Biotic-abiotic transformations of chromium in long-term tannery waste contaminated soils: Implications to remediation

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Dedication

I sincerely dedicate this thesis to my beloved parents,
S. Kamaludeen and K. Meherrunisa Begum
for all their efforts

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Abstract

In the past tannery industries have generated considerable amounts of chromium (Cr)-rich wastes, which has been disposed onto soil and water ecosystems. In soils Cr exists primarily as either hexavalent Cr [Cr(VI)] or trivalent Cr [Cr(III)] species. Soluble Cr(VI) is extremely toxic and exhibits mutagenic and carcinogenic effects on biological systems due to its strong oxidising nature. Hence, the major focus in the current remediation practices for Cr is on irreversible reduction of Cr(VI) to non-toxic and relatively immobile Cr(III) species. In this work efforts were made to determine (i) the effect of Cr on soil microbial community and its activity, (ii) biotic-abiotic mechanisms involved in Cr oxidation, and (iii) phytostabilisation of Cr using plants and organic amendment in a long-term tannery waste contaminated soil.

Studies on the ecotoxic impact of tannery waste revealed a significant ($p < 0.05$) inhibition of microbial activity and distinct alterations in PLFA patterns with increased contamination. Soil samples from all three contaminated locations (high, medium and low) had elevated pH, EC, organic carbon (OC), total Cr and Cr(VI). The highest total Cr concentration was 110 g kg^{-1} with 0.92 mg L^{-1} in a bioavailable/water soluble form. More than 50% of the soluble Cr was present as Cr(VI) (0.54 mg L^{-1}). DHA was more pronounced in highly contaminated soil samples and showed a significant linear relationship with OC ($r^2=0.91$, $p < 0.01$), but not with total Cr, water soluble Cr, Cr(VI) or EC. The high OC in highly contaminated soil samples appeared to mask the adverse effect of Cr on DHA since DHA normalised for OC showed a significant ($p < 0.05$) negative relationship with Cr contamination.

Long-term contamination impacted not only the microbial activity, but also on microbial populations, as indicated by phospholipid fatty acid (PLFA) patterns. A distinct shift in microbial community was observed in highly contaminated soil, compared to low contaminated soil. The PLFAs specific for bacteria (i15:0, a15:0, 15:0, i16:0, a17:0 and cy17:0) decreased significantly ($p<0.01$) with an increase in Cr contamination. Bacterial specific PLFAs, 15:0, i16:0 and a17:0 also showed a negative correlation with soluble Cr(VI). This indicated that the inhibitory effects of Cr associated with the tannery wastes persisted in these soils even though disposal had ceased 25 years ago.

The soil at this site was highly alkaline and Cr(VI) was detected in soil solutions

at concentrations exceeding the maximum permissible level of 0.05 mg L^{-1} (USEPA guidelines) for drinking water. The reason for the presence of Cr(VI), despite the favourable thermodynamic conditions (pH-Eh) and high OC for reduction, is not clear. In highly contaminated soils, both soluble and exchangeable Cr(VI) fractions were significantly correlated ($r^2 > 0.8$, p< 0.01) with total Mn, the prime factor governing Cr oxidation in soils.

Biogenically formed Mn oxides from cultures of a pure bacterial strain (SKMn2) and a consortium (SKCons2) provided evidence for the involvement of Mn oxides in Cr(III) oxidation in these contaminated soils. The Mn oxidising bacterial population inherent in the contaminated soil was sensitive to Cr(VI) even at 1.0 mg L^{-1}. The bacterium (SKMn2) and the consortium (SKCons2) developed from the contaminated soil could oxidise 80 and 60% of the spiked Mn(II) (50 mg L^{-1}) in a culture broth within 25 d to Mn(IV) in a 2-step process. The oxidation commenced after 3 weeks with initial deposition of rhodochrosite. After 3 months, brown bixbyite (Mn_2O_3) was deposited in the culture broth and after 6 months disordered Mn oxides minerals were observed. XRD characterisation of the microbial Mn oxides revealed that the microbial Mn oxide formed at 6 months was a mixture of bixbyite and manganese oxides [both Mn(III) and Mn(IV)].

Although microbially produced Mn oxides could oxidise Mn(II) in Cr spiked broth they could not oxidise Cr(III) during Mn oxidation. Interestingly, dried microbial Mn oxides were able to oxidise Cr(III). Kinetic studies showed that microbial Mn oxides were capable of chemically oxidising Cr(III) to Cr(VI). At pH 4, the oxidation of aqueous Cr(III) catalysed by microbial Mn oxides was non-linear with time and maximum oxidation (5%) occurred within 12 hours. The reaction between Cr(III) and microbial Mn oxides resulted in the simultaneous formation of Cr(VI) and Mn (II) with a linear relationship (r^2=0.62 p< 0.05). Since the Mn oxides were a mixture of Mn(III) and Mn(IV), the Cr oxidation was triggered by both these oxides and hence the stoichiometry of the reaction was complex. Further extension of this hypothesis on Mn-assisted Cr oxidation in soil extract of contaminated site revealed that the Mn oxide formed in soil extract was hausmannite (Mn_3O_4). Spiking this soil solution with Cr(III) (20 mg L^{-1}) resulted in 15-20% oxidation within 5 hours releasing Cr(VI) and Mn(II). These studies indicated that the nature and amount of biogenically formed Mn oxides would determine the rate of Cr oxidation in soils with Mn oxides.

Remediation of anionic Cr(VI) in alkaline soils is difficult as high pH favours Cr

oxidation and the presence of highly mobile Cr(VI). Phytostabilisation studies using an organic amendment with pea and clover exerted a significant effect on Cr(VI) reduction during initial phase (5 weeks). Cow manure, applied at 25 and 50 tonnes ha^{-1}, appeared to reduce the toxicity of the contaminated soil to peas and clover. Though Cr(VI) decreased initially, plant-mediated release of Cr(VI) was subsequently observed, especially with pea plants. However, the organic amendment retarded the intensity of plant-mediated release of Cr(VI). Microbial activity (DHA) increased with the rate of cow manure application and over time. Likewise, DHA increased 3-fold in the rhizosphere soil compared to the bulk soil and followed the order: peas > clover > unplanted. The relationship between Cr(VI) concentration and the DHA in the rhizosphere soil was significantly correlated (r^2=0.54, p< 0.05). This study indicated that it might be possible to harness the Cr-reducing properties of plants, rhizosphere microorganisms and organic amendment for the stabilisation of Cr in this contaminated soil, but this needs further research on a long-term basis.

Statement

This thesis contains no material, which has been accepted for the award of any other degree or diploma in any university or tertiary education. To the best of author's knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis, when deposited in the university of Adelaide library, being available for photocopying and loan.

DATE...15.03.02....   SIGNED ....

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I thank god for giving me courage and endurance to do this piece of research work and complete my studies in Australia.

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Publications/ conference papers arising from this thesis

1. Kamaludeen Sara P B., Megharaj M., Sethunathan, N., Juhasz, A and Naidu, R. 2002. Chromium toxicity to microbial activity and potential remediation strategies. Reviews of Environmental Contamination and Toxicology (accepted).

2. Kamaludeen Sara P B., Megharaj M., Naidu, R., Singleton, I., Juhasz, A and Hawke, B.G. 2002. Chromium bioavailability in long-term tannery waste contaminated soils: Implications for soil microbial activity and populations. Submitted to Ecotoxicology and Environmental Safety (communicated).

3. Kamaludeen, Sara P. B., Naidu, R., Megharaj, M., Merrington, G. and Juhasz, A. L. 2001. Bioavailability of Cr (VI) during phytostabilisation of long-term tannery waste contaminated soil. Bioavailability 2001, Chemical Bioavailability in the Terrestrial Environment, Adelaide, Australia (Nov 18-20). (oral presentation)

4. Kamaludeen, Sara P. B., Naidu, R., Megharaj, M., Juhasz, A. L. and Merrington, G. 2001. Biotic-abiotic coupling reactions influence chromium (III) oxidation in long-term tannery waste contaminated soil. Sixth International Conference on the Biogeochemistry of Trace Elements, Guelph, Canada (July 29-Aug 2).

5. Kamaludeen Sara P B., Naidu, R., Megharaj M., Singleton, I. and Juhasz, A. 2000. Effect of chrome rich tannery wastes on phospholipid fatty acid patterns (PLFA) of soil microbial communities. Proceedings of the "2000 contaminated site remediation conference. From Source zones to Ecosystems" held in Melbourne, Australia (Dec 4-8). (oral presentation)

6. Kamaludeen Sara P B., Naidu, R., Singleton, I., Juhasz, A and Megharaj M. 2000. Fate and dynamics of chromium at long-term tannery waste contaminated sites: VI. Effects on soil microbial activity and diversity. Abstracts of "Annual meetings of ASA-SSSA-CSSA", held in Minneapolis, MN" (Nov 5-9).

7. Kamaludeen Sara P B., Naidu, R., Juhasz, A., Megharaj M and Hawke, B.G. 2000. Effect of chrome-rich tannery wastes on soil microbial communities. Proceedings of the "Fifth International Symposium on Environmental Biotechnology" held in Kyoto, Japan (July 9-13).

8. Kamaludeen Sara P B., Naidu, R., Singleton, I., Juhasz, A and Megharaj M. 1999. Do tannery wastes have long-term inhibitory effect on soil microbial activity? Proceedings of the "Second International Conference on Contaminants and Soil Environment in the Australasia – Pacific region" held in New Delhi, India (Dec 12-17) pp 327-328. (oral presentation)

Introduction

No one is given a wish without also being given the power to make it
true
- Richard Bach -
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CHAPTER 1

General Introduction

Soil contamination by heavy metals is extremely pernicious because of their prolonged persistence in the environment. Unlike most organic contaminants, metals are generally refractory and cannot be readily degraded or detoxified, and therefore they pose a long-term pollution problem.

The total annual global consumption of chromium (Cr) is approximately 20,000 tonnes of which leather industries are responsible for the major share (Hughes, 1988). About 40% of the Cr used for processing leather hides is disposed to landfill as dried sludge waste (Lollar, 1986). Generally, tannery wastes contain Cr(III) as the predominant species of Cr together with high concentrations of organic carbon derived from hides. Because of the perceived thermodynamic stability of Cr(III), wastes containing Cr in excess of 30,000 mg kg^{-1} had been commonly disposed onto land and water bodies. The long-term disposal of tannery wastes has led to extensive contamination of soil and groundwater in several countries, including Australia, Bangladesh, Brazil, China, India, Nepal, Spain, and Pakistan. The major contaminant of concern in tannery waste contaminated sites is the soluble and readily bioavailable hexavalent chromium [Cr(VI)], which is a known mammalian carcinogen (USEPA, 1996a). At contaminated sites in these countries, soil solution Cr(VI) concentrations ranging from < 1 mg L^{-1} to > 8 mg L^{-1} have been recorded in surface and ground water (Mahimairaja et al., 2000; Naidu et al., 2000b). Although Cr(VI) is only a minor constituent of tannery wastes, the potential transformation of non-toxic Cr(III) to toxic and mobile Cr(VI) in contaminated sites under certain conditions is also possible. Such Cr transformations have led to considerable uncertainty in setting guidelines for disposal of tannery wastes to land and water (Chaney et al., 1996). Hence, most remediation strategies for Cr-contaminated sites are aimed at reducing the Cr(VI) to insoluble and less toxic Cr(III).

Current case study is an extension of a project on the effect of long-term disposal of tannery wastes to soil at Mount Barker, Adelaide, South Australia. This area had been used by the nearby tannery for disposal of the solid and liquid wastes until 25

years ago. The land (approximately 7 ha) has been declared contaminated by the city council and extensive research has been done on the extent of Cr contamination (Naidu et al., 2000b), the chemistry (ACIAR, 2000) and desorption (Avudainayagam et al., 2001) of Cr.

Chromium(VI) is detected in the deeper layers of the soil and leachate at this site, at concentrations far above the permissible levels, even though it has been >2 decades since the last waste input. Despite favourable thermodynamic conditions and high organic matter for its reduction, the detection of Cr(VI) in the soil solution is of great concern to human and environmental health. The reasons for the persistence of Cr(VI) under these conditions are not yet clear.

The major challenge facing the remediation of soils contaminated with Cr-rich wastes is the potential transformation of non-toxic Cr(III) to carcinogenic Cr(VI) under certain conditions. This has also led to considerable difficulties in setting guidelines for disposal of these wastes to land and water ecosystems.

One of the reasons for the presence of Cr(VI) at Mount Barker site is the possible oxidation of the dominant Cr(III). There is strong evidence for the chemical oxidation of Cr(III) by Mn oxides in high Mn-containing soils (Bartlett and James, 1979; Eary and Rai, 1987; Fendorf and Zasoski, 1992; Chung et al., 1994; Milacic and Stupar, 1995; Kozuh et al., 2000), but not in long-term tannery waste contaminated soil. That the formation of Mn oxides in soil is mainly a microbial process is well known (Bromfield and Skerman, 1950; Ghiorse, 1984a,b). Biogenic Mn oxides, in turn, may play an important role in Cr(III) oxidation in soil, in normal or contaminated soils.

An effective strategy for the removal of Cr(VI) from contaminated soil is to irreversibly reduce it to Cr(III) (James, 1996). In acidic conditions, the reduction product [Cr(III)] is not amenable for reoxidation; however, alkaline conditions favour the prevalence of Cr(VI). Addition of organic matter to contaminated soil would serve a dual purpose of favouring Cr(VI) reduction and preventing reoxidation of Cr(III). Apart from organic amendments, other environmental friendly remediation approaches include bioremediation and phytostabilisation (Higgins et al., 1997; Berti and Cunningham, 2000). Bioremediation has been used for remediation of Cr-contaminated soils, but so far, there are no reports specifically targeting Cr associated with long-term tannery waste contamination. Generally, revegetation practices are followed for highly contaminated and barren lands. By revegetating the contaminated soils, the surface properties could be changed by the active root system, with plant-microbe interactions

in the metabolically active rhizosphere possibly aiding the phytostabilisation of metals. Vegetation with an appropriate crop, and with normal agricultural practices certain heavy metals can be made inert in soil by locking them in organic colloids or other inert matrices in soil. This phytostabilisation technique has been used for remediation of cations, such as Pb, Cu, Zn and Cd (Berti and Cunningham, 1994, 1997; Chaney et al., 1997; Didier et al., 1997; Hartman, 1997; Mel Lytle et al., 1998; Berti and Cunningham, 2000). However, this approach is yet to be tried in Cr-contaminated soils. While the chemical characteristics of the soils at the contaminated site have been studied in detail, there is a dearth of information on the microbial processes impacted by long-term contamination or governing Cr transformations at this site. Indeed, while it has been shown that chemical processes such as pH, Eh, etc. can influence the speciation of Cr, the role of biological processes in Cr transformations has been studied to a less extent. Development of sustainable remedial practices for cleaning Cr contaminated soils requires a thorough understanding of both the abiotic and biotic processes that influence Cr transformation in contaminated soils.

To this end, this study, using the long-term tannery waste contaminated site at Mount Barker, was designed to investigate:

(a) the dynamics of Cr at the tannery waste contaminated site and their impact on microbial processes,

(b) the presence of Cr(VI) in these soils with focus on the biotic oxidation of Mn coupled with oxidation of Cr(III) by biogenic Mn oxides, and

(c) the possible phytostabilisation of Cr in the contaminated soil using plants combined with organic amendment.

Learning is finding out what you already know
- Richard Bach -

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CHAPTER 2

Review of Literature

2.1   INTRODUCTION

The increasing urbanisation and human population worldwide has generated an ever-increasing amount of wastes, domestic and industrial. Such wastes have generally been disposed onto land for centuries relying on the soil's capacity to decontaminate waste materials by biological and physico-chemical means and render them harmless by adsorption or precipitation of potential pollutants in the wastes (Martin and Parkin, 1985). Likewise, disposal of tannery wastes onto soil have been a common practice before enactment of stringent regulations in many countries including Australia. Vast areas of agricultural land adjacent to leather industries has been affected by the solid and liquid wastes, especially in the developing countries of Asia and in a few places in Australia, this has led to the destruction of agricultural land (ACIAR, 2000). Leather industries generate enormous amount of wastes that may be grouped into three categories: (i) solid wastes from splitting and trimming of hides, (ii) sludges from dehairing, pickling and chrome tanning, and (iii) liquid wastes from all stages. The vast quantity of sludge produced can be gauged from the fact that 40% of the total weight of the hides ends up as dried waste (Lollar, 1980).

One of the major problems encountered with disposal of tannery wastes is the presence of chromium (Cr), a heavy metal, in the waste. Chromium is widely used in the metallurgic, refractory, chemical and tannery industries. Chrome plating, the deposition of metallic Cr, imparts a refractory nature to materials rendering them resistant to microbial attack and flexible over extended periods of time (Barnhart, 1997). More than five million tonnes of Cr waste are discharged to the environment annually as a consequence of industrial activities. Of the total Cr used in the processing of leather, 40% is retained in the sludge. The disposal of this sludge to soil has led to an increase in Cr levels as high as 30,000 mg kg^{-1} or more in contaminated soils (Naidu et al., 2000b).

Chromium is of great concern, because the soluble Cr species, Cr(VI), is a mammalian respiratory carcinogen (USEPA, 1996a). Cr(VI) is a powerful oxidising agent and causes damage to tissues. In contaminated soils, in the absence of reducing agents, Cr(VI) is soluble in alkaline environment, posing a threat to surface and groundwater quality. For these reasons, regulatory authorities monitoring the decontamination have placed considerable emphasis on rehabilitation of Cr-polluted soils. Most of the techniques for decontamination of Cr-contaminated soils (Higgins et al., 1997) are based on transforming the toxic Cr(VI) to non-toxic Cr(III) species. However, there is still a danger that detoxified forms may later revert to toxic forms due to changes in soil properties, different farming techniques or climatic variables. Hence, it has been difficult to set guidelines and the rapid transformations of Cr have further complicated the task of determining whether Cr-bearing waste or waste contaminated soil is hazardous as recorded in Federal Register, 1991 (James et al., 1997).

This review highlights the

- Cr transformations in soil,

- abiotic and biotic factors governing Cr transformations in soil,

- effect of Cr on soil microbial activities, and

- remediation of Cr-contaminated soils with special emphasis on bioremediation and phytostabilisation techniques.

2.2   FORMS OF CHROMIUM

Chromium, categorised under heavy metals, is the 24th element in the periodic table and is prevalent in nature with chromite ($FeOCr_2O_3$), as the only major commercial product. Chromium occurs in oxidation states ranging from Cr(II) to Cr(VI), as given in Table 2.1.

Generally, Cr(III) has a low toxicity, while Cr(VI) is a skin and mucous membrane irritant and produces an allergic contact dermatitis known as eczema. Cr(VI) is also recognized by the International Agency for Research on Cancer and by the US Toxicology Program as a pulmonary carcinogen (Barceloux, 1999).

Table 2.1 Characteristics of Cr oxidation states

Cr oxidation states	Characteristics	Common forms and solubility
Cr(II)	Reducing agent and readily reduced to Cr(III)	
Cr(III)	Stable form of Cr if in equilibrium with organic carbon in soils. Has affinity to oxide and hydroxides. Forms complexes with ligands and non labile. Replaces Fe(III) in soil because of similar configuration.	Cr_2O_3 [i] Cr(III) phosphate [s] Acetate, oxalate and nitrate salts [s]
Cr(IV)	Less stable and less common with short half lives. React as metal ion in solutions.	
Cr(V)	Long lived anion and stable than Cr(IV) Behave as ligands towards metal ions and derived from chromate mainly	CrO_4^{3-} [s]
Cr(VI)	Strong oxidising anion existing only as oxo species. Stable form of Cr in soils more than NO_3 if we consider equilibrium with atmospheric O_2. Unstable in acid solution in presence of electron donors such as Fe(II), H_3AsO_3, HSO_3 and all organic molecules with oxidisable groups (alkanes, alkenes, alcohols, aldehydes, ketones, carboxylic acids, mercaptans, etc). Less reactive at high pH. Behave as ligands	CrO_3, CrO_2Cl_2, CrO_4^{2-}

(i - insoluble; s- soluble :Ref: (Nieboer and Jusys, 1988)

2.3   SOURCES OF CHROMIUM IN SOIL

Chromium, first discovered in crocoite in 1798, was named after the bright colours of Cr compounds. Chromium occurs not only naturally in soil, but is also added to the soil due to anthropogenic activities.

2.3.1   Natural sources

Chromium is found preferentially in ultrabasic and basic rocks, feldspar materials in particular. On average, the earth crust contains 3700 mg Cr kg^{-1}, most of which resides in the core and mantle (Nriagu, 1988). The Cr concentration of the inner

core is about 12,100 mg kg^{-1} (Liu, 1982). Most of the chrome ores are located in three major places: deposits of bushved complex of South Africa, the great dyke in Zimbabwe and the kemi intrusion of Finland (DeYoung et al., 1984).

2.3.2 Anthropogenic sources

Chromium compounds are widely used in many industries, the major ones being in metallurgical, refractory and chemical manufacture. These industries use low grade chromite ores for numerous applications including pigment manufacture, metal finishing, corrosion inhibition, organic synthesis, leather tanning and wood preservation (USEPA, 1988).

The annual Cr consumption in different industries is given in Fig. 2.1. A large proportion of the worldwide Cr usage (40%) is by the leather industry. Large scale disposal of tannery wastes has significantly contributed to Cr contamination in soils and water worldwide. Most of the Cr reaches the soil by improper disposal of industrial wastes, spills or faulty storage containers (USEPA, 1984). Approximately 50,000 tonnes of Cr-rich solid waste is disposed onto land annually from tannery industries alone. Extensive areas of agricultural land have been wasted due to the disposal of tannery wastes in Asian countries such as India and Bangladesh and also in Australia. About 50,000 hectares of land were rendered barren due to this activity in India and Bangladesh (ACIAR, 2000).
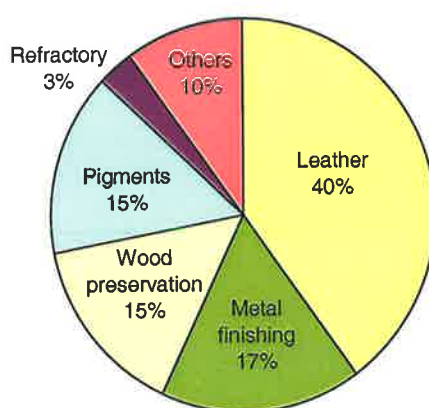
Fig. 2.1 Chromium consumption in different industries.

Studies on a contaminated site around Mount Barker (South Australia) revealed the presence of Cr at concentrations as high as 70,000 to 100000 mg kg^{-1} soil even 20 years after cessation of tannery waste disposal.

2.4 CHROMIUM TRANSFORMATIONS IN SOIL

In soils, Cr(III) and Cr(VI) are the more common forms of Cr. Because of its high mobility, Cr(VI) easily contaminates both groundwater and soil. In contrast, Cr(III) compounds are sparingly soluble and relatively stable in the environment (McGrath and Cegarra, 1992). Following addition of Cr-rich wastes to soil, Cr undergoes rapid transformations and attains a dynamic equilibrium between Cr(III) ⇔ Cr(VI) through a combination of physical, chemical and biological processes. The major processes governing Cr transformations in soils include adsorption or desorption, redox conditions and precipitation or dissolution (Nieboer and Jusys, 1988).

2.5 PHYSICO-CHEMICAL FACTORS GOVERNING CHROMIUM TRANSFORMATIONS IN SOIL

Soil is a complex and dynamic system that harbours a variety of chemical and biological species. Several physical, chemical and biological factors influence Cr transformations in soils.

2.5.1 Soil physical factors

The major soil physical factors are soil texture, soil structure, bulk density, particle density and porosity. Influence of soil physical factors in relation to Cr transformations is not documented in detail, except for a few reports. The adsorption and desorption processes are governed largely by the bulk and particle density of the soils. In peat soils, the Cr(III) was more prevalent owing to its binding to organic and mineral fractions. However, in sandy and clay soils, Cr(III) tended to oxidise because of its solubility in easily extractable fractions of soil solution (Milacic and Stupar, 1995).

Chromium behaves both as anion and cation depending on the speciation. The chemical factors dominate and play a major role in Cr transformations in the soil. Some of the important chemical and biological factors are discussed hereunder.

2.5.2 Soil pH

Soil pH is an important factor governing the nature of Cr species and their transformations. Numerous investigators (Bartlett and James, 1988; Losi et al., 1994c) have demonstrated that soil pH plays a significant role in controlling the dynamics of Cr redox reactions. Low pH favours the formation of stable cationic Cr(III) species whereas at higher pH, especially in alkaline soils, anionic Cr(VI) formation is favoured. At low pH, Cr(III) precipitates or tightly binds to a variety of ligands such as hydroxyls, humates and phosphates present in soil. Organically complexed Cr(III) may remain soluble, whereas the free Cr(III) metal ion would quickly become adsorbed or hydrolysed and precipitated in the presence of soluble complexing ligands. Soil pH along with redox potential (Fig. 2.2) determine the nature of Cr prevalent in the soil (Rai et al., 1989). Cr(VI) exists as anion or cation depending on pH: H_2CrO_4 at pH between 0 to 6.0 and CrO_4^{2-} beyond 6.0.
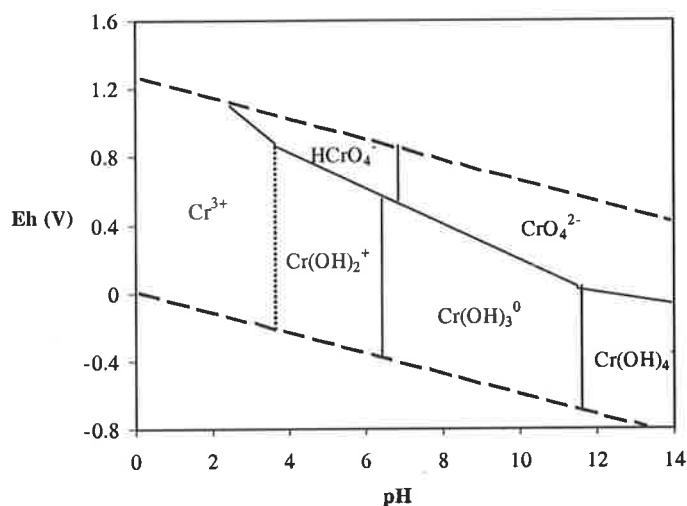
Fig. 2.2 Stability diagram showing speciation of Cr at various Eh and pH values (Rai et al., 1989)

2.5.3 Organic matter

Humic substances and organic matter play a major role in the reduction of Cr(VI) to Cr(III). Organic matter during its oxidation reduces Cr(VI) (Bartlett and Kimble, 1976b). Citric and fulvic acids and water soluble extracts of air-dried soils form soluble complexes with Cr(III) (James and Bartlett, 1983a). Organically complexed Cr(III) may remain soluble whereas metal ions are quickly adsorbed and precipitated.

Complexed Cr(III) hydroxides differ in their solubility. Freshly precipitated Cr(III) such as $CrCl_3$ or $Cr(OH)_3$ are highly soluble compared to aged precipitates and hence become amenable to oxidation. Cr oxidation occurs in the following order: freshly precipitated $Cr(OH)_3$ > Cr-citrate > aged $Cr(OH)_3$ in citrate > aged $Cr(OH)_3$ (James and Bartlett, 1983b). The solubility of these forms of Cr determines the extent of oxidation, which also plays a role in their access to microorganisms for further oxidation. In soils, low in organic matter, incorporation of organic-rich waste amendment has been recommended in order to favour the reduction of Cr(VI) (Bartlett and Kimble, 1976b).

2.5.4   Iron (Fe)

Chromium transformations are, however, influenced by other ionic species released during changes in pH. For example, at low pH, the presence of ferrous (Fe(II)) iron increased the rate of Cr reduction (Palmer and Wittbrodt, 1991). The reduction of Cr(VI) to Cr(III) in the aqueous (aq) phase was rapid, as described by the following reaction:.

$$Cr(VI)\ (aq)\ +\ 3Fe(II)\ (aq)\ \longrightarrow\ Cr(III)\ (aq)\ +\ 3\ Fe(III)\ (aq)$$

At pH values greater than 4.0, brown precipitates were observed (Pettine, 1994), hypothetically *via* the following reaction:

$$xCr(III)\ +\ (1-x)\ Fe(III)\ +\ 3H_2\ \longrightarrow\ (Cr_xFe_{1-x})\ (OH)_3\ (s)\ +\ 3H^+$$

where x varied between 0 and 1. The precipitate formed, presumably $Cr_{0.25}Fe_{0.75}(OH)_3$, was not conclusively identified (Patterson et al., 1997). At low pH, even the addition of small amounts of iron alone can increase the rate of Cr reduction.

The ratio of Fe(II) oxidised was proportional to the amount of Cr reduced (approximately 1.0) (Weng et al., 1996). Two mechanisms have been proposed to explain the chemical reduction of Cr by Fe. Firstly, humic substances convert Fe(II) to Fe(III) which in turn reduces Cr. Secondly, Fe(II) can form $FeCrO_4$ which complexes with chromate. Amorphous iron sulphide minerals like mackinawite (FS_{1-x}) have the potential to reduce large quantities of Cr(VI) (85 - 100%) and form stable Cr, $Fe(OH)_3$ solids (Patterson et al., 1997). Partially oxidised iron was also effective and widely used

for subsurface remediation. In subsoils, a combination of Fe(II), organic matter and low pH (4.2 - 4.3) govern the reduction of Cr(VI) (Powell et al., 1995).

2.5.5 Manganese

Bartlett and James (1979) were the first to report the oxidation of Cr in soils with a pH above 5.0, provided the soil was fresh and moist. The amount of Cr oxidised was proportional to the amount of Mn reduced (exchangeable) and also to the amount of Mn reducible by hydroquinone. However, earlier reports showed no oxidation (Bartlett and Kimble, 1976a) as the analyses were done in dry soils. The minimum amount of MnO_2 necessary for complete oxidation of Cr in soil is not known. A knowledge of the energetics of the reaction in relation to the availability of oxidisable Cr(III) would be desirable.

For optimum oxidation of Cr(III) by manganese oxides, the surface of the latter must be relatively free of specifically adsorbed Mn(II) and other heavy metals. The Cr(III) gets close to a receptive surface, gets quickly oxidised to the anionic form and then repelled by the like negative charges.

The rate and amount of Cr(III) oxidised depend on the soil type, pH, organic matter content, nature of Cr(III) present in soil and quantity of Mn oxides available for oxidation.

a) pH

Manganese-mediated Cr oxidation is dependent on soil pH and is normally observed in any soils with a pH of 5.0 and above. Up to pH 5.5, Cr(III) oxidation was increased by naturally occurring δ–MnO_2 (Fendorf and Zasoski, 1992). However, Cr oxidation by β–MnO_2 increased with decreasing pH. Oxidation of Cr(III) occurs not *via* surface-catalysed reaction with dissolved O_2, but by direct reaction with synthetic β–MnO_2. The extent of Cr(III) oxidation at lower pH is limited by the strong adsorption of anionic Cr(VI), thereby inhibiting contact between active oxidising sites on β–MnO_2 (Eary and Rai, 1987).

Chromium (III) oxidation by naturally occurring δ–MnO_2 was suppressed as pH and Cr(III) concentration increased simultaneously. The reaction products, Mn(II) or Cr(VI), were not limiting for further oxidation. At pH > 3.5, Cr(III) induced alteration

in Mn oxide surface, thus limiting the extent of oxidation. However, the oxidation was also dependent upon Cr(III) concentration, pH, initial surface area and ionic strength (Fendorf and Zasoski, 1992).

Oxidation of Cr(III) by a MnO_2 preparation proceeded rapidly at pH 5.5 and 7.5 with identical rates, slowly at pH 3 and very slowly at pH 1 (Amacher and Baker, 1982). In addition to pH-dependent charge characteristics of oxide minerals, Mn oxide surfaces might also have permanent negative charges as substitution of Mn(II) and Mn(III) for Mn(IV) occurs during their oxidation. Because of the rapid redox transformations and specific adsorption continually taking place on manganese oxide surfaces, these charges will be temporary. Exchangeable Cr(III) is not found in soils with pHs greater than 4.5 or 5. As the pH of a 1 μM Cr(III) solution was lowered, amount of oxidation by a dilute soil suspension ranged from 20% at pH 7.5 to 100% at pH 3.2. In some systems, the effects of pH on charge characteristics and surface behaviour of manganese oxides can mask the effects of pH on Cr speciation and solubility.

b) Cr(III)

The oxidation of Cr(III) is directly related to concentration of Cr(III) in the soils. However, oxidation is also dependent on the moisture content of soils. For instance, James and Bartlett (1983a) observed that soils incubated under moist conditions released 0 – 41 μmol of Cr(VI) L^{-1} after four years of incubation. In 1 m^2 field plots containing 1000 mg Cr kg^{-1}, 0.04 % of the Cr was leached as Cr(VI). In contrast, Ciavatta and Sequi (1989) did not observe oxidation when Cr-rich leather meal was applied to the soil as a fertilizer. The addition of Cr(VI) resulted in its immediate transformation to Cr(III).

A Possible Chromium Oxidation Score (PCOS) was calculated based on the four important factors, waste oxidation potential (WOP), soil oxidation potential (SOP), soil reduction potential (SRP) and soil-waste pH modification value (PMV) as per the formula: PCOS = WOP + SOP + SRP + PMV. Higher values indicated a greater possibility for Cr oxidation in soils. This can be used as a quick screening tool to determine the oxidative ability of soils. Recently, the nature of Cr precipitates on goethite (a crystalline ferric oxide) and silicon dioxide was studied using the scanning force microscope. This study demonstrated a new concept; that the ability of the

precipitating phase to fit the crystal lattice structure of the material on which it is precipitating can cause a significant change in the form and stability of Cr precipitates. Chromium precipitated on goethite spread evenly on the surface and had a low extractability with oxalate compared to Cr precipitating on silica as clumps of Cr(OH)$_3$ which were extracted more rapidly with oxalate (Fendorf et al., 1996) Thus, studies of the chemical nature of Cr precipitated in different soils might also add to our understanding of how soil chemistry can influence potential oxidation of Cr(III) (Chaney et al., 1996).

c) Type of soil

Studies conducted in whole soils to observe the Cr oxidation have shown that the extent of Cr(III) oxidation varied with clay content and the proportion of waste materials containing Cr(III) (Bartlett, 1985, 1986). Land disposal of Cr and associated health effects have been fully reviewed by (Chaney et al., 1981). Behaviour of Cr(VI) in organic waste is similar to slow release nitrate fertilisers and chromium in sludge could release low levels of Cr(VI) over a period of years. Also the Cr associated with high molecular weight ligands is not readily oxidised (Amacher and Baker, 1982). These authors report that sludge borne Cr was not oxidised even for four years; however, moist samples showed phosphate extractable Cr(VI).

2.5.5.1 Mechanisms of Cr(III) oxidation by Mn oxides

Manganese oxides have a high adsorptive capacity for metal ions, thus potentially providing local surface environments in soil. The oxidation of Cr(III) to Cr(VI) occurs after adsorption of Cr to the Mn, with simultaneous formation of Mn(II). The overall reaction is given below:

$$Cr(OH)_2^+ + 1.5\,MnO_2 + H_2O \longrightarrow HCrO_4^- + 1.5\,Mn^{2+} + H_2$$

The rate of transformation is, however, governed by the mineralogy of Mn, soil pH and the form and solubility of Cr(III) in soil (Bartlett and James, 1988; Milacic and Stupar, 1995).

Dissolved oxygen has no effect on Cr(III) oxidation by Mn oxides. There was a proportional increase in oxidation of Cr(III) as the surface of Mn oxide increased. Pyrolusite (β–MnO$_2$) in solution oxidised 15.6% of the spiked Cr(III) (96 μM) at pH 3.0

after 400 h (Eary and Rai, 1987). Acidic pH favoured the dissolution of Mn oxides and enhanced the oxidation of Cr(III). As the pH increased from 3 to 4.3, the rate of Cr oxidation decreased.

$$\beta-MnO_2(s) \ + \ 2H^+ \ \longrightarrow \ Mn^{2+} \ + \ H_2O \ + \ ½ \ O_2 \ (aq)$$

Birnessite ($\delta-MnO_2$), the predominant Mn oxide in soils, effected more than 90% Cr(III) oxidation. The reaction was also faster (24 h) with birnessite than with pyrosulite ($\beta-MnO_2$). pH had a similar effect on oxidation i.e., acidic pH favoured more oxidation. There was no difference in rate of oxidation until pH 4.0 whereas beyond this pH, there was a decrease up to pH 5.2. Cr oxidation was also observed at pH 6.3, 8.3 and 10.1 in spite of the low solubility of Cr(III) at these pH levels. Increases in Cr(III) concentration (200 – 800 μM) retarded its oxidation. The overall reaction for $\delta-MnO_2$ was given as

$$Cr^{3+} \ + \ 1.5 \ \delta-MnO_2 \ + \ H_2O \ \longrightarrow \ HCrO_4^- \ + \ 1.5 \ Mn^{2+} \ + \ H^+$$

At pH 5.0,

$$CrOH^{2+} \ + \ 1.5 \ \delta-MnO_2 \ \longrightarrow \ HCrO_4^- \ + \ 1.5 \ Mn^{2+}$$

Stoichiometry reactions indicate that 1.5 moles of Mn(II) was produced for every mole of Cr(VI) formed. Generally, oxidation of Cr(III) decreased with increasing pH and Cr(III) concentration (Eary and Rai, 1987) and several hypotheses have been postulated to explain the inhibition.

During the Cr(III) and MnO_2 reaction, only a portion of MnO_2 is available for oxidation. There is evidence that Mn(II) and Cr(VI) inhibit Cr(III) oxidation. The more Mn(II) formed the lower the chance for Cr(III) to react due to poisoning of the surface. In acidic pH, the MnO_2 has a negative charge and Mn(II) and Cr(III) compete for binding sites. However, with an increase in binding of Mn(II) the negative charge on MnO_2 surfaces is lowered. This in turn increases the pH of the surface due to –OH ions and Mn(II) gets auto-oxidised by atmospheric oxygen. When Mn(II) gets auto-oxidised, the surface becomes more negative and is available for further Cr(III) oxidation. But, Fendorf et al. (1993) showed that oxidation of Cr(III) was not inhibited by the addition of Mn(II) to the system.

In contrast, Eary and Rai (1987), proposed that Cr(VI) formed was a limiting factor in Cr(III) oxidation. The initial Cr(III) oxidation was instantaneous and Cr(VI) formed gets strongly bound to β–MnO$_2$ surfaces, especially at acidic pHs. Moreover, this adsorption also retarded the dissolution of MnO$_2$. The dissolution of MnO$_2$ was more in Cr-free solution than with Cr in the solution (Eary and Rai, 1987). At neutral and alkaline pHs, Cr(OH)$_3$ precipitates. The difference in mechanism between δ– and β–MnO$_2$ was attributed mainly to the differences in zero point charges (pH 2.3 for δ–MnO$_2$ and 7.3 for β–MnO$_2$). Formation of MnOOH as the intermediate at higher pHs in β–MnO$_2$ can also lead to decreased oxidation of Cr(III).

$$Cr(OH)^{2+} + 3\,\beta\,MnO_2(s) + 3\,H_2O \longrightarrow HCrO_4^- + 3\,MnOOH(s) + 3\,H^+$$

γ-MnOOH is formed during the reduction or oxidation of Mn oxides as an intermediate (Johnson and Xyla, 1991) The oxidation kinetics of Cr(III) to Cr(VI) on the surface of manganite (γ–MnOOH) is a function of Cr(III) and manganite concentration, pH, ionic strength and temperature. The reaction is first order with respect to the manganite adsorption density and also Cr(III) concentration up to a critical adsorption density (0.2 μmol m^{-2}). Above this concentration, the reaction is inhibited. The reaction is independent of pH and ionic strength.

Considering the chemical speciation, the overall reaction at pH 4.5 can be written as

$$Cr(OH)^{2+} + 3\,MnOOH \longrightarrow HCrO_4^- + 3Mn^{2+} + 3OH$$

The oxidation rate of Cr(III) is 10 - 10,000 times faster with γ-MnOOH than with other Mn-oxides. Such fast rates of Cr(III) oxidation by γ-MnOOH contribute significantly in the cycling of Cr in natural water systems.

Nakayama et al., (1981) found that, in sea water, only 10% of a 10^{-5} M solution of Cr(III) was oxidised with 30 mg γ–MnOOH L^{-1} in 100 h. The low oxidation may be linked to the organic substances in natural waters. Experiments with salicylate clearly indicated the inhibition of Cr(III) oxidation reaction with γ-MnOOH by organic ligands.

2.6 MICROBIOLOGICAL FACTORS GOVERNING CHROMIUM TRANSFORMATIONS IN SOIL

Similar to chemical factors, biological factors also play a major role in the mobilisation of Cr in soils through ingestion. Since Cr(III) is known to have little nutritional value few soil macrofauna utilise Cr. The important microbial factors influencing Cr transformations are dealt in this section.

2.6.1 Chromium bioreduction

A wide variety of heterotrophic microorganisms are known to be involved in the reduction of Cr(VI) to Cr(III), aerobically or anaerobically depending on the organism (Lovley and Phillips, 1994). Screening of a variety of Cr-contaminated and pristine soils indicated that Cr(VI) reducers are ubiquitous and are able to reduce Cr(VI) under ideal physicochemical conditions (Turick et al., 1996a). Chromium-tolerant microbes have been isolated from water sediments (Luli et al., 1983) and soil (Losi et al., 1994a). Some of the bacterial isolates reported are listed in Table 2.2.

In soils, microbial Cr reduction may occur directly or indirectly. In the direct mode, Cr is taken up by the microbes and enzymatically reduced (Komori et al., 1990b; Losi et al., 1994c; Lovley and Coates, 1997), while in the indirect mode, reduction products of microbial decomposition in the soil such as H_2S reduce Cr(VI) (DeFilippi and Lupton, 1992).

2.6.1.1 Direct reduction

Biosorption is the non-directed physico-chemical complexation reaction between dissolved metal species and charged cellular components. The precipitation or crystallisation of metals can take place at or near the cell. Also, insoluble metal species can be physically entrapped in microbially produced extracellular matrix. Many extracellular matrices consist of neutral polysaccharides, uronic acids, hexosamines and organically bound phosphates that are capable of complexing metal ions. Metabolically mediated accumulation is usually intracellular and linked to the control of plasmid linked genes (Shumate II and Strandberg, 1985). The reduction process is believed to involve a specific, plasmid mediated enzyme released during growth by certain microorganisms (Bopp and Ehrlich, 1988).

Table 2.2 Cr(VI) reducing microbial isolates

Identification	Source	Cr tolerance level	Cr reducing efficiency	Mechanism	References
Consortium of sulphate reducing bacteria (SRB)	Metal refining wastewaters	2500 ppm Cr(VI)	80 - 95% from 50 - 2000 ppm	Indirect involving H_2S	(Fude et al., 1994)
Enterobacter Cloacae HO1	Activated sludge sample	10 mM	90 % removal	Membrane associated and CrO_4^{2-} as terminal electron acceptor	(Wang et al., 1989; Komori et al., 1990a)
Bacillus sp. QC1 - 2	Soil and water samples near chromium processing factory	0.33 mM	100 % within 22h	Cr(VI) reductase enzyme (soluble NADH dependent)	
Agrobacterium radiobacter EPS - 916	-	0.5 mM	100% removal within 6 h	-	(Llovera et al., 1993)
Saccharomyces cerevisiae	-	1.9 ppm	100% reduction	-	(Krauter et al., 1996)
Escherichia coli ATCC 33456	Activated sludge	0.3 mM	100\% reduction in 12 h	Enzymatic reduction	(Shen and Wang, 1993)
Desulfovibrio vulgaris	-	-	-	-	(Lovley and Phillips, 1994)
Candida sp	Sewage/ tannery-treatment plant tannery wastes	10 mM	No reduction	-	(Baldi et al., 1990)

Strains of *Pseudomonas, Oscillatoria, Arthrobacter, Agrobacter, Chlorella* and *Zoogloea* have been reported to enzymatically reduce Cr(VI) (Losi et al., 1994b). *Pseudomonas ambigua* and *P. fluorescens* reduced 50 - 60 % of the 150 mg of Cr(VI) L^{-1} over 36 h (Horitsu et al., 1987) and an *Enterobacter cloacae* strain reduced 90 - 95% of 208 mg of Cr(VI) L^{-1} (Ohtake and Silver, 1995). (Cifuentes et al., 1996) reported that organic amendments enhanced the reduction of Cr by indigenous microflora. Anaerobic chromate reducing strains are prevalent in subsurface soils and probably enhance Cr reduction in this environment (Turick et al., 1996b).

2.6.1.2 Indirect reduction

Microbes can also indirectly promote Cr(VI) reduction when products of microbial metabolism such as Fe(II) and sulphide, abiotically reduce Cr(VI) (Lovley, 1995). Gaseous bioreduction is an important mechanism where anaerobic conditions prevail, as in flooded compacted soils. For instance, H_2S, produced by sulphate reducing bacteria causes effective reduction of Cr(VI) (Losi et al., 1994c). Recently, (Wielinga et al., 2001b) elucidated the reduction of Cr(VI) by a closely biotic-abiotic coupling reduction using Fe reductive pathway. They used dissimilatory Fe reduction by *Shewanella alga* for reduction of Cr(VI) injected into the bioreactor.

2.6.2 Chromium biooxidation

Though extensive literature exists on microbial reduction of Cr(VI), biological oxidation of Cr(III) has not been reported.

2.6.2.1 Direct oxidation

No convincing evidence exists so far for direct oxidation of Cr(III) to Cr(VI) by microorganisms. Since Cr(VI) is toxic to most microorganisms due to its high oxidising nature, oxidation reactions may not be a predominant process.

