THE IMPACT OF HEAVY METALS ON THE AEROBIC BIODEGRADATION OF 1,2-DICHLOROETHANE IN SOIL

BY

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Submitted in fulfilment of the academic requirements for the degree of Master of Science (MSc) in the Discipline of Microbiology, School of Biochemistry, Genetics and Microbiology, Faculty of Science and Agriculture at the University of KwaZulu-Natal (Westville Campus).

As the candidate's supervisor, I have approved this dissertation for submission.

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ABSTRACT

1,2-Dichloroethane (1,2-DCA), a short chain chlorinated aliphatic compound, is one of the most hazardous toxic pollutant of soil and groundwater, with an annual production in excess of 5.44×10^9 kg. The major concern over soil contamination with 1,2-DCA stems largely from health risks. Owing to their toxicity, persistence and potential for bioaccumulation, there is a growing interest in technologies for their removal. Many sites are, however, co-contaminated with a complex mixture of 1,2-DCA and heavy metal contaminants. Co-contaminated environments are considered difficult to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently. Therefore, the objective of this study was to evaluate the aerobic biodegradation of 1,2-DCA by autochthonous microorganisms in soil co-contaminated with 1,2-DCA and heavy metals, namely; arsenic (As^{3+}), cadmium (Cd^{2+}), mercury (Hg^{2+}) and lead (Pb^{2+}), via a direct and quantitative measurement of the inhibitory effects of heavy metals in a microcosm setting. Effects of various metal concentrations and their combinations were evaluated based on the following: (i) degradation rate constants; (ii) estimated minimal inhibitory concentrations (MICs) of metals; (iii) concentrations of heavy metals that caused biodegradation half-life doublings (HLDs); and (iv) heavy metal concentrations that caused a significant effect on biodegradation ($> 10\%$ increase in $t_{1/2}$ of 1,2-DCA). The effects of biostimulation, bioaugmentation and the addition of treatment additives on the biodegradation process were evaluated. The presence of heavy metals was observed to have a negative impact on the biodegradation of 1,2-DCA in both clay and loam soil samples, with the toxic effect being more pronounced in loam soil for all heavy metal concentrations except for Hg^{2+}, after 15 days. Heavy metal concentrations of 75 mg/kg As^{3+}, 840 mg/kg Hg^{2+}, and 420 mg/kg Pb^{2+}, resulted in 34.24%, 40.64%, and 45.94% increases in the $t_{1/2}$ of 1,2-DCA, respectively, in loam soil compared to clay soil. Moreover, the combination of four heavy metals in loam soil resulted in 6.26% less degradation of 1,2-DCA compared to clay soil, after 15 days. Generally, more than 127.5 mg/kg Cd^{2+}, 840 mg/kg Hg^{2+} and 420 mg/kg of Pb^{2+} was able to cause a $>$ 10% increase in the $t_{1/2}$ of 1,2-DCA in clay soil, while less than 75 mg/kg was required for As^{3+}. An increased reduction in 1,2-DCA degradation was observed with increasing concentration of the heavy metals. In clay soil, a dose-dependant relationship between k_1 and metal ion concentrations in which k_1 decreased with higher initial metal concentrations was observed for all the heavy metals tested except Hg^{2+}. Ammonium nitrate-extractable fractions of bioavailable As^{3+} and Cd^{2+} concentrations varied greatly, with approximately $< 2.73\%$ and $< 0.62\%$ of the total metal added to the system being bioavailable, respectively. Although bioavailable heavy metal fractions were lower than the total metal concentration added to the system, indigenous microorganisms were sensitive to the heavy metals. Biostimulation, bioaugmentation and amendment with treatment additives were all effective in enhancing

the biodegradation of 1,2-DCA in the co-contaminated soil. In particular, biostimulation with fertilizer, dual-bioaugmentation and amendment with $CaCO_3$ were most efficient in enhancing 1,2-DCA degradation resulting in 41.93%, 59.95% and 51.32% increases in the degradation rate constant of 1,2-DCA in the As^{3+} co-contaminated soil, respectively, after 20 days. Among all the treatments, dual-bioaugmentation produced the highest 1,2-DCA degrading population of up to 453.33×10^7 cfu/ml in the Cd^{2+} co-contaminated soil. On comparison of the As^{3+} and Cd^{2+} co-contaminated soil undergoing either biostimulation or dual-bioaugmentation, similarity in the denaturing gradient gel electrophoresis (DGGE) banding patterns was observed. However, the banding patterns for the different bioremediation options demonstrated a difference in bacterial diversity between the fertilized and dual-bioaugmented samples. DGGE profiles also indicate that while numerous bands were common in the fertilized co-contaminated soils, there were also changes in the presence and intensity of bands due to treatment and temporal effects. Dehydrogenase and urease activities provided a more accurate assessment of the negative impact of heavy metals on the indigenous soil microorganisms, resulting in up to 87.26% and 69.58% decreases in activities, respectively. In both the biostimulated and bioaugmented soil microcosms, dehydrogenase activity appeared biphasic with an initial decrease followed by an increase in the treated soils over time. Results from this study provide relevant information on some alterations that could be introduced to overcome a critical bottle-neck of the application of bioremediation technology. In conclusion, the bioremediation strategies adopted in this study may be used as a rational methodology for remediation of sites co-contaminated with 1,2-DCA and heavy metals, subject to a thorough understanding of the microbial ecology and physico-chemical parameters of the site.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal (Westville Campus), Durban, South Africa from January 2008 to December 2009, under the supervision of Dr. A. O. Olaniran and the co-supervision of Professor B. Pillay.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

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ABSTRACT

1,2-Dichloroethane (1,2-DCA), a short chain chlorinated aliphatic compound, is one of the most hazardous toxic pollutant of soil and groundwater, with an annual production in excess of 5.44×10^9 kg. The major concern over soil contamination with 1,2-DCA stems largely from health risks. Owing to their toxicity, persistence and potential for bioaccumulation, there is a growing interest in technologies for their removal. Many sites are, however, co-contaminated with a complex mixture of 1,2-DCA and heavy metal contaminants. Co-contaminated environments are considered difficult to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently. Therefore, the objective of this study was to evaluate the aerobic biodegradation of 1,2-DCA by autochthonous microorganisms in soil co-contaminated with 1,2-DCA and heavy metals, namely; arsenic (As^{3+}), cadmium (Cd^{2+}), mercury (Hg^{2+}) and lead (Pb^{2+}), via a direct and quantitative measurement of the inhibitory effects of heavy metals in a microcosm setting. Effects of various metal concentrations and their combinations were evaluated based on the following: (i) degradation rate constants; (ii) estimated minimal inhibitory concentrations (MICs) of metals; (iii) concentrations of heavy metals that caused biodegradation half-life doublings (HLDs); and (iv) heavy metal concentrations that caused a significant effect on biodegradation (> 10% increase in $t_{1/2}$ of 1,2-DCA). The effects of biostimulation, bioaugmentation and the addition of treatment additives on the biodegradation process were evaluated. The presence of heavy metals was observed to have a negative impact on the biodegradation of 1,2-DCA in both clay and loam soil samples, with the toxic effect being more pronounced in loam soil for all heavy metal concentrations except for Hg^{2+}, after 15 days. Heavy metal concentrations of 75 mg/kg As^{3+}, 840 mg/kg Hg^{2+}, and 420 mg/kg Pb^{2+}, resulted in 34.24%, 40.64%, and 45.94% increases in the $t_{1/2}$ of 1,2-DCA, respectively, in loam soil compared to clay soil. Moreover, the combination of four heavy metals in loam soil resulted in 6.26% less degradation of 1,2-DCA compared to clay soil, after 15 days. Generally, more than 127.5 mg/kg Cd^{2+}, 840 mg/kg Hg^{2+} and 420 mg/kg of Pb^{2+} was able to cause a > 10% increase in the $t_{1/2}$ of 1,2-DCA in clay soil, while less than 75 mg/kg was required for As^{3+}. An increased reduction in 1,2-DCA degradation was observed with increasing concentration of the heavy metals. In clay soil, a dose-dependant relationship between k_1 and metal ion concentrations in which k_1 decreased with higher initial metal concentrations was observed for all the heavy metals tested except Hg^{2+}. Ammonium nitrate-extractable fractions of bioavailable As^{3+} and Cd^{2+} concentrations varied greatly, with approximately < 2.73% and < 0.62% of the total metal added to the system being bioavailable, respectively. Although bioavailable heavy metal fractions were lower than the total metal concentration added to the system, indigenous microorganisms were sensitive to the heavy metals. Biostimulation, bioaugmentation and amendment with treatment additives were all effective in enhancing

the biodegradation of 1,2-DCA in the co-contaminated soil. In particular, biostimulation with fertilizer, dual-bioaugmentation and amendment with $CaCO_3$ were most efficient in enhancing 1,2-DCA degradation resulting in 41.93%, 59.95% and 51.32% increases in the degradation rate constant of 1,2-DCA in the As^{3+} co-contaminated soil, respectively, after 20 days. Among all the treatments, dual-bioaugmentation produced the highest 1,2-DCA degrading population of up to 453.33×10^7 cfu/ml in the Cd^{2+} co-contaminated soil. On comparison of the As^{3+} and Cd^{2+} co-contaminated soil undergoing either biostimulation or dual-bioaugmentation, similarity in the denaturing gradient gel electrophoresis (DGGE) banding patterns was observed. However, the banding patterns for the different bioremediation options demonstrated a difference in bacterial diversity between the fertilized and dual-bioaugmented samples. DGGE profiles also indicate that while numerous bands were common in the fertilized co-contaminated soils, there were also changes in the presence and intensity of bands due to treatment and temporal effects. Dehydrogenase and urease activities provided a more accurate assessment of the negative impact of heavy metals on the indigenous soil microorganisms, resulting in up to 87.26% and 69.58% decreases in activities, respectively. In both the biostimulated and bioaugmented soil microcosms, dehydrogenase activity appeared biphasic with an initial decrease followed by an increase in the treated soils over time. Results from this study provide relevant information on some alterations that could be introduced to overcome a critical bottle-neck of the application of bioremediation technology. In conclusion, the bioremediation strategies adopted in this study may be used as a rational methodology for remediation of sites co-contaminated with 1,2-DCA and heavy metals, subject to a thorough understanding of the microbial ecology and physico-chemical parameters of the site.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1   Introduction

In tandem with rapid industrial and economic advancement, human activities have lead to widespread pollution of the natural global environment (Ang *et al*., 2005). In recent years, concern about the presence, disposition, and persistence of chlorinated organic pollutants in the environment (air, soil, and water systems) has increased since most of the important classes of these chemicals have been shown to be carcinogenic in experimental animals thus posing a potential human health risk (Cerniglia, 1984; Cerniglia and Heitkamp, 1989). In addition, some have been shown to be toxic and carcinogenic to humans (van den Wijngaard *et al*., 1993). Chlorinated organic solvents are among the most widespread organic contaminants present in the groundwaters and subsurface soils of many contaminated sites. The physico-chemical properties of these compounds, particularly when they are classified as dense non-aqueous phase liquids (DNAPLs), make them difficult to remove once they have entered the subsurface and they remain among the most complex contaminants to remediate in the environment (Yoshida *et al*., 2005).

Among the short chained chlorinated aliphatic compounds, 1,2-dichloroethane (1,2-DCA) represents one of the world's most important toxic chlorinated aquifer pollutant (Marzorati *et al*., 2007), and it is produced industrially in larger volumes than any other halogenated compound (Laturnus, 2003). The major use of 1,2-DCA (more than 90% of production) is as a chemical intermediate in the synthesis of a number of chlorinated hydrocarbons, in particular; vinyl chloride which is used in making a variety of plastic and vinyl products including polyvinyl chloride (PVC) pipes (De Wildeman and Verstrate, 2003; IARC, 1999), trichloroethylene and tetrachloroethane (DEA, 2002; Hage and Hartmans, 1999). The widespread use of 1,2-DCA has resulted in serious environmental contamination (Hughes *et al*., 1994), and has resulted in its inclusion in the E. U. and U. S. priority lists of contaminants (Marzorati *et al*., 2005; USEPA, 1982). In addition, 1,2-DCA has been found in at least 570 of the 1585 National Priorities List sites identified by the U. S. Environmental Protection Agency (USEPA). Depending on environmental conditions, the estimated half-life of 1,2-DCA can range from months to decades due to its relatively high water solubility, potential for migration in soil, and very long persistence in anoxic groundwater (Barbee, 1994; Vogel *et al*., 1987). There is therefore, a growing interest in technologies for its removal. Bioremediation is a possible option to destroy contaminants completely or render them innocuous by using natural biological activity (Singh *et al*., 2008; Vidali, 2001). Microbial degradation has been proposed as an efficient strategy for organic waste removal, with distinct advantages over physico-chemical remediation methods; it uses relatively low cost, low technology techniques, and may be carried out on site to achieve the complete degradation of organic pollutants without collateral destruction of the site material or its indigenous flora and fauna (Timmis and Pieper, 1999). Also, biological processes and biodegradation of organic contaminants to innocuous end products (CO_2, cell

mass, water) minimizes the environmental impact and residual contamination (Kovalick Jr, 1992; NRC, 1994). Microorganisms have evolved several pathways for the biodegradation and/or transformation of various toxic pollutants (Singh *et al*., 2008). However, the presence of heavy metals in the environment can negatively influence the effectiveness of bioremediation strategies (Baldrian *et al*., 2000).

Forty percent of hazardous waste sites on the Environmental Protection Agency's (EPA's) National Priority List (NPL) are characterized by the concomitant presence of both organic contaminants and heavy metals, and bioremediation of these sites poses a complex problem because of the multiplicity of contaminants (Sandrin and Maier, 2003). Co-contamination often causes a synergistic cytotoxic effect on microorganisms, and the two components often must be treated differently (Hoffman *et al*., 2005; Sandrin *et al*., 2000; Sandrin and Maier, 2003). Such concerns have heightened the need for novel and advanced bioremediation techniques to effectively remove organic pollutants from a variety of co-contaminated environmental media including water, sediments and soil (Ang *et al*., 2005; Duran and Esposito, 2000). Metals most frequently found at USEPA Superfund sites are divided into two categories: *cationic* metals (metallic elements in soil with positively charged cations) and *anionic* compounds (elements in soil which are combined with oxygen and are negatively charged). The most common problem-causing cationic metals are mercury, cadmium, lead, nickel, copper, zinc and chromium, whereas the most common anionic compound is arsenic (NRCS, 2000). Common organic pollutants at these sites include petroleum, polycyclic aromatic hydrocarbons (PAH), chlorinated solvents, herbicides and pesticides (Amor *et al*., 2001; Hoffman *et al*., 2005). Few reports have focused on the adverse effects of heavy metals on biodegradation in co-contaminated environments under both aerobic and anaerobic conditions. These effects include extended acclimation periods, reduced biodegradation rates and failure of the degradation of the target compound (Kuo and Genthner, 1996; Said and Lewis, 1991). Complications of the effects of metal toxicity on organic pollutant biodegradation in co-contaminated soil and water environments stem from the fact that heavy metals may be present in a variety of chemical and physical forms, namely, ionic solutes, soluble complexed species and soil adsorbed species (Sandrin and Maier, 2003). Further impediments arise due to the effect of environmental conditions on the physical and chemical state of the metals. These conditions include pH, redox potential of the water phase as well as soil properties (ion exchange capacity, clay type and organic matter content) (Sandrin and Maier, 2003).

The clean-up of soil contaminated with both 1,2-DCA and heavy metals is a contemporary remediation issue as most of the current techniques are directed at the removal of individual contaminants (Gregor, 2001; Hirschorn *et al*., 2007; Janssen *et al*., 2005). Previous studies have focused extensively on the biodegradation of 1,2-DCA in several contaminated soil and water environments (van der Wijngaard *et al*., 1993), and toxic effects of heavy metals on soil microorganisms have also been studied (Rajapaksha *et al*., 2004). However, few reports exist on the biodegradation of 1,2-DCA in the presence

of heavy metals (Olaniran *et al*., 2009). Since heavy metals and 1,2-DCA are found together in most co-contaminated sites, it is necessary to evaluate the biodegradation of 1,2-DCA in co-contaminated soil and ascertain the effects of heavy metals on 1,2-DCA degradation. Also, due to the widespread use and release of organic pollutants and heavy metals, determining the combined effect on microbial activity and community composition is essential.

1.2   Chlorinated hydrocarbons

1.2.1   Classes

Chlorinated hydrocarbons are an important class of chemicals containing one or more chlorines in their molecular structures (Ma and Wang, 2009). In particular, chlorinated aliphatic compounds are a diverse group of synthetic industrial chemicals which play a significant role as environmental pollutants in subsurface soils and groundwaters of many contaminated sites. These chlorinated hydrocarbons are subdivided into chloroalkanes, chloroalkenes and chloroalkynes. Among the chloroalkanes, chloroethanes are further divided into the lower and higher chlorinated ethanes based on the differences in biodegradability (Sutherson, 1997). Examples of lower chlorinated ethanes include 1,1-dichloroethane and 1,2-dichloroethane which may be utilized as primary growth substrates by aerobic microorganisms but only cometabolized by anaerobic microorganisms (Sutherson, 1997). Higher chlorinated ethanes include 1,1,2-trichloroethane and tetrachloromethane (Sutherson, 1997). Most prominent with regard to industrial use, environmental persistence, toxicity and potential carcinogenicity are the chlorinated one-carbon, two-carbon and three-carbon compounds (Leisinger, 1996).

1.2.2   Properties and uses

Chlorinated hydrocarbons comprise the strong C-Cl bond, which confers high stability to these organic compounds. They have general physico-chemical characteristics: denser than and minimally soluble in water and volatile with variable vapor pressure (Sutherson, 1997). Most chlorinated compounds are classified as DNAPLs which tend to sink and accumulate on the non-permeable layer at the bottom of confined aquifers (Sutherson, 1997). This property in particular makes them complex to remove once they have entered the subsurface and they remain among the most difficult contaminants to remediate in the environment. When released to surface or subsurface systems, DNAPLs migrate through unsaturated zones until they reach groundwater tables. They then disperse throughout the saturated soils, sorbing strongly with soil organics and minerals and dissolving in groundwater (Ferguson and Pietari, 2000), creating long-term sources of contaminant plumes moving in the general direction of groundwater flow, thus potentially entering drinking-water supplies and threatening human health. Owing to their stability, several chlorinated hydrocarbons have been synthesized and used extensively for many years in industrial, commercial and agricultural applications (Fetzner, 1998). These compounds have widespread use in industry as solvents, degreasing agents, chemical feedstocks, and in some cases as pesticides.

1.2.3　Environmental and health effects

Chlorinated hydrocarbons are of major environmental concern since these contaminants are often found in groundwater, soil and the atmosphere due to improper disposal of wastes, accidental spillage, or deliberate release. Many of the chlorinated compounds are of public health concern since they are considered as suspected carcinogens or mutagens and potentially toxic to humans and microorganisms (Bouwer and McCarty, 1983). This concern is further compounded by the ability of these organochlorines to accumulate in the tissues of living organisms, a phenomenon known as bioaccumulation (Philips, 1993). Relatively small amounts of organochlorines present in water may be preferentially transferred and accumulated in the fats of aquatic plants and animals, with resulting concentrations being as much as 500 000 times or more than the surrounding waters. Living organisms may also accumulate chlorinated hydrocarbons through the food chain via a process termed biomagnification. As a result, concentrations of organochlorines may increase with trophic levels, with the highest concentrations being observed in higher consumers including certain fish, marine mammals, birds, or humans (Phillips, 1993).

1.3　1,2-Dichloroethane

1.3.1　Properties

1,2-Dichloroethane, commonly known as ethane dichloride, is a short chained chlorinated aliphatic compound which appears colourless and oily with a sweet taste and pleasant chloroform like odour (IPCS, 1995). The chemical formula for 1,2-DCA is $C_2H_4Cl_2$ and it has a molecular weight of 98.96 g/mol. It is a volatile liquid with a density of 1.2351 and vapour pressure of 8.5 kPa at 20°C, i.e., it evaporates quickly (ATSDR, 1993; IPCS, 1995). As a pure phase, 1,2-DCA is a DNAPL with moderate solubility in water (8,624 g/L at 20°C) and soluble in most organic solvents (HSDB, 2000; IARC, 1999; IPCS, 1998).

1.3.2　Production and uses

1,2-Dichloroethane is generally considered to be largely produced from anthropogenic sources, i.e., a synthetic manufactured chemical. However, 1,2-DCA can in low concentrations originate from natural sources (de Rooij *et al*., 1998). It was reported as the first chlorinated hydrocarbon to be synthesized (IARC, 1979), and is manufactured via the catalytic vapor-phase or liquid-phase chlorination of ethylene, or by oxychlorination of ethylene (Archer, 1979). In commercial ethylene oxychlorination reactors, gaseous ethylene, hydrogen chloride, and air react with catalysts at a temperature range of 473-573K and a pressure of 0.4-0.6 MPa (Magistro and Cowfer, 1986; Mallikarjunan and Hussain, 1983; Naworski and Velez, 1983). Currently, more than 17.5 million tons are produced annually in the United

States, Western Europe and Japan (Field and Sierra-Alvarez, 2004), and with a global capacity for vinyl chloride monomer of 35 million metric tons in 2005, production continues.

1,2-Dichloroethane is used almost exclusively as a feedstock for the manufacture of vinyl chloride (VC) monomers, primarily PVC (ATSDR, 2006; Bejankiwar *et al.*, 2005; Davis *et al.*, 2009). Polyvinyl chloride is one of the most mass produced thermoplastics in chemical industries of the world (Go *et al.*, 2010), and hence the increased production of 1,2-DCA across the globe. Smaller amounts of 1,2-DCA are used in the production of vinylidene chloride, 1,1,1-trichloroethane, trichloroethene, tetrachloroethene, ziridines, ethylene diamines and chlorinated solvents (ATSDR, 2001). In the past it has been widely used as an insect fumigant for stored grains and in mushroom houses, a soil fumigant in peach and apple orchards, metal degreasers, varnish removers and soaps (IARC, 1999). 1,2-Dichloroethane has also been used as a lead scavenger in fuels to prevent engine lead fouling (Falta, 2004).

1.3.3   Release

The widespread use of 1,2-DCA in a variety of products and in manufacturing processes has resulted in its frequent occurrence in sites contaminated with organic chemicals (DEA, 2002; Hage and Hartmans, 1999). The largest fraction of all releases of 1,2-DCA into the environment is the result of atmospheric emissions from industrial processes, manufacturing, handling, storage, and inappropriate disposal of the compound (IARC, 1999; van den Wijngaard *et al.*, 1993). In addition, fugitive emissions contribute significantly. In the air, 1,2-DCA degradation proceeds mainly by reaction with hydroxyl radicals, or by reacting with other compounds formed by sunlight. However, it can stay in the air for more than 5 months (between 47-182 days) before being broken down. It may also be removed from air by rain or snow. Releases to soils and surface waters are frequently detected at several tens of micromolars (ATSDR, 1999; Gotz *et al.*, 1998; IPCS, 1995; Yamamoto *et al.*, 1997), which is above the natural background level of 5 µmol in non-industrialized areas (de Rooij *et al.*, 1998). In these environmental settings, 1,2-DCA is expected to volatilize rapidly into the atmosphere, with subsequent photo-oxidation, typically within four months (WHO, 1996). The presence of methane can increase the rate of aerobic degradation of 1,2-DCA in soils, although, where volatilization is restricted, the lifetime in groundwater is expected to be in the order of years (ATSDR, 2001; IPCS, 1998). It has been estimated that 1,2-DCA has an environmental half-life of approximately 50 years (Vogel *et al.*, 1987).

1.3.4   Human exposure

Human exposure to 1,2-DCA has usually occurred when the chemical has been improperly disposed of, or spilled onto the ground. Humans are exposed to 1,2-DCA mainly by inhalation or by drinking water that contains 1,2-DCA. Numerous factors determine whether an individual will be harmed after contact with 1,2-DCA. These factors include the dose, duration, the way in which the contact occurred, as well as the presence of other chemicals (ATSDR, 2001). Humans can also be exposed to low levels of 1,2-DCA through the skin or air by contact with old products made with 1,2-DCA, such as pesticides, cleaning agents, and adhesives used to glue wallpaper and carpets. Besides these environmental exposures, occupational exposures may occur for workers involved in the manufacture or use of chemicals containing 1,2-DCA (NPI, 2005).

1.3.5   Environmental and health effects

1,2-Dichloroethane is one of the most prevalent xenobiotic compounds present in industrial wastewater discharges, groundwater and soil (Kocamemi and Çeçen, 2009). Because of the impact on the environment and human health, 1,2-DCA has been placed on the USEPA Priority List of Pollutants (USEPA, 1982) and on the United Kingdom „Red list'' of priority pollutants (Edwards, 1992). Industrial emissions of 1,2-DCA can produce elevated, but still low-level concentrations in the atmosphere around the source, however, it can travel for long distances. Spillages to the ground result in soil contamination and secondary contamination of groundwater via 1,2-DCA leaching. 1,2-Dichloroethane has slight acute (short-term) toxicity and slight chronic (long-term) toxicity to aquatic life. However, it is not expected to concentrate in fish. 1,2-Dichloroethane has also been found to cause injury to woody trees (ATSDR, 1999).

1,2-Dichloroethane is a toxic and potentially carcinogenic compound, and so its emissions have to be minimized by following strict environmental regulations (Baptista *et al*., 2006; IARC, 1999). The maximum contaminant level (MCL) for 1,2-DCA in drinking water is 5.0 μg/L (Henderson *et al*., 2008). Based on its physical properties and on case reports of deaths arising from oral or inhalation exposures, 1,2-DCA is likely to be absorbed by humans through any form of exposure (ATSDR, 2001). The analysis of „several' tissues of oral poisoning victims indicated a wide distribution of 1,2-DCA throughout the body (IARC, 1999). 1,2-Dichloroethane also appeared in the placenta, and has been detected in human milk following occupational exposure (WHO, 2003). The lethal oral dose in humans is estimated to be in the range of 20-50 ml (about 300-750 mg/kg bw) (IPCS, 1998). According to the ATSDR, the "minimal risk level" (MRL) for oral exposure to 1,2-DCA for a year is 0.2 mg/kg bw per day.

In acute toxicity, the ingestion of large single doses results in pulmonary edema, heart arrhythmias, bronchitis, depression, and changes in the brain tissue which eventually lead to death in most

cases (IPCS, 1995). Acute inhalation exposure of humans to 1,2-DCA can induce neurotic, nephrotoxic, and hepatoxic effects, as well as respiratory distress (ATSDR, 1993). From case studies of poisoning incidents, the lethal dose of 1,2-DCA is estimated to be 40-80 ml (ATSDR 1992). Other toxic symptoms include central nervous system depression, vomiting, and diarrhoea, and the consequences associated with kidney and liver injury (ATSDR, 2001; IPCS, 1995; 1998). In animals, clouding of the cornea and eye irritation have also been observed and are thought to be the result of vapour contact with the eyes. Acute animal tests, such as the lethal concentration (LC_{50}) and lethal dose (LD_{50}) tests in rats, mice and rabbits, have demonstrated that 1,2-DCA has moderate acute toxicity from inhalation or dermal exposure and moderate to high acute toxicity from oral exposure (USDHHS, 1993). In rodents and rabbits, 1,2-DCA demonstrated a moderate acute oral toxicity with LD_{50} values in the range of 413-860 mg/kg bw (IPCS, 1995).

Female workers who were using 1,2-DCA as solvents in a rubber processing plant were reported to have an increase in spontaneous abortions, premature births and pre-eclamptic toxaemia (GDCh-BUA, 1997). Also, increased rates of premature births were observed in female workers and in the wives of male workers in a synthetic fibre factory. In inhalation studies of rats, decreased fertility and increased embryo mortality have been observed (ATSDR, 1993). Human studies on 1,2-DCA as a cause of cancer have been considered inadequate. In 1999, an IARC Working Group assigned 1,2-DCA a Group 2B cancer classification ("possibly carcinogenic to humans") (IARC, 1999). However, in men over 55 years of age exposed to 1,2-DCA in drinking water an increased incidence of colon and rectal cancer has been reported (ATSDR, 1993). In animals, increases in the occurrence of stomach, mammary gland, liver, lung, and endometrium cancers have been seen following inhalation, oral, and dermal exposure (ATSDR, 2001). Administration by stomach tube to groups of 50 male and 50 female rats and mice, 5 day/week for 78 weeks produced multiple tumor (including alveolar/bronchiolar adenomas, endometrial stromal polyps and sarcomas, hepatocellular carcinomas, and mammary adenocarcinomas) types in both species (ATSDR, 1993; ATSDR, 2001).

1.3.6 Biodegradation of 1,2-dichloroethane

There is sufficient evidence that confirms that 1,2-DCA is susceptible to both abiotic (Gerritse, 1999; Lee *et al*., 1999; Nobre and Nobre, 1998; 1999; Stucki and Thuer, 1995) and biological transformation (Barbash and Reinhard, 1989; Belay and Daniels, 1987; Egli *et al*., 1987; Holliger *et al*., 1990a,b; Janssen *et al*., 1985; Jeffers *et al*., 1989; Stucki *et al*., 1987; 1983; Vandenbergh and Kunka, 1988; van den Wijngaard *et al*., 1992). However, microbial enzyme systems capable of its degradation have not evolved sufficiently to make the compound widely biodegradable (van der Zaan *et al*., 2009).

