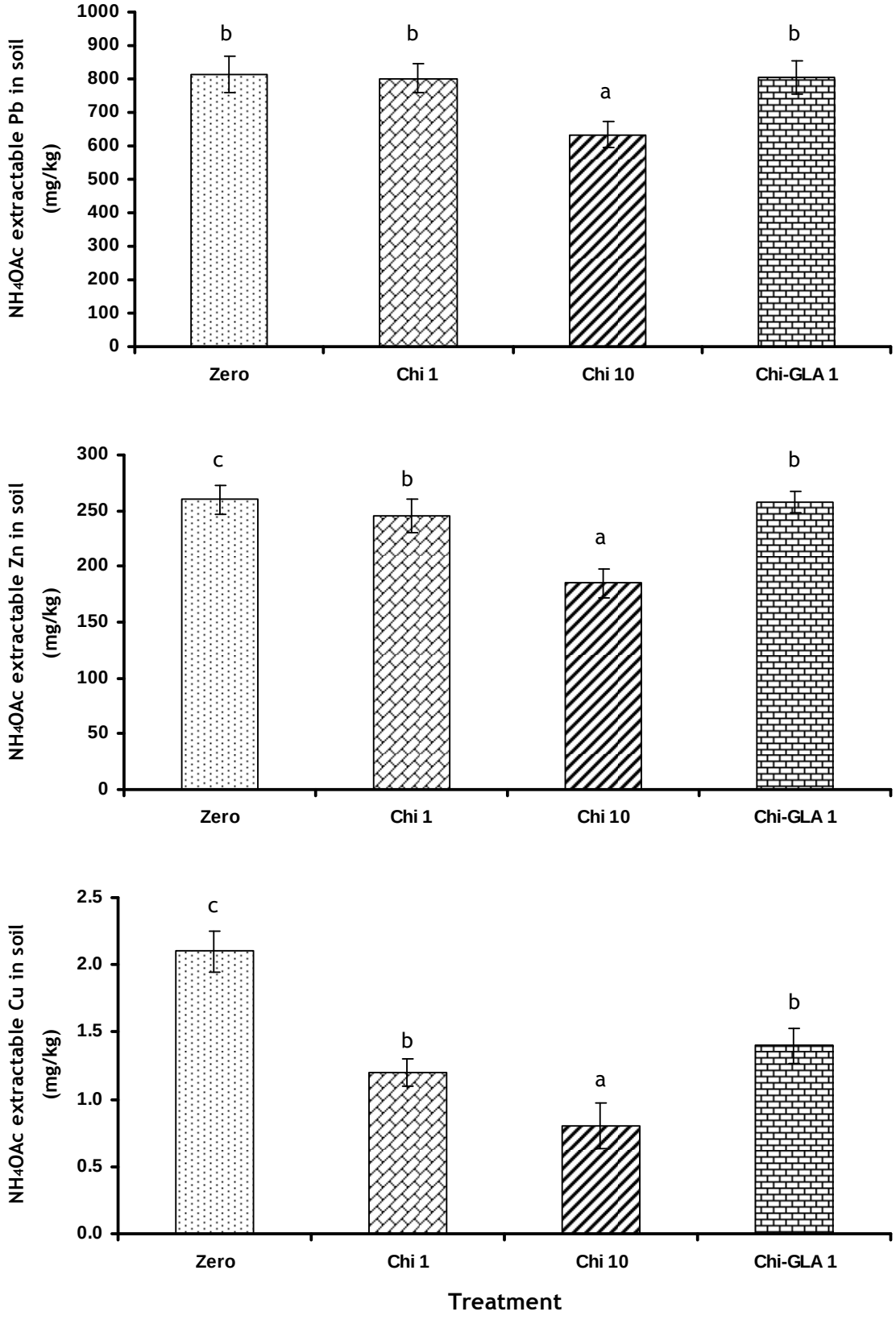


Figure 5.18 Ammonium acetate extractable metals in soil after 8 weeks of pot trial (rapeseed). Values represent mean of 18 replicates ± standard deviation. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$). Chi 1: Chitosan 1% (w/w), Chi 10: Chitosan 10% (w/w), and Chi-GLA 1: Chitosan-GLA 1% (w/w).

5.4.4 Soil pH

The effect of chitosan and chitosan-GLA amendment on soil pH is given in Table 5.19. Addition of both amendments to contaminated soil increased the soil pH by 0.3 to 0.5 units. A slight increase (0.3 to 0.7 units) was also observed in the first pot experiment (Table 5.7, Section 5.2.4), when both amendments were applied at the rates of 0.5% and 1.0% (w/w). An increase in the application rate of amendments up to 10% (w/w) did not change the soil pH.

Table 5.19 Effect of chitosan and chitosan-GLA amendment on soil pH.

Treatment	pH[a]	pH[b]
Bulk soil (before pot experiment)	4.5 ± 1.2	4.5 ± 0.3
Zero	4.8 ± 0.7	4.8 ± 0.9
Chitosan 1%	4.9 ± 0.5	4.8 ± 0.8
Chitosan 10%	4.9 ± 1.4	4.9 ± 0.5
Chitosan-GLA 1%	4.8 ± 0.6	5.0 ± 1.1

Values represent mean of three replicates ± standard deviation. [a] perennial ryegrass; [b] rapeseed.

5.5 Summary

Amending contaminated soil with chitosan (0.5-10.0% w/w) and chitosan-GLA (0.5-1.0% w/w) improved plant growth and increased biomass yield. Elemental analysis on the shoots of perennial ryegrass and rapeseed revealed that there was an increase in the nitrogen content following the application of amendments. Chitosan and chitosan-GLA contain 7-8% of nitrogen (Section 3.3.2) that can provide plant nitrogen nutrition for growth and survival. However, addition of chitosan-GLA at 10% (w/w) caused a toxicity effect on plants (Section 5.4.1). This effect was likely due to glutaraldehyde (GLA), the chemical used in cross-linking treatment of chitosan (Section 2.4). No toxicity symptoms were observed on plants grown on untreated contaminated soil, suggesting both plants have great tolerance to high metal concentrations.

The first pot experiment showed that application of amendments at 0.5% and 1.0% (w/w) increased the removal of Pb and Zn by perennial ryegrass (Table 5.5). Zn was the metal most removed, while Cu was the least. The BCF and TF values of Pb (Tables 5.3 and 5.4) estimated at the second harvest were found to be greater than the values obtained at the first harvest. This suggests that the ability of perennial ryegrass to accumulate and translocate Pb in its tissues increased after the first harvest. Harvesting may have an effect in enhancing metal uptake by plants. Following the harvest, plants

seek nutrients for re-growth. There was a possibility that metals may have also extracted into the plant tissues during the uptake of nutrients.

In the second pot experiment, amendments were applied at the rates of 1% and 10% (w/w). Their effects on plant growth and metal accumulation in plant tissue were determined using perennial ryegrass and rapeseed. Results obtained from the second pot experiment (Table 5.16) confirmed the earlier findings observed in the first pot experiment, for which application of chitosan and chitosan-GLA at 1% (w/w) enhanced the removal of Pb and Zn by perennial ryegrass (Table 5.5). Based on the off-take values estimated in the second pot experiment (Tables 5.16 and 5.17), it is clear that the removal of Pb and Zn by perennial ryegrass and rapeseed reduced with chitosan 10% (w/w) treatment.

The ammonium acetate and EDTA extractable metals in soil were found to decrease after the first pot experiment. However, no great difference was obtained in amount of metal extracted from untreated and treated contaminated soils (Figures 5.6 and 5.7). The decrease in metal concentration of soil can be attributed to immobilisation effect by the amendments, as well as plant uptake and leaching. The determination of metal concentration in soil after the pot experiment was influenced by the presence of fine root tissues. Soil analysis after the second pot experiment showed that the bioavailability of heavy metals decreased with chitosan 10% (w/w) treatment (Figures 5.17 and 5.18). A lower effect in reducing the bioavailability of heavy metals was observed when amendments were applied at 1% (w/w). The reduction in metal concentration of soil following the treatments confirmed the results obtained from the sorption study (Chapter 4), which showed that chitosan and cross-linked chitosans able to bind metal.

Overall, results from the pot experiments highlight the potential of chitosan and chitosan-GLA for reducing metal uptake by plants, as well as enhancing phytoextraction. The effectiveness of amendments for metal immobilisation is mainly affected by application rate of amendment and plant species. It is apparent that perennial ryegrass and rapeseed have poor ability to take up Cu. As a result, the ability of amendments to immobilise and to reduce Cu uptake by the plants was not very clear. Depending on plant species, the use of chitosan and chitosan-GLA at 0.5% and 1.0% (w/w) can be promising in enhancing the efficiency of phytoextraction. Chitosan 10% (w/w) treatment showed the best effect in improving plant growth, increasing biomass yield, reducing plant metal uptake and lowering ammonium acetate extractable metals in soil.

6 BIODEGRADATION AND METAL BIOAVAILABILITY STUDIES

In Chapter 5, the ability of amendments to reduce plant metal uptake and immobilise heavy metals in a contaminated soil was investigated. Although such aspects are important, the effectiveness of amendments also relies on their stability in soil (Farrell *et al.*, 2010; Tandy *et al.*, 2009). It has been hypothesised that the degradation of amendments in soil may release metals that previously bound to them (Farrell *et al.*, 2010; Doelsch *et al.*, 2010). Therefore, a biodegradation study was carried out to understand the nature of microbial breakdown of amendments in contaminated soil. In addition, the influence of biodegradation on metal bioavailability was also studied.

There are two main sections in this chapter. The first section reports the effect of metal addition to soil on biodegradation of chitosan and cross-linked chitosans. The second section reports the results obtained for metal bioavailability study.

6.1 Objectives of the study

1. To determine the biodegradation of chitosan and cross-linked chitosans in metal contaminated soil.
2. To study the effect of biodegradation on metal bioavailability.

6.2 Biodegradation study

It has been estimated that about 20-40% of the carbon added to the soil is assimilated by microorganisms (Ajwa and Tabatabai, 1994), while the rest is lost as CO_2, mainly as a product of microbial respiration (Machulla *et al.*, 2004; Usman *et al.*, 2004). As further discussed by Nwachukwu and Pulford (2011), Tejada and Gonzalez (2006) and Montemurro *et al.* (2009), CO_2 evolution can be used as an effective measure of biodegradation of a material in soil.