2.6.2.2 Indirect oxidation

Biotic oxidation of Cr may be possible through indirect pathways where chemical species capable of oxidising Cr(III) are produced by microbial action. Such transformations could in the soil involve either Mn or Fe reducing microorganisms (Fig 2.3) that may indirectly facilitate chemical Cr oxidation.
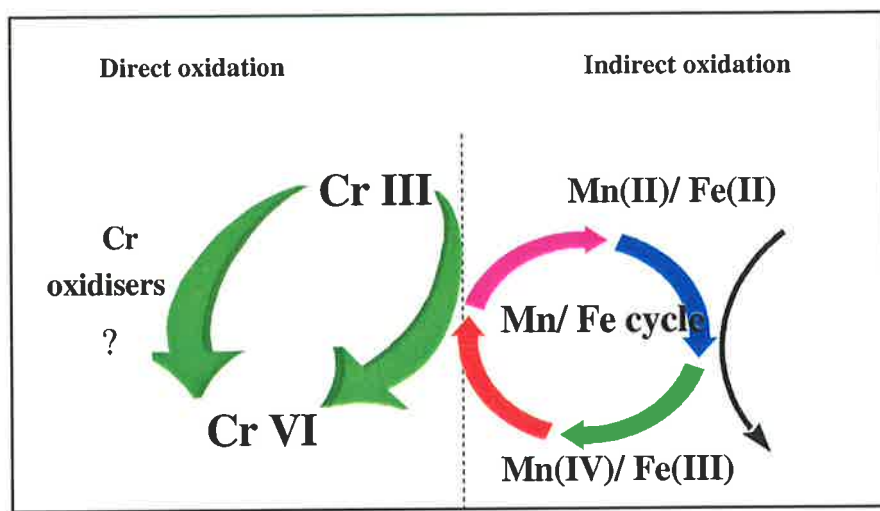
Fig 2.3 Possible direct and indirect Cr oxidation reactions in soil

A diverse range of Fe(III) and Mn(IV) oxide forms is potentially formed by microorganisms to utilise energy during redox reactions and also to prevent the accumulation of Fe/Mn at toxic concentrations in the environment. Fe(III), available in significant quantities in the soil, is used as an electron acceptor in the degradation of organic pollutants (Lovley and Phillips, 1988).

Although it is speculated that both Fe and Mn redox systems could be involved in Cr oxidation reactions, the following review focuses mainly on Mn systems, as the role of Mn in chemical oxidation of Cr in soils is well established (Section 2.5.5). The nature and amount of Mn oxides formed in the soils determine the rate of Cr oxidation. However, oxidation of Mn(II) to Mn(IV) in the soil is mediated essentially by microorganisms. The following literature discusses the processes of microbial Mn oxidation in soils, mechanisms of Mn oxidation and the nature of Mn oxides formed given that such oxides could play a significant role in Cr(III) oxidation in contaminated soils.

2.6.3 Microbial oxidation of manganese

The oxidation of Mn(II) to its higher oxides in natural environments such as soils (Leeper and Swaby, 1940), fresh water lakes (Tipping et al., 1985) and estuarine waters and sediments (Edenborn et al., 1985) is mediated by a range of heterotrophic microorganisms (Ghiorse, 1988). The microorganisms involved are capable of oxidising Mn(II) far more effectively at neutral pH levels (pH 6-8) than any non-biological system. Oxidation of Mn(II) to Mn(IV) is an exergonic reaction, yielding approximately

-18.2 kcal mol^{-1} at 1 M concentrations of the reactants which can be used as an energy source by microorganisms; but also there are many contradictory reports (Ghiorse, 1984b). That this reaction minimises the toxic effect of Mn(II) by transforming it to insoluble oxides (that cannot enter the cell) is yet another advantage (Bromfield, 1976).

The heterotrophic microbial populations which can effect the conversion of soluble Mn to solid Mn oxides include bacteria, prosthecate bacteria, sheathed bacteria, fungi, algae and their synergistic combinations (Ehrlich, 1981; Nealson, 1983; Nealson et al., 1988). Mn oxidation mechanisms may be either direct or indirect. The direct mechanisms involve either catalysis or specific binding of cell associated materials, which enhance auto-oxidation later. Indirect mechanisms relate to microbially promoted changes in the cell's microenvironment that later lead to non-biological oxidation of Mn(II) (Greene and Madgwick, 1991)

2.6.3.1 Microbial Mn oxidisers

A wide range of microbial cultures exhibiting Mn oxidising ability in water and soil matrices include: *Bacillus* SG1 (Rosson and Nealson, 1982; Mandernack et al., 1995a), *Arthrobacter* sp., *Leptothrix* sp. (Ghiorse, 1984b), *Pseudomonas* sp. S 36 (Nealson, 1983), *Oceanospirillum* sp. (Ehrlich, 1982), and *Pedomicrobium* sp. (Larsen et al., 1999). Fungal cultures capable of oxidising Mn include the white rot fungus *Phanerochaete chrysosporium* (Kirk and Farrell, 1987) and an isolate of *Streptomyces* (Bromfield, 1979). Algae such as *Scenedesmus* (Knauer et al., 1999) and *Chlamydomonas* (Greene and Madgwick, 1991; Stuetz et al., 1996) are also known to oxidise Mn.

2.6.3.2 Mechanisms of Mn oxidation

As discussed above, the oxidation of Mn is mediated either enzymatically or by its binding to other extracellular secretions (Table 2.3). The majority of the Mn oxidising systems are extracellular as in cultures of fungi and a *Streptomyces* wherein the oxidising factors are diffused into the surrounding environment (Bromfield, 1979). The Mn oxides formed in fungus are accumulated mainly in the hyphae, resulting in black coloured colonies. Take all fungus (*Gaeumannomyces* graminis var. tritici) has been reported to oxidise Mn in soils (Graham et al., 1984). *Bacillus* and *Leptothrix discophora* have been extensively studied for their mode of Mn oxidation. In *Bacillus*

SG 1, the spore coats bind and oxidise Mn(II) in a similar manner like whole spores (deVrind et al., 1986).

Greene and Madgwick (1991) have reported that a *Pseudomonas* strain in association with an alga *Chlamydomonas* oxidised 5 g Mn(II) L^{-1} to disordered semi-pure γ–MnO_2 and manganite (γ–$MnOOH$) as an intermediate. Indirect Mn oxidation, where the changes in pH and/or Eh of an environment are modified as a result of metabolism and growth of microorganisms, is well documented. Bacteria and fungi have long been recognised to catalyse this type of non-specific manganese oxidation (Ehrlich, 1976).

Table 2.3 Microorganisms capable of oxidising manganese.

Organism	Source	Oxidising part	Optimum temperature for oxidation	pH	Reference
Bacillus SG1	Shore sediments	Mature dormant spores	4 - 45°C	7.8	(Rosson and Nealson, 1982)
Leptothrix discophora	Fresh water sediments	Protein in sheaths (110kD Mr)	7.8 - 28°C		(Ghiorse, 1984b)
Pseudomonas S-36	Marine sediment enrichment	Extracellular glycocalyx			(Nealson, 1978)
Pedomicrobium sp.	Fresh water	Extracellular enzymes			(Larsen et al., 1999)
Scenedesmus sp.	Fresh water	Extracellular	25	7.9	(Knauer et al., 1999)

2.6.3.3 Microbial Mn oxides

Though microbial Mn(II) oxidation has been investigated extensively, less attention has been given to the characterisation of the microbially formed Mn oxides (Greene and Madgwick, 1991). Generally, the identification of Mn oxides presents problems, because of their complex nature. The most common methods used for characterisation of Mn oxides are X-ray diffraction and FTIR (Fourier Transform Infrared) spectroscopy (Murray et al., 1984). Microbial Mn oxides include hausmannite (Mn_3O_4), fietknechite (β–MnOOH), manganite (γ–MnOOH) (Mandernack et al., 1995b), todokorite (Takematsu et al., 1988) and γ–MnO_2 (Greene and Madgwick, 1991).

In pure bacterial cultures, Mn is oxidised initially to a low valence state, predominantly hausmannite which later disproportionates to MnO_2 (Hem and Lind, 1983), in a two-step process:

$$3\ Mn^{2+} + 3H_2O + 1/2\ O_2 \longrightarrow Mn_3O_4 + 6H^+$$

$$Mn_3O_4 + 4\ H^+ \longrightarrow MnO_2 + 2\ Mn^{2+} + 2\ H_2O$$
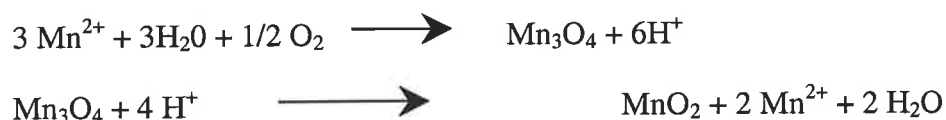
Similar results have been reported with *Bacillus* SG-1 (deVrind et al., 1986). SEM studies showed that microbial Mn oxides are not highly crystalline and are amorphous re-crumpled, and sheety microcrystalline solids. Prolonged incubation of cultures for a few weeks or months resulted in more crystalline forms of Mn oxides (Mandernack et al., 1995b). However, Murray et al. (1985) reported that even after 8 months the reaction did not proceed beyond γ–MnOOH. The reaction was also rapid in initial stages and once the cells and sheaths were covered with an excess of Mn oxides, autooxidation predominated over bacterial oxidation. The morphology of Mn oxides formed by bacterial pure cultures was very similar to that of many naturally occurring Mn precipitates surrounding microbes in environmental samples (Ghiorse, 1984b).

2.6.3.4 Factors governing Mn oxidation and oxides formed

The type of oxide formed can vary according to the type of microorganisms and changes in chemical, physical and growth conditions of cultures (Table 2.4). *Metallogenium* cultures catalysed the deposition of disordered Mn oxides like vernadite (δ–MnO_2) in association with a Mn oxidising fungus (Emerson et al., 1982).

Table 2.4 Mn oxides formed by microorganisms

Organisms	Mn oxides formed	Reference
Bacillus SG1	Hausmannite, manganite	(Mandernack et al., 1995b)
Leptothrix sp.	Hausmannite, manganite	(Adams and Ghiorse, 1988)
Pseudomonas sp.	Manganite	(Nealson et al., 1988)
Pseudomonas & Chlamydomonas	Manganite γ–MnO_2	(Greene and Madgwick, 1991)
Pedomicrobium	Manganite	(Larsen et al., 1999)

In general, low Mn oxide concentration (nM-μM) and low temperature promoted direct oxidation of Mn(II) to Mn(IV) (Table 2.5) without any intermediate steps, as reported earlier by (Rosson and Nealson, 1982).

Table 2.5 Mn oxides formed by *Bacillus* spores in a culture broth as a function of Mn(II) concentration and temperature.

Initial Mn(II)	3°C		25°C		50°C	
	Mineral	Final Mn(II) (μM)	Mineral	Final Mn(II) (μM)	Mineral	Final Mn(II) (μM)
10 μM	manganate	0.2	manganate	0.2	manganate	ND
100 μM	Buserite	0.3	buserite	1.8	manganate	89.8
1 mM	manganate	889	manganate feitknechite	455	manganate	3.6
10 mM	amorphous	7700	manganate feitknechite	8800	manganate & hausmannite	4600

(modified from (Mandernack et al., 1995b)

2.6.3.5 Mn oxides in soil

In soils, the commonly occurring Mn oxides are birnessite and vernadite (McKenzie, 1988). Birnessite is mainly formed in neutral and alkaline soils whereas in acid soils, the coprecipitates may be manganites. In flooded soils, the intermediate oxides, $Mn(Fe)_2O_3$ (bixbyite), $3(MnFe)_2O_3.MnSiO_3$ (braunite), $(Mn^{+2}Fe)(Mn^{+3}Fe)_2O_4$ (jacobsite), and $Mn_3O_4.Fe_3O_4$ (vrendenburgite) and perhaps γ-MnOOH (manganite) and Mn_3O_4 (hausmannite) may be present. When the flooded soils are drained, co-precipitates of iron and manganese are probably formed (Ponnamperuma et al., 1969). Table 2.6 shows the common forms of Mn oxides prevalent in the stated pH range.

Table 2.6 Manganese oxides at different pH values.

Manganese forms	pH
δ-MnO$_2$	3.7 - 4.2
γ-MnO$_2$	4.2 - 4.6
γ-Mn$_2$O$_3$	6.5 - 7.2
γ-MnOOH	6.4 - 7.0
Mn$_3$O$_4$	6.3 - 6.9

Most of the Mn oxides, present in the soil, especially manganate and birnessite (the highly reactive forms of Mn oxides in Cr oxidation), are also produced by microbial cultures (Section 2.5.5).

2.7 IMPLICATION OF CHROMIUM TRANSFORMATIONS ON MICROORGANISMS

There is abundant literature on the effects of heavy metals on soil microbes and microbial processes (Doelmann and Haanstra, 1986). Chromium is also lethal to many organisms with several reports of human poisoning worldwide.

2.7.1 Effect on microorganisms

Many microbial cells showed negative response upon contact with Cr. Genotoxic effects in microbial cells are mainly produced by Cr(VI) resulting in frameshift mutations (Petrilli and deFlora, 1977) and lethal DNA damage. Cr(VI) can diffuse across the microbial cell membrane and hence is more toxic than Cr(III) (DeFlora et al., 1984). Accumulation of Cr resulting in cell sequestering is a major phenomenon in most of the microbes to combat its inhibitory effect.

Microorganisms differ in their tolerance to Cr(VI) (Table 2.7). Some of the important changes in bacteria and fungi due to Cr are summarised in Table 2.8. Gram-positive bacteria are more resistant than Gram-negative bacteria (Ross et al., 1981).

2.7.2 Effect on soil microbial populations

Phospholipid fatty acids are a good indicator of environmental disturbance. The technique is based upon the fact that different subsets of microbial community differ in their fatty acid composition. Membrane lipids and their associated fatty acids are particularly useful biomarkers as they are essential components of every living cell and have greater structural diversity, coupled with high biological specificity. Also, by using the phospholipid composition, only one can ensure that the measurement is on the living part of the microflora, since phospholipids are assumed to decompose quickly after the organism dies. The PLFA pattern can therefore be viewed as an integral measurement of all living organisms present in that sample, reflecting both species composition and relative species abundance (Baath, 1989).

Table 2.7 Tolerance of bacteria to Cr(VI).

Organism	Tolerable Cr(VI) concentration (mg L^{-1})	References
Pseudomof different onas K 21	5, 356	(Shimada, 1979)
P. fluorescens	>400	(Bopp, 1980)
Arthrobacter sp.	450	(Coleman and Paran, 1983)
Agrobacterium sp.	100	(Coleman and Paran, 1983)
Escherichia coli	66	(Thompson and Watling, 1984)

Table 2.8 Effect of chromium in microbes

Organism	Cr species and concentration.	Effects	Reference
Staphylococcus aureus, S. epidermis, Bacillus cereus, B. subtilis	1×10^{-2}M, $K_2Cr_2O_7$	Small sized colonies, cell elongation, cell enlargement, results in filamentous forms	(Bondarenko, 1981)
Shigella sonnei, Shigella flexneri, Salmonella typhosa, Proteus mirabilis and *Escherichia coli*	3.2×10^{-2}M, $K_2Cr_2O_7$	Small sized colonies	(Bondarenko, 1981)
E. coli	Cr(VI)	Filamentous forms observed	(Theotou, 1976)
Arthrobacter sp.	Cr(VI) 0-200 mg l^{-1}	Depressed growth rate. MGT[†] increased from 2.1 - 10.2 h	(Coleman, 1983)
Agrobacterium sp.	0-100 mg l^{-1}	MGT increased from 1-3 h	(Coleman, 1983)
Serratia marcescens	Chromous nitrate 0.37 mM	Inhibit prodigiosin production	(Furman, 1984)
Pseudomonas dechromaticans		Utilise chromates and dichromates, results in clumping of cells	(Romanenko, 1977)
Photobacterium phosphoreum	Cr(VI) twice toxic than Cr(III)	Reduction in light emission	(Qureshi, 1984)

[†] MGT - Mean Generation Time

Phospholipid fatty acids have been useful in distinguishing the abundance and structure of microbial communities in soils (Zelles, 1999). There are several reports on the shift in microbial populations in short- and long-term contaminated soils with Cu, Pb, Zn and Ni as compared to uncontaminated soils (Frostegård et al., 1993; Pennanen et al., 1996; Griffiths et al., 1997; Kelly et al., 1999a, b). In most cases, the multivariate PCA differentiates the PLFA patterns of polluted soils from those of unpolluted soils. Sludge (44.5 Cd, 512 Cr, 341 Cu, 159 Ni, 337 Pb and 1506 Zn in mg kg^{-1}) amended soils showed a difference in PLFA patterns (Kelly et al., 1999a). In sludge amended soils, counts of culturable bacteria significantly increased, in contrast to > 20-fold decrease in DHA. There was a relative decrease in fatty acids, 18:010Me, 16:1ω5c, 18:2ω6c and 20:2ω6c. Such relative decreases in several fatty acids in sludge amended soils suggested inhibition of several specific populations of soil microorganisms. However, there is no report hitherto on PLFA patterns and shift in microbial populations in soils freshly spiked with Cr alone or in long-term tannery waste contaminated soils with Cr as the major pollutant.

2.7.3 Effect of chromium on soil microbial processes and activities

Usually, concentration of Cr in soil varies from 100-300 mg kg^{-1}; however, the concentration of Cr available to soil microflora is low. The toxic effects of Cr are mainly governed by the speciation rather than by the total Cr concentration. In alkaline soils, Cr(VI) in solution is dominant, resulting in an increased inhibition; but, in acidic soils, most of the Cr(VI) complexes with organic matter and gets reduced to Cr(III), leading to its decreased toxicity.

Dehydrogenase activity (DHA) is one of the important parameters widely used to study the ecotoxic effects of metals and organic contaminants. The main advantage of this method is that it reflects the overall microbial activity of the active microbial populations in the soil to provide the current status of soil health.

Generally, in sewage sludge amended soils, a decrease in DHA was observed and since sewage sludge contains a mixture of heavy metals, it is difficult to identify the metal responsible for the specific effects. In contrast, an increase (18-25%) in DHA has also been reported in soils amended with sewage sludge containing 220 μg of Cr g^{-1} of soil. This increase was more pronounced in sandy loam than in loam or clay loam

(Hassan Dar, 1996). Reports on the effect of Cr on different microbial processes are summarised in Table 2.9.

Soil factors such as pH moisture content and CEC (Doelmann and Haanstra, 1979) influence the DHA in soils. Soil pH determines the amount of metal available to the microbes in soil solution and thereby its eventual effect on DHA. Likewise, moisture at field capacity might mask the effect of heavy metals on DHA.

Long-term incubation of soils with heavy metals has a great impact on DHA. However, in general, Brendecke et al., (993) found that DHA and soil respiration were little affected by sewage sludge containing multimetals even after four years of its application. A decreased toxicity was observed in most cases as the exposure time increased. This was attributed to the elimination of the sensitive microbial populations by the chronic effects of the heavy metals with a concomitant shift toward the dominance of tolerant microorganisms. The increased abundance of tolerant organisms in polluted environments can be due to genetic changes, physiological adaptations or replacement of metal sensitive species with species that already are tolerant for that heavy metal. Bacterial cultures e.g. *Pseudomonas* could tolerate maximum Cr(VI) concentrations of about 5,356 mg L^{-1}. Thus, a distinct shift in population can occur in contaminated soils, especially under long-term impact. Several techniques such as phospholipid fatty acid (PLFAs), degenerative gradient gel electrophoresis (DGGE) and thymidine incorporation (Diaz-Ravina and Baath, 1996) have been used recently to determine the microbial populations in agricultural soils and in soils polluted with organics and inorganics. Among these techniques, PLFA has been used widely to determine the impact of metals on microbial communities in soils.

2.8   REMEDIATION OF CHROMIUM CONTAMINATED SOILS

Remediation of soils, water and sediments, contaminated with metal and organic pollutants has been studied extensively in the last two to three decades. Several treatment techniques are available for remediation of soils contaminated with chrome wastes. Traditional and innovative methods to manage Cr(VI) contaminated soils have been reviewed by (Higgins et al., 1997). The techniques chosen are mainly based on the feasibility and cost at that particular location and the concentration of Cr(VI) present in the polluted soils. Though the total Cr concentration is important, in remediation technologies utmost consideration is given to Cr(VI) levels, because of its carcinogenic

Table 2.9 Effect of chromium on soil microbial processes

Soil type	Cr Concentration (μg g-1)	Effects, Parameter measured, % inhibition	References
Silt loam 1.31% C+ 1% dry sludge and 1% alfalfa	8.6	-, CO_2, 10%	Chang and Broadbent (1981)
Sandy pH 7.0, 1.6% OM and CEC 1-2	400	-, CO_2, 17%	(Doelmann, 1985)
pH 6.2, 64% OM	260	-, CO_2, 15%	Lighthart et al. (1983)
pH 6.7, 3.1% OM	26	-, CO_2, 10%	Lighthart et al. (1983)
Forest sandy loam pH 7.0	50	-, CO_2, 20%	Skujins et al. (1986)
Forest Humus pH 3-4.2	10000	0, N mineralisation	Ruhling and Tyler (1979)
Silt loam pH 6.9 + 1% sludge and alfalfa	400	-, N mineralisation, 40%	Chang and Broadbent (1982)
	100	-, nitrification, 40%	Chang and Broadbent (1982)
Soil litter, agricultural pH 5.8-7.8 2.6-5.5% C	260	-, N mineralisation, 18% -, nitrification	Liang and Tabatabai (1977)
Agricultural, pH 5.8-7.8 2.6 - 5.5 % C	269	-, nitrification, 81%	Liang and Tabatabai (1978)
Sandy loam	500 Cr + 1000 Ni	-, nitrification	Morrissey (1985)
Forest sandy clay pH 7.0	200	-, nitrification, 26%	Skujins et al. (1986)
	50	-, N fixation, 93%	Skujins et al. (1986)
Sandy loam pH 6.6, 0.84 % C	0.32Cd+5.6Cu+7.2Pb+ 30.7Zn+0.76Cr	0, nitrification	Wilson (1977)

Soil	Concentration	Effect	Reference
	2.24Cd+7.5Cu+47Pb+148Zn+15.7Cr	-, nitrification	Wilson (1977)
	0.56Cd+1.88Cu+12Pb+37Zn+3.9Cr	+, lag nitrification	Wilson (1977)
Forest Humus pH 3.6-4.1	200 Cr+Ni+Mo	-, phosphatase, 20%	Ruhling and Tyler (1979)
pH 6.2-7.0, 2.7-5.3% C	130	-, aryl sulfatase, 19%	Al-Khafaji and Tabatabai (1979)
Sandy pH 5.7, 2.8% OM	1500	-, dehydrogenase, 40%	(Doelmann and Haanstra, 1979)
Clay pH 7.5, 3.2 % OM	7500	0, dehydrogenase	(Doelmann and Haanstra, 1979)
Peat pH 5.7, 46 % OM	7500	0, dehydrogenase	(Doelmann and Haanstra, 1979)
Sandy pH 7.0, 1.6 % OM	390-1880	-, urease, 10%	(Doelmann and Haanstra, 1986)
Sandy peat pH 4.4, 12.8 % OM	360	- urease, 10%	(Doelmann and Haanstra, 1986)
Agricultural soil 1.3 % OM	150	-, dehydrogenase, 83%	Rogers and Li (1985)
Forest sandy loam, pH 7.0	200	-, urease, 28%	Skujins et al. (1986)
Agricultural soil 2.5% OC	269	-, urease, 17-50 %	Tabatabai (1977)
Silt loam pH 6.0, 2.1 % OM	1.0	-, CFU bacteria 20%	Zibilske and Wagner (1982)
	1.0	-, ATP, 60%	
	556	0, altered fungal community	

(modified after Baath, 1989)

nature. The risk-based soil cleanup level guideline (USEPA, 1996c) is 390 mg Cr kg^{-1} based on the ingestion pathway and the soil screening level is 270 mg Cr(VI) kg^{-1} for human exposure by inhalation (USEPA, 1996b). But, there is no comparable soil screening level for Cr(III) as such. Also the permissible limit for Cr(VI) in potable water is 0.05 mg L^{-1} as per (USEPA, 1996a).

The selection of the remediation depends on: 1) the size, location and history of the site, 2) soil characteristics like structure, texture, pH etc., 3) the type, physical and chemical state of the contaminants, 4) the degree of contamination, 5) the desired final land use and 6) the technical and financial means available.

Advances in understanding the chemistry and toxicity of Cr compounds have led to efforts to remediate Cr-contaminated soil. Some of the important techniques used are excavation and disposal, soil washing, soil flushing, solidification (*ex situ* and *in situ*), vitrification, chemical and biological reduction and phytoremediation. The advantages of each method and the cost involved are given in Table 2.10.

All the methods listed have their own advantages and disadvantages (Higgins et al., 1997). The selection of the most appropriate technology is based on the concentration of Cr(VI) present in the polluted soils, nature of contamination, feasibility and cost at that particular location. Of all these methods, bioremediation and phytoremediation have been the most widely used, because they are economical and do not release further waste into the environment. The main aim of current remediation techniques is irreversible reduction of Cr(VI) to Cr(III) and its hydroxides. Reduction of Cr(VI) can be achieved by incorporation of organic matter, Fe-containing salts and organic acids (James, 1996). The Cr(VI) reduction reactions include the following:

1. Reduction with Fe and Fe compounds

$$Fe + CrO_4^{2-} + 0.5\,H_2O \longrightarrow Fe(OH)_3 + 0.5\,Cr_2O_3$$
$$6\,Fe^{2+} + 2\,CrO_4^{2-} + 13\,H_2O \longrightarrow 6\,Fe(OH)_3 + Cr_2O_3 + 8H^+$$
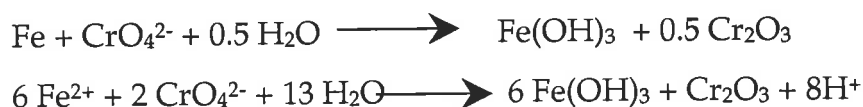
2. Reduction by organic compounds (e.g., hydroquinone)

$$1.6\,C_6H_6O_2 + CrO_4^{2-} + 2H^+ \longrightarrow 0.5\,Cr_2O_3 + 1.5\,C_6H_4O_2 + 2.5H_2O$$
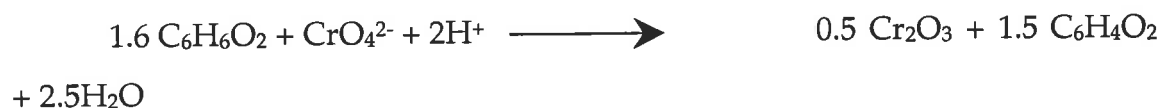
Table 2.10 Methods for remediation of chrome contaminated soils: Advantages and disadvantages

Method	Advantages	Disadvantages	Cost (US $/ tonnes)
Excavation and offsite disposal	Appropriate for small volumes of soil and quick	Makes Cr(VI) airborne and hence related health hazard, can be expensive especially for deep materials	100-200
Soil washing	Used where there is a high concentration of Cr	Makes Cr(VI) airborne, generates contaminated water	50-200
Soil flushing	*In situ* technique used for spills	Generates contaminated water	75-200
Solidification	Relatively inexpensive	Cr(VI) should be reduced first, may require soils dewatering	40-100
Vitrification	Reduces and immobilises Cr (VI)	Very expensive, high energy requirement	350-400
Chemical reduction	Mainly *ex situ* processes	Require high quantity of reducing agents, sometimes generate lots of chemical waste	75-100
Biological reduction	*In–situ*, applicable for sites where there is Cr (VI) leaching	Does not remove Cr, required controlled conditions and process is slow	20-100
Phytoremediation	*In situ* remediation	Does not remove the Cr	

(Higgins et al., 1997)

2.8.1 Bioremediation

A wide range of microorganisms has been demonstrated to have Cr reducing ability (Section 2.6.1). These properties are harnessed in bioremediation wherein the microbial strains are multiplied to desired population and pumped into soil/sediments to promote Cr reduction. The efficiency can be enhanced if the organic matter content and nutrient availability of the soil are sufficient to promote the growth of the introduced microflora. In *in situ* techniques, nutrients are pumped along with aeration to promote the Cr reduction. Some Cr-reducing bacteria and algae have been efficiently used in the treatment of Cr-rich waste water (Fude et al., 1994; Losi et al., 1994c; Cifuentes et al., 1996). However, success has been limited in complex soils.

Recently, for treatment of Chromite Ore Processing Residue (COPR), a technique involving the use of organic-rich acidic manure along with chrome reducing microbes to effectively reduce the Cr(VI) in the waste has been developed (Fig 2.4). This layer is positioned below the Cr rich waste and Cr(VI) leaching out of the waste, is effectively reduced in the organic layer, thereby preventing further contamination of groundwater (James, 1996; Higgins et al., 1997).
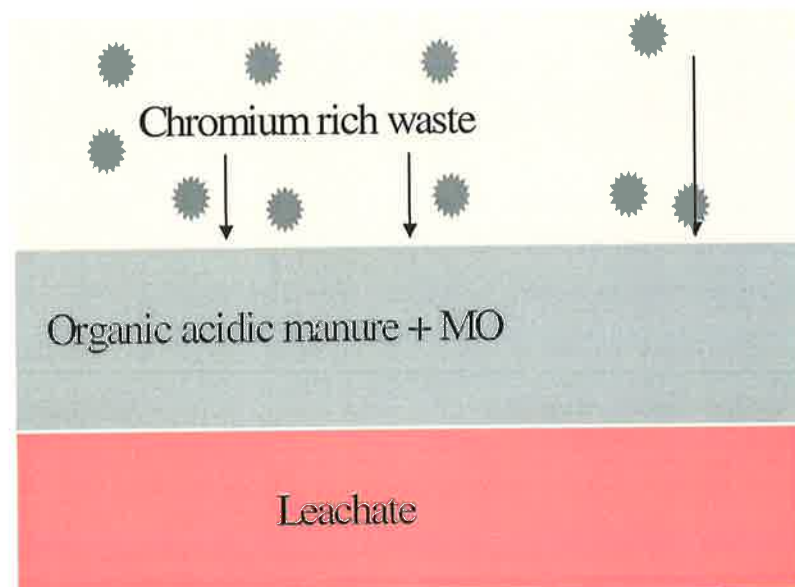
Fig. 2.4 Bioremediation of COPR contaminated soil using organics and microorganisms

As described by Losi et al., (1994c), the bioremediation of the soil is achieved by either direct or indirect biological reduction of Cr(VI). Most of the direct microbial reduction would be expected on surface soils where aeration favours the enzymatic reduction. In the subsurface layers, indirect biological reduction of Cr(VI) involving H_2S is predominant and very effective. The H_2S, diffused into inaccessible soil pores, promotes the reduction of Cr(VI) and also Mn oxides, involved in reoxidation. *In situ* stimulation of sulphate reducing bacteria may be achieved by addition of sulphate and nutrients. This method has shown some promise for remediation of Cr(VI) contaminated soils when applied to an anaerobic bioreactor system (Losi et al., 1994c). (Turick et al., 1996a) have confirmed the usefulness of anaerobic chromate reducing strains in the reduction and sedimentation of tannery wastes (Smillie and Loutit, 1982).

There is evidence to suggest that organic contaminants such as aromatic compounds are suitable electron donors for Cr(VI) reduction (Shen et al., 1996). Chromium-reducing microbes may then be able to simultaneously remediate organic contaminants as well. The success of bioremediation processes mainly depends on the efficiency of the microorganisms and also the level of contamination.

2.8.2 Phytostabilisation

Phytoremediation techniques, currently available for metal contaminated soils, include: phytoextraction, phytovolatilisation, rhizofiltration and phytostabilisation. Phytoextraction uses metal accumulating plants capable of transporting and concentrating metals from the soil to the roots and aboveground shoots (Cunningham, 1995). Phytovolatilisation uses plants to remove metals from soil by converting them to volatile forms and is mainly achieved by both plant and plant-microbe interactions (Terry and Zayed, 1993). Rhizofiltration is use of the roots of various plants to remove toxic metals (Dushenkov et al., 1995).

Phytostabilisation is an *in situ* metal inactivation process using vegetation with or without soil amendment (Vangronsveld and Cunningham, 1998). This technique employs the principles of metal stabilisation and using plants to further improve the soil and to reduce the risk in a contaminated soil by decreasing metal bioavailability using a combination of plants and amendments. This technique has been mainly used for remediation of mine spoils around smelters where chemical amendments are often added to establish plant growth. *In situ* inactivation or immobilisation is an interesting technique for low contaminated soils to minimise the plant uptake and metal transfer to

higher trophic levels.

Some of the advantages of *in situ* phytostabilisation are that it:

- stabilises wastes and prevents exposure pathways *via* wind and water erosion of metals,

- prevents the vertical migration of contaminants to groundwater (hydraulic control), and

- physically and chemically immobilises contaminants by binding in roots and organic matter in soils and by chemical fixation with soil amendments including lime, phosphate rock, aged manure and other materials (Berti and Cunningham, 2000).

Compared to other plant-based techniques, phytostabilisation has recently gained importance, as it is cost effective (US$ 0.02 – 1.0 m^{-3} per year) (Cunningham et al., 1995) and has other advantages. Firstly, since it is an *in situ* method, it is applicable for heavily and large contaminated sites. Appropriate vegetation and soil amendments with organic sources are standard, natural environment friendly practices without generating any further undesirable wastes unlike many chemical amendments. Most of the amendments and practices followed are basic farming practices, such as amendments with phosphorus, lime or organic sources. Apart from remediation, phytostabilisation initiates a new, healthy ecosystem at the site and it is also aesthetically more preferable than a barren contaminated site (Vangronsveld and Cunningham, 1998). Though literature exists on the phytostabilisation of metals like Pb, Zn, Ni and Cu, no literature exists on phytostabilisation of Cr(VI) or Cr in soils. This review, hence, concentrates on the details of this technique and successful case studies on remediation of other metals. This can be useful to formulate strategies for Cr contaminated soils.

2.8.2.1 Type of soil amendments

Amendments added to the soil should convert the soluble and pre-existing high soluble phases to more geochemically stable solid phases, resulting in reduced biological ability and phytotoxicity of heavy metals. Metals are mainly altered through precipitation, humification and redox transformation. The amendments commonly used include phosphates (triple calcium phosphate, hydroxyapatite) liming agents, metal

oxyhydroxides and organic materials (sludges and composts). Recent research has also used synthetic zeolites (aluminosilicates), a modified aluminosilicate (beringite) and steel shots for the potential inactivation of metals (Mench et al., 1998).

a) Lime and phosphatic fertilisers

Liming has been used as an ameliorant in agriculture and adapted for immobilisation of cationic metals in soil solution by increasing the soil pH. Phosphate minerals have the potential to sorb or coprecipitate trace metals (Ma et al., 1993) and for instance with Pb contamination, hydroxy pyromorphite was mainly formed. Apatite was successfully used to reduce the release of Pb, Zn and Cd (Mench et al., 1998). Lime and phosphatic fertilisers may not be appropriate for tannery waste contaminated sites. The tannery waste contaminated soils are mainly alkaline, because of the lime used during processing. Also, application of phosphates will lead to exchange of more Cr(VI) into soil solution. However, the phosphates could possibly be used to release the Cr(VI) and increase its bioavailability in solution. Later, this pool can be reduced using other amendments.

b) Hydrous Fe and Mn oxides

Hydrous Fe and Mn oxides sorb a variety of trace metals and contaminants on their oxide surface exchange sites. Coprecipitation and formation of contaminant-Fe complexes are common. Fe and Mn hydroxides are effectively used for metals such as As, Cd, Cu, Pb and Zn (Manceau et al., 1992). The potential use of Fe in Cr(VI) reduction is well known. Irreversible reduction of Cr(VI) by Fe to insoluble Fe-Cr(III) hydroxides is used as the major remediation strategy for chromate contaminated soils. Hence, this could be used as amendment in the stabilisation processes. Hydrous Mn oxides play a crucial role in redox and adsorption processes of many trace metals in soil. The birnessite mineral is widely prevalent and is effective in reducing the bioavailability of Cd and Zn in sludged soils (Mench et al., 1998). Mn oxides, albeit very effective in reducing the bioavailability of most metals, are not suitable for Cr contaminated soil, as they are the major oxidants of Cr(III) to Cr(VI) (Johnson and Xyla, 1991; Kim et al., 2002).

c) Aluminosilicates

Montmorillonite, Al-montmorillonite and gravel sludge have been effectively used for immobilisation of Zn and Cd (Hartman, 1997). Beringite, a modified

aluminosilicate from the fluidised bed burning of coal refuse, immobilises metals by precipitation, ion exchange and crystal growth. Another added advantage of this amendment is the long-term retention of mobile metals as opposed to a chemical immobilisation using lime (Vangronsveld et al., 1995). These amendments can be used after Cr(VI) reduction to Cr(III) to further immobilise the Cr(III) fractions.

d) Organic amendments

Organic manures and composts have been used for immobilisation of Zn (Li and Chaney, 1998). Addition of organic sources to soils enhances the formation of insoluble Zn organic complexes with humic acids, decreases the phytoavailability of Zn and thereby its toxicity to plants (Kirkham, 1977). Compost application can also improve the stability of low organic soils by improving the bulk density, cation exchange capacity and aggregate stability. However, very little information is available on the use of organics for phytostabilisation processes (Mench et al., 1998).

Organic amendments appear promising for Pb contaminated soils. However, after an extended duration, mineralisation of organics may lead to the release of the organically bound Pb to soil solution. Hence, use of organics should be considered carefully before their application to specific sites (Berti et al., 1998).

Organics, as biosolids or other sludge materials, provides a diverse inoculum of microbes that can enhance Cr(VI) reduction. Conversely, organic sources have been used for Cr(VI) reduction extensively not in the context of phytostabilisation, but as an amendment to aid reduction processes (James and Bartlett, 1983a; Buerge and Hug, 1998). Losi et al.(1994a) applied different levels (0, 12 and 50 tons ha^{-1}) of cow manure to the soil with and without alfalfa plants to effectively reduce the Cr(VI) in the soil and reduce its transort to the irrigation water. Data revealed that Cr(VI) reduction (51-98%) increased with an increase in organic matter loadings and the contact time with the organics. More than 90% of the Cr was rendered immobile and less than 0.5% was taken up by the test plant, alfalfa, minimising the transport of Cr(VI) to the drainage water.

2.8.2.2 Type of plant species

In phytorestoration, plants perform the principal functions of protecting the contaminated soil from wind and water erosion, and reducing water percolation through the soil to prevent the leaching of contaminants. Agricultural crop plants have been used

along with amendments to stabilise Zn, Cd and Pb. Some examples are given in Table 2.11. It is essential that the right cultivar, one tolerant to the particular metal contaminant, be chosen since mostly revegetation of highly polluted soil is problematic due to its phytotoxicity. Turf grass (*Agrostis stolonifera*) with its extensive vegetative cover was tolerant to soils contaminated with Pb and Zn wastes and Cu-rich wastes. Red fescue has been used for Zn contaminated soils (Li and Chaney, 1998). Several plant species, known to be Cr-tolerant, have been used for phytoremediation of Cr-contaminated soils. Plants such as *Plantago lancelota* have been recently reported to take up high levels of Cr from a tannery waste contaminated field in Italy. *Silene vulgaris*, an excluder plant (Bini et al., 2001), effectively reduced Cr(VI) to Cr(III) and restricted the less bioavailable fractions of Cr in the surface soils. In a study on the uptake and translocation of Cr(III) and Cr(VI) in rice plants, Cr(VI) reduction was attributed to the plant-microbe interactions in the rhizosphere (Mishra et al., 1997). Rhizosphere plays a significant role in harbouring different microorganisms aiding the release or stabilisation of metals. The role of EDTA in the concentration and stabilisation of metals like Ni and Cu (Madrid et al., 2002) and Pb (Huang et al., 1997) in contaminated soils has been reported. The accumulation of Pb by roots is well known and only a small portion of absorbed Pb is accumulated in the shoots..

2.8.2.3 Applicability of phytostabilisation to Cr contaminated soil

Given the literature available on phytostabilisation of metals and also through correspondence with researchers specialised on phytostabilisation, it was evident that no attempt has been made to stabilise Cr in soils. However, there are reports on the use of plants and chemical amendments for the reduction of Cr(VI) to Cr(III). In another study, the main focus was to reduce Cr(VI) to Cr(III) using prevalent techniques (Chaney et al., 1997) and then to stabilise the Cr(III) formed as with any other cation such as Pb, Cu, Zn or Cd. Amendments with Fe bearing minerals along with organics could be effectively used for reduction of Cr(VI) and precipitation to Cr(III). A schematic diagram on the phytostabilisation of Cr(VI) is given in Fig. 2.5. In general, several details like site characteristics and possible risk assessment have to be assessed before implementing the appropriate technique to the field.

Table 2.11 Soil amendments and plants used in phytostabilisation of different metal species

Amendments	Plants	Target metal	Stabilisation/ Decreased bioavailability	Uptake by plants	References
Al-smectite	Radish, lettuce, carrot, potato	As	75% decrease in water soluble pool	50% reduction in uptake	Mench, 1998
Fe oxides	Maize, Barley, Radish	Zn	++	**	Chlopecka, 1996
Hydroxyapatite	Maize	Cd, Zn		**	
Steel shots	Radish	As	++	**	Mench, 1998
Apatite	Barley	Zn		**	
Gravel sludge	-	Cd, Zn	++		
Zeolite	Lettuce	Cd		**	Gworek, 1992
Zeolite	Barley, strawberries, cherries	Zn		**	Miinyev, 1990
Zeolite + fertiliser (N:P:K)	*Lolium perenne*	Zn, Cd, Cu		**	Mench, 1998
Steel shots + basic slag		Zn, Cd	++		Mench, 1998
Beringite + bentonite			++		Mench, 1998

+ + reduction in bioavailability; ** reduction in uptake
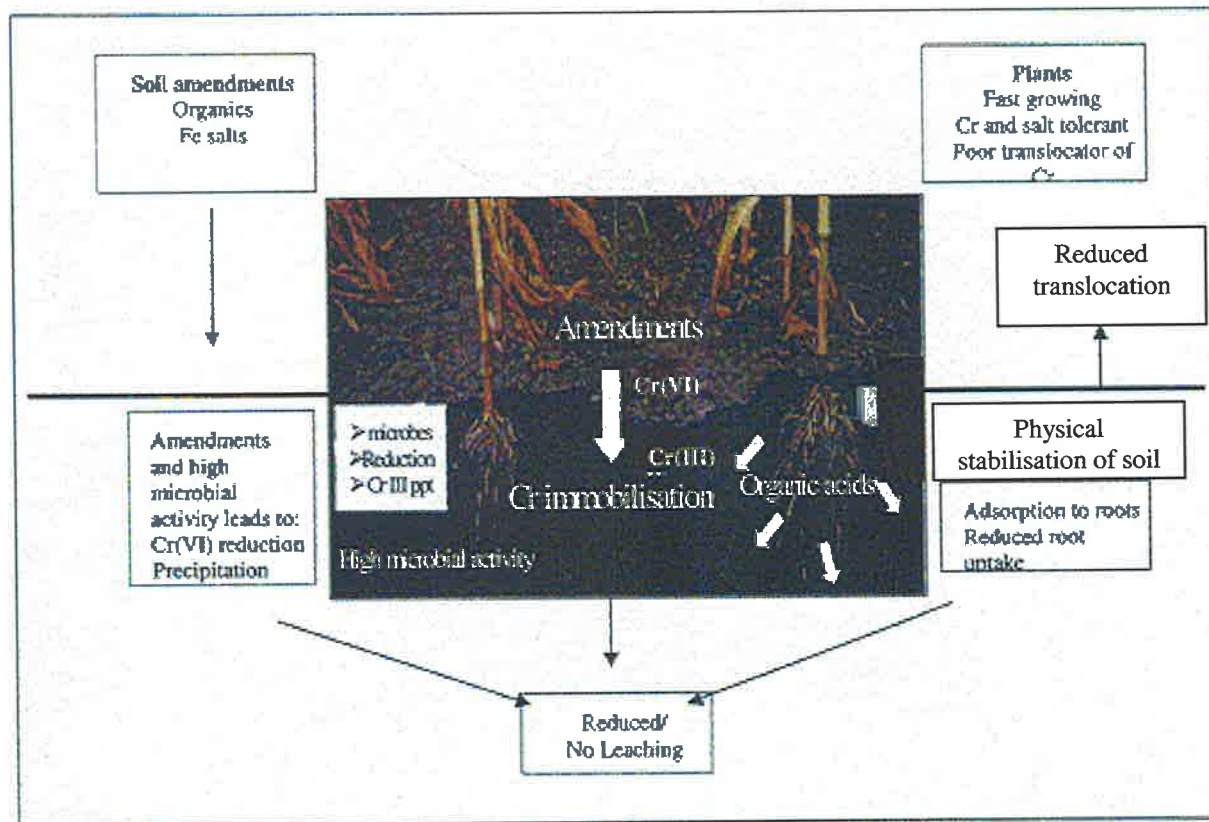
Fig. 2.5 Phytostabilisation of Cr contaminated soils: Possible role of amendments and plants (modified from Berti and Cunningham, 2000)

2.9   CHALLENGES

As rightly stated by James (1996), the complex chemistry involved in Cr transformations causes unique measurement and regulatory challenges. Remediation becomes complicated in heterogeneous wastes wherein the transformation reactions are rapid and interchanging. Though there exist treatment technologies for remediation of Cr in soils, as discussed in the individual sections, there are few setbacks that need to be resolved.

In a complex soil system, both biotic and abiotic processes play a significant role in determining the success of the remediation. One of the major problems encountered in using Cr reduction as a remediation option is the Mn-assisted reversible oxidation of Cr(III) to Cr(VI) at later stages due to changes in soil conditions or by natural weathering processes. An indirect role of microorganisms in Cr(III) oxidation can be envisaged when microbially produced Mn oxides mediate the chemical oxidation of Cr(III). In this regard, it is necessary to understand the mechanisms behind Cr oxidation in Mn-rich Cr-contaminated soils.

Being both environmental friendly and cost effective, bioremediation and phytostabilisation techniques are gaining popularity in the field of remediation. Bioremediation, especially for Cr contaminated soils, has been investigated; however, few detailed reports exist on targeting Cr associated with tannery wastes, especially in the long-term disposal sites. Future research should be directed towards the stability of Cr(III) formed using long-term contaminated soils as an excellent model. Phytostabilisation techniques are emerging as one of the options for remediation of metal contaminated sites, but not for sites contaminated with Cr. This technique involves the use of appropriate vegetation and soil amendments (organic manure, phosphatic fertilisers etc.) for immobilisation of the metals. Similar techniques can possibly be used also for Cr contaminated soils. Most of the plant species capable of taking up Cr may harbor rhizosphere microflora to facilitate the solubilisation of Cr complexes through dynamic plant-microbe interactions. Overall, there is a need to include and understand the major biotic-abiotic mechanisms governing Cr transformation in order to develop effective remediate technologies for complex Cr contaminated soils. Some of the major challenges are addressed in this research. The major objectives include the effect of tannery waste contamination on soil microbial

activity and populations, the biotic-abiotic interactions involved in Cr oxidation, and applicability of phytostabilisation techniques for Cr(VI) contaminated soils.