1.3.6.1 Abiotic transformation of 1,2-dichloroethane

Abiotic degradation has been well documented, however, under these conditions, dissolved 1,2-DCA is transformed slowly and the resulting products may even be more toxic than 1,2-DCA itself (Gallegos *et al.*, 2007). Under abiotic and alkaline hydrolysis, 1,2-DCA is transformed to vinyl chloride, whereas reactions at neutral pH favor a hydrolytic substitution reaction, yielding ethylene glycol as a product (Jeffers *et al.*, 1989; Lehmicke and Mukherjee, 1999). The half-life for the reaction at pH 7 and 25°C has been reported to be > 72 years (Jeffers *et al.*, 1989). It has been documented, however, that abiotic reaction rates can be enhanced by the presence of certain anions frequently encountered in aqueous environments. For example, Barbash and Reinhard (1989) reported that the half-life of 1,2-DCA at 25ºC decreased to 37 years in the presence of 50 mM phosphate buffer, and to 6 years in reactions containing 50 mM phosphate buffer and 0.67 mM sodium sulfide.

1.3.6.2 Aerobic biodegradation of 1,2-dichloroethane

To date, most studies on aerobic biodegradation of 1,2-DCA have employed enriched or pure cultures (Hage and Hartmans, 1999; Hirschorn *et al.*, 2007; Hunkeler and Aravena, 2000; Inguva and Shreve, 1999; Janssen *et al.*,1985; Klečka *et al.*, 1998; Stucki *et al.*,1983), rather than soils from contaminated sites. The aerobic transformations are usually biotic and occur through oxidation. Several members of the genera *Xanthobacter*, *Ancylobacter* and *Pseudomonas* that are able to utilize 1,2-DCA have been isolated under laboratory conditions from polluted sites.

The aerobic biodegradation of 1,2-DCA has been comprehensively studied with cultures of *Xanthobacter autotrophicus* GJ10, *Ancylobacter aquaticus* AD20, AD25, and AD27, *Pseudomonas* sp. strain DCA1. These microorganisms are capable of utilizing 1,2-DCA as a sole carbon and energy source resulting in complete mineralization of the compound, forming carbon dioxide, inorganic chloride and water (Janssen *et al.*, 1985; Stucki *et al.*, 1983; Vandenbergh and Kunka, 1988; van den Wijngaard *et al.*, 1992). The best-studied 1,2-DCA degrading organism is *X. autotrophicus* GJ10, was reported to degrade 1,2-DCA via hydrolytic dehalogenation as illustrated in Figure 1.1 (Janssen *et al.*, 1989). In this microorganism, 1,2-DCA is initially dehalogenated by the substitution of one of the two terminal chlorine atoms by a hydroxyl group to form 2-chloroethanol. This reaction is catalyzed by the constitutively produced haloalkane dehalogenase (DhlA) enzyme (Janssen *et al.*, 1994; 1989; 1985). The intermediate 2-chloroethanol is then oxidized via two sequential dehydrogenation steps to monochloroacetic acid, catalysed by two inducible dehydrogenases, the pyrrolo-quinoline quinone-containing alcohol dehydrogenase (Mox) and NAD-dependent aldehyde dehydrogenase (Ald) (Janssen *et al.*, 1987; 1985). The rate at which *X. autotrophicus* can degrade 1,2-DCA is limited by the rate at which the 2-chloroacetaldehyde and monochloroacetic acid is metabolized as these intermediates are more toxic

than 1,2-DCA and are utilized poorly (Baptista *et al*., 2006; Tardiff *et al*., 1991; Van der Ploeg *et al*., 1994; 1995). Although monochloroacetic acid may be potentially highly toxic to the bacterium, it is converted to glycolic acid by a second, constitutively produced hydrolytic dehalogenase, haloacetate dehalogenase (DhlB) (Van der Ploeg *et al*., 1991). Glycolic acid is taken up in the central metabolic route and used for the generation of energy and cell components. A similar catabolic pathway is present in the other 1,2-DCA degraders. Interestingly, the two enzymes, namely the alcohol dehydrogenase and the haloacetate dehalogenase, appear to be common in nature, and the haloalkane dehalogenase and aldehyde dehydrogenase appear to be specifically adapted for the degradation of xenobiotic substrates (Klečka *et al*., 1998). Based on several extensive biochemical as well as genetic analysis of the enzymes involved, the authors speculate that evolution of organisms with the ability to grow on 1,2-DCA requires a number of steps (Janssen *et al*., 1995). However, in some cases at concentrations above 5 mM 1,2-DCA, the bacteria produce extracellular polysaccharides as a protective barrier to reduce uptake (van den Wijngaard *et al*., 1993) thus limiting their use for *in situ* bioremediation.
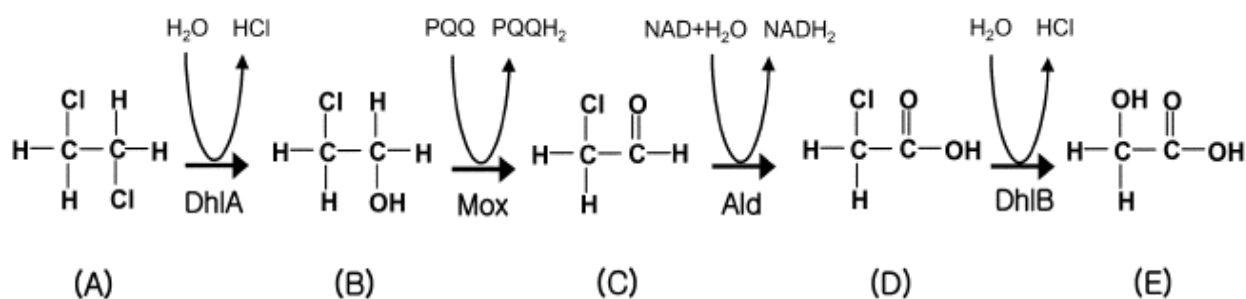
Figure 1.1: Proposed catabolic pathway of 1,2-DCA in *X. autotrophicus* GJ10. (A), 1,2-DCA; (B), 2-Chloroethanol; (C), 2-Chloroacetaldehyde; (D), Monochloroacetic acid; (E), Glycolic acid (Song *et al*., 2004).

1.3.6.3 Anaerobic biodegradation of 1,2-dichloroethane

The biotransformation of 1,2-DCA has also been reported under anaerobic conditions. The anaerobic biotic processes are generally reductions that involve either hydrogenolysis reductive dechlorination, the substitution of a chlorine atom from the molecule by a hydrogen atom, or dihaloelimination, where two adjacent chlorine atoms are removed, leaving a double bond between the respective carbon atoms (Bosma *et al*., 1998; McCarty and Semprini, 1994). Belay and Daniels (1987) and Egli *et al*. (1987), have described the biotransformation of 1,2-DCA to ethene by pure cultures of sulfate reducing or methanogenic bacteria. In contrast, Holliger *et al*. (1990b) observed that cell suspensions of methanogenic bacteria reductively dechlorinated 1,2-DCA via two different reaction mechanisms: a dihaloelimination reaction yielding ethene as well as two consecutive hydrogenolysis reactions yielding chloroethane and ethane. 1,2-Dichloroethane may also be oxidized anaerobically under

nitrate reducing conditions, with nitrate as the electron acceptor (Gerritse *et al.*, 1999). Abiotic transformation of 1,2-DCA under anaerobic conditions includes hydrolytic substitution yielding ethylene glycol (Lehmicke and Mukherjee, 1999). Furthermore, anaerobic bacteria can also reductively dechlorinate 1,2-DCA to chloroethane which can either be further dechlorinated to ethane or abiotically hydrolyzed to ethanol (Vogel *et al.*, 1987).

1.3.6.4 Halorespiration of 1,2-dichloroethane

Halorespiration of 1,2-DCA was demonstrated using *Dehalococcoides ethenogens* strain 195 and *Dehalococcides* strain BAV1. The growth of these halorespiring bacteria occurred on 1,2-DCA, which served as a terminal electron acceptor and hydrogen as the electron donor (He *et al.*, 2003; Maymo-Gatell *et al.,* 1999). Additionally, the bacterium *Desulfobacterium dichloroeliminans* strain DCA1was isolated and reported to utilize 1,2-DCA as a terminal electron acceptor with formate or hydrogen as the electron donor (De Wildeman *et al.,* 2003). In these transformation pathways ethane was the primary product of the conversion, and traces of vinyl chloride were also present (De Wildeman *et al.*, 2003; Maymo-Gatell *et al.*, 1999).

1.4 Metal toxicity and microbial resistance mechanisms

1.4.1 Toxic effects of heavy metals

Metals play an essential role in the life processes of microorganisms. Some metals, such as calcium, chromium, cobalt, copper, iron, magnesium, manganese, nickel, potassium, sodium and zinc, serve as micronutrients and are used for redox-processes: to stabilize molecules through electrostatic interactions; as cofactors in various enzymes and electron transport chains; and for regulation of osmotic pressure (Bruins *et al.*, 2000). Thus, metal ions may play important roles as "trace elements" in sophisticated biochemical reactions. Many other metals (e.g. silver, aluminium, arsenic, cadmium, gold, lead and mercury) have no biological role, are non-essential (Bruins *et al.*, 2000), and are potentially toxic to microorganisms. At higher concentrations these heavy metal ions form unspecific complex compounds within the cell, which leads to toxic effects, making them too dangerous for any physiological function (Nies, 1999). Toxic levels of metals may result in the production of free radicals that disrupt nucleic acids, proteins, and phospholipids (Halliwell and Gutteridge, 1984; 1985). Metals may also displace metal enzyme cofactors, disrupting the structural integrity and function of enzymes (Stadtmann, 1993; Stohs and Bagchi, 1995). Toxicity of non-essential metals occurs through the displacement of essential metals from their native binding sites or through ligand interactions (Bruins *et al.*, 2000; Nies, 1999). For example, Hg^{2+}, Cd^{2+} and Ag^{2+} tend to bind to sulfhydryl (SH-) groups of enzymes essential for microbial

metabolism, and thus inhibit the activity of sensitive enzymes (Nies, 1999). To have a physiological or toxic effect, most metal ions have to enter the microbial cell. Many divalent heavy metal cations (e.g. Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) are structurally very similar. Also, the structure of oxyanions such as chromate resembles that of sulfate, and the same is true for arsenate and phosphate (Nies, 1999). In such cases, these toxic metal cations may substitute for physiological essential cations within an enzyme rendering the enzyme non-functional. Thus, to be able to differentiate between structurally similar metal ions, the microbial uptake systems have to be tightly regulated.

Most microorganisms have solved this problem by using two types of uptake mechanisms for heavy metal ions. One is quick, unspecific, constitutively expressed and driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria (Nies, 1999). The second is inducible, has high substrate specificity, is slower, often uses ATP hydrolysis as the energy source, and is only produced by the cell in times of need, starvation or a special metabolic situation (Nies and Silver, 1995). Even though the constitutively expressed unspecific system is more energy efficient, it results in an influx of a wider variety of heavy metals. When these metals are present in high concentrations, they are more likely to have toxic effects once inside the cell (Nies and Silver, 1995). High levels of both essential and non-essential metals can damage cell membranes; alter enzyme specificity; disrupt cellular functions; and damage the structure of DNA (Bruins *et al*., 2000). In addition, concentrations of elevated levels of heavy metals impose oxidative stress on microorganisms (Kachur *et al*., 1998).

1.4.2   Mechanisms of microbial resistance to metals

To survive under metal-stressed conditions, some microorganisms have been forced to develop metal-ion homeostasis factors and metal-resistance determinants to tolerate the uptake of virtually all toxic metals via selective pressures from the metal containing environment (Hoostal *et al*., 2008; Bruins *et al*., 2000; Nies, 1999; Nies and Silver, 1995; Rouch *et al*., 1995). Bacteria have adapted to metals through a variety of chromosomal, transposon, and plasmid-mediated resistance systems which are capable of being spread throughout a bacterial community by lateral gene transfer through conjugation and transduction (Coombs and Barkay, 2004; Martínez *et al*., 2006). The primary difference between chromosomal and plasmid based metal resistance systems is that chromosome based systems are more complex and are usually required for essential metal resistance. On the other hand, plasmid-encoded systems are usually toxic ion efflux mechanisms (Bruins *et al*., 2000). This suggests that ion efflux mechanisms are more likely to be plasmid-borne because they can be quickly mobilized to other organisms, and they reduce the gene carrying load since they are only needed on certain occurrences (Silver and Walderhaug, 1992). The extent of resistance in a microorganism is determined by several factors: the role each metal plays in normal metabolism; the type and number of mechanisms for metal

uptake; and the presence of genes located on plasmids, chromosomes, or transposons that control metal resistance (Bruins *et al*., 2000). The main mechanism by which microorganisms affect changes in the speciation and mobility of metals is well described by van Hullebusch *et al*. (2005).

Six possible metal resistance mechanisms exist: exclusion by permeability barrier; intra- and extra-cellular sequestration; active transport efflux pumps; enzymatic detoxification; and reduction in the sensitivity of cellular targets to metal ions (Bruins *et al*., 2000; Carine *et al*., 2009; Ji and Silver, 1995; Nies, 1999; Nies and Silver, 1995; Rensing *et al*., 1999). One or a combination of these resistance mechanisms allows microorganisms to function in metal co-contaminated environments.

1.4.2.1 Arsenic

Arsenic, which is a heavy metalloid with metallic and non-metallic properties, is toxic to bacteria, as well as other domains of life (Bruins *et al*., 2000; Nies, 1999). Arsenic commonly occurs as As(V) in AsO_4^{3-}, arsenate, and as As(III) in AsO_2^-, arsenite (Nies, 1999). Arsenate is an analogue of phosphate, thus, its main toxicity results from its interference with the metabolism of the major bio-element phosphorus via phosphate transporters (Nies, 1999; Nies and Silver, 1995). Arsenate usually enters the cell in periods of phosphate abundance through the *Pit* system, which is a constitutively expressed, nonspecific, nutrient transport system (Bruins *et al*., 2000). In times of phosphate depletion, a more specific *Pst* system is induced. This system is 100 times more specific for phosphate than arsenate. The microorganism has the capacity to increase As(V) tolerance by inactivating the *Pit* system in favor of *Pst* (Nies and Silver, 1995). Several mechanisms for resistance to arsenic have been identified. The best studied example is the plasmid mediated efflux mechanism of the arsenical resistance of *E. coli* (Chen *et al*., 1986). Expression of the As(V) efflux pump is coded for by a family of genes called the *ars* operon (Tsutomu and Kobayashi, 1998). The number of genes in this operon can vary from three to five (*arsR*, *arsA*, *arsD*, *arsB*, and *arsC*) (Rouch *et al*., 1995). Operons in *Staphylococcus* plasmids and the chromosome of *E. coli* do not contain the *arsD* and *arsR* genes. The gene products of *arsR* and *arsD* regulate the operon. Therefore, loss of *arsD* does not seem to affect resistance to arsenicals (Rouch *et al*., 1995; Tsutomu and Kobayashi, 1998). The nucleotide sequence of a fragment of DNA containing the *ars* operon was studied (Chen *et al*., 1986), and three genes, *arsA*, *arsB* and *arsC*, were found to encode for the proteins ArsA, ArsB and ArsC, respectively. ArsA is a protein with ATPase activity and thus is involved in translocation of the metal ions across the cell membrane. ArsB interacts with ArsA to form an $ArsA_2B$ complex on the inner membrane of the cell, and the two proteins form the arsenite pump which is driven chemiosmotically and by ATP (Dey and Rosen, 1995). Since anion export from bacterial cells is always driven by the chemiosmotic gradient, simple arsenic efflux systems may be composed of just one efflux protein, the ArsB product (Wu *et al*., 1992). Typical examples are the plasmid-encoded system

from *S. xylosus* (Rosenstein *et al*., 1992) and the chromosomally encoded system in *E. coli* (Diorio *et al*., 1995). Detoxification of arsenate is the initial step required to differentiate it between phosphates. This step involves the reduction of arsenate to arsenite (Ji *et al*., 1994; Ji and Silver, 1992). For the resistance determinant in *E. coli*, arsenate reduction by the ArsC protein is coupled to glutathione (Oden *et al*., 1994) via glutaredoxin (Gladysheva *et al*., 1994; Liu and Rosen, 1997). For ArsC from *S. aureus*, the electron donor is thioredoxin and NADPH (Ji *et al*., 1994). Thus, ArsC is only required for tolerance to arsenate, and ArsA and ArsB are required for tolerance to both species of arsenic.

1.4.2.2 Cadmium

Cadmium is a non-essential heavy metal that is toxic at low concentration (Bruins *et al*., 2000). Numerous studies have demonstrated the toxicity of cadmium to microorganisms, however, specific mechanisms have yet to be defined (Dopson *et al*., 2003). The toxic effects of cadmium generally include thiol-binding and protein denaturation, interaction with calcium and zinc metabolism, loss of a protective function (Bruins *et al*., 2000; Nies, 1999) and single-strand breakage of DNA in *E. coli* (Trevors *et al*., 1986). Sensitive bacteria have been reported to accumulate 3 to 15 times more Cd^{2+} than resistant bacteria (Laddaga *et al*., 1985; Trevors *et al*., 1986). Several bacteria such as *Staphylococcus aureus*, *Bacillus subtillis*, and *Escherichia coli* demonstrate resistance to Cd^{2+} (Cohen *et al*., 1991; Laddaga and Silver, 1985; Smith and Novick, 1972). Cadmium resistance occurs through all of the biochemical resistance mechanisms with the exception of enzymatic detoxification (Bruins *et al*., 2000). Resistance to Cd^{2+} in bacteria is based mainly on cadmium efflux pumps (Nies, 1999), a system widely distributed in a number of microorganisms. For example, the Czc system is driven by a resistance, nodulation, cell division (RND-driven) system and a P-type ATPase pump (CadA) in Gram-negative and Gram-positive bacteria, respectively (Dopson *et al*., 2003; Nies, 1999). Bacteria that naturally form an extracellular polysaccharide coating also demonstrate the ability to bioabsorb metal ions and prevent them from interacting with fundamental cellular components (Scott and Palmer, 1990; Scott *et al*., 1988). A protective layer of exopolysaccharide improved the survival of *K. aerogenes* strains in Cd^{2+} solutions (Scott and Palmer, 1990). The extracellular capsule of *K. aerogenes* prevented the entry of up to 1 nM of Cd^{2+} when compared to non-encapsulated forms (Mergeay, 1991). *P. putida* can bind 100% of Cd^{2+} added to broth at a concentration of 2.5 mg/L (Scott *et al*., 1988). Strains of *A. viscous* can accumulate 30 mg/g of Cd^{2+} when added to broth at 100 mg/L (Scott and Palmer, 1988). In both cases, binding was pH dependent and the optimum was between pH 4 and 9 (Scott *et al*., 1988). This protective layer appears to prevent uptake, keeping metal ions away from sensitive cellular components. Metal resistance based on extracellular sequestration has been demonstrated in *S. cerevisiae* whereby cadmium is bound by glutathione, and the resulting cadmium-bisglutathionato complex is transported via an ABC transporter

into the vacuole (Li *et al*., 1997; 1996). Other organisms, such as yeast or *Citrobacter* sp., form insoluble complexes of cadmium phosphate to confer resistance (McEntee *et al*., 1986). A strain of *K. aerogenes* has been shown to exhibit the ability to remove Cd^{2+} ions from the surrounding environment by excreting sulfur to limit metal influx by external precipitation (Scott and Palmer, 1990). In some species of *S. aureus*, penicillinase plasmids can mediate resistance by changing cell membrane permeability to Cd^{2+} as well as to other metals (Bruins *et al*., 2000). This is usually low-level resistance in the range 0.01 to 0.1 nM of Cd^{2+} (McEntee *et al*., 1986). Furthermore, cyanobacteria contain metallothioneins (Olafson *et al*., 1979). The metal resistance system in *Synechococcus* sp. consists of two genes: *smtA* and *smtB*. Amplification of the smt metallothionein locus increases cadmium resistance (Gupta *et al*., 1992), and deletion of it decreases resistance (Gupta *et al*., 1993; Turner *et al*., 1993; 1995). The SmtB protein acts as a transacting transcriptional repressor turning off *smtA* expression and metallothionein production (Huckle *et al*., 1993; Morby *et al*., 1993; Turner *et al*., 1996). Since cyanobacteria contain a diversity of RNA- and P-type transport systems, transport may also be important for cadmium resistance in these bacteria (Nies, 1999).

1.4.2.3 Mercury

Mercury is considered the heavy metal with the strongest toxicity (Nies, 1999). Due to the strong affinity of Hg^{2+} to thiol groups, mercury is most commonly found in cinnabar (HgS). The solubility product of HgS is 6.38×10^{-53}, indicating it is a highly insoluble compound (Dopson *et al*., 2003; Nies, 1999). Resistance to mercury is based, however, on its unique peculiarities: metallic mercury has an extraordinarily low melting/boiling point for a metal (melting point -39°C, boiling point 357°C) and the electrochemical potential of Hg^{2+}/Hg^0 at pH 7 is +430 mV (Weast, 1984). Mercury resistance is based primarily on enzymatic detoxification of the metal to a less toxic form, and this system is regarded as a model example of resistance via detoxification in microorganisms (Bruins *et al*., 2000). Resistance to mercury has been demonstrated in both Gram-positive (*S. aureus, Bacillus* sp.) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Serratia marcescens,* and *Acidithiobacillus ferrooxidans*) (Misra, 1992). A mercury resistance system involves the reduction of Hg^{2+} via a Hg^{2+}-reducing flavoprotein producing Hg^0 which volatilizes out of the cell by passive diffusion (Silver, 1996; Silver and Phung, 1996). Some bacteria contain a set of five to six genes that form an Hg^{2+} (*mer*) resistance operon, which has been studied extensively in the plasmids of several microorganisms (Misra, 1992). This operon not only detoxifies Hg^{2+} but also transports and self-regulates resistance (Ji and Silver, 1995; Misra, 1992; O'Halloran, 1993). The *mer* transport genes consist of *merC*, *merT* and *merP*; which code for proteins that manage Hg^{2+} transport within the bacteria. MerP is a periplasmic protein whereas MerT and MerC gene products are cytoplasmic membrane proteins. It has been demonstrated that both

*merT a*nd *merP* are required for full Hg^{2+} resistance (Hamlett *et al*., 1992). In Gram-negative bacteria, the first step in detoxification is the binding of Hg^{2+} to the MerP (Qian *et al*., 1998). Thereafter, MerP possibly delivers the toxic cation to the mercury transporter MerT for transport into the cytoplasm (Hobman and Brown 1996). Once inside the cell, Hg^{2+} is reduced with NADPH to Hg^0 by the MerA protein (Schiering *et al*., 1991). In addition to MerTP, another uptake route which involves the MerC protein exists (Hamlett *et al*., 1992; Sahlman *et al*., 1997). Although it has been linked to transport of Hg^{2+}, the function of MerC remains unclear (Hamlett *et al*., 1992). Furthermore, mercury-resistant organisms can be divided into two categories: narrow spectrum and broad spectrum. Narrow spectrum organisms lack the gene which encodes for MerB organomercurial lyase and, therefore, are not resistant to most organomercurials. Broad-spectrum organisms have both enzymes and are resistant to most mercury-containing compounds (Bruins *et al*., 2000). However, it has been postulated that the high toxicity of organomercurials and other methylated and alkylated heavy metal compounds makes it very unlikely that these kinds of chemical modification of heavy metals are metal-resistance mechanisms (Nies, 1999).

1.4.2.4 Lead

In the natural world, lead is a ubiquitous but biologically non-essential element (Ewers and Schlipköter, 1991). Contamination of the environment with lead has increased as it has become an essential material for many industries. Due to its low solubility (especially in the form of lead phosphate), its biologically available concentration is low (Nies, 1999). Furthermore, Heinrichs and Mayer (1980) considered lead as one of the least soluble metals with a very long retention time. The toxicity of lead is a consequence of the ability of Pb^{2+} to interfere with several enzymes (Ewers and Schlipköter, 1991). Isolation of lead-tolerant bacteria has been reported (Trajanovska *et al*., 1997), and precipitation of lead bound phosphate within bacterial cells has also been observed (Levinson and Mahler 1998; Levinson *et al*., 1996). Resistance to lead has also been postulated to be based predominantly on metal efflux (Nies, 1999), since in *Ralstonia* sp. CH34 it has been shown that resistance to lead is mediated by a P-type ATPase (Borremans and van der Lelie, unpublished observation). In addition, the CadA P-type ATPase is also able to transport Pb^{2+} (Rensing *et al*., 1998).

1.5 Metal speciation and bioavailability

Heavy metals may inhibit organic pollutant biodegradation through the interaction with enzymes involved in general metabolism or those directly involved in biodegradation (Sandrin and Maier, 2003). The ionic form of the metal mediates inhibition of enzymes involved in pollutant degradation in heavy metal contaminated environments (Angle and Chanley, 1989), indicating that metal toxicity is related to

the concentration of bioavailable metal rather than the total or even total soluble metal concentration. Metals have been reported to inhibit organic pollutant biodegradation and affect degradation rates; however, widely varying degrees and patterns of inhibition have been reported, due to the lack of consistent methods to characterize metal toxicity (Hoffman *et al*., 2005). Most commonly, reports on metal inhibition of biodegradation have been related to the total concentration of a metal in the test system. However, this may not be the most appropriate measure as it has been well established that some metal species are more bioavailable than others (Hughes and Poole, 1991; Knotek-Smith *et al*., 2003; Roane *et al*., 2001; Traina and Laperche, 1999).

Speciation can broadly be defined as the identification and quantification of the different, defined species, forms or phases in which an element occurs (Tack and Verloo, 1995), while bioavailability is the fraction of the total amount of a metal in a specific environmental compartment that, within a given time span, is either available or can be made available for uptake by microorganisms from the direct surrounding of the organism. Metal speciation and the resulting bioavailability rather than total metal concentration determine the overall physiological and toxic effects of a metal on biological systems (Hughes and Poole, 1991; Morrison *et al*., 1989; Traina and Laperche, 1999). Unfortunately, few studies investigating the impact of metals on biodegradation have provided metal speciation and bioavailability data (Sandrin and Maier, 2003). Traditionally, the environmental risk caused by heavy metal pollution is determined by quantification of total metal concentration using conventional analytical methods (Rodriguez-Mozaz *et al*., 2004). However, conventional analytical procedures are not able to distinguish between available (potentially hazardous) and non-available (potentially non-hazardous) fractions of metals to biological systems (Rasmussen *et al*., 2000). This is of particular interest with respect to solid environments, e.g. soils, because of the great adsorption capability of heavy metals to solid phase (Vanhala and Ahtiainen, 1994). In the water phase, the chemical form of a metal determines the biological availability and chemical reactivity (sorption/desorption, precipitation/dissolution) to other components of a system. Often overlooked in metal toxicity studies is the importance of the pH of buffer used in microbiological media and the time at which a metal is added to a given medium (Lage *et al*., 1996; Vasconcelos *et al*., 1998). Also, the level of inhibition depends on the concentration and availability of the heavy metals which in turn are dependent on complex processes controlled by multiple factors including the nature of the metals and microbial species (Amor *et al*., 2001; Goblenz *et al*., 1994; Hashemi *et al*., 1994; Olasupo *et al*., 1993; Tomioka *et al*., 1994). Some of the factors affecting metal speciation and bioavailability will be discussed below.

1.5.1 Binding components and physico-chemical characteristics in medium and soil

Many pH buffers are often present at higher concentrations than other medium components used in test systems (Hughes and Poole, 1991; Teresa *et al*., 2000; Vasconcelos and Leal, 2002), and are able to complex and precipitate metals, thus affecting metal speciation and bioavailability. For example, phosphate, probably the most common buffer used in the majority of studies (Amor *et al.,* 2001; Benka-Coker and Ekundayo, 1998; Birch and Brandl, 1996; Nakamura and Sawada, 2000), is well known for its ability to precipitate many metals and reduce their bioavailability (Hughes and Poole, 1991). Phosphate readily sequesters metals and reduces their bioavailability via the formation of insoluble metal phosphate species, even at neutral to mildly acidic pH values (Sandrin and Hoffman, 2007). In a predictive model of the concentrations of free ionic metals as a function of phosphate concentration in the Bushnell Haas medium (Difco™, Sparks, MD), commonly used in biodegradation studies, 44% less free ionic cadmium existed in the medium containing a relatively low phosphate concentration of 2.27 mM compared to the same medium not containing phosphate. Some metals are more sensitive to phosphate precipitation than others. Cobalt bioavailability was predicted to remain high (95%) in the free, ionic form as the phosphate concentration was raised to 15 mM; while free, ionic nickel was predicted to fall to 21% of its concentration in the medium free of phosphate (Sandrin and Hoffman, 2007).

The metal-complexing capabilities of some zwitterionic buffers incorporated into microbiological media [e.g., HEPES (4-2-hydroxyethyl-1-piperazine-ethanesulfonic acid), MES (2-4-morpholino-ethanesulfonic acid), MOPS (3-N-morpholino-propansulfonic acid), PIPES (1,4-piperazine-bis-ethanesulfonic acid)] have been reported (Hoffman *et al*., 2005). However, metals tend to remain more bioavailable in the presence of zwitterionic buffers than in the presence of phosphate buffers, due to the fact that former buffers do not interact with metals as strongly as phosphate buffers. Mash *et al*. (2003) indicated that MES and MOPS (50 mM) did not complex copper, while HEPES (35 mM) strongly complexed copper at pH 7.2. PIPES buffer has been reported to complex lead but not cadmium or copper (Soares and Conde, 2000; Vasconcelos *et al*., 1998).

Although available data suggests that Tris-base (2-amino-2-hydroxymethyl-1,3-propanediol) complexes many metals, limited quantitative complexation data has been gathered (Twiss *et al.*, 2001). Hoffman *et al*. (2005) reported that the degrees of inhibition of cadmium on naphthalene (NAPH) biodegradation by *Comamonas testosteroni* were different in each of three chemically-defined minimal salts medium (MSM) tested. Biodegradation was completely inhibited by 100 μM total cadmium in PIPES-buffered MSM and by 500 μM total cadmium in Tris- and PIPES-buffered MSM. However, neither of the cadmium concentrations completely inhibited biodegradation in Bushnell-Haas medium.