The biodegradation study was carried out twice. The first trial was performed in June 2010 using chitosan only, while the second trial was conducted in September 2010 on chitosan and cross-linked chitosans. In both trials, the effect of metal addition on biodegradation of amendments was studied at three concentrations namely 5, 10 and 50 mg/kg. The microbial breakdown of the amendments in non-contaminated soil (zero

metal treatment) was also studied. Experiments were carried out for a total of 12 weeks, and the procedure is fully described in Section 2.12.

6.2.1 First trial

The amount of CO_2 evolved per week and cumulative CO_2 evolved for biodegradation of chitosan in soil during 12 weeks of the study are shown in Figure 6.1. From Figure 6.1, it is apparent that the biodegradation of chitosan was affected by the 50 mg/kg Zn treatment, whereas no obvious effect was obtained for other metal treatments. In the first week, the amount of CO_2 evolved ranged from 1040 to 1520 mg C per kg. The fact that there was no great difference in the amount of CO_2 evolved suggests that the microorganisms had similar level of activity.

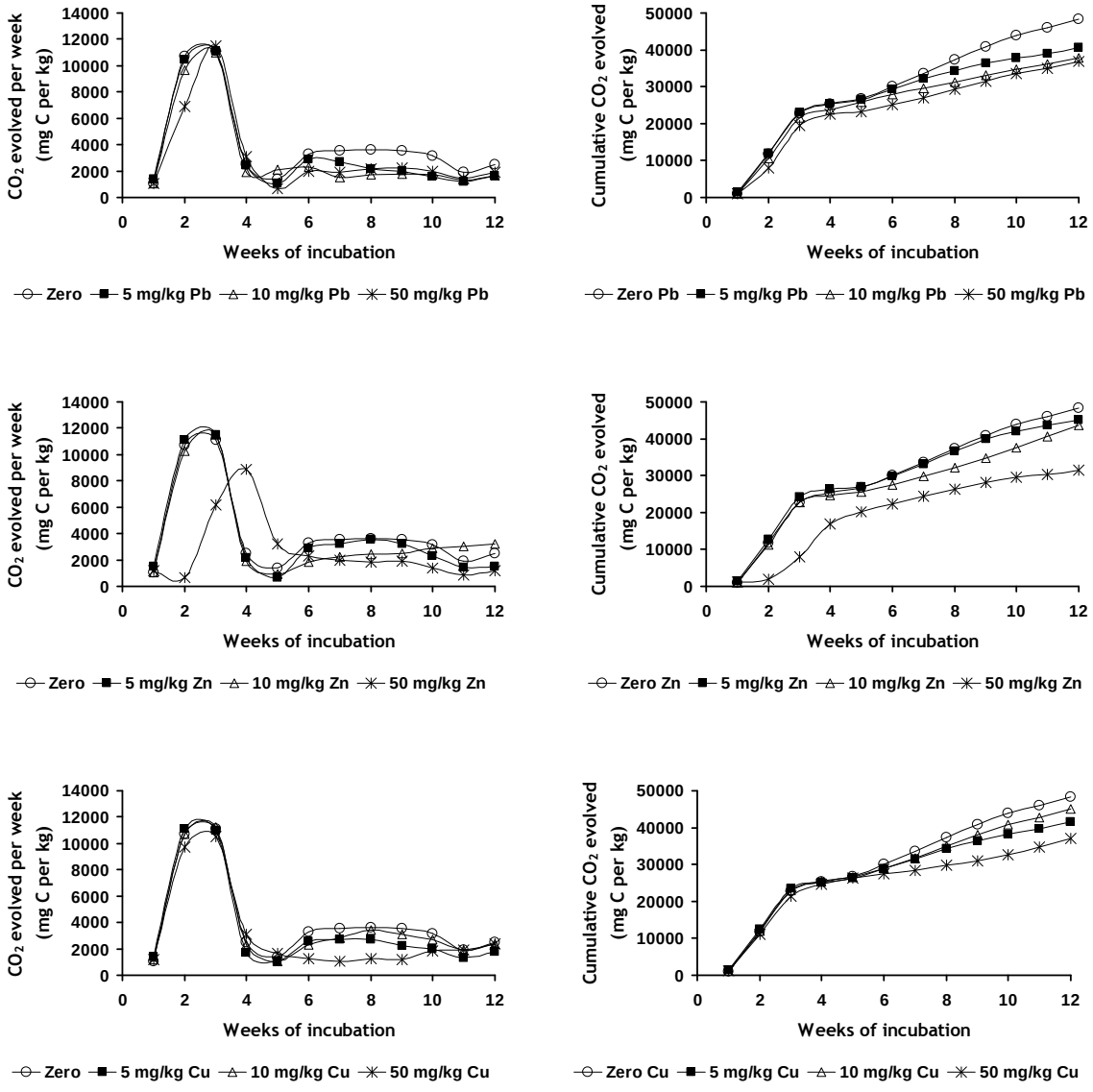


Figure 6.1 Amounts of CO_2 evolved per week and cumulative CO_2 evolved (as mg C per kg of chitosan) (first trial).

For the second week, except for soil treated with 50 mg/kg Zn, soils showed a marked increase in the amount of CO_2 evolved. However, the CO_2 released from soils treated with Cu and Pb at 50 mg/kg was found to be lower than soil which had received no metal treatment. Meanwhile, the lowest amount of CO_2 evolved was measured for soil treated with 50 mg/kg Zn. The amount of CO_2 evolved (mg C per kg) at week 2 was determined as 10,720 for zero metal treatment, 9760 for 50 mg/kg Cu, 6880 for 50 mg/kg Pb, and 640 for 50 mg/kg Zn. Low relative amounts of CO_2 evolved can be attributed to metal toxicity, and Zn was particularly pronounced in this effect.

The CO_2 evolution was found to increase up to week 3 of the study. In fact, microorganisms seemed to survive with Cu and Pb treatments at 50 mg/kg. This indicates that microorganisms were able to overcome the toxicity effect of both metals. Only 50 mg/kg Zn caused an inhibitory effect on chitosan degradation at week 3. The amount of CO_2 evolved determined at week 3 ranged from 11,000 to 11,520 mg C per kg for the treatments. In the case of 50 mg/kg Zn, the highest amount of CO_2 evolved was achieved at week 4 with an amount of 8880 mg C per kg.

However, soils showed a marked reduction in CO_2 evolution after 4 and 5 weeks of the study. This was also observed for soil which had received no metal addition. Therefore, such reduction was not due to metal toxicity. Instead, the readily degradable fractions of chitosan were exhausted. Except for 50 mg/kg Zn treatment, the amounts of CO_2 evolved at week 5 ranged from 680 to 2120 mg C per kg, which were quite close to the values obtained at the first week of the study. This indicates that the microbial activity at this stage was similar to that at the beginning of the degradation process (week 1). A slight increase in CO_2 evolution was obtained at week 6. This observation might be due to degradation of the more resistant fractions of chitosan by microorganisms.

During weeks 6 to 10, each treatment released rather similar amounts of CO_2 implying that a steady state system was established. However, a marginal increase in CO_2 evolution was observed for 10 mg/kg Zn, while 50 mg/kg Zn showed a gradual decrease. During the same period, non-contaminated soil released the highest amount of CO_2, while the lowest was observed for soil contaminated with Cu and Zn at concentration of 50 mg/kg.

As shown in Figure 6.1, the cumulative curves show three distinct stages of CO_2 evolution, initially rapid at weeks 1-3, relatively constant at weeks 3-5 and gradually increasing at weeks 5-12. However, this observation was less apparent for soils treated with 50 mg/kg metals. The breakdown of the easily degradable fractions of chitosan by a population of microorganisms was highly efficient at weeks 2 and 3. This contributed

to the rapid increase in cumulative CO_2 evolved observed at weeks 1-3. As the most readily degradable chitosan was used up, it may be that the initial microbial population decreased, and was replaced by a different population that was able to utilise the more resistant chitosan. This second population built up in weeks 3-5. The rate of CO_2 evolution was lower during this period than in weeks 1-3. The increase in the rate of CO_2 evolved observed at weeks 5-12 was likely due to degradation of the more resistant fractions of chitosan by the second population of microorganisms, which had built to an optimum during weeks 3-5.

The weekly evolution rate of CO_2 and the corresponding R^2 values are given in Table 6.1. The evolution rate was estimated for each stage of the CO_2 evolution, as mentioned earlier. From Table 6.1, except for soil treated with 50 mg/kg Zn, all soils showed high rates of CO_2 evolution during weeks 1-3 with values ranging from 9200 to 11,300 mg CO_2-C per kg per week. The R^2 values obtained during weeks 1-3 were between 0.9792 and 1.0000, suggesting linear rate of CO_2 production. This observation is in agreement with the results obtained for CO_2 evolved at weeks 2 and 3, which clearly showed that there was a marked increase in CO_2 evolution from week 1 to weeks 2 and 3. In the case of 50 mg/kg Zn, the greatest rate of CO_2 production was achieved at weeks 3-5 with a value of 6040 mg CO_2-C per kg per week. Following 50 mg/kg Zn treatment, microorganisms suffered Zn toxicity at week 2, which caused a delay in biodegradation of chitosan. As a consequence, the CO_2 production peaked at week 4 of the study.

Based on the cumulative curves of CO_2 evolution, the effect of Pb and Zn addition on biodegradation of chitosan in soil was in the order (least to greatest effect) 0 mg/kg < 5 mg/kg < 10 mg/kg < 50 mg/kg. In the case of Cu, the order (least to greatest effect) was found to be 0 mg/kg < 10 mg/kg < 5 mg/kg < 50 mg/kg. From the cumulative curves, it is clear that 50 mg/kg Zn had a significant effect on microbial breakdown of chitosan, as compared to Cu and Pb at the same concentration. The total amount of CO_2 evolved (mg CO_2-C per kg) during 12 weeks of the biodegradation study was estimated as 48,400 for zero metal treatment, 37,000 for 50 mg/kg Cu, 36,800 for 50 mg/kg Pb and 31,480 for 50 mg/kg Zn. Meanwhile, for 5 and 10 mg/kg metal treatments the total amount of CO_2 released for chitosan biodegradation ranged from 37,720 to 45,040 mg CO_2-C per kg.