Materials and Methods

To invent, you need a good imagination and a pile of junk
- Thomas A. Edison -

ℒ

CHAPTER 3

General Methods and Materials

3.1   SOILS

Soil samples were collected from a long-term tannery waste contaminated site at Mount Barker, located 35 km east of Adelaide, South Australia (Fig. 3.1). This site is highly contaminated as it was used for the disposal of tannery sludge and effluent 20-25 years ago. Disposal ceased due to implementation of strict regulations. A previous survey of this site revealed that the soils had a total Cr concentration ranging from 249 to 69,500 mg kg^{-1} (Naidu et al., 2000b). Mostly the soil Cr levels at Mount Barker site were above the Australian National Health and Medical Research Council guidelines of 100 mg kg^{-1} (NHMRC, 1996). Piezometer and lysimeter studies also showed the presence and mobility of Cr as Cr(VI) in subsurface soils (Naidu et al., 2000b). Relative to total Cr, the Cr(VI) concentrations in surface and subsurface soils were also higher than the USEPA recommended levels of 0.05 mg L^{-1} (USEPA, 1996a). Although many reviews discuss Cr contamination in soils, there is a dearth of information on the long-term impact of Cr on soil chemical and biological processes.

Based on the previous study by (Naidu et al., 2000b) of chemical characteristics of soils at Mount Barker site, three sites representing a range of Cr and Mn concentrations were chosen for this study. The soils were designated as 'low' (< 2,000 mg kg^{-1}), 'medium' (2,000-50,000 mg kg^{-1}) and 'highly' (>50,000 mg kg^{-1}) contaminated based on the total Cr concentration.

Adjacent uncontaminated (UC) site was selected and used as a reference soil for comparative purposes. Since the main objective of the study was to determine the effect of Cr on soil processes, uncontaminated soil was taken from the site where there was no exposure to Cr contamination. However, since contamination was not uniform across the site and was particularly widespread there was difficulty in choosing a real control soil.

Location of Mt Barker (Courtesy: Adelaide maps)

Aerial photograph of the contaminated site (Courtesy: Avudainayagam, 2001)
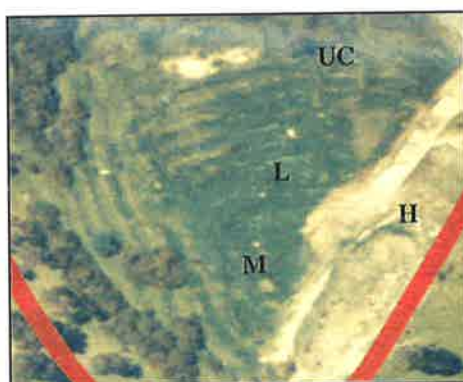
Fig. 3.1 Location of Mount Barker, aerial view of the contaminated site at Mt Barker and location of the uncontaminated (UC), low (L), medium (M), highly (H) contaminated sites used in this study.

Hence, an uncontaminated site (pseudocontrol/reference soil) close to the contaminated site was chosen for comparative studies. The locations of the different sites are given in Fig. 3.1. The selection of problems associated with obtaining a true control was further compounded because the contaminated site had received historically large applications of tannery sludge containing considerable amounts of organic matter and salts. Changes to the soil at this site, irrespective of Cr contamination, may therefore be expected to be considerable, making a true control soil (soil with the same characteristics without Cr) prohibitively difficult to find.

From each site (low, medium, high and UC), five replicate soil core samples were taken up to 1 m depth. Soil samples from different depths (0-10, 10-20, 20-30, 30-50 and 50-100 cm) of all the five replicates were also pooled and bulked (1 kg). From this representative sample, subsamples were used for chemical and microbiological analyses. For chemical analysis, soils were air-dried in a fan-forced oven (40°C) and passed through a stainless steel 2-mm sieve. For microbiological analyses, field moist soils were passed through a 2-mm sieve and then stored at 4°C prior to use.

3.2 CHEMICAL METHODS

Soil chemical characteristics were studied using standard procedures with slight modification as required. Soil pH and electrical conductivity (EC) were measured in 1:5 soil water extracts after equilibrating the soils with deionised water for 18 h in an end over end shaker (10 rpm; 25°C) (Rayment and Higginson, 1992). Water-soluble Cr and Cr(VI) were also measured in these extracts immediately after extraction..

Exchangeable Cr and Cr(VI) were determined in soil solution following end-over-end equilibration of the soil with 10 mM K_2HPO_4 for 2 hours (James and Bartlett, 1983a). Total Cr, Mn and Fe concentrations were estimated in soils digested with fresh aqua regia (100°C for 2 h) (Merry and Zarcinas, 1980). Chromium concentrations in these extracts were quantified using an Atomic Absorption Spectrophotometer (AAS) after filtration through 0.45 μm disposable filters. Moroccan rock phosphate containing 250 mg Cr kg^{-1} (BCR reference material No. 32) was used as the reference during all AAS analyses (recovery, >95%). Cr(VI) was quantified as per Method 7199 (USEPA, 1996a) by Ion chromatography equipped with Dionex Ion pack AS7 column. Soil organic carbon (OC) was determined using the method of Walkley and Black (Allison, 1965). All analyses were performed in duplicate using ANALAR grade reagents and

chemicals.

3.3 MICROBIAL METHODS

All microbial analyses were maintained with field moist soils equilibrated uniformly to 60% moisture content. The soils were stored at 4°C after usage. General aseptic conditions were followed throughout the study of microbial cultures. Unless specified, media and standard stock solutions were prepared in distilled water and sterilised by autoclaving at 121°C for 20 minutes. Chromium stock solutions were filter sterilised using 0.2 μm sterile disposable filters (Sartorius) before their addition to media.

3.3.1 Bold's medium

$NaNO_3$ 250 mg, $CaCl_2.2H_2O$ 25 mg, $MgSO_4.7H_2O$ 75 mg, K_2HPO_4 75 mg, KH_2PO_4 175 mg and NaCl 25 mg. Trace elements: KOH 31 mg, $FeSO_4.7H_2O$ 5 mg, H_3BO_3 11.4 mg, $ZnSO_4.7H_2O$ 8.8 mg, $MnCl_2.4H_2O$ 1.44 mg, MoO_3 0.71 mg, $CuSO_4.5H_2O$ 1.57 mg, $Co(NO_3).6H_2O$ 0.49 mg and EDTA 50 mg. All the constituents were dissolved in 1L of water and pH was adjusted (6.5-6.8) before sterilisation (Bischoff and Bold, 1963).

3.3.2 Mn enrichment broth

Peptone 2.0 g, yeast extract 1.0 g, KH_2PO_4 50 mg, $CaCl_2$ 100 mg, NaCl 200 mg, NH_4SO_4 100 mg and $MgSO_4$ 20 mg in 1 L of deionised water (pH 7.0). Unless otherwise stated 0.1% Mn was added to broth as filter sterilised $MnSO_4$ stock solution.

3.3.3 Mn oxidising broth

Peptone 0.25 g and yeast extract 0.25 g in 1 L of deionised water (pH 7.0). Unless otherwise stated $MnSO_4$ 0.5 g per litre was used.

3.3.4 NDY broth

$NaNO_3$ 2 g, KH_2PO_4 .5 g, KCl 0.5 g, $FeSO_4$ (10 ml of 1% solution), yeast extract 0.5 g and sucrose 30 g in 1 L of deionised water

3.3.5 PYG medium

Peptone, yeast extract and glucose 0.25 g, $MgSO_4.7H2O$ 0.5 g and $CaCl_2$ 0.01g

in 1 litre of deionised water (Ghiorse, 1994)

3.3.6 TSY broth

Tryptone soy agar 15g, soyto ne 5 g and NaCl 15 g in 1 litre of distilled water (pH 7.0) (Difco manual)

3.4 DETAILS OF ANALYTICAL TECHNIQUES

The total metal concentrations (Cr and/or Mn) in the aqua regia digests and water extracts were measured using a Flame Atomic Absorption Spectrophotometer (FAAS, GBC 906 AA, GBC Scientific, Melbourne, Australia). Samples containing Cr concentration up to 0.1 mg L^{-1} were measured in flame AAS and lower metal concentrations were quantified using graphite furnace AAS (GFAAS, Varian SpectrAA 400 plus, GTA 96) with detection limit of 5 μg L^{-1}. Standard conditions used during the analyses are given in Table 3.1.

Table 3.1 Conditions of AAS during the analyses of Cr and Mn

Conditions	Cr	Mn
Lamp current (mA)	6.0	5.0
Wavelength (nm)	357.9	403.1 or 279.5
Slit width (nm)	0.2	0.2
Slit height	Normal	Normal
Instrument mode	Absorbance background off	Absorbance background off
Measurement mode	Integration	Integration
Sampling mode	Manual	Manual
Flame type	Air-acetylene (reducing)	Air-Acetylene (oxidising)
	Air (12.8 L min^{-1})	Air (12.8 L min^{-1})
	Acetylene (2.75 L min^{-1})	Acetylene (2.0 L min^{-1})
	Burner angle (3.0)	Burner angle (6.0)
Data collection		
Replicates	3	3
Read time (s)	2.0	2.0
Time constant (s)	1.0	0.4
Expansion factor	1.0	1.0

Cr(VI) was measured using either Ion chromatography (USEPA, 1996) or a modified spectrophotometer method (Templeton, 1997). Unless otherwise stated, the soil/soil solution Cr(VI) was measured using Ion chromatographic method as dissolved organics in soils interfered with the absorbance in the spectrophotometer method using diphenyl carbazide. Samples were analysed for Cr(VI) immediately to prevent its possible transformation to Cr(III).

3.4.1   Ion chromatographic method

Dionex Ion chromatograph (Dionex AI 450) was used in the present study. An aqueous sample was filtered (0.45 μm) and pH adjusted to 9 - 9.5 before a measured volume (50-250 μl) was introduced into the ion chromatograph. A guard column (Dionex Ionpac NG1), used to remove organic compounds from the sample, preceded the anion exchange separator column (Dionex Ionpac AS7). Post column derivatisation of the Cr(VI) with diphenyl carbazide was followed by detection of the coloured complex at 530 nm. The detection limit was 10 μg L^{-1}. Standard conditions are given in Table 3.2.

Table 3.2 Standard ion chromatographic conditions

Columns	Guard column – Dionex Ionpac NG1
	Separator column – Dionex Ionpac AS7
Eluant	250 mM ammonium sulphate
	100 mM ammonia
	Flow rate = 1.5 ml min^{-1}
Post column reagent	2 mM diphenyl carbazide
	10% v/v HPLC grade methanol
	1 N sulphuric acid
Detector	Visible 530nm

Eluants and post column reagents were degassed with N$_2$ prior to use. Samples were filtered (0.45 μm) and adjusted to pH 9-9.5 using buffer (Dissolve 33 g of

ammonium sulphate in 75 ml of milli Q water and add 6.5 ml of ammonium hydroxide. Dilute with 100 ml water and degass the solution).

The instrument was calibrated using a calibration blank and three calibration standards. Calibration standards were prepared from Cr(VI) stock standards by appropriate dilution using milli Q water (pH adjusted to 9-9.5). All the standards and samples were maintained in the same matrix. Standards were immediately stored in polypropylene containers.

3.4.2 Spectrophotometer method

A set of calibration standards (0 - 2.0 mg L^{-1}) was prepared in 50 ml volumetric flasks from a 4 mg L^{-1} Cr(VI) stock solution. A known aliquot of filtered samples were taken in 50 ml volumetric flasks in the range of 0 - 2.0 mg L^{-1} of Cr(VI) by appropriate dilution. To this 10 ml of 1 N sulphuric acid and 4 ml of diphenyl carbazide solution (DPC) were added. The volume was made up to 50 ml and the pink colour developed was read at 530 nm in double beam spectrophotometer (GBC UV/VIS 918, GBC Scientific, Melbourne, Australia). A 0.96 mg Cr(VI) L^{-1} standard produced an absorbance of approximately 0.8. From the absorbance of known standard solutions, the concentration of Cr(VI) in the samples was quantified. This method had a detection limit of 10 $\mu g\,L^{-1}$.

The DPC solution was prepared by dissolving 0.25 g of diphenyl carbazide and 4.0 g of phthalic anhydride in 100 ml of ethanol. The solid was dissolved with continuous stirring and during this time the bottle remained capped to prevent evaporation. After the solid was completely dissolved, the solution bottle was wrapped with aluminium foil and stored at 4°C.

All the reagents were of analytical grade and prepared in oven dried glassware that had been acid washed (soaked in 2 N HCl overnight and rinsed twice with distilled water).

3.5 STATISTICAL ANALYSES

All statistical analyses were performed using GENSTAT 5 (4th edition) from Lawes Agricultural Trust, Rothamsted, UK. One way and two-way analysis of variance (ANOVA) was used to test the significant difference among the samples. Significant differences were represented as follows: * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$), n.s ($p > 0.05$).

Chapter 4

If the fact do not fit the theory, change the facts
- Albert Einstein -

CHAPTER 4

Impact of Tannery Wastes on Soil Microbial Activity and Populations

4.1 INTRODUCTION

The disposal of tannery wastes onto soil, as a means of waste disposal, was a common practice before enactment of strict environmental regulations in Australia and many other countries throughout the world. Dumping to soil was preferred over disposal to water bodies given (a) the proximity of land based disposal sites relative to rivers, estuaries and ocean bodies and (b) the higher soil buffering capacities, which allow greater volumes of waste to be dumped into this matrix. Both tannery effluent and solid wastes are rich in organic matter from proteinaceous hides. These wastes also contain very high levels of other nutrients such as phosphorus and calcium (Naidu et al., 2000a). Although the application of tannery wastes to low productive soils may have some beneficial effects due to the high carbon and nutrient content, their impact on soil ecosystem also has to be considered carefully due to their high chromium (Cr) and salt content.

Previous research has shown that the disposal of Cr-rich tannery wastes to land caused significant changes to the physico-chemical properties of the soil (James and Bartlett, 1983a). A similar effect was found in a long-term tannery wastes contaminated site located at Mount Barker, South Australia (Naidu et al., 2000b). At this site, Cr was found to be mobile resulting in contamination of the subsurface horizon even 20 years after the waste disposal had ceased. Much of this mobile Cr was in the hexavalent form, Cr(VI) (Naidu et al., 2000b), which has serious health implications due to its carcinogenic effect (USEPA, 1996a).

Although considerable literature exists on the effect of tannery wastes on soil chemical properties, information on its impact on soil microbial properties is scant. It is likely that changes in the physico-chemical properties of soil might directly or indirectly influences microbial populations and their activities. These impacts can be measured through changes in microbial biomass, enzyme activities and respiration rates of

microorganisms in the soil (Valsecchi et al., 1995). Dehydrogenase activity (DHA), a measure of total microbial activity, is considered as a useful tool in assessing the harmful effect of metals on microorganisms (Ohya et al., 1988). However, a contradictory report also exists on the reliability of DHA as a measure of microbial activity in copper contaminated soils (Chander and Brookes, 1991).

Generally, short-term exposure of metal contamination affects the overall activity of microbial populations (Barnhart and Vestal, 1983), whereas, in long-term contaminated soils, normally a population shift occurs towards the prevalence of resistant microorganisms due to elimination of sensitive organisms. Such population shifts can lead to changes in phospholipid fatty acid (PLFA) patterns of the soil. The PLFA assay is used to characterise changes in the microbial community structure by evaluating shifts in PLFAs extracted from whole environmental samples. Because PLFAs constituting cell membranes vary with different subgroups of microorganisms, shifts in PLFA profiles are indicative of changes in the overall microbial community structures (Frostegård et al., 1993). Changes in microbial community structure, as determined by changes in PLFA profiles, have been reported in Cu, Ni and Zn contaminated soils (Frostegård et al., 1993; Pennanen et al., 1996; Kelly et al., 1999a). However, no reports exist on the effect of Cr-rich wastes on microbial communities. Given the recent move towards bioremediation techniques, information on the effect of contaminants on soil microbial community is considered critical as the efficiency of indigenous microorganisms plays a vital role in the success of this technique. For this reason, we investigated the long-term impact of tannery wastes on

a)   the physico-chemical properties of the soil, and

b)   the associated changes on population structure (PLFAs) and microbial activity (DHA) of the soil.

4.2   MATERIALS AND METHODS

4.2.1   Soils

Replicate soil samples from long-term tannery waste contaminated sites with low, medium and high levels of Cr contamination collected from different depths (0 - 10, 10 - 20, 20 - 30, 30 - 50 and 50 - 100 cm) were used in this study (Section 3.1). Soil samples from an adjacent uncontaminated site were also used for comparison.

Following sampling, soils were dried using a fan forced oven at 40°C, crushed to pass through a stainless steel 2-mm sieve and stored in polyethylene containers for subsequent studies (Section 3.2).

4.2.2 Chemical characteristics

Air-dried soil samples (< 2mm) were used to estimate the pertinent soil chemical properties. The details of the methods were described in Section 3.2.

4.2.3 Dehydrogenase activity (DHA)

Soil DHA was determined based on the dehydrogenation reaction of triphenyl tetrazolium chloride (TTC) to triphenyl formazan (TPF) by microbial dehydrogenases (Alef, 1995). DHA activity was determined in duplicate on field moist soils. Soil samples (5 g) were incubated with 5 ml of TTC in Tris buffer (0.6%, pH 7.8) at 30°C in the dark. The control contained 5 ml of Tris buffer without TTC. After 24 h, 40 ml of acetone was added to the soil-TTC mixture, contents were shaken thoroughly and further incubated in the dark for 2 h. The TPF formed was extracted from soils using acetone. The extracts were filtered (0.45 μm) and TPF was quantified by measuring the absorbance at 546 nm in a spectrophotometer. The concentration of TPF was quantified from a standard curve constructed using authentic TPF (Sigma) (0 – 30 μg). Total DHA activity was expressed as μg TPF g^{-1} of oven dry soil or normalised to organic carbon (OC) as μg TPF g^{-1} OC. TTC and TPF solutions were prepared daily for the DHA assay and maintained in dark to prevent oxidation.

The reliability of DHA as a measure of microbial activity is questionable especially in Cu contaminated soils (Chander and Brookes, 1991). This is because Cu reacts directly with TTC and reduces it to TPF. In order to study the direct effect of Cr on TTC, Cr at different concentrations (0, 5 and 10 mg L^{-1}) as Cr(VI) (K$_2$Cr$_2$O$_7$) and Cr(III) (CrCl$_3$) was incubated with TTC. TPF formed was quantified as described above for soils.

4.2.4 Phospholipid fatty acid (PLFA) analyses

PLFA analyses were performed in all soil samples (Section 4.2.1) except samples from 50 - 100 cm depth. Samples at this depth were excluded as no appreciable microbial activity (DHA) was detected.

4.2.4.1 Lipid extraction and fractionation

The PLFAs were extracted from field moist soils and analysed according to the method of (Bossio and Scow, 1998). Soil (12 g) was extracted overnight with 75 ml of one phase buffer containing a 1:2:0.8 ratio of chloroform, methanol and phosphate buffer (8.7 g L^{-1} K_2HPO_4; pH 7.4). The soil and solvent mixture was kept static overnight in airtight conical flasks. The mixture was filtered through fast ash-less filter paper into a separating funnel. The soil remaining on the filter was washed twice with one phase buffer (2 x 20 ml) and the eluate pooled in a separating funnel along with 25 ml each of chloroform and phosphate buffer. Samples were shaken and the solvent phases were allowed to separate overnight. The chloroform layer was decanted and evaporated to dryness on a rotary evaporator at 32°C. The dried extract was dissolved in chloroform (3 - 5 ml) and phospholipids were separated using a silicic acid column conditioned with chloroform. Neutral lipids were eluted with chloroform (10 ml) and glycolipids with acetone (20 ml). Phospholipids were eluted with methanol (10 ml) and dried under a gentle stream of nitrogen at 30°C.

4.2.4.2 Alkaline methanolysis and GC analysis

Polar lipids were subjected to overnight acid methanolysis (60°C) with 1.5 ml of methanolic H_2SO_4 (1% H_2SO_4 in methanol) where 100 μL of $C_{19:0}$ (methyl nonadecanoate) was used as an internal standard. Resulting fatty acid methyl esters (FAMEs) were then extracted with petroleum ether (2 x 5 ml) and dried under nitrogen at room temperature. The dried extract was redissolved in hexane (100 μL) and FAMEs were separated by gas chromatography (HP 5890 using a 25 m x 0.2 mm fused silica capillary column) equipped with flame ionisation detector following the MIS Eukary method (MIS, Microbial ID, US). The HP 3365 chemstation software operated the sampling, analysis and integration of the chromatographic samples. Samples were run for 38 min with the temperature program ramped from 170°C to 250°C at 5°C min^{-1}. Hydrogen was the carrier gas. Injector and detector were maintained at 170 and 300°C, respectively. Flow rates of hydrogen, nitrogen and air were 30, 30 and 400 ml min^{-1} respectively. Individual FAMEs were identified using the peak naming table component of the microbial identification system (MIDI), quantified using the $C_{19:0}$ internal standard and expressed as μg g^{-1} of soil sample. Five replicates were used for each sample.

4.2.4.3 Fatty acid nomenclature

Fatty acids are designated in terms of total number of carbon atoms: number of double bonds, followed by the position of the double bond from the methyl end of the molecule. The prefixes a and i indicate anteiso and iso branching, and cy indicates a cyclopropane fatty acid. Methyl branching (Me) is indicated as the position of the methyl group from the carboxyl end of the chain.

Individual specific PLFAs indicative of total bacteria, Gram-positive bacteria, Gram-negative bacteria and fungi are given below based on previous reports (Zelles, 1999).

Total bacteria: i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0 and cy19:0

Gram-positive bacteria: i15:0, a15:0, 15:0, i16:0, i17:0 and a17:0

Gram-negative bacteria: cy17:0 and cy19:0

Fungi: 18:2ω6c

4.2.5 Statistical analyses

Two way analyses of variance of the soil chemical and microbiological soil variables were performed using GENSTAT version 5.0. For analyses of PLFAs, only FAMEs with carbon lengths 10 - 21, which cover the range of fatty acids normally found in bacteria (Haack et al., 1994), were used. Minor peaks, present in quantities of less than 0.25% by weight, were not included in the analyses. Conventional two-mode principal component analyses (PCA) were performed on the PLFA data.

4.3 RESULTS

Disposal of tannery wastes to soil at Mount Barker has resulted in extensive contamination with varied Cr concentrations across this site (Naidu et al., 2000b). Based on this earlier report, three sites were chosen for chemical and microbiological characterisation and were designated as low, medium and high based on their Cr concentration.

Initial studies were focused on the comparison of chemical and microbial characteristics of the contaminated (low, medium and high) and an uncontaminated site. Soil samples (0-100 cm) from uncontaminated soil profile were acidic in reaction (pH 5.5 - 6.0) and low in OC (0.1 - 1.6 %) with a background Cr concentration of 0.01 g kg^{-1}

soil. As described in Chapter 3 (Section 3.1), collection of a true control was not possible in practice owing to the heterogeneous nature of the contaminated site. Also, since pH and OC were the main factors governing the release of Cr in solution, it was unrealistic to compare a low organic-acidic uncontaminated site with a high organic-alkaline contaminated site. Analyses of chemical and microbial characteristics of the uncontaminated site revealed significant variation in these attributes with depth (Appendix I). Compared to the uncontaminated site, there was a significant increase in all measured chemical parameters in contaminated soils; a major problem being the large dilution of soils with the long-term loading of solid waste from tannery industries. This led to significant differences in pH, OC and salt content of surface and subsurface soils. Hence, comparison between the uncontaminated and contaminated soils was not deemed ideal due to the confounding variation in the chemical variables, especially pH (more details in Appendix I).

Therefore, only contaminated soils (low, medium and high) were compared amongst each other to observe major changes in chemical and microbial characteristics due to increase in contamination by tannery wastes.

4.3.1   Effect of tannery waste disposal on chemical characteristics of soil

The disposal of tannery wastes to soil led to distinct long-term changes in the soil's chemical properties. There was a significant ($p < 0.001$) increase in pH, electrical conductivity (EC), OC, total Cr, water soluble Cr and exchangeable Cr content with increase in level of contamination at all depths (Tables 4.1). However, all tested parameters decreased with depth except EC.

Of special significance is the marked increase in OC and Cr content of the surface (0-20 cm) soil. Both these parameters were major constituents of the tannery wastes. Organics were mainly proteinaceous from the trimmed hides and hairy material during processing and Cr was mainly from chrome tanning process where Cr(III) salts were used.

There was a six-fold increase in OC content (15.7%) in surface soil samples from the highly contaminated site compared to 2.5% at the corresponding low contaminated site. The OC content decreased with soil depth in all soil samples at all three sites and a minimum of 0.1% was observed beyond 30 cm depth in soil samples from low and medium contaminated sites. However, in highly contaminated soil, the

Table 4.1 Chemical characteristics of contaminated soils at different depths

Soils*	Depth	pH	EC	Organic carbon	Total metals (g kg^{-1})			Soluble metals (mg kg^{-1})			
	(cm)		(dS m^{-1})	(%)	Cr	Mn	Fe	Cr	Cr(VI)	Ex. Cr[a]	Ex. Cr (VI)[b]
Low	0-10	6.8	0.07	2.5	1.5	0.04	14.9	1.1	< 0.01	0.3	0.1
	10-20	7.2	0.03	0.7	0.7	0.03	4.4	0.5	0.1	0.2	0.1
	20-30	7.4	0.02	0.1	0.1	0.02	2.6	0.1	0.1	< 0.01	< 0.01
	30-50	8.4	0.06	0.1	0.1	0.04	11.7	0.6	< 0.01	< 0.01	< 0.01
	50-100	8.6	0.40	0.1	0.1	0.09	22.3	0.4	< 0.01	0.6	< 0.01
Medium	0-10	8.2	0.25	9.8	47.8	0.34	7.7	4.2	2.7	6.1	3.4
	10-20	8.3	0.17	1.6	8.8	0.06	14.3	1.9	1.5	3.8	2.1
	20-30	8.6	0.11	0.4	1.1	0.01	10.4	0.4	0.2	0.5	0.2
	30-50	8.7	0.07	0.1	0.3	0.01	8.4	0.1	0.1	0.1	< 0.01
	50-100	8.7	0.05	<0.1	0.1	< 0.01	9.9	0.1	< 0.01	< 0.01	< 0.01
High	0-10	8.1	0.31	15.7	102	0.36	8.3	4.6	2.7	6.8	4.0
	10-20	7.9	0.63	12.7	112	0.57	9.6	3.4	3.5	14.9	9.0
	20-30	7.9	1.00	7.9	102	0.29	10.8	3.1	2.8	6.5	5.8
	30-50	8.2	1.04	5.8	103	0.18	7.4	1.1	1.1	2.3	2.3
	50-100	7.7	1.53	0.5	8	0.01	10.0	0.5	0.3	0.5	0.4
s.e.d (p<0.001)		0.14	0.02	0.77	1.79	0.01	0.42	0.17	0.02	0.31	0.14

Soils were designated low, medium and high based on the total Cr concentration in surface soils s.e.d. = standard error of deviation. Soluble metals are measured in 1:5 soil water extracts shaken for 16 h. a - exchangeable Cr, b - exchangeable Cr(VI) measured in phosphate extracts.

OC content was significantly high (5.8 %) beyond 30 cm (Table 4.1).

Total Cr concentration (0 - 10 cm) ranged between 1.5 g Cr kg^{-1} in low and 102 g Cr kg^{-1} in the highly contaminated soils. This trend was also reflected in the subsurface soils although the Cr concentration decreased with depth in soil samples from the low and medium contaminated sites. There was no decrease in total Cr up to a depth of 50 cm (102 – 112 g Cr kg^{-1}) in samples from highly contaminated soil, beyond which a minimum of 8 g Cr kg^{-1} was recorded in 50 – 100 cm depth. This concentration of Cr was still significantly higher than the total Cr recorded at this depth in low and medium contaminated soils.

Total soluble and exchangeable Cr released into soil solution increased with an increase in contamination. In low contaminated surface soils (0-10 cm), the soluble Cr was 1.1 mg L^{-1} and in highly contaminated soil it was 4.6 mg L^{-1}. The exchangeable Cr was 0.3 mg L^{-1} in low and 6.8 mg L^{-1} in highly contaminated soils (Table 4.1). In surface soils (0 - 10 cm), more than 50% (2.7 mg L^{-1}) of the soluble Cr (5.6 mg L^{-1}) existed as the Cr(VI) species, whereas with increased depths more than 90 % existed as Cr(VI). Exchangeable Cr concentration was 20 – 22 fold higher in medium and highly contaminated soil compared to low contaminated soil. As was observed for soluble Cr, more than 50% (4.0 mg L^{-1}) of exchangeable Cr existed as Cr(VI) species. With increasing depth there was a significant decrease in the soluble and exchangeable Cr fractions in all soil samples. Although the amount of Cr(VI) present beyond 30 cm depth was negligible in the low and medium contaminated soils, significantly high concentration of water soluble (1.1 mg Cr kg^{-1}) and exchangeable (2.3 mg Cr kg^{-1}) Cr(VI) species was detected in the highly contaminated soils.

Similar to variations in Cr content of the soils, the pH of the soil samples varied from 6.8 - 8.6 in low, 8.2 – 8.7 in medium and 7.7 – 8.1 in highly contaminated soil samples. The EC was higher in highly contaminated soil samples at deeper layers, where a maximum of 1.53 dS m^{-1} was observed. Total Mn in the low, medium and highly contaminated soils varied with depth and ranged from 0.04 – 0.09, 0.01 – 0.34 and 0.01 –0.57 g kg^{-1}, respectively. Maximum concentration of total Mn was observed in highly contaminated soils. However, there was no consistent trend in total Mn content with depth.

A significant correlation existed between total Cr and Cr released as soluble and

exchangeable pools into the soil solution (Fig. 4.1) in the order of soluble Cr(VI) (r^2=0.71***) > exchangeable Cr(VI) (r^2=0.61***) > soluble Cr(r^2=0.55**) = exchangeable Cr(r^2=0.54**). The OC, total Cr, total Mn and all soluble Cr fractions had significant positive correlation (r^2 = 0.6**). Other variables such as pH and EC values were almost constant across all the samples and hence no correlation with other parameters was observed for these variables.

4.3.2 Effect of tannery waste disposal on soil microbial characteristics

4.3.2.1 Dehydrogenase activity

Dehydrogenase activity in soil is normally measured based on the intensity of TTC reduced to TPF by microbial dehydrogenases. However, DHA is not always a reliable measure of microbial enzyme activity as some metals, such as Cu, can directly reduce TTC to TPF (Chander and Brookes, 1991). This chemical reduction interferes with the measurement of DHA due to microbial processes. It was not previously known if high concentrations of Cr would interfere with microbial DHA determination. However, initial tests with Cr(III), Cr(VI) and TTC showed no reduction to TPF within 24 hours.

Maximum DHA, in terms of μg TPF g^{-1} dry soil was observed in surface soil samples (0 - 10 cm) of low (275), medium (120) and highly (366) contaminated sites (Fig. 4.2A). The activity varied significantly ($P < 0.05$) between soils, and there was no proportional increase in activity with increased level of contamination. Indeed, medium contaminated soil showed lower activity than the low and highly contaminated soils. There was also no relationship with the level of Cr and OC.

Subsurface soil samples (10 - 50 cm) showed a significant increase in DHA with increase in contamination. The highest activity was found in highly contaminated soil containing higher concentration of Cr and also OC. In these subsurface soils, there existed a linear relationship between DHA and OC (r^2=0.91***) content indicating that the increased DHA observed was mainly due to OC.

To nullify the effect of OC and to determine the effect of Cr on DHA, DHA values were normalised to OC and expressed as μg TPF g^{-1} OC (Fig. 4.2B). This indicated clearly that the inhibition observed was mainly due to Cr contamination. In medium contaminated surface soil, 89% (1227) inhibition in DHA was observed
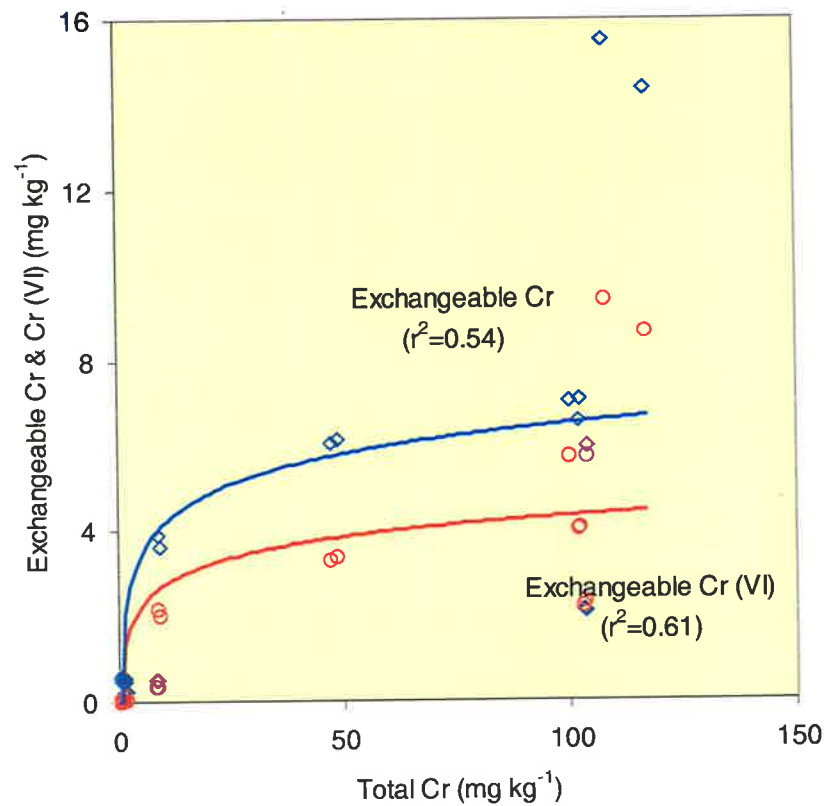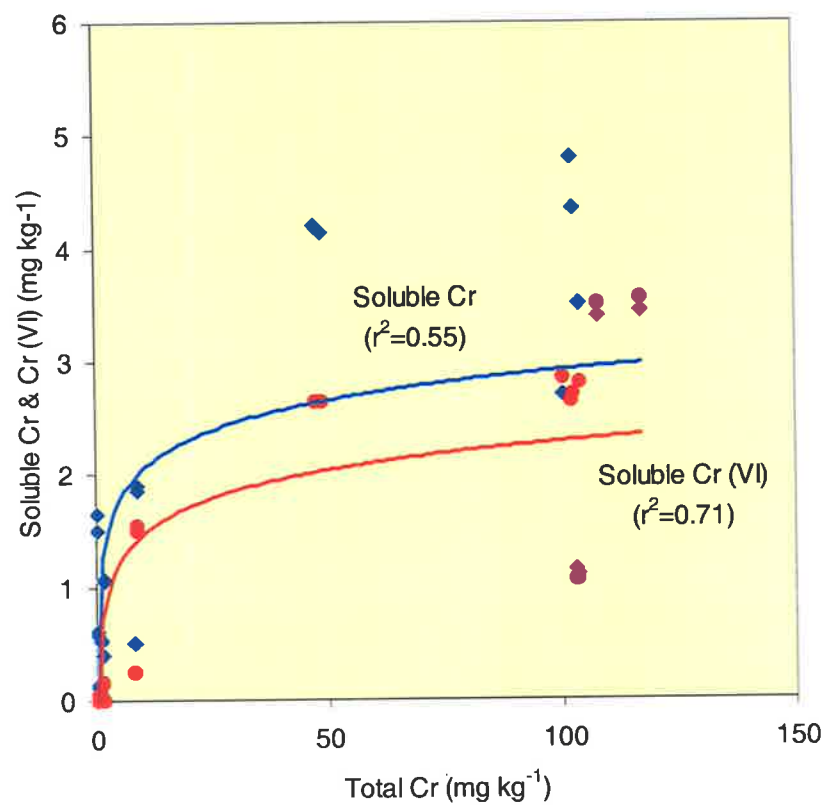
Fig. 4.1 Relationship between total Cr and Cr released into soil solution. Symbols indicate soluble Cr (●), exchangeable Cr (◆), soluble Cr (VI) (○) and exchangeable Cr (VI) (◇)
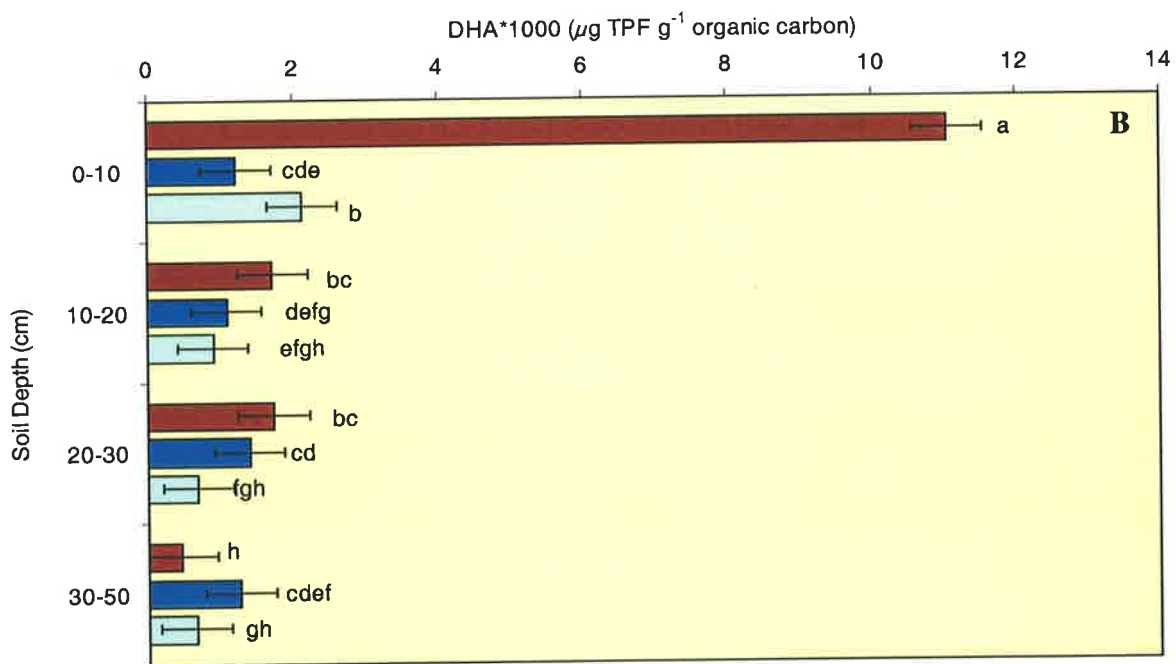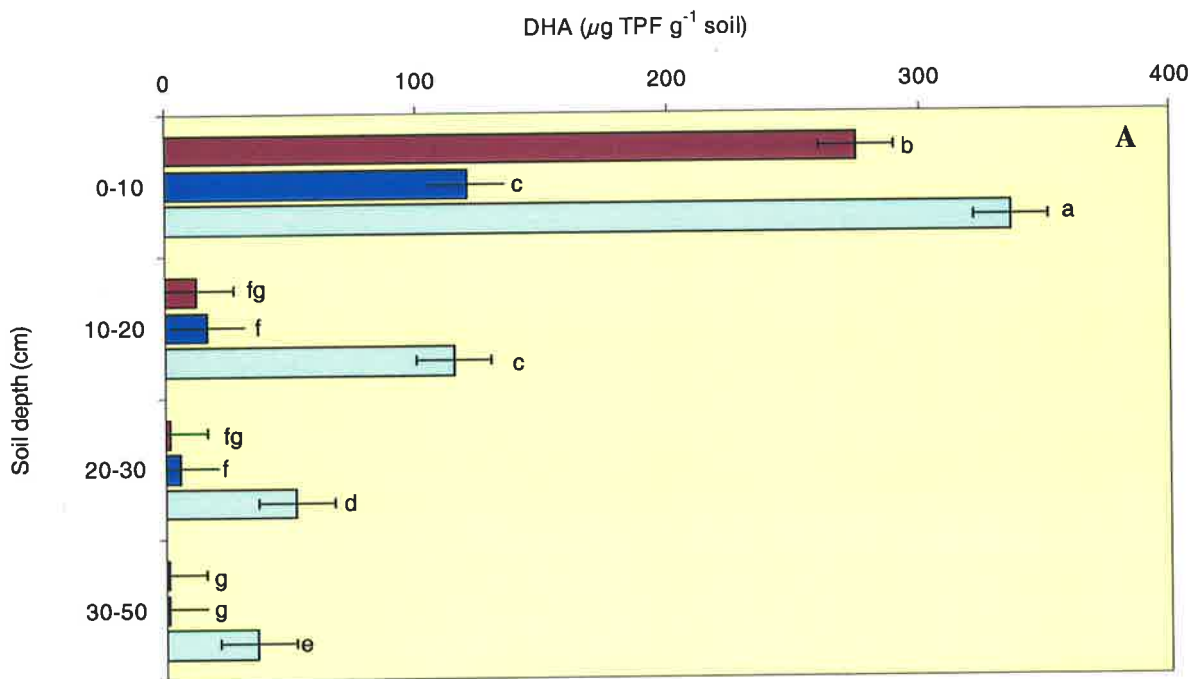
Fig.4.2 Dehydrogenase activities of contaminated soils at different depths (A – DHA in μg TPF g[-1] soil and B- DHA in μg TPF g[-1] OC). Error bars indicate sed and same alphabet represents they do not differ significantly (p > 0.05)

■ Low ■ Medium ☐ High

compared to low contaminated soil (11047). In the highly contaminated site there was 81% (2138) inhibition. A similar trend was observed in soil samples from other depths (10 - 20, 20 - 30 and 30 - 50 cm). Compared to the surface soils, the per cent inhibition in subsurface soils was less (20 - 60%). As a general trend, the DHA decreased with depth and no activity was detected below 50 cm.

4.3.2.2 Phospholipid fatty acid analyses

Phospholipid fatty acids extracted from all soil samples at 0 - 50 cm depth contained a variety of saturated, unsaturated, branched, cyclopropane and hydroxyl fatty acids. Most of the fatty acids were identified from the PLFA profile of standards and peak naming component of the MIDI program. A total of 123 fatty acids were separated in soil samples. Based on the literature cited (Pankhurst et al., 2001), the relevant peaks were identified and represented in Table 4.2. The concentration of majority of the fatty acids was significantly higher ($p < 0.001$) in highly contaminated soil compared to low contaminated soil at all depths. The increase, however, was not proportional to the increase in contamination.

The concentration of unsaturated (except 18:2ω6c) and branched fatty acids was higher in medium and highly contaminated soils than in low contaminated soils. In surface soils, the unsaturated fatty acid, 16:1ω7c increased from 0.71, 2.0 to 1.35 μg g^{-1} soil in low, medium and highly contaminated soils respectively. Fatty acid concentrations decreased with depth. Fatty acids 18:2ω6c, 18:1ω9c, i14:0 and i19:0 were not detected beyond 20 cm in low and medium contaminated soils. However, in highly contaminated soils, all the fatty acids were detected up to 50 cm. The hydroxyl fatty acids and an unidentified fatty acid (X, with relative retention time of 17.82) were found at higher concentrations in medium (0.75 μg g^{-1}) and highly contaminated soils (2.16 μg g^{-1}) compared to low contaminated soils (0.68 μg g^{-1}). These fatty acids were observed only in 0 - 10 cm soil samples and were not detected beyond this depth. As observed with DHA, the increase in PLFA concentration in highly contaminated soils was due to the increased OC present in these soils. Hence, subsequent data of PLFAs were expressed as μg g^{-1} OC of the soil samples.

When corrected to OC, individual PLFAs specific for bacteria (i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0 and cy19:0) and fungi (18:2ω6c) decreased with

Table 4.2 Concentration of PLFAs ($\mu g \, g^{-1}$ soil) in contaminated soils at different depths

Fatty Acid	Low				Medium				High				Significance[b]		
	0-10	10-20	20-30	30-50	0-10	10-20	20-30	30-50	0-10	10-20	20-30	30-50	Site	Depth	SitexDepth
Saturated															
14:0	0.40	0.17	0.08	0.12	0.67	0.47	0.10	0.06	0.43	0.36	0.59	0.26			***
15:0	0.20	0.06	0.02	0.04	0.28	0.17	0.04	0.01	0.15	0.13	0.18	0.11			***
16:0	3.17	0.88	0.40	0.52	4.15	2.49	0.74	0.51	3.05	2.62	3.53	1.94			***
17:0	0.16	0.07	0.04	0.05	0.25	0.16	0.06	0.04	0.28	0.25	0.34	0.20			***
18:0	0.67	0.25	0.18	0.21	1.17	0.68	0.26	0.23	1.32	0.93	1.57	0.80			***
20:0	0.27	0.11	0.05	0.06	0.37	0.31	0.09	0.05	0.33	0.21	0.00	0.00	*	***	ns
Unsaturated															
16:1ω7c	0.71	0.00	0.00	0.00	2.00	0.82	0.00	0.00	1.35	1.06	1.21	0.56			
16:1ω5c	0.45	0.00	0.00	0.00	0.97	0.30	0.00	0.00	0.65	0.30	0.31	0.08			
18:3ω6c	0.24	0.21	0.19	0.21	0.35	0.27	0.22	0.20	0.34	0.24	0.26	0.20			***
18:2ω6c	0.31	0.01	0.00	0.00	0.38	0.08	0.00	0.00	0.34	0.14	0.15	0.06	*	***	ns
18:1ω9c	0.61	0.00	0.00	0.00	1.36	0.47	0.00	0.00	1.35	0.80	0.44	0.41	***	***	ns
Branched															
i14:0	0.26	0.04	0.00	0.00	0.35	0.19	0.03	0.00	0.19	0.15	0.20	0.13			***
i15:0	1.94	0.38	0.10	0.09	2.97	1.63	0.30	0.10	1.17	1.34	1.72	1.00			***
a15:0	1.26	0.31	0.11	0.11	1.99	1.40	0.30	0.13	0.84	0.78	0.94	0.60			***
i16:0	0.83	0.20	0.04	0.06	0.85	0.51	0.12	0.04	0.39	0.29	0.41	0.21			***
i17:0	0.67	0.14	0.02	0.04	0.94	0.43	0.11	0.04	0.41	0.29	0.47	0.24			***
a17:0	0.50	0.13	0.04	0.04	0.63	0.42	0.10	0.04	0.32	0.27	0.39	0.24			***
i19:0	0.12	0.01	0.00	0.00	0.40	0.16	0.00	0.00	0.20	0.12	0.14	0.04			***
Cyclopropane															
cy17:0	0.70	0.10	0.01	0.02	0.66	0.39	0.11	0.02	0.40	0.31	0.40	0.25			***
cy19:0	0.70	0.24	0.07	0.05	1.01	0.63	0.16	0.06	1.22	0.91	1.06	0.79			***
Hydroxyl															
10:03OH	0.01	0.00	0.00	0.00	0.04	0.02	0.00	0.00	0.12	0.13	0.06	0.04	***	***	*
16:03OH	0.12	0.02	0.00	0.00	0.33	0.24	0.05	0.00	0.14	0.17	0.32	0.16			***
X^a	0.68	0.00	0.00	0.00	2.48	0.75	0.00	0.00	2.16	1.22	1.20	0.61			***

Significant effects were calculated by two-way analysis of variance * ($p<0.01$), *** ($p<0.001$), ns ($p>0.05$). a - X indicates an unidentified fatty acid methyl esters. b - statistical significance using two way ANOVA.

increasing levels of contamination (Fig. 4.3A,C). PLFA profiles of low, medium and highly contaminated soils varied significantly among each other and also at different depths. In highly contaminated surface soils (0 - 10 cm), the signature fatty acids of both bacteria and fungi decreased by more than 50% compared to those observed in low contaminated soil. The inhibition was significant ($p < 0.05$) in all the bacterial specific fatty acids. The fatty acid, 18:2ω6c, specific for fungi did not differ significantly between low and medium contaminated soils; however it was significantly inhibited in the highly contaminated soil. At 10 - 20 cm depth (Fig. 4.3B), medium contaminated soils harboured significantly ($p < 0.05$) higher concentration of PLFAs compared to the low contaminated soils.