The physico-chemical properties of soil can widely influence metal speciation and consequently, its mobility, bioavailability and toxicity (Irha *et al*., 2003; McLean and Bledsoe, 1992). Metals may be

distributed among many components of soil solids and may be associated with them in different ways (ion exchange, adsorption, precipitation, complexation or present in the structure of minerals). Irha *et al*. (2003) modified the dehydrogenase enzyme activity (DHA) assay using resazurin (oxidoreduction dye) for toxicity analysis of combined mixture of heavy metals and polycyclic aromatic hydrocarbons in soil. The method was modified to take into account possible interaction of resazurin with complex soil matrix (adsorption on the soil components, influence of inorganic substances and humic acids). Results showed that the sensitivity of soils to contamination correlated reasonably well with organic matter, calcium and amorphous phase content. These tallied with the investigations of other researchers (Alexander and Alexander, 2000; Bååth, 1989; Eriksson, 1988; Sauve *et al*., 2000). In soil with relatively low organic matter content and significant quantity of amorphous phase, high sensitivity to contamination by heavy metals and persistence of organic pollutants were observed. Organic matter content has a strong influence on cation exchange capacity, buffer capacity as well as on the retention of heavy metals. Thus, metals present in organic soils contaminated with a combination of heavy metals are less mobile and less bioavailable than metals present in mineral soils (Balasoiu *et al*., 2001). Time and moisture effects are also important factors that need to be considered when interpreting short-term toxicity studies and when making predictions concerning possible long-term effects of heavy metals in the soil environment, as the retention of copper in dry soil has been observed to be much less pronounced in soils with higher moisture content (Tom-Petersen *et al*., 2004).

1.5.2 pH and redox potential

At high pH, metals tend to form insoluble metal mineral phosphates and carbonates, whereas at low pH they tend to be found as free ionic species or as soluble organo-metals and are more readily bioavailable (Naidu *et al*., 1997; Rensing and Maier, 2003; Sandrin and Hoffman, 2007; Twiss *et al*., 2001). At acidic pH, more protons (H^+) are available to saturate metal-binding sites; therefore metals are less likely to form insoluble precipitates with phosphates when the pH of the system is lowered because much of the phosphate has been protonated (Hughes and Poole, 1991). Under basic conditions, metal ions can replace protons to form other species such as hydroxo-metal complexes (Babich and Stotzky, 1985; Collins and Stotzky, 1992; Ivanov *et al*., 1997). In some cases the hydroxo-metal complexes, such as those formed with cadmium, nickel, and zinc, are soluble; while those formed with chromium and iron are insoluble. A small change in pH can decrease metal solubility and bioavailability by several orders of magnitude, e.g., the solubility of cadmium was reduced 8.8-fold by an increase in pH from 6 to 7 in 1.3 mM phosphate (Rensing and Maier, 2003). The dependence of metal bioavailability on pH varies between different metals. For example, a rapid decline in the concentrations of the free, ionic species of copper

and zinc in minimal media was observed at pH values higher than 5, while the free, ionic form of cobalt remained prevalent until the pH value was higher than 8 (Sandrin and Hoffman, 2007).

Many studies have shown that pH mediates metal toxicity (Babich *et al.,* 1985; Franklin *et al.,* 2000; Korkeala and Pekkanen, 1978). Babich and Stotzky (1982; 1983) found that increasing pH reduces the toxicity of nickel to a variety of different organisms, including bacteria (*Serratia marcescens*), filamentous fungi (*Arthrobotrys conoides*, *Penicillium vermiculatum*, *Rhizopus stolonifer*), and yeast (*Cryptococcus terreus*). Under mildly basic conditions (pH 8.5), much of the nickel may not be bioavailable because it forms complexes with various ligands. More commonly, increasing pH has been shown to increase the toxicity of zinc, copper, and uranium to certain algal species (Franklin *et al.,* 2000; Hargreaves and Whitton, 1976) and of cadmium to various bacteria (*Bacillus subtilis, Escherichia coli, Micrococcus luteus, Streptococcus bovis*), actinomycetes (*Micromonospora chalcea, Nocardia corallina, Streptomyces flavovirens*), and fungi (*Saccharomyces cerevisiae, Schizosaccharomyces octosporus*) (Korkeala and Pekkanen, 1978). This may be due to cells being able to take up or adsorb more of the metal ions under high pH conditions (Rudd *et al.,* 1983; Sandrin and Maier, 2002). Also, various functional groups associated with the membrane of microorganisms would be protonated under acidic conditions, reducing the electrostatic attraction between the metal cations and the membrane. A third possibility is that metals are removed from the cell more efficiently under acidic conditions by efflux pumps that are driven by the proton motive force (Sandrin and Maier, 2002). Studies examining the effect of metal toxicity on biodegradation usually use a buffer that has a neutral to mildly acidic pH range (Amor *et al.,* 2001; Sandrin and Maier, 2003; Said and Lewis, 1991). The operational pH range is determined by the pKa of the buffer, which is the pH at which half of the weak acid used for buffering is protonated. When the pH is beyond the operational range of a buffer, even small additions of acid, such as the excretion of acidic metabolic end products by microbes, may drastically change the pH and can result in unanticipated metal speciation events (Hughes and Poole, 1991; Twiss *et al.,* 2001).

The redox potential (Eh) of an environment also influences metal speciation. Redox potential is established by oxidation-reduction reactions that tend to be relatively slow, particularly in soil environments (Rensing and Maier, 2003). However, microbial activity can dramatically influence the rate and establishment of redox potential in soil. Reducing conditions (negative Eh) found in anaerobic media can result in metal precipitation with media components. Kong (1998) reported that the soluble metal concentration in sediment slurries initially amended with 20 mg/L cadmium, copper, or chromium were below detection limits of 0.03-0.04 mg/L. Furthermore, at 100 mg/L added metal, only 1 mg/L cadmium and < 0.12 mg/L copper and chromium were found in the aqueous phase. Under positive Eh (oxidizing) conditions, metals are more likely to exist in their free ionic form and exhibit increased water solubility.

Moreover, pH may decrease slightly or even dramatically under oxidizing conditions (Rensing and Maier, 2003).

1.6 Measurement of bioavailable metal

Measurement of bioavailable metal concentrations is a vital step towards determining the effects of metals on organic pollutant biodegradation, since the environmental risk caused by heavy metal pollution is traditionally determined by quantification of total metals (Liao *et al*., 2006; Sandrin and Hoffman, 2007). The development of sensitive, effective, and inexpensive methods that can efficiently monitor and determine the presence and amount of hazardous heavy metals is still in its infancy. Common analytical techniques used are ion chromatography, ion-selective electrodes and polarography (Durrieu and Tran-Minh, 2002). However, these methods are not able to distinguish between available and non-available fractions of metals to biological systems (Rasmussen *et al*., 2000). According to the standards, measurements and testing programme of the European Commission, the most suitable approach for certification of a soil sample to characterize the bioavailable fraction of metals was a single step procedure using EDTA and acetic acid. Conventionally, single step extraction procedures are mainly applied to soil samples to identify the bioavailable fraction, using a number of different reagents able to extract all or part of the metals from soil (Žemberyová *et al*., 2007). Conventional methods are reaching the highest accuracy with low detection limits (Rodriguez-Mozaz *et al*., 2004), but are expensive, time consuming, and require highly trained personnel. However, the main drawback of chemical methods is the question of the transfer of the results obtained on non-biological systems to biological ones (Liao *et al*., 2006). The current tendency to carry out field monitoring has driven the development of bioassays, biomarkers, and biosensors as new analytical tools able to provide fast, reliable, and sensitive measurements with lower cost, many of them aimed at on-site analysis. These tools have also gained much attention since they integrate all aspects of bioavailability, including exposure, accumulation, and toxic effects at the receptor level (Peijnenburg and Jager, 2003). Risk assessments of metal contaminated soils obviously require a comprehensible protocol for testing metal bioavailability and mobility. Such a test should ideally be applicable with minimum perturbation of the soil, without disrupting the equilibrium between solid and solution phases and that is sensitive to prevailing conditions (Editorial, 2003).

1.6.1 Bacterial biosensors

Recombinant bacterial sensors have been constructed and used for the determination of the bioavailability of specific metals. Ivask *et al*. (2002) used recombinant luminescent bacterial sensors for the determination of the bioavailable fraction of cadmium, zinc, mercury, and chromium in soil. In their study, two bacterial recombinant heavy metal sensors were constructed based on two different receptor-reporter systems: one was inducible by Zn^{2+}, Cd^{2+}, and Hg^{2+}, and the other by Cr^{6+} and Cr^{3+}. The bacterial sensors used were not perfectly specific to one heavy metal, but responded to some "non-target" metals as well (Ivask *et al*., 2002). In another example, the mer-lux gene fusion in *E. coli* was used to estimate bioavailable mercury in soil. The mer-promoter was activated when Hg^{2+}, present in the cytoplasm of the biosensor bacterium, binds to *MerR*, resulting in transcription of the *lux* genes and subsequent light emission (Rasmussen *et al*., 2000). The luminescence-based bacterial sensor strains, *Pseudomonas fluorescens* OS8 (pTPT11) and *Pseudomonas fluorescens* OS8 have also been used for mercury and arsenite detection, respectively, in soil extracts (Petanen and Romantschuk, 2002). Other biosensors have been designed, based on bioengineered proteins. In these cases, the biosensor monitors conformational changes caused by the binding of the metal ion to the engineered protein (Ziegler and Göpel, 1998). Bontidean *et al*. (2003) used mercuric ion-binding regulatory proteins as the biological component of the biosensor, MerR. The conformational change resulting from the binding of the metal ion to the protein caused a change in the capacitance, which was proportional to the concentration of the metal ions determined.

1.6.2 Immunoassays and bioreporters

Numerous promising tools are being developed that use biological systems to quantify solution phase and bioavailable metal concentrations. One of the most attractive features of these tools is that they can be used in complex systems such as microbiological media and soil. Immunoassays, which can detect solution phase metal concentrations in the low μg/L range, have been developed for cadmium, lead, cobalt, nickel, and zinc. An immunoassay for mercury is commercially available (Blake *et al*., 1998; Khosraviani *et al*., 1998). Bioavailable metal fractions have also been measured using whole cell bioreporters that produce a protein with measurable activity (e.g., LacZ) or light in response to bioavailable metal. Bioreporters for detection of mercury have been produced using both the lacZ system (Rouch *et al*., 1995) and the luminescent lux system (Corbisier *et al*., 1999; Selifonova *et al*., 1993). However, it should be emphasized that measurement of bioavailable metal can vary, as it is dependant on the metal resistance mechanisms of the bioreporter system used. A review of applications, advantages and limitations of immunoassays and bioreporters for metal detection is available (Neilson and Maier, 2001).

1.6.3 Geochemical modeling software

In addition to biological-based approaches, geochemical modeling software, such as MINEQL+ (Environmental Research Software, Hallowell, ME) or MINTEQA2, has been employed to predict metal speciation patterns as a function of ionic strength and pH (Pardue *et al.*, 1996). These programs take into account equilibrium constants for each ion in solution and accurately calculate the concentration of any metal species under specified conditions. The accuracy of programs such as MINEQL+ has been verified experimentally. For example, Sandrin and Maier (2002) used a cadmium ion-selective electrode to determine the concentration of divalent cadmium ion in a minimal salts medium over the pH range from 4 to 7. The experimental concentrations were comparable to those predicted by the modeling software. These programs do not take into account all organic ligands present in complex media, so they are more comparable to experimental situations in minimal media (Sandrin and Maier, 2002). In complex media, it is difficult to calculate the concentrations of all components because the composition of complex ingredients (e.g., yeast extract, beef extract) differs slightly in every batch (Sandrin and Maier, 2002). At least three computational models have been developed to predict the impact of metals on organic biodegradation (Amor *et al.*, 2001; Jin and Bhattacharya, 1996; Nakamura and Sawada, 2000). None of these models incorporates metal speciation and bioavailability. Thus, data generated by these models may only be meaningful for the medium or soil that was used to develop the model (Sandrin and Hoffman, 2007).

1.6.4 Diffusive gradients in thin-films

Recently, a diffusion-based *in situ* technique known as diffusive gradients in thin-films (DGT) has been proposed for the measurement of labile metal species in soils (Hooda *et al.*, 1999). DGT has been developed on the premise that metal speciation in conventional methods of testing soil solutions may change during sampling and extraction. The potential of DGT use in assessing metal bioavailability was further demonstrated when Cu uptake by plants grown on a large number of soils was linearly correlated to DGT measurements, while soil solution concentrations predicted a non-linear relationship (Zhang *et al.,* 2001).

1.7 Influence of heavy metals on microbiological processes involved in the biodegradation of chlorinated organic compounds

The influence of heavy metals on microbial processes, of individual strains and communities, such as respiration (Codina *et al.*, 2000; Fliessbach *et al.*, 1994; Hattori, 1992; Insam *et al.*, 1996; Khan and Scullion, 1999; Witter *et al.*, 2000), luminescence (Campbell *et al.*, 2000; Chaudri *et al.*, 2000; Lappalainen *et al.*, 2000; Paton *et al.*, 1995; Sousa *et al.*, 1998; Villaescusa *et al.*, 2000), and N_2

transformations (Heckman *et al*., 1986; Ibekwe *et al*., 1995; McGrath *et al*., 1988; Obbard and Jones, 1993) has been extensively reviewed. The impact of heavy metals on microorganisms has also been reviewed (Bååth, 1989; Giller *et al.,* 1998; Tyler, 1981; Weiner *et al.,* 1999; Wright and Mason, 2000). However, there is a marked dearth of information on the impacts of heavy metals on the biodegradation of chlorinated organic pollutants. Thus, additional studies that incorporate a variety of benchmark chlorinated organic chemicals and various manipulations of environmental factors that affect metal speciation and bioavailability are necessary.

A few research efforts that aimed at addressing the issue of co-contamination under aerobic conditions are listed in Table 1.1. below. Van Zwieten *et al*. (2003) reported that the natural breakdown of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT), a persistent organochlorine pesticide, was inhibited in an arsenic co-contaminated soil resulting in an increased persistence of DDT in the soil environment studied. The intrinsic breakdown of DDT to 1,1-dichloro-2,2-bis (4-chlorophenyl) ethane (DDD) in the presence of 2000 mg/kg arsenic resulted in a 50% reduction in the concentration of DDD compared to background arsenic of 5 mg/kg. Thus, it was demonstrated that arsenic co-contamination has an inhibitory effect on the breakdown of DDT via DDD, and that, as arsenic concentrations increased, the DDT:DDD and DDT:1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene (DDE) ratios also increased (Van Zwieten *et al*., 2003). The biodegradation of 2,4-dichloro-phenoxyacetic acid methyl ester (2,4-DME) in two microbial samples, namely, sediment and aufwuchs (floating mats of filamentous algae), from lakewater was inhibited in the presence of Cu, Hg, Zn, Cd and Cr (Said and Lewis, 1991). Minimal inhibitory concentrations (MIC) varied according to the metals and the type of microbial sample tested and did not necessarily follow the toxicity patterns observed for the metal concentrations required for significant effects on maximum degradation rates (V_{max}) and half-lives ($t_{½}$). Zinc was the most toxic in sediment samples with an MIC of 0.006 mg total zinc/L, whereas mercury was most toxic in Aufwuch samples with an MIC of 0.002 mg total mercury/L. Metal inhibition has also been observed during the biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) in cadmium contaminated systems (Roane *et al*., 2001). Degradation by *Ralstonia eutropha* JMP134, a cadmium-sensitive 2,4-degrader, occurred in the presence of up to 24 mg/L cadmium in mineral salts medium containing cadmium-resistant isolate and 0.060 mg/g cadmium in amended soil microcosms and field-scale soil bioreactors (Roane *et al*., 2001). Experiments also indicated that 10^4 colony forming units of *Ralstonia eutropha* JMP134/ml alone in the presence of > 3 mg/L cadmium in mineral salts medium did not degrade 2,4-D due to cadmium toxicity (Roane *et al*., 2001).

Reported metal concentrations that cause inhibition of anaerobic biodegradation of halogenated organic contaminants are listed in Table 1.2. below. Kuo and Genther (1996) demonstrated three effects of Cd^{2+}, Cu^{2+}, Cr^{6+} and Hg^{2+} ions on dechlorination and biodegradation of 2-chlorophenol (2-CP) and 3-

chlorobenzoate (3-CB), including extended acclimation periods, reduced dechlorination or biodegradation rates, and failure to dechlorinate or biodegrade the target compound. It was suggested that the concentration at which these effects were observed was characteristic of the metal ion added, the target compound studied and the consortium being used. The biodegradation of 3-CB was shown to be most sensitive to Cd^{2+} and Cr^{6+} whereas 2-CP consortium was considered most sensitive to added Cd^{2+} and Cu^{2+}. Since 2-CP and 3-CB were dechlorinated by distinct bacterial species, differences in metal sensitivity may have been specific to the dechlorinating species or the dechlorinating enzymes themselves. Interestingly, with Hg^{2+} at 1.0 to 2.0 ppm, 2-CP and 3-CP were biodegraded 133 to 154% faster than controls after an extended acclimation period, suggesting adaptation to Hg^{2+}, perhaps via removal or transformation of mercury by mercury-resistant bacterial species (Kuo and Genther, 1996).

Table 1.1: Reported metal concentrations that cause inhibition of biodegradation of chlorinated organic contaminants under aerobic conditions (Sandrin and Maier, 2003).

Metal	Organic	Lowest metal concentration reported to reduce biodegradation	Microbe(s) studied	Environment	pH	Reference
As^{3+}	DDT	5 mg/kg[a]	Indigenous community	Former co-contaminated soil	NR	Van Zwieten *et al.* (2003)
Cu^{2+}	2,4-DME	0.027 mg/L[a]	Indigenous community	Aufwuchs (microcosm)	5.0	Said and Lewis (1991)
Cu^{2+}	2,4-DME	0.076 mg/L[a]	Indigenous community	Sediment (microcosm)	6.1	Said and Lewis (1991)
Cu^{2+}	4-CP, 3-CB, 2,4-D	< 14.3-71.6 mg/L[a, b]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)
Cd^{2+}	2,4-D	0.060 mg/g[a]	*Ralstonia eutropha* JMP134	Soil microcosms	8.2	Roane *et al.* (2001)
Cd^{2+}	2,4-D	0.060 mg/g[a]	*Ralstonia eutropha* JMP134	Field-scale bioreactors	8.2	Roane *et al.* (2001)
Cd^{2+}	2,4-DME	0.100 mg/L[a]	Indigenous community	Sediment (microcosm)	6.5	Said and Lewis (1991)
Cd^{2+}	2,4-DME	0.629 mg/L[a]	Indigenous community	Aufwuchs (microcosm)	5.6	Said and Lewis (1991)
Cd^{2+}	2,4-D	> 3 mg/L[a]	*Ralstonia eutropha* JMP134	Mineral salts medium	6.0	Roane *et al.* (2001)
Cd^{2+}	2,4-D	24 mg/L[a]	*Ralstonia eutropha* JMP134	Mineral salts medium containing cadmium-resistant isolate	6.0	Roane *et al.* (2001)
Cd^{2+}	4-CP, 3-CB, 2,4-D	< 25.3-50.6 mg/L[a, b]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)
Co^{2+}	4-CP, 3-CB, 2,4-D	< 13.3-1.330 mg/L[a, b]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)
Cr^{3+}	2,4-DME	0.177 mg/L[a]	Indigenous community	Aufwuchs (microcosm)	6.1	Said and Lewis (1991)
Cr^{6+}	4-CP, 3-CB, 2,4-D	< 131 mg/L[a, b]	*Alcaligenes* spp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)

Hg^{2+}	2,4-DME	0.002 mg/L[a]	Indigenous community	Aufwuchs (microcosm)	6.8	Said and Lewis (1991)
Hg^{2+}	4-CP, 3-CB, 2,4-D	< 45.2-226 mg/L[a, b]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)
Ni^{2+}	4-CP, 3-CB, 2,4-D	5.18-10.3 mg/L[a, b]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)
Zn^{2+}	2,4-DME	0.006 mg/L[a]	Indigenous community	Sediment (microcosm)	6.4	Said and Lewis (1991)
Zn^{2+}	2,4-DME	0.041 mg/L[a]	Indigenous community	Aufwuchs (microcosm)	5.6	Said and Lewis (1991)
Zn^{2+}	4-CP, 3-CB, 2,4-D	< 29.5-736 mg/L[a, b]	*Alcaligenes* sp., *Pseudomonas* spp., *Moraxella* sp.	Tris-buffered minimal medium plates	7.0	Springael *et al.* (1993)

Abbreviations: 3-CB, 3-chlorobenzoate; 4-CP, 4-chlorophenol; 2,4-D, 2,4-dichlorophenoxyacetic acid; DDT, 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane; 2,4-DME, 2,4-dichloro-phenoxyacetic acid methyl ester; MTC, maximum total concentration; NR, not reported.

[a]Value represents total metal added to system. [b]Value represents MIC calculated by multiplying MTC by a factor of 2.25 (Sandrin and Maier, 2003).

Table 1.2: Reported metal concentrations that cause inhibition of biodegradation of chlorinated organic contaminants under anaerobic conditions (Sandrin and Maier, 2003).

Metal	Organic	Lowest metal concentration reported to reduce biodegradation	Microbe(s) studied	Environment	pH	Reference
Cd^{2+}	TCA	0.01 mg/L[a]	Indigenous community	Laboratory soil microcosms containing rice paddy and bottomland hardwood soils	6.9-7.4	Pardue *et al*. (1996)
Cd^{2+}	TCA	0.2 mg/L[a]	Indigenous community	Laboratory soil microcosms containing organic matter- rich soil	6.8	Pardue *et al*. (1996)
Cd^{2+}	2-CP, 3-CB	0.5-1.0 mg/L[b]	Indigenous community	Aqueous sediment enriched in anaerobic growth medium	7.0	Kuo and Genther (1996)
Cd^{2+}	2-CP, 3-CP	20 mg/L[b]	Indigenous community	Sediment slurry	7.0	Kong (1998)
Cr^{6+}	2-CP, 3-CB	0.01-0.5 mg/L[b]	Indigenous community	Aqueous sediment enriched in anaerobic growth medium	7.0	Kuo and Genther (1996)
Cu^{2+}	2-CP, 3-CB	0.1-1.0 mg/L[b]	Indigenous community	Aqueous sediment enriched in anaerobic growth medium	7.0	Kuo and Genther (1996)
Cu^{2+}	2-CP, 3-CP	20 mg/L[b]	Indigenous community	Sediment slurry	7.0	Kong (1998)
Cr^{6+}	2-CP, 3-CP	20 mg/L[b]	Indigenous community	Sediment slurry	7.0	Kong (1998)
Pb^{2+}	HCB	0.001 mg/g[b]	Indigenous community	Microcosms containing contaminated sediment	NR	Jackson and Pardue (1998)
Hg^{2+}	2-CP, 3-CB	0.1-1.0 mg/L[b]	Indigenous community	Aqueous sediment enriched in anaerobic growth medium	7.0	Kuo and Genther (1996)
Zn^{2+}	PCP	2 mg/L[b]	Indigenous community	Anaerobic digester sludge in a liquid medium containing 0.6 mM phosphate	NR	Jin and Bhattacharya (1996)
Zn^{2+}	PCP	8.6 mg/L[b]	Indigenous community	Anaerobic enrichment cultures in serum bottles	NR	Majumdar *et al*. (1999)

Abbreviations: 3-CB, 3-chlorobenzoate; 2-CP, 2-chlorophenol; 3-CP, 3-chlorophenol; HCB, Hexachlorobenzene; PCP, Pentachlorophenol; NR, not reported; TCA, Trichloroaniline.
[a]Value represents solution-phase concentration of metal present in system. [b]Value represents total metal added to system (Sandrin and Maier, 2003).

1.8   Relationships between metal concentration and inhibition of biodegradation

The total metal concentration in a system has been the most commonly employed indicator of metal inhibition of biodegradation. Clearly, other factors are also involved, considering the large disparities between minimum inhibitory concentrations among studies (Sandrin and Hoffman, 2007). The most common trend observed in most of the data presented indicates that inhibition increases progressively as the concentration of bioavailable metal in co-contaminated system increases. Numerous research efforts have indicated that this is not always the case, and literature contains reports that metals do not inhibit some biodegradative processes (Baldrian *et al*., 2000; Delaune *et al.,* 1998; Riis *et al*., 2002). When metals inhibit biodegradation, their effects are not always dose-dependent and there is evidence for two semi-dose dependent patterns of metal effects on organic biodegradation.

1.8.1   Semi-Dose Dependent Pattern 1: low metal concentrations stimulate biodegradation; high metal concentrations inhibit biodegradation

Several studies suggest that metals stimulate activity until a maximum level of stimulation is reached and, thereafter, metal toxicity increases with increasing metal concentration (Sandrin and Maier, 2003). It is important to note that all of these studies used consortia, not single isolates. Thus, it is likely that this pattern results from differential toxicity effects, where one population that is sensitive to metal stress competes in some way with a second metal-tolerant population expressing the activity of interest (e.g., biodegradation). Inhibition of the more sensitive population reduces competition for resources needed by the metal tolerant population expressing the activity of interest. Capone *et al*. (1983) provided evidence supporting this explanation, and reported that copper and cadmium (both at 0.01 mg total metal/L) increased 2-chlorophenol biodegradation rate by 168%, while mercury (1-2 mg total mercury/L) increased the biodegradation rates of 2-CP and 3-CP by 133-154%. Hughes and Poole (1989) and Sterritt and Lester (1980) reported similar results with various consortia, and suggested that the stimulatory effect may be due to reduced competition for reducing equivalents or nutrients between metal-resistant degraders and metal-sensitive non-degraders. Capone *et al.* (1983), Kuo and Genthner (1996) and Roberts *et al*. (1998) also reported that the impact of metals on microbially mediated processes may be due mainly to effects of metals on a population other than the one carrying out the process of interest.

1.8.2 Semi-Dose Dependent Pattern 2: low metal concentrations inhibit biodegradation; high metal concentrations inhibit less

Some studies have shown a second semi-dose dependent pattern, in which low concentrations of metals increasingly inhibit activity until a maximum level of inhibition is reached and, thereafter, metal toxicity decreases with increasing metal concentration. The data published by Said and Lewis (1991) generally showed that 2,4-DME biodegradation decreased in a dose-dependent trend. A closer examination of the data revealed that the maximal degradation rate (V_{max}) of 2,4-DME was less in the presence of 10 μM cadmium (0.61 ± 0.03 Mg 2,4-DME/L/min) than in the presence of 100 μM cadmium (0.74 ± 0.00 Mg 2,4-DME/L/min). In a later study, Roane and Pepper (1997) identified a similar pattern of inhibition as populations of 2,4-D degraders in a cadmium contaminated soil were more resistant to cadmium toxicity at a higher concentration of cadmium (40 mg total cadmium/L) than at a lower concentration of cadmium (20 mg total cadmium/L). The pattern 2 responses to metals might be explained by microbial community dynamics. High metal concentrations may create selective pressure for metal-resistant, organic-degrading microorganisms that reduced competition from metal-sensitive non-degrading microorganisms, thus increasing biodegradation at higher metal concentrations. It has been suggested that at the level of single cells, it is possible that high metal concentrations may more rapidly induce a metal resistance mechanism important in cadmium detoxification (e.g., an efflux pump) than low metal concentrations (Sandrin and Hoffman, 2007).

In summary, the existence of semi-dose dependent patterns of metal effects on biodegradation complicates understanding and predicting metal toxicity in the environment. As demonstrated by the patterns described above, metals may impact both the physiology and ecology of pollutant degrading microorganisms. For this reason, models designed to predict the impact of metals on biodegradation may fail to do so accurately unless they include both physiological and ecological effects of metals on organic-degrading microorganisms (Sandrin and Hoffman, 2007).

1.9 Bioremediation

By definition, bioremediation is the employment of biological activities to degrade and/or detoxify contaminants for alleviation (and wherever possible complete elimination) of the noxious effects, both to human health and the environment, caused by organic and inorganic pollutants in contaminated sites (Iwamoto and Nasu, 2001). It is a managed treatment process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities (Smets and Prichard, 2003). de Lorenzo (2008) noted that bioremediation is an intervention aimed at mitigating pollution, and therefore stated that the field belongs to the sphere of biotechnology. Depending on the extent of such intervention, bioremediation is

commonly considered to include natural attenuation (little or no human action on the contaminated site), biostimulation (addition of nutrients, and electron donors/acceptors to promote the growth or metabolism of particular microorganisms), or bioaugmentation (the deliberate addition of natural or engineered microorganisms with the desired catalytic capabilities) (El Fantroussi and Agathos, 2005; Van Dillewijn *et al*., 2007). Bioremediation is gradually making inroads for applications in environmental clean-up of co-contaminated environments because it has been established as a versatile, efficient, economical, easy to apply, simple to maintain, environmentally sound treatment and leads to complete destruction of a wide variety of contaminants with little secondary pollution compared to other techniques (Lee *et al*., 2008; Norris, 1994; Park *et al*., 2008).