Assuming there is no change in rate of CO_2 evolution after 12 weeks of the study, the total number of years required for biodegradation of chitosan in both non-contaminated and contaminated soils can be estimated (Table 6.2). The estimation was based on the three rates of CO_2 evolution identified above. From Table 6.2, the degradation of chitosan in non-contaminated soil will be complete in 6 years. It is clear that a longer

period is needed to breakdown the chitosan in such contaminated soil and this can be directly related to the effect of metal toxicity on microbial activity.

Table 6.1 Evolution rate of CO_2 for biodegradation of chitosan in soil (first trial).

Week	Metal (mg/kg)	Evolution rate (mg CO_2-C per kg per week)					
		Pb	R^2	Cu	R^2	Zn	R^2
1-3	Zero	10,920	0.9999	10,920	0.9999	10,920	0.9999
	5	10,800	0.9997	11,040	1.0000	11,300	0.9999
	10	10,320	0.9986	10,980	0.9998	10,880	0.9988
	50	9200	0.9792	10,140	0.9995	3420	0.8195
3-5	Zero	1960	0.9735	1960	0.9735	1960	0.9735
	5	1720	0.9505	1360	0.9819	1420	0.9170
	10	2020	0.9992	1660	0.9556	1420	0.9603
	50	1900	0.8792	2380	0.9688	6040	0.9314
5-12	Zero	3155	0.9919	3155	0.9919	3155	0.9919
	5	2000	0.9789	2192	0.9869	2685	0.9793
	10	1685	0.9969	2742	0.9949	2598	0.9932
	50	1964	0.9976	1494	0.9781	1625	0.9786

Table 6.2 Number of years required for chitosan biodegradation (first trial).

Metal	Concentration (mg/kg)	Years required
Zero	Zero	6
Pb	5	9
	10	11
	50	10
Cu	5	9
	10	7
	50	12
Zn	5	7
	10	7
	50	12

6.2.2 Second trial

Figure 6.2 presents the amount of CO_2 evolved per week and cumulative CO_2 evolved for biodegradation of chitosan in soil (second trial). In general, the CO_2 evolution for biodegradation of chitosan observed in the second trial showed a similar trend to that of obtained in the first biodegradation trial (Figure 6.1). Results from both degradation studies suggest that the breakdown of the readily degradable fractions of chitosan

occurred at weeks 2 and 3, while the degradation of the more resistant fractions of chitosan began at week 6 of the incubation study.

From Figure 6.2, it is apparent that the readily degradable fractions of chitosan were broken down by microorganisms at weeks 2 and 3. However, microbial activity was inhibited by the addition of metals at a concentration of 50 mg/kg. As a consequence, the microbial breakdown of the readily degradable fractions of chitosan was delayed. Zn was pronounced in this effect, with the biodegradation of chitosan peaking at week 6 of the study. A similar observation was reported by Leita *et al*. (1995). They found that Zn caused a significant inhibitory effect on microbial respiration, as compared to Pb.

In the first week, the amount of CO_2 evolved ranged from 1200 to 1920 mg C per kg, comparable to the values estimated for the first week of the first biodegradation trial (1040 to 1520 mg C per kg). The fact that there was no great difference in amount of CO_2 released from the soils indicates that microorganisms in non-contaminated and contaminated soils had a similar ability to breakdown chitosan. This observation also suggests that there was no obvious difference in microbial activity within 7 days of incubation.

Except for soil treated with 50 mg/kg Zn, the CO_2 evolution for biodegradation of chitosan in soil increased at week 2. A slight increase in amount of CO_2 released was obtained for soil treated with 50 mg/kg Pb, for which the amount increased from 1200 to 1360 mg C per kg. Little difference in CO_2 evolution was observed for 50 mg/kg Cu treatment, whereby the amount of CO_2 evolved increased from 1200 to 1280 mg C per kg. The 50 mg/kg Zn treatment reduced the amount of CO_2 released from 1260 (week 1) to 280 mg C per kg at week 2. Although other treatments caused a difference in the amount of CO_2 evolved at week 2, no clear trend was observed. For example, soil which had received 10 mg/kg Pb released a higher amount of CO_2 than the zero metal and 5 mg/kg Pb treatments. In the case of Cu and Zn treatments, 5 mg/kg metal treatment showed the highest amount of CO_2 evolved, whereas a lower amount was measured for the zero metal and 10 mg/kg metal treatments. According to Leita *et al*. (1999) and Kiikkilä *et al*. (2001), an unclear pattern in CO_2 evolution observed at early period of incubation indicates that microorganisms are in the adaptation stage.

The biodegradation of chitosan in soils treated with metals at concentrations of 0 mg/kg, 5 mg/kg and 10 mg/kg peaked at week 3, with no obvious difference in the amount of CO_2 evolved by the various soils. The amount of CO_2 released ranged from 9480 to 10,800 mg C per kg. Although soils treated with Pb and Cu at a concentration of 50 mg/kg also showed an increase at week 3, the amount of CO_2 released was found to

be lower than the amount determined for the other treatments. 50 mg/kg Zn treatment continued to cause an inhibitory effect on microbial breakdown of chitosan, whereby the amount of CO_2 evolved increased slightly from 280 (week 2) to 840 mg C per kg estimated at week 3.

Marked reductions in CO_2 evolution were observed at weeks 4 and 5. Except for soils treated with 50 mg/kg metals, a slight increase in microbial breakdown was obtained at week 6 and soils released rather similar amount of CO_2 during the period of weeks 6 to 12. These observations are consistent with the trend observed in the first biodegradation trial. As previously discussed, the reduction in amount of CO_2 evolved at weeks 4 and 5 can be attributed to the change in the microbial population, while an increase in the CO_2 evolution at week 6 can be related to the degradation of the more resistant fractions of chitosan.

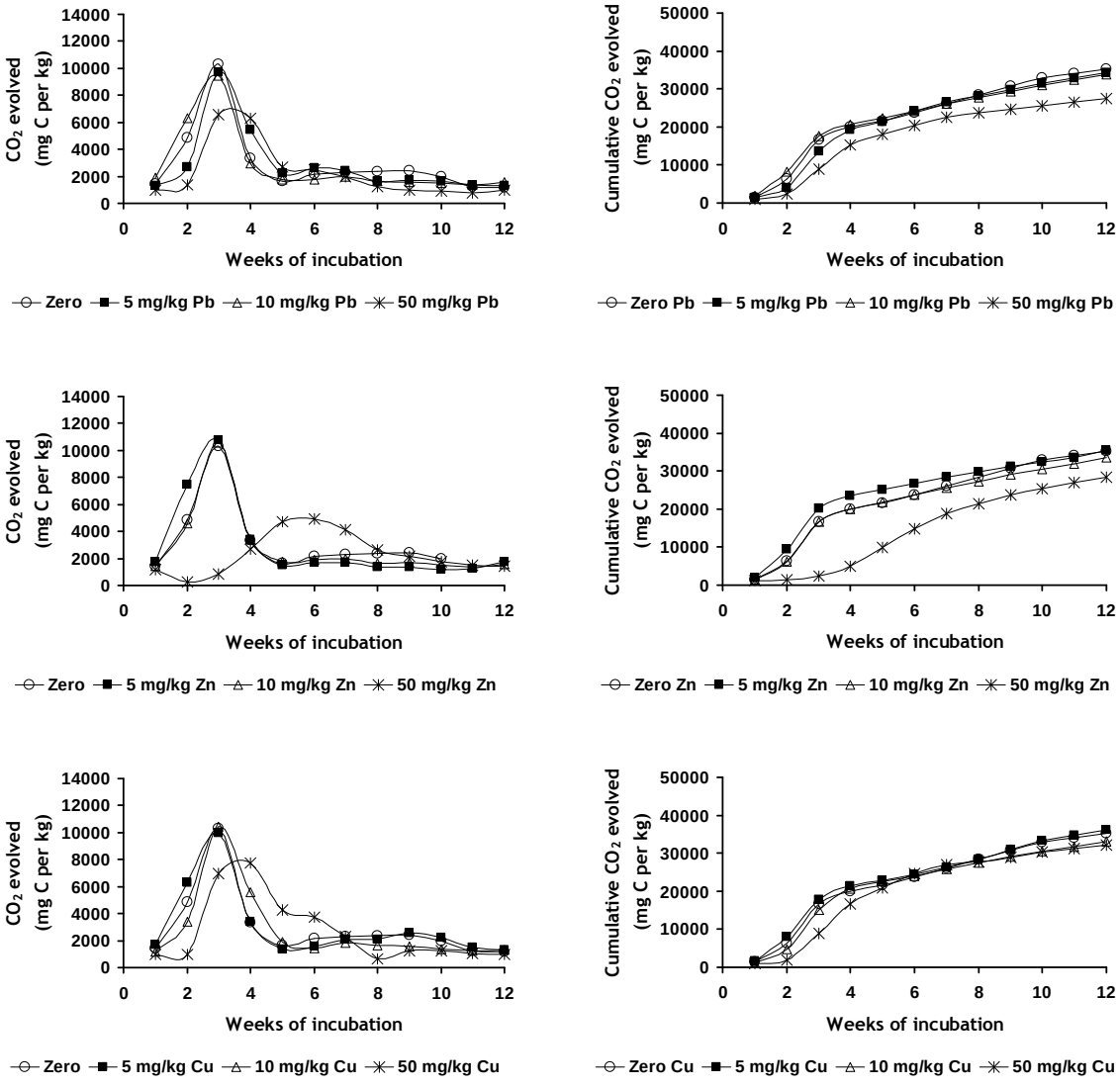


Figure 6.2 Amounts of CO_2 evolved per week and cumulative CO_2 evolved (as mg C per kg of chitosan) (second trial).