There was an increased proportion of Gram-negative bacteria in highly contaminated soil compared to low and medium contaminated soils at all depths (Fig. 4.4). This increase was significant ($p < 0.05$) at all depths except 20 - 30 cm indicating that there was a population shift towards greater proportion of Gram-negative bacteria.

The total amount of bacterial PLFAs that were chosen to represent the bacterial part of the biomass was affected by increases in contamination. The amount of the bacterial PLFAs in low contaminated surface soil samples (0 - 10 cm) was 2728 μg g^{-1} OC, which decreased to 950 and 312 μg g^{-1} OC in medium and highly contaminated soils, respectively. Corresponding values for the fungal biomass (18:2ω6c) were 125, 39 and 21 μg g^{-1} OC. The decrease in bacterial biomass was also relative to the increase in exchangeable Cr, Cr(VI), soluble Cr and Cr(VI). However, there was no relationship between bacterial biomass and DHA of soil samples.

The PLFA data of all detected fatty acid concentrations normalised to OC, was subjected to a PCA (Fig. 4.5A). PCA of the soil samples from different sites showed that the PLFA composition varied with site, where the first principal component explained 87.5% and the second component explained 4.5% of the variation of this data. Soil samples from lower depths (low 20 - 50, medium 30 - 50) having low metal concentrations were found to the right and other samples scattered on the left hand side. Careful examination of the data indicated that the values for soil samples from low (20 - 50 cm) and medium (30 - 50 cm) depth had very high values because of their very low OC. This had artificial inflating effects and hence these samples were eliminated in all further analyses.
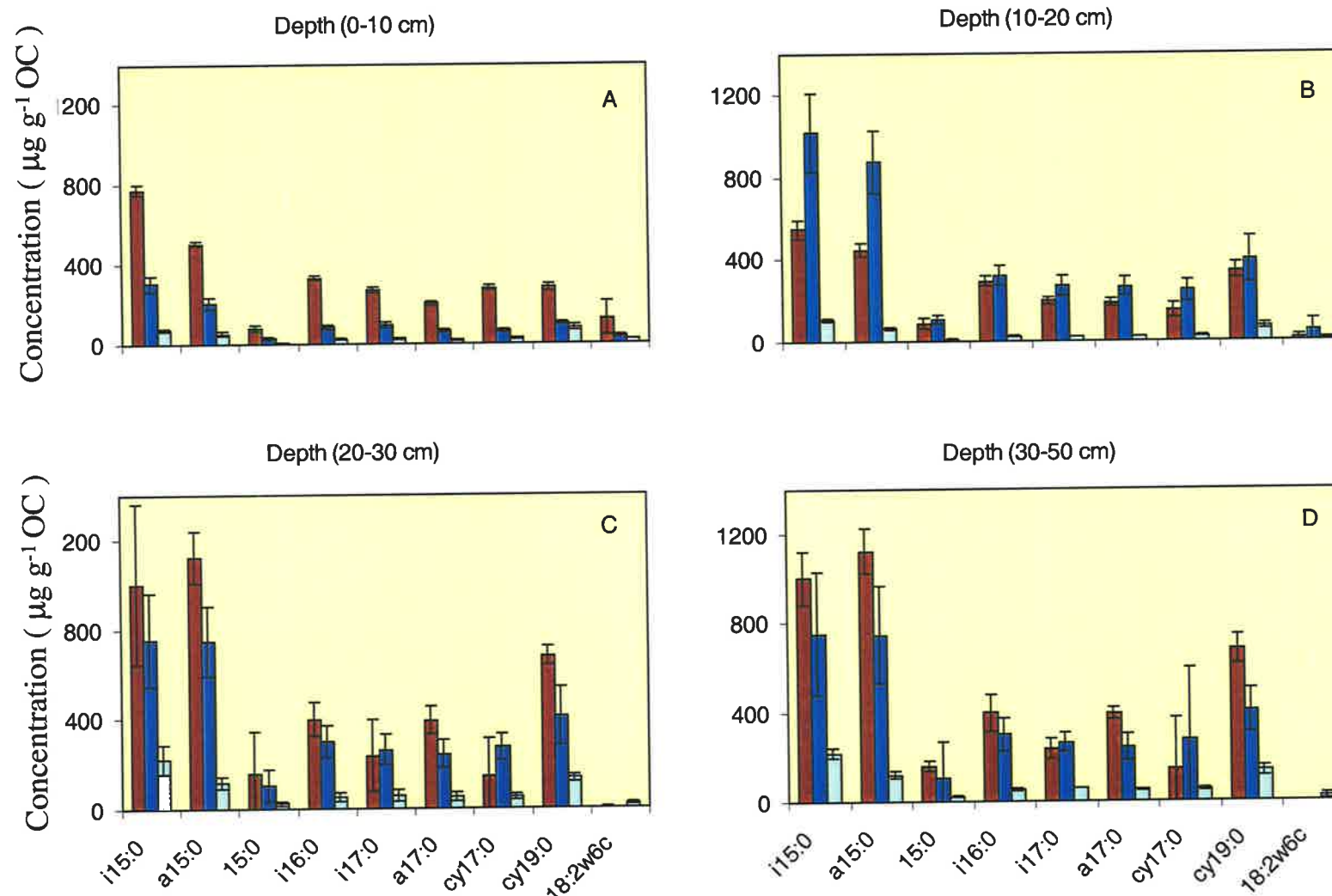
Fig. 4.3 Concentration of bacterial and fungal specific fatty acids in contaminated soils at different depths
■ Low ■ Medium □ High)
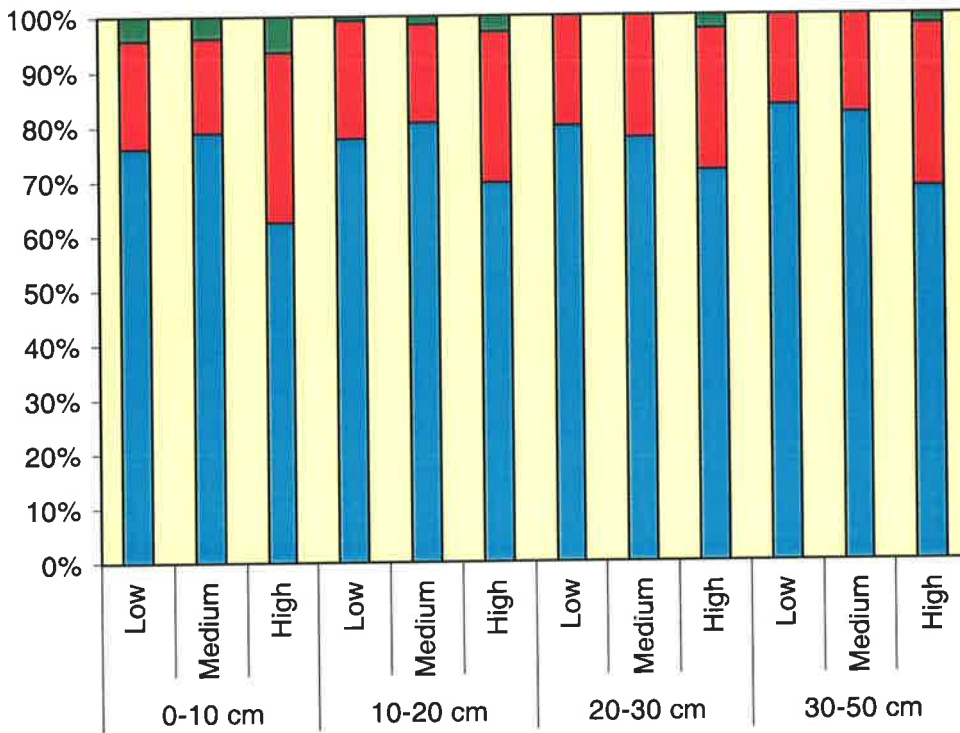
Fig. 4.4 Proportion of Gram positive ▮ ,Gram negative ▮ and fungal ▮ specific PLFAs of contaminated soils at different depths
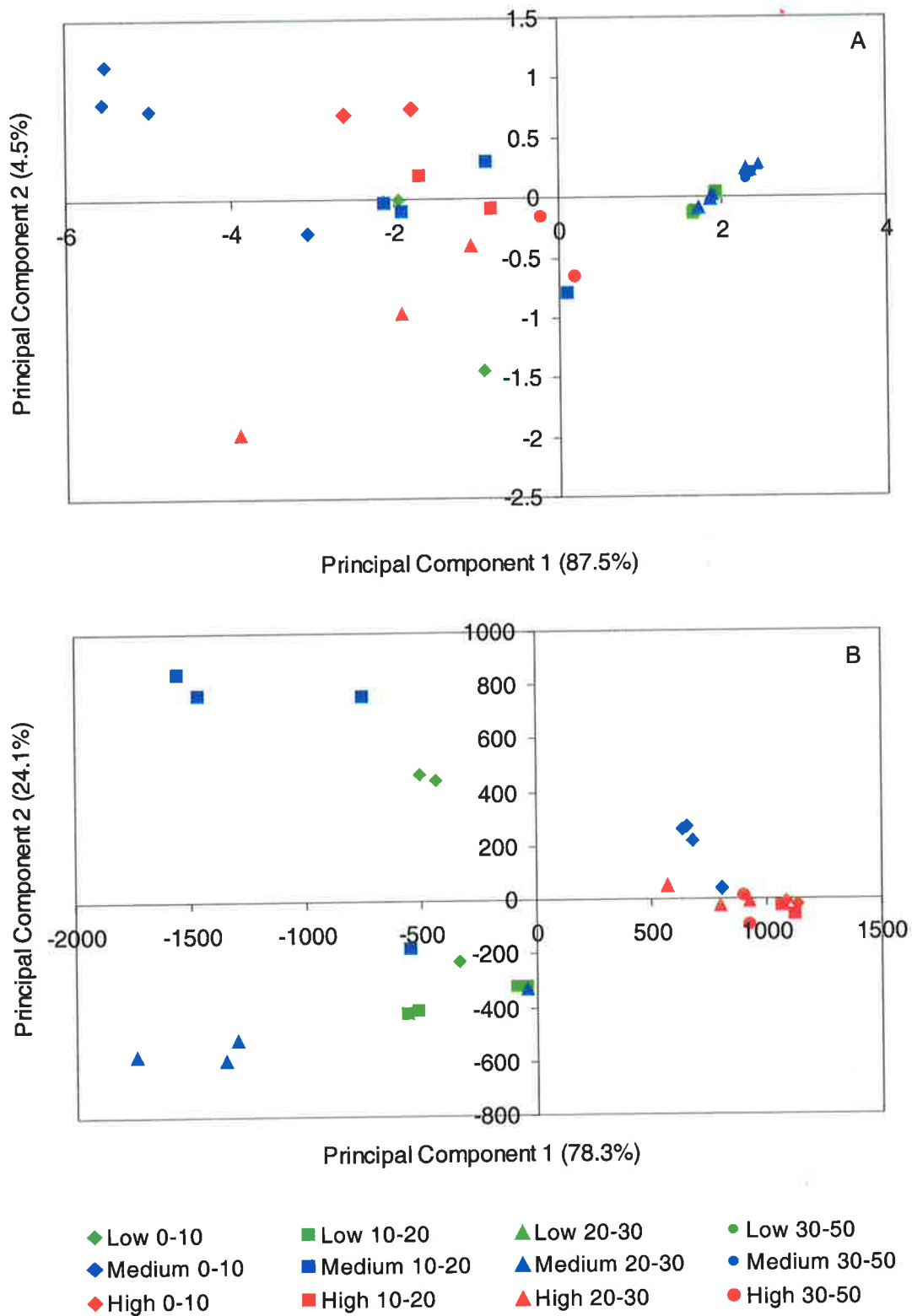
Fig. 4.5 Principal component analysis of PLFAs of contaminated soils from all depths (A) and at specific depths (B). Samples having similar microbial populations group together and dissimilar samples did not group.

In the second analysis, the PC1 accounted for 78.3% of the variance that discriminated between low and highly contaminated soils (Fig 4.5B). Samples from 0 - 10 cm depth of medium contaminated soil having high concentration also grouped with highly contaminated soil on the right. Most of the variation in PLFA pattern due to contamination was explained by the first component. The second component, PC2 showed discrimination between the depths among the low and medium contaminated soils. Samples from low 0 - 10 cm, medium 10 - 20 cm were seen above the PC2 axis, whereas, low 10 - 20 cm and medium 20 - 30 cm grouped below the PC2 axis.

The changes in PLFA pattern due to contamination was further confirmed by the positive relationship between the scores of PC 1 and total Cr and soluble Cr fractions in soil solution (Fig 4.6). As the metal concentration increased, there was an increase in scores of PC1 indicating more changes in PLFA patterns. Highest scores were observed for highly contaminated soil and lowest scores for low contaminated soil. From all the graphs, it was evident that low and highly contaminated soils varied significantly among each other compared to low and medium contaminated soils.

4.4   DISCUSSION

Chromium is ubiquitous in the environment, occurring naturally in water, rocks and soil. Its use in various manufacturing processes, such as stainless steel, electroplating, dyes, wood preservation and leather tanning, and the disposal of wastes from such industries, has contributed to higher concentrations of Cr in the environment. Environmental conditions (physical, chemical and biological) govern the oxidation states of Cr and therefore the potential hazards associated with its presence in the environment. The hexavalent form of Cr is highly mobile and is a mammalian carcinogen, however, Cr (III) is immobile and relatively non-toxic. Although it is known that the presence of metals, such as Cr, in the environment can influence soil physico-chemical properties, little is known about the long-term effects of Cr contamination on soil microbial populations.

In this study, the addition of tannery wastes to soil has resulted in significant changes in chemical characteristics especially increase in pH, OC and heavy metal, Cr. These changes have resulted in inhibition of soil microbial activity and changes in microbial populations.
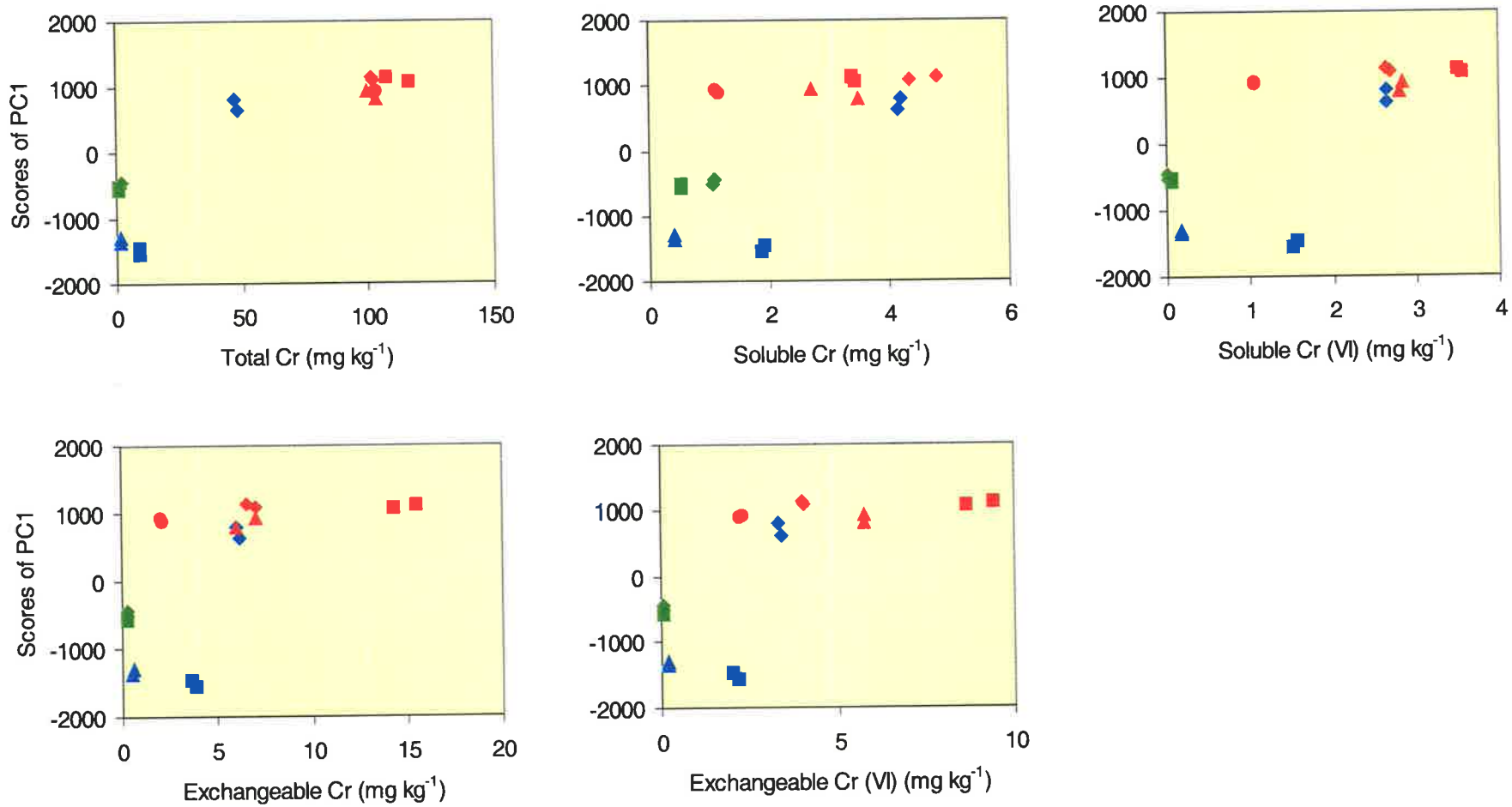
Fig 4.6 Scores of the principal component (PC1) from the analysis in Fig 4.5B in relation to the soil total Cr, soluble Cr, soluble Cr (VI), exchangeable Cr and exchangeable Cr (VI) showing the pollution effect on the PLFA pattern. Red – High, Blue- Medium, Green – Low contaminated soils. More values indicate more effect of contamination and changes in microbial populations.

4.4.1 Changes in total, soluble and exchangeable Cr

Surface and subsurface soil samples from the contaminated site showed very high concentrations of Cr with a maximum of 112 g kg^{-1} in highly contaminated surface soil. Of major concern at highly contaminated site is the presence of Cr(VI), a known respiratory carcinogen (USEPA, 1984; IARC, 1990; ATSDR, 1994) both in soluble and exchangeable form at significantly (p< 0.05) higher levels even after 20 years of ageing. Although the soluble and exchangeable Cr(VI) was only 0.003% and 0.006% of the total Cr (112000 mg kg^{-1}), this concentration is higher than the maximum permissible level of 0.05 mg Cr L^{-1} (USEPA, 1996a). Also more than 90% of this easily available Cr fraction existed as Cr(VI) species.

The fact that a significant amount of Cr(VI) was detected even at 1-m soil depth suggested that these extremely labile fractions of Cr(VI) were leaching down the soil profile. Naidu et al., (2000b) also reported the presence of Cr(VI) at deeper soil profiles at Mount Barker. The amount of Cr released into soil solution and exchangeable Cr(VI) is a function of total Cr and soil properties, especially Eh-pH relationship. At this site, theoretically the thermodynamic conditions (pH-Eh diagram) and high OC should favour Cr(VI) reduction at this site. Despite these reasons and given that disposal of tannery waste had ceased 25 years ago, the presence of Cr(VI) in the surface and subsurface soils makes this contaminated site unique.

Earlier mineralogical studies on soils from this site revealed high density of Cr on calcareous mineral surfaces (Avudainayagam et al., 2001). Further, Cr speciation studies using Minteqa2 by these investigators suggested the presence of sparingly soluble calcium chromate salt. It is likely that in the presence of moisture, there is a gradual dissolution of this sparingly soluble salt releasing Cr(VI) in soil solution. Also according to these authors the aromaticity of OC present in this site makes OC more refractile, hence, less available for Cr(VI) reduction in these soils. Generally, high organic matter content with adequate moisture would stimulate microbial activity in the soil leading to oxygen stress conditions conducive for reduction reactions including Cr reduction (Bartlett and James, 1988; Bartlett and James, 1993). However, the prevalence of Cr(VI) in the Mount Barker soils in spite of 15.7 % OC in surface soils, contradicts previous observations recorded by Bartlett and James (1988, 1993).

4.4.2 Changes in microbial activity and populations

Although the OC did not influence Cr(VI) reduction, it had a significant (p< 0.05) positive effect on microbial activity (DHA) of the subsurface soils and a linear relationship existed between DHA and OC (r^2=0.9***). In surface soils, where high microbial activity was recorded, organic carbon did not show any relationship with DHA due to the fact that the surface soil was comprised of sludge. The high OC from the sludge might be used for growth of microorganisms or act as buffer withholding the toxicity of Cr to microbes.

The inhibition of DHA due to Cr contamination was evident only when the values were normalised to their corresponding OC values. This indicates that OC plays a major role in DHA, as carbon source for higher microbial activity and it might be important to compare the OC normalised DHA values in heavy metal contaminated soils to counteract the masking effect of OC. The inhibitory effect of Cr(VI) an microorganisms have been proven, as a strong oxidising agents such as Cr(VI) can diffuse into cell the membrane and can damage the DNA (DeFlora et al., 1984) leading to inhibition in microbial activities. The low contaminated soils showed an exceptional increase in DHA g^{-1}OC. While this needs further investigation, it is likely that there is critical Cr concentration, which might be stimulatory for microorganisms. Also, presumably the Cr concentration in low contaminated soil might be stimulatory compared to the high Cr concentrations encountered in medium and highly contaminated soils. A critical concentration of Cr less than 50 μg L^{-1} stimulated photosynthesis in unicellular green alga, *Chlorella* sp.; however, 10 mg Cr L^{-1} was inhibitory (Petria, 1978).

When compared to low contaminated soil, the medium and highly contaminated soils showed decreases in DHA (Fig. 4.2B). DHA was inhibited by total and/or bioavailable Cr/Cr(VI) in contaminated soils. No previous reports exist on the inhibitory effect of very high concentrations of Cr on DHA especially in long-term contaminated soils. But soil studies with freshly spiked Cr (29 mg kg^{-1}) indicated that Cr(VI) was slightly more toxic than Cr(III) as EC50 (mg kg^{-1}) of Cr(VI) (71) > Cr(III) (75). The reverse was true for water-soluble Cr species, Cr(III) (0.62) > Cr(VI) (78.1) (Welp, 1999). Other studies have demonstrated that elevated concentration of metals such as Cd, Cr, Cu, Ni, Pb and Zn in combination decreased DHA (Obbard et al., 1994). In all the sites, DHA decreased with depth, which is a general function of the microbial density, oxygen status and easily available organic matter (Lavahun et al., 1996).

Similar to DHA assays, PLFA studies also revealed that there was a significant shift in microbial populations as a result of tannery waste application. The shift in microbial community structure resulted in a decrease in signature bacterial and fungal PLFAs. The majority of the fatty acids constituting PLFA patterns decreased in highly contaminated soil samples compared to low contaminated soil samples. However, the inhibition was not directly proportional to the increase in contamination.

The observed changes in PLFA patterns were due to Cr contamination as evidenced by the first principal component of PCA, which revealed 78.3% of the variation. Also the PLFA loadings were higher for highly contaminated soils compared to the low contaminated soils (Fig 4.6), and hence the changes in microbial community structure could be primarily due to Cr contamination, not the OC. Similar observation of increased PLFA loadings were reported in soils contaminated by Cu from smelters. Soils collected from near vicinity of the smelters had higher concentrations of Cu and had higher PLFA loading values compared to the soils collected from further away from the smelters (Pennanen et al., 1996).

In general, the bacterial and fungal populations were sensitive to the contamination by tannery wastes as both bacterial and fungal specific fatty acids were inhibited in the highly contaminated site. As the concentration of total Cr and Cr(VI) in the surface soil increased, signature PLFAs for bacteria decreased. The negative relationship correlated significantly with the increase in total Cr and Cr(VI) concentrations (r^2=0.60*) which indicated that some of the indigenous soil bacteria are sensitive to Cr. Bacterial fatty acids were reported to be either increased or decreased depending on the soil and the heavy metal. (Frostegård et al. (1993) reported that bacterial PLFAs 15:0 and 17:0 increased in arable soil while they were unaffected in forest soils.

The population shift was towards Gram-negative bacteria as indicated by a proportional increase in PLFAs specific for Gram-negative bacteria (Fig 4.4) with a decrease (30 %) in iso and anteiso branched fatty acids (i15:0, a15:0, i16:0) specific for Gram-positive bacteria soils from highly contaminated site. Gram-negative bacteria were predominantly reported in metal contaminated soils compared to Gram-positive bacteria (Doelmann and Haanstra, 1979; Duxbury and Bicknell, 1983; Doelmann, 1985). Also Gram-negative bacteria were associated with the rhizosphere (Gilbert et al., 1994). However, contradictory reports also exist suggesting that Gram-negative bacteria

are more sensitive to Cr than gram-positive bacteria (Ross et al., 1981).

Fungi were observed only in surface soils and they were more inhibited (decrease in PLFA, 18:2ω6c) compared to the total bacterial biomass. However, 16:1ω5c, a signature PLFA for vesicular arbuscular mycorrhiza seems to be not inhibited by the tannery waste and did not show any relationship with Cr concentration. Olsson et al. (Olsson et al., 1995) also reported that 16:1ω5c was not affected by metal contamination. (Pennanen et al. (1996) reported similar results to those described above for Cu contaminated soils. Mycorrhizal association in plants growing in metalliferous soils are studied. In metal contaminated soils, most of the metal tolerant grasses are often strongly colonised with arbuscular mycorrhizae (Shetty et al., 1994).

Although many reports exist on the effect of Cu (Frostegård et al., 1993; Pennanen et al., 1996; Griffiths et al., 1997), Pb (Frostegård et al., 1993) and Zn (Kelly et al., 1999b) and sewage sludge amended soils (Sandaa et al., 2001) on microbial populations, there is no published record of the inhibitory effect of tannery waste and associated Cr on microbial population as determined by PLFA patterns. Since tannery wastes is a composite mixture of organics and inorganics this means that microbial responses in soils contaminated with such wastes may differ from that in soils contaminated with metal alone.

4.4.3 Chemical and microbial changes due to tannery wastes: Implications to Cr(VI) – Cr(III) transformation

Increased DHA in highly contaminated soil samples indicated enhanced microbial activity and availability of carbon sources for this activity. Such increased microbial activity under field moist conditions prevailing in the contaminated site may not necessarily generate reducing conditions favourable for Cr(VI) reduction. Since there was inhibition in microbial activity nullifying the OC effect, there might not be enough Cr(VI) reducing microorganisms. Conversely, low moisture, hence, aerobic conditions may promote oxidation of Cr(III) to Cr(VI). Also, these soils are high in total Mn, 0.57 g Mn kg^{-1} at the highly contaminated site and there existed a significant (p< 0.05) linear relationship between the total Mn and soluble Cr(VI) released in soil solution (Fig. 4.7). Manganese present in this site should be predominantly insoluble Mn oxides as no detectable soluble Mn (Mn II) was observed in the soil solution. The role of Mn oxides on Cr oxidation has been widely reported in soils (Bartlett and James,
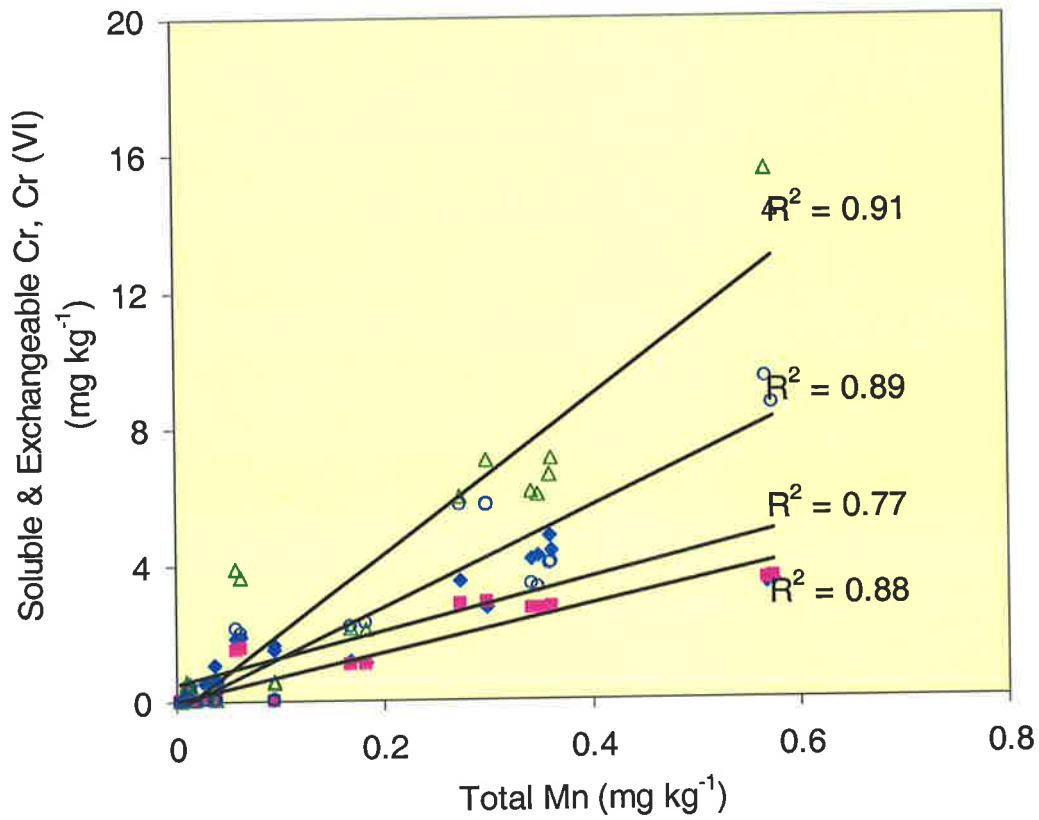
Fig. 4.7 Linear relationship between total Mn and Cr released in the soil solution of highly contaminated soils (◆ soluble Cr, ■ soluble Cr (VI), Δ exchangeable Cr and O exchangeable Cr (VI))

1979; Fendorf and Zasoski, 1992; Milacic and Stupar, 1995). The presence of Mn oxides coupled with alkaline pH might favour Cr oxidation in these soils. Details of Mn mediated Cr oxidation are discussed in subsequent chapters (Chapters 5, 6).

4.5 CONCLUSION

Present investigations demonstrate that even after 25 years of waste disposal, ecotoxicity effects of Cr(III)/Cr(VI) still persists. This toxicity was evident from the inhibition of microbial activity (DHA) and alterations in microbial community structure (PLFAs).

The inhibition also indicated a poor soil health in terms of microorganisms available for Cr(VI) reduction. Given that Cr(III) complexes are thermodynamically more stable than Cr(VI) there is a general acceptance that the presence of organic matter and other species contributing to electron transfer reactions in soils would rapidly convert toxic Cr(VI) to non toxic Cr(III). However, in this particular site, the presence of Cr(VI) despite high organic matter warrants further investigation of factors that either enhance Cr(III) to Cr(VI) transformation or the stability of Cr(III) in this environment. The following chapter reports the reason behind the presence of Cr(VI) in this contaminated soils.

Chapter 5

"I haven't failed. I've found 10,000 ways that don't work"
- Thomas A Edison -
⌀

"I sincerely dedicate this chapter to my supervisor, Ravi Naidu, for the core idea
and constant encouragement during this work to make it happen. It worked."

CHAPTER 5

Microbial Manganese Oxides and Chromium Oxidation in Tannery Waste Contaminated Soil

5.1   INTRODUCTION

From the previous chapter, it is evident that Cr(VI) is present in long-term tannery waste (LTTW) contaminated soils despite the relatively high organic carbon content available for its reduction. The presence of Cr(VI) in subsurface soil (>50 cm) indicates that it is mobile and may potentially leach down the soil profile and impact upon groundwater quality. The presence of Cr(VI) in contaminated soils has been attributed to the presence of manganese (Mn) oxides that catalyse Cr oxidation and the oxidation of Cr(III) by naturally occurring Mn oxides in soils has been studied extensively (Bartlett, 1979; Eary, 1987; Fendorf, 1992; Milacic, 1995). Manganese oxides have a high adsorption capacity for metal ions (Murray, 1975), thus providing a local surface environment in soils where the coupled processes of aqueous Cr(III) oxidation and Mn oxide reduction are promoted.

$$CrOH^{2+} + 1.5\ MnO_2\ (s) \longrightarrow HCrO_4^- + 1.5\ Mn^{2+} \qquad (1)$$

The rate of Cr oxidation is, however, governed by the nature of Mn oxides, soil pH and the solubility of Cr(III) (James and Bartlett, 1983; Milacic and Stupar, 1995). While extensive research has been published on abiotic processes governing Cr oxidation, no reports exist on either the direct or indirect role of biotic processes that influence Cr oxidation in soil.

Although Cr(III) oxidation by Mn oxides is a chemically catalysed/abiotic process, the formation of Mn oxides in soil and fresh water environments is predominantly a microbial/biotic process (Leeper and Swaby, 1940; Tyler and Marshall, 1967). Evidence suggests that Mn oxidation may occur due to non-biological processes

in soil (Ross and Bartlett, 1981). However, abiotic processes may only contribute to a fraction of the total amount of Mn oxides produced. Mn oxidising microorganisms convert soluble Mn(II) to insoluble Mn oxides such as birnessite. However, the environmental conditions and the number of Mn oxidising microorganisms present will influence the type, rate and volume of the Mn oxides formed.

In the LTTW contaminated soil used in this study, a significant correlation existed between the total concentration of Mn present in the soil and the concentration of Cr(VI) released into the soil solution. This suggests that Mn oxides may potentially play a significant role in catalysing the oxidation of Cr at this site and this may account for the elevated levels of Cr(VI) in soil solution. The influence of Mn oxidising microorganisms, and the Mn oxides produced, may therefore impact significantly on abiotic processes that influence Cr oxidation. The hypotheses to be tested in this Chapter is that a biotic-abiotic coupling reaction exists in which microbial oxidation of Mn(II) results in the synthesis of Mn(IV) oxides that trigger abiotic oxidation of Cr(III). The reaction scheme is described below. In the second reaction Mn^{2+} acts as a catalyst.

$$Mn^{2+} \longrightarrow Mn^{4+} + 2e \qquad \text{(biotic)}$$

$$Mn^{4+} + Cr(III) \longrightarrow Mn^{2+} + Cr(VI) \qquad \text{(abiotic)}$$

The general aims of this chapter were to investigate the influence of microbially synthesised Mn oxides on Cr(III) oxidation and to verify the validity of the above hypothesis for the LTTW contaminated site. Specifically the aims included:

1. Determination of the distribution of Mn oxidising microorganisms in LTTW contaminated soil;

2. Determination of the rate of Mn oxidation by isolated pure cultures and microbial consortia;

3. Characterisation of microbially synthesised Mn oxides;

4. Determination of the rate of Cr oxidation by microbially synthesised Mn oxides; and

5. Assessment of the biotic-abiotic coupling reactions leading to Cr oxidation.

5.2   MATERIALS AND METHODS

5.2.1 Mn oxidising microorganisms in contaminated soil

5.2.1.1 Distribution of Mn oxidising bacteria

The distribution of Mn oxidising bacteria in LTTW contaminated soil was determined using a plate count method. Highly contaminated soil (102 g total Cr kg^{-1}, 2.7 mg Cr(VI) kg^{-1} in soil solution; Table 4.1) (10 g) was serially diluted (10 folds) in sterile phosphate buffered saline (Appendix II) to a dilution of 10^{-2} using the pour plate method (Zuberer, 1994). In addition, the tolerance of Mn oxidising bacteria to Cr was assessed by adding filter sterilised Cr(VI) (0.5 and 1.0 mg L^{-1}) to molten PYG mineral medium (Section 3.3.5) prior to plate pouring. Four replicates were maintained for each dilution. Plates were incubated in the dark at 27°C for up to three weeks. After incubation, plates were flooded with leuco-crystal violet (LCV) reagent to determine the number of Mn oxidisers (Appendix II). Colonies of Mn oxidising bacteria were identified and counted based upon the development of a blue colour around them indicative of the reaction of Mn oxides with LCV.

5.2.1.2 Enrichment and isolation of Mn oxidising bacteria and microbial consortia

Soil solution from LTTW contaminated soil (collected from different depths: 0-10, 10-20, 20-30, 30-50 and 50-100 cm) was used as the inoculum for the enrichment and isolation of Mn oxidising bacteria and microbial consortia. Soil solutions (5 ml) were used to inoculate Mn enrichment broth (50 ml) (Section 3.3.2) containing 1000 mg Mn(II) L^{-1} as MnSO$_4$. Enrichment cultures were incubated at 25°C in the dark with shaking at 100 rpm for three weeks.

For the isolation of Mn oxidising pure cultures, aliquots of enrichment cultures (0.1 ml) were plated onto PYG mineral medium agar containing Mn (Section 3.3.5) and incubated at 25°C in the dark. After a two-week incubation, Mn oxidising bacteria were visualised by flooding the plates with a solution of LCV. Individual Mn oxidising colonies were removed and streaked onto PYG mineral medium and further incubated. Once the purity of the isolated colonies was established (after 3 subcultures on PYG mineral medium), colonies were removed from agar plates and transferred to liquid

PYG mineral medium containing 1000 mg Mn(II) L^{-1} as $MnSO_4$. Ten bacterial cultures were purified, of which three were used for further studies.

5.2.2 Mn oxidising potential of microbial cultures

5.2.2.1 Mn oxidation by bacterial cultures and consortia

Three bacterial Mn oxidising pure cultures (designated SKMn1, SKMn2 and SKMn3) and five microbial consortia (designated SKCons1, SKCons2, SKCons3, SKCons4 and SKCons5), isolated from LTTW-contaminated soil (Section 5.2.1.2) were examined for their Mn oxidising potential.

Inocula for Mn oxidising experiments were prepared by growing microbial consortia and pure cultures in a Mn enrichment medium (100 ml) (Section 3.3.2) for two weeks at 30°C, with continuous shaking at 100 rpm. After reaching the stationary phase, cells were harvested by centrifugation (10,000 g for 20 minutes), washed three times with phosphate buffer (pH 7.0) and resuspended in mineral salts medium (25 ml), resulting in a cell suspension with an optical density (600 nm) of 1.2.

The Mn oxidising potential of pure cultures and microbial consortia was assessed by inoculating the respective cultures (5 ml) into Mn oxidising broth (75 ml) (Section 3.3.3) containing 50 mg Mn(II) L^{-1} as $MnSO_4$ (in duplicate). The production of Mn oxides was measured after three weeks of incubation. Culture media (5 ml) were routinely sampled and the concentration of Mn oxides was determined by the method of (Lovley and Phillips, 1988) (Section 5.2.6). Controls consisted of uninoculated culture broth containing Mn(II).

After initial screening of the cultures for Mn oxidising potential, the bacterial culture SKMn2 and the microbial consortium SKCons2 were selected for further study due to their high efficiency of Mn oxidation. Mn oxidation studies were repeated (as outlined above) using SKMn2 and SKCons2. In these experiments, the concentration of Mn(IV) in culture media was determined by binding with LCV (Kessick et al., 1972) (Section 5.2.6) while microbial growth was measured in terms of protein concentration (Bradford, 1976).

5.2.2.2 Mn oxidation by fungal cultures

Previous research demonstrated the potential of Take-all fungus (*Gaeumannomyces graminis* var *tritici*, Ggt) to oxidise Mn (Pedler et al., 1996). Based on this study, Ggt isolates from the CSIRO Land and Water culture collection (courtesy of Dr P. Harvey) were screened for their ability to oxidise Mn. Mn oxidation was assessed by growing Ggt strains on NDY broth (Section 3.3.4) amended with 50 mg Mn(II) L^{-1} as $MnSO_4$. Filter sterilised $MnSO_4$ was added to the media before pouring plates.

Small blocks (3 x 3 mm^2) of actively growing hyphae (Ggt) were transferred aseptically to the centre of Petri plates and incubated in the dark for 10 d at 20°C. After incubation, fungal hyphae from Mn amended and unamended medium were compared for the intensity of brown precipitate formed around the hyphae. Brown precipitates were confirmed as Mn oxides based on the colour reaction with LCV (Kessick et al., 1972).

Fungal isolates positive for Mn oxide production were selected for further experimentation to determine the rate and extent of Mn oxidation. NDY broth (25 ml) amended with $MnSO_4$ (20 mg Mn L^{-1}) was inoculated with strains of GAN30, TW27, TC24, TC25 and 500 by aseptically adding small blocks of agar (3 x 3 mm^2) covered with actively growing hyphae. Duplicates were maintained for each strain while uninoculated medium served as the control. Cultures were incubated without shaking in the dark at 20°C for 2 weeks before fungal mats were harvested. Biomass was harvested by suction filtration using sterile Whatman #1 filters. Filter papers with fungal mats were folded, placed in a sealable bag and immersed in liquid nitrogen before storage at

-20°C. The control medium was also passed through the filter paper and stored in a similar manner. The concentration of Mn oxides produced was determined by digesting samples (fungi and filter paper) in aqua regia prior to quantification by AAS (Section 3.2).

5.2.2.3 Mn oxidation by alga

An algal culture (strain MMA1), isolated from LTTW contaminated soil (Algal culture collection of Megharaj M, CSIRO Land and Water, Adelaide), was screened for its ability to oxidise Mn(II). The inoculum for Mn oxidation experiments was prepared

by growing strain MMA1 in Bold's medium (Bischoff and Bold, 1963; Section 3.3.1) for 10 d at 25°C shaking at 100 rpm.

Mn oxidation experiments were prepared by inoculating Strain MMA1 (5 ml) into Bold's medium (45 ml) spiked with 100 mg Mn(II) L^{-1} as $MnSO_4$. Cultures were incubated under static conditions for five weeks at room temperature. Aliquots of the culture medium (5 ml) were removed periodically and analysed for Mn oxides by the method of (Lovley and Phillips, 1988)(Section 5.2.6).

5.2.3 Identification of Mn oxidising bacteria

Mn oxidising bacteria, isolated from highly contaminated soil, were identified based on the fatty acid composition of the cell walls using the MIDI-FAME technique. Fatty acid methyl esters (FAME) were extracted by the method of Glucksman et al. (2000). A loop of bacterial culture, grown in TSY broth (Section 3.3.6), was transferred to a sterile glass tube with a teflon lined cap. To this 1 ml of saponification reagent (45 g of NaOH in 150 ml of methanol and 150 ml of water) was added and vortexed for 5-10 sec. This was incubated at 100°C (5 min), vortexed and again heated at 100°C (25 min). The saponified mixture was then methylated by adding 2 ml of methylation reagent (325 ml of 6 N HCl in 275 ml of reagent grade methanol), vortexed for 5-10 sec, heated at 80°C (10 min) and cooled rapidly under ice cubes. The fatty acids were extracted in the third step by adding 1.25 ml of extraction solvent (200 ml of hexane in 200 ml of methyl tert butyl ether, HPLC grade), mixed end over end (10 min) and the lower phase was removed. To the top phase, 3.0 ml of dilute NaOH (10.8 g in 900 ml distilled water) was added, mixed end over end (5 min) and centrifuged (2000 rpm; 3 min). The top 2/3 phases was removed using a Pasteur pipette and transferred to GC vials. The fatty acids were analysed by GC-FID using standard reference, MIDI TSBA 40 for calibration. The temperature ramping was 170 – 270°C at 5°C per minute on a 20 minute cycle.

5.2.4 Scanning electron microscopy (SEM) of Mn oxidising bacteria

Scanning electron microscopy coupled with X-ray analyses (EDAX) was used to characterise Mn oxides associated with bacterial cells. Bacterial cell suspensions (1 ml) were fixed with equal volumes of 5% glutaraldehyde in 100 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) buffer (pH 6.8) containing 2 mM $MgCl_2$ (final glutaraldehyde concentration, 2.5%). The mixture was incubated for 4 h

before a drop of this suspension was diluted with 40 ml of filtered (0.1 μm) deionised water. The suspension was filtered (2 μm nucleopore filter, 13 mm) and rinsed with filtered deionised water. The nucleopore filter was removed carefully, air-dried and coated with either gold or carbon. Where imaging of the surface topography was required, specimens were sputter coated with 20 nm of gold to provide electrical conductivity and maximize Secondary Electron (SE) signal yield. Specimens, where imaging of the composition was required, were evaporatively coated with 30 nm of carbon to provide electrical conductivity and maximize phase contrast of the Backscattered Electron (BE) signal. Carbon coating also minimizes extraneous X-ray peaks from the characteristic X-ray spectrum.

The specimens were placed into a "Phillips" XL30 Scanning Electron Microscope (SEM) for examination, using a primary electron beam energy of 10 KeV. Imaging was performed using the Secondary Electron (SE) signal, were information about fine surface topography was required. Energy Dispersive X-ray (EDX) analysis was done using the characteristic X-ray signals at selected positions using an "EDAX" DX4 energy dispersive X-ray (EDX) system.

5.2.5 Microbial Mn oxides

5.2.5.1 Production of microbial Mn oxides

Bacterial Mn oxides for characterisation were produced by growing SKMn2 and SKCons2 in 1 L of Mn oxidising broth (Section 3.3.3) containing 50 mg Mn L^{-1} as $MnSO_4$. Cultures were incubated at 30°C, in a shaker at 100 rpm and harvested at monthly intervals. The brown Mn oxides precipitated were collected by centrifugation at 5000 g for 10 min. The precipitates were washed three times with distilled water and dried at 40°C. Samples were ground to a fine powder using an agate mortar and pestle before characterisation of Mn oxides by XRD, ICP-AES and Quantasorb surface area analyser (Section 5.2.5.2).