1.9.1   Natural attenuation

Natural attenuation, as a management approach for contaminated environments, hinges on the notion that there exist *in situ* transformation processes (involving dispersion, sorption, biotic and abiotic degradation of target compounds) that are possibly, self-sustaining, appropriate with regard to type and sufficient in magnitude to control the risk associated with the resident pollutants (Davis *et al*., 2003; Smets and Pritchard, 2003). In virtually all situations, microbial reactions are the dominant processes driving the natural attenuation of both organic and inorganic contaminants (Smets and Pritchard, 2003). Numerous case studies have reported natural attenuation of soil (Chaineau *et al*., 2003; Kastanek *et al*., 1999; Margesin and Schinner, 2001). For example, Chaineau *et al*. (2003) noted a 56% removal of the hydrocarbons via natural attenuation in a 480-day field experiment contaminated with 18,000 mg hydrocarbon/kg soil, compared to 70% to 81% with fertilization. Natural attenuation is advantageous as it avoids damaging ecologically sensitive microbial habitats (Dowty *et al*., 2001). Intrinsic bioremediation, however, is often a long term process because of low population sizes of the indigenous degrading microorganisms (Forsyth *et al*., 1995; Yu *et al*., 2005).

1.9.2   Biostimulation

Biostimulation is considered the most extensively used bioremediation procedure. This practice is employed for the proliferation of indigenous microorganisms by addition of nutrients and/or a terminal electron acceptor as well as making appropriate pH adjustments (Margesin and Schinner, 2001; Salanitro *et al*., 1997). However, prior to initiating biostimulation protocols, it is important to establish the potential of indigenous microorganisms and to assess the limiting factors to be controlled during treatment (Menendez-Vega *et al*., 2007). It has been established that the nutrient requirements for microbes are approximately the same as the composition of their cells (Sutherson, 1997), with carbon being an exception as it is required at larger quantities and can be supplied by the contaminant for heterotrophic

microorganisms (Liebeg and Cutright, 1999). Nutrient requirements of microorganisms are divided into three categories (macro, micro, and trace nutrients) which are based largely on the essential need and quantity required by the microorganism. For example, the macronutrients carbon, nitrogen, and phosphorus comprise approximately 50, 14, and 3% dry weight of a typical microbial cell, respectively (Liebeg and Cutright, 1999). Based on this criterion, the optimal C:N:P mole-ratio recommended for bioremediation applications is 100:10:1 (Cookson Jr, 1995; Norris *et al*., 1994). Contaminated sites consisting of large quantities of organic pollutants tend to depletion of the available pools of major macronutrients, N and P. It is not surprising; therefore, that numerous studies of the effects of biostimulation with N-P-K fertilizers have reported positive effects (Margesin and Schinner, 2001). Moreover, redox potential is another important aspect which must be considered prior to supplementation with nutrients. It defines electron availability and affects the oxidation states of hydrogen, carbon, nitrogen, oxygen, sulfur, manganese, iron, etc. (Liebeg and Cutright, 1999). Therefore, careful consideration must be taken in determining the quantity and type of nutrients to add so that the optimal redox potential is maintained. For an optimal aerobic environment, the redox potential must be greater than 50 millivolts (Norris *et al*., 1994). Currently, there are no precise protocols for determining the exact nutrient sources to employ at a contaminated site. The specific ratio depends, inter alia, on the rate and extent of degradation of the chemicals present, the bioavailability of soil nutrients, the soil types, and the presence of oxygen or other electron acceptors (Liebeg and Cutright, 1999). The nutrients inherent to the particular environment, as well as those supplemented, can interact with the contaminant, bacteria, soil, and terminal electron acceptor. These interactive effects will significantly impact the successful implementation of biostimulation. However, understanding the effects of various nutrients and quantity may enable comparisons to be drawn across different sites, hence accelerating the bioremediation process (Liebeg and Cutright, 1999).

1.9.3 Bioaugmentation

Bioaugmentation entails the addition of indigenous and/or non-indigenous laboratory grown microorganisms capable of biodegrading the target contaminant (Vogel, 1996; Widada *et al.,* 2002) or serving as donors of catabolic genes (Top *et al*., 2002). In cases where indigenous communities of bacteria are unable to carry out the desired reactions, degrading the contaminant at rates that are too low, inhibited by the presence of multiple contaminants or killed as a result of drastic (abiotic) remediation techniques, bioaugmentation can be introduced to hasten the degradation process (Widada *et al.,* 2002). An extensive review by Gentry *et al*. (2004) details several new approaches that may increase the persistence and activity of exogenous microorganisms and/or genes following introduction into the environment. The major advantage of bioaugmentation over other techniques is that when a specific

microbial population is injected the degradation process can start immediately, whereas during biostimulation, for example, a delay after injection of nutrients occurs as the microbial population propagates and also nutrients are not specific, so that all microbes will potentially propagate, diluting the effect of the nutrients (Weston and Balba, 2003). The success/failure of most bioaugmentation experiments depends highly on the survival of the inoculated cells under stress conditions inherent in the soil environment, including competition from indigenous microorganisms. Bento *et al*. (2003) concluded that the best approach for bioremediation was the bioaugmentation performed by inoculating indigenous microorganisms pre-selected from their own environment, as these microbes are more likely to survive and propagate when reintroduced into the site. Inoculating the contaminated site with microorganisms carrying self-transmissible plasmids containing genes involved in resistance and/or degradation is an alternative approach to bioaugmentation (Newby *et al*., 2000a). These plasmids may be transferred to indigenous microorganisms that possess the characteristics necessary for growth and survival in the soil environment and, thus, establish a stable array of hosts for the plasmids (Daane *et al*., 1996; Newby *et al*., 2000b; Top *et al*., 1998). This bioremediation approach may be of particular interest for sites that contain both organic and metal contaminants, as the presence of metals has been shown to significantly reduce, if not inhibit, organic degradation (Said and Lewis, 1991; Olaniran *et al*., 2009)

1.10 Bioremediation strategies for increasing biodegradation in co-contaminated environments

Several approaches aimed at reducing the extent to which metals inhibit chlorinated organic biodegradation have focused specifically on lowering bioavailable metal concentrations and/or increasing metal resistance. Approaches include inoculation with metal-resistant microorganisms and the addition of treatment amendments that can reduce metal bioavailability. Phytoremediation has also shown promise as an emerging alternative clean-up technology for co-contaminated environments, and is currently under investigation. The various approaches are discussed below.

1.10.1 Metal-resistant bacteria

In the case of co-contamination, the double stress imposed on the soil bacterial communities means that for effective *in situ* bioremediation of the organic contaminant, there must be metal-resistant microbes with appropriate degradative genes, or consortia of metal-resistant microbes with suitable catabolic capabilities (Pepper *et al.,* 2002). Previously, bioaugmentation studies focused on the introduction of a microorganism that was both metal-resistant and capable of organic degradation. However, under environmental conditions such an approach is often unsuccessful, probably due to the high energy requirements needed to maintain concurrent metal resistance and organic degradation (Roane

et al., 2001). Recent approaches have demonstrated the use of a dual-bioaugmentation strategy and the role of cell bioaugmentation in the remediation of co-contaminated systems (Pepper *et al.,* 2002; Roane *et al*., 2001).

Unlike organics, metals cannot be degraded, and thus most biological heavy metal remediation approaches rely on the detoxification and immobilization of the metal both to reduce the biological toxicity and to retard metal transport (Roane *et al*., 2001). Many factors influence the survival of organisms exposed to toxic levels of heavy metals, including lateral gene transfer (LGT) for the dissemination of resistance phenotypes throughout microbial communities (Coombs and Barkay, 2004; 2005; Osborn *et al*., 1997) and changes in active-site residues that influence substrate specificity of metal homeostasis proteins (Tong *et al*., 2002). Although metals are thought to inhibit the ability of microorganisms to degrade chlorinated organic pollutants, several microbial systems of resistance to metals are known to exist (Nies, 1992; 1999; Rosen, 1996; Saier Jr *et al*., 1994; Silver, 1996; Silver and Phung, 1996). However, there are only three possible mechanisms by which these systems operate. Firstly, the accumulation of the particular ion can be diminished by efflux, an active extrusion of the heavy metal from the cell (Nies and Silver, 1995), which include: members of the resistance-nodulation-cell division (RND) protein family-export superfluous cations; cation diffusion facilitators (CDF family) which serve as secondary cation filters in bacteria; P-type ATPases-basic defence against heavy metal cations; and CHR protein family, NreB, CnrT. Secondly, cations, especially the "sulfur lovers", can be separated into complex compounds by thiol-containing molecules; and thirdly, some metal ions may be reduced to a less toxic oxidation state. A detailed review is available that describes modes of efflux-mediated heavy metal resistance in prokaryotes (Nies, 2003).

Most aerobic cells have a physiological redox range (-421 mV to + 808 mV); therefore, to be detoxified by reduction the redox potential of a given heavy metal should be between this range. Thus, heavy metals such as Hg^{2+} (+430 mV), arsenate (+139 mV), and Cu^{2+} (-268 mV) may be reduced by the cell, but Zn^{2+} (-1.18 V), Cd^{2+} (-824 mV), and Ni^{2+} (-678 mV) may not (Nies, 1999). In the case of many metals, resistance and homeostasis involve a combination of two or three of the basic mechanisms mentioned. Roane *et al*. (2001) investigated dual-bioaugmentation involving inoculation with both metal-detoxifying and organic-degrading bacteria to facilitate organic degradation within a co-contaminated system. Soil microcosms were constructed using uncontaminated sandy loam soil amended with 500 μg of 2,4-D/ml, and co-contaminated with 60 μg of cadmium to a final concentration of 60 μg/ml. This was followed by inoculation with *Ralstonia eutropha* JMP134, a 2,4-D degrader and *Pseudomonas* H1, a cadmium-resistant strain. Based on the results obtained, it was concluded that dual-bioaugmentation with metal-detoxifying and organic-degrading microbial populations is effective for remediation of co-contaminated soil; however, reducing bioavailable metal concentrations via sequestration prior to

inoculation with the organic-degrading population will promote increased degradation. In another study, Pepper *et al.* (2002) investigated the role of cell bioaugmentation and gene bioaugmentation in the remediation of co-contaminated soil. *Escherichia coli* D11, which contains plasmid pJP4, but does not have the chromosomal genes necessary for the transformation of 2-chloromaleylacetate to succinic acid, was used for gene bioaugmentation. The observation from this study suggests that the indigenous transconjugant population generated from *E. coli* D11 inoculation was better suited for subsequent 2,4-D degradation than the *R. eutropha* JMP134-inoculated soil, in which the presence of the 2,4-D degrading inoculant repressed transconjugant growth. However, the ultimate choice of cell or gene bioaugmentation will depend on the relative health potential of the recipient population, the degree of contamination and the time frame available for remediation.

1.10.2   Treatment amendments

Many studies have been carried out to evaluate the ability of different chemical amendments to immobilize heavy metals in polluted environments. These additives include organic materials, phosphate rocks, iron and manganese oxides and oxy-hydroxides, and waste by-products rich in these oxides as well as alkaline agents such as lime and zeolite (Basta *et al.*, 2001; Boisson *et al.*, 1998; Chen *et al.*, 2000; Edwards *et al.*, 1999; García-Sánchez *et al.*, 1999; Gworek, 1992; Hodson *et al.*, 2000; Lehoczky *et al.*, 2000; Li *et al.*, 2000; Mench *et al.*, 2000; Shuman *et al.*, 2002). In general, these treatments prove to have an ameliorative effect on reducing the metal mobility or bioavailability. Panuccio *et al.* (2009) evaluated cadmium sorption in three different minerals (vermiculite, zeolite and pumice). Results indicated that zeolite and vermiculite reduced soluble cadmium concentrations by 90% and that the metal sorbed on zeolite was mainly present in the non-exchangeable form (70%) at the lowest cadmium concentration (30-120 μM). Furthermore, it was reported that the percentage of cadmium sorption in zeolite and vermiculite was independent of the initial cadmium concentration, and the mineral sorption capacity was closely dependent upon pH. In particular, cadmium adsorption on pumice was raised from 20% to 90% with an increase of pH from 4 to 7.5. Phosphate amendments, in particular, have been given much attention for the treatment of Pb-contaminated environments (Brown *et al.*, 2005; Chen *et al.*, 2000; 2003; Li *et al.*, 2000; Shuman *et al.*, 2002). Despite the well-documented ability of treatment amendments to reduce metal mobility and bioavailability, not much attention has been directed towards determining microbial endpoints after the treatment of contaminated environments. Brown *et al.* (2005) examined the effect of lime, phosphorus, red mud, cyclonic ashes, biosolids and water treatment residuals on the toxicity of cadmium, lead and zinc in an international inter-laboratory study. Each participating laboratory selected a common soil material from mine wastes and common treatments. Nitrogen (N) transformation and a measure of the total soil microbial biomass were chosen as microbial endpoints. The N transformation test

was designed to measure nitrate formation in soils after the addition of an organic substrate. The formation of nitrate is an indicator of microorganisms degrading the C-N bonds in the organic substrate and recycling nutrients within the soil. Of the amendments tested by the participating laboratories, P added as either triple sugar phosphate or H_3PO_4 appeared to be the most effective. Phosphorus addition to the soil resulted in reduced soil solution and extractable metals, reduced bioavailability of soil Pb, and increased microbial activity based on the two measures. These promising results suggest that the use of treatment amendments may be an effective means to increase chlorinated organic pollutant biodegradation in the presence of toxic levels of heavy metals.

1.10.3   Clay minerals

The use of clay minerals to reduce metal bioavailability and resulting toxicity in groundwater and sub-soils has been successful for the remediation of heavy metal polluted environments (Boenigk *et al*., 2005; Sandrin and Maier, 2003). Clays differ in chemical and physical properties and have a comparatively high ion exchange capacity of 5 to > 50 meq/100 g clay (montmorillonite > illite > kaolinite). Charged substances attach easily to clay particles. Sorption of heavy metals on clays has been studied for montmorillonite (Barbier *et al*., 2000), illite (Echeverria *et al*., 2002), kaolinite (Coles and Yong, 2002; Sarkar *et al*., 2002), and bentonite and vermiculite (Kamel, 1986; Panuccio *et al*., 2009). These clays are chosen to avoid pollutant release into the environment owing to their high specific surface areas, low cost and ubiquitous presence in most soils (Bailey *et al*., 1999). In particular, the evaluation of the total capacity of Na-montmorillonite shows that this clay is a good sorbent towards a variety of metals (Abollino *et al.,* 2003), and generally has a higher sorption of heavy metals than kaolinite (Barbier *et al*., 2000). This clay mineral adsorbs heavy metals via two different mechanisms: (1) cation exchange in the inter layers resulting from the interactions between ions and negative permanent charge; and (2) formation of inner-sphere complexes through Si-O⁻ and Al-O⁻ groups at the clay particle edges (Kraepiel *et al*., 1999; Mercier and Detellier, 1995; Schindler *et al*., 1976). Abollino *et al*. (2003) reported that the adsorption of metal ions on Na-montmorillonite decreases with decreasing pH and is also influenced by the presence of ligands. At low pH values (2.5-3.5), the hydrogen ion competes with the heavy metals towards the superficial sites and, moreover, the Si-O⁻ and Al-O⁻ groups are less deprotonated and they form complexes with bivalent and trivalent ions in solution with greater difficulty (Abollino *et al*., 2003). This effect was particularly evident for Cu^{2+} (as aqua ion $[Cu(H_2O)_6]^{2+}$) which has a distorted geometry, and for Pb^{2+} and Cd^{2+} that have a lower electrostatic attraction versus the clay because of their lower charge density (Abollino *et al*., 2003). For these reasons, the adsorption of these ions is unsupported by cation exchange mechanism and, hence, they are influenced more by pH variations. Therefore, the pH effect on each metal tested was different and, at pH ≤ 3.5, the studied metals were adsorbed in increasing

entity in the following order: $Cu^{2+} < Pb^{2+} < Cd^{2+} < Zn^{2+} \leq Mn^{2+} \approx Cr^{3+} \approx Ni^{2+}$ (Abollino *et al*., 2003). This result was a function of the ligand and metal considered but the formation of metal-ligand complexes in solution altogether hinders the adsorption of the metal ions on the clay. In this case, the metal adsorption increased in the following order: $Cr^{3+} < Cu^{2+} < Ni^{2+} < Zn^{2+} \leq Cd^{2+} \leq Pb^{2+} \leq Mn^{2+}$ (Abollino *et al*., 2003). The result indicated that the sorption capability of Na-montmorillonite towards each metal ion examined was different under various conditions, and was a function of both pH and of the ligand present in solution. It is therefore necessary to consider both these factors in studying a real soil/solution system and effectively predicting the fate of heavy metals in the environment. From the results it was evident that the total capacity of Na-montmorillonite towards the investigated metals increases in the order: $Pb^{2+} = Cd^{2+} < Cu^{2+} < Zn^{2+} < Mn^{2+} < Ni^{2+} < Cr^{3+}$ (Abollino *et al*., 2003). In a similar study, Boenigk *et al*. (2005) reported that LC_{50} of *Spumella* sp in solution systems contaminated with Cd^{2+} decreased by 71% and 64% in the presence of clay and silicate beads, respectively.

1.10.4 Chelating agents

Chelating agents increase metals diffusion in the soil solution and keep them in plant available forms by forming large, less reactive ions, by increasing the concentration of these larger chelated ions in solution, and by decreasing the ability of the free ions to react with the soil (Žemberyová *et al*., 2007). Chelating agents offer great promise for assessing readily available micronutrient cations in soils (Žemberyová *et al*., 2007). These agents adhere with free metal ions in solution forming soluble complexes and thereby reduce the activities of the free metal ions in solution. In response, metal ions desorb from soil particles or dissolve from labile solid phases to replenish the free metal ions in solution. Chelating agents, such as ethylenediamine-tetraacetic acid (EDTA), have been employed to reduce metal toxicity to organic-degrading microorganisms. EDTA was shown to reduce the toxicity of cadmium to *Chlorella* sp. (Upitis *et al*., 1973), of nickel to algae (Spencer and Nichols, 1983), and of copper to bacteria and algae (Sunda and Guillard, 1976). However, the toxicity of EDTA to many microorganisms and its limited biodegradability reduce its suitability for application to the bioremediation of co-contaminated environments (Borgmann and Norwood 1995; Braide, 1984; Ogundele, 1999). For this reason, the development of several surfactant-modified clay complexes to reduce metal toxicity has sparked greater interest.

Malakul *et al*. (1998) have demonstrated the potential application of surfactant-modified clay adsorbents in mixed-waste biotreatment, in which toxic organics and heavy metals co-exist. In this study, the toxicity of cadmium to *Pseudomonas putida* was greatly reduced by the addition of a surfactant modified-clay complex and a commercially available chelating resin (Chelex 100; Biorad, Hercules, CA) during the biodegradation of naphthalene. Surfactant modified-clay complexes are prepared through a

simple surface modification method of grafting metal-chelating ligands in order to impart a higher metal capturing capacity and selectivity to the base clays.

1.10.5   Biosurfactants

Biosurfactants are amphiphilic compounds which can reduce surface and interfacial tensions by accumulating at the interface of immiscible fluids and increase the solubility, mobility, bioavailability and subsequent biodegradation of hydrophobic or less soluble organic compounds (Makker and Rochne, 2003), such as polychlorinated biphenyls (Van Hamme *et al.*, 2006). Biosurfactants are produced extracellularly or as part of the cell membrane by bacteria, yeasts and fungi, from various substrates including sugars, alkanes, oils and waste (Mulligan, 2005). Many studies of biosurfactant-enhanced bioremediation have employed small, well-characterized biosurfactants such as *Pseudomonas aeruginosa* rhamnolipids (Maier and Soberon-Chavez, 2000; Mulligan, 2005), *Candida apicola* sophorose lipids (Hommel *et al.*, 1994), *Rhodococcus erythropolis* trehalose dimycolipids (Kanga *et al.,* 1997), *Bacillus* sp. lichenysins (Jenny *et al.*, 1991), and *Bacillus subtilis* surfactin (Awashti *et al.,* 1999; Makker and Cameotra, 1997; Mulligan *et al.*, 2001a).

Important here is the advantage of such compounds at co-contaminated sites, since microorganisms have long been shown to produce potent surface-active compounds that enhance the rate of degradation by emulsification or solubilization of the hydrophobic hydrocarbon (Noriyuki *et al.,* 2002). Exploiting this property, Berg *et al.* (1990) described the potential use of a *P. aeruginosa* UG2-produced biosurfactant to significantly increase the solubility and dissolution of hexachlorobiphenyl into the aqueous phase. In tests with the emulsifying agent in soil slurries, 31% of the added hexachlorobiphenyl was recovered in the aqueous phase. In a similar study, Van Dyke *et al.* (1993) surveyed a variety of biosurfactants for removal of hexachlorobiphenyl from soil. Out of 13 biosurfactants tested, seven removed hexachlorobiphenyl more efficiently compared to controls. Two strains of *P. aeruginosa* and one strain of *A. calcoaceticus* RAG-1 produced the most efficient biosurfactant.

Furthermore, biosurfactants may also enhance the desorption of heavy metals from soils via 2 approaches: firstly, complexation of the free form of the metal residing in solution which decreases the solution phase activity of the metal and, therefore, promotes desorption according to Le Chatelier's principle; secondly, direct contact of biosurfactant to sorbed metal at solid solution interface under conditions of reduced interfacial tension, which allows biosurfactants to accumulate at solid solution interface (Miller, 1995). Considerable work has been done on rhamnolipid biosurfactant produced by various *P. aeruginosa* strains capable of selectively complexing cationic metal species such as Cd^{2+}, Pb^{2+}, and Zn^{2+} (Herman *et al.*, 1995; Tan *et al.*, 1994; Torrens *et al.*, 1998), thus increasing the bioavailability of substrates with limited aqueous solubilities (Herman *et al.*, 1997; Zhang *et al.*, 1997; Zhang and Miller,

1995), and also increasing cell surface hydrophobicity (Al-Tahhan *et al*., 2000; Zhang and Miller, 1994). Research has shown that rhamnolipids complexes more preferentially with toxic metals such as Cd and Pb than with normal soil metal cations such as Ca and Mg, for which it has a much lower affinity (Said and Lewis, 1991). Also, metals such as lead and cadmium have stronger affinities for rhamnolipids than for many of the soil components to which they are bound in contaminated soils (Sandrin *et al.,* 2000).

Due to the foaming property of the biosurfactant, metal-biosurfactant complexes can be removed by addition of air to cause foaming and then the biosurfactant can be recycled through precipitation by reducing the pH to 2 (Wang and Mulligan, 2004). Anionic biosurfactants were found to be more effective where metals are the agents to be sequestered. Surfactin, rhamnolipid, and sophorolipids, all anionic biosurfactants, were able to remove copper and zinc from a hydrocarbon-contaminated soil (Mulligan *et al*., 1999). One advantage in case of co-contaminated soil is that biosurfactants can potentially be produced *in situ* using the organic contaminants as substrates for their production, which subsequently would lead to both the remediation of the contaminants and a great reduction in remediation costs (Abraham *et al.,* 2002). The efficiency of biosurfactants for stimulating biodegradation of contaminants is uncertain given the specificity observed between biosurfactant and organism. Addition of biosurfactant can stimulate some organisms but also can inhibit some microorganisms; a strategy suitable for effective remediation would therefore be to stimulate biosurfactants produced by indigenous population, or to use commercial biosurfactants produced by organisms found to be already present at the contaminated site (Abraham *et al.,* 2002). Furthermore, delivery of a biosurfactant into co-contaminated sites for *in situ* treatment may be more environmentally compatible and more economical than using modified clay complexes or metal chelators such as EDTA (Gray and Wilkinson, 1965; Kamel, 1986; Sandrin *et al*., 2000).

1.10.6 Phytoremediation

In situ bioremediation is gaining momentum as a low-cost and effective method for restoration and remediation of many contaminated sites. In particular, the use of plants for rehabilitation of heavy metal contaminated environments is an emerging area of interest because it is ecologically sound and safe (Lin *et al*., 2008; Wu *et al*., 2006). Although phytoremediation is a slow process, improvement of efficiency and thus increased stabilization or removal of heavy metals from soils is an important goal (Göhre and Paszkowski, 2006), especially in the case of co-contamination. Plants use the following mechanisms to facilitate remediation, these include: phytostabilization, phytoextraction, phytovolatilization, phytopumping, phytotransformation/degradation, and rhizodegradation (Singh and Jain, 2003; Susarla *et al*., 2002). The biomass production of a few hyperaccumulator plants has been judged sufficient for phytoremediation; for example, the brake fern *Pteris vittata* accumulated up to 7500

μg/g As on a contaminated site without showing toxicity symptoms (Ma *et al.,* 2001). Numerous research efforts have focused on the use of these techniques as viable alternatives to mechanical and chemical approaches in remediation of metal contaminated soils or as a final, consummating step in high-level organic contamination (Jones, 1991; Leigh *et al.,* 2002; Singh *et al.,* 2004; Zhu *et al.,* 2001). However, an alternative approach, which focussed on the dissipation mechanisms by *Lolium perenne* L (ryegrass) and *Raphanus sativus* (radish) for pentaphlorophenol (PCP) in copper co-contaminated soil, indicated that with an initial PCP concentration of 50 mg/kg, plants grew better with the increment of soil Cu level (0, 150, 300 mg/kg). This inferred that combinations of inorganic and organic pollutants sometimes exerted antagonistic effects on plant cytotoxicity (Lin *et al.,* 2006). In copper co-contaminated soil with an initial PCP concentration of 100 mg/kg, however, both plant growth and microbial activity were inhibited with the increment of soil Cu level, implying that the soil phytotoxicity was increased in the presence of Cu (Lin *et al.,* 2006). The reduced mass flow and lowered degrading activity of microorganisms were probably responsible for the significantly lower levels of PCP dissipation in the soil co-contaminated with copper. As mentioned previously, heavy metals are bound to soil components in varying degrees, depending on soil conditions such as pH, clay content, organic matter, redox potential (Sandrin and Hoffman, 2007; Sandrin and Maier, 2003). Natural chelating agents, such as citric and acetic acid, released by plant roots make the ions of both nutrients and contaminants more mobile in the soil. Plants can usually break the chelation bond, take up the metal, and release the chelant back into the soil solution. Since most organic-degrading microorganisms are sensitive to the toxic effects of heavy metals, a successful strategy to address this mixed-waste situation requires the use of microorganisms that will survive and thrive in soil polluted with heavy metals. An attractive feature of using rhizoremediation in such a situation is the flexibility of utilizing different engineered rhizobacteria to remediate mixed-waste co-contaminated soil (Khan, 2005). To provide a modified rhizoremediation system, the rhizosphere bacterial community can be specifically engineered to target various pollutants at co-contaminated sites (Wu *et al*., 2006). Furthermore, specific plant species and biodegradation genes can be selected in accordance with the contaminants present and plant growth conditions at the toxic sites (Wu *et al*., 2006).

1.11   Assessment of microbial diversity in contaminated soil environments

Traditionally, characterization of microbial ecosystems in contaminated soils focused mainly on analyzing numbers of culturable bacteria, measuring biomass and processes such as respiration (Bloem *et al*., 1995; De Leij *et al*., 1993; Evdokimova, 2001). Unfortunately, these methods only provide data about processes or bacterial numbers and are not suitable for the analysis of microbial community composition or diversity. Culturing colony forming units on different media was the most popular method for investigating microbial diversity. However, most bacteria targeted for isolation from environmental samples are difficult to culture due to constraints imposed by the selectivity of artificial media and conditions at which they are to be grown (Kirk *et al*., 2004). It has been estimated that the microbial community in one gram of soil may contain over one thousand different bacterial species (Rossello-Mora and Amann, 2001), but less than 1% of these have been reported to grow on artificial media (Kirk *et al*., 2004; McCaig *et al*., 1999; Sekiguchi *et al*., 2002; Stephen *et al*., 1999; Von Wintzingerode *et al*., 2002; Wayne *et al*., 1987).

Modern molecular techniques offer an exciting opportunity to overcome the requirement for culturing microorganisms and have therefore greatly increased our understanding of microbial diversity and activity in the environment (Malik *et al*., 2008). These methods rely on the characterization of cellular constituents such as nucleic acids, fatty acids, proteins, and other taxonomic specific compounds (Borneman *et al*., 1996; Nakatsu *et al*., 2000; Rossello-Mora and Amann, 2001). The first culture-independent estimate of prokaryotic organisms in soil indicated the presence of 4600 distinct genomes in one gram of soil (Kent and Triplett, 2002). Extracted DNA or RNA can, via molecular genetic techniques, facilitate coupling of microbial community analysis with phylogeny (Blackwood *et al*., 2003). The uncultured diversity reflects species closely related to known cultured organisms and also species from virtually uncultured lineages (Blackwood *et al*., 2003). Molecular methods usually involve the separation of polymerase chain reaction (PCR) amplicons on the bases of DNA nucleotide sequence differences, most often the 16S rRNA gene. The 16S rDNA regions are useful for such studies since these genes are present in all bacteria and comparison of sequences of 16S rDNA fragments has been well established as a standard method for the identification of species of bacteria (Gürtler and Stanisich, 1996). The success of this method does not depend on the physiological state of the cells from which the DNA is extracted. The only requisites are that cells are lyzed by the extraction buffer and that all the 16S rRNA genes are equally accessible for amplification (Li *et al*., 2006). Culture-independent approaches include denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), single strand conformation polymorphism (SSCP), restriction fragment length polymorphism (RFLP)/amplified ribosomal DNA restriction analysis (ARDRA), terminal restriction fragment length polymorphism (T-

RFLP), and ribosomal intergenic spacer analysis (RISA)/automated ribosomal intergenic spacer analysis (ARISA).

The application of molecular fingerprinting techniques holds great promise in assessing microbial diversity in co-contaminated environments. The presence of multiple contaminants presents extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community (Shi *et al*., 2002), therefore, only microbes that tolerate both heavy metals and toxic levels of hydrocarbons may survive. However, the combined effect of metals and organic carbon pollutants on microbial diversity and activity are unclear since few studies have addressed this issue (Nakatsu *et al*., 2005).