The amount of CO_2 evolved per week and cumulative CO_2 evolved for biodegradation of cross-linked chitosans in soil are shown in Figures 6.3 to 6.5. From these figures, it is obvious that the breakdown of the readily degradable fractions of cross-linked chitosans occurred within 7 days of the start of the incubation study. The amount of CO_2 evolved in the first week ranged from 7560 to 9840 mg C per kg for chitosan-GLA, 9200 to 10,760 mg C per kg for chitosan-ECH, and 7400 to 10,640 mg C per kg for chitosan-EGDE. As discussed earlier, results obtained from the first and second biodegradation trials have shown that the breakdown of the easily degradable fractions of chitosan occurred at weeks 2 and 3 of the study (Figures 6.1 and 6.2). The cross-linked chitosans were fine powders, ≤ 500 μm (Section 2.4), whereas the chitosan had a size of ≤ 1000 μm. Therefore, the breakdown of the readily degradable fractions of cross-linked chitosans by microorganisms appeared to be more rapid than chitosan as a result of the greater surface area exhibited (Section 3.3.1).

Marked reductions in CO_2 evolution were obtained for week 2 of the study, which indicate that the easily degradable fractions of cross-linked chitosans are exhausted. In some cases, soils showed a slight increase in the amount of CO_2 evolved at week 3. This observation can be related to degradation of the more resistant fractions of cross-linked chitosans. Except for soil treated with 50 mg/kg Pb (Figure 6.3), a gradual decrease in CO_2 evolution for cross-linked chitosans was observed from week 4 to week 8 of the study. No great difference in CO_2 evolution was obtained during the period of weeks 8 to 12. In fact, the amount of CO_2 released from the soils was found to be rather similar, suggesting that a steady state for the system was established. A similar observation was made for chitosan (Figure 6.2). During this period, the amount of CO_2 evolved ranged from 680 to 2440 mg C per kg for chitosan. Meanwhile, 80 to 2720 mg C per kg was estimated for cross-linked chitosans.

From the cumulative curves of CO_2 evolution for chitosan-GLA biodegradation (Figure 6.3), it was found that the addition of Pb and Zn at a concentration of 50 mg/kg caused an increase in CO_2 evolution, particularly from week 7 to week 12 for 50 mg/kg Pb treatment, and from week 6 to week 12 for 50 mg/kg Zn. In fact, soils treated with both treatments released higher amounts of CO_2 as compared to soil which had received no metal treatment. These observations contradict the earlier findings, which clearly showed that biodegradation of chitosan was affected by the 50 mg/kg metal treatments (Figures 6.1 and 6.2). A similar trend was observed for biodegradation of chitosan-ECH (Figure 6.4). From Figure 6.4, it can be seen that soils treated with Pb and Zn at a concentration of 50 mg/kg produced more CO_2 than soils which had received both metals at 5 and 10 mg/kg levels. A different trend was observed for chitosan-EGDE.

Cumulative curves for chitosan-EGDE (Figure 6.5) revealed that the addition of metals at 50 mg/kg inhibited microbial breakdown of chitosan-EGDE.

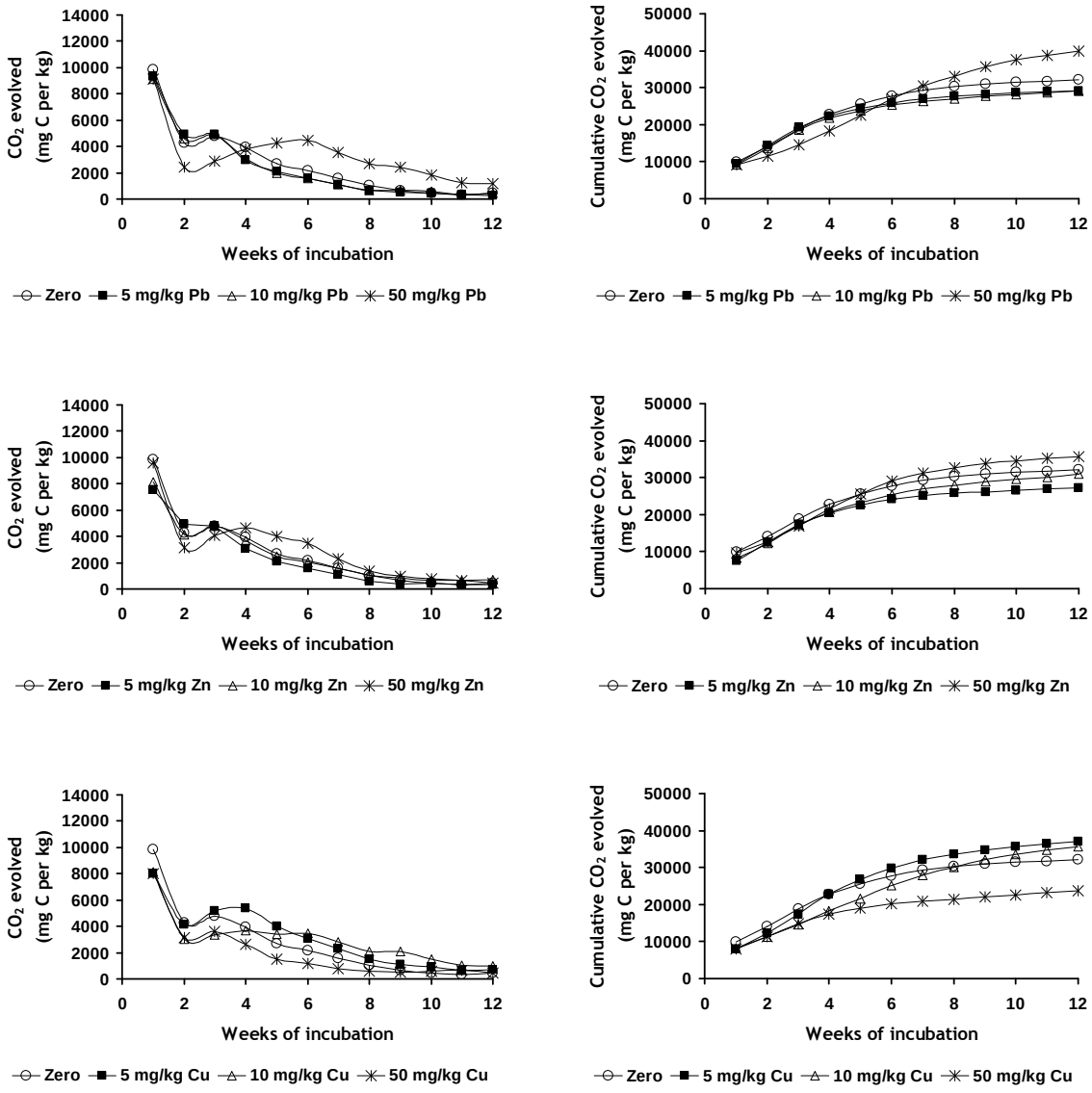


Figure 6.3 Amounts of CO_2 evolved per week and cumulative CO_2 evolved (as mg C per kg chitosan-GLA).

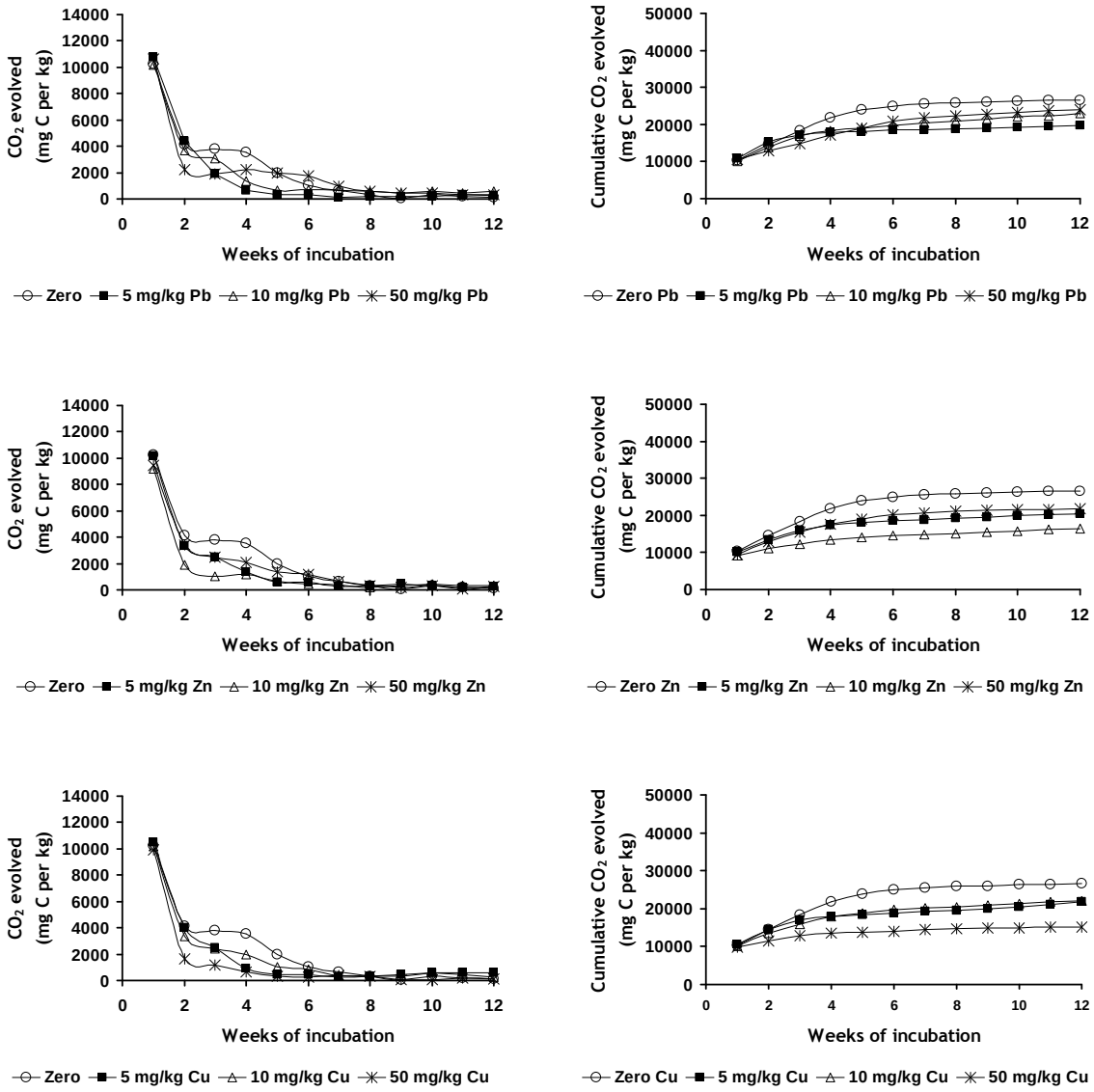


Figure 6.4 Amounts of CO_2 evolved per week and cumulative CO_2 evolved (as mg C per kg chitosan-ECH).