5.2.5.2 Characterisation of Mn oxides

X-ray diffraction (XRD) analyses

Microbial Mn oxides were characterised by powder X-ray diffraction. Individual samples were ground in an agate mortar and pestle and lightly pressed into aluminium sample holders for analysis. XRD patterns were recorded with a Philips PW microprocessor controlled diffractometer using Co Kalpha radiation, variable divergence slit and graphite monochromator. The diffraction patterns were recorded in steps of 0.05° 2 theta with a 3.0 second counting time per step and logged to data files for analysis.

For comparison, known standard Mn oxides such as MnO_2 (manganese oxide technical, MW 86.94, Ajax Chemicals, Melbourne) and Mn_2O_3 (manganese III oxide, FW 157.87, d 4.5, -325 mesh, 99% pure, Sigma-Aldrich) were also analysed.

Elemental composition

Microbial Mn oxides were digested in freshly prepared aqua regia at 100°C for 2 h, and metal concentrations were determined by Inductively Coupled Plasma – Atomic Emission Spectrophotometry (ICP-AES, spectro flame modula, Spectroanalytical Instruments GMBH, Germany).

Surface area

The specific surface area of air-dried microbial Mn oxides was determined by N_2 gas adsorption in a Quantasorb surface area analyser. The nitrogen surface area is a measurement of the external surface of material based on the adsorption of nitrogen onto the surface of the sample at the temperature of liquid nitrogen. The Quantasorb analyser is used to determine the volume of nitrogen adsorbed, which is then used to calculate the specific surface area of the sample expressed as m^2 g^{-1}, using a model developed by Brunauer-Emmet-Teller and their equation.

B.E.T surface area calculations:

1. Volume adsorbed, desorbed (Va, mls)

Va = (desorption counts/calibration counts) (calibration volume) (273/temp)(BP/760)

Where BP – Barometric pressure

2. Relative pressure of nitrogen (P/Ps) where

$$P \text{ (partial pressure)} = (\text{fraction } N_2) (BP)$$

$$P/Ps = P/785$$

3. y co-ordinate

$$y = (P/Ps) / [Va(1-P/Ps)]$$

4. $Vm = 1/(y/x)$

5. Specific surface area

$$SA \ (m^2 \ g^{-1}) = (4.38)(Vm) / \text{ sample wt. (g)}$$

5.2.6 Determination of Mn oxide concentration

The concentration and speciation of Mn in culture supernatants was determined by the method of (Lovley and Phillips, 1988). Total Mn was determined by dissolving culture suspension (1 ml) in 0.25 N hydroxylamine hydrochloride (5 ml). After incubation at room temperature for 15 minutes, samples were filtered (0.45 μm filter) and the concentration of Mn determined by AAS (Section 3.4). Mn(II) was determined by adding 5 ml of 0.5 N HCl to 1 ml of sample. After 15-minute incubation at room temperature, solutions were filtered and Mn(II) concentrations were determined by AAS. The concentration of Mn(IV) was calculated by the difference between the total Mn and Mn(II) concentrations. The extraction efficiency of Mn was determined using selected concentrations of Mn salts (MnO$_2$, MnSO$_4$ and MnCl$_2$) and was found to be ± 5 mg L^{-1}.

The quantity of Mn oxides produced was also determined by the colorimetric method of Kessick *et al.* (1972). Aliquots of culture fluid (5 ml) were added to 50 ml of 1 mM NaHCO$_3$, 2 ml of LCV solution (0.1% LCV in 0.1 N perchloric acid) and 5 ml of 0.1 M acetate buffer (pH 4.0) (Acetate buffer was prepared by mixing 16.6 ml of 0.1 M sodium acetate with 83.4 ml of 0.1 M acetic acid) and the volume adjusted to 100 ml. The concentration of Mn oxides was determined by measuring the absorbance at 591 nm against a reagent blank in a double beam spectrophotometer (GBC Scientific, Melbourne). The Mn oxide concentration was calculated using a standard curve prepared with a MnO$_2$ colloid slurry (Ehrlich, 1975). Mn(IV) oxide slurry was prepared

by dissolving 3.16 g of $KMnO_4$ and 3.2 g of NaOH in 1L of distilled water. In another 1L of water 5.94 g of $MnCl_2$ was dissolved and this $MnCO_3$ solution was slowly added to the basic $KMnO_4$ solution while mixing constantly with a magnetic stirrer. The resulting brown floc was washed free of salts, suspended in distilled water and stored at 4°C. The detection limit of the Kessick method was 0.2 mg L^{-1}.

5.2.7 Chromium oxidation by Mn oxidising microorganisms

The main objective of this experiment was to determine whether Cr(III) is oxidised during growth and deposition of Mn oxides in liquid broth by Mn oxidising microorganisms. The bacterial strain SKMn2 and microbial consortium SKCons2, isolated from LTTW contaminated soil, were used in these Cr-Mn oxidation-reduction experiments. Inocula were prepared as described previously (Section 5.2.2.1) in Mn oxidising broth (Section 3.3.3) (75 ml) containing 1000 mg Mn L^{-1} (as $MnSO_4$) and 5 mg Cr L^{-1} (as Cr sulphate, Cr III) was inoculated with 2 ml of the respective inocula (duplicate cultures). A Cr(III) concentration of 5 mg L^{-1} was chosen for these experiments as this reflected the concentration of soluble Cr at the Mount Barker site (Table 4.2 pg 81). An uninoculated medium served as the control. Inoculated and uninoculated cultures were incubated at 30°C with shaking at 100 rpm. Aliquots were sampled at weekly intervals and analysed for the presence of Cr(VI) using the DPC method (Section 3.4.2).

In another study, Mn oxidising broth was spiked with 1000 mg Mn L^{-1} and inoculated with SKMn2 and SKCons2. Cultures were incubated at 30°C with shaking at 100 rpm. After the appearance of brown Mn oxides (approximately 6 weeks), Cr(III) (5 mg Cr L^{-1}, as Cr sulphate) was added. The concentration of Cr(VI) in solution was monitored with time by periodically withdrawing culture fluid (5 ml) and analysing for Cr(VI) by DPC method (Section 3.4.2).

5.2.8 Chromium oxidation by microbial Mn oxides

Experiments were conducted to test the effect of microbially produced Mn oxides on the oxidation of Cr(III) in the absence of bacterial cells. XRD results showed that the microbial Mn oxides were a mixture of Mn(III) (Mn_2O_3, bixbyite) and Mn(IV) (MnO_2) which could potentially oxidise Cr (Eary and Rai, 1987). For comparison commercially available reagent grade Mn(IV) (MnO_2) and Mn(III) (Mn_2O_3) (Section 5.2.5.2) were examined for their ability to oxidise Cr(III). Microbial Mn oxides used in

this study, represented the oxides collected during the growth of consortium (SKCons2) in Mn oxidizing broth (Section 3.3.3) spiked with 50 mg Mn(II) L^{-1} (as MnSO$_4$) in 1-L flasks. This consortium was preferred over strain SKMn2 for the production of Mn oxides as the quantum of Mn oxides formed was greater in this consortium. After 6 months, the brown Mn oxide precipitates were harvested by centrifugation (10,000 g for 20 minutes) and subsequently washed (3-4 times) with deionised water, air dried, and finely ground with an agate mortar and pestle.

5.2.9 Batch Studies

Cr oxidation studies were conducted by adding either 10 mg of microbially produced or reagent grade Mn oxide to Cr(III) solution (10 mg L^{-1}). The Cr(III) solution was prepared by dissolving chromic chloride in deionised water and adjusting the pH to 4.0 using 0.1 N HCl. An acidic pH was chosen in this experiment as preliminary studies indicated that no observable Cr oxidation occurred at pH 7.0 by microbial Mn oxides following a 24-h incubation period. Furthermore, Eary and Rai (1987) reported that an acidic pH favoured the oxidation of Cr(III) owing to the solubility of Cr(III) in acidic solutions.

Reactions were initiated by adding the Cr(III) solution (10 ml) to 10 mg of solid Mn oxide in a 50-ml polypropylene tube. Tubes were shaken in an end over end shaker (10 rpm) at 25°C. Duplicate tubes were removed at regular intervals and solutions were immediately filtered through a 0.2 μm sterile disposable Millipore filter (Sartorius). Filtered solutions were analysed for Cr(VI) by a spectrophotometric method (Section 3.4.2) and for Mn(II) by atomic absorption spectroscopy.

In the above experiment, although equal amounts of the various Mn oxides were added to Cr oxidation experiments, the molar amounts of Mn varied considerably. In order to compare the Cr oxidising potential of microbial and reagent grade Mn oxides, experiments were repeated using equimolar concentrations of Mn. Microbial and reagent grade Mn oxides (1500 μM) were mixed with 1000 μM of Cr(III) solution. The 1:1.5 ratio of Cr(III) to Mn were calculated on the basis of the stoichiometric equation reported in the literature (Fendorf and Zasoski, 1992).

$$CrOH^{2+} + 1.5\ MnO_2\ (s) \longrightarrow HCrO_4^{1-} + 1.5\ Mn^{2+} \qquad (1)$$

A 1000 µM (52 mg L^{-1}) Cr(III) solution was prepared by dissolving chromic chloride (CrCl$_3$) in deionised water. Cr(III) solution (10 ml) was added to 6, 2.6 and 2.4 mg of solid microbial Mn oxides, Mn(IV) and Mn(III), respectively to provide 1500 µM of Mn in the reaction mixture. Cr(III) solution without the addition of Mn oxides served as the control. As described in the above experiments, the reaction mixtures were contained in 50 ml polyethylene tubes and shaken at 25°C in an end over end shaker (10 rpm). After definite time intervals, duplicate samples were sacrificed and the solutions analysed for Cr(VI) (DPC method) and Mn(II) (AAS).

5.2.10 Mn oxidation in contaminated soil solution

The effect of oxidation of Mn and its implication in Cr oxidation was assessed in soil extract from the LTTW contaminated soil.

Soil water extracts were prepared by end-over-end overnight equilibration of LTTW contaminated surface soil with deionised water (1:10; soil:water) at 100 rpm. The resulting solution was filtered through a muslin cloth, dispensed (200 ml) into conical flasks (500 ml) and was spiked with filter sterilised Mn(II) as MnSO$_4$ (50 mg Mn L^{-1}). Soil solutions without the addition of Mn(II) served as controls. Three replicates were maintained for each treatment. Soil solutions with and without Mn(II) were incubated at 30°C in a shaker at 100 rpm for 6 months.

During this time, samples were periodically removed and analysed for pH, Mn(II), Mn(IV), Cr(III) and Cr(VI) concentrations. Mn(IV) was measured directly in soil solution by the LCV method (Section 5.2.6). Samples were then filtered (0.2 µm sterile filter: Sartorius) and analysed for Mn(II) and Cr(III) by AAS and Cr(VI) by Ion chromatography (Section 3.4.1).

The Cr oxidation potential of the Mn oxides formed in soil solution was studied by spiking the Cr(III) solution. After the formation of Mn oxides in soil solution (5 weeks), 10 ml of Cr(III) solution (5 mg Cr L^{-1}) as CrCl$_3$ (pH 7.0) was added. Cr oxidation, in terms of Cr(VI) formed in soil solution was monitored by periodically withdrawing aliquots from flasks, filtering and analysing for Cr(VI) (Section 3.4.1). Since no Cr oxidation with 12-week old soil solution was apparent, the soil solution was incubated for a prolonged period of 6 months for subsequent ageing of microbial Mn

oxides. After 6 months, the soil solution was again spiked with 20 mg Cr L^{-1} as $CrCl_3$ (pH 7.0). The Cr(III) solution was adjusted to pH 7.0 prior to its addition as the pH of the soil solution varied between 7.0 – 7.6. Soil solution samples were withdrawn periodically and analysed for Cr(VI) and Mn(II).

5.3   RESULTS

5.3.1   Distribution of Mn oxidizing bacteria in contaminated soil

Enumeration of Mn oxidising bacteria in LTTW contaminated soil at different soil depths revealed that the highest number of Mn oxidisers were located in the surface soils (0-20 cm) (Fig. 5.1). An initial increase in the number of Mn oxidisers was observed at a soil depth of 10-20 cm (5.4 x 10^3 CFU g^{-1}) compared to the top 10 cm (4.7 x 10^3 CFU g^{-1}). However, microbial numbers decreased with soil depth beyond 20 cm and at a depth of 50-100 cm a population of only 0.4 x 10^3 Mn oxidising microorganisms per gram of soil were enumerated. A similar decrease in total Mn content was recorded in soils at this contaminated site. When Cr(VI) was added to the enumeration medium (0.5 and 1.0 mg L^{-1}) for Mn oxidising microorganisms, the growth of Mn oxidisers was significantly inhibited (Fig. 5.1). In surface soils (0-20 cm) where the highest number of Mn oxidisers were observed, over 95% of the population was inhibited by the addition of 0.5 mg Cr(VI) L^{-1}. The growth of Mn oxidisers was further inhibited in the presence of higher concentrations of Cr(VI) (1.0 mg L^{-1}) (1 CFU g^{-1} soil). The population of Mn oxidising microorganisms at depths below 20 cm was almost completely inhibited by the presence of Cr(VI).

5.3.2   Isolation and identification of Mn oxidising microorganisms

Three Mn oxidising pure cultures of bacteria (designated SKMn1, SKMn2 and SKMn3) were isolated from the consortium developed from LTTW contaminated soil. Distinct colonies were removed, re-streaked onto Mn containing medium and tested for Mn oxide production. Once the purity of the cultures was established, colonies were inoculated into Mn oxidation liquid medium. The colony morphology of the isolated cultures varied from a light creamy colour to a slightly brownish colour. Strain SKMn1 was creamy and had hairy growth around the colonies. SKMn2 and 3 were pink, with crinkled margins and exhibited very slow growth in PYG mineral medium containing Mn. All three strains tolerated 0.1 % of Mn(II) in the medium and oxidised Mn(II) in culture broth.
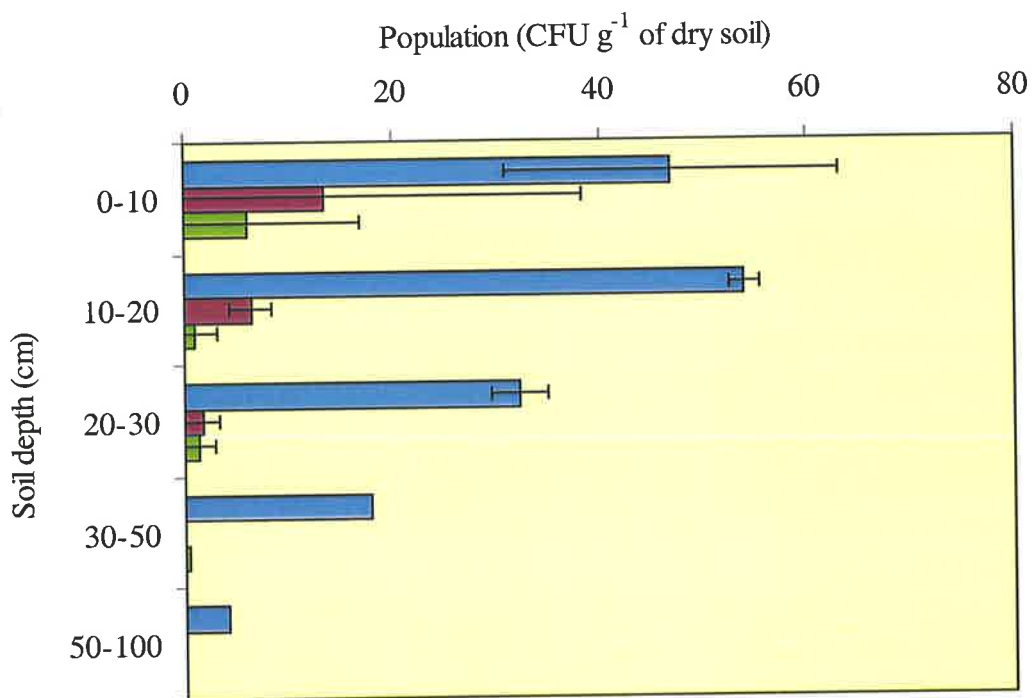
Fig. 5.1 Population of Mn oxidising bacteria in contaminated soil at different depths (■ CFU*100). The number of Mn oxidising bacteria tolerant to Cr (VI) at 0.5 mg L^{-1} (■) and 1.0 mg L^{-1} (■) was also estimated.

The bacterial strain, SKMn2 was selected for detailed studies, based on its efficiency for Mn(II) oxidation. Fatty acid methyl ester (FAME) analysis of the cell wall components of strain SKMn2 were analysed for identification. After extraction and analysis of fatty acids the FAME profile of SKMn2 was compared with the MIDI database. The cell wall composition of strain SKMn2 best matched the FAME profile of *Rhodococcus* sp., but with only a 58% correlation. The major fatty acids detected include 14:0, 15:0, 16:0, 16:0 10methyl, 17:1ω8c, 17:1ω5c, 17:0, 17:0 10 methyl, 18:1ω9c and 18:0. Strain SKMn2 had an aerial mycelium with Gram-positive rods, 1.5 μm x 0.54 μm in size covered with mucilaginous material. A scanning electron micrograph of strain SKMn2's cell structure is shown in Fig. 5.2.

5.3.3 Manganese oxidation by bacteria, fungi and algae

The Mn oxidising potential of three bacterial strains, five consortia (SKCons1, SKCons2, SKCons3, SKCons4 and SKCons5) and an algal strain isolated from LTTW contaminated soil was determined in medium supplemented with 30-50 mg Mn(II) L^{-1} after 3 weeks. In addition, the Mn oxidising potential of Ggt strains was also determined for comparison. These initial Mn oxidation tests were conducted as a screening procedure for the selection of 'efficient' Mn oxidising microorganisms for use in following experiments. Mn oxidation by bacterial isolates varied between 5.3 mg L^{-1} (10.5%) and 6.1 mg L^{-1} (12.2 %) of the spiked Mn(II) (from an initial concentration of 50 mg L^{-1}) (Fig. 5.3). When Mn oxidation was standardised per mg of protein, bacterial strain SKMn2 exhibited a greater efficiency for Mn oxidation: 7.4 mg Mn(IV) mg protein^{-1} was oxidised compared to 6.5 and 6.7 of Mn(IV) formed mg^{-1} of protein in cultures, SKMn1 and SKMn3. Microbial consortia, enriched from different soil depths, also varied in their Mn oxidising potential. Consortium SKCons2, isolated from surface soil (10-20 cm) exhibited the greatest potential for Mn oxidation, oxidising 6.3 mg Mn (mg protein)$^{-1}$. The extent of Mn oxidation decreased for consortia developed from soil depth beyond 10-20 cm. Consortium, SKCons5, isolated from a depth of 50-100 cm only oxidised 1.7 mg Mn mg protein^{-1} (2.8 mg L^{-1}).

All tested fungal strains of Ggt were able to oxidise Mn (Fig. 5.3). However, the Mn oxidising efficiency of the fungal strains was less than that of the consortia and bacterial isolates. Mn oxidation by fungal isolates started after a lag phase of approximately three weeks. Following growth, brown Mn oxides precipitated on the
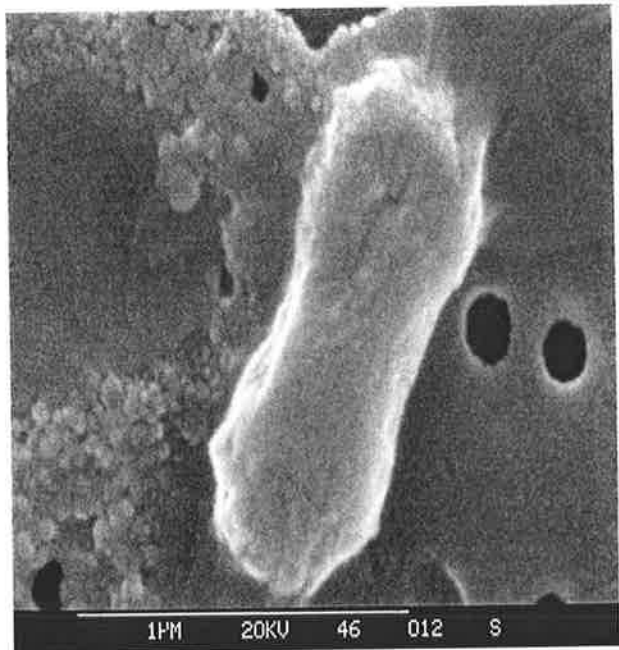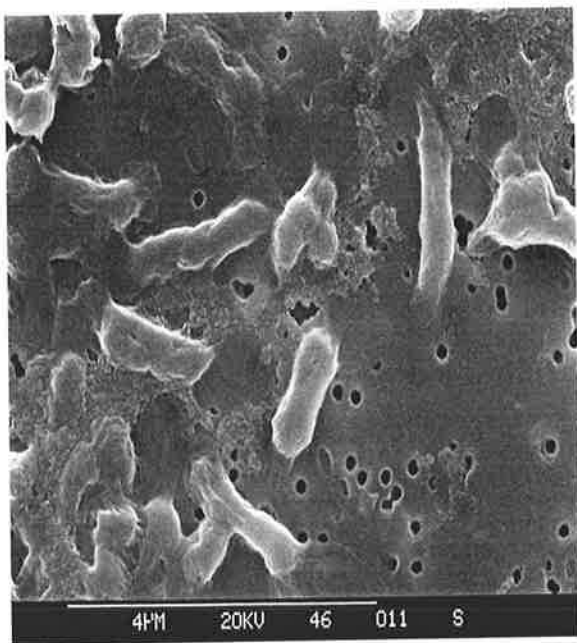
Fig. 5.2 Scanning electron micrograph of Mn oxidising bacterial strain, SKMn2. Note the cells are covered with mucilaginous material.
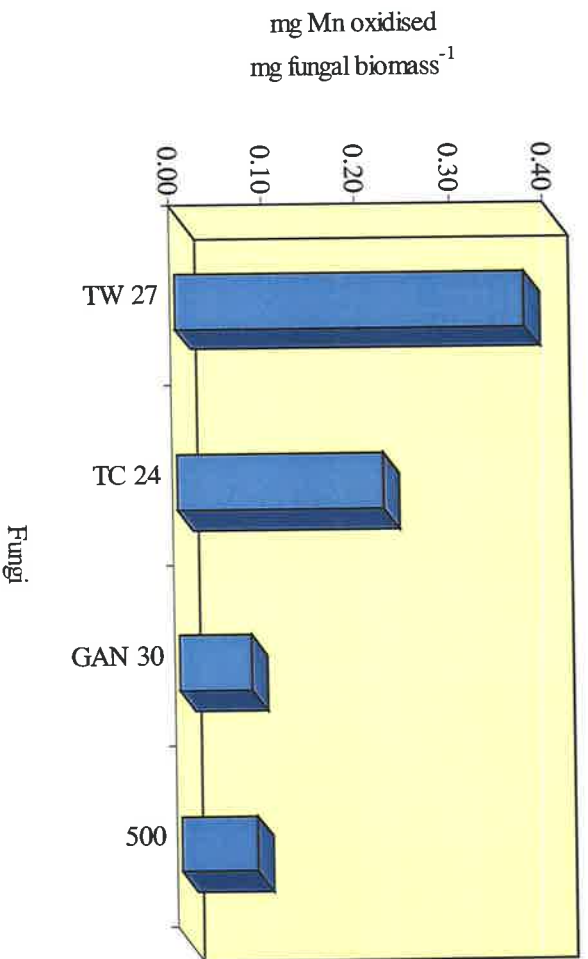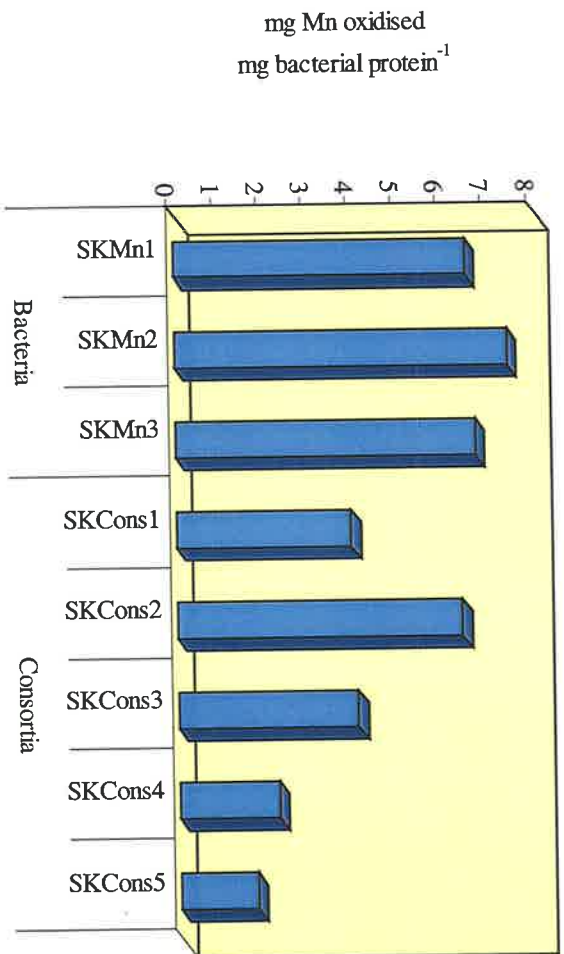
Fig 5.3 Mn oxidising potential of bacteria and consortia from the contaminated soil in comparison with known fungal cultures (Ggt strains)

fungal hyphae. Ggt strains were able to oxidise 2.5 mg L^{-1} (5 %) to 7.5 mg L^{-1} (15 %) of the spiked Mn(II). When Mn oxidation was standardised on a per mg^{-1} of dry biomass basis, strains TW27, TC24, GAN30 and 500 and oxidised 0.4, 0.2, 0.08 and 0.08 mg Mn mg^{-1} of dry biomass.

The unicellular green alga was unable to oxidise Mn(II) even after an incubation period of 5 weeks. Based on their relatively high efficiency to oxidise Mn(II), the bacterial strain SKMn2 and the consortium, SKCons2 were selected for further studies.

5.3.4 Manganese oxidation during microbial growth

The kinetics of Mn oxidation by strain SKMn2 and microbial consortium SKCons2 was examined in Mn oxidising broth inoculated with these cultures by monitoring the change in protein and Mn(IV) concentrations over time. Initial studies based on visual examination of Mn oxide formation indicated that Mn oxidation was faster in shaken cultures (100 rpm) than in static cultures. In addition, the optimum temperature for Mn oxidation was 30°C (10.5 mg Mn(II) L^{-1} was oxidised at 30°C compared to 6.5 and 8.5 mg L^{-1} at 25°C and 50°C, respectively).

Growth of strain SKMn2 and microbial consortium SKCons2 started after a lag period of 5 d. Rapid growth occurred between 5 and 15 d resulting in increases in protein concentrations from 0.3 to 0.5 mg L^{-1} and from 0.3 to 0.9 mg L^{-1} for SKMn2 and SKCons2, respectively (Fig. 5.4). The stationary phase for SKMn2 was reached after 18 d while the stationary phase for SKCons2 was reached after 22 d.

Mn oxides were visualised (as brown precipitates) in both cultures after three weeks of incubation. After 32 d of incubation, 32 and 41 mg Mn(IV) L^{-1} were accumulated in the medium of SKCons2 and SKMn2, respectively (64 and 82% oxidation of MnSO$_4$). However, rapid oxidation of Mn(II) occurred during the exponential growth phase of the organisms (between day 5 and 12). The bacterium SKMn2 and SKCons2 oxidised 7.6 and 3.2 mg Mn mg protein^{-1} d^{-1} during this period.
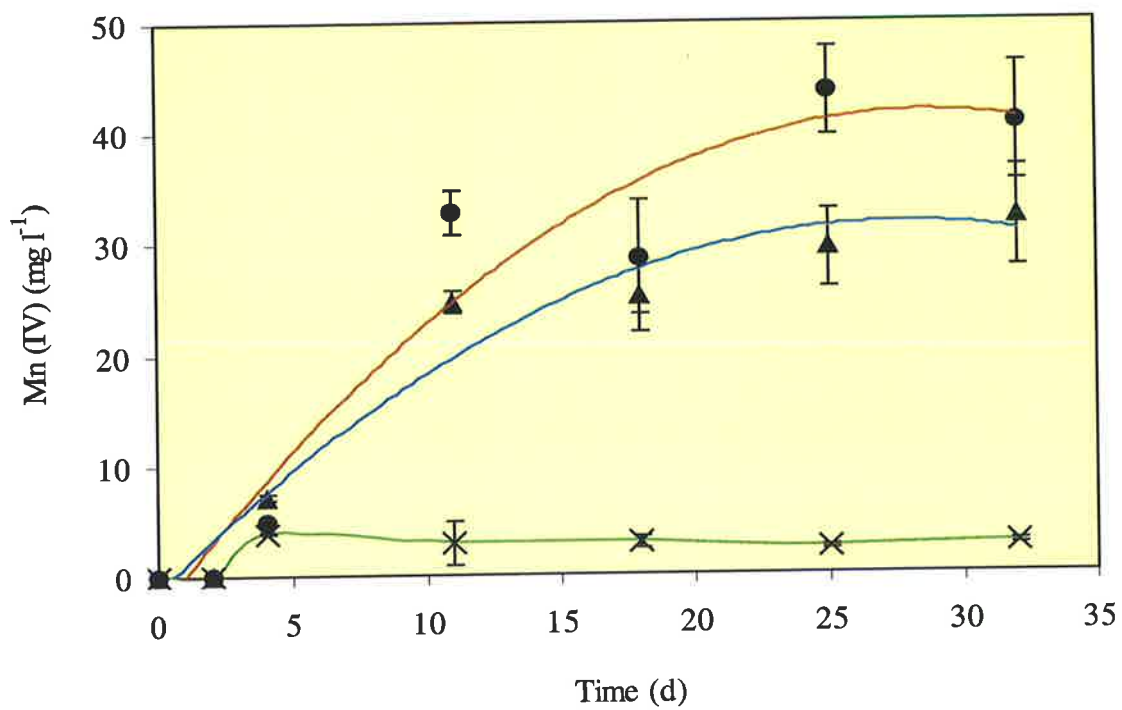
Fig. 5.4 Mn oxidation by bacterial strain SKMn2 (●) and consortium, SKCons2 (▲). Initial Mn (II) spiked was 50 mg Mn L^{-1}. Control (x).

5.3.5 Characterisation of microbial Mn oxides

Mn oxide precipitates from cultures containing $MnSO_4$ and strain SKMn2 or microbial community SKCons2 were produced in large volumes. For chemical characterisation, precipitates were collected after 1, 3 and 6 months of incubation and were initially characterised by XRD. Characterisation of Mn oxides using XRD suggested the accumulation of rhodochrosite ($MnCO_3$, Mn II) in the culture medium of strain SKMn2 after one-month incubation (Fig. 5.5A). Major peaks (d spacing of 3.64, 2.85 and 1.77) were characteristic of the mineral rhodochrosite.

In a 3-month-old culture, the final product was disordered manganese (III) oxide (Mn_2O_3, bixbyite) (Fig. 5.5B). This XRD was identical to the three major peaks (d spacing of 2.72, 1.66, 1.41) of reagent grade manganese (III) oxide (Fig. 5.6A). Prolonged incubation of cultures for 6 months resulted in poorly crystalline mixtures of manganese dioxide (MnO_2) and birnessite ($\beta-MnO_2$) (Fig. 5.5C), comparable with the XRD pattern of commercial Mn(IV) (Fig. 5.6B) This indicated that strain SKMn2 was able to oxidise Mn(II) to Mn oxides with Mn_2O_3 as the predominant intermediary product before its conversion to more crystalline, birnessite and manganese oxide minerals.

The microbial Mn oxides were initially brown but later turned blackish (Fig. 5.7). SEM/EDAX (Fig. 5.8) of bacterial cells at this stage showed that Mn was the major constituent of the cell and cell surfaces. XRD analysis was also performed on Mn oxides produced by microbial consortium (SKCons2); however, these oxides were rich in organic material, which interfered with XRD analysis. In an attempt to remove the organic material, Mn oxides were washed with distilled water and 0.03 M H_2SO_4. However, this procedure was unable to adequately remove the organic matter interference. FTIR techniques, used to characterise the microbial Mn oxides from the consortium, were not successful due to interference by protein and polysaccharide components and consequent masking of spectra.
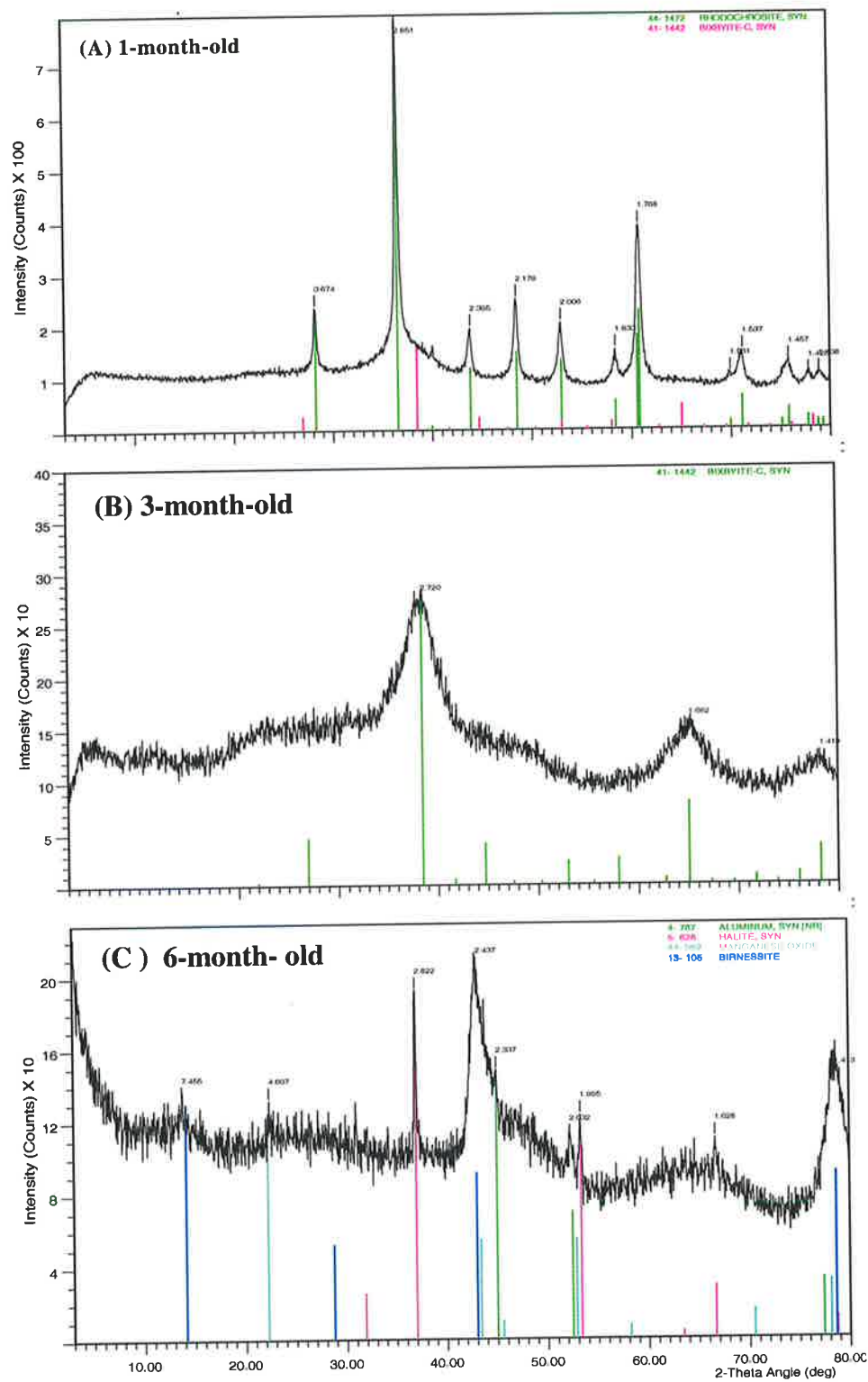
Fig. 5.5 Xray diffraction (XRD) spectra of oxides formed in one (A), three (B) and six-month old (C) culture medium of bacterial strain, SKMn2
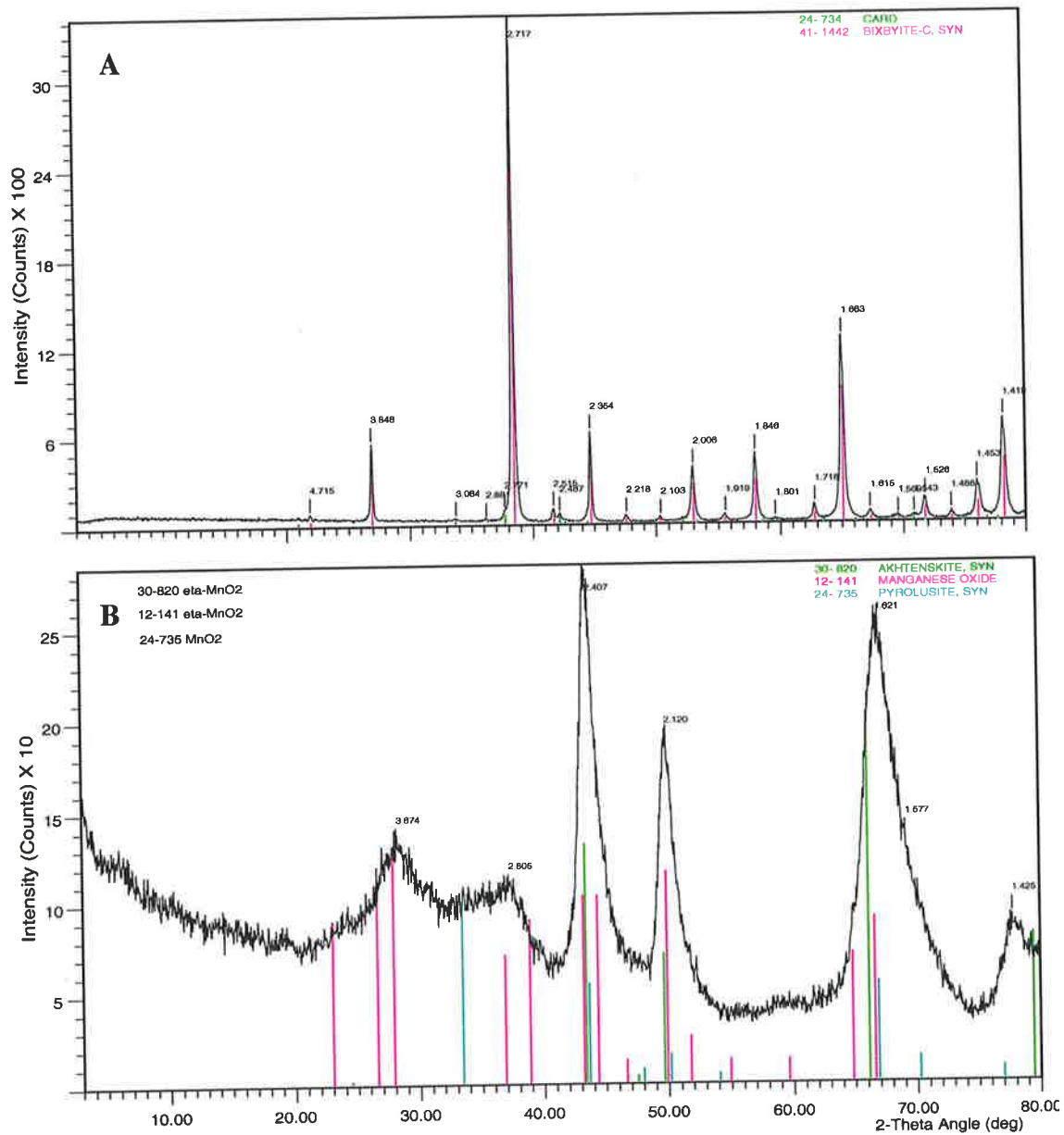
Fig 5.6 XRD spectrum of reagent grade manganese (III) oxide (Mn_2O_3) (A) and manganese (IV) oxide (B).
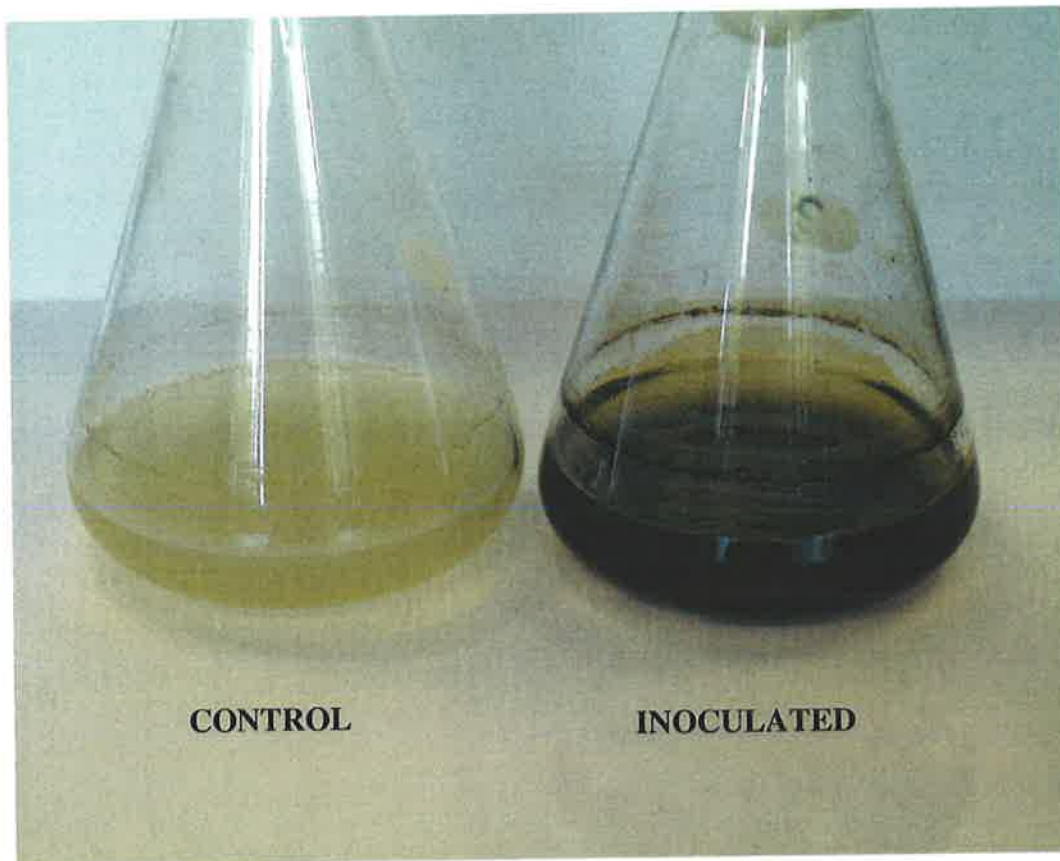
Fig. 5.7 Photograph showing dark brown Mn oxides deposition in the culture broth of strain, SKMn2 after 6 months of incubation
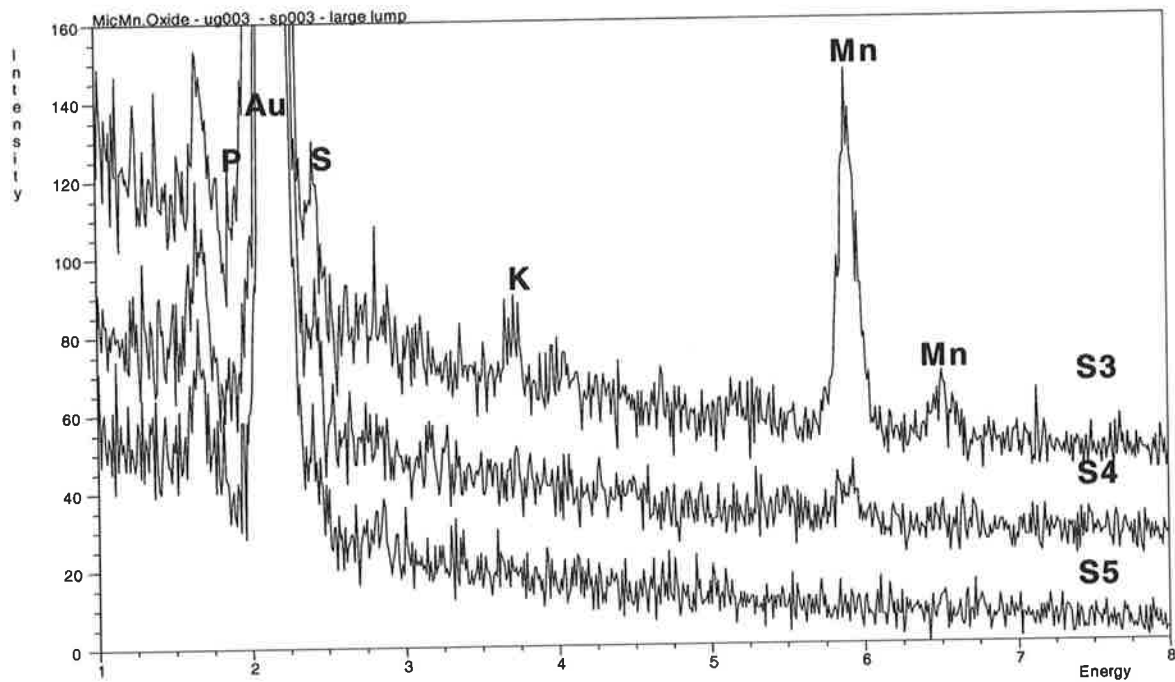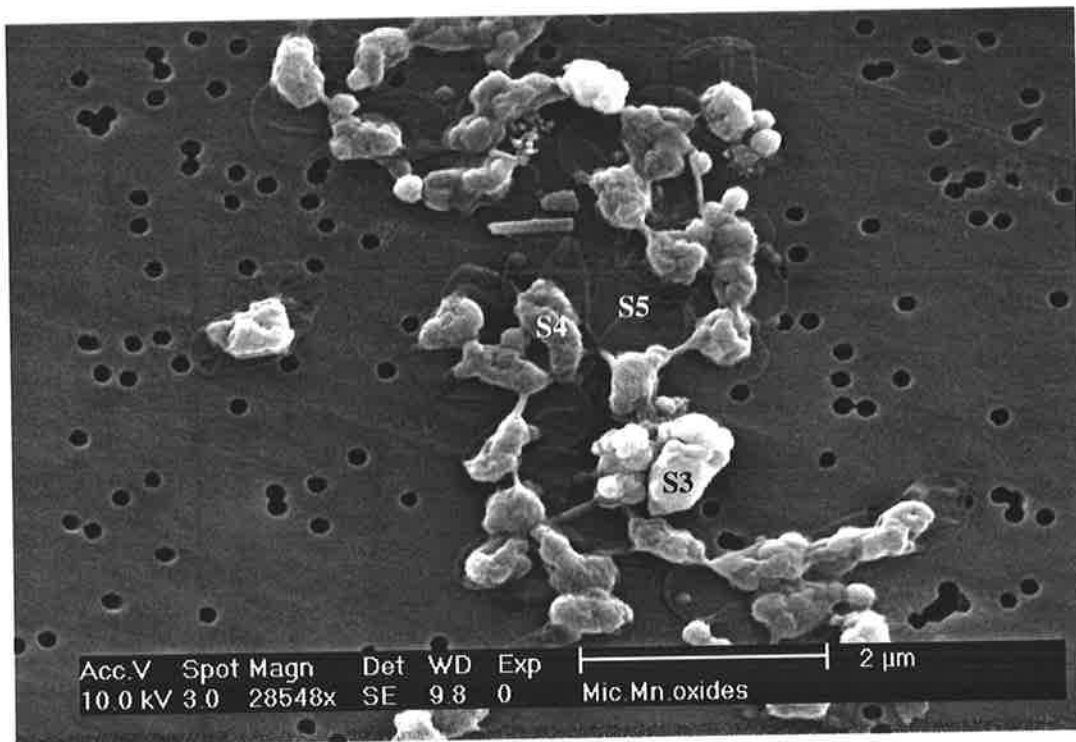
Fig. 5.8 SEM and EDAX of bacterial cells strain SKMn2 showing the accumulation of Mn oxides on the surface (S3 – Mn observed outside the cell; S4 – Mn inside the cell; S5 – background)

The microbial Mn oxides had a total Mn content of 306 µg g^{-1}, and S, P and K at concentrations of 50, 14 and 6.8 µg g^{-1}, respectively (Table 5.1). The specific surface area of microbial Mn oxides was 9.7 ± 2.4 m^2 g^{-1}. The specific surface area of reagent grade Mn$_2$O$_3$ and MnO$_2$ was also measured for comparison. MnO$_2$ had a higher surface area of 73 ± 7 m^2 g^{-1} and Mn$_2$O$_3$ has a lower surface area of 2.5 ± 0.6 m^2 g^{-1} than that of microbially synthesised oxides.