1.12 Denaturing gradient gel electrophoresis

Denaturing gradient gel electrophoresis (DGGE) is a highly sensitive technique which provides information on the diversity and composition of mixed microbial communities (Banks and Allemen, 2002; Hayes *et al*., 1999; Koizumi *et al*., 2002). This molecular technique allows a high number of samples to be screened simultaneously, thus facilitating much broad-spectrum analysis of microbiological diversity (Muyzer, 1999). The technique is based upon differential melting of double-stranded DNA molecules in a polyacrylamide gel with an increasing gradient concentration of denaturant (urea and formamide) (Hayes *et al*., 1999). DNA is extracted from the environmental samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The 16S rRNA genes are most commonly used to give an overall indication of the bacterial species composition of the sample (Muyzer, 1999). The PCR-amplified DNA fragments are generally limited in size to 500 bp and are separated on the basis of sequence differences, not variation in length (Malik *et al*., 2008). To prevent complete strand dissociation and to facilitate the detection of mutations in the higher melting domains, the 5´-end of the forward primer contains a GC-clamp of 35-40 base pairs. The GC-clamp theoretically increases the percentage of single base changes detectable by DGGE to 100% (Abrams *et al*., 1990; Sheffield *et al*., 1989). DGGE separates DNA fragments according to their melting behaviour; therefore the absence of a GC-clamp would result in the DNA denaturing into single strands. On denaturation, DNA melts in domains which are sequence specific, and will migrate differentially through the polyacrylamide gel (Muyzer, 1999). Theoretically, double stranded DNA molecules differing by only a single base substitution in their lowest melting domain show different melting behaviour patterns (Miller *et al.,* 1999). As a consequence, melting at different positions along a denaturing gradient gel generates a genetic fingerprint of the entire community being examined (Gillan, 2004). Resulting gel images can be digitally captured and used for species identification when samples are run against known standards (Temmerman *et al*., 2003). Analysis of DGGE profiles often involves the use of principal component analysis (Ogino *et al*., 2001; Widmer *et al*., 2001) or hierarchical cluster analysis to demonstrate

similarities in the data, and the results are invariably presented in the form of dendrograms (Boon *et al*., 2000; 2002; Eichner *et al*., 1999; Van Der Gucht *et al*., 2001).

DGGE has been widely used for the assessment of microbial community structure in contaminated soil, and is primarily aimed at studying the evolution of microbial communities (Avrahami and Conrad, 2003; Morris *et al*., 2002; Nicol *et al*., 2003). Kourtev *et al*. (2006) reported on the selection of microorganisms in a soil microcosm setting. Glucose and protein amendments were selected for different bacterial communities, and this selection was modified by the addition of Cr(VI), since some DGGE bands were intensified and new bands appeared in Cr(VI)-amended microcosms. DGGE allows for determining total community as well as specific community or gene diversity without further analysis and without elucidating particular individuals. It has been used in the identification of sequence variations in multiple genes among several organisms simultaneously (Muyzer *et al*., 1993). Moreover, functional genes, having more sequence variation, can be used to discriminate between closely related but ecologically different communities. For example, catabolic genes, such as methane manooxygenase has been targeted for DGGE analysis (Fjeilbirkeland *et al*., 2001; Knief *et al*., 2003). This provided information on the diversity of specific groups of microorganisms competent in a defined function such as pollutant degradation. For environmental or contaminated source samples where microbial diversity is largely unknown (Amann *et al*., 1995), DGGE provides the opportunity for the identification of the microbial population through the excision and sequencing of bands (Forney *et al*., 2004).

1.12.1 Advantages

- The main advantage of DGGE is that it enables the monitoring of the spatial/temporal changes in microbial community diversity and provides a simple view of the dominant microbial species within a sample (Malik *et al*., 2008).
- It is relatively easy to use and amenable to the rapid comparison of multiple samples (Neufeld and Mohn, 2005) i.e. large number of samples can be analyzed simultaneously.
- It is a rapid technique and relatively inexpensive (Neufeld and Mohn, 2005).

1.12.2 Disadvantages

- Sequence information derived from microbial populations is limited to 500 bp fragments of 16S rRNA sequences, which may lack the specificity required for phylogenetic inferences as well as for probe design (Gilbride *et al*., 2006).
- Due to the existence of multiple copies of rRNA in an organism, multiple bands for a single species may occur (Nubel *et al*., 1997).

- Band intensity may not truly reflect the abundance of microbial population (strong band may just mean more copies) and perceived community diversity may be underestimated (Malik *et al*., 2008).

- Co-migration of DNA fragments can be a problem for retrieving clean sequences from individual bands (Muyzer and Smalla, 1998).

- There is a lack of consensus regarding standards for normalization as gradients formed in different gels are somewhat variable making gel-to-gel comparisons difficult (Ferrari and Hollibaugh, 1999; Moeseneder *et al*., 1999).

1.13   Scope of the present study

1,2-Dichloroethane incessantly enters the soil environment mainly due to atmospheric emissions from industrial processes and inappropriate disposal of the compound. The major concern over soil contamination with 1,2-DCA stems primarily from health risks, both of direct contact and from secondary contamination of water supplies. Furthermore, heavy metals are routinely introduced into the environment through the discharge of toxic metal wastes from defence-related activities, municipal waste disposal in landfill sites or by accidental release. These practices have inspired a growing interest in bioremediation strategies for their removal. However, co-contamination poses serious challenges worldwide, as it is considered complicated to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently.

Previous research efforts have focused extensively on the biodegradation of 1,2-DCA in several contaminated soil and water environments and several microorganisms have been characterized for their ability to metabolize 1,2-DCA under various conditions. Furthermore, the toxic effects of heavy metals on soil microorganisms have also been studied. Although research based on single-pollutant exposures provides fundamental knowledge about individual pollutants under carefully controlled conditions, they do not mimic real world exposures. Moreover, approximately 40% of the hazardous waste sites currently on the National Priorities List of the USEPA are co-contaminated with organic and metal pollutants. Few studies have addressed the issue of co-contamination, and the impact of heavy metals on organic pollutant biodegradation. Accordingly, this study was undertaken to assess the impact of heavy metals on the aerobic biodegradation of 1,2-DCA by autochthonous microorganisms in soil co-contaminated with 1,2-DCA and heavy metals, via a direct and quantitative measure of the inhibitory effects of heavy metals in a microcosm setting. Denaturing gradient gel electrophoresis, dehydrogenase activity and urease activity were used to assess the impact of heavy metals on the microbial population, biodiversity and activity during 1,2-DCA degradation in the co-contaminated soil. The potential of biostimulation, bioaugmentation and treatment additives to enhance the degradation process was also investigated.

1.13.1 Hypotheses tested

It was hypothesized that the presence of heavy metals will have a negative impact on 1,2-DCA degradation in co-contaminated soil. It was further hypothesized that the addition of nutrients, treatment additives and heavy metal-resistant and 1,2-DCA-degrading microorganisms may accelerate the degradation of 1,2-DCA in such co-contaminated soils.

1.13.2 Objectives

The following objectives were established to test the above hypothesis:

a) To evaluate the biodegradation of 1,2-DCA in soil co-contaminated with 1,2-DCA and heavy metals and ascertain the effects of heavy metals on 1,2-DCA degradation.

b) To investigate the effects of biostimulation, bioaugmentation and treatment additives on the biodegradation of 1,2-DCA in the co-contaminated soil samples.

c) To profile 1,2-DCA degrading microbial populations, monitor microbial activity and diversity in the co-contaminated soil microcosms during 1,2-DCA degradation in the co-contaminated soil.

1.13.3 Experimental design

In order to achieve the stated objectives, this research was divided into the relevant chapters described below.

▪ **Chapter Two**

This chapter focuses on the quantitative assessment of the toxic effects of various metal concentrations (singly and in combination) on the biodegradation of 1,2-DCA. Toxic effects were evaluated based on the following: (i) degradation rate constants; (ii) estimated MICs; (iii) concentrations that caused biodegradation half-life doublings (HLDs); and (iv) heavy metal concentrations that caused a significant effect on biodegradation (> 10% increase in $t_{1/2}$ of 1,2-DCA).

▪ **Chapter Three**

This chapter investigated the effects of biostimulation and bioaugmentation on the aerobic biodegradation of 1,2-DCA in soil co-contaminated with As^{3+} and Cd^{2+}. Both traditional methods of soil dehydrogenase and urease enzyme assays and advanced molecular PCR-DGGE techniques were used to evaluate soil microbiological activity and diversity in the co-contaminated soils.

- **Chapter Four**

This chapter specifically addresses the efficiency of different inorganic treatment additives, to reduce the bioavailability of heavy metals and to assess its impact on 1,2-DCA degradation in soil co-contaminated with arsenic and cadmium. Moreover, the effects of such treatment additives on soil urease and dehydrogenase activities were also investigated.

- **Chapter Five**

This chapter places the entire research in perspective, thereby providing an overview of the significant findings reported in each of the various chapters of this dissertation. It also identifies the possible limitations or shortcomings of the study and provides a scope for future directions of the study.

CHAPTER TWO

QUANTITATIVE ASSESSMENT OF THE TOXIC EFFECTS OF HEAVY METALS ON THE AEROBIC BIODEGRADATION OF 1,2-DICHLOROETHANE IN CO-CONTAMINATED SOIL

2.1 Introduction

The increase in agricultural, industrial and urban activities, many of which inevitably involve the use of chemicals has given rise to a number of environmental problems due to the release of large quantities of toxic organic pollutants (Bhattacharya *et al.*, 2002; Collins and Stotzky, 1992; Laturnus, 2003; Mulligan *et al.*, 2001b; Weissenhorn *et al.*, 1995). In particular, chlorinated organic pollutants are introduced into the environment by effluent from pulp and paper industries, bleaching plants and chlorination procedures used in the treatment of water (Yu and Welander, 1995). Among the short-chained chlorinated aliphatic compounds, 1,2-dichloroethane (1,2-DCA) is one of the world's most hazardous toxic chlorinated aquifer pollutant (Marzorati *et al.*, 2007). Its annual production is in excess of 5.443×10^9 kg, a quantity larger than that of any other industrial halogenated chemical (Janssen *et al.*, 1989; Laturnus, 2003). As was noted in Chapter one, the widespread use of 1,2-DCA in a variety of products and manufacturing processes has resulted in its ubiquitous presence in most sites contaminated with organic pollutants (DEA, 2002; Hage and Hartmans, 1999) and it has been found in at least 570 of the 1585 National Priorities List (NPL) sites identified by the U. S. Environmental Protection Agency (USEPA). Soil contaminated with 1,2-DCA poses serious health risks, and its toxicity, persistence and potential for bioaccumulation (Squillace *et al.*, 1999) has inspired a growing interest in technologies for their removal (Baptista *et al.*, 2006). Remediation of hydrocarbon contaminated soils is usually difficult, due to a number of limiting factors such as inappropriate pH and moisture content, nutrient and oxygen content and availability, and, importantly, bioavailability and bioaccessibility of the chemicals (Ehlers and Luthy, 2003; Semple *et al.*, 2004; 2003).

Metals are also routinely introduced into the environment through the discharge of toxic metal wastes from defence-related activities, industry, and municipal waste disposal in landfill sites or by accidental release (Stephen *et al.*, 1998). These practices have resulted in surface contamination problems, transport to groundwater, and/or bioaccumulation of radionuclides and toxic metals in soils (Stephen *et al.,* 1998). Heavy metals (HMs) include a range of metals and metalloids which are commonly associated with pollution and toxicity, but also include elements (e.g., Zn, Cu and Ni) which are essential for the metabolism of living organisms, albeit at low concentrations (Wong *et al.*, 2005). All heavy metals are known to be potentially toxic to soil microorganisms at high concentrations and can hinder the biodegradation of organic contaminants (Amor *et al.*, 2001; Bååth, 1989; Benka-Coker and Ekundayo, 1998; Riis *et al.*, 2002, Roane *et al.*, 2001; Sokhn *et al.*, 2001; White and Knowles, 2000; 2003). Thus, co-contaminated matrices represent a further problem in bioremediation processes (Said and Lewis, 1991). Heavy metals appear to affect organic pollutant biodegradation through interference with both the physiology and ecology of organic degrading microorganisms, thus imposing a double stress on the microbial populations (Roane *et al.*, 2001). Therefore, the presence of multiple contaminants may

present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community required for the degradation process.

Sites co-contaminated with organic and metal pollutants raise far more serious concerns as the two components often causes a synergistic effect on cytotoxicity to microorganisms (Lin *et al*., 2006). Previous studies assessing toxicity of heavy metals have typically relied on physiologically restrictive processes such as, sulfate reduction, methanogenesis, [^{14}C] glucose uptake, and [^{3}H]thymidine incorporation, all of which may not relate to organic chemical biodegradation rates (Said and Lewis, 1991). Therefore, the effects of metal toxicity on organic pollutant biodegradation in co-contaminated soil environments have not been adequately defined quantitatively or qualitatively (Sandrin and Maier, 2003). This is partly due to the fact that metals can exist in a range of different physical and chemical forms such as colloidal solutions, soluble complexed species or organic solutes. Moreover, complications arise because the physical and chemical state of metals is affected by environmental conditions such as pH and ionic strength of the water phase, as well as soil properties including clay type and content, ion exchange capacity, and organic matter content (Sandrin and Maier, 2003). Metal toxicity is most commonly attributed to the tight binding of metal ions to sulfhydryl (-SH) groups of enzymes essential for microbial metabolism (Sandrin and Maier, 2003). Pollutant biodegradation may be inhibited through interaction with enzymes directly involved in biodegradation or those involved in general metabolism, thus rendering the enzyme non-functional (Angel and Chaney, 1989).

The effects of metals on biodegradation processes have not been well-characterized, although broad ranges of heavy metal concentrations have been reported to inhibit biodegradation. In addition, different patterns of inhibition have also been reported (Kuo and Genthner, 1996; Roane and Pepper, 1997; Sandrin *et al*., 2000). Delaune *et al*. (1998) reported that chromium (0-5000 µg/g) and lead (0-2500 µg/g) had no effect on the biodegradation of petroleum hydrocarbons from clay sediments. Other studies indicated that metals inhibited biodegradation specifically in a dose dependant manner; higher metal concentrations inhibited biodegradation more than lower concentrations. For example, the rate of toluene biodegradation by a *Bacillus* sp. was reduced by 55%, 61%, and 100% by 0.4, 0.8, and 1 mM nickel, respectively (Amor *et al*., 2001). Furthermore, non-dose-dependent inhibitions in which higher metal concentrations are less inhibitory than lower metal concentrations have also been reported (Chang *et al*., 2004; Gonzalez-Gil *et al*., 1999, Kuo and Genthner, 1996; Said and Lewis, 1991). For example, Chang *et al*. (2004) reported that the lag phase of *Desulfovibrio vulgaris* during sulfate respiration was 20 h shorter when exposed to 1 mM copper than when exposed to 0.1 mM. The existence of different degrees and patterns of inhibition may be due to lack of standardized protocols to characterize metal toxicity to the microorganisms (Hoffman *et al*., 2005).

Approximately 40% of the hazardous waste sites currently on the NPL of the USEPA are co-contaminated with organic and metal pollutants (Cheng, 2003; Fierens *et al*., 2003; Norena-Barroso *et al*., 2004; Sandrin *et al*., 2000; Sandrin and Maier, 2003). Co-contaminated environments are considered difficult to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently (Roane *et al*., 2001). Therefore, the issue of co-contamination is regarded as a serious one. Previous research efforts have focused extensively on the biodegradation of 1,2-DCA in several contaminated soil and water environments (van den Wijngaard *et al*., 1993), and several microorganisms have been characterized for their ability to metabolize 1,2-DCA under various conditions (De Wildeman *et al*., 2003; He *et al*., 2003; Janssen *et al*., 1985; Maymo-Gatell *et al*., 1999; Stucki and Leisinger, 1983). Furthermore, the toxic effects of heavy metals on soil microorganisms have also been studied separately (Rajapaksha *et al*., 2004). Also, most of the knowledge about the ecological effects of heavy metals on soil microorganisms is based on data collected for a few metals such as Cu and Zn or on data collected from sewage sludge that contains a broad mixture of heavy metals at relatively low concentrations for a relatively short period of time. Most studies incorporated axenic cultures isolated from environmental samples and determined the effects of heavy metal toxicities on these organisms in defined media (Said and Lewis, 1991). Such methods are limited in their application to field situations, because very rarely, if ever, are conditions in the environment such that only one microbial species is active nor are conditions in nature comparable with the conditions on defined laboratory media. Since heavy metals and 1,2-DCA are found together in most co-contaminated sites there is a need to evaluate the biodegradation profiles of 1,2-DCA in co-contaminated soil to ascertain the effects of heavy metals on 1,2-DCA degradation. The objective of this study, therefore, was to evaluate the aerobic biodegradation of 1,2-DCA by autochthonous microorganisms, in soil co-contaminated with 1,2-DCA and heavy metals, via a direct and quantitative measure of the inhibitory effects of the heavy metals in a microcosm setting. Four of the eight heavy metals of concern in the USEPA's priority list of pollutants (Sandrin and Maier, 2003); arsenic, cadmium, mercury and lead were used in this study. Effects of various metal concentrations and their combinations were evaluated based on the following: (i) degradation rate constants; (ii) estimated minimal inhibitory concentrations (MICs); (iii) concentrations that caused biodegradation half-life doublings (HLDs); and (iv) heavy metal concentrations that caused a significant effect on biodegradation (> 10% increase in $t_{1/2}$ of 1,2-DCA).

2.2   Materials and methods

2.2.1   Sample collection and handling

Clay and loam soils were collected from specific sites in the Westville area in Durban, KwaZulu-Natal, to obtain a representative sample of the autochthonous microbial community living attached to the sand grains. The soil samples were collected from the surface to a depth of about 0.4 m in an approximately 2 m^2 plot. These soils had no known history of chlorinated hydrocarbon contamination. Thereafter, the soils were sieved using a 1.7 mm lab test sieve to obtain a homogeneous texture, and stored at 4°C prior to use in the microcosm set-up (Olaniran *et al.*, 2006).

2.2.2   Experimental design and microcosms set-up

Microcosms were set-up by artificially co-contaminating the soil sample with 1,2-DCA and heavy metals to study the effects of four heavy metals, individually and in combination, on the biodegradation of 1,2-DCA. Sixteen treatments were used in total, comprising positive and negative controls, three concentrations of metals and eleven different combinations, with each treatment conducted in triplicate. For the experiments, soil microcosms were constructed in a laminar flow cabinet using sterile screw-capped 250 ml serum bottles (Wheaton). The synthetic groundwater was prepared as described by Klier *et al.* (1999), and contained 1.5 mM $MgCl_2$, 0.12 mM KCl, 0.03 mM NH_4NO_3, 1.0 mM $CaCl_2$, 1.5 mM $Ca(OH)_2$, and 8.5 mM $NaHCO_3$, in de-ionized water at pH 7.8. The reaction mixtures were prepared in the sterile bottles by combining 100 g of soil and 75 ml of synthetic groundwater. The heavy metals were added as salts, i.e., As_2O_3 (> 99.0%, Fluka), $CdCl_2$ (98%, Sigma), $HgCl_2$ (99.6%, Sigma) and $Pb(NO_3)_2$ (98%, Merck). Heavy metal concentrations used were based on the regulatory limit (RM) stipulated by the Natural Resources Conservation Services (NRCS), United States Department of Agriculture, Soil Quality-Urban Technical Note No. 3 (NRCS, 2000). Three levels of heavy metal contamination were incorporated; RM was referred to as low concentration, 1.5 × RM the moderate concentration and 2 × RM the high concentration. The eleven combinations were prepared using the moderate concentration of heavy metals. The soils were mixed manually using a sterile glass rod to distribute the heavy metals as homogeneously as possible. The headspace in each bottle was made up of approximately 75 ml of air. An aerobic condition was maintained by purging the reaction mixture with pure oxygen gas during the preparation, using a 0.2 µm filter. The microcosms were quickly sealed with sterile Teflon-lined butyl-rubber stoppers immediately after spiking with 20 µl of 1,2-DCA [% purity (GC) ≥ 99.5%, Merck]. In order to determine the initial concentration of 1,2-DCA, the bottles were shaken for 2 h on a rotary shaker at 150 rpm at 25°C to allow for the equilibration of 1,2-DCA between the gas and aqueous phases. Thereafter, the bottles were incubated at 25°C with no shaking for the course of the experiment. Biological inhibited controls were prepared using soil samples which were autoclaved

four times prior to microcosms set-up at 121ºC and 120 kPa for 20 min, to measure abiotic losses of 1,2-DCA. The microcosms were sampled every 5 days to measure 1,2-DCA degradation, as described below.

2.2.3   Analytical procedures

1,2-Dichloroethane is a volatile organic compound and equilibrium between its gas and liquid concentrations is maintained through rapid mass transfer between the gas and aqueous phases. Changes in liquid-phase concentration due to biological reactions are slow when compared to the mass transfer rate between the gas and liquid phase (Lin *et al*., 2007). Therefore, gas-phase measurements closely reflect liquid substrate concentrations for biodegradation experiments (Coleman *et al*., 2002). As such, 500 µl headspace samples were periodically collected from each microcosm using a gas tight syringe (Hamilton) and injected into a gas chromatograph (GC) (Varian model 3700) equipped with a flame ionization detector. The samples were analyzed with the injector and flame ionization detector at 200°C and a packed capillary column at 100°C. Ultra high purity nitrogen was used as the carrier gas at a flow rate of 10 ml/min. The 1,2-DCA concentrations were quantified by comparison with standard curves derived from known quantities of the compounds in serum bottles with the same gas and liquid volumes as the experimental bottles. The GC peak areas were substituted into the linear regression equation to obtain the concentration of 1,2-DCA at the different sampling times.

2.2.4   Quantitative analysis

Pseudo-first-order transformation rate coefficients, k_1, were calculated from plots of the natural logarithm of substrate concentration versus time according to the integrated first-order rate equation:

$$\ln (C_t/C_0) = -k_1 . t$$

where C_0 and C_t are the concentrations at time 0 and t, respectively (Said and Lewis, 1991). Percentage reductions in microbial degradation rate coefficients were calculated on the basis of comparisons with control clay and loam samples (unamended with the metal salts) as follows.

% Reduction in k_1 = [(control k_1 - treatment k_1)/control k_1] × 100 (Said and Lewis, 1991).

Half-lives were calculated as $0.693/k_1$. The metal concentrations at which a doubling in 1,2-DCA degradation half-lives ($t_{1/2}$) occurred were calculated from linear regressions of the percentages of reductions in k_1 versus logarithms of metal salt concentrations, i.e., a 50% reduction in k_1 equaled one doubling of $t_{1/2}$. These values were referred to as half-life doubling concentrations (HLDs). Minimal inhibitory concentrations (MICs) were determined from abscissa intercepts of plots of percent reduction in pseudo-first-order rate coefficients versus logarithmic concentrations (mg/kg) of metals (Said and Lewis, 1991).

2.2.5 Soil analysis

2.2.5.1 Moisture content

The moisture content was determined by drying a known quantity of the soil samples at 70ºC for 72 h, until a constant weight was obtained. The difference between the initial and final dry weight of the soil gave the moisture content of the soil (Olaniran *et al*., 2009).

2.2.5.2 pH

Ten grams of soil sample were added to 25 ml of distilled water in one beaker, while another 10 g of soil sample was added to 25 ml of 0.01 M calcium chloride in a separate beaker. Both were stirred for one minute and left to stand for one hour (Black *et al*., 1965). The pH of the soil samples was determined using a Beckman 50 pH meter.

2.2.5.3 Nutrients

Soil samples were analyzed for calcium, magnesium, sodium, potassium, iron, nitrate, sulphate, soluble organic carbon, phosphate and total Kjeldhal nitrogen at Umgeni Water Laboratory Services using standard methods.

2.3 Results

2.3.1 Soil characterization

The physico-chemical properties of both clay and loam soil samples used for the microcosm experiments are shown in Table 2.1. The pH of the two soil samples was slightly acidic and ranged from 6.10 to 6.59, while the moisture content of the clay soil was found to be about 20% higher than that of the loam soil. Soluble nitrate was estimated to be approximately < 0.5 μg/g in both clay and loam soil samples. Calcium concentration was found to be about 22.41%, soluble organic carbon about 34.23%, iron about seven-hundred and ninety-eight-fold, phosphate about two-fold and sulphate about fifteen-fold higher in loam soil than in clay soil. However, magnesium concentration was found to be about 78.35%, sodium about 40%, potassium about two-fold and Total Kjeldahl nitrogen concentration about nineteen-fold higher in clay soil than in loam soil.

Table 2.1:   Physico-chemical properties of the soil samples.

Determinant	Clay soil	Loam soil
Calcium (µg/g)	6047	7402
Magnesium (µg/g)	2035	1141
Sodium (µg/g)	273	< 195
Potassium (µg/g)	1510	662
Iron (µg/g)	< 3.88	3097
Nitrate (soluble) (µg/g)	< 0.5	< 0.5
Phosphate (mg/g)	647.2	1400
Sulphate (soluble) (µg/g)	83.9	1225
Soluble organic carbon (µg/g)	1110	1490
Total Kjeldahl nitrogen (µg/g)	115	2125
pH	6.10	6.59
Moisture content (%)	17.05	14.20

2.3.2   Impact of different concentrations of heavy metals on 1,2-DCA biodegradation in soil

The biodegradation profiles of 1,2-DCA in both contaminated soil types, in the presence and absence of heavy metals, are illustrated in Figure 2.1. 1,2-Dichloroethane was observed to be readily degraded in both contaminated soil samples with up to 66.05% degradation (above that of the autoclaved soil) observed in loam soil after 20 days in the absence of heavy metals (Figure 2.1b), which is, about 17.60% more than the degradation observed in clay soil (Figure 2.1a), at the same period. The degradation rate constants of 1,2-DCA, as indicated in Table 2.2, ranged between $0.049 – 0.078$ day^{-1} in clay soil and between $0.074 – 0.114$ day^{-1} in loam soil. In all cases, except for soil co-contaminated with 1,2-DCA and 420 mg/kg Pb^{2+}, higher degradation rate constants were observed in loam soil compared to the clay soil. Furthermore, a decline in 1,2-DCA degradation rate constant was observed from the lower to the higher concentration of each of the heavy metals in clay soil. However, this trend was observed only in the presence Hg^{2+} in loam soil. The presence of heavy metals was observed to have a negative impact on the biodegradation of 1,2-DCA, with the toxic effect being more pronounced in loam soil for all heavy metal concentrations except for mercury, after 15 days. An increased reduction in 1,2-DCA degradation was observed with increasing concentration of the heavy metals. In clay soil, 11.88%, 5.50%, 6.62% and 5.46% decrease in 1,2-DCA degradation occurred in the presence of 150 mg/kg As^{3+}, 170 mg/kg Cd^{2+}, 1680 mg/kg Hg^{2+} and 840 mg/kg Pb^{2+}, respectively, compared to the lower concentration of

75 mg/kg As^{3+}, 85 mg/kg Cd^{2+}, 840 mg/kg Hg^{2+} and 420 mg/kg Pb^{2+}. Similarly, in loam soil the same trend was observed. For example, a 24.38% reduction in 1,2-DCA degradation was observed in the presence of 150 mg/kg As^{3+} after 15 days, while a 23.28% reduction occurred at 75 mg/kg As^{3+} concentration. Thus, inhibitory effects increased with increasingly higher concentrations of the metal salts.
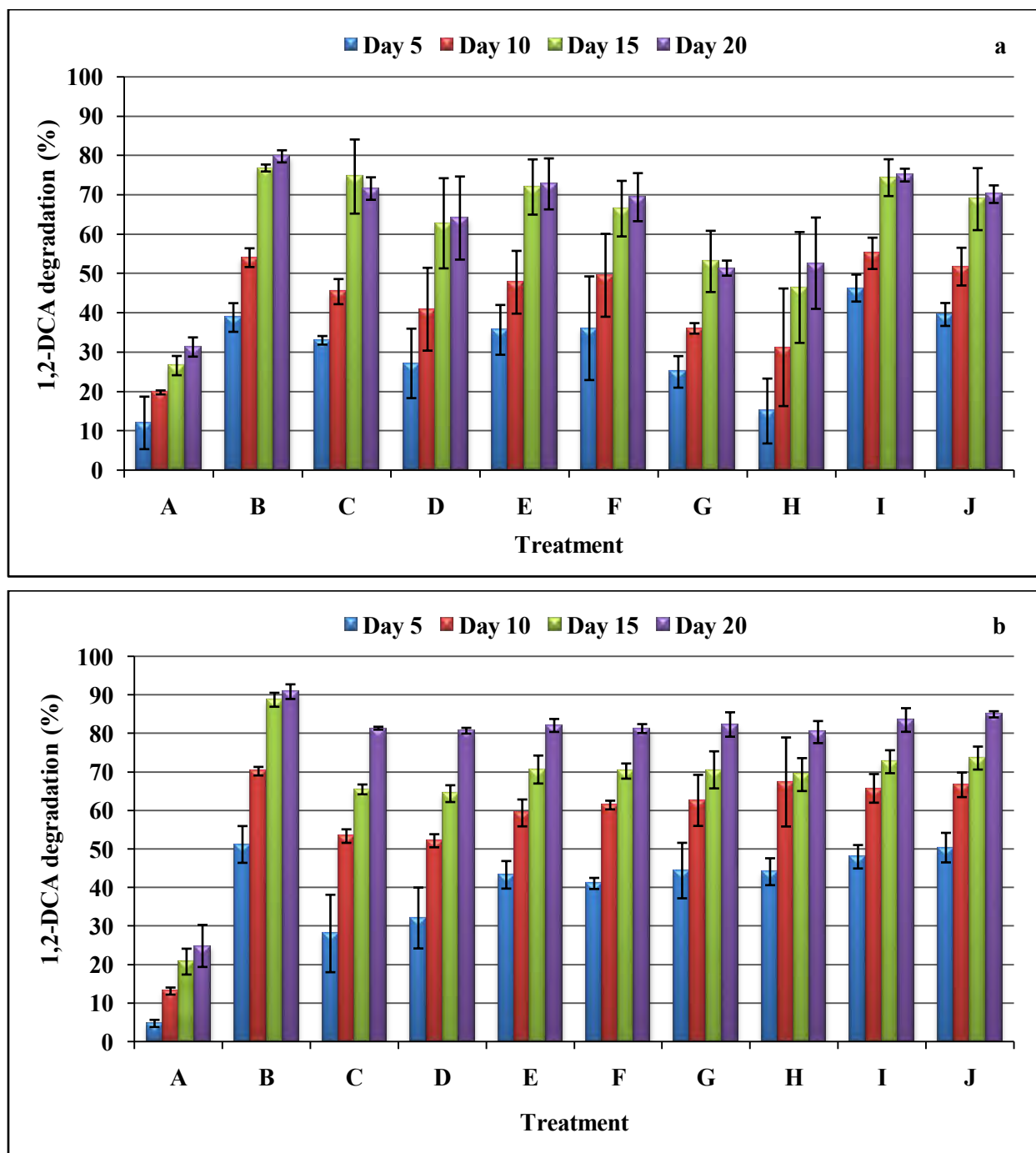
Figure 2.1: Biodegradation profiles of 1,2-DCA in (a) clay soil and (b) loam soil co-contaminated with various concentrations of heavy metals. A = autoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA; C = soil + 1,2-DCA + 75 mg/kg As^{3+}; D = soil + 1,2-DCA + 150 mg/kg As^{3+}; E = soil + 1,2-DCA + 85 mg/kg Cd^{2+}; F = soil + 1,2-DCA + 170 mg/kg Cd^{2+}; G = soil + 1,2-DCA + 840 mg/kg Hg^{2+}; H = soil + 1,2-DCA + 1680 mg/kg Hg^{2+}; I = soil + 1,2-DCA + 420 mg/kg Pb^{2+}; J = soil + 1,2-DCA + 840 mg/kg Pb^{2+}. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

Table 2.2:   Biodegradation rate constants (day^{-1}) of 1,2-DCA in soil microcosms co-contaminated with different concentrations of heavy metals.