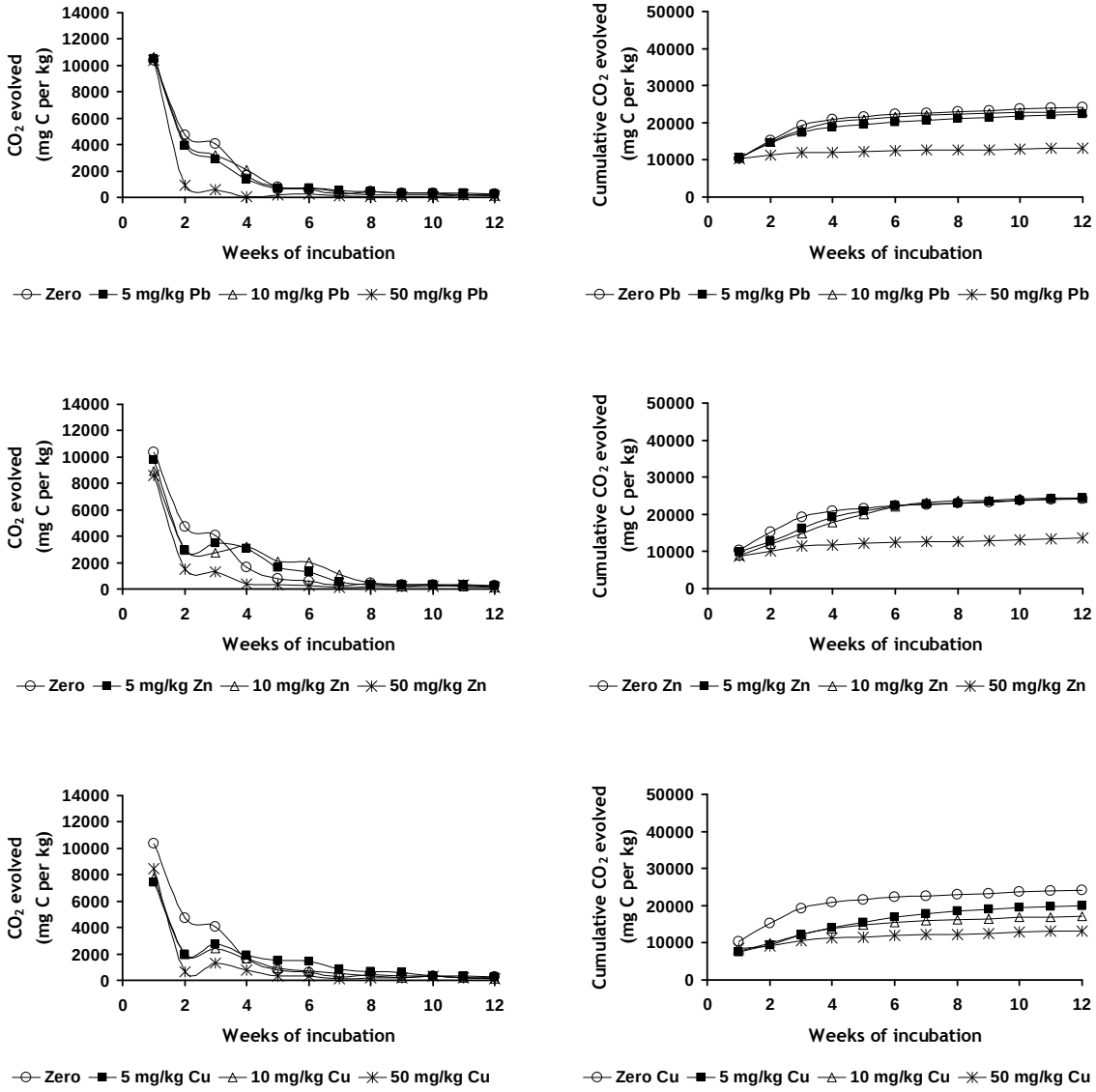


Figure 6.5 Amounts of CO_2 evolved per week and cumulative CO_2 evolved (as mg C per kg chitosan-EGDE).

Leita *et al*. (1995) reported a significant increase in CO_2 evolution after addition of Pb and Zn to soil. Meanwhile, Chander and Brookes (1991) measured more CO_2 evolved in soil amended with sewage sludge, containing heavy metals, than in unamended soil. It has been postulated by Leita *et al*. (1995) and Chander and Brookes (1991) that the increase in amount of CO_2 evolved in the contaminated soils may also emphasize the need of microorganisms to consume more energy to survive. In this research, it can be speculated that the increase in CO_2 evolution is due to a combination of toxicity effect of metals and chemicals used for cross-linking treatment of chitosan (Section 2.4). This effect can be linked to the observation obtained from the second pot experiment (Chapter 5), where plants grown on soils treated with 10% (w/w) chitosan-GLA died after three weeks of growth (Section 5.4.1).

Since microorganisms have a different level of sensitivity to metal contamination (Moreno *et al*., 2009), in contaminated soil, different microorganisms respond with different energy requirements and possibly death rates (Leita *et al*., 1995; Cardelli *et al*., 2009). The behaviour of microorganisms in contaminated soil is also influenced by the nature of soil amendments. For example, Mühlbachová (2009) reported that the addition of EDTA to metal contaminated soils decreased CO_2 evolution. Meanwhile, a three year field study by Kiikkilä *et al*. (2001) found that application of mulch consisting of a mixture of compost and woodchips to a contaminated soil enhanced CO_2 evolution. Nwachukwu and Pulford (2011) reported a significant increase in microbial respiration when coir and wood bark were added to a Pb/Zn contaminated soil.

As shown in Figure 6.2, the cumulative curves for biodegradation of chitosan show two stages of CO_2 evolution, namely at weeks 1-3 and weeks 3-12. However, this observation was less apparent for soils treated with 50 mg/kg levels of metal. The cumulative curves for cross-linked chitosans did not show a clear pattern, but, in most cases two stages of CO_2 evolution were observed, namely at weeks 1-6 and 6-12 (Figures 6.3 to 6.5). Tables 6.3 to 6.6 present the weekly evolution rate of CO_2 and the corresponding R^2 values. The rates were estimated for each stage of the CO_2 evolution, as described earlier. From Table 6.3, except for soil treated with 50 mg/kg Zn, soils showed high rates of CO_2 evolution for chitosan degradation during weeks 1-3 with values ranging from 3980 to 9140 mg CO_2-C per kg per week. In the case of soil treated with 50 mg/kg Zn, the greatest rate of CO_2 production was achieved at weeks 3-12 with a value of 2999 mg CO_2-C per kg per week.

Similar to chitosan, cross-linked chitosans also showed a high initial flush of CO_2 evolution. During weeks 1-6, the rate of CO_2 released for chitosan-GLA biodegradation ranged from 2462 to 4515 mg CO_2-C per kg per week and the R^2 values obtained were

between 0.9516 and 0.9993 (Table 6.4), suggesting a linear production of CO_2. During the same period, the greatest rate of CO_2 evolution for chitosan-ECH biodegradation was found to be 2999 mg CO_2-C per kg per week (Table 6.5), while 2653 mg CO_2-C per kg per week was determined for chitosan-EGDE breakdown (Table 6.6). Soils showed a lower rate of CO_2 evolved during weeks 3-12 (chitosan) and 6-12 (cross-linked chitosans), which can be attributed to the breakdown of the more resistant fractions of the chitosans. However, in the case of chitosan-GLA breakdown at weeks 6-12, soils treated with Pb and Zn at 50 mg/kg levels released CO_2 at a higher rate as compared to other treatments (Table 6.4). As discussed earlier, the high amount of CO_2 evolved can be related to the toxicity effect of metal and glutaraldehyde (GLA).

As shown in Figures 6.2 to 6.5, the total amount of CO_2 evolved (mg CO_2-C per kg) from soils which had received no metal treatment was found to be 35,320 for chitosan, 32,200 for chitosan-GLA, 26,600 for chitosan-ECH and 24,080 for chitosan-EGDE. In non-contaminated soil, a lower cumulative CO_2 evolved determined for cross-linked chitosans can be attributed to the adverse effect of cross-linking reagents, which affect the microbial activity in soil. As a consequence, the biodegradation of cross-linked chitosans occurred at a slower rate than chitosan.

Table 6.3 Evolution rate of CO_2 for biodegradation of chitosan in soil (second trial).

Week	Metal (mg/kg)	Evolution rate (mg CO_2-C per kg per week)					
		Pb	R^2	Cu	R^2	Zn	R^2
1-3	Zero	7580	0.9583	7580	0.9583	7580	0.9583
	5	6200	0.9030	8120	0.9832	9140	0.9891
	10	7880	0.9864	6920	0.9206	7580	0.9510
	50	3980	0.8738	3980	0.8426	560	0.9231
3-12	Zero	2109	0.9912	2109	0.9912	2109	0.9912
	5	2122	0.9589	2023	0.9938	1579	0.9829
	10	1742	0.9921	1794	0.9537	1801	0.9884
	50	1801	0.8825	2249	0.8543	2999	0.9556

Table 6.4 Evolution rate of CO_2 for biodegradation of chitosan-GLA in soil.