Table 5.1 Characteristics of microbial Mn oxides of consortium SKCons2 harvested after 6 months

Mn species present	Elemental composition								Total C	Surface area
	Mn (mg g^{-1})			Others (µg g^{-1})					(%)	(m^2 g^{-1})
	Total	Mn(II)	Mn(IV)	S	P	K	Na	Ca		
MnO$_2$ and β–MnO$_2$	306	160	146	50	14	6.8	5.9	0.8	13.7	9.7 (± 2.4)

5.3.6 Chromium oxidation during growth of Mn oxidising bacteria

Bacterial strain SKMn2 and microbial consortium SKCons2 could not oxidise Cr(III) during their growth in Mn oxidising medium containing 5 mg Cr L^{-1}. Additional experiments were conducted where the Mn oxidising microorganisms were initially grown in Mn medium and after the accumulation of Mn oxides (3 weeks) Cr(III) was then added into the cultures. No Cr(III) oxidation occurred.

5.3.7 Chromium oxidation by microbial Mn oxides

The oxidation of Cr(III) by equal quantities (10 mg) of microbially produced Mn oxides and reagent grade oxides of Mn(IV) and Mn(III) is shown in Fig. 5.9. At pH 4.0, the rate of Cr(III) oxidation by microbial oxides was slow compared to commercially produced Mn(IV) and Mn(III) oxides. After 6 h, only 6.3 µM of Cr(VI) was formed in the reaction mixture (representing 0.33 % of the total Cr(III) added) compared to 108 µM Cr(VI) (57% oxidation) with reagent grade Mn(IV) and 42 µM
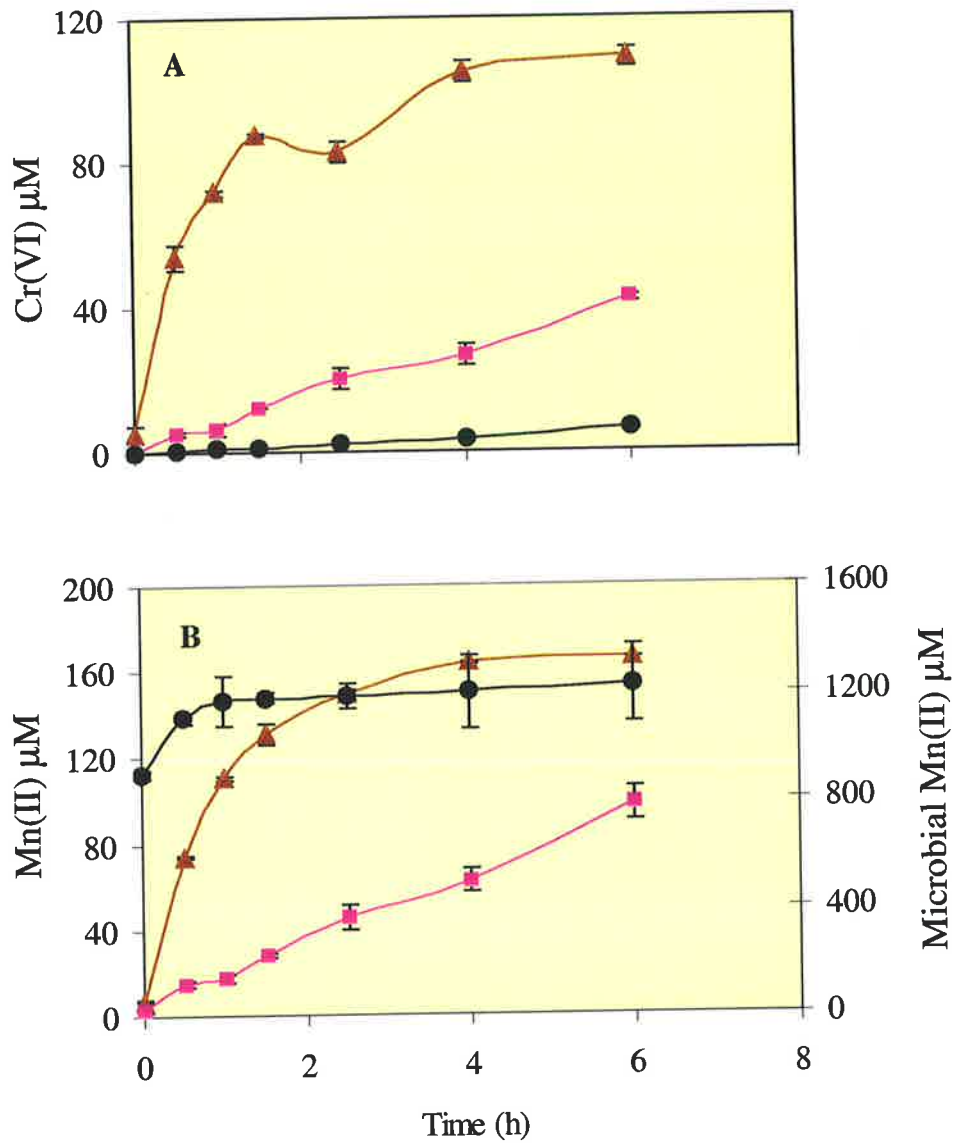
Fig. 5.9 Concentration of Cr (VI) (A) and Mn (II) (B) released during oxidation of Cr (III) by microbial (●) and commercial Mn oxides, Mn (IV) (▲), Mn (III) (■). The reaction mixture had an initial Cr (III) concentration of 1920 μM and 1 g L^{-1} Mn oxides (pH 4.0).

Cr(VI) (22%) with Mn(III). In all cases, concomitant with increase in Cr(VI) concentration over time, the concentration of Mn(II) in solution increased. Oxidation by microbial oxides released from 903 to 1230 μM of Mn(II) to solution.

When the molar amounts of Cr(VI) released were regressed against the molar amounts of Mn(II) formed, the regression indicated that the microbial Mn oxides did not show any relationship between Cr(VI) and Mn(II) formed, probably due to the narrow range of Mn(II) released during Cr oxidation (Fig. 5.10). A different trend was observed for Mn(III) and Mn(IV). For the three Mn oxides considered, more Mn(II) was released into solution per mole of Cr(VI) released (4.7 μM of Mn(II) per mole of Cr(VI) released) during oxidation by Mn(III). This indicated that the variation observed in Cr(III) oxidation might be due to the varying molar concentrations of Mn in the oxides tested even though the same weight of Mn oxides were used. From the results obtained from the above experiment, Cr oxidation studies were repeated using equimolar concentrations of Mn.

The microbial Mn oxides had 306 mg Mn kg^{-1} of sample on a dry weight basis. Based on the 1:1.5 stoichiometry, 1000 μM of Cr(III) were reacted with 1500 μM of Mn (microbial oxides, Mn(IV) and Mn(III). Fig. 5.11 shows the rate of Cr(III) oxidation by the three Mn oxides at pH 4.0. Microbial Mn oxides oxidised Cr to a greater extent than commercially produced Mn(IV) and Mn(III). After 8 h, 36, 22 and 36 μM of Cr(VI) was released into solution by microbial Mn oxides, reagent grade Mn(IV) and Mn(III) respectively. After 8 h, there was no significant increase in Cr oxidation by the reagent grade Mn oxides. However, Cr oxidation by the microbial produced Mn oxide continued albeit at a slower rate and after 48 h, 61 μM of Cr(VI) was in solution (Fig. 5.11A). Concomitant with the formation of Cr(VI) for all Mn oxides was the accumulation of Mn(II) in solution (Fig. 5.11B). There was a sharp increase in the Cr oxidation rate up to 8 h, beyond which the increase was statistically insignificant. In microbial oxides, the rate of oxidation varied between 0.6 – 4.9 % [6.0 – 49.4 μM Cr(VI)] within the first 12 h. This increased later, but at a slow rate, 6.1 % [61 μM Cr(VI)] in 48 h.

For microbial Mn oxides, reagent grade Mn(IV) and Mn(III), the Mn(II) released into solution and Cr(VI) formed (Fig. 5.12) were linearly related. Cr oxidation by Mn(IV) released 1.3 moles of Mn(II) for every mole of Cr(VI) released. This
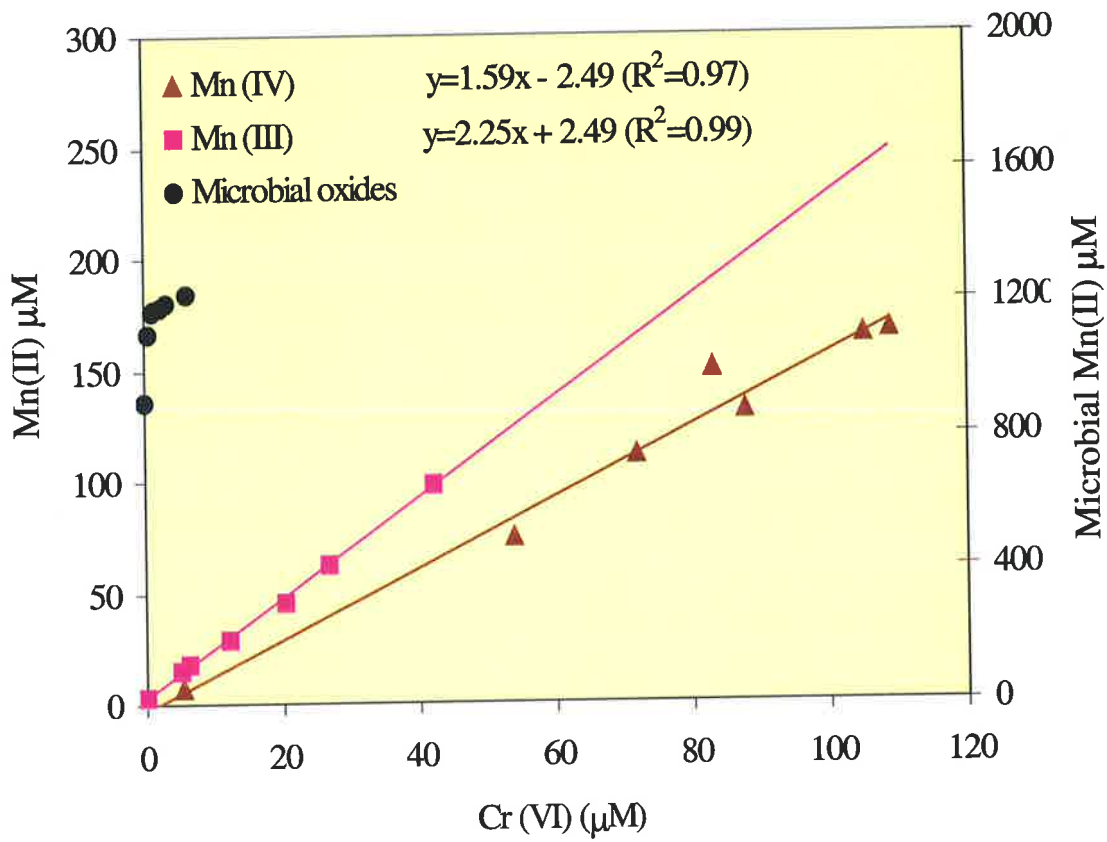
Fig. 5.10 Rate of Mn (II) formed for each mole of Cr (VI) produced during the oxidation of Cr (III) by microbial (●) and reagent grade Mn (III) (■) and Mn (IV) (▲) oxides.
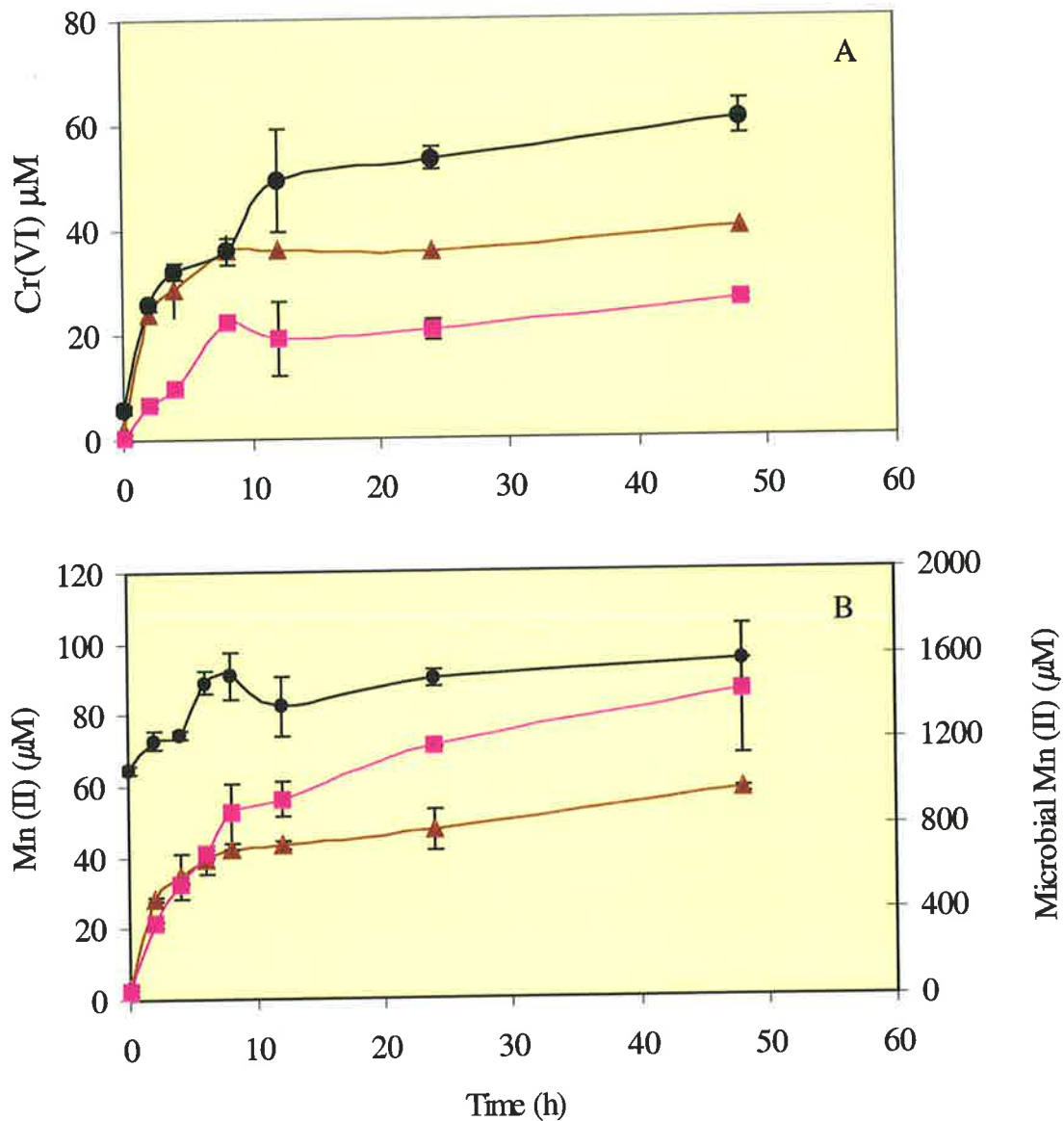
Fig. 5.11 Concentration of Cr (VI) (A) and Mn (II) (B) released during oxidation of Cr (III) by equimolar concentration of microbial (•) and commercial Mn oxides, Mn (IV) (▲), Mn (III) (■). The reaction mixture had an initial Cr (III) concentration of 1000 μM and 1500 μM of Mn oxides (pH 4.0).
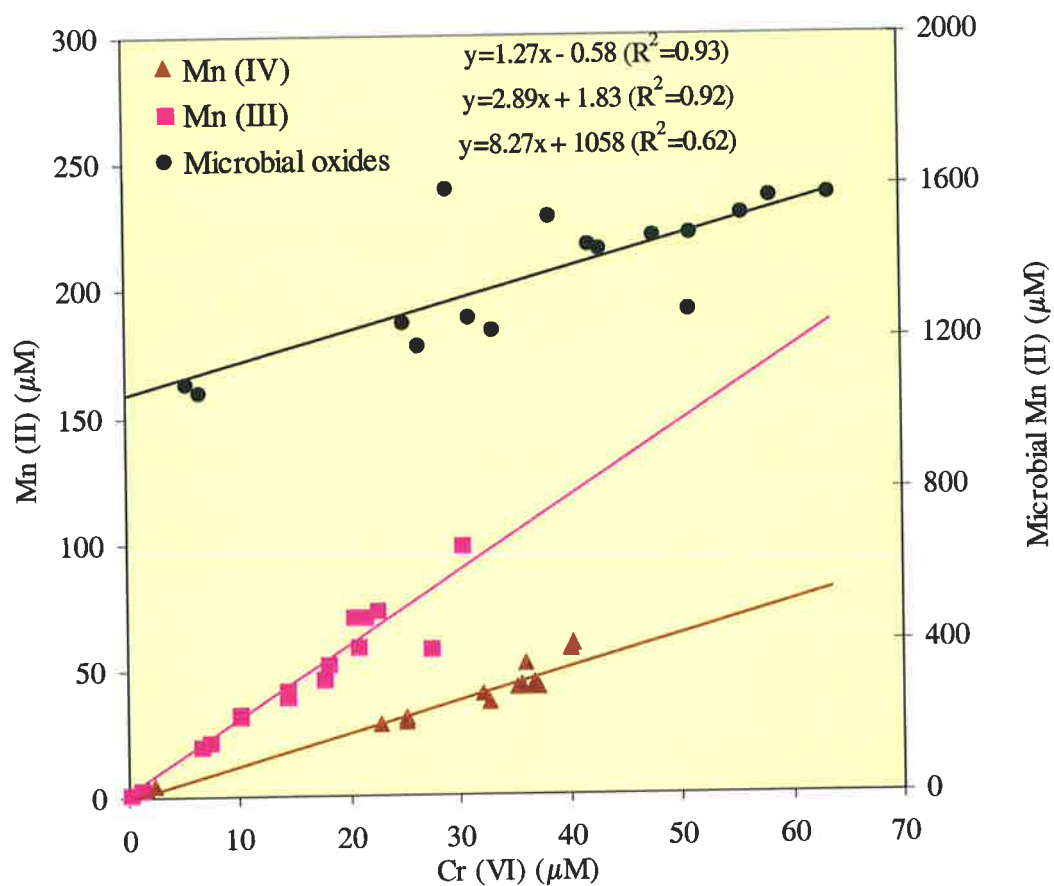
Fig. 5.12 Rate of Mn (II) formed for each mole of Cr (VI) produced during the oxidation of Cr (III) by microbial (●) and reagent grade Mn (III) (■) and Mn (IV) (▲) oxides.

compared favourably with the stoichiometry suggested by equation 1 that 1.5 moles of Mn(II) should form for each mole of Cr(VI) produced. For Mn(III), 2.89 moles of Mn(II) were released per mole of Cr(VI) produced. Compared to reagent grade Mn oxides, microbial Mn oxides released more Mn(II) into solution (8.3 moles) during Cr oxidation. The high concentration of Mn(II) observed in solution might be due to the elevated concentration of Mn(II) in microbially produced Mn oxides at the start of the experiment.

5.3.8 Mn oxidation and Cr oxidation in contaminated soil extract

The results outlined in this chapter thus far have described experiments conducted in 'pure systems', i.e. microorganisms added to defined media containing Mn and Cr(III). In the soil environment, conditions are not as simple as those encountered in the laboratory since soil is a heterogeneous matrix. With the information gained from initial pure system experiments, additional tests were undertaken to determine Mn oxidation in contaminated soil extracts and its effect on Cr oxidation. Oxidation of Mn was examined in soil extracts prepared using LTTW contaminated soil. Soil solution had a pH value of 7.6 ± 0.2 with a soluble Cr concentration of 0.4 mg Cr L^{-1} and a Cr(VI) concentration of 0.2 mg Cr L^{-1}. No soluble Mn(II) and Mn(IV) were detected in soil extracts. Soil extract experiments were initiated by the addition of 50 mg Mn(II) L^{-1}.

Over the 12-week incubation period, Mn oxidation was observed in soil extracts (Fig. 5.13). No Mn oxidation occurred in control flasks, where Mn(II) was omitted. Mn oxidation was preceded by a lag phase of approximately two to three weeks. After this lag phase, Mn oxidation proceeded in soil extracts.

After the formation of Mn oxides (5 weeks), Cr(III) solution (10 ml of a 10 mg Cr L^{-1} solution as CrCl$_3$) was spiked into incubated soil extracts to study the effect of Mn oxides on Cr oxidation. The oxidation of Cr(III) to Cr(VI) was not observed in soil extracts over the five hour reaction time.

Soil extracts experiments were also conducted with an extended incubation period preceding Cr(III) addition. After a 6-month incubation, the concentration of Mn oxides formed in soil extracts was 42 mg L^{-1}. Over the initial 6-month incubation period, no significant change in pH (7.6 ± 0.4) or Mn oxidation was observed in control soil extracts. As described in the above experiments, 10 ml of
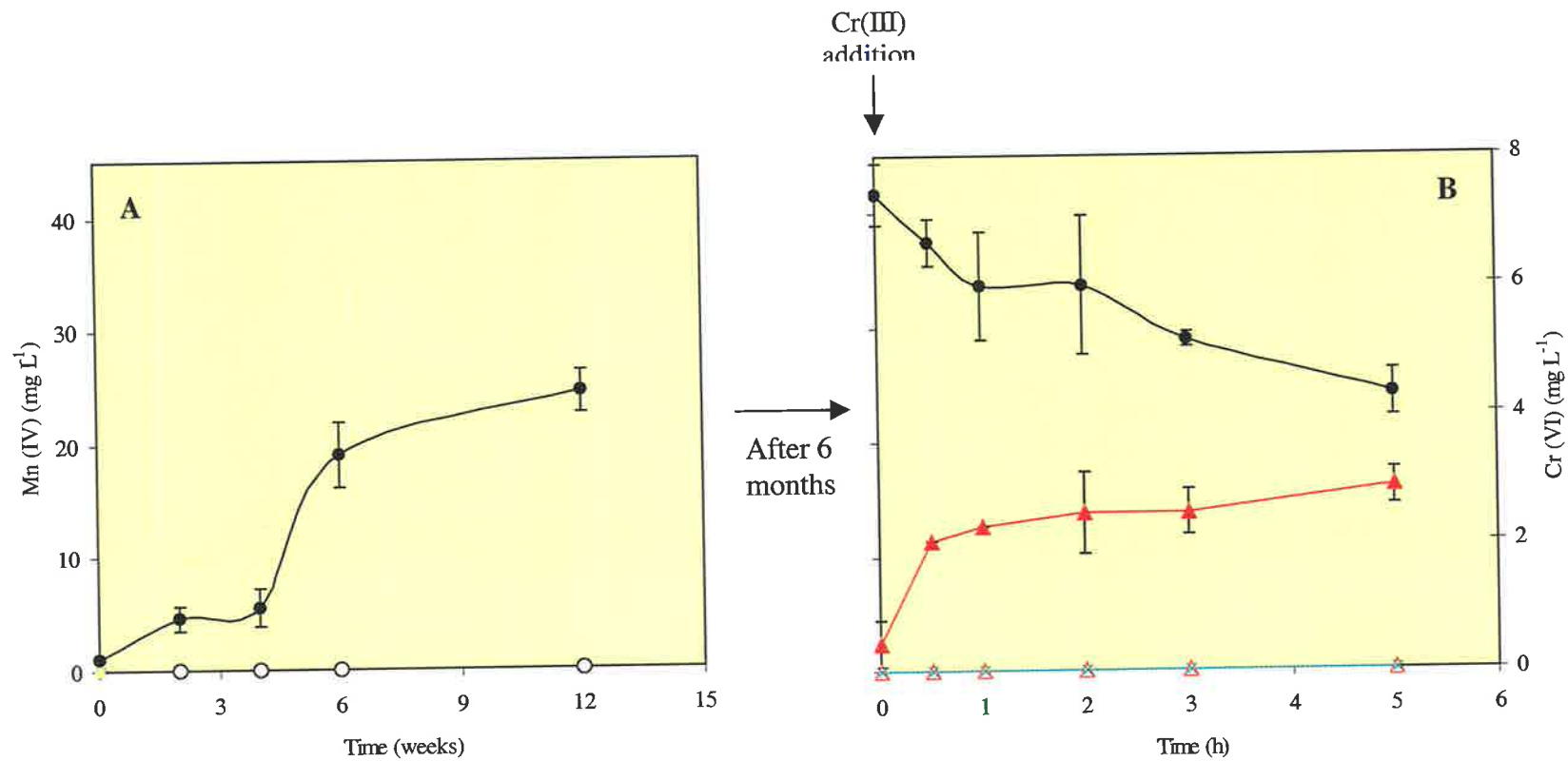
Fig. 5.13 Biotic Mn oxidation in contaminated soil extract (●) (A) and associated Cr(III) oxidation (B). Cr(III) as CrCl₃ was spiked at 12 weeks (No Cr oxidation occurred) and after 6 months. Cr(III) oxidation to Cr(VI) (▲) occurred in 6-month-old Mn oxides. Symbols (O) and (Δ) indicate control samples containing soil extract without Mn(II). Error bars indicate s.e

Cr(III) solution (20 mg Cr L^{-1} as $CrCl_3$) was spiked into soil extracts after the 6 month pre-incubation and the concentration of Cr(VI) was monitored. In solutions containing aged Mn oxides, Cr(III) oxidation, in terms of Cr(VI) formed, was observed in soil extracts (Fig. 5.13B). Cr(VI) concentrations presented in the graph are concentrations determined after subtracting background Cr(VI) concentrations in soil extracts (0.2 mg Cr L^{-1}). The Cr oxidation reaction was rapid resulting in the maximum release of 2.9 mg Cr(VI) L^{-1} after 3 h. As Cr(III) oxidation proceeded, there was a simultaneous decrease in Mn oxide concentration in soil extracts from 42 to 29 mg L^{-1}. The Mn oxides present in soil extracts were a mixture of hausmannite (Mn_3O_4) and manganese oxide hydrate (MnO) (Fig 5.14).

5.4   DISCUSSION

Oxidation of Cr(III) to Cr(VI) in soils causes a significant environmental hazard due to the greater mobility and toxicity of Cr(VI) (Makino et al., 1998). Most of the oxidation-reduction reactions of Cr in soil and natural water are dependent on the presence of oxidants and reductants. The main oxidants of Cr(III) to Cr(VI) in soils are Mn oxides (Bartlett and James, 1988) which play a major role in controlling the dynamics of Cr(III)-Cr(VI) transition in soils.

The chemical oxidation of Cr(III) to Cr(VI) by Mn oxides is well known in both pure chemical systems (Eary and Rai, 1987) and in soils (Amacher and Baker, 1982). Likewise, oxidation of Mn(II) to the higher oxidation state, Mn(IV), mediated by microorganisms, is a common and widespread occurrence in soils (Bromfield and Skerman, 1950). But, coupling of these two processes *viz.*, microbial oxidation of Mn(II) and chemical oxidation of Cr(III) by Mn oxides generated by microbial action, in soil system has not been described, especially in LTTW contaminated soil.

5.4.1   Mn oxidising microorganisms

Transformation of Mn(II) to Mn(IV) has been widely studied in freshwater, marine and soil ecosystems (Ghiorse, 1988). Mn oxidising bacteria are ubiquitous in nature, with populations ranging from 0.01 to 1.0 % of the total bacterial population (Ghiorse, 1994). In this contaminated soil, the Mn oxidising bacterial population in surface soil accounted for 4 % of the total bacterial population. The relatively high
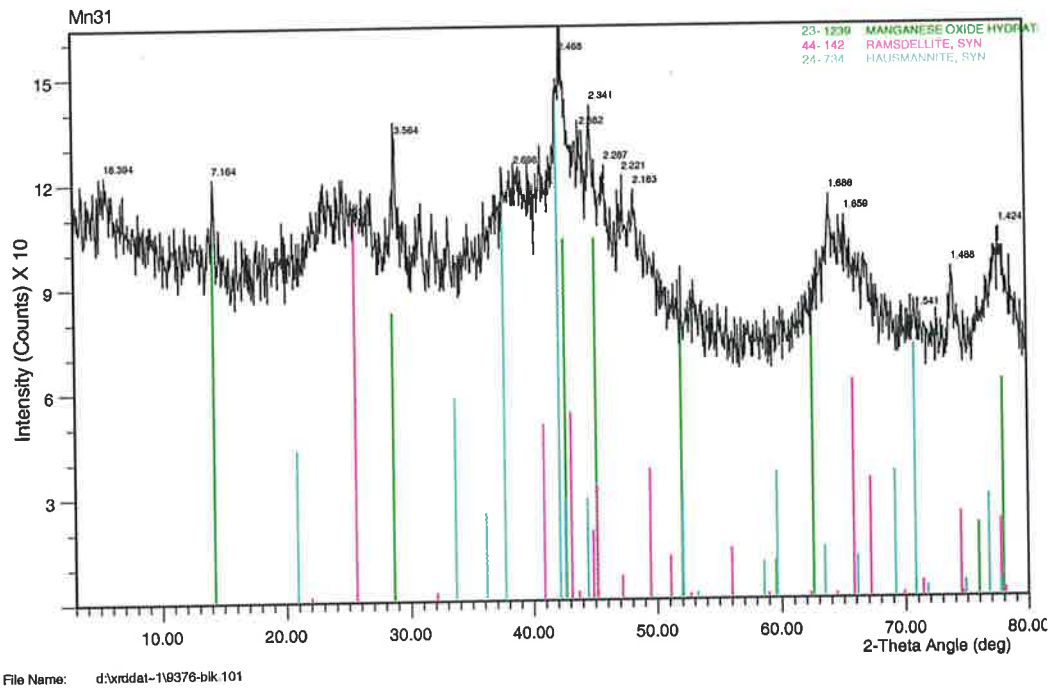
Fig. 5.14 XRD spectrum of the Mn oxides formed in soil extracts after 6 months of incubation showing the Mn (III) (hausmannite) and Mn (IV) (manganes oxide hydrate).

population of Mn oxidisers may be due to elevated Mn content in this contaminated soil (> 300 mg kg^{-1}). Although results showed that Mn oxidising bacteria were highly sensitive to Cr(VI) in agar media, no such inhibitory relationship was found in soils. This might be due to buffering capacity of soil and hence the direct toxicity will not be prominent.

The bacterium, SKMn2 and consortium, SKCons2 isolated from this contaminated site, had Mn oxidising potential ranging from between 82% (40.8 mg L^{-1}) and 65% (32.3 mg L^{-1}) of the Mn(II) oxidised. Earlier reports on bacteria showed that more than 95% of added Mn(II) (100 μM) was oxidised by *Bacillus* SG1 spores (Mandernack et al., 1995a), *Pseudomonas* sp. (Kepkay and Nealson, 1987) and alga *Scenedesmus* sp. (Knauer et al., 1999) (Fig. 5.15). Also in the current study, maximum Mn(II) oxidation occurred during exponential phase of growth of the cultures and diminished towards the stationary phase. Similarly, in *Pedomicrobium* sp. maximal Mn oxidation was observed between early and mid-exponential phases of growth (Larsen et al., 1999). However, Mn oxidation can also occur during stationary phase as in cultures of *Pseudomonas* sp. and *Leptothrix* sp (Nealson et al., 1989). Mn oxidation occurs at different phases of growth cycle depending on the mechanism of Mn oxidation. In *Bacillus* it was mainly observed in stationary phase as the major oxidising agents are the spores, which are formed later in the growth cycle. In some cultures, it occurs in stationary phases, wherein the release of enzymes will govern the Mn oxidation. The rate of Mn oxidation exhibited varied with bacterial genera, media composition, pH, temperature and initial concentration of Mn spiked into the medium. Apart from the rate of oxidation, these factors also govern the nature and mineralogy of Mn oxides formed by the microorganisms (Mandernack et al., 1995a).

Identification of strain SKMn2, isolated from surface contaminated soil was performed based on the fatty acid composition of cell walls. Although the cell wall composition of SKMn2 best matched a *Rhodococcus* sp. the percentage similarity was only 58% based on the current MIDI database. FAME profiles can be an effective method for the identification of some bacteria (Dunfield et al., 1999) and these authors successfully used this technique to characterize *Rhizobium* in the legume root nodules. The technique has also been used to identify food pathogens (Neyts et al., 2000). However, the data in MIDI database is predominantly based on pathogens with very few profiles of environmental isolates. Hence, unequivocal identification of

environmental isolates may not be possible by this method. However, FAME profiles may assist in the characterization of the microorganisms providing a fatty acid signature of the cell wall components. Further characterisation of strain SKMn2 is required for its complete identification. Such characterization might include sequencing of the 16SrRNA gene of the strain to identify the genus and species and was beyond the scope of the current thesis.
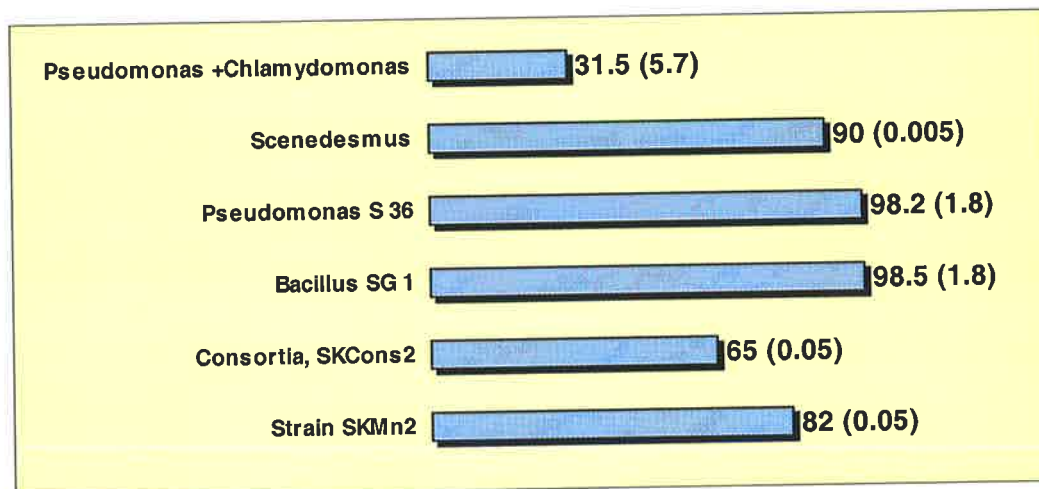
Fig. 5.15 Mn oxidising efficiency of known bacteria and algae compared to bacterium, Strain SKMn2 and SKCons2 used in the present study. The legend values the maximum Mn(II) oxidised (%). The values in paranthesis indicate the initial Mn(II) concentration used in the respective studies.

5.4.2 Microbial Mn oxides – Formation and characteristics

The main Mn oxides formed during microbial Mn oxidation are usually manganite and hausmannite (Mandernack et al., 1995a). The specific type of Mn oxide formed being mainly determined by the initial concentration of Mn(II) and the organisms involved.

Low temperature (3°C) and low Mn(II) concentrations (10-100μM) favour manganate/ buserite formation whereas high temperature (50°C) and high Mn(II) concentration favour manganate/ hausmannite formation. In general, low Mn oxide concentration (nM-μM) and low temperature promoted direct oxidation of Mn(II) to Mn(IV) without any intermediate steps, as reported earlier by Rosson and Nealson (1982).

At pH 7.0 and at 30°C, bacterial strain SKMn2, oxidised Mn(II) to disordered Mn oxides, a mixture of birnessite and other manganese oxides, but the rate of oxidation was slow. Initially, within a month, the bacterium deposited $MnCO_3$ (rhodochrosite) and the major intermediate formed was Mn_2O_3 (bixbyite) before disordered Mn oxides were formed at 6 months (Fig. 5.5). It is important to note that Mn_2O_3 has not been previously reported as an intermediate in Mn oxidising bacterial cultures. However, there is evidence for accumulation of MnOOHs (manganite) (Greene and Madgwick, 1991; Larsen et al., 1999) and Mn_3O_4 (hausmannite) (Tipping et al., 1984; de Vrind et al., 1986) as the major intermediates during microbially mediated Mn oxidation. $MnCO_3$ was occasionally formed by strain *Bacillus* SG1 at temperatures > 20°C (Mandernack et al., 1995b).

The Mn oxides formed were deposited on the surface of the cells of SKMn2 after prolonged incubation as shown by SEM and EDAX measurements (Fig. 5.8). There have also been some reports of deposition of Mn oxides on algal cell surfaces (Greene and Madgwick, 1991) and the ability to oxidise Mn(II) and deposit Mn oxide extracellularly is characteristic of several heterotrophic bacteria and fungi (Leeper and Swaby, 1940; Bromfield and Skerman, 1950). These reports tend to support the current observations of cell surface deposition. Although the majority of the Mn oxidising heterotrophs do not derive energy from oxidation reactions (Ghiorse, 1984b) this activity can be beneficial since in excluding Mn oxides outside the cells the toxicity from high concentrations of Mn(II) can be relieved.

5.4.3 Microbial Mn oxides aided Cr oxidation in pure systems

The microbial Mn oxides generated by strain SKMn2 and consortium, SKCons2 were capable of oxidising Cr(III), however, no Cr oxidation was observed in culture broth (pH 7.0) during growth. Alternatively, decomposable products of organics present in the growth medium could reduce the Cr(VI), if formed, back to Cr(III). Low solubility of Cr(III) at near neutral to alkaline pH was reported to severely inhibit the rate of oxidation by Mn oxides even in pure solution studies (Rai et al., 1986; Eary and Rai, 1987). These authors observed higher oxidation at acidic pH and at pH 8 and 10, the rate of oxidation was slow and insignificant and this was mainly related to the solubility of Cr(III). However, Cr(III) solubility could be significantly higher at the root:soil interface, especially in soils with rhizosphere effects. Under these conditions the dynamics of Cr(III) may be very different from the bulk soil.

Microbial Mn oxides when dried and used in pure solution studies oxidised Cr(III) at pH 4.0. An acidic pH increases the solubility of Cr(III) and its oxidation by Mn oxides is consequently generally greater under acidic conditions (Eary and Rai, 1987). Mn oxides [microbial, reagent grade Mn(IV) and Mn(III)] when reacted with aqueous Cr(III) solution at a 1:1.5 ratio (Cr:Mn) showed that microbial Mn oxides were able to oxidise more Cr(VI) compared to the reagent grade Mn(IV) and Mn(III) in solution. The rate of oxidation is normally attributed to the surface characteristics of the Mn oxides, the greater the surface area, the greater the Cr oxidation. This would suggest a more rapid oxidation in the presence of reagent grade Mn oxides given their larger surface area. Therefore, the reason for the observed lower rate of oxidation of Cr(III) in the presence of reagent grade Mn oxides is not at present clear and further investigations need to be conducted to investigate the reasons for the greater rate of oxidation in the presence of microbial Mn oxides. Ageing of Mn oxides also seems to influence the reactivity of the microbially sythesised Mn oxides and this may be attributed to the greater crystallinity of long-term aged oxides. The initial rapid oxidation of Cr(III) followed by a slow but continued oxidation has been observed earlier and the retardation was attributed to the competition by reaction products (Mn (II) and Cr (VI)) for the Mn oxides surfaces (Fendorf and Zasoski, 1992). Also, at acidic pH, the adsorption of anionic Cr(VI) to Mn surface reduces the number of active sites available for further oxidation.

The linear plots showing the relationship between the products formed, Cr(VI) and Mn(II) were compared to the stoichiometry of reaction 1 (pg 91). Since the plots are linear, the slope determines the amount of Mn (II) released per mole of Cr (VI) formed. According to the reaction stoichiometry, in pure solution, for each mole of Cr(VI) released 1.5 moles of Mn(II) should be released (Eary and Rai, 1987). At pH 4.0, the slope of the reaction with microbial Mn oxides was 8.3 (Fig. 5.12) indicating that more moles of Mn(II) were released into solution per mole of Cr(VI) formed than stoichiometry would allow. However, the Mn(IV) and Mn(III) had lower slopes approaching 1.5. The reasons for the increased slope in our study may be due to:

(a)   release of Mn(II) entrapped within the microbially synthesized oxides during the Cr(VI) oxidation process; or

(b) simultaneous reaction between the different forms of Mn oxides bixbyite and Mn oxides present in the microbial oxides and Cr(III) and this could release stoichiometrically different amounts of Mn(II) compared to pure oxides.

5.4.4 Microbial Mn oxides mediated Cr oxidation in soils

Manganese was effectively oxidised in the contaminated soil extracts. As observed in pure culture studies with SKMn2, disordered Mn oxides were also formed in soil extracts after 6 months, but the intermediary product formed was different. In the pure culture study, bixbyite (Mn_2O_3) was the major intermediate while in soil extracts hausmannite (Mn_3O_4) was formed. It can be inferred that both bixbyite and hausmannite might be formed by different groups of Mn oxidising microorganisms in this soil, which later becomes highly crystalline MnO_2. Hausmannite (Mn_3O_4), as an intermediate, is commonly observed in Mn oxidising systems (Mandernack et al., 1995b). Some of the major Mn oxides present in the soils included birnessite, pyrolusite, todokorite and hausmannite (Post, 1992, 1999) where formation was influenced by pH.

These microbial Mn oxide deposits also oxidised the spiked Cr(III) (20 %). This is highly significant as the amount of Cr(VI) released (4 mg L^{-1}) was higher than the recommended permissible level. It is also evident that the oxidation of Cr(III) is dependent on its initial concentration in solution as concentrations below 20 mg L^{-1} did not show evidence of Cr(VI) in the solution. It is likely that oxidation occurs but the concentration of Cr(VI) is below the detection limit of the analytical technique used for the speciation of Cr(VI) in solution. Amacher and Baker (1982) observed a similar rate of Cr oxidation (20 %) in soil solution at pH 7.5 when spiked with 1 μM of Cr(III). They also reported that decreasing the pH increased the rate of oxidation. However, the process was mainly determined by soil factors, pH, surface area of manganese oxides and the solubility of Cr(III) (James, 1996). Chromium oxidation in soil depends on equiponderance among the chemical availabilities of Cr(III), manganese oxides and reducing organic substances (Bartlett and James, 1988). (Amacher and Baker, 1982)) reported the oxidation of Cr(III) in whole soils and was found that Cr(III) oxidation was proportional to the exchangeable Mn oxides (Ross and Bartlett, 1981). The type of soil also plays a major role. For instance, Andisols pose less threat of Cr (III) oxidation (Makino et al., 1998) compared to organic soils rich in low molecular weight compounds, wherein the Cr(III) binds to organic component and is more prone to oxidation (Bartlett and James, 1988).

Since the pH of the contaminated soil used in the present study is alkaline, Cr(III) oxidation is favoured. However, studies on the soil's oxidation potential showed that only 0.2-0.3 % of spiked Cr(III) (1mM) was oxidised within 1 h. These values were significantly lower than the reduction capacity, 85-88% of spiked 0.1mM Cr(VI) for the same period. This clearly indicated that the reducing capacity of these soils was higher than the oxidation potential. Nevertheless Cr(VI) was still detected in the contaminated soils. The Cr oxidation potential for tannery sludges was reported to be 1.4 (Eutrochrept). Soils at field moist conditions produced about 1 mg kg^{-1} of Cr(VI) in soil and released it relatively slowly (Bartlett, 1985). There is also some evidence that Cr(III) complexed with high molecular weight organic ligands is not readily disassociated or oxidised. Under optimum conditions, Cr in the sludge acts like a slow release fertilizer and is released into the system (Bartlett and James, 1988).

5.4.5   Implications of biotic-abiotic coupling reactions

The presence of Cr(VI) in this particular LTTW contaminated soil can be attributed to the presence of Mn oxides. The mere abundance of Mn oxides (300 mg kg^{-1}) might suggest that Mn may play a significant role in oxidation. However, studies on soil solutions indicated that these processes were very slow. Cr oxidation might be a possibility in this soil driven as a result of coupled biotic-abiotic reaction in which Mn oxides deposited by microbes catalyse Cr(III) oxidation. After oxidation with the Mn surfaces, Cr(VI) formed would be released into soil solution and be relatively persistent due to the favourable alkalinity of the soil. Hence, in Mn rich soil it is essential to take into account these biotic-abiotic processes before formulating a viable remediation technique. Biotic-abiotic processes have previously proven to be a significant tool for Cr (VI) reduction (Wielinga et al., 2001a) in bioremediation techniques by using the biotic [Fe(II)→Fe (III)] reactions to convert Cr(VI) to Cr(III) abiotically.

5.5 CONCLUSION

The results of this study suggest that biotic - abiotic coupling reactions are likely to play a key role in the oxidation of Cr(III). This would explain the presence of Cr(VI) at significant levels even after 25 years of waste disposal. Although this hypothesis seems to be possible at this site, the thermodynamic conditions showed that reduction capacities of these soils were higher than the oxidation potential. In the following chapter, the main objective was to examine the potential for the reduction and

stabilisation of persistent Cr(VI) at this site using plants and chemical amendments.