Treatment	Clay soil	Loam soil
Autoclaved soil control	0.015 ± 0.004	0.014 ± 0.003
Unautoclaved soil control	0.076 ± 0.000	0.114 ± 0.013
As^{3+} (75 mg/kg)	0.066 ± 0.009	0.075 ± 0.003
As^{3+} (112.5 mg/kg)	0.065 ± 0.018	0.081 ± 0.009
As^{3+} (150 mg/kg)	0.053 ± 0.015	0.076 ± 0.002
Cd^{2+} (85 mg/kg)	0.070 ± 0.011	0.075 ± 0.005
Cd^{2+} (127.5 mg/kg)	0.071 ± 0.013	0.082 ± 0.007
Cd^{2+} (170 mg/kg)	0.056 ± 0.009	0.075 ± 0.001
Hg^{2+} (840 mg/kg)	0.069 ± 0.006	0.075 ± 0.007
Hg^{2+} (1260 mg/kg)	0.056 ± 0.007	0.074 ± 0.007
Hg^{2+} (1680 mg/kg)	0.049 ± 0.011	0.074 ± 0.005
Pb^{2+} (420 mg/kg)	0.078 ± 0.010	0.077 ± 0.006
Pb^{2+} (630 mg/kg)	0.072 ± 0.020	0.100 ± 0.027
Pb^{2+} (840 mg/kg)	0.069 ± 0.006	0.086 ± 0.005

2.3.3   Kinetics of 1,2-DCA degradation in the presence of heavy metals

The effects of the different concentrations of the heavy metals on the half-lives ($t_{1/2}$) of 1,2-DCA are represented in Table 2.3. The relative $t_{1/2}$ in clay soil ranged from 0.99 – 1.63 days, whereas in loam soil it ranged from 1.17 – 1.53 days in the presence of the different concentrations of the heavy metals. Generally, more than 127.5 mg/kg Cd^{2+}, 840 mg/kg Hg^{2+} and 420 mg/kg of Pb^{2+} was able to cause > 10% increase in the $t_{1/2}$ of 1,2-DCA in clay soil, while less than 75 mg/kg was required for As^{3+}. In clay soil, Cd^{2+} at 127.5 mg/kg resulted in an 8.66% increase in the $t_{1/2}$ of 1,2-DCA, whereas the $t_{1/2}$ was increased by 38.50% in loam soil, at the same concentration, indicating a much more pronounced effect of Cd^{2+} in loam soil compared to clay soil. Similarly, the presence of 75 mg/kg As^{3+}, 840 mg/kg Hg^{2+} and 420 mg/kg Pb^{2+} resulted in 34.24%, 40.64% and 45.94% increase in the $t_{1/2}$ of 1,2-DCA, respectively, in loam soil compared to clay soil. Therefore, $t_{1/2}$ determinations indicated that various metals caused different levels of inhibitory effects on biodegradation rates depending on the metal and soil type. By using these criteria as a measure of significant effects, indigenous microorganisms in loam soil were more sensitive to heavy metals than in clay soil samples. Since a non-linear response for higher metal concentrations was observed in loam soil, percentage decreases in k_1 values plotted as a function of the logarithms of metal concentrations could not be subjected to linear regression analysis to estimate the MICs and HLDs. However, in clay soil, a dose-dependant relationship between k_1 and metal ion concentrations in which k_1 decreased with higher initial metal concentrations was observed for all the heavy metals tested except Hg^{2+} (Figure 2.2). When percentage decreases in k_1 were plotted as a function of the logarithms of metal concentrations, a linear relationship was obtained for a range of the metal concentrations tested in clay soil. Because HLDs were not within the linear range of observed inhibition levels, non-linear regression analysis of the data was carried out in this study. Percentage decreases in k_1 values plotted as a function of the logarithms of metal concentrations were subjected to linear regression analysis to estimate HLDs and MICs and to compare correlation (r) and regression coefficients (slopes) and the data summarized in Table 2.4. The MIC was lowest for As^{3+} (62.78 mg/kg) and highest for Hg^{2+} (665.73 mg/kg). In clay soil, MICs were below the stipulated regulatory limit for all of the heavy metals except for Pb^{2+}. The MICs varied according to the metals tested, and did not necessarily follow the toxicity patterns observed for the metal concentrations required for significant effects on $t_{1/2}$. Moreover, biodegradation rates were very sensitive to increases in the concentration of some heavy metals.

Table 2.3: Effects of heavy metals on the half-lives of 1,2-DCA in the soil samples.

Metal ion[a], soil type, and metal concn. (mg/kg)	$t_{1/2}$ (days) (Average ± SD)		Relative $t_{1/2}$[b]
	Untreated soil	**Treated soil**	
Clay	9.12		
As^{3+}			
75		10.58 ± 1.42	1.16
112.5		11.18 ± 2.77	1.23
150		13.61 ± 3.38	1.49
Cd^{2+}			
85[c]		10.01 ± 1.58	1.10
127.5[c]		9.91 ± 1.61	1.09
170		12.52 ± 2.07	1.37
Hg^{2+}			
840		10.04 ± 0.83	1.10
1260		12.45 ± 1.74	1.37
1680		14.84 ± 3.88	1.63
Pb^{2+}			
420[c]		9.04 ± 1.29	0.99
630		10.21 ± 2.92	1.12
840		10.04 ± 0.83	1.10
Loam	6.13 ± 0.67		
As^{3+}			
75		9.21 ± 0.38	1.50
112.5		8.62 ± 0.88	1.41
150		9.16 ± 0.19	1.49
Cd^{2+}			
85		9.27 ± 0.63	1.51
127.5		8.49 ± 0.76	1.38
170		9.24 ± 0.12	1.51
Hg^{2+}			
840		9.24 ± 0.80	1.51
1260		9.39 ± 0.98	1.53
1680		9.35 ± 0.56	1.53
Pb^{2+}			
420		9.00 ± 0.78	1.47
630		7.20 ± 1.66	1.17
840		8.11 ± 0.47	1.32

[a] As^{3+} added as an oxide salt; Pb^{2+} added as nitrate salt; Cd^{2+} and Hg^{2+} added as the chloride salts.
[b] Value for metal-treated soil divided by value for untreated soil. [c] Did not cause >10% increase in $t_{1/2}$.
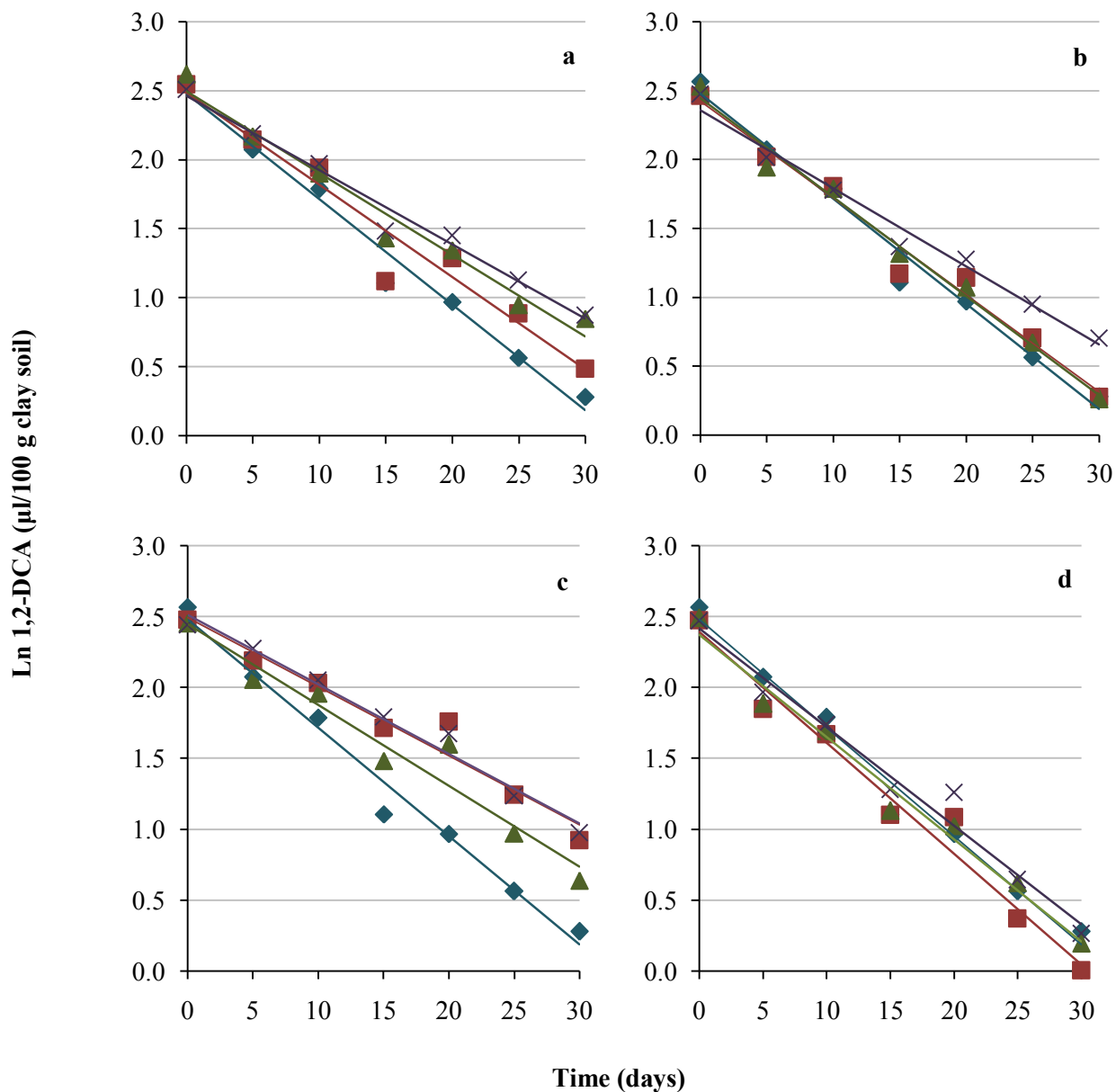
Figure 2.2: Inhibitory effects of (a) As^{3+}, (b) Cd^{2+}, (c) Hg^{2+} and (d) Pb^{2+} on pseudo-first-order 1,2-DCA biodegradation rates in clay soil. The slopes of regression lines of the natural logarithms of 1,2-DCA concentration versus time equal the rate coefficients, k_1. Treatments shown are: (◆) Unautoclaved soil control, (■) Low concentration of HM, (▲) Moderate concentration of HM and (X) High concentration of HM.

Table 2.4: Concentrations of heavy metals inhibiting 1,2-DCA biodegradation in clay soil.

Metal ion[a]	Correlation coefficient (*r*)	Slope	MIC[b] (mg/kg) Average ± SD	HLD[c] (mg/kg) Average ± SD
As^{3+}	0.91	72.81	62.78 ± 10.91	305.14 ± 2.48
Cd^{2+}	0.98	60.28	65.54 ± 39.86	442.59 ± 35.83
Hg^{2+}	0.10	90.78	665.73 ± 132.39	2,366.46 ± 618.19
Pb^{2+}	0.98	36.97	469.79 ± 167.33	10,575.48 ± 521.81

[a] As^{3+} added as an oxide salt; Pb^{2+} added as nitrate salt; Cd^{2+} and Hg^{2+} added as the chloride salts.
[b] MICs caused no reduction in k_1 values. Numerically, these are abscissa intercepts.
[c] HLD, concentration that caused half-life doubling.

2.3.4 Impact of different combinations of heavy metals on 1,2-DCA biodegradation in soil

The patterns of biodegradation of 1,2-DCA in both soil types, co-contaminated with different combinations of heavy metals, are illustrated in Figures 2.3 and 2.4. The degradation rate constants of 1,2-DCA, as indicated in Table 2.5, ranged between 0.045 – 0.115 day^{-1} in clay soil and between 0.050 – 0.078 day^{-1} in loam soil, in the presence of the different combinations of heavy metals. In clay soil, combinations of $As^{3+} + Hg^{2+}$ and $As^{3+} + Cd^{2+}$ were observed to be the most toxic, resulting in 32.46% and 21.14% decrease in 1,2-DCA degradation after 15 days, respectively (Figure 2.3a). However, in loam soil the combinations of $As^{3+} + Pb^{2+}$ and $As^{3+} + Hg^{2+}$ resulted in the greatest reduction in 1,2-DCA degradation with 21.90% and 15.50% decrease, respectively, observed over the same time period (Figure 2.3b). Furthermore, an increase of 21.08% and 14.50% in 1,2-DCA degradation was observed for $As^{3+} + Cd^{2+} + Hg^{2+}$ and $As^{3+} + Hg^{2+} + Pb^{2+}$, respectively, compared to $As^{3+} + Hg^{2+}$, in clay soil. A similar trend occurred in loam soil with the combination of $As^{3+} + Pb^{2+}$, where a corresponding increase of 11.45% and 13.74% was observed for $As^{3+} + Cd^{2+} + Pb^{2+}$ and $As^{3+} + Hg^{2+} + Pb^{2+}$. In addition, the effect of the combination of four heavy metals in both clay and loam soils were less pronounced than the combinations of two heavy metals which resulted in the most significant decreases in 1,2-DCA degradation. In clay soil, the combination of four heavy metals resulted in a 28.66% increase in 1,2-DCA degradation compared to the combination of $As^{3+} + Hg^{2+}$. The same trend was observed in loam soil whereby an 11.84% increase in 1,2-DCA degradation was observed in the presence of four heavy metals compared to the combination of $As^{3+} + Pb^{2+}$. Moreover, the combination of four heavy metals in loam soil resulted in 6.26% less degradation of 1,2-DCA compared to clay soil, after 15 days.
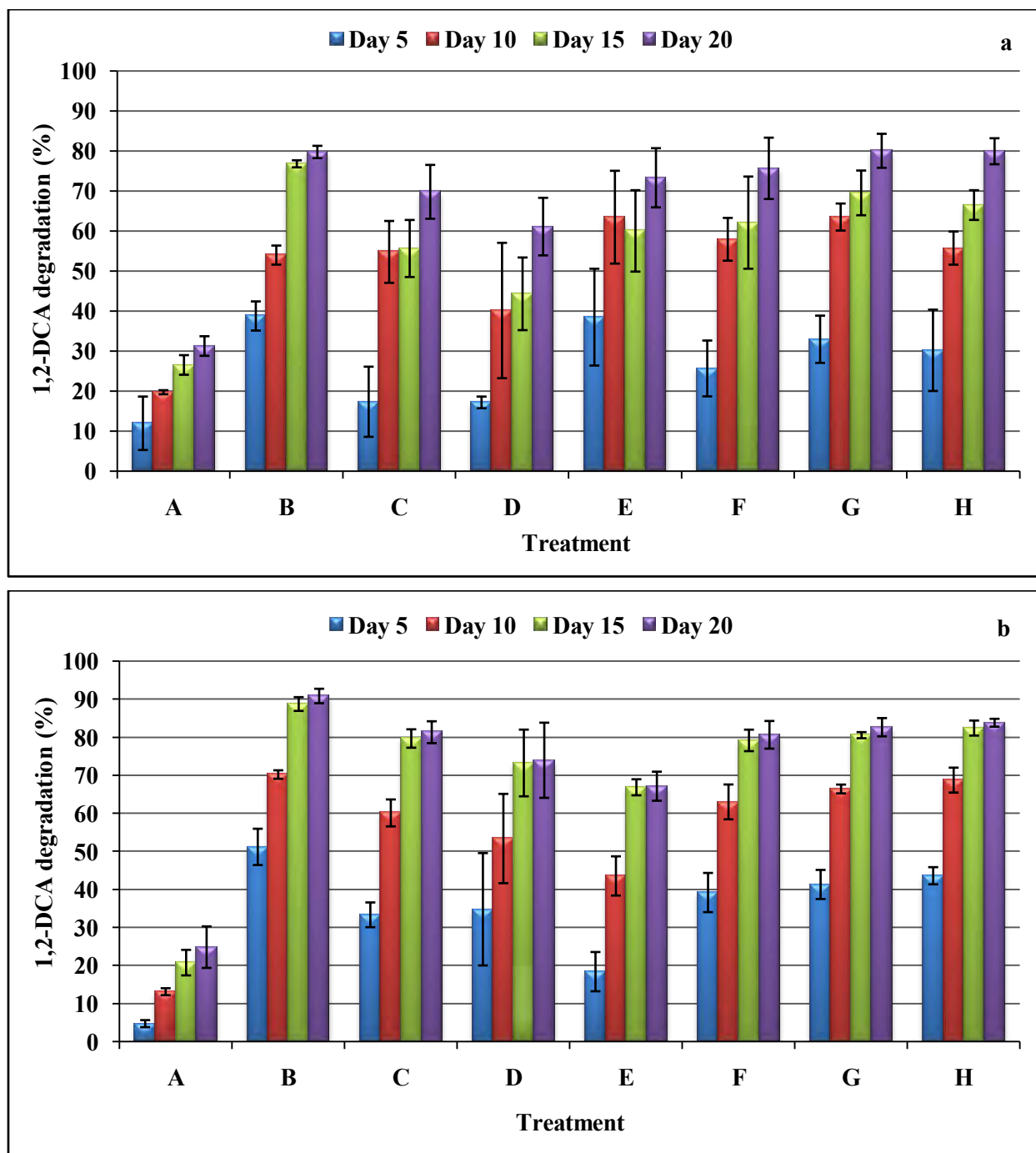
Figure 2.3: Biodegradation profiles of 1,2-DCA in (a) clay and (b) loam soil co-contaminated with binary combinations of heavy metals (As^{3+} = 112.5 mg/kg, Cd^{2+} = 127.5 mg/kg, Hg^{2+} = 1260 mg/kg, and Pb^{2+} = 630 mg/kg). A = autoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA; C = soil + 1,2-DCA + As^{3+} + Cd^{2+}; D = soil + 1,2-DCA + As^{3+} + Hg^{2+}; E = soil + 1,2-DCA + As^{3+} + Pb^{2+}; F = soil + 1,2-DCA + Cd^{2+} + Hg^{2+}; G = soil + 1,2-DCA + Cd^{2+} + Pb^{2+}; H = soil + 1,2-DCA + Hg^{2+} + Pb^{2+}. Bars indicate the average of triplicate samples while the error bars show the standard deviation.
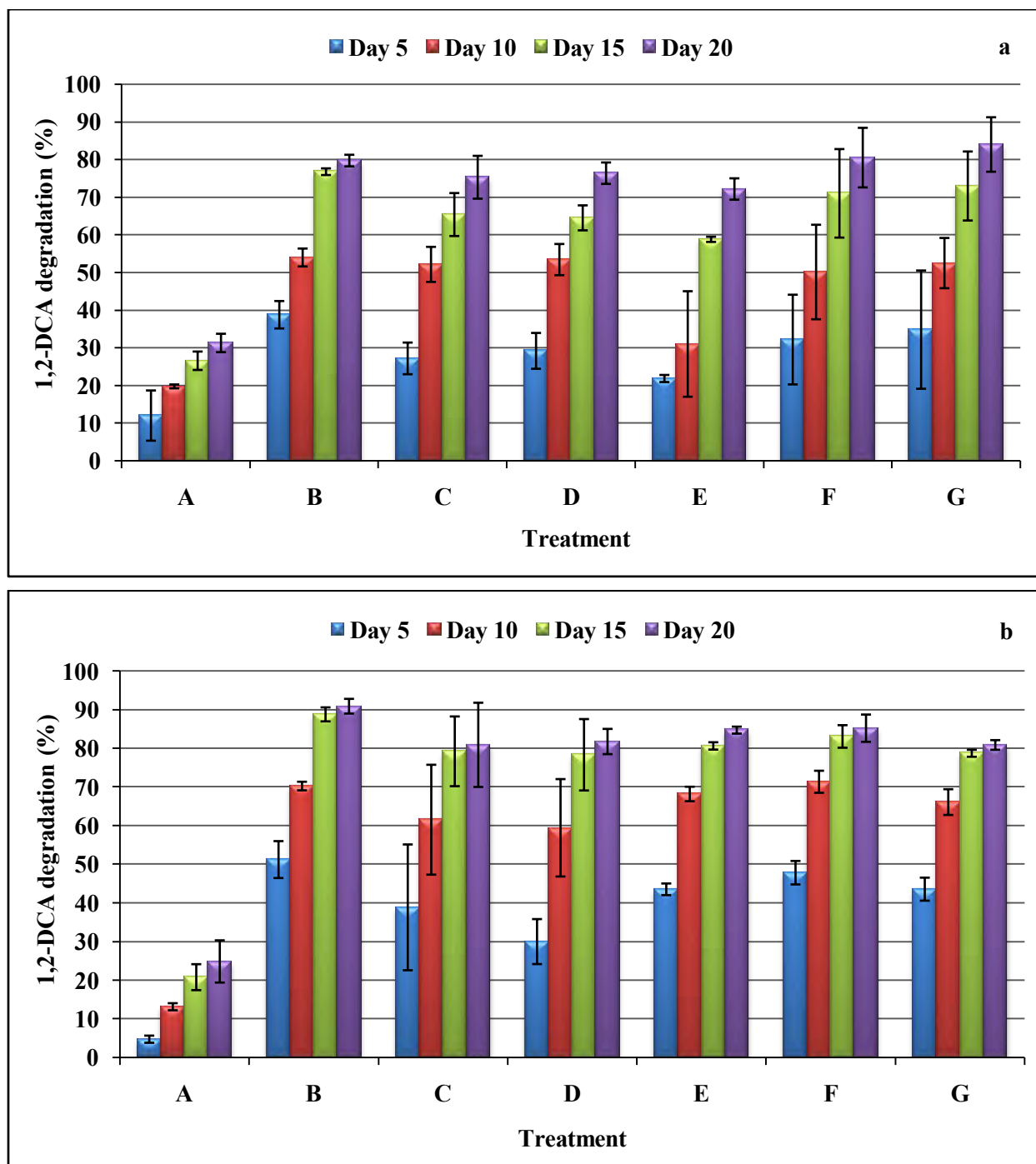
Figure 2.4: Biodegradation profiles of 1,2-DCA in (a) clay and (b) loam soil co-contaminated with combinations of three and four heavy metals (As^{3+} = 112.5 mg/kg, Cd^{2+} = 127.5 mg/kg, Hg^{2+} = 1260 mg/kg, and Pb^{2+} = 630 mg/kg). A = autoclaved soil control + 1,2-DCA; B = unautoclaved soil control +1,2-DCA; C = soil + 1,2-DCA + As^{3+} + Cd^{2+} + Hg^{2+}; D = soil + 1,2-DCA + As^{3+} + Cd^{2+} + Pb^{2+}; E = soil + 1,2-DCA + As^{3+} + Hg^{2+} + Pb^{2+}; F = soil + 1,2-DCA + Cd^{2+} + Hg^{2+} + Pb^{2+}; G = soil + 1,2-DCA + As^{3+} + Cd^{2+} + Hg^{2+} + Pb^{2+}. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

Table 2.5: Biodegradation rate constants (day^{-1}) of 1,2-DCA in soil microcosms co-contaminated with different combinations of heavy metals.

Treatment	Clay soil	Loam soil
Autoclaved soil	0.015 ± 0.004	0.014 ± 0.003
Unautoclaved soil	0.076 ± 0.000	0.114 ± 0.013
$As^{3+} + Cd^{2+}$	0.057 ± 0.010	0.073 ± 0.005
$As^{3+} + Hg^{2+}$	0.045 ± 0.005	0.059 ± 0.016
$As^{3+} + Pb^{2+}$	0.059 ± 0.018	0.050 ± 0.002
$Cd^{2+} + Hg^{2+}$	0.070 ± 0.013	0.071 ± 0.003
$Cd^{2+} + Pb^{2+}$	0.080 ± 0.012	0.077 ± 0.002
$Hg^{2+} + Pb^{2+}$	0.072 ± 0.006	0.075 ± 0.005
$As^{3+} + Cd^{2+} + Hg^{2+}$	0.069 ± 0.013	0.066 ± 0.025
$As^{3+} + Cd^{2+} + Pb^{2+}$	0.069 ± 0.006	0.057 ± 0.006
$As^{3+} + Hg^{2+} + Pb^{2+}$	0.062 ± 0.005	0.074 ± 0.005
$Cd^{2+} + Hg^{2+} + Pb^{2+}$	0.088 ± 0.036	0.078 ± 0.012
$As^{3+} + Cd^{2+} + Hg^{2+} + Pb^{2+}$	0.115 ± 0.046	0.069 ± 0.003

As^{3+} = 112.5 mg/kg; Cd^{2+} = 127.5 mg/kg, Hg^{2+} = 1260 mg/kg, and Pb^{2+} = 630 mg/kg.

2.4 Discussion

Microcosms were constructed to gain a better understanding of the effects of heavy metals on the aerobic biodegradation of 1,2-DCA by indigenous microorganisms in clay and loam soil samples. The results obtained indicate that 1,2-DCA was readily degraded by indigenous microbial populations in both soil types without heavy metal contamination, with loam soil exhibiting greater degradation than clay soil. The presence of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} was observed to have adverse effects on the biodegradation of 1,2-DCA in both soil samples. Metals exert their toxic effects on microorganisms via different mechanisms, including substitution of toxic metal ions for physiologically essential ions within an enzyme, thus rendering the enzyme non-functional (Nies, 1999); and imposition of oxidative stress on microorganisms (Kachur *et al*., 1998). Furthermore, heavy metal ions are able to form unspecific complex compounds in microbial cells, resulting in inhibitory toxic effects on microbial activities (Roane *et al*., 2001). Thus, co-contamination with metal ions may inhibit microorganisms involved in the degradation of organic compounds and consequently affect biodegradation rates (Amor *et al*., 2001; Hattori, 1992). The level of inhibition, by heavy metals, depends on the concentration and availability of the heavy metal and the action of complex processes controlled by several factors, including the nature of metals, and indigenous microbial species (Chen and Lin, 2001). When considering inhibition data, it is also important to take into account the possible effects of various environmental factors such as nutrient availability,

oxygen, pH, temperature, water retention, quantity and bioavailability of contaminants, salinity, and soil properties (Margesin *et al.*, 2000; Olaniran *et al.*, 2006; Said and Lewis, 1991). Since such confounding factors often manifested in contaminated sites, it is essential to understand the influence they have on contaminant removal and partitioning with respect to assessing bioremediation potential (Hickman *et al.*, 2008). Interestingly, it was observed that the degradation rates were higher in loam soil, compared to clay soil, in the absence of heavy metals. This could be attributed to the higher concentrations of inorganic trace elements (e.g., nitrogen, iron and calcium) in loam soil, as increased levels of trace elements have been demonstrated to enhance biodegradation rates in both marine and terrestrial ecosystems considerably (Atlas and Bartha, 1992). Clay has been shown to decrease metal bioavailability and toxicity to bacteria (Boenigk *et al.*, 2005), due to the high cation exchange capacity and adsorption of the metals to the clay particles causing uneven distribution of the heavy metals within the soil matrix. It is therefore not surprising that the presence of the heavy metals in clay soil has a lesser impact on the biodegradation of 1,2-DCA.