Week	Metal (mg/kg)	Evolution rate (mg CO_2-C per kg per week)					
		Pb	R^2	Cu	R^2	Zn	R^2
1-6	Zero	3641	0.9807	3641	0.9807	3641	0.9807
	5	3302	0.9516	4515	0.9936	3305	0.9535
	10	3254	0.9540	3432	0.9993	3490	0.9769
	50	3597	0.9871	2462	0.9621	3998	0.9975
6-12	Zero	697	0.9150	697	0.9150	697	0.9150
	5	530	0.9385	1156	0.9477	496	0.9485
	10	607	0.9667	1739	0.9660	890	0.9728
	50	2137	0.9637	586	0.9974	1057	0.9324

Table 6.5 Evolution rate of CO_2 for biodegradation of chitosan-ECH in soil.

Week	Metal (mg/kg)	Evolution rate (mg CO_2-C per kg per week)					
		Pb	R^2	Cu	R^2	Zn	R^2
1-6	Zero	2999	0.9604	2999	0.9604	2999	0.9604
	5	1367	0.7705	1559	0.8279	1624	0.8907
	10	1857	0.8890	1857	0.8890	1038	0.9533
	50	2049	0.9990	791	0.8910	2104	0.9630
6-12	Zero	260	0.9323	260	0.9323	260	0.9323
	5	221	0.9898	466	0.9816	311	0.9894
	10	526	0.9978	393	0.9927	337	0.9946
	50	491	0.9605	183	0.9246	277	0.9394

Table 6.6 Evolution rate of CO_2 for biodegradation of chitosan-EGDE in soil.

Week	Metal (mg/kg)	Evolution rate (mg CO_2-C per kg per week)					
		Pb	R^2	Cu	R^2	Zn	R^2
1-6	Zero	2303	0.8668	2303	0.8668	2303	0.8668
	5	1837	0.8845	1953	0.9866	2574	0.9719
	10	2166	0.9031	1569	0.9566	2653	0.9936
	50	369	0.8611	729	0.9468	729	0.8706
6-12	Zero	307	0.9881	307	0.9881	307	0.9881
	5	354	0.9855	487	0.9473	333	0.9902
	10	213	0.9765	273	0.9637	359	0.8674
	50	114	0.9792	213	0.9901	209	0.9920

The total number of years required for biodegradation of chitosan and cross-linked chitosans in soil was estimated based on the assumption that there is no change in rate of CO_2 evolution after 12 weeks of the study. From Tables 6.7, it is found that the biodegradation of chitosan is more rapid than cross-linked chitosans. In non-contaminated soil, approximately 9 years would be required to complete the breakdown

of chitosan, whereas a longer period, 27 to 73 years, would be needed to degrade cross-linked chitosans. As discussed in Section 3.2, cross-linked chitosans have more rigid structures than chitosan. Therefore, they are more recalcitrant in soil.

The breakdown of a material in soil is influenced by its physical and chemical properties. A study by Ajwa and Tabatabai (1994) found that the decomposition of animal manures in soils was greatly influenced by the fibrous texture and N content, whereas the biological breakdown of crop residues was highly affected by the content of carbohydrates and lignin. For example, the high amount of cumulative CO_2 determined for pig manure was related to its non-fibrous texture and high N content. Meanwhile, horse manure was reported to contain large amount of fibrous materials and low content of N, and therefore, had the lowest amount of cumulative CO_2. Crop residues with high contents of carbohydrates were found to evolve more CO_2 than residues containing high lignin. As further discussed by Leifeld *et al*. (2002) and Hofmann *et al*. (2009), carbohydrates and proteins are regarded as easily degradable plant compounds, while lignin is a more recalcitrant compound.

Martinez and Tabatabai (1997) noted that the sugar content in biotechnology by-products such as spent fermentation broth and hydrolysed soybean oil meal had a significant contribution to decomposition of the materials in soils. They obtained a linear relationship between the rate of decomposition and the total sugar content in the materials. In a study investigating the biodegradation process of seven solid wastes, Komilis and Ham (2003) found that wastes with high cellulose content had a greater degradation rate, while a slower rate was observed for wastes containing high lignin content. Cellulose is a simple glucose polymer which can be easily broken down by soil microorganisms (Nwachukwu and Pulford, 2011). Due to its polyphenolic structure, lignin is considered more resistant to microbial breakdown, and therefore, persists in soil (Rasse *et al*., 2006; Leifeld *et al*., 2002; Montemurro *et al*., 2009).

The degradation of materials in soil depends on several soil parameters such as pH, temperature, oxygen and moisture content (Ajwa and Tabatabai, 1994; Tejada and Gonzalez, 2006). For example, CO_2 evolution and decomposition of organic matter in soil are diminished by acidification treatment using H_2SO_4 (Persson *et al*., 1989). Borken *et al*. (2002) studied biodegradation of mature compost under field conditions in temperate forest soils for a period of two years. They found that the rate of CO_2 emission correlated reasonably well with the soil temperature. A field study by Davidson *et al*. (1998) found that the microbial breakdown of organic matter was affected by soil drought.

Table 6.7 Number of years required for chitosan (second trial), chitosan-GLA, chitosan-ECH and chitosan-EGDE biodegradation.

Metal	Concentration	Years required			
	(mg/kg)	Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Zero	Zero	9	27	73	62
Pb	5	9	36	86	54
	10	11	31	36	89
	50	11	9	39	168
Cu	5	9	16	41	39
	10	11	11	48	70
	50	8	32	105	90
Zn	5	12	38	61	57
	10	10	21	57	53
	50	6	18	69	92

6.3 Metal bioavailability study

The metal bioavailability study was carried out in order to investigate the influence of biodegradation on the bioavailable fraction of heavy metals in soil. This experiment was performed for a total of 12 weeks and was concurrent with the second biodegradation trial. However, only chitosan was assessed in this study. The ammonium acetate (1.0 mol/L, pH 7.0) extractable metal concentrations in soil were determined at three periods, namely 1 day, 6 weeks and 12 weeks after the metal treatment, and are shown in Figure 6.6 (see Appendix D, Table D1 for full data set). The procedure is fully described in Section 2.13.

Results obtained from biodegradation study suggest that the breakdown of readily degradable fractions of chitosan in soil occurred within 3 weeks of incubation. Meanwhile, the degradation of the more resistant fractions took place during weeks 3-12. From Figure 6.6, no increase in percentage of metal bioavailability was obtained during 12 weeks period of the study. Instead, results from this study suggest that the bioavailability of heavy metals in soil decreased with time. Therefore, it can be speculated that the biodegradation process does not affect the behaviour and ability of chitosan to bind heavy metals in soil.

Results from this study confirm the results obtained for the second pot experiment (Chapter 5), which showed that application of chitosan at rates greater than 1% (w/w) reduced metal uptake by plants and decreased ammonium acetate extractable metals in soil (Sections 5.4.2 and 5.4.3).

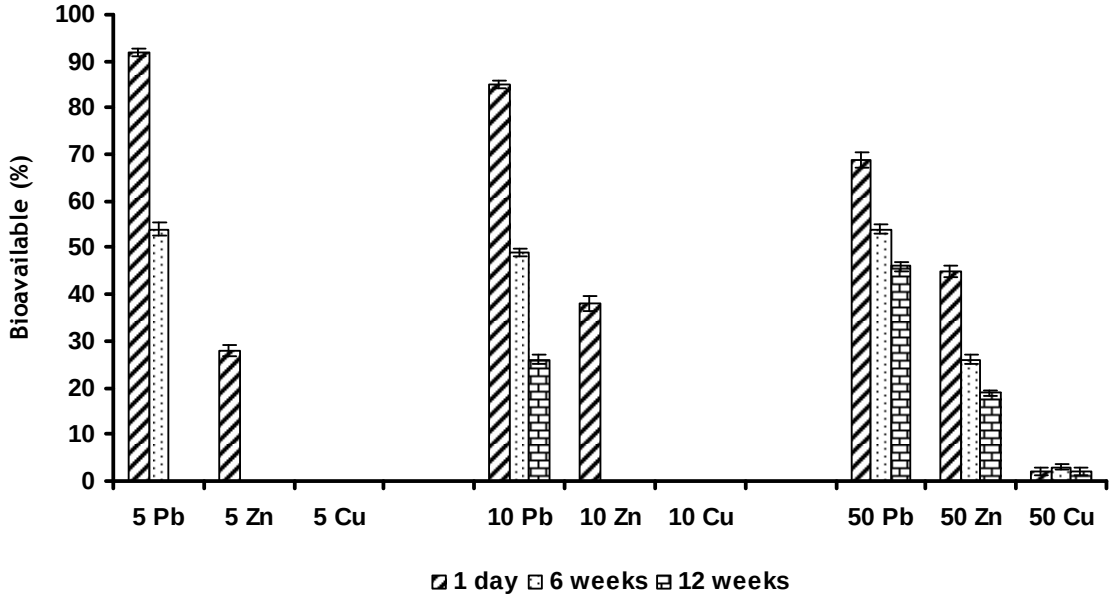


Figure 6.6 Ammonium acetate extractable metals in soil, as a percentage of metal added, during the biodegradation study. Error bars are ± standard deviation of 2 replicates.

As shown in Figure 6.6, it is obvious that chitosan has different binding affinity for heavy metals in soil. Although results from the sorption study (Chapter 4) have shown that chitosan has a greater binding affinity for Pb as compared to Cu and Zn, this study revealed that Pb was the least sorbed by chitosan. When added at a concentration of 5 mg/kg, 92% of Pb was extracted 1 day after the treatment. The percentage of bioavailable Pb decreased from 92% to 54% at week 6, and no bioavailable fraction was determined at the end of the study. For 10 and 50 mg Pb/kg treatments, the percentage of bioavailable reduced with time. However, smaller reductions in percentage of bioavailable metal were obtained for 50 mg Pb/kg treatment as compared to 10 mg Pb/kg. At week 12, 46% of the total 50 mg/kg Pb was available.

One day after the metal treatment, the percentage of bioavailable Zn was found to be 28% for 5 mg/kg, 38% for 10 mg/kg and 45% for 50 mg/kg. At weeks 6 and 12, no bioavailable fraction of Zn was determined for soils treated with 5 and 10 mg Zn/kg. Soils which received 50 mg Zn/kg treatment showed a decreased in the percentage of bioavailable Zn with time. When compared to the 50 mg Pb/kg treatment over 12 weeks of the study, the reduction in percentage of bioavailable metal was found to be greater for 50 mg Zn/kg, suggesting a better affinity for chitosan. Cu had the lowest percentage of bioavailable metal over 12 weeks of the study. In fact, Cu was not extracted by ammonium acetate when added at concentrations of 5 and 10 mg/kg. However, when applied at 50 mg/kg, 2-3% of Cu was extracted and the percentage remained constant until week 12. This suggests that Cu was highly bound and strongly retained by chitosan.