Chapter 6

The value of an idea lies in the using of it
- Thomas A Edison -

∅

CHAPTER 6

PHYTOSTABILISATION OF CHROMATE IN TANNERY WASTE CONTAMINATED SOILS

6.1 INTRODUCTION

Developing health based cleanup standards and remediation strategies for Cr contaminated soils based on the hexavalent form of this heavy metal is a complex and controversial task (James, 1996). Oxidation and reduction reactions can convert Cr(III) to Cr(VI) and *vice versa*. The potential for this interconversion has therefore complicated the task of determining whether Cr bearing waste or contaminated soil is hazardous (Federal Register, 1991).

The results, presented in Chapter 5, indicated that Cr(VI) was persistent in LTTW contaminated soil at concentrations exceeding permissible limits even 20 years after waste disposal to this site had ceased. This is probably due to the alkaline nature of the soil and presence of significant concentrations of Mn oxides. It was also evident that biotic-abiotic coupling processes may play a significant role in the formation and persistence of Cr(VI) in contaminated soil and these processes need to be considered before formulating a bioremediation strategy for this site.

A number of *in situ* and *ex situ* techniques including excavation and disposal, soil washing, soil flushing, solidification, vitrification, chemical and biological reduction and phytoremediation/phytostabilisation have been used for the remediation of Cr contaminated soil. Although each of these techniques has its own merits (Chapter 2, Table 2.10), a number of them would not be applicable for the remediation of the Mount Barker site because of the high levels of Cr in the soil (often exceeding 10% in surface and 0.5% in subsurface soils), the nature of the soil, the large volume of the soil to be remediated or removed as well as the prohibitive costs associated with many of these remediation techniques.

The important factors to be considered before formulating a remediation strategy for this contaminated site include:

    a) the extensive area of contaminated land (approximately 7 ha),

    b) the alkaline pH of the soil, and

    c) the presence of Mn oxides

Based upon economic rationales alone remediation of large areas of Cr contaminated land using *in situ* remediation techniques, such as chemical and biological reduction or phytoremediation/phytostabilisation are more economically viable than *ex situ* physical-chemical methods. One simple *in situ* approach could involve the incorporation of organic matter, iron containing salts and organic acids to the contaminated soil, which should facilitate the irreversible reduction of Cr(VI) to Cr(III) and its hydroxides (James, 1996).

Efforts to remediate Mount Barker soil using ferrous sulphate has shown that approximately 3% $FeSO_4$ was needed to completely reduce Cr(VI) to Cr(III). (Avudainayagam, 2002). Even then, the effectiveness of this process was short-lived due to the high buffering capacity of the soil (and therefore high uneconomical doses of Fe required), the depth of contamination (which exceeds one metre) and the recalcitrant nature of organic matter present (which rendered it unavailable for Cr reduction) (Avudainayagam, 2002).

Cr reduction may also be achieved through microbial processes (Ishibashi et al., 1990; Deleo and Ehrlich, 1994; Fude et al., 1994; Shen and Wang, 1995; Shen et al., 1996; Chirwa and Wang, 1997; Schmieman et al., 1997). Soil characterisation studies showed that LTTW-contaminated soil from Mount Barker harboured bacteria capable of reducing Cr(VI) (Megharaj et al., 2001). But DHA and PLFA studies on this soil (Chapter 4) indicated that the activity of these organisms was significantly inhibited by elevated concentrations of Cr. Therefore, attempts to stimulate indigenous microorganisms, or to augment existing microbial populations with Cr reducing microorganisms may prove unsuccessful due to the inhibitory effect of high concentrations of Cr(VI).

Another remediation option for the Mount Barker site is phytoremediation/ phytostabilisation. Of the phytoremediation techniques, phytostabilisation has recently gained recognition as a remediation strategy for metal-contaminated soil due to its low cost compared to traditional physico-chemical techniques and its applicability to heavily polluted sites (Vangronsveld, 1998 #2). Phytostabilisation (phytorestoration), is a site stabilisation technique that reduces the risk of soil contaminants through the use of soil amendments that induce the formation of insoluble contaminant species. Since this technique combines the use of plants and stabilising amendments for the immobilisation of metals, it offers an effective *in situ* remediation strategy for the reduction of metal mobility and bioavailability. Amendments such as phosphate minerals, hydrous Fe

oxides, organic materials and inorganic clay minerals may all be used depending on the contaminant. Although many reports exist on the use of phytostabilisation for Pb, As, Cu and Cd contaminated soils (Berti and Cunningham, 2000), no studies have yet demonstrated the effectiveness of phytostabilisation for Cr contaminated soils. In addition, most of the research in this area has focused on the immobilisation of metals by amendments, thereby reducing the plant uptake. But the role of the rhizosphere and microbial activity in the effectiveness of phytostabilisation processes is not well documented.

As described above, considering the vast area of Cr contaminated land to be remediated, the phytostabilisation technique offers the most appropriate strategy for long-term remediation. Potentially phytostabilisation may immobilise Cr(VI), thereby preventing leaching to groundwater and uptake by plants (such as crops or fodder). As a result of amendment and crop growth, microbial activity will be stimulated in the soil (and also in the rhizosphere) resulting in the release of electrons available for Cr reduction processes. The organic acids released around the plants will result in dissolution of Mn oxides (reducing the occurrence of surface oxidation of Cr) and will also cause direct reduction of Cr(VI).

The research described in this Chapter was an attempt to explore the feasibility of phytostabilisation of LTTW-contaminated soil. The specific aims were to determine,

a) the effectiveness of organic amendments on chemical reduction and immobilisation of Cr(VI);

b) the effect of crop plants [peas (*Pisum sativum*) and clover (*Trifolium repens*)] and organic amendments on Cr(VI) reduction; and

c) the role of rhizosphere and associated microbial activity on Cr(VI) reduction.

6.2   MATERIALS AND METHODS

6.2.1   Materials and Treatment Structure

Surface soil (0-10 cm) (75 kg) was collected from the LTTW contaminated site (Section 4.2.1), dried in a fan forced oven (30°C), composited, passed through a 2-mm sieve and weighed (1 kg) into polyethylene containers (20 cm wide and 25 cm deep) each containing a single rhizon sampler. Rhizon samplers are thin, long and porous 0.2 μm filters connected to nylon tubing specifically designed for sampling soil solution

without disturbing the soil. Rhizon samplers were obtained from Rhizosphere Research Products (RRP, Equipment for Soil, Rhizosphere and Root Research, Dolderstraat 62, NL-6706 JG Wageningen, The Netherlands) (Meijboom and Noordwijk, 1992).

The soil amendment for phytostabilisation experiments consisted of organic manure in the form of dried cow manure. An organic amendment was chosen despite 15% OC in this soil since the OC present at this site was not easily available for Cr(VI) reduction (Avudainayagam, 2002). Cow manure was chosen in preference to other organic manures based on its ability to reduce Cr(VI) (Losi, 1994) and to stimulate plant growth. Commercially available cow manure was air-dried and sieved (< 2 mm) before use.

Two application rates of cow manure (25 and 50 tons ha^{-1}) were chosen for the phytostabilisation studies based on previous work that had shown optimum Cr(VI) reduction at these levels (Losi et al., 1994b). The amount of cow manure required was calculated based on the bulk density of the contaminated soil, 0.6 g cm^{-3}. Cow manure (37 and 74 g equivalent to 25 and 50 tons ha^{-1}) was added to contaminated soil (total dry weight of 1 kg) and thoroughly combined with shaking in an inverting rotating shaker (100 rpm) for 30 minutes. The soil mixtures were then transferred to polyethylene pots, rhizon samplers inserted and irrigated with distilled water (with constant mixing) to attain 75% water holding capacity (450 ml kg^{-1} dry soil). Pots were incubated at this moisture holding capacity for two weeks to attain equilibrium before sowing of test plant species. Peas (*Pisum sativum*) and clover (*Trifolium repens*) were chosen for phytostabilisation experiments because of their intense rhizosphere activity and tolerance to Cr-contaminated soil during preliminary germination tests. Phytostabilisation treatments (with and without cow manure application) consisted of fallow soil and soil planted with peas or clover. Seven replicates were prepared for each soil treatment and the complete treatment structure used is outlined in Table 6.1.

Five seeds of the respective plant types were sown per pot. However, after germination only three plants per pot were maintained. Plants were grown in a glasshouse (25 ± 3°C) for up to 15 weeks. Soil moisture was maintained at 75% water holding capacity by the daily addition of distilled water as required. Over the experimental period, total Cr, Cr(VI), DOC and pH were monitored in soil solution and soil samples while the yield of peas and clover was determined upon termination of the experiment.

Table 6.1 Treatment structure used in glasshouse experiment

CO	Contaminated soil, no plants
C25	Contaminated soil + cow manure (25 tons ha^{-1}), no plants
C50	Contaminated soil + cow manure (50 tons ha^{-1}), no plants
C0-P	Contaminated soil + Peas
C25-P	Contaminated soil + cow manure (25 tons ha^{-1}) + Peas
C50-P	Contaminated soil + cow manure (50 tons ha^{-1}) + Peas
C0-C	Contaminated soil + Clover
C25-C	Contaminated soil + cow manure (25 tons ha^{-1}) + Clover
C50-C	Contaminated soil + cow manure (50 tons ha^{-1}) + Clover

6.2.2   Sampling of soil solution

Soil solution was sampled every week from the pots by applying suction to the rhizon samplers. Pots were irrigated to the desired moisture (75%) in the morning, allowed to equilibrate, and suction applied using syringes (20 ml) in the evening. The following morning, soil solution collected in the syringes was transferred to sterile plastic tubes and immediately analysed for Cr(VI), total Cr (Section 3.4.), DOC and pH.

Initial characteristics of the soil solution (prior to the start of the experiment) were performed on soil solution collected from pore water after centrifuging the soil (at 75% moisture) (Fotovat et al., 1997). Moist soil was packed into a syringe containing glass wool to prevent the escape of soil from the syringe end. The syringe was then placed in round-bottomed plastic centrifuge tube (25 ml) with a ceramic tube (spacer) between the base of the centrifuge tube and the syringe. This entire assembly was centrifuged at 3000 rpm for 30 minutes and soil solution which collected in the larger outer centrifuge tube was filtered (0.5 μm, Sartorius filters) and analysed for pH, EC, DOC, soluble Cr (AAS) and Cr(VI) (IC).

6.2.3   Sampling of soil

Soil samples were collected at definite time intervals (30, 60 and 90 d) and analysed for its chemical characteristics and microbial activities. One pot from each treatment was sacrificed and the soil was collected, homogenised and analysed for soluble and exchangeable Cr(VI). Soluble Cr(VI) was measured by shaking soil (1.5 g)

with distilled water (30 ml) for 6 h at 25 ± 2°C in an end over end shaker. Soil solution was collected after centrifugation at 10,000 rpm for 20 minutes and filtration through 0.5 μm filters (Sartorius). To the remaining soil, 30 ml of 0.01 M K_2HPO_4 was added and shaken again for 16 h at 25 ± 2°C in an end over end shaker. The soil solution was then centrifuged, filtered and designated the exchangeable fraction. Both fractions were analysed for Cr(VI) using IC (Section 3.4.1).

Microbial activity, as determined by DHA (Section 4.2.3) was also performed on moist soils. After establishment of pea and clover crops, soil samples taken from within the pots were classified into two categories: rhizosphere soil, i.e. soil surrounding the roots of the plants and bulk soil (non-rhizosphere soil away from the root). Rhizosphere soil adhering to the roots was collected carefully by brushing. Both these soils were homogenised separately and analysed for chemical and microbiological characteristics as described above.

6.2.4 Sampling of plants

At the end of the experimental period, the yield of peas and clover was determined. Pea yield was expressed as the fresh weight of pods (g) pot^{-1}, while clover yield was expressed as the fresh weight of foliage (g) pot^{-1}. Clover leaves were chopped off at soil level and weighed.

6.2.5 Analytical Methods

Contaminated soil and cow manure were analysed, prior to the start of the experiment to determine their nutrient status (N and P), pH, EC, chemical characteristics (total carbon, OC, total Cr, Fe and Mn, soluble Cr, Cr(VI), Fe and Mn) and microbial activity (DHA).

Soil total N was determined by Dumas Combustion method using a Leco 2000 analyser. NH_4-N/NO_3-N (Method 7C2) and P as bicarbonate extractable P (Method 9B1) were also determined (Rayment and Higginson, 1992).

Metals (Cr, Fe and Mn) were determined in aqua regia digests as described in Section 3.2. Soil solution samples collected from rhizon samplers were analysed for pH Cr(VI), IC (Section 3.4.1) and DOC.

6.3 RESULTS

6.3.1 Chemical characteristics of contaminated soil and cow manure

Initial characterisation of the contaminated surface soil (C0) revealed that it was high in total carbon (18.5%) (Table 6.2) with a DOC of 311 mg kg^{-1} and a pH of 8.4. The total N content was 2.5% and the plant available NH$_4$-N was only 11.7 mg kg^{-1} and (NO$_3$ + NO$_2$)–N was 30 mg kg^{-1}. Bicarbonate P was 44.5 mg kg^{-1}. The total Cr concentration in the soil was 23 g kg^{-1} with the water soluble Cr(VI) and exchangeable Cr(VI) being 10.9 and 9.6 mg kg^{-1}, respectively.

Cow manure (pH 8.1) had a total carbon content of 18.5% and very high DOC (4100 mg L^{-1}) and extractable P (870 mg P kg^{-1}). The NH$_4$-N and (NO$_3$ + NO$_2$)-N were 704 and 1.9 mg kg^{-1}, respectively. Cr in cow manure was negligible with background concentration of only 0.02 g kg^{-1}. The cow manure used in this study had a similar pH to the soil, but had a higher EC (3.95 dS m^{-1}).

The soil solution characteristics of the contaminated soil mixed with cow manure at 0, 25 and 50 tons ha^{-1} are given in Table 6.3. Addition of cow manure to soil increased the DOC and EC significantly, but no distinct changes were noticed in pH, soluble Cr and Cr(VI) concentrations. DOC increase was 1.36 and 2.16 times higher in soil amended with 25 and 50 tons ha^{-1} of cow manure, respectively compared to the untreated soil.

6.3.2 Effect of organic manure on Cr(VI) reduction

The main objective of this experiment was to evaluate the use of cow manure as an organic amendment for Cr(VI) reduction and immobilisation.

Soil solution collected in rhizon samplers was analysed for Cr(VI) by IC and total Cr by AAS (Sections 3.4.1 and 3.4.2 respectively). Analyses showed that more than 95% of solution Cr existed as the Cr(VI) species. The soil solution pH in all treatments varied between 7.7 and 8.2 during the 15-week incubation.

Table 6.2 Initial nutrient status and chemical characteristics of contaminated soil and cow manure used for glasshouse experiments

	pH	EC	Total carbon	DOC	N			P	Fe		Mn	
					Total N	NH_4-N	NO_3+ NO_2 N	Total	Total	Water soluble	Total	Water soluble
		(dS m^{-1})	(%)	(mg L^{-1})	(%)	(mg kg^{-1})	(mg kg^{-1})	(mg P kg^{-1})	(g kg^{-1})	(mg L^{-1})	(g kg^{-1})	(mg L^{-1})
Contaminated soil	8.4	0.34	18.6	311	2.5	11.7	30	44.5	8.0	0.04	0.3	nd
	(0.0)	(0.03)	(0.22)	(13)	(0.02)	(0.06)	(4.5)	(1.7)	(0.1)	(0.01)	(0.02)	
Cow manure	8.1	3.95	18.5	4100	1.44	704	1.9	870	8.1	nd	0.2	nd
	(0.04)	(0.21)	(0.71)	(106)	(0.03)	(18)	(0)	(46)	(0.4)		(0.02)	

	Cr concentration in different fractions			
	Total Cr (g kg^{-1})	Water soluble Cr (VI) (mg kg^{-1})	Exchangeable Cr (VI) (mg kg^{-1})	Organically bound Cr (g kg^{-1})
Contaminated soil	23 (0.64)	10.9 (0.4)	9.6 (0.9)	14.7 (0.54)
Cow manure	0.02 (0.01)	n.d	n.d	0.01 (0.01)

Figures in the parenthesis indicate sd values of 3 or more replicates.

Table 6.3 Initial pore water characteristics of amended and unamended contaminated soil

Treatment	pH	EC	DOC (mg L^{-1})	Soluble Cr (mg L^{-1})	Soluble Cr (VI) (mg L^{-1})
C0	8.7	0.34	311	1.5	1.4
	(0.02)	(0.03)	(31.2)	(0.3)	(0.2)
C25	8.6	0.51	424	1.5	1.4
	(0.01)	(0.01)	(38.9)	(0.1)	(0.13)
C50	8.6	0.66	672	1.7	1.5
	(0.07)	(0.04)	(151)	(0.1)	(0.18)

Figures in the parenthesis indicate sd values of 3 replicates.

Analysis of soil solution for Cr(VI) during incubation without plants showed that Cr(VI) concentrations in soil solution decreased with an increase in incubation time until 5 weeks (Fig 6.1A). The initial Cr(VI) concentration was 1.50 and 1.67 mg L^{-1} in soil solutions of organic amendments, C25 and C50, respectively, as compared to 1.2 mg L^{-1} in unamended (C0) control (Fig 6.1A, B&C). The higher levels of soil solution Cr(VI) in organic amendments, at the start of the experiment, were probably due to the increased P content of cow manure that may exchange the Cr(VI) from soil to solution. The Cr(VI) reduction reached the maximum at week 5. The addition of cow manure at 25 and 50 tons ha^{-1} to the contaminated soil resulted in a distinct decrease in soil solution Cr(VI) over that in the unamended control at 2 weeks. The addition of cow manure did not have any appreciable effect at 4 and 5 weeks when Cr(VI) concentration continued to decline, reaching similar levels, in soil solution for both unamended and cow manure amended treatments. After the week 5, the Cr(VI) concentration increased (up to week 10) in all the treatments, after which an equilibrium was reached. Though reduction in Cr(VI) of 30 – 50% (of the initial, 1.2-1.7 mg L^{-1}) was observed after the 15-week experimental period, Cr(VI) was not completely reduced/removed from the soil solution.

As Cr(VI) in the soil solution decreased with time there was a corresponding decrease in DOC. The initial DOC (208 mg L^{-1}) increased with the rate of cow manure applied, C25 (287 mg L^{-1}) and C50 (386 mg L^{-1}) (Fig 6.1A, B&C). However, DOC declined rapidly between 2 and 3 weeks and reached almost the same level at 4 weeks (98-110 mg L^{-1}), irrespective of the rate of cow manure application. DOC decreased to 148, 205 and 190 mg L^{-1} in C0, C25 and C50, corresponding to 29, 29 and 51% reduction from the initial DOC levels. The DOC concentrations steadily decreased with incubation time and at week 10 the values were insignificant ($p < 0.05$) in all the treatments (10 mg L^{-1}).

Both Cr(VI) and DOC data showed that DOC had a significant role in Cr(VI) reduction during the initial stages (6 weeks) when DOC was higher in soil solution, there was a rapid decline in Cr(VI).

6.3.3 Effect of organic manure and plants on Cr(VI) reduction

Peas and clover were used as test crops to assess their ability to reduce Cr(VI) in the presence and absence of cow manure.
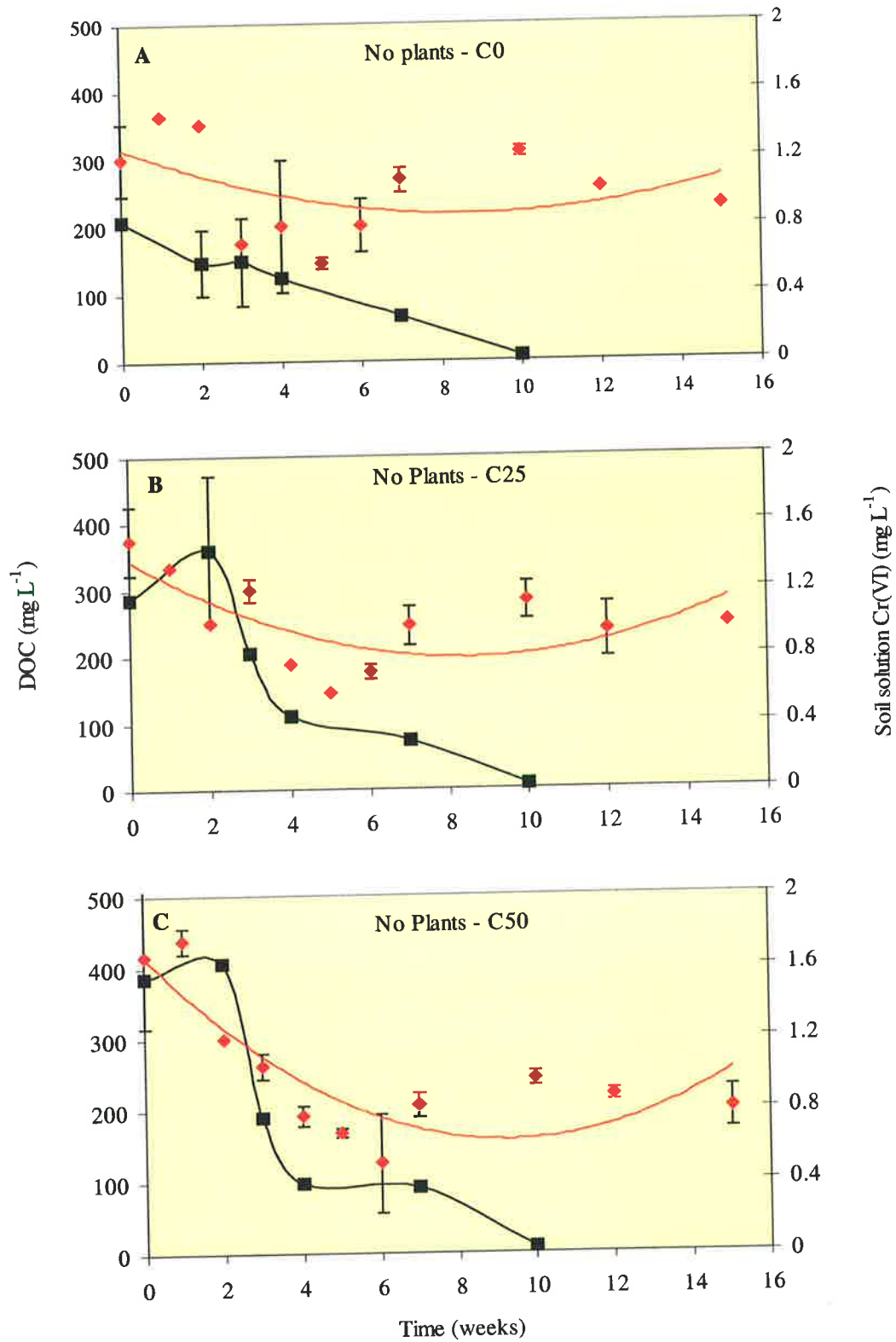
Fig. 6.1 Changes in Cr (VI) (◆) and DOC (■) concentrations in soil solution of contaminated soil unamended (A) and amended with cow manure at rate of 25 (B) and 50 (C) tons ha⁻¹. The red line indicates the exponential trendline for Cr(VI).

6.3.3.1 Peas

There was an initial decline in Cr(VI) concentrations in soil solution of unamended soils from 1.7 – 0.8 mg L^{-1} up to week 6 (Fig 6.2A). Similar trends were observed in amended soils in which the Cr(VI) concentration declined to 0.8 mg L^{-1} in C25 and 0.6 mg L^{-1} in C50 on week 5 (Fig. 6.2B&C). After this initial decline, as was observed for unplanted treatments (Section 6.3.2.1), there was a steady increase in Cr(VI) into soil solution between 6 and 15 weeks in both unamended and amended treatments. Pea plants effected a significant increase in solution Cr(VI) in the presence of cow manure at 25 tons ha^{-1} (C25) over that in C0 especially at week 10. But, generally, cow manure (except at week 7 and 10 with cow manure 25 tons ha^{-1}) at both 25 and 50 tons ha^{-1} retarded the release of Cr(VI) into soil solution as at the end of the experimental period, only 1.1 mg L^{-1} (C25) and 0.9 mg Cr(VI) L^{-1} (C50) was observed compared to 1.7 mg L^{-1} in unamended soils (C0).

There was a corresponding decrease in DOC concentration with time and the maximum decrease was observed at week 6 in all the treatments (Fig. 6.2A, B&C). Compared to C0, the rate of DOC decrease was more in C25 and C50 treatments in which DOC decreased by 64% (88 mg L^{-1}) and 60% (136 mg L^{-1}) respectively. The concentration continued to declined to undetectable levels after week 10 in all treatments.

6.3.3.2 Clover

The variation in Cr concentrations for clover was similar to the variations observed for peas. Initially, Cr(VI) decreased with time followed by an increase in Cr(VI) concentrations at later stages (Fig. 6.3A, B&C). In the initial 5 weeks, the Cr(VI) concentration decreased from 1.8 to 0.8 mg L^{-1} in C0, 1.9 to 0.8 in C25 and 1.9 to 0.6 in C50. In general, the trend in Cr(VI) concentrations was identical in both clover and unplanted treatments. (Fig. 6.1). After 6 weeks, a slight increase in Cr(VI) concentration was observed up to week 10 and a steady state was reached afterwards. At the end of 15 weeks, there was 1.1 (C0), 1.1 (C25) and 0.9 (C50) mg Cr(VI) L^{-1} in soil solution. The DOC declined sharply (> 50%) at week 3 from initial values in C0 (181 to 60 mg L^{-1}), C25 (289 to 121 mg L^{-1}) and C50 (472 to 250 mg L^{-1}). The decrease later was gradual and reached minimum at week 10.

Fig. 6.2 Changes in Cr (VI) (◆) and DOC (■) concentrations in soil solution of contaminated soil unamended (A) and amended with cow manure at rate of 25 (B) and 50 (C) tons ha⁻¹ and grown with peas. The red line indicates the exponential trendline for Cr(VI).
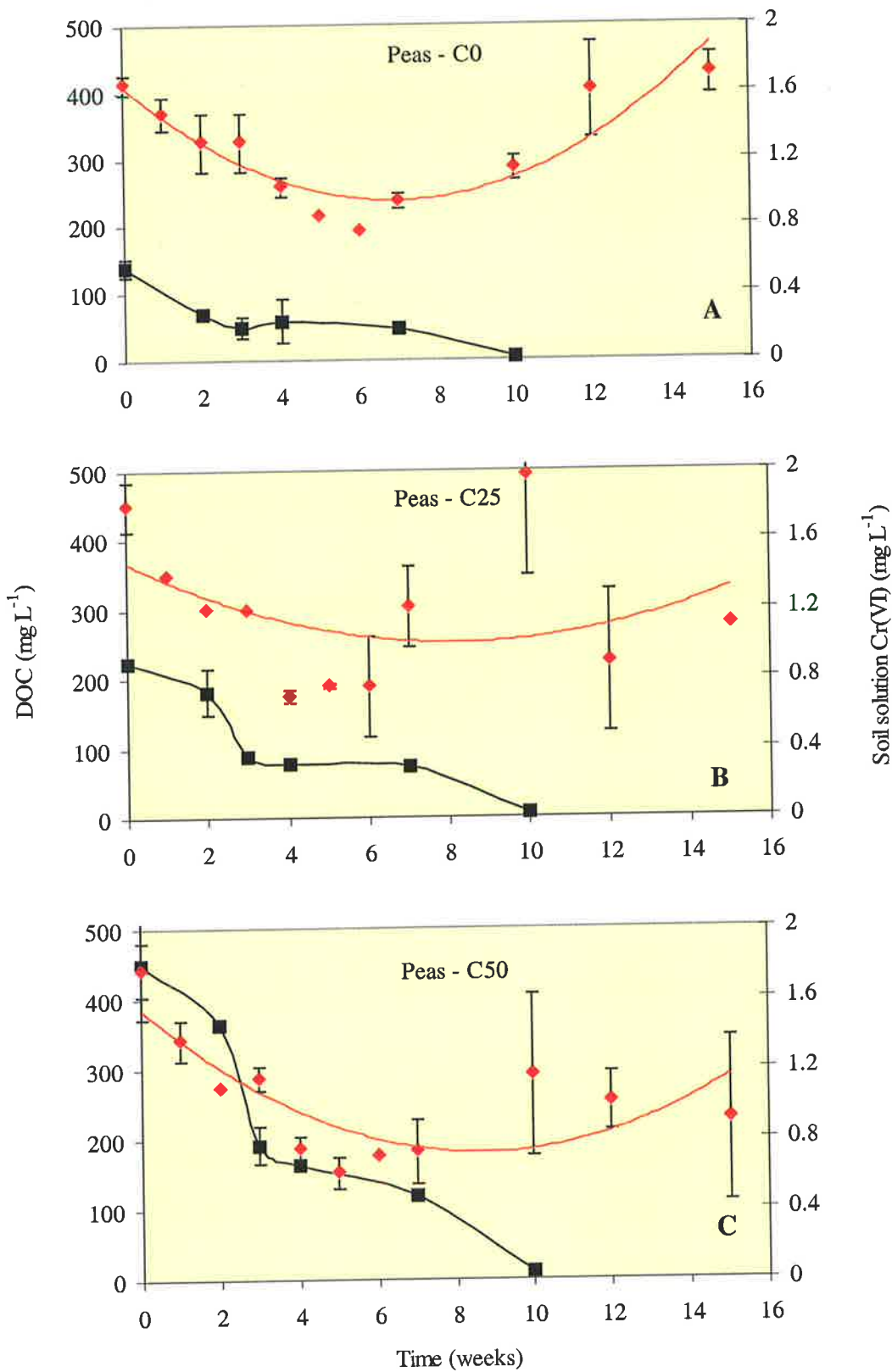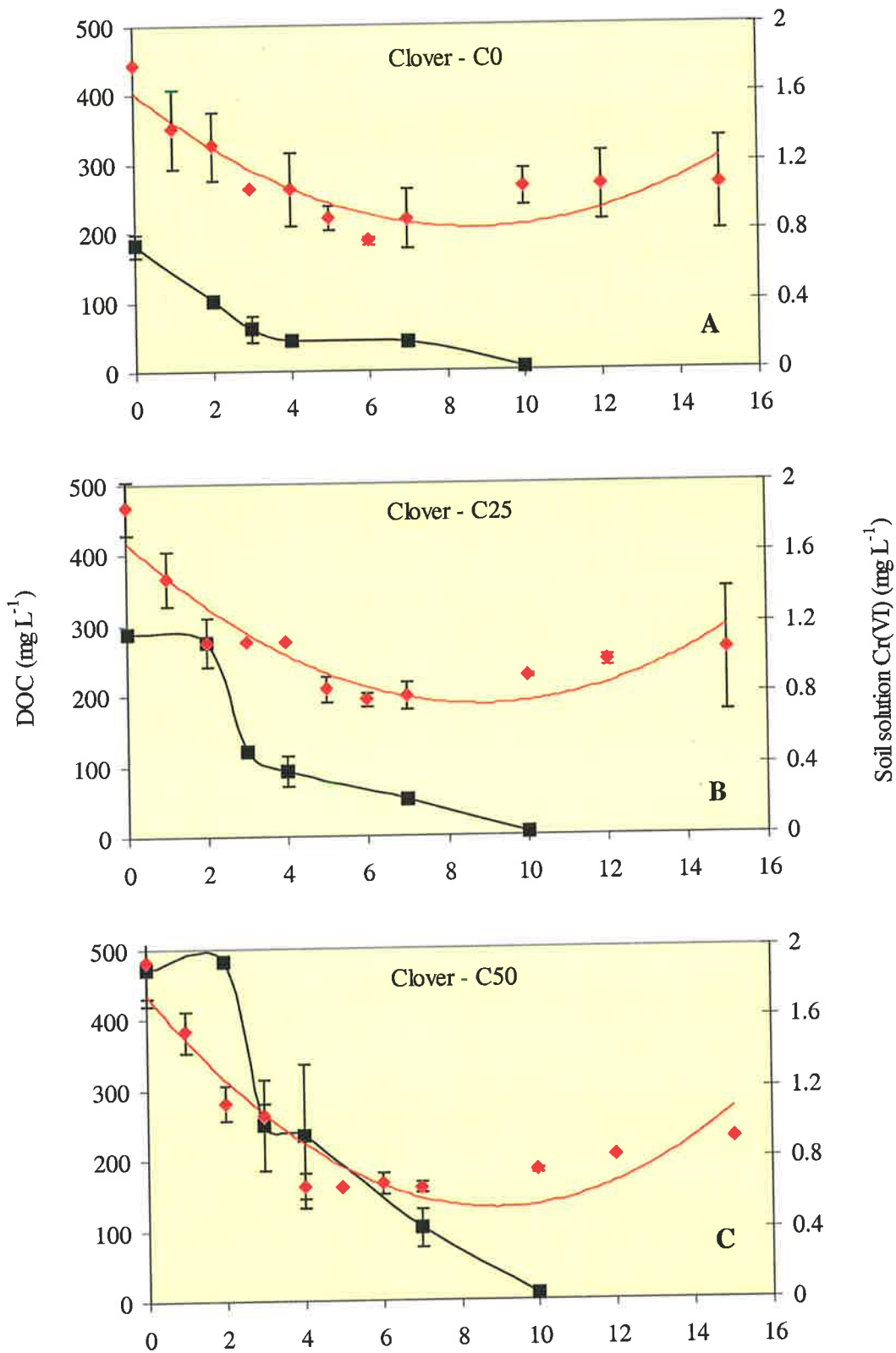
Fig. 6.3 Changes in Cr (VI) (◆) and DOC (■) concentrations in soil solution of contaminated soil unamended (A) and amended with cow manure at rate of 25 (B) and 50 (C) tons ha^{-1} and grown with clover. The red line indicates the exponential trendline for Cr(VI).

Overall, the addition of organic amendment (cow manure) at higher rates (50 tons ha^{-1}) retarded the release of Cr(VI) into soil solution compared to unamended treatments in both planted and unplanted treatments (Fig 6.1, 6.2 & 6.3). Compared to initial one, during most of the incubation periods, C50, recorded the minimum Cr(VI) concentration in soil solution. Peas resulted in increased release of Cr(VI) in soil solution during the active growth period, but addition of cow manure retarded this process. Compared to peas, clover released less Cr(VI) in soil solution.

6.3.4   Cr(VI) versus DOC

Organic manure was used as an amendment in these experiments to enhance the availability of organics for microbial activity. The organic matter oxidation releases electrons aiding Cr reduction in these soils. From Fig. 6.1, 6.2 and 6.3, it was evident that maximum Cr reduction was observed within 5 weeks and DOC decrease commenced after 2 weeks. It is likely that an increased DOC would have reduced Cr(VI) to Cr(III) and would have complexed some Cr forming Cr-organic complexes. A scatter plot (Fig. 6.4) relating depleted DOC to depleted Cr(VI) shows a marked increase in the depletion of Cr(VI) with increasing depletion of DOC. This suggests that DOC plays a significant role in Cr(VI) transformation in the amended soils. It is evident from Fig. 6.4 that there existed a significant relationship between DOC depleted (initial DOC − DOC at time t) to Cr(VI) reduced in all the treatments. This relationship was drawn only for the period when the Cr(VI) reduction was higher (0-7 weeks). As the DOC depletion increased more Cr(VI) was reduced and it was linear in treatments with no plants (r^2=0.81, p< 0.05), peas (r^2=0.59, p< 0.05) and clover (r^2=0.51, p< 0.1).

6.3.5   Microbial activity and Cr(VI) reduction during phytostabilisation

Microbial activity, in terms of DHA, and Cr(VI) concentrations (soluble and exchangeable fractions) in the soil, were determined after 3, 6 and 10 weeks of incubation. The soil was collected from all treatments (sacrificed during the specified period), homogenised and analysed for DHA. Sequential extraction method was used to extract soluble and exchangeable Cr(VI). All microbial activity and Cr(VI) concentrations were determined at 75 % moisture content used in the experiment (Fig. 6.5).

Fig. 6.4 Relationship between DOC depletion and Cr (VI) reduction in 5 weeks
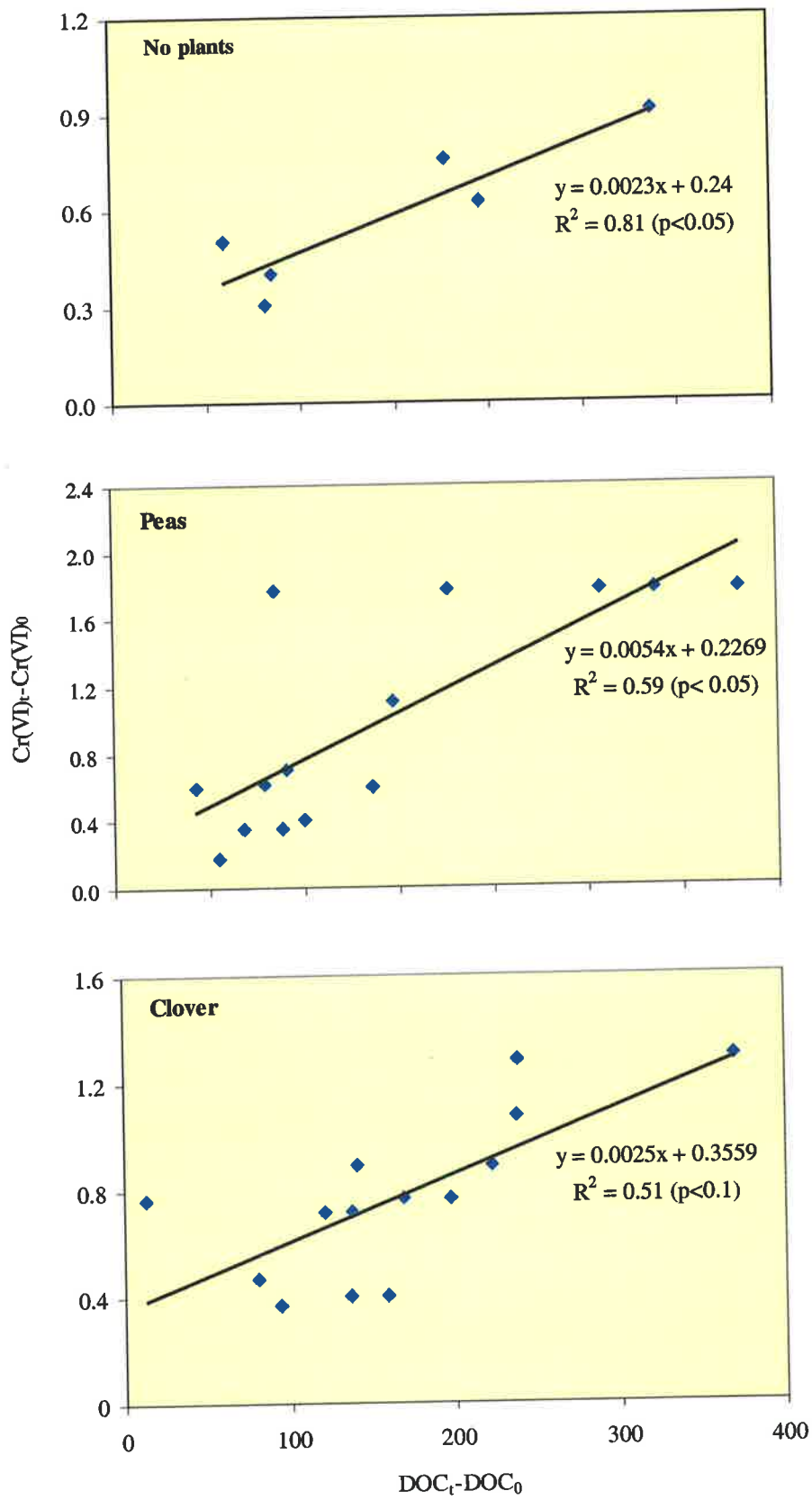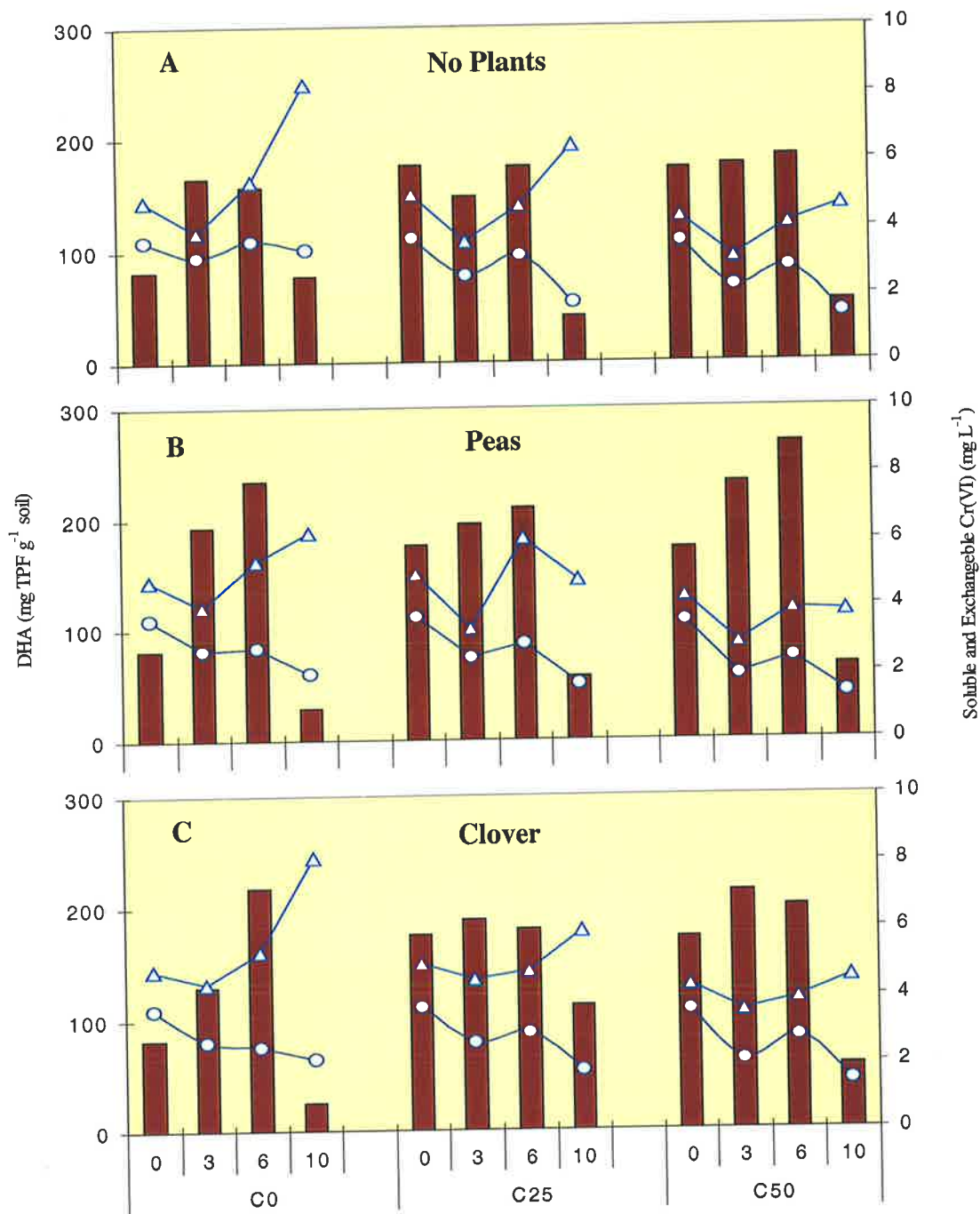
Fig 6.5 Effect of different rates of organic manure on DHA (■), soluble (●) and exchangeable (▲) Cr (VI) during incubation.

6.3.5.1 Cr(VI) reduction

Addition of cow manure to contaminated soil without plants accelerated the decrease in solution Cr(VI) (Fig. 6.5A). Cr(VI) reduction increased with increase in the rate of cow manure applied. Thus, the concentration of soluble and exchangeable Cr(VI) in organic amended soil was generally less at 50 tons ha^{-1} than at 25 tons ha^{-1}. There existed a direct relationship between cow manure amendment and Cr(VI) reduction. At 10 weeks, there was a sharp increase in exchangeable Cr(VI) over that in earlier weeks.

In treatments containing plants also Cr(VI) reduction was higher in C50-P treatment than in C0-P (Fig. 6.5B). A concomitant Cr(VI) reduction with increase in organic manure was observed. As was observed with unplanted treatments maximum reduction of soluble Cr(VI) was noticed at week 10, 55 and 62% in C25-P and C50-P compared to 44% in C0-P. The Cr(VI) reduction in clover also behaved in a similar manner and also the maximum Cr(VI) reduction on 10th week was comparable to values noticed in peas (53% in C25-C and 60% in C50-C) (Fig. 6.5 C).

6.3.5.2 Microbial activity (DHA)

The addition of cow manure increased the DHA significantly. Two times more DHA was measured in C25 (176 µg TPF g^{-1} dry soil) and C50 (173 µg TPF g^{-1} dry soil) treatments compared to C0 (81 µg TPF g^{-1}) at the start of the experiment (Fig 6.5A). In treatment without plants, there was no significant change in DHA up to week 6, however there was a significant decrease on week 10. Treatment C25 (41 µg TPF g^{-1} dry soil) and C50 (54 µg TPF g^{-1} dry soil) had lower DHA than C0 (78 µg TPF g^{-1} dry soil) on week 10.

DHA was higher in planted treatments than that in unplanted treatments and also DHA increased with increasing levels of cow manure. DHA increased with time, reaching a maximum at week 6 and declined on week 10. In peas, DHA increased with time during plant growth and maximum DHA activity was noticed at 6 week in all the treatments, C0-P (235 µg TPF g^{-1} dry soil), C25-P (210 µg TPF g^{-1} dry soil) and C50-P (267 µg TPF g^{-1} dry soil). In treatments with clover, DHA activity behaved similar to pea treatments and maximum activity was observed in C50-C (267 µg TPF g^{-1} dry soil) followed by C0 (235 µg TPF g^{-1} dry soil) and C25 (210 µg TPF g^{-1} dry soil).

An insignificant inverse relationship existed between the level of organic manure and Cr(VI) in soil solution. Also correlation trials showed that overall no relationship existed between DHA and Cr(VI) concentrations.

6.3.6   Bulk versus rhizosphere soils

The microbial activity (DHA) and the soluble Cr(VI) of the bulk soil, as discussed above, were compared with those in rhizosphere soils to determine the effect of intense microbial activity in the root zone on Cr(VI) reduction. The soil adhering to the roots represented the rhizosphere soil while soil collected away from the roots was used as bulk soil (non-rhizosphere soil).