In this study, all the heavy metals tested had an inhibitory effect on 1,2-DCA degradation in both soil types, with mercury found to have the highest inhibitory effect in clay soil, and arsenic in loam soil. Mercury is well known to have no beneficial function in biological processes and it is the heavy metal with the strongest toxicity due to the strong affinity of Hg^{2+} to thiol groups (Nies, 1999). Hong *et al.* (2007) reported that the growth of *Sphingomonas wittichi* RW1 and the degradation of dibenzofuran were strongly inhibited by 1 mg/L of mercury. The biodegradation of 2,4-dichloro-phenoxyacetic acid methyl ester in two microbial samples, namely, sediment and aufwuchs, from lakewater was inhibited in the presence of Hg and Cd (Said and Lewis, 1991). Mercury in the ionic form (Hg^{2+}) was most toxic in aufwuch samples, with a MIC of 0.002 mg total mercury/L. Van Zwieten *et al.* (2003) reported that the natural breakdown of 1,1,1-trichloro-2,2-bis (4-chlorophenyl) ethane (DDT) was inhibited in arsenic co-contaminated soil resulting in an increased persistence of DDT in the soil environment studied. The intrinsic breakdown of DDT to 1,1-dichloro-2,2-bis (4-chlorophenyl) ethane (DDD) in the presence of 2000 mg/kg arsenic resulted in a 50% reduction in the concentration of DDD compared to background arsenic of 5 mg/kg. Thus, it was demonstrated that arsenic co-contamination has an inhibitory effect on the breakdown of DDT via DDD, and that, as arsenic concentrations increases, the DDT:DDD and DDT:1,1-dichloro-2,2-bis (4-chlorophenyl) ethylene (DDE) ratios also increased. Furthermore, Roane *et al.* (2001), reported that degradation by *Ralstonia eutropha* JMP134, a cadmium-sensitive 2,4-dichlorophenoxyacetic acid (2,4-D)-degrader, occurred in the presence of up to 24 mg/L cadmium in mineral salts medium containing cadmium-resistant isolate and 60 mg/kg cadmium in amended soil microcosms. Experiments also indicated that 10^4 colony forming units of *Ralstonia eutropha* JMP134/ml alone in the presence of > 3 mg/L cadmium in mineral salts medium did not degrade 2,4-D due to cadmium toxicity.

A non-linear response for higher metal concentrations was observed for mercury and lead in clay and loam soil, respectively, after 20 days. This could be due to the non-availability of these metals in the soil matrix at high concentrations as they can either exist in precipitates, bound to the soil matrix (by adsorption or cation exchange) (Riis *et al.*, 2002), or are masked by organic or inorganic materials in the soil sample (Konopka *et al.*, 1999). This corroborates the findings of Baldrian *et al.* (2000), who found no inhibitory effect on polycyclic aromatic hydrocarbon degradation in soil containing 10-100 mg/kg Cd or Hg. Furthermore, some studies have shown that low concentrations of metals increasingly inhibit activity until a maximum level of inhibition is reached, and thereafter, metal toxicity decreases with increasing metal concentration (Said and Lewis, 1991). Roane and Pepper (1997) reported that populations of 2,4-D degraders in cadmium-contaminated soil were more resistant to cadmium toxicity at a higher concentration (40 mg total cadmium/L) than at a lower concentration (20 mg total cadmium/L). These responses to heavy metals may be explained by microbial community dynamics wherein high metal concentrations create selective pressure for metal-resistant, organic-degrading microorganisms. This selective pressure might have reduced competition from metal-sensitive, non-degrading microorganisms, thus increasing biodegradation at higher metal concentrations (Sandrin and Maier, 2003). The combinations of metals also resulted in non-linear responses, whereby combinations of two heavy metals resulted in a greater negative impact on 1,2-DCA degradation compared to the combinations of three or four heavy metals. A similar trend was observed by Benka-Coker and Ekundayo (1998) who reported that the toxicity of 0.5 mg total zinc/L on crude oil biodegradation was reduced by addition of 0.5 mg total copper, lead, and manganese/L.

In both clay and loam soil, lead (840 mg/kg) had the least inhibitory effect on 1,2-DCA degradation, thus confirming that Pb^{2+} has a less adverse effect on biodegradation processes. Lead is also a heavy metal toxic to a range of microorganisms, and is one of the most ubiquitous pollutants; released from chemical industry and various other inventories (Nies, 1999). However, owing to its low solubility, (especially as lead phosphate) its biologically available concentration is low (Hughes and Poole, 1991). This could be a possible reason for lower toxicity of Pb^{2+} to indigenous bacteria, since both soils contained high concentrations of phosphate. This is in agreement with other recent studies which showed that dibenzofuran degradation by *S. wittichii* RW1 remained unaffected even at a relatively high concentration of lead in the phosphate buffered condition (Hong *et al.*, 2007). Moreover, Shi *et al.* (2002) reported that much larger inputs of Pb were required to inhibit microbial activity in soil than that found when microbes were removed from soil particles. Approximately 10,000 mg of Pb per kg of soil was required to reduce $^{14}CO_2$ production by a factor of 2 in soil, whereas only about 400 mg of Pb per kg of soil could reduce microbial activity by 50% in a suspension of bacteria. This tallies with the results obtained in this study as the concentration of Pb^{2+}, approximately 10,575.48 mg/kg, caused a HLD of 1,2-DCA degradation in clay soil.

In summary, it can be inferred that the rate of 1,2-DCA biodegradation in co-contaminated soils was retarded by the presence of As^{3+}, Cd^{2+}, Hg^{2+}, and Pb^{2+}, and that the level of inhibition is dependent on soil types and their nutritional composition. Based on the MICs, As^{3+} and Cd^{2+} were the most toxic to indigenous soil microorganisms. Therefore, these two heavy metals were used to establish effective strategies in abating the toxic effects of heavy metals on 1,2-DCA degradation in such co-contaminated soils and results are presented in Chapter Three. However, reporting bioavailable metal concentrations to characterize metal inhibition represents a key step in standardizing methods to quantify metal impacts on biodegradation. Thus, relating inhibition results to dissociated metal concentrations may be needed for enhanced correlations of metal concentrations with toxicity effects.

CHAPTER THREE

ENHANCED BIODEGRADATION OF 1,2-DICHLOROETHANE IN SOIL CO-CONTAMINATED WITH HEAVY METALS UNDER DIFFERENT BIOREMEDIATION STRATEGIES

3.1 Introduction

Advances in science and technology have resulted in the exploitation of natural resources by humans to a great extent, generating unprecedented disturbances in the natural global environment (Lin *et al.*, 2006). In many countries, attempts are underway to control the release of contaminants (Schnoor *et al.*, 1995) and to accelerate the removal or breakdown of existing contaminants by appropriate remediation techniques, since accumulation of pollutants is of concern for both human and ecosystem exposures. In particular, the clean-up of soil co-contaminated with both chlorinated aliphatic hydrocarbons and heavy metals (HMs) is a contemporary subject of remediation efforts considering the fact that most of the current techniques are directed towards the removal of a specific group of contaminants (Ehsan *et al.*, 2007). Bioremediation, the use of biological activity for remediation, is one such highly appealing technology (Farhadian *et al.*, 2008; Volpe *et al.*, 2009; Widada *et al.*, 2002), and some promising results suggest that these techniques might become feasible alternatives to mechanical and chemical approaches in remediating soils co-contaminated with chlorinated organics and heavy metals (Mulligan *et al.*, 2001b; Romantschuk *et al.*, 2000; Singh *et al.*, 2008; Watanabe, 2001).

Indigenous microorganisms with natural degradation potentials are widely distributed in soil media; therefore the activation of the degradation potentials of these organisms is currently the challenge facing the implementation of bioremediation strategies, in addition to the fact that these microbes are usually present in very small numbers (Alexander, 1999). Moreover, autochthonous microorganisms capable of degrading specific organic contaminants often needs to be induced and the presence of heavy metals often inhibits the biodegradation processes in co-contaminated matrices (Alisi *et al.*, 2009). Biostimulation and/or bioaugmentation are such technologies which offer possible ways to overcome these limitations. Biostimulation involves the alteration of physico-chemical parameters to stimulate the growth of indigenous degraders by the addition of nutrients or other growth-limiting co-substrates. In particular, the presence of dissolved oxygen and nutrients, such as nitrate and phosphate, is essential (Li *et al.*, 2007). Previous studies have demonstrated that nitrogen concentrations ranging from 2.0 to 10.0 mg/L are sufficient for near-maximum growth of hydrocarbon-degrading microorganisms (Boufadel *et al.*, 1999; Wrenn *et al.*, 2006). On the other hand, bioaugmentation offers to provide specific microbes, a known degrader and/or heavy metal-resistant bacteria, in sufficient numbers to supplement the existing indigenous microbial population. A review by Gentry *et al.* (2004) provides an extensive survey of new bioaugmentation strategies. However, due to the site-specific characteristics of remediation technologies, numerous research efforts have come to heterogeneous results regarding the most feasible approach in enhancing the degradation of chlorinated organic pollutants in co-contaminated environments. More recently, bioaugmentation of contaminated sites using microorganisms pre-selected from their own environment showed great promise, as these microbes are more likely to survive and propagate when re-introduced into the site (Alisi *et al.*, 2009; Bento *et al.*, 2003).

Soil enzymatic activities have been recognized as sensitive indicators of any natural and anthropogenic disturbance (Hinojosa *et al*., 2004). Moreover, soil enzyme assays have been considered as one of the easiest and cheapest techniques used to integrate information concerning microbiological community status in contaminated environments (Baum *et al*., 2003; Shen *et al*., 2005). In particular, the dehydrogenase and urease enzyme assays have been established and recognized as a reliable indicator of the overall measure of the intensity of microbial metabolism, in soils contaminated with both organic and inorganic contaminants (Mathew and Obbard 2001; Rossel *et al*., 1997; Shen *et al*., 2005; von Mersi and Schinner 1991). Dehydrogenases are intracellular enzymes of the parent microbial cell, and could provide an indication of the oxidative potential of a soil which in turn provides a good overall indication of microbial activity (Gianfreda *et al*., 2005; Obbard, 2001; Trasar-Cepeda *et al*., 2000). Although the origin of ureases is also microbial, its activity is extracellular (Bremner and Mulvaney, 1978). Soil microbial activity and its products, such as intra and/or extracellular enzymes, have shown considerable differences associated with their sensitivity to heavy metal toxicity (Giller *et al*., 1998). Nannipieri *et al*. (1990) suggested that the simultaneous assessment of various enzymes in soil may be more valid than that of a single enzyme to evaluate overall microbial activity and its response to environmental stress. However, a few studies have focused on the combined effects of organic and inorganic pollutants on soil enzymes (Irha *et al*., 2003; Maliszewska-Kordybach and Smreczak, 2003). In addition, investigating the link between soil enzymatic activities and microbial community structure changes using advanced molecular biological techniques is necessary in order to provide a proper understanding of microbial dynamics in the co-contaminated soil environment (Trasar-Cepeda *et al*., 2000). Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) has recently received much attention in assessing changes in soil microbial community structure (Demanou *et al*., 2006; He *et al*., 2005; Li *et al*., 2006; Ros *et al*., 2008; Shi *et al*., 2005). This technique is based on the decreased electrophoretic mobility of a partially melted double stranded DNA molecule in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) (Muyzer and Smalla, 1998). DGGE of PCR amplified 16S rDNA fragments are used to profile microbial community complexity and changes in response to environmental stresses (Li *et al*., 2006; Mette and Neils, 2002).

Accordingly, the present study investigated the effects of biostimulation and bioaugmentation on the aerobic biodegradation of 1,2-DCA in soil co-contaminated with As^{3+} and Cd^{2+} in a soil microcosm setting. Furthermore, the structural diversity of bacterial communities in such treated soils was assessed by analyzing the 16S rDNA fingerprints, resolved by DGGE. In addition, the functional status of the microbial community was investigated by measuring the soil microbial urease and dehydrogenase activities.

3.2 Materials and methods

3.2.1 Enrichment cultures and isolation of heavy metal-resistant bacteria

One gram of co-contaminated loam soil sample was added to a 1000 ml flask containing 200 ml of a culture medium, comprising; 2.5 g of D-glucose (Merck, Saarchem), 2.5 g of yeast extract (Merck, Biolab) and 5 g tryptone (Difco), per litre of double-distilled water (Pepi *et al*., 2007). Stock solutions of arsenic oxide (As^{3+}) (> 99.0%, Fluka) and cadmium chloride (Cd^{2+}) (98%, Sigma) were added separately to each flask to obtain a final concentration of 300 mg/kg and 340 mg/kg, respectively. Control flasks containing the heavy metals with no inoculum were also included. Flasks were mixed and incubated at 30°C, without shaking, in the dark. After two weeks of incubation, cultures showing turbidity were subcultured by streaking onto agar plates containing the same enrichment culture medium but solidified with 1.6% of agar (Bacto-Agar, Difco), and amended with increasing concentrations of As^{3+} and Cd^{2+} (ranging from 75 mg/kg up to 340 mg/kg) to determine the minimal inhibitory concentration (MIC) of the heavy metal-resistant microorganisms. The inoculated culture media plates were incubated at 30°C for 72 h. MIC was defined as the lowest concentration of the heavy metals that causes the total growth inhibition of the organisms. In order to discriminate among resistant and sensitive strains, a reference heavy metal sensitive *Escherichia coli* strain was used. Colonies different in colour, shape, and margins appearing on inoculated plates were 4-way streak purified on the same culture medium, in the presence of the same concentrations of heavy metals, and used for dual-bioaugmentation studies.

3.2.2 Bacterial cultures

Bioaugmentation was performed using *Xanthobacter autotrophicus* GJ10, a well-known 1,2-DCA degrader, and a native heavy metal-resistant strain (selected as described above). The bacterial strain, *Xanthobacter autotrophicus* GJ10, was obtained from the culture collection of the Department of Microbiology, University of KwaZulu-Natal (Westville), and thereafter preserved on nutrient agar (Merck, Biolab) plates at 4°C as a working stock culture.

3.2.3 Standardization of the bacterial cultures

Pure cultures of the above-mentioned microorganisms were inoculated into 40 ml sterile nutrient broth and incubated for 72 h at 30°C on a rotary shaker at 150 rpm. Thereafter, the cultures were centrifuged (Beckman, USA, Model J2-21) at 4000 rpm for 15 min, followed by washing twice in 20 ml phosphate buffered saline (pH 7.4) containing 8 g NaCl, 0.2 g KCl, 3.58 g $Na_2HPO_4.12H_2O$ and 0.24 g KH_2PO_4 per litre of double-distilled water and re-suspended in the same solution. The cultures were then standardized to an optical density value of 1.0 at an absorbance of 600 nm (Sarret *et al*., 2005), using the biochrom, Libra S12 UV-Visible Spectrophotometer.

3.2.4   Experimental design and microcosm set-up

Soil samples were collected and used in setting up the microcosms as described in Chapter Two with the following amendments. Microcosm experiments were designed using artificially co-contaminated loam soil to study the effects of arsenic and cadmium on the biodegradation of 1,2-DCA over a 20 day period. Arsenic and cadmium stock solutions were incorporated into the groundwater, prior to the microcosm set-up, to obtain final concentrations of 150 mg/kg and 170 mg/kg, respectively. For the biostimulation experiments one and a half grams of D-glucose (Merck, Saarchem), fructose (Merck, Saarchem) and KOMPEL fertilizer (Chemicult products, Pty Ltd.), an agricultural fertilizer with N:P:K ratio of 3:1:6 were added separately to the microcosms. Bioaugmentation was carried out by inoculating 1 ml of standardized *X. autotrophicus* GJ10, and dual-bioaugmentation by adding 1 ml of a mixed culture inoculum prepared from the standardized pure cultures of both *X. autotrophicus* GJ10 and the isolated native heavy metal-resistant strain to the respective bottles. In addition to autoclaving, the biological inhibited controls prepared for each series of reaction mixtures were amended with $HgCl_2$ to achieve a final concentration of 500 mg/kg. The degradation profile of 1,2-DCA under the different conditions was determined using gas chromatographic analysis of the headspace as described in Chapter Two.

3.2.5   Enumeration of total 1,2-DCA degrading populations

Total 1,2-DCA degrading bacterial populations was determined at different sampling times using a standard spread plate technique (Gerhardt *et al*., 1991). The co-contaminated soil was homogenized manually using a sterile glass rod prior to pipetting 1 ml of the slurry for the initial 10-fold dilution. Appropriate culture dilutions were carried out using normal physiological saline solution (0.85% NaCl), thereafter 100 μl of the culture dilutions were plated on a minimal salts medium containing 1.36 g KH_2PO_4, 5.37 g $Na_2HPO_4.12H_2O$, 0.5 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4.7H_2O$ and 12 g Bacteriological agar (Bacto-Agar, Difco) per litre of double-distilled water (Janssen *et al*., 1984) spiked with 5 μl 1,2-DCA as the carbon source. The plates were incubated at 30°C for 48 h prior to estimating the bacterial population.

3.2.6   Soil analysis

The physico-chemical properties of the loam soil sample were determined as described in Chapter Two.

3.2.7 Soil enzyme assays

3.2.7.1 Dehydrogenase activity

Soil dehydrogenase activity was determined using 1 g of homogenized co-contaminated soil slurry and 5 ml of sterile distilled water. The samples for metabolic measurements were vortexed and incubated at room temperature for 30 min (Mosher *et al*., 2003). Thereafter, 5 ml of 1.08 mM 2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution was added and vortexed; these were incubated at room temperature for 60 min. All activity measurements were performed under dark room conditions, as the INT is light sensitive. Metabolic activity was stopped by adding 30 ml of acetonitrile (Merck, Germany) and INT formazan (INTF) was extracted at room temperature for 10 min. The samples were filtered through a 0.45 μm filter and the sediment washed with 10 ml of acetonitrile. The absorbance of the combined filtrate was determined with a biochrom, Libra S12 UV-Visible Spectrophotometer at 490 nm. Control samples were killed with 30 ml acetonitrile before the addition of INT to prevent reduction. Then, 5 ml of 1.08 mM INT was added and allowed to extract for 10 min prior to filtering. INTF (red-colored formazan) concentrations were determined from the linear least squares best-fit line from a standard curve of INTF solutions in acetonitrile. Dehydrogenase activity was expressed as μg INTF/g dwt soil/h (von Mersi and Schinner, 1991).

3.2.7.2 Urease activity

Soil urease activity was determined by the buffered method of Kandeler and Gerber (1988). In this procedure, 2.5 ml of 0.48% urea solution and 20 ml of borate buffer (pH 10) were added to 5 g of soil in 100 ml hermetically sealed Erhlenmeyer flasks in triplicate, and incubated for 2 h at 37°C. This was followed by a shaking incubation at 150 rpm for 30 min subsequent to the addition of 30 ml 1 M KCl solution. The ammonium content of the filtered (0.45 μm) extracts was determined using a colorimetric method. Briefly, 5 ml freshly prepared Na-salicylate/NaOH, 2 ml of 0.1% Na-dichloroisocyanide and 9 ml of sterile distilled water was added to 1 ml of clear filtrate and allowed to stand at room temperature for 30 min prior to measuring the optical density at 690 nm. The Na-salicylate solution was prepared by dissolving 17 g Na-salicylate and 0.12 g sodium nitroprusside in distilled water and brought up to 100 ml with distilled water. Na-salicylate/NaOH solution was prepared by mixing equal volumes of NaOH, Na-salicylate solutions, and distilled water. Blank controls were prepared with 2.5 ml sterile distilled water to determine the ammonium produced in the absence of added urea. The calibration curve was constructed using appropriate dilutions of a stock solution of ammonium chloride (100 μg NH_4-N/ml) using 1 M KCl and sterile distilled water to obtain final concentrations between 0.1-5 μg NH_4-N/ml. Urease activity was expressed as μg NH_4-N/g dwt soil/2h (Kandeler and Gerber, 1988).

3.2.8   DNA extraction and PCR

3.2.8.1 DNA extraction and PCR amplification of 16S rDNA region

Total DNA from the co-contaminated loam soil was isolated at different sampling times using an UltraClean^{TM} Soil DNA Isolation Kit (MOBIO, USA), following the manufacturer's protocol for maximum yields, but slightly modified, due to high humic acid content in the soil samples. The DNA was washed times with an ethanol based solution to remove residues of salts and humic acids, prior to eluting the DNA. DNA concentration was quantified using the NanoDrop 1000 Spectrophotometer (Thermo Scientific) and appropriate dilutions thereafter were carried out to standardize the DNA prior to PCR. This was subsequently used as template for the amplification of the 16S rDNA region using the 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'- GGCGGWGTGTACAAGGC-3') universal bacterial primer sets (Marchesi *et al.,* 1998). Each amplification reaction mixture (25 µl) contained 2.5 µl of 10 × PCR buffer, 1 µl of 25 mM MgCl$_2$, 1 µl each of the forward and reverse primers (10 µM), 1 µl of 1 mM deoxynucleoside triphosphate (dNTPs), 0.5 U of SuperTherm *Taq* DNA polymerase (Southern Cross Biotech), 1 µl of template DNA (0.2-3.8 ng/µl) and 17 µl of sterile double-distilled water. PCR was performed using the PE Applied Biosystems GeneAmp PCR System 9700 (Perkin-Elmer) and was programmed to implement an initial denaturation at 95°C for 5 min followed by 30 cycles of annealing and extension at 95°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final extension at 72°C for 5 min. The amplicons were analyzed by electrophoresis on 1% (w/v) agarose (SeaKem) gels in 1 × TAE running buffer with an applied voltage of 90 V for 90 min. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide (Sigma) for 20 min and visualized by UV transillumination (Chemi-Genius[2] BioImaging System, Syngene).

3.2.8.2 PCR amplification of V3 to V5 region

PCR for bacterial 16S rDNA region was performed using the universal DGGE primer set F341-357-GC (CCTACGGGAGGCAGCAG) with a 5′ GC-clamp: CGCCCGCCGCGCCCCGCGCCC GTCCCGCCGCCCCCGCCCG and R907-926 (CCGTCAATTCMTTTGAGTTT) (Casamayor *et al*., 2000). A GC-clamp was attached to the forward primer to prevent complete separation of the strands during DGGE (Muyzer *et al*., 1993). For PCR, 2 µl DNA extract (between 0.2-3.8 ng/µl) was added to the PCR amplification reaction mixture (50 µl) containing 5 µl 10 × PCR-buffer, 2 µl of 25 mM MgCl$_2$, 2.5 µl each of F341-GC and 907R (10 µM), 5 µl of 2 mM dNTPs, 30.5 µl sterile double-distilled water and 0.5 U of SuperTherm *Taq* DNA polymerase (Southern Cross Biotech). PCR was performed using the GeneAmp PCR System (Version 2.25, Perkin Elmer). A modified form (Muyzer *et al*., 1993) of the touchdown thermal profile technique (Watanabe *et al*., 1998) was used: an initial denaturation (94°C, 5 min), followed by annealing via 10 cycles of 94°C, 1 min; 65°C, 1 min with a decrease in temperature of

1°C per cycle; and 72°C, 3 min. This was followed by 20 cycles of 94°C, 1 min; 55°C, 1 min; 72°C, 3 min and a final 5 min extension step at 72°C. The amplification of the correct product size of 585 bp was confirmed by electrophoresis in a 2% (w/v) agarose gel in a 1 × TAE running buffer with an applied voltage of 90 V for 120 min. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide and visualized by UV transillumination (Chemi-Genius[2] BioImaging System, Syngene).

3.2.9 Denaturing Gradient Gel Electrophoresis (DGGE)

PCR amplicons were separated by DGGE using the D-Code Universal Mutation Detection System (BioRad) (Muyzer *et al.,* 1997). Firstly, 0% and 100% denaturing solutions were prepared, filtered through 0.45 µm pore size GN-6 Metricel membrane filters (Pall, 47 mm) and stored in brown bottles at 4°C. The DGGE gel was cast by preparing 20 ml each of low (40%) and high (70%) density solutions containing 20 µl TEMED and 200 µl of 10% ammonium persulphate, for gradient formation. The density solutions were applied to the gradient delivery system to cast the perpendicular 6% acrylamide DGGE gels (dimensions: 200 mm by 200 mm by 1 mm). Prior to sample loading, a pre-run was performed at a constant voltage of 150 V at 60°C for 30 min to facilitate sample migration out of the wells during the electrophoretic run. Following the pre-run, samples were loaded into the gel (5 µl gel loading buffer : 20 µl PCR amplicons) and DGGE was conducted at a constant voltage of 60 V in 1 × TAE buffer at 60°C for 16 hrs. After electrophoresis, the gel was stained in 0.5 µg/ml ethidium bromide (BioRad) for 20 min, destained in the same volume of 1 × TAE buffer for a further 20 min and thereafter visualized by UV transilluminator (Chemi-Genius[2] BioImaging System, Syngene).

3.2.10 Statistical analysis

Analysis of the biodegradation results was carried out using student's (paired) *t*-test, 2 tails distribution with significance level of $p < 0.05$.

3.3 Results

3.3.1 Effect of biostimulation and bioaugmentation on the biodegradation of 1,2-DCA in co-contaminated soil

Microcosm studies using co-contaminated loam soil moistened with synthetic groundwater were performed to evaluate the biodegradation profiles of 1,2-DCA upon the addition of supplemental substrates/nutrients or inoculation with bioaugmentation cultures. The biodegradation profile of 1,2-DCA in co-contaminated soil microcosms undergoing biostimulation and bioaugmentation, in the presence of As^{3+} and Cd^{2+} are illustrated in Figures 3.1 (a) and (b), respectively. In the autoclaved soil control amended with $HgCl_2$, abiotic loss of 1,2-DCA was observed, but to a lesser extent. In the unautoclaved

positive control not contaminated with heavy metals, the initial 1,2-DCA concentration of 15.79 μl/100 g soil was reduced to 1.64 μl/100 g soil, corresponding to 89.78% elimination of 1,2-DCA, a value which is significantly ($p < 0.05$) higher than that of the sterile control, correlating with up to 60% above that of the sterile control, after 20 days. The presence of heavy metals was observed to have a negative impact on the biodegradation of 1,2-DCA resulting in a significant ($p < 0.05$) decrease of 9.30% and 5.86% in degradation, in soil co-contaminated with As^{3+} and Cd^{2+}, respectively, indicating the more pronounced toxic effect of As^{3+} compared to Cd^{2+}, after 20 days. The degradation rate constants of 1,2-DCA, ranged between $0.081 – 0.204$ day^{-1} and between $0.091 – 0.216$ day^{-1} in As^{3+} and Cd^{2+} co-contaminated soils, respectively (Table 3.1). The degradation rate constants of 1,2-DCA in the biostimulated and bioaugmented co-contaminated microcosms were higher than in the unautoclaved soil control containing no heavy metals. In all cases, except for biostimulation with fructose, higher degradation rate constants were observed in soil co-contaminated with Cd^{2+} compared to As^{3+}. In addition, the degradation rate constants of 1,2-DCA in bioaugmented soil samples were higher than the biostimulated microcosms in both As^{3+} and Cd^{2+} co-contaminated soil samples. Bioaugmentation with *X. autotrophicus* GJ10 resulted in a 2.5-fold and 2.3-fold increase in the rate of 1,2-DCA degradation in As^{3+} and Cd^{2+} co-contaminated soil, respectively compared to the untreated co-contaminated soil. All supplemented substrates which included; glucose, fructose and fertilizer, enhanced the biodegradation of 1,2-DCA in soil co-contaminated with both heavy metals. After 15 days, biostimulation with fertilizer resulted in the greatest increase in 1,2-DCA degradation with 4.94% and 6.85% achievd in both the As^{3+} and Cd^{2+} co-contaminated soil, respectively. In the Cd^{2+} co-contaminated soil a significant ($p < 0.05$) increase of 12.02%, 10.37% and 12.37% in 1,2-DCA degradation occurred in the presence of glucose, fructose and fertilizer, respectively after 20 days. Compared to the Cd^{2+} co-contaminated soil, an additional 3.01%, 4.50% and 2.55% increase in degradation was observed in the As^{3+} co-contaminated soil in the presence of glucose, fructose and fertilizer, respectively over the same period. 1,2-Dichloroethane degradation was significantly ($p < 0.05$) lower in unfertilized than in fertilized soil resulting in up to 14.92% increase in degradation in As^{3+} co-contaminated soil after 20 days. Both amendments and time had a significant influence on soil decontamination. After 5 days, 10.40% and 5.03% more degradation occurred in the dual-bioaugmented soil compared to that bioaugmented with only *X. autotrophicus* GJ10, in the As^{3+} and Cd^{2+} co-contaminated soil, respectively. Thereafter, a similar profile was observed in both the As^{3+} and Cd^{2+} co-contaminated soil up until day 15.
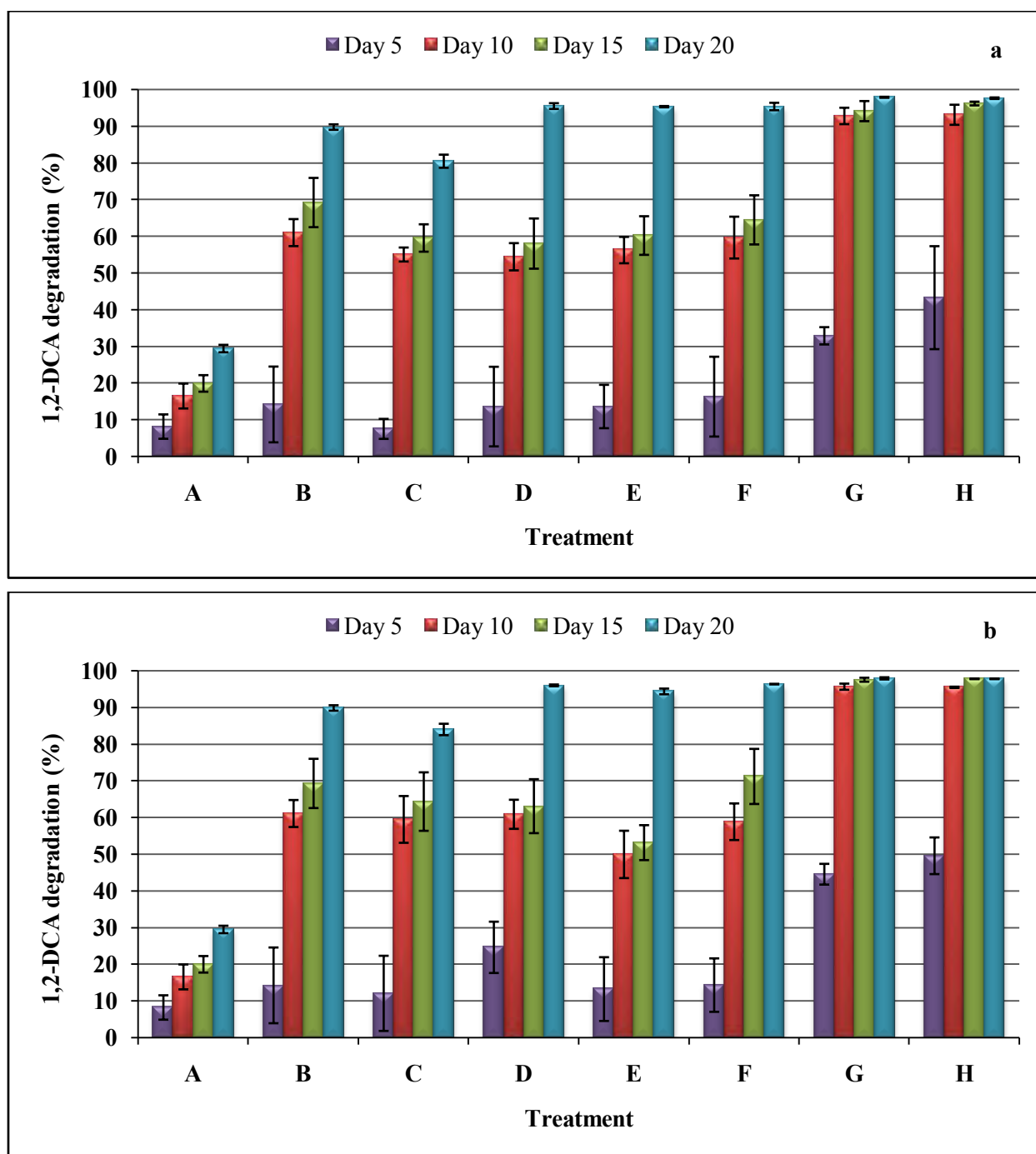
Figure 3.1: Effects of biostimulation and bioaugmentation on the biodegradation of 1,2-DCA in soil co-contaminated with (a) 150 mg/kg As^{3+} and (b) 170 mg/kg Cd^{2+}. A = autoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA; C = unautoclaved soil control + 1,2-DCA + HM; D = soil + 1,2-DCA + HM + glucose; E = soil + 1,2-DCA + HM + fructose; F = soil + 1,2-DCA + HM + fertilizer; G = soil + 1,2-DCA + HM + *X. autotrophicus* GJ10; H = soil + 1,2-DCA + HM + dual-bioaugmentation. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

Table 3.1: Biodegradation rate constants (day^{-1}) of 1,2-DCA in soil co-contaminated with arsenic (150 mg/kg) and cadmium (170 mg/kg), undergoing biostimulation and bioaugmentation.