6.4 Summary

Biodegradation of chitosan and cross-linked chitosans in soils was affected by the presence of heavy metals, especially when applied at a concentration of 50 mg/kg. Zn was pronounced in inhibiting microbial activity. It reduced the amount of CO_2 evolved and caused a delay in microbial breakdown. However, results also showed that the addition of heavy metals at 50 mg/kg enhanced the evolution of CO_2 for biodegradation of cross-linked chitosan (Figure 6.3). As discussed by Leita *et al*. (1995) and Chander and Brookes (1991), the increase in CO_2 evolution in the contaminated soils may also indicate the need of microorganisms to consume more energy to survive. Since such an increase was not observed for chitosan (Figures 6.1 and 6.2), the increase in the amount of CO_2 released from biodegradation of cross-linked chitosans in metal contaminated soil can be related to the combination of the toxicity effect of metals and cross-linking reagents.

The soil microorganisms decomposed the easily degradable fractions of chitosan within 3 weeks, while the same fractions of cross-linked chitosans were broken down within 7 days of the incubation period. The fine structure of cross-linked chitosans favoured the breakdown of the readily degradable fractions by microorganisms. The evolution rate of CO_2 was high at weeks 1-3 for chitosan and at weeks 1-6 for cross-linked chitosans. Lower rate of CO_2 evolution was obtained during weeks 6-12 and this can be attributed to the breakdown of the more resistant fractions of the chitosans.

Assuming the rate of CO_2 evolution after 12 weeks of the study is constant, the total number of years required for biodegradation of chitosan and cross-linked chitosans in soil was estimated. The biodegradation of chitosan was found to be more rapid than cross-linked chitosans. In non-contaminated soil, approximately 6 to 9 years (Tables 6.2 and 6.7) is required to complete the breakdown of chitosan, whereas 27 to 73 years (Table 6.7) is needed to degrade the cross-linked chitosans. There was no clear effect of metal contamination on the total time required to complete the breakdown of the amendments. A longer period required for cross-linked chitosans can be attributed to their more rigid structure as affected by the cross-linking treatment (Section 3.2).

From the metal bioavailability study, it was found that the binding behaviour of chitosan for heavy metals in soils was not affected by the biodegradation process. In fact, the bioavailability of heavy metals in soil decreased with time, confirming the feasibility of chitosan as an immobilising agent for heavy metals in soils (Sections 5.4.2 and 5.4.3). The binding affinity of chitosan for heavy metals in soil was in the order of

Cu > Zn > Pb, which is not in agreement with the order established for metal binding by chitosan from aqueous solution, Pb > Cu > Zn, discussed in Chapter 4 (Sorption Study).

7 CONCLUSIONS AND FUTURE RESEARCH

7.1 Conclusions

Chitosan and cross-linked chitosans were effective in removing Ag, Cd, Cu, Pb and Zn from the model soil solutions, simulating soil conditions. The binding capacity of chitosan and cross-linked chitosans were greater than other low-cost materials used as soil amendments. For example, the maximum amount of Pb (mg/g) that can be bound to chitosan, chitosan-EGDE, crab shells and furnace sludge was estimated as 133, 156, 19.83 (Dahiya *et al.*, 2008) and 92.51 (Naiya *et al.*, 2009), respectively. Chitosan is naturally rich with amino (NH_2) and hydroxyl (OH) groups that can form stable complexes with metal ions. This is an important key factor for the success of metal immobilisation in contaminated soil.

Besides their great ability for binding metals, chitosan and chitosan-GLA amendments were beneficial as growing media through improvement of soil fertility and provision of nitrogen to plant. However, application of chitosan-GLA at 10% (w/w) caused a toxicity effect on plants. This effect was due to the glutaraldehyde (GLA) used in the cross-linking treatment of chitosan.

The effectiveness of chitosan and chitosan-GLA for metal immobilisation was confirmed with the reduction in metal uptake by rapeseed. Unexpectedly, a different effect was observed on perennial ryegrass, in which application of amendments at the rates of up to 1% (w/w) increased metal uptake, whereas a significant reduction in metal uptake was achieved when chitosan was applied at 10% (w/w). This highlights their potential for enhancing phytoextraction, an alternative remediation strategy for metal contaminated land.

Amending contaminated soil with chitosan and chitosan-GLA decreased the ammonium acetate extractable metals, as anticipated. An increase in the rate of application resulted in a greater reduction in metal bioavailability.

Chitosan and cross-linked chitosans are not easily decomposed by microorganisms in the soil environment. From a soil stabilisation remediation strategy viewpoint, this is important for the long-term success of metal immobilisation. An amendment that is only slowly degradable is normally able to retain metals for a long period of time and so further application of amendment is not necessary. From an economic aspect, this is a great advantage. However, there is a great concern with regard to the risk of

groundwater contamination following application of highly persistence soil amendments. A soil amendment such as EDTA is known to be effective in chelating metal ions. Due to its persistence in soil, however, it may result in contamination of groundwater. Therefore, this aspect should be considered when applying the chitosans to a contaminated site.

7.2 Future research

Due to the difference in climate and soil properties, it is necessary to further investigate the efficacy of chitosan and cross-linked chitosans as immobilising agents for the remediation of metal contaminated land in Malaysia.

The effects of the chitosans on plant growth and metal accumulation in plant tissue should be determined on a range of plant species including grass, vegetables, fruits and ornamental plants, available in Malaysia. This perhaps will give valuable information on the feasibility of the use of chitosan and chitosan derivatives under realistic field conditions. For example, chitosan treatment along with an appropriate choice of plant species may be promising for phytoextraction and phytostabilisation remediation strategies.

The effectiveness of the chitosans in reducing bioavailability of heavy metals in soil, as well as decreasing metal uptake by plants should be compared with other waste-based materials available in Malaysia. This can be evaluated by both single and in-combination of applications.

It is important to conduct a comprehensive study assessing the risk of groundwater contamination following application of the chitosans.

Since cost is a critical aspect, a study focusing on cost versus benefit should be carried out in detail. This should entail the extraction of chitin from crustacean shells, the production of chitosan from chitin, cross-linking treatment of chitosan and some logistical issues relating to production and delivery of chitosans.

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APPENDICES

Appendix A – Sorption-desorption Study

Table A1 Percentage of metal ions retained on the surface of sorbents (C_o = 100 mg/L).

Metal ion	Dilution factor	Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Ag^{+}	0	98, 96, 103	97, 100, 99	98, 102, 101	100, 98, 99
	1.5	99, 101, 97	98, 99, 101	100, 99, 98	97, 101, 98
	2.0	96, 98, 100	96, 102, 98	99, 97, 100	99, 99, 100
	3.5	99, 99, 95	97, 96, 102	98, 102, 97	97, 98, 99
	6.0	97, 96, 101	95, 98, 97	96, 98, 96	96, 97, 99
	8.5	95, 98, 99	93, 97, 96	95, 97, 96	97, 96, 96
	11.0	96, 96, 98	94, 95, 97	93, 92, 97	95, 97, 96
Pb^{2+}	0	96, 100, 98	96, 98, 96	248, 250, 250	99, 98, 97
	1.5	91, 93, 97	95, 99, 98	214, 217, 216	96, 97, 98
	2.0	92, 92, 94	97, 98, 91	208, 209, 212	95, 96, 99
	3.5	85, 90, 88	96, 94, 95	199, 199, 201	97, 96, 98
	6.0	77, 79, 80	92, 93, 96	159, 163, 164	97, 96, 95
	8.5	74, 72, 72	94, 94, 95	155, 154, 156	95, 96, 98
	11.0	67, 64, 63	92, 96, 94	120, 118, 120	94, 94, 97
Cu^{2+}	0	115, 116, 119	104, 101, 106	157, 156, 160	104, 103, 105
	1.5	109, 113, 120	99, 103, 99	143, 146, 148	100, 102, 103
	2.0	112, 108, 115	98, 100, 99	129, 131, 132	99, 102, 103
	3.5	109, 110, 104	96, 99, 97	130, 132, 129	100, 99, 98
	6.0	98, 105, 102	93, 94, 90	128, 130, 129	96, 98, 97
	8.5	103, 100, 95	90, 91, 93	124, 125, 126	94, 93, 93
	11.0	97, 100, 101	85, 89, 86	117, 114, 115	91, 90, 94
Cd^{2+}	0	88, 92, 90	88, 84, 89	162, 164, 163	92, 91, 93
	1.5	85, 84, 89	77, 76, 78	149, 151, 150	86, 85, 87
	2.0	81, 82, 85	73, 74, 77	120, 120, 118	83, 83, 84
	3.5	75, 79, 77	69, 71, 65	113, 114, 115	69, 70, 68
	6.0	70, 68, 77	59, 60, 60	87, 86, 82	59, 57, 58
	8.5	64, 65, 69	52, 55, 54	82, 81, 80	55, 57, 56
	11.0	60, 58, 61	49, 52, 51	59, 60, 60	46, 47, 48
Zn^{2+}	0	73, 75, 69	64, 66, 65	70, 71, 69	64, 66, 65
	1.5	68, 72, 69	60, 64, 66	69, 67, 70	59, 60, 61
	2.0	63, 61, 58	56, 57, 61	64, 64, 60	51, 53, 52
	3.5	54, 48, 51	54, 50, 53	55, 53, 53	49, 46, 45
	6.0	44, 47, 35	45, 44, 47	26, 24, 25	33, 36, 34
	8.5	32, 38, 37	49, 50, 51	18, 19, 15	30, 32, 31
	11.0	18, 20, 21	28, 34, 31	4, 3, 3	28, 27, 25

Table A2 Percentage of metal ions retained on the surface of sorbents (C_o = 500 mg/L).