6.3.6.1 Cr(VI) and DHA

The Cr(VI) concentrations and DHA were measured in bulk and rhizosphere soils of pea plants after 3, 6 and 10 weeks of incubation. The data revealed that the Cr(VI) concentration in the rhizosphere was significantly ($p < 0.05$) and consistently less than that in bulk soil (Fig. 6.6). Thus, during all the three samplings, the Cr(VI) concentration in the rhizosphere was 36 - 60% less than that in the bulk soil. This decrease in Cr(VI) levels in the rhizosphere was more pronounced at weeks 3 and 6 than at week 10. Correspondingly, rhizosphere soil registered significantly higher DHA ($p < 0.05$) than did the bulk soil. On week 3, rhizosphere DHA increased with an increase in level of cow manure applied. Thus, at 3 weeks, rhizosphere DHA was 2.7 (515 µg TPF g^{-1} dry soil), 4.5 (873 µg TPF g^{-1} dry soil) and 4.1 (943 µg TPF g^{-1} dry soil) times higher than that in corresponding bulk soil at C0 (193 µg TPF g^{-1} dry soil), C25 (195 µg TPF g^{-1} dry soil) and C50 (232 µg TPF g^{-1} dry soil), respectively. The same trend was observed at week 10.

In clover, similar studies were performed on rhizosphere samples collected at 6 and 10 weeks. Since the roots of clover were very small, collection of sufficient rhizosphere soil for analyses was not possible on 3rd week. At week 6, rhizosphere soil contained 45 – 65% less Cr(VI) in rhizosphere soil compared to bulk soil. And the DHA values in C0, C25 and C50 were 1.2, 1.9 and 1.9 times higher in rhizosphere soil than in bulk soil. The rhizosphere activity of clover was comparable to that of peas. The rhizosphere activity at week 10 was less than that at week 6.

6.3.7   Effect of organic manure on plant growth and yield

Both peas and clover were able to establish in the contaminated soil, but were stunted and chlorosis was observed. However, plants grew better in the organic
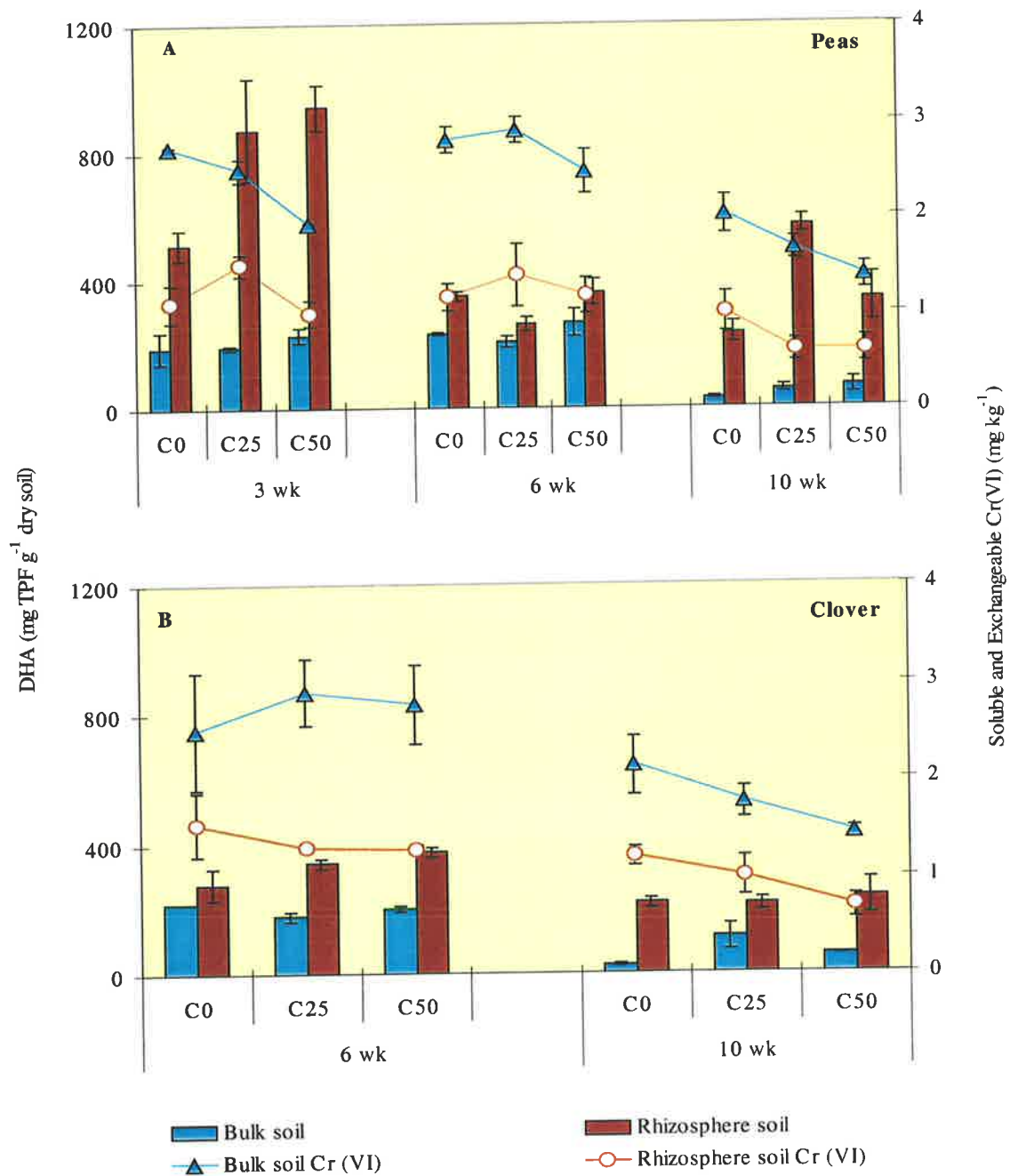
Fig. 6.6 DHA and soluble Cr (VI) concentration in bulk and rhizosphere soils of peas and clover (Error bars indicate ± sd)

amended pots with no symptoms of chlorosis. Again, the increasing rate of cow manure had a positive effect on growth of peas and clover (Fig 6.7).

Initially, the growth of peas was better than that of clover. Clover took nearly 4 weeks to establish. The number of flowers and pods formed in pea plants were more in C50 treatment followed by C25 and C0. The increased availability of nutrients from cow manure would have had an alleviating effect on toxicity exerted by this contaminated soil to the plants.

After 10 weeks, the height of plants, number of pods and fresh weight of the pods were recorded per pot in peas (Table 6.4). Compared to unamended soil (C0), the height and yield of peas were significantly higher in C25 and C50. There was 6.5 and 5.7 times higher yield in C25 (15.2 g) and C50 (14.3 g) than C0 (9.1 g). Similar trends were observed for clover with 2.4 (3.1 g) and 2.5 (3.3 g) times higher yield in C25 and C50, respectively.

6.3.8 Chromium uptake by the plants

The Cr concentration in shoot and roots of peas and clover was measured after the experimental period and results are given in Table 6.5. Roots of peas and clover accumulated more Cr than shoots. The addition of organic manure increased the amount of Cr in shoots that varied from 27.1 (C0), 42.6 (C25) and 49 (C50) mg kg^{-1}. However, clover did not accumulate significantly high levels of Cr except C25 (27.5 mg kg^{-1}).

The increase in organic amendment increased the amount of Cr accumulated in clover roots, however, this trend was not proportional in peas. In clover roots, the Cr increased from 780 (C0) to 4067 (C50) mg kg^{-1}. In peas, the root accumulation was higher in C25 treatment (1410 mg kg^{-1}) than in C0 (750 mg kg^{-1}) and C50 (922 mg kg^{-1}). It has to be noted that adhesion of fine sludge particles which cannot be completely removed by repeated washing probably contributed to high values of Cr in roots to some extent.

6.4 DISCUSSION

In situ remediation using soil amendments and plants is rapidly gaining acceptance as a cost-effective and environment friendly technique for cleaning highly contaminated soils. The major problem encountered at the Mount Barker, Adelaide, contaminated site is the presence of Cr (VI) at concentrations exceeding Australian National Environment Protection measures. Presence of Cr(VI) in the subsurface soils and ground water led to
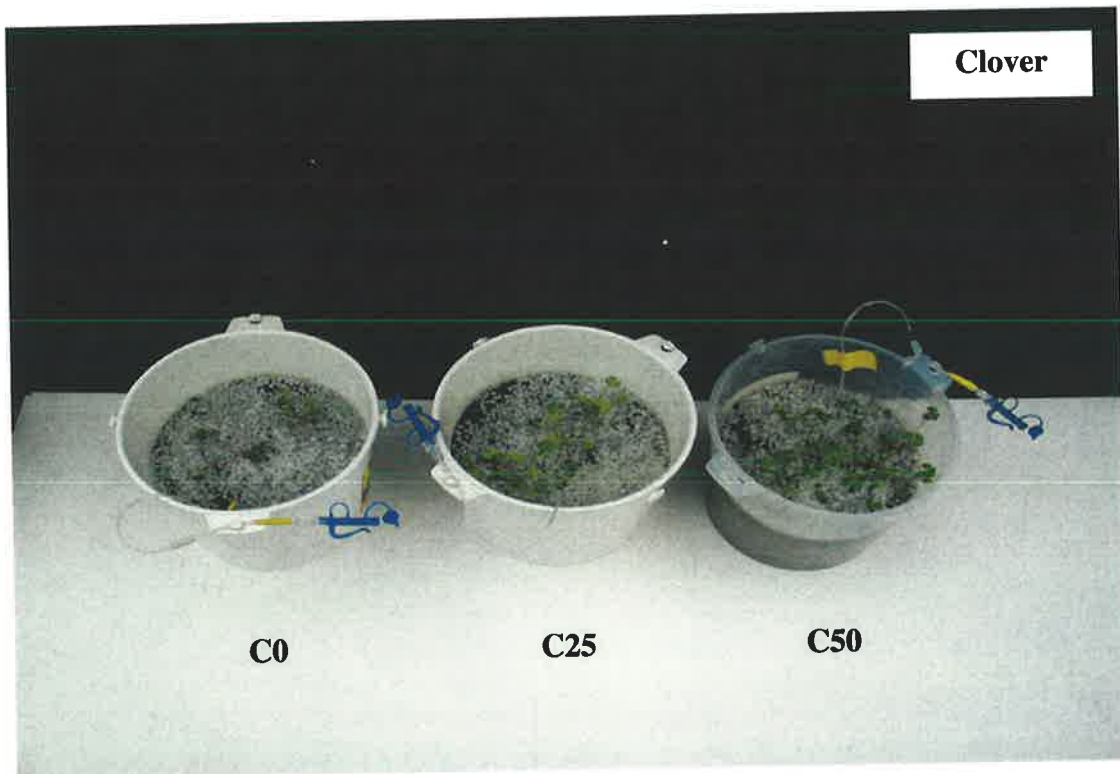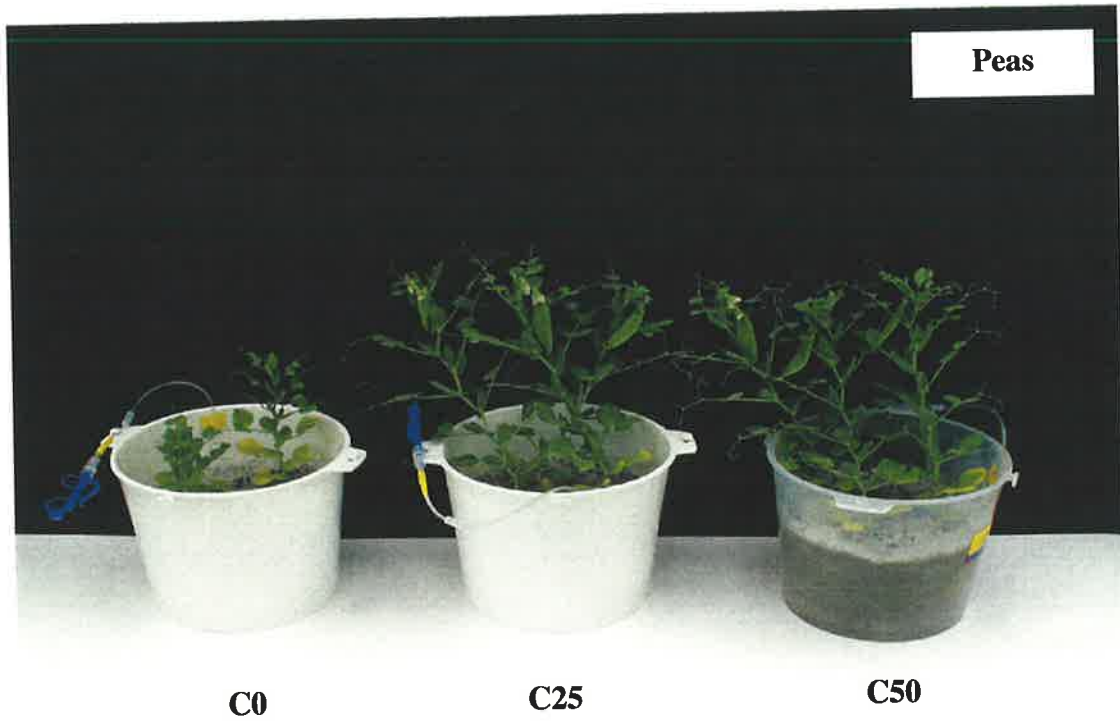
Fig. 6.7. Photographs of peas and clover grown in cow manure amended (C25, C50) and unamended (C0) contaminated soils.

Table 6.4 Yield of peas and clover per pot grown on contaminated soil unamended and amended with cow manure

Treatment	Peas			Clover
	Height (cm)	No. of pods	Wt (g)	Wt (g)
C0	14.1 (2)	3.0 (0)	9.1 (1.9)	0.8 (0.4)
C25	21.3 (1)	4.3 (1)	15.2 (1.9)	3.1 (0.8)
C50	20.5 (1)	3.0 (0)	14.3 (0.8)	3.3 (0.8)

Figures in the parenthesis indicate sd of 4 replicates

Table 6.5 Cr concentration in the root and shoot of the peas and clover plants

Treatment	Peas		Clover	
	Shoot	Root	Shoot	Root
C0	27.1 (16.4)	750 (353)	ND	780 (413)
C25	42.6 (27.5)	1410 (176)	27.5 (15)	2713 (638)
C50	49.0 (15.9)	922 (526)	ND	4067 (704)

Figures in the paranthesis indicate sd of 4 replicates ND – Not detected, Values < 0.1 mg kg^{-1}

major concerns amongst the public and regulators prompting the need for remediation of the contaminated sites. Given the large volume of contaminated soils and the high cost of excavation and transportation to prescribed landfill sites, this study examined techniques for converting Cr(VI) into Cr(III); the Cr(III) being thermodynamically stable and also the form that binds tightly to soil colloidal fraction. Hence, the major focus for remediation was to convert the Cr (VI) to Cr (III) followed by immobilisation of the latter.

6.4.1 Organic amendments for Cr(VI) reduction

Cow manure as an organic amendment decreased the release of Cr(VI) in the soil solution over unamended control C0. Organic amendments are known to be effective in Cr(VI) reduction (Losi et al., 1994a). Also, organics can serve as a nutrient source to both microbes and plants and thus, may alleviate toxicity in contaminated soil. A significant positive relationship existed between the decrease in soil solution Cr(VI) concentration and DOC depleted during the first 6 weeks. The DOC depletion could be attributed to the increased microbial activity, as reflected in increased DHA, which in turn contributed to the reduction of Cr(VI).

Recently, it has been shown that soil amendments with glucose (Turick et al., 1998) or molasses (Rege et al., 1997; Turick et al., 1997) enhanced the microbial activity, aiding Cr(VI) reduction compared to unamended soils (Turick et al., 1998).

In soil solution, there was a decrease in Cr(VI), but no Cr(III) was detected. This indicated that any Cr(III) formed was tightly bound to soil colloidal material. Higher amounts of organic matter have been used to lock up metals as stable complexes to organic colloids (Harrison, 1992).

Generally, pH plays an important role in determining the Cr speciation, Cr(III) forms complexes with organics around pH 7 (James, 1983), but in this particular soil since the pH was close to 8, Cr hydroxides were likely to be formed (Losi et al., 1994b).

Furthermore, the decrease in soluble and exchangeable Cr(VI) fractions in amended treatments (Fig. 6.5), indicated that organics as an amendment play a major role in transformation of easily bioavailable Cr(VI) fractions to less bioavailable fractions as $Cr(OH)_3$. Fractionation studies on Cr(VI) spiked soil after addition of organics as *Pinus radiata* bark was reported to increase the retention of Cr (VI) in soil,

thus decreasing the uptake by sunflower (Bolan and Thiagarajan, 2001). Also the bark helped in conversion of Cr(VI) to Cr(III) and also decreasing the soluble Cr(VI) fraction in the soil solution. At later stages, these amendments resulted in more Cr in organic-bound, oxide-bound and residual fractions than in soluble and exchangeable fractions. Liming of soils decreased the release and uptake of Cr(III), probably due to the formation of insoluble Cr hydroxides.

In the process of precipitation and immobilisation in the present study, no such increase in Cr was observed in organic bound fraction with time (remaining constant at initial value, 13.8 ± 1 g kg^{-1}) subsequent to the decrease in soluble Cr(VI) fraction. Only a small fraction of Cr(VI) (mg kg^{-1}) in soluble pool gets reduced compared to the very high concentration of organic bound Cr (g kg^{-1}) and even if the precipitated Cr(III) was bound to less mobile organic fraction, it would be practically undetectable.

The effect of test plants, peas and clover on Cr(VI) concentration in soil solution, albeit a decrease, was significant until week 6 ($p < 0.05$). As observed for the unplanted treatments, the planted treatments also decreased soluble and exchangeable Cr(VI) fractions in soil solution (Fig 6.5). Evidently, cow manure had a major role in decreasing the concentration of Cr(VI) in soil solution ($p < 0.05$), compared to plants. The cow manure also had a positive effect on growth of these crops both pea and clover (Table 6.4). Clearly, the high nutrient content in cow manure alleviated the phytotoxicity exerted by high Cr concentrations in contaminated soil. But, clover was more sensitive than peas to the toxicity in this tannery waste contaminated soil probably due to the increased uptake of Cr (Table 6.5).

6.4.2 Stabilisation and immobilisation of Cr(VI)

Though the organic manure showed a positive effect in Cr(VI) reduction, this process was only short lived in this soil. It can be speculated that the Cr(VI) observed in due course might be due to Cr oxidation by Mn oxides; however, as discussed before (Chapter 5), the Cr oxidising potential of this soil is less compared to its reduction capacity. Since the cow manure contained a very high concentration of phosphate, this would have released more Cr(VI) from the exchangeable fraction. But this argument might not be valid, as increase in the organic amendment in fact released less Cr(VI) in soil solution. Likewise, (Losi, 1994) found the decrease in the concentration of Cr(VI) in the drainage water from the unamended soil was higher than that from soils amended with 12 Mg OM ha^{-1} and 50 Mg OM ha^{-1}. The Cr(VI) release from the contaminated

soil in the present study, following an initial decline is not surprising given the large pool of Cr(VI), 40 mg kg^{-1} (Avudainayagam, 2002) prevalent in this soil. The preliminary study described here suggests that amount of organic matter added was presumably not sufficient for complete transformation of labile Cr(VI) to non-toxic Cr(III) in this soil.

Use of chemical amendments like FeSO$_4$ to this contaminated soil also showed a similar effect of initial reduction followed by a release of Cr(VI) (Avudainayagam, 2002). The reappearance of Cr(VI) in soil solution was attributed to the nature of Cr present at this site, CaCrO$_4$, a sparingly soluble salt (Avudainayagam, 2002 #11). The slow release of Cr(VI), similar to slow-release fertilisers, is characteristic of old sludge materials (Bartlett and James, 1988). The increased proportion of exchangeable Cr(VI) fraction compared to soluble fraction further confirms that ageing resulted in release of Cr(VI) (Fig. 6.8).

Evidently, Cr(VI) was not completely removed from soil by organic amendment and/or plants. Apart from organic manure, the non-availability of O$_2$ (complete reducing conditions) is known to promote the complete reduction of Cr(VI) (Bloomfield and Pruden, 1980). The soil in this experiment was always held at 75% WHC which would provide aerobic conditions. Submergence of amended and unamended contaminated soil after harvest of the plants for a week, in the present study, effected almost complete disappearance of Cr(VI) (<0.1 mg L^{-1}) in soil solution. Soil submergence decreases the redox potential and addition of organic matter hastens this reduction (Ponnamperuma, 1972). There is evidence that redox potential of around –200 mV favours the reduction of Cr(VI) by resting cells of *Agrobacterium radiobacter* EPS-(916) (Llovera et al., 1993). Chromate reduction correlated with the redox potentials of the resting cells.

6.4.3 Effect of plant and rhizosphere on stabilisation of Cr(VI)

The release of Cr(VI) into soil solution is governed by the crop plant grown. For instance, pea plants released more Cr(VI) than clover. Plants are known to differ in their root distribution system and root exudate pattern. Peas with a more extensive root system than clover probably released more organic acids causing greater dissolution of more metals, as noticed with Cr(VI), in the soil solution which in turn can enhance the availability of Cr(VI) for uptake by the plants. The high concentration of Cr associated with pea roots at C25 was due to the increased availability of Cr(VI) in soil solution (Table 6.5).
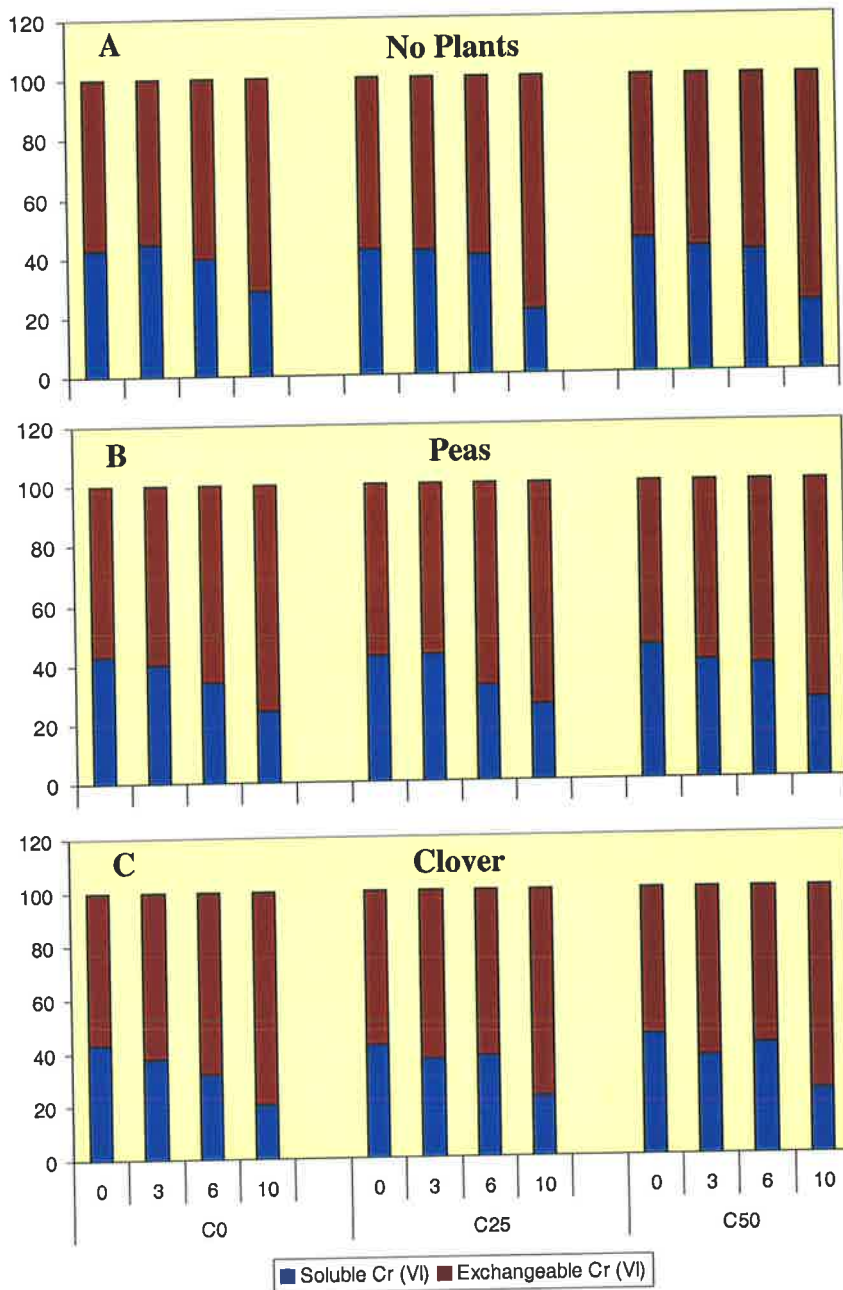
Fig 6.8 Changes in soluble and exchangeable Cr(VI) fractions estimated by a sequential extraction method for no plants (A), peas (B) and clover (C).

Dissolution of essential elements for plant growth by plant root exudates is well known. But again, release of anionic and soluble Cr(VI) by organic acids might not be practically feasible.

The data presented in this study demonstrate that plant-mediated release of Cr(VI), as noticed with peas, can increase the mobility of toxic and soluble Cr(VI) to groundwater. Amendment with cow manure alleviated, though not completely, the release of Cr(VI) into soil solution. But, it is not clear whether the reduced concentration of Cr(VI) in soil solution in the presence of both plants and organic manure was due to decreased plant-mediated release or enhanced reduction of released Cr(VI). Treatments with C50 always resulted in less release of Cr(VI) proving the essential role of organic amendment. Likewise, in low organic soils amended with 12 tons ha^{-1} of cow manure, a more significant reduction of Cr(VI) occurred in treatments without plants than in treatments with plants (Losi et al., 1994a). Plant roots and associated microorganisms are responsible for an active modification of the chemical and physical properties of the adjacent soil and thus have an important influence on the bioavailability of elements (Mench et al., 1998). Though there existed an increased release of Cr(VI) by peas into soil solution, increased microbial activity around the rhizosphere significantly reduced the Cr(VI) in the soil. The significant relationship between the DHA and Cr(VI) in rhizosphere soil (r^2=0.54, p< 0.05) (Fig. 6.8) further confirms this observation. The role of microbial reduction of Cr(VI) has been widely reported and has been harnessed for bioremediation of contaminated soils (Deleo and Ehrlich, 1994; Fude et al., 1994; Turick et al., 1997; Turick et al., 1998). Microbial immobilisation of Cr(III) formed by the cell wall components (Gadd, 2000) and its adsorption could further reduce the release of Cr(VI) in soil solution. The role of rhizosphere microorganisms and its association has been widely studied in phytoremediation research that enhances the degradation/ detoxification of metals. Plants interact specifically or non-specifically to determine the microbial communities harbouring the soil. Such plant-microbe interactions are important in the detoxification of the metals and their toxicity to plants (Siciliano and Germida, 1998).

In the vicinity of the rhizosphere, the pH is usually two or more units lower than the surrounding non-rhizosphere, thus favouring the reduction of Cr(VI) as reported in the case of wheat rhizosphere (Chen et al., 2000). The Cr(VI) reduction experiments were studied using rhizobox system and it revealed that 10 mg kg^{-1} of Cr(VI) was

reduced near vicinity of wheat rhizosphere as against 2 mg kg^{-1} reduction in bulk soils. The reduction of Cr(VI) by plants roots has been proven both in terrestrial (Chen et al., 2000) and aquatic plants (Mel Lytle et al., 1998). Apart from pH, Cr(VI) reduction in the rhizosphere was affected by aqueous Fe(II), organic compounds and H_2S (Bartlett and Kimble, 1976a; Eary and Rai, 1991). Application of acidifying fertilizers were also effective in enhancing Cr(VI) reduction (Chen et al., 2000) around the rhizosphere.

6.5   CONCLUSION

Phytostabilisation trials to immobilise anionic Cr(VI) in an alkaline soil showed that organic manure could be used as an effective amendment to prevent the release of Cr(VI) in a long-term contaminated soil. Apart from the selection of manure, the selection of crop plants is also crucial. Since pea plants enhanced the release of Cr(VI), probably due to high root biomass, pea plants alone may not be a suitable option for phytostabilisation of this site. However, it must be mentioned here that the pea plants showed a positive rhizosphere effect on decreasing the Cr(VI) concentration. More importantly the results indicated, that at this site, the total pool of Cr(VI) and its nature govern the prevalence of Cr(VI). More trials on various amendments and different crops are necessary to formulate a phytostabilisation programme for this long-term tannery waste contaminated sites.

CHAPTER 7

SUMMARY AND CONCLUSIONS

Chromium contamination resulting from land-based disposal of tannery wastes is one of the most serious environmental contamination problems confronting regulatory bodies and the public throughout the world. In India alone, over 55,000 ha of highly productive land has been declared unsafe due to the presence of high levels of Cr(VI). At this site Cr(VI) (> 8 mg L^{-1}) has also been detected in ground water. Similar contamination has been reported in Bangladesh, China, Nepal, Sri Lanka and a number of countries in Europe and South America (ACIAR, 2000; Naidu et al., 2000b). In developed countries such as Australia, extensive contamination has been reported at old tannery waste disposal sites but the number of sites is not as prolific as in developing countries.

Numerous investigators have conducted extensive studies concerning the fate and behaviour of Cr in the soil environment and the potential options for managing Cr contaminated soils (Chapter 2). However, most of these studies have focused on freshly contaminated soil and the outcomes may therefore not be directly relevant to long-term contaminated soils. Unlike freshly contaminated soils, in which contaminant bioavailability is high, long-term contaminated soils show appreciable decline in bioavailability with time. The present study focused on the impact of Cr present in tannery wastes on soil biota and considered the potential options for managing such contaminated sites. The focus of this study was a long-term tannery waste contaminated site at Mount Barker, Adelaide. The key findings from this study follow:

- Preliminary investigations at the tannery waste disposal sites revealed extensive contamination of both surface and subsurface soils with Cr. Based on Cr content alone, the soils could be subdivided into low (Cr content), medium (Cr content) and highly (Cr content) contaminated soils. Tannery waste disposals had led to subsurface contamination and major changes in soil pH (6.8 - 8.7) and OC content (0.1 - 15%). The most surprising observation made at this site was the presence of elevated levels of Cr(VI) in soil solution in highly contaminated soils (0.3 mg L^{-1}), even at 50-100 cm depths.

- Further detailed studies on the effect of contamination on soil biological properties revealed that contamination led to a distinct inhibition in the activities and distribution of microbial populations. Microbial populations, as determined by phospholipid fatty acid (PLFA) analyses, showed a major shift in associated microflora with increased contamination. As the level of contamination increased, bacterial (i15:0, a15:0, 15:0, i16:0, i17:0, a17:0, cy17:0, cy19:0) and fungal (18:2ω6c) growth was inhibited the most. In addition, dehydrogenase activity (DHA, an indicator of overall microbial activity) was also inhibited due to contamination. These changes in biological properties appear to be due to the toxic effect of total Cr and soluble Cr(VI) at this site.

- In addition to impacting biological soil biological properties, tannery waste disposals had also led to elevated levels of Mn (0.3 - 0.6 g kg^{-1}), mainly as insoluble Mn oxides since no soluble Mn(II) was detected in the soil solution. The presence of Mn was not unexpected since Mn salts were added to minimise sulphide production at the effluent disposal sites. Correlation studies revealed a significant linear relationship between soil solution Cr and exchangeable Cr(VI); indicating the possibility of Mn mediated Cr oxidation in these soils.

The presence of Cr(VI) in these highly contaminated soils with organic matter exceeding 11% was unexpected since the thermodynamics of Cr speciation would suggest rapid transformation of any Cr(VI) to Cr(III) in the presence of high organic matter. For this reason, this thesis examined other possible soil biochemical processes that could influence Cr chemistry in these contaminated soils. Hence, the potential coupling reaction involving biotic-abiotic transformation of Cr [Mn(II)-Mn(IV)---Cr(III)-Cr(VI)] was investigated.

- Investigations revealed a predominance of Mn oxidising bacteria in these soils (4% of total bacterial population). One pure bacterial strain (SKMn2) and consortium (SKCons2) were examined in detail for their Mn oxidising potential. Both the pure culture and enrichment could oxidise > 60 % of the spiked Mn(II) in the culture broth. Maximum Mn oxidising activity coincided with the lag phase of the growth cycle of these cultures.

- Deposition of Mn oxides by microbial Mn oxidisers was a slow process.

Detailed XRD investigations revealed that rhodochrosite ($MnCO_3$) was initially formed in the Mn oxidising broth (pH 7.0, 30°C) and was later oxidised to bixbyite (Mn_2O_3). These oxides further crystallised to manganese oxide (MnO_2) after 6 months. Characterisations of these oxides revealed that they constituted 306 mg g^{-1} of Mn and had a surface area of 10.7 m^2 g^{-1}.

- Microbially formed Mn oxides were capable of oxidising Cr(III) in concentrations higher than commercial Mn(IV) and Mn(III). Further detailed kinetic studies using solutions containing a mixture of 1000 μM Cr(III) and 1500 μM of microbial Mn oxides led to oxidation of 0.6 – 4.9% of Cr(III). There was an increase in Mn(II) concentration with a concomitant increase in Cr(VI). There existed a linear relationship between Cr(VI) formed and also Mn(II) formed. Though the reaction stoichiometry could be calculated for reagent grade Mn oxides, it was difficult to calculate a definitive reaction stoichiometry in the case of microbial oxides because of impurities in these oxides such as organic matter and residual Mn(II) concentrations.

- Mn oxides in contaminated soil extract were able to oxidise 5 mg Cr(III) L^{-1} to Cr(VI) in solution and the majority of the Mn oxides formed were hausmannite (Mn_3O_4).

Following detailed investigations of the impact of tannery wastes on soil biota and potential factors controlling Cr(III) dynamics in these contaminated soils, this thesis examined possible options for remediating the Cr contaminated soils. Following a literature review and evaluation of both chemical and biological techniques previously examined, an attempt was made to study the potential for phytoremediation of these soils using phytostabilisation techniques. The current investigation focused on establishment of plants in the contaminated soils with and without cow manure amendment. Crops used during the study included peas and clovers and rate of cow manure amendment was 25 and 50 tons ha^{-1}. Cow manure was added to assist with the difficult task of establishing most crops in the presence of toxic levels of Cr(VI). Remediation of this site using this *in situ* phytostabilisation technique revealed that cow manure amendment had indeed alleviated toxicity and helped to establish both crops. Higher rates of organic manure decreased the amount of Cr(VI) leached into the soil solution. Specifically, the results indicated that,

- Contaminated soil amended with organic manure at the rate of 50 tons ha^{-1} was initially more effective in Cr(VI) reduction and also retarded the long-term release of Cr(VI) into soil solution.

- Plants, peas and clover contributed to Cr(VI) reduction, however the reduction was not irreversible. Although clover was more sensitive to the contamination than peas, as indicated by plant establishment, clover released less Cr(VI) to soil solution in the long-term. Since peas released more Cr(VI) to soil solution they might be an unsuitable crop choice for the phytostabilisation technique.

- Plants aided significant Cr(VI) reduction around the rhizosphere due to increased microbial activity (as indicated by DHA). The microbial activity was significantly ($p < 0.05$) higher in rhizosphere soils of both clover and peas and this correlated with low Cr(VI) concentrations.

- Fractionation studies revealed that as remediation progressed there was a depletion in the soluble Cr(VI) pool and a corresponding increase in the exchangeable Cr(VI) pool. This indicated that Cr(VI) dissolution from tannery sludge in this soil was occurring but was a slow process.

Future research needs and directions

This research focused primarily on biotically mediated processes that controlled Cr(III)-Cr(VI) dynamics in long-term tannery contaminated soils. However, there still remains many unanswered questions. Some specific suggestions for future research follow.

- There is a dearth of research on long-term tannery waste contaminated soils. Future research should concentrate on factors that influence Cr(VI) reduction and stability and should study the long-term changes in Cr(VI) transformations in field based studies.

- Although there are a few studies on the effect of short-term exposure of Cr on

microbial activities in soils and some soil spike studies, only few reports exist on the long-term effect of tannery waste contamination on microbial activity and populations. The short-term studies cannot be extrapolated to these complex long-term contaminated soils. To our knowledge, the impact of tannery wastes on microbial populations as studied here is the first such attempt. More detailed studies will enable researchers to better understand shifts in microbial populations due to Cr contamination. Artificial spiking studies of Cr to soil and related microbial activity also do not realistically represent the Cr transformations happening in contaminated soils.

- Another major factor to be considered is the biotic-abiotic coupling reactions in soil. It is evident from the current study that major Cr transformations are governed by both factors. Extension of this study on biotic Mn oxidation and abiotic Cr oxidation to different soil types will provide more insight into the Cr oxidation processes in soils.

- From a practical point of view, efficient but cost effective technologies should be tried for remediation. As mentioned earlier, phytostabilisation techniques are becoming popular for remediation of heavy metal contaminated soils. Preliminary investigations performed here reveal that there is a good possibility for utilisation of this technique for Cr contaminated soils. Different amendments such as combination of Fe and organic matter could also be tested to completely immobilise Cr(VI). Also screening of Cr tolerant plants for Cr stabilisation needs further study. Furthermore, suitable plants may also be used to release more Cr(VI) to soil solution by exudation of organic acids from their roots, which can later be more readily reduced using appropriate amendments. This process will help to reduce Cr(VI), even from exchangeable pool, thereby depleting the total pool of Cr(VI) in soils.

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Appendix I

The chemical and microbial characteristics of soil samples collected from an adjacent uncontaminated site (UC) are included.

From the Table 1, it is evident that the uncontaminated site is acidic in nature and had low organic matter and no background chromium. Also the measured DHA was very low when compared to the contaminated sites. The decrease may be significantly due to the low pH as earlier reports suggest that DHA in acidic soils are lower than the alkaline soil.

The concentration of bacterial and fungal specific PLFAs in uncontaminated soil samples was lower compared to the low contaminated sites (0-10 cm depth) (Figure 1). However, the concentration was higher than the medium and highly contaminated sites. Principal component analysis (Figure 2) showed that microbial population is the uncontaminated site was similar to the low contaminated soils as they grouped together, however, the contamination may not be a major role as it had other variables like low pH and also OC. Since the main objective of the first phase of study (chapter 4) is to identify the effect of Cr contamination, it may not be relevant to compare with this particular uncontaminated soil.

Table 1. Chemical and microbial characteristics of uncontaminated soil site

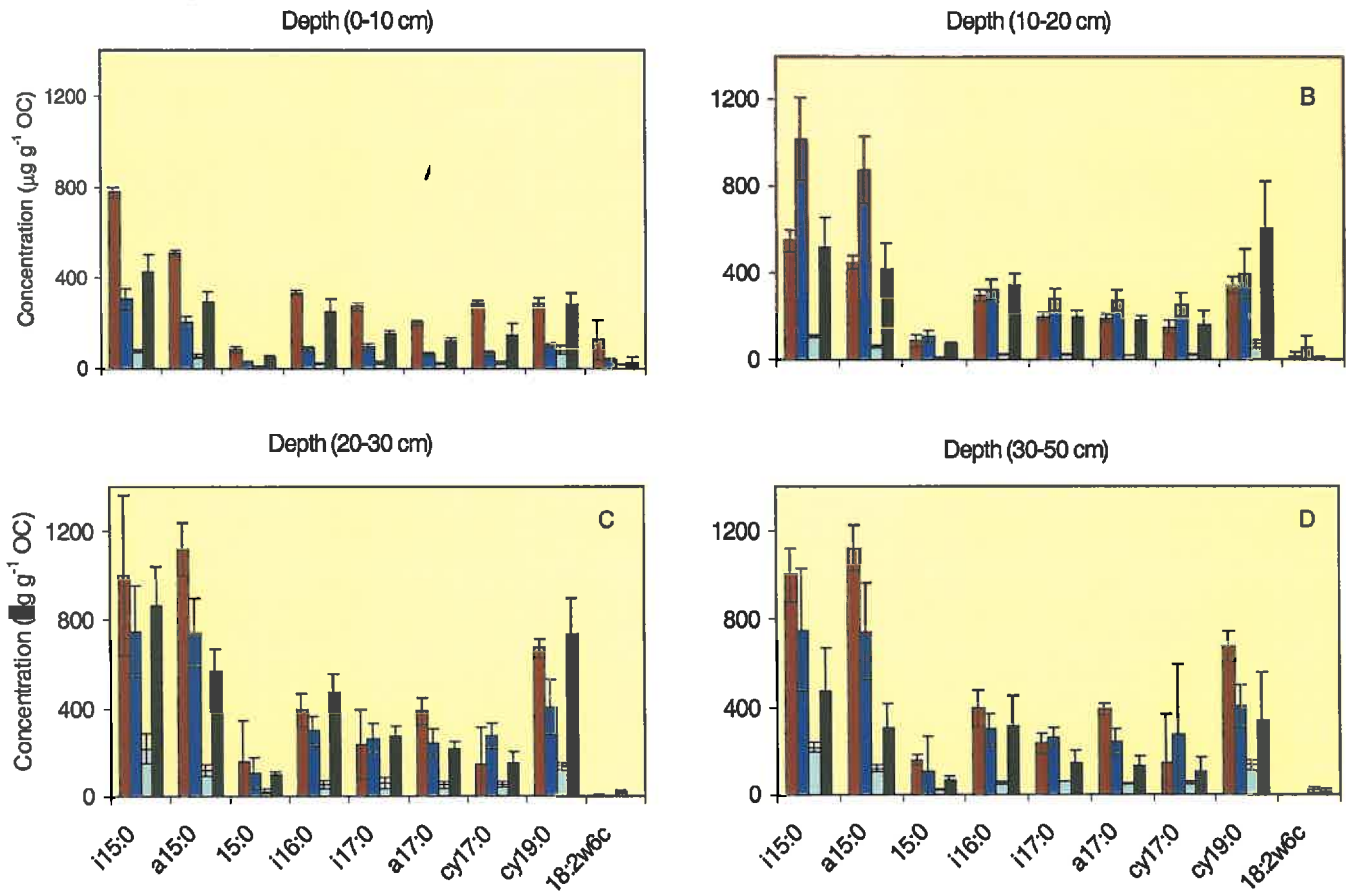| Depth (cm) | pH | EC (dS m^{-1}) | Organic carbon (%) | Total metals | | | DHA (μg TPF g^{-1} soil) | DHA (μg TPF g^{-1} OC) |
				Cr (g kg^{-1})	Mn (g kg^{-1})	Fe (g kg^{-1})		
0-10	6.0	0.05	1.6	0.012	0.10	5.4	32	1973
10-20	5.5	0.05	0.9	0.007	0.07	5.8	10	1127
20-30	5.6	0.04	0.6	0.009	0.05	4.6	3	577
30-50	5.5	0.05	1.0	0.009	0.05	5.3	3	340
50-100	5.7	0.03	0.3	0.011	0.09	7.6	0	0

Figure 1 Concentration of bacterial and fungal specific PLFAs of uncontaminated and contaminated sites ■ Low ■ Medium □ High ■ UC
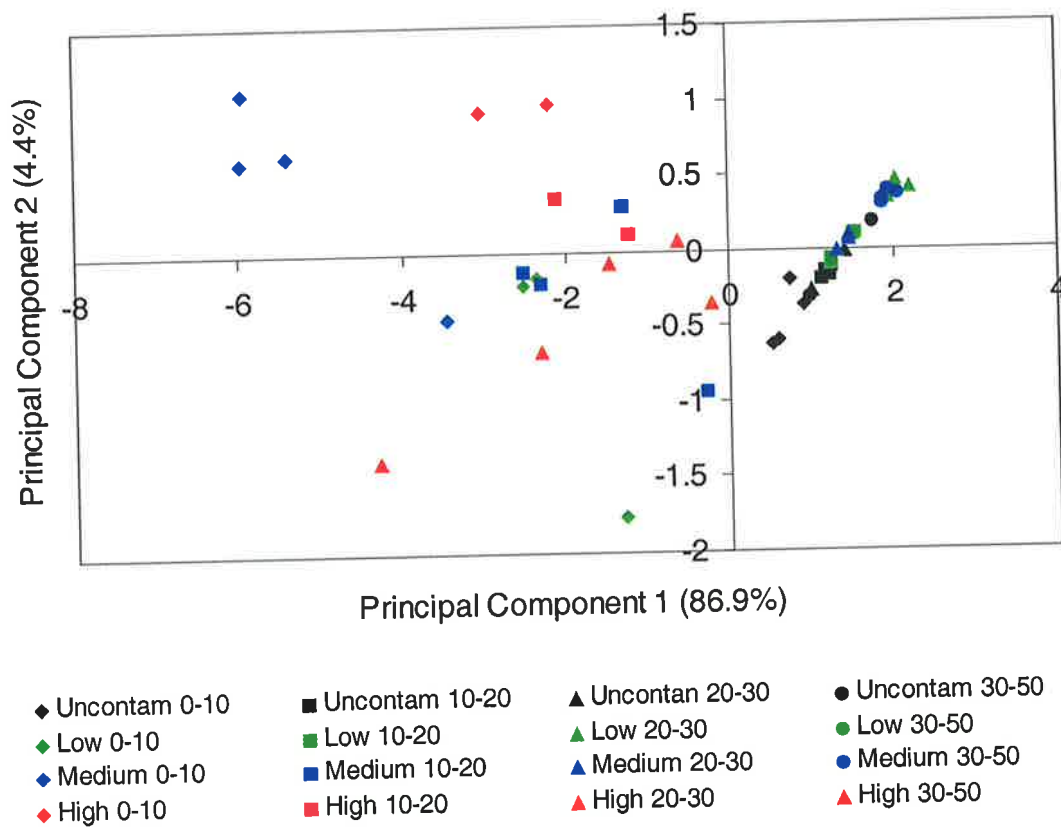
Figure 2 PCA of PLFAs from UC, low, medium and highly contaminated soils Note that UC soils group along with low contaminated soils but the changes in population might not be due to contamination. This might be influence of low pH and also low OC.

Appendix II

Leuco-crystal violet reagent (Kessick et al., 1972)

Dissolve 0.1% leucocrystal violet in distilled water. Dilue 2 ml of a 0.1% leucocrystal violet in 0.1N perchloric acid with 5 ml 0.1M acetic acid acetate buffer (final pH 4.0). This reagent was used in detection and quantification of Mn oxides as it turns blue after reacting with oxides. This solution is stored at 4°C.

Phosphate buffer saline

Dissolve 1.18 g of Na_2HPO_4, 0.22 g of $NaH_2PO_4.H_2O$ and 8.5 g of NaCl in 1000 ml of water. If necessary, adjust the pH to 7.2. This is used as diluent in enumeration of bacteria