Metal ion	Dilution factor	Chitosan	Chitosan-GLA	Chitosan-ECH	Chitosan-EGDE
Ag^{+}	0	102, 101, 103	108, 106, 105	101, 105, 102	104, 105, 98
	1.5	99, 102, 103	99, 100, 98	100, 98, 104	99, 96, 101
	2.0	100, 99, 101	97, 97, 96	96, 99, 102	98, 99, 100
	3.5	98, 97, 97	98, 99, 97	97, 96, 99	96, 95, 99
	6.0	97, 96, 98	95, 97, 96	95, 98, 97	94, 97, 93
	8.5	97, 95, 96	96, 95, 96	98, 101, 93	95, 96, 93
	11.0	95, 95, 96	93, 95, 94	96, 95, 99	92, 98, 96
Pb^{2+}	0	115, 117, 117	106, 105, 107	98, 96, 100	113, 114, 110
	1.5	116, 114, 117	103, 104, 105	85, 87, 85	109, 110, 113
	2.0	113, 112, 116	101, 103, 102	83, 86, 85	104, 108, 108
	3.5	109, 113, 112	102, 104, 101	79, 83, 81	100, 98, 99
	6.0	107, 106, 108	90, 92, 91	63, 63, 64	92, 91, 96
	8.5	102, 104, 101	88, 80, 89	50, 52, 47	87, 86, 82
	11.0	100, 102, 98	76, 75, 82	45, 44, 49	77, 81, 83
Cu^{2+}	0	104, 105, 101	123, 126, 125	107, 108, 104	107, 104, 105
	1.5	99, 97, 98	117, 120, 120	82, 86, 87	103, 106, 103
	2.0	98, 96, 99	119, 117, 119	71, 67, 68	101, 100, 105
	3.5	95, 93, 99	112, 116, 114	60, 65, 63	100, 102, 96
	6.0	93, 96, 94	110, 109, 113	57, 62, 61	98, 97, 99
	8.5	91, 92, 94	106, 104, 103	42, 45, 41	92, 96, 95
	11.0	90, 91, 88	101, 105, 104	43, 40, 46	85, 89, 90
Cd^{2+}	0	91, 92, 92	87, 84, 85	97, 95, 44	95, 91, 90
	1.5	94, 95, 96	96, 94, 100	94, 98, 95	102, 97, 101
	2.0	95, 97, 94	95, 97, 91	92, 93, 95	90, 95, 98
	3.5	94, 91, 95	83, 89, 94	89, 86, 87	92, 90, 93
	6.0	90, 89, 93	77, 81, 80	80, 79, 82	85, 84, 88
	8.5	76, 77, 78	75, 78, 75	70, 74, 73	79, 81, 85
	11.0	74, 72, 75	67, 72, 70	69, 70, 66	72, 70, 73
Zn^{2+}	0	79, 78, 80	82, 78, 80	72, 67, 71	82, 79, 80
	1.5	75, 77, 76	75, 76, 72	63, 65, 69	73, 75, 72
	2.0	64, 66, 65	71, 66, 69	62, 65, 66	69, 70, 66
	3.5	63, 64, 60	62, 66, 64	50, 54, 52	63, 66, 62
	6.0	57, 56, 55	58, 59, 53	49, 47, 43	41, 45, 44
	8.5	52, 53, 55	47, 53, 50	39, 40, 35	40, 38, 44
	11.0	42, 43, 45	42, 40, 37	36, 34, 30	33, 37, 36

Appendix B – Pot Experiment 2009

Table B1 Metal concentration in the shoot and root tissues of perennial ryegrass (pot experiment 2009).

Treatment	Concentration (mg/kg)					
	Pb		Zn		Cu	
Weeks	12	24	12	24	12	24
Shoot						
Zero	138 a	425 a	815 b	795 a	18 a	13 a
Chitosan 0.5%	214 c	592 b	801 b	900 b	19 a	14 b
Chitosan 1.0%	216 c	578 b	675 a	843 ab	21 ab	16 b
Chitosan-GLA 0.5%	179 b	379 a	905 b	870 ab	19 a	15 b
Chitosan-GLA 1.0%	164 b	361 a	1083 c	1029 c	23 b	18 c
LSD	19	69	109	100	4	1
Root						
Zero		1758 a		1952 a		105 a
Chitosan 0.5%		2677 d		2129 b		135 c
Chitosan 1.0%		2492 c		1840 a		127 bc
Chitosan-GLA 0.5%		2467 c		2152 b		122 b
Chitosan-GLA 1.0%		2309 b		2064 ab		121 b
LSD		157		168		9

Values represent mean of 18 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table B2 Ammonium acetate extractable metals in soil after pot experiment 2009.

Treatment	Concentration (mg/kg)			Decrease (%)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	402 b	134 b	1.1 ab	49.8	47.7	72.5
Chitosan 0.5%	378 ab	115 a	1.1 ab	52.8	55.1	72.5
Chitosan 1.0%	381 ab	109 a	0.9 a	52.4	57.4	77.5
Chitosan-GLA 0.5%	391 ab	110 a	1.2 b	51.1	57.0	70.9
Chitosan-GLA 1.0%	362 a	100 a	1.3 b	54.6	60.9	67.5
LSD	34	18	0.2			

Values represent mean of 18 replicates. Letters a and b show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table B3 EDTA extractable metals in soil after pot experiment 2009.

Treatment	Concentration (mg/kg)			Decrease (%)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	1378 a	464 b	9.7 a	11.4	19.7	25.4
Chitosan 0.5%	1342 a	428 a	10.2 a	13.7	26.0	21.5
Chitosan 1.0%	1282 a	417 a	10.2 a	17.6	27.9	21.5
Chitosan-GLA 0.5%	1309 a	419 a	10.5 a	15.8	27.5	19.2
Chitosan-GLA 1.0%	1339 a	405 a	10.0 a	13.9	29.9	23.1
LSD	107	27	0.8			

Values represent mean of 18 replicates. Letters a and b show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Appendix C – Pot Experiment 2010

Table C1 Metal concentration in the shoot and root tissues of perennial ryegrass (pot experiment 2010).

Treatment	Concentration (mg/kg)					
	Pb		Zn		Cu	
Weeks	9	20	9	20	9	20
Shoot						
Zero	28 b	55 b	474 b	527 b	15 a	13 b
Chitosan 1%	56 d	85 d	688 c	830 d	20 b	18 c
Chitosan 10%	10 a	12 a	198 a	209 a	14 a	11 a
Chitosan-GLA 1%	42 c	63 c	694 c	689 c	20 b	18 c
LSD	10	4	50	46	1	1
Root						
Zero		1416 b		2136 b		114 b
Chitosan 1%		1780 c		2269 b		122 b
Chitosan 10%		961 a		1492 a		84 a
Chitosan-GLA 1%		1563 b		2323 b		118 b
LSD		165		244		14

Values represent mean of 18 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table C2 Metal concentration in the shoot and root tissues of rapeseed (pot experiment 2010).

Treatment	Concentration (mg/kg)					
	Shoots			Roots		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	134 c	2003 c	13 b	1061 b	687 b	117 ab
Chitosan 1%	86 b	1075 b	9 a	1335 c	793 c	118 ab
Chitosan 10%	67 a	577 a	12 ab	818 a	564 a	105 a
Chitosan-GLA 1%	137 c	1139 b	11 ab	1535 d	849 c	121 b
LSD	13	108	4	109	72	14

Values represent mean of 18 replicates. Letters a, b, c and d show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table C3 Ammonium acetate extractable metal in soil after pot experiment 2010 (perennial ryegrass).

Treatment	Concentration (mg/kg)			Decrease (%)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	711 b	239 c	1.8 c	24.8	13.7	64.0
Chitosan 1%	692 b	217 b	1.4 b	26.8	21.7	72.0
Chitosan 10%	625 a	176 a	0.9 a	33.9	36.5	82.0
Chitosan-GLA 1%	732 b	229 bc	1.2 b	22.5	17.3	76.0
LSD	48	12	0.2			

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Table C4 Ammonium acetate extractable metal in soil after pot experiment 2010 (rapeseed).

Treatment	Concentration (mg/kg)			Decrease (%)		
	Pb	Zn	Cu	Pb	Zn	Cu
Zero	815 b	270 c	2.1 c	13.8	2.5	58.0
Chitosan 1%	802 b	246 b	1.2 b	15.1	11.2	76.0
Chitosan 10%	633 a	185 a	0.8 a	33.0	33.2	84.0
Chitosan-GLA 1%	806 b	258 b	1.4 b	14.7	6.9	72.0
LSD	42	10	0.1			

Values represent mean of 18 replicates. Letters a, b and c show the significant differences between the soil treatments, where letter a represents the lowest mean. Different letters indicate significant statistical differences (Tukey's test at $p < 0.05$).

Appendix D – Metal Bioavailability Study

Table D1 Ammonium acetate extractable metals in soil, as a percentage of metal added, during the biodegradation study.

Metal added (mg/kg)	1 day after treatment		6 weeks after treatment		12 weeks after treatment	
	Metal extracted* (mg/kg)	Bioavailable (%)	Metal extracted* (mg/kg)	Bioavailable (%)	Metal extracted* (mg/kg)	Bioavailable (%)
5 Pb	4.6	92	2.7	54	0	0
5 Zn	1.4	28	0	0	0	0
5 Cu	0	0	0	0	0	0
10 Pb	8.5	85	4.9	49	2.6	26
10 Zn	3.8	38	0	0	0	0
10 Cu	0	0	0	0	0	0
50 Pb	34.3	69	26.8	54	22.8	46
50 Zn	22.3	45	13.1	26	9.7	19
50 Cu	1.1	2	1.6	3	0.9	2

*Values represent means of two replicates.

Appendix E – List of Publications

1. Kamari, A., Pulford, I.D., Hargreaves, J.S.J. 2011. Chitosan as a potential amendment to remediate metal contaminated soil – A characterisation study. Colloids and Surfaces B: Biointerfaces 82, 71-80.

2. Kamari, A., Pulford, I.D., Hargreaves, J.S.J. 2010. Chitosan-assisted phytoextraction of heavy metal from lead/zinc tailings using *Lolium perenne* – A preliminary study. Proceedings of 15th International Conference on Heavy Metals in the Environment, 19-23 September 2010, Gdańsk Poland. ISBN 978-83-928986-5-8.