Treatment	Arsenic	Cadmium
Controls		
Autoclaved soil	0.016 ± 0.001	0.016 ± 0.001
Unautoclaved soil	0.111 ± 0.003	0.111 ± 0.003
Unautoclaved soil + HM	0.081 ± 0.003	0.091 ± 0.003
Biostimulation		
Glucose	0.139 ± 0.008	0.142 ± 0.004
Fructose	0.138 ± 0.003	0.127 ± 0.006
Fertilizer	0.140 ± 0.010	0.154 ± 0.003
Bioaugmentation		
X. autotrophicus GJ10	0.204 ± 0.012	0.216 ± 0.007
Dual-bioaugmentation	0.203 ± 0.007	0.213 ± 0.003

3.3.2   Total 1,2-DCA degrading bacterial population dynamics in the co-contaminated soil

The total 1,2-DCA degrading bacterial population dynamics in the As^{3+} and Cd^{2+} co-contaminated loam soil undergoing biostimulation and bioaugmentation is shown in Tables 3.2 and 3.3, respectively. An alteration in the population of the autochthonous 1,2-DCA degrading microbial community was observed subsequent to the addition of As^{3+} and Cd^{2+}, resulting in an approximate seventeen-fold and thirteen-fold decrease in population after 10 days, respectively. In the As^{3+} co-contaminated soil biostimulated with glucose and fertilizer an ten-fold and three-fold increase in the total 1,2-DCA degrading bacterial population was observed after 10 days, correspondingly. In the dual-bioaugmented treatments, the total number of culturable 1,2-DCA degraders detected increased from the initial inoculum level of 6.47×10^6 to 111.67 and 403.33×10^6 in the As^{3+} and Cd^{2+} co-contaminated soil, respectively after 10 days. In the biostimulation experiment, the highest 1,2-DCA degrading population of 36.33×10^7 cfu/ml (in the fertilizer-amended microcosm) was obtained and 34.86×10^7 cfu/ml (in the glucose-amended microcosm) for the soil co-contaminated with As^{3+} and Cd^{2+}, respectively after 20 days. In both cases, dual-bioaugmentation produced the highest 1,2-DCA degrading population.

Table 3.2: Total 1,2-DCA degrading bacterial population in arsenic (As^{3+}) co-contaminated soil undergoing biostimulation and bioaugmentation.

Treatment	1,2-DCA degrading bacterial population	
	Day 10 (cfu/ml × 10^6)	Day 20 (cfu/ml × 10^7)
Controls		
Autoclaved soil	0	0
Unautoclaved soil	48.33 ± 7.77	2.91 ± 0.10
Unautoclaved soil + As^{3+}	2.90 ± 0.34	0.26 ± 0.06
Biostimulation		
Glucose	28.98 ± 13.55	33.90 ± 3.77
Fructose	2.87 ± 0.52	2.83 ± 0.06
Fertilizer	7.37 ± 1.11	36.33 ± 5.51
Bioaugmentation		
X. autotrophicus GJ10	31.03 ± 4.98	291.50 ± 23.34
Dual-bioaugmentation	111.67 ± 3.51	430.00 ± 91.65

Values are averages of triplicate data ± standard deviation.
The initial 1,2-DCA degrading bacterial population was 6.47×10^6 cfu/ml.

Table 3.3: Total 1,2-DCA degrading bacterial population in cadmium (Cd^{2+}) co-contaminated soil undergoing biostimulation and bioaugmentation.

Treatment	1,2-DCA degrading bacterial population	
	Day 10 (cfu/ml × 10^6)	Day 20 (cfu/ml × 10^7)
Controls		
Autoclaved soil	0	0
Unautoclaved soil	48.33 ± 7.77	2.91 ± 0.10
Unautoclaved soil + Cd^{2+}	3.75 ± 0.83	0.29 ± 0.02
Biostimulation		
Glucose	3.33 ± 0.69	34.86 ± 10.62
Fructose	1.91 ± 0.20	2.74 ± 0.11
Fertilizer	4.20 ± 0.36	32.90 ± 5.93
Bioaugmentation		
X. autotrophicus GJ10	82.00 ± 10.15	410.00 ± 56.57
Dual-bioaugmentation	403.33 ± 80.21	453.33 ± 105.04

Values are averages of triplicate data ± standard deviation.
The initial 1,2-DCA degrading bacterial population was 6.47×10^6 cfu/ml.

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3.3.3 PCR-DGGE analysis of bacterial community diversity

In addition to the culture-dependent approach in assessing total 1,2-DCA degrading bacterial population dynamics, DGGE analysis of PCR-amplified 16S rDNA fragments was used to investigate the effect of biostimulation and bioaugmentation on the co-contaminated loam soil microbial communities. Fertilizer treatments which appeared to cause significant increases in 1,2-DCA degradation, as reflected in 1,2-DCA biodegradation profile, were selected for DGGE analysis. Distinct DGGE profiles were observed for both the fertilized and dual-bioaugmentated samples as shown in Figures 3.2 and 3.3, respectively. The bands denoted by the arrow (except band A3) were all shown to be present throughout the degradation period. On comparison of the As^{3+} and Cd^{2+} co-contaminated soil undergoing either biostimulation or dual-bioaugmentation, the banding patterns observed seemingly exhibited a great similarity. However, the banding patterns for the different bioremediation options, demonstrated much difference in bacterial diversity between the fertilized and dual-bioaugmented samples. DGGE profiles indicate that while numerous bands were common in the fertilized co-contaminated soils, there were also changes in band presence and relative intensity due to treatment and temporal effects. In both the As^{3+} and Cd^{2+} co-contaminated soil biostimulated with fertilizer, bands A5 and A6 representing dominant degrading populations became brighter by day 20, indicating that these organisms where involved in the degradation of 1,2-DCA in such co-contaminated environments. These bands were present on day 0, albeit at very low concentrations, thus appearing faintly. On the other hand, band A3 was absent on day 0 and was observed on day 5 in both the As^{3+} and Cd^{2+} co-contaminated soils, whereas bands A1, A2 and A4 were present throughout the degradation period, however the band intensity remained unchanged. All the bands mentioned above maintained its dominance (as indicated by the brightness of the band) until day 20 of the degradation process. Meanwhile, in the dual-bioaugmented microcosms, the native heavy metal-resistant strain band intensity increased on day 15 in the As^{3+} co-contaminated soil, however, in the Cd^{2+} co-contaminated soil, band intensity remained relatively unchanged up until day 15. In both the As^{3+} and Cd^{2+} co-contaminated soil, *X. autotrophicus* GJ10 (B3) band brightness increased slightly over time.
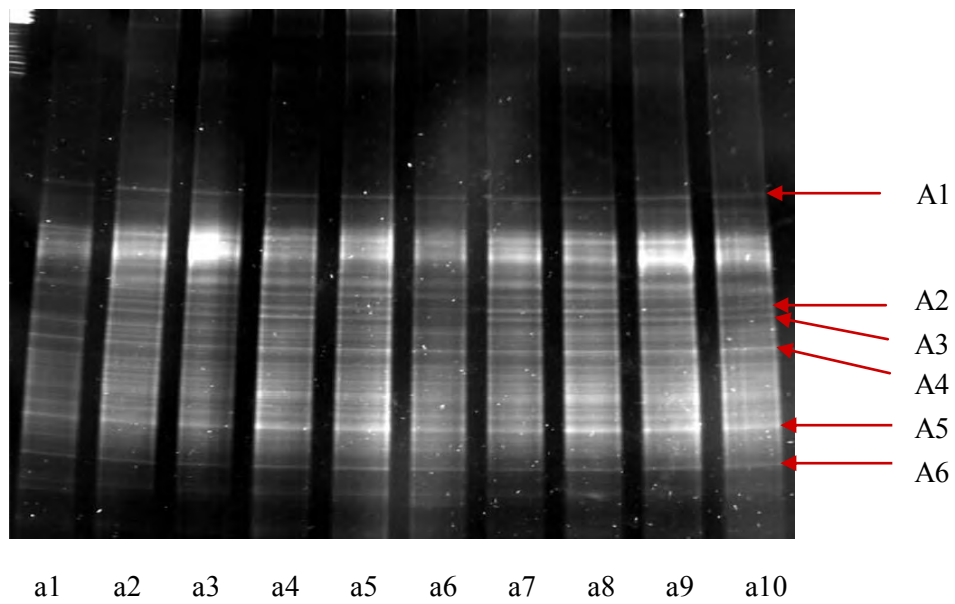
Figure 3.2: DGGE profiles of 16S rRNA gene fragments from co-contaminated loam soil samples biostimulated with fertilizer. Lanes: a1, a2, a3, a4 and a5 represent days 0, 5, 10, 15 and 20 of the As^{3+} co-contaminated soil and a6, a7, a8, a9 and a10 represent days 0, 5, 10, 15 and 20 of the Cd^{2+} co-contaminated soil.
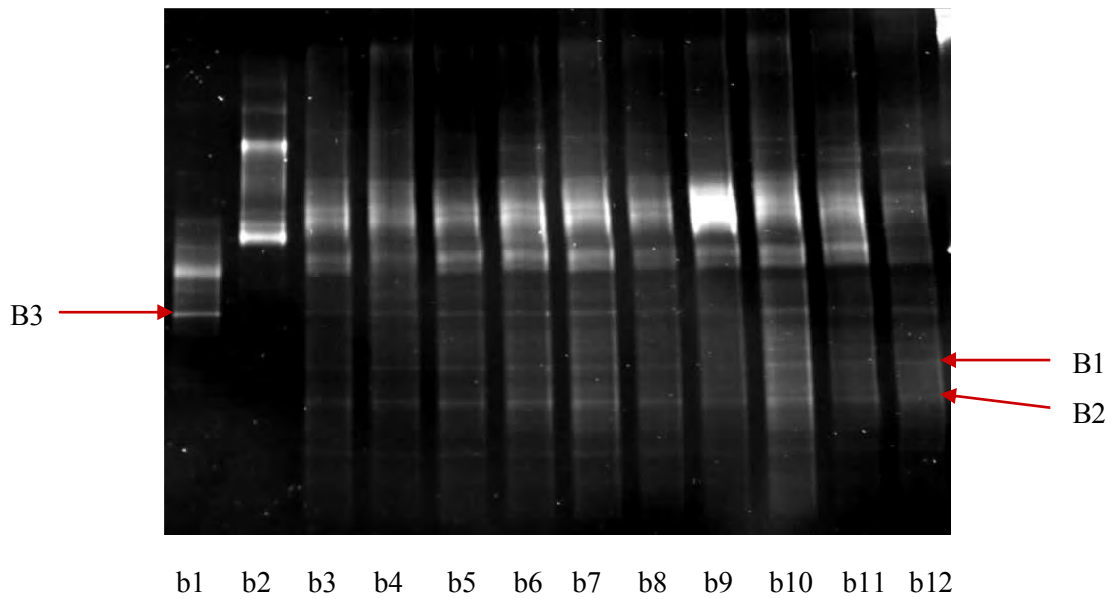
Figure 3.3: DGGE profiles of 16S rRNA gene fragments from co-contaminated loam soil samples undergoing dual-bioaugmentation. Lanes: b1 represents *X. autotrophicus* GJ10, b2 represents native HM resistant strain, b3, b4, b5, b6 and b7 represent days 0, 5, 10, 15 and 20 of the As^{3+} co-contaminated soil and b8, b9, b10, b11 and b12 represent days 0, 5, 10, 15 and 20 of the Cd^{2+} co-contaminated soil.

3.3.4 The combined effect of 1,2-DCA and heavy metals on soil microbial activities

The combined effect of 1,2-DCA and heavy metals on soil microbial enzyme activities varied depending on the heavy metal tested. The changes in dehydrogenase activity in all treatments are shown in Figures 3.4 and 3.5. In both the As^{3+} and Cd^{2+} co-contaminated soil there was a significant decrease of 87.26% (Figure 3.4a) and 86.33% (Figure 3.4b) in the dehydrogenase activity after 20 days, respectively. In both the biostimulated and bioaugmented soil microcosms, dehydrogenase activity appeared biphasic with an initial decrease followed by a progressive increase in the treated soils over time. Due to the different supplemental substrates, dehydrogenase activity differed in the three biostimulated microcosms. In all cases, except for the Cd^{2+} co-contaminated soil amended with fructose, higher dehydrogenase activity was observed in the biostimulated soil compared to the untreated co-contaminated soil. The greatest increase in dehydrogenase activity, in both the As^{3+} and Cd^{2+} co-contaminated soil was observed in the glucose amended soil, resulting in 86.52% and 84.18% increase, respectively after 20 days, compared to the untreated co-contaminated soil (Figure 3.4). Moreover, the supplemental substrates appeared to follow a similar pattern in both As^{3+} and Cd^{2+} co-contaminated soil; the trend, in ascending order of increased dehydrogenase activity was fructose, fertilizer and glucose. In addition, 2.41% and 6.42% increase in dehydrogenase activity was observed in the As^{3+} co-contaminated soil amended with glucose and fructose, respectively after 20 days, compared to Cd^{2+}. Bioaugmentation with only *X. autotrophicus* GJ10 resulted in a greater increase in dehydrogenase activity compared to dual-bioaugmentation, with an increase of 85.18% (Figure 3.5a) and 80.95% (Figure 3.5b) observed in the As^{3+} and Cd^{2+} co-contaminated soil, respectively after 20 days. However, these values are 1.34% and 3.23% less than the dehydrogenase activity observed in the soil microcosms biostimulated with glucose in the As^{3+} and Cd^{2+} co-contaminated soil, respectively. Soil urease activity was inhibited in the presence of heavy metals, resulting in a 7.66% (Figure 3.6a) and 20.17% (Figure 3.6b) decrease in the presence of As^{3+} and Cd^{2+}, respectively after 20 days. In the case of supplemental substrates/nutrients, a decline in urease activity was observed in the glucose and fructose amended soil microcosms in both heavy metal co-contaminated soils. On the other hand, fertilizer was observed to significantly ($p < 0.05$) increase urease activity resulting in approximately 79% increase in both heavy metal co-contaminated soils after 20 days compared to the untreated soil. Both bioaugmentation and dual-bioaugmentation was observed to result in higher urease activity within the first 10 days in As^{3+}, followed by a decrease. Bioaugmentation with *X. autotrophicus* GJ10 was also noted to decrease urease activity in both As^{3+} and Cd^{2+} co-contaminated soil resulting in 25.35% (Figure 3.7a) and 35.95% (Figure 3.7b) decrease, respectively after 20 days. These values correspond to a 2.57% and 10.95% less pronounced effect on urease activity in dual-bioaugmented soil.
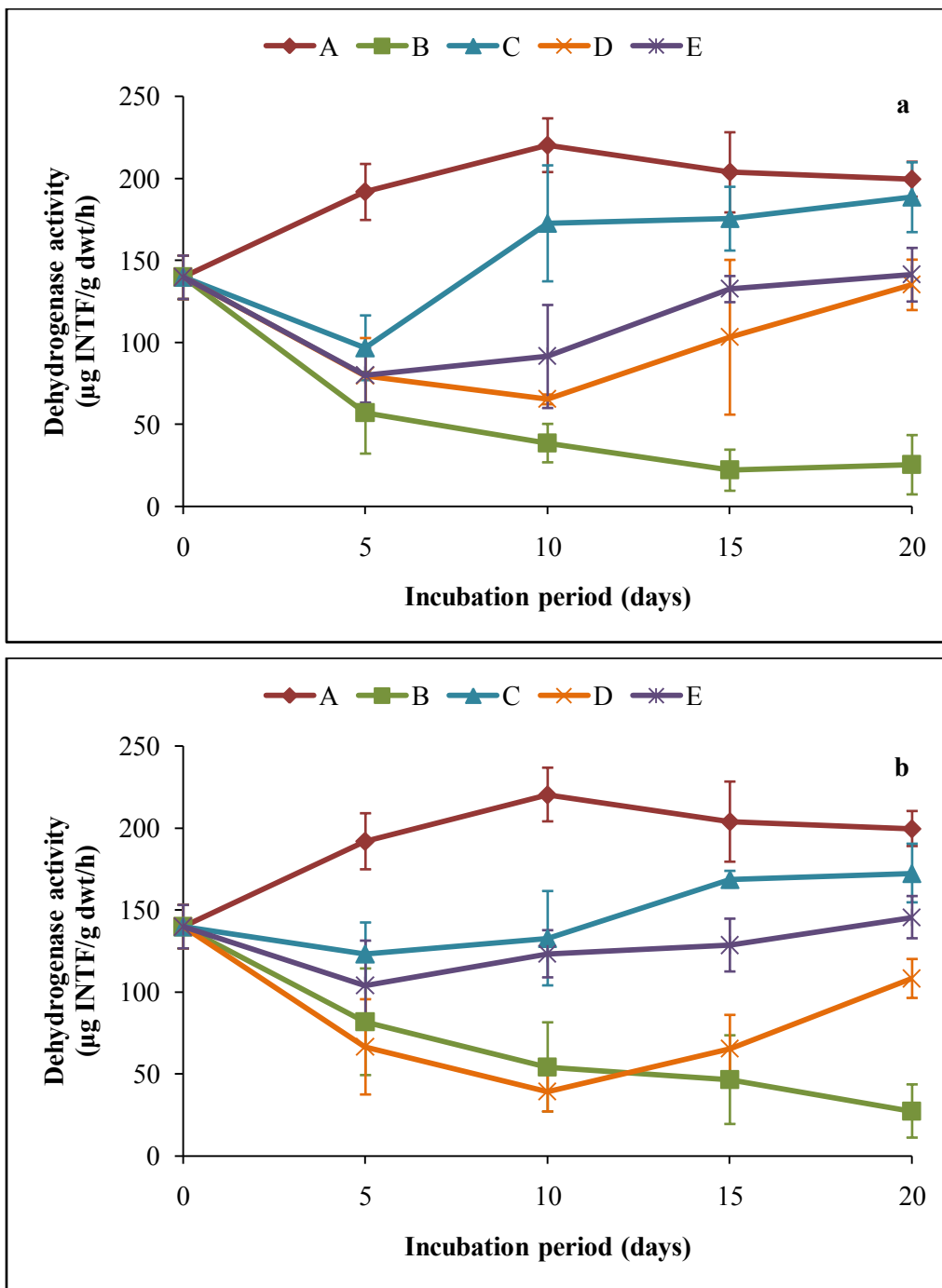
Figure 3.4: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial dehydrogenase activity in microcosms undergoing biostimulation. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + glucose; D = soil + 1,2-DCA + HM + fructose; E = soil + 1,2-DCA + HM + fertilizer. The results indicate the average of triplicate samples while the error bars show the standard deviation.
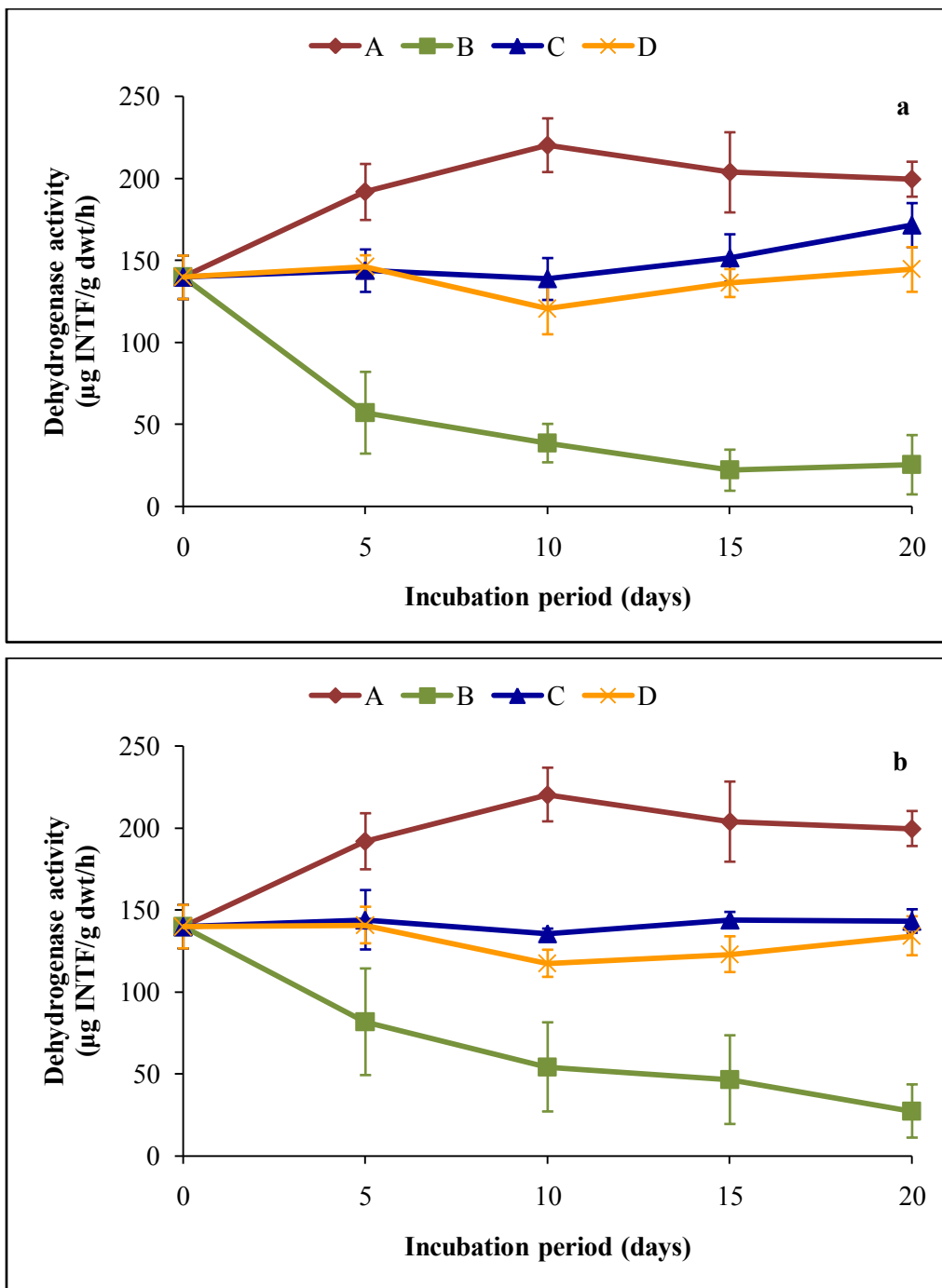
Figure 3.5: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial dehydrogenase activity in microcosms undergoing bioaugmentation. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + *X. autotrophicus* GJ10; D = soil + 1,2-DCA + HM + dual-bioaugmentation. The results indicate the average of triplicate samples while the error bars show the standard deviation.
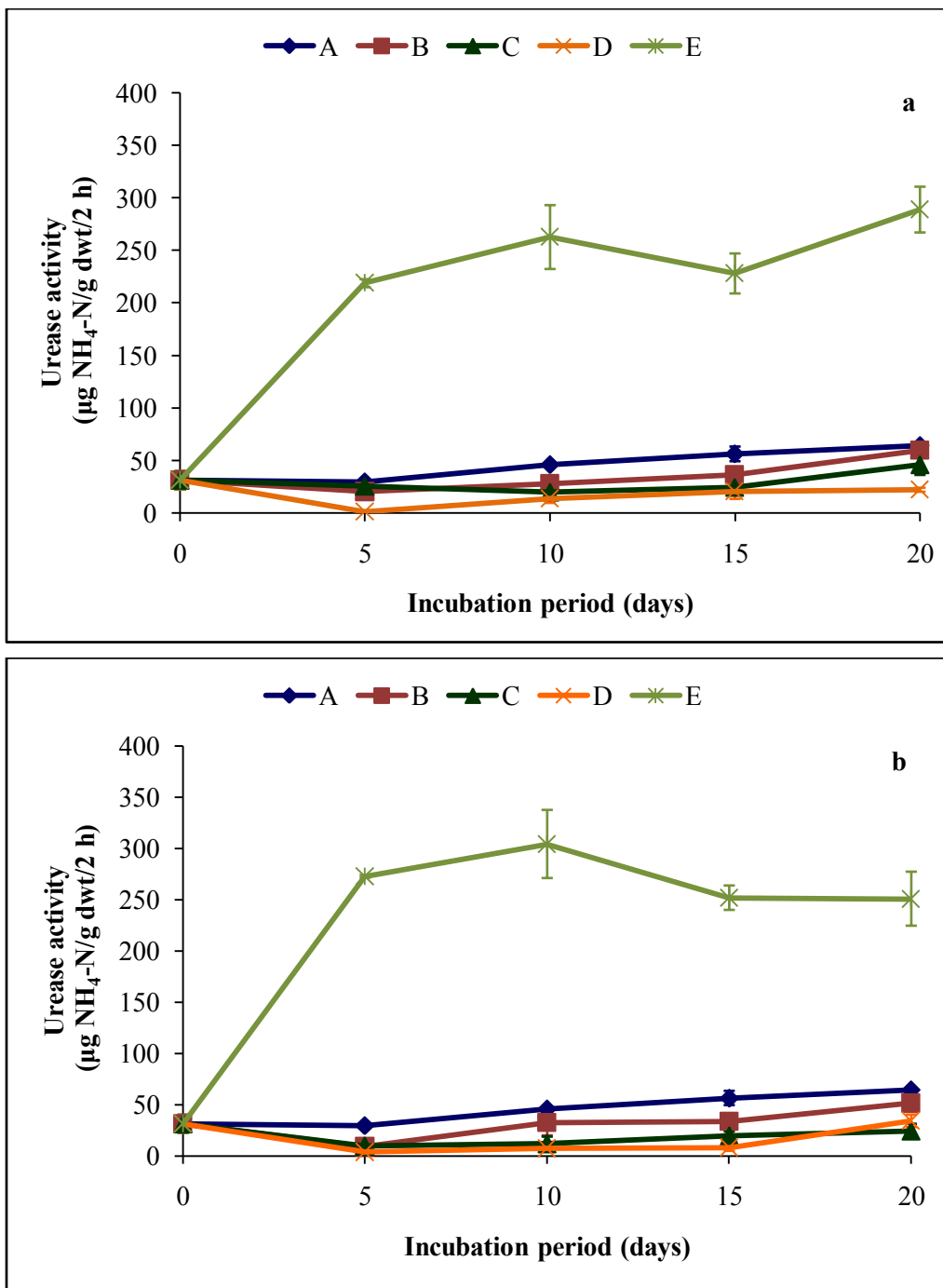
Figure 3.6: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial urease activity in microcosms undergoing biostimulation. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + glucose; D = soil + 1,2-DCA + HM + fructose; E = soil + 1,2-DCA + HM + fertilizer. The results indicate the average of triplicate samples while the error bars show the standard deviation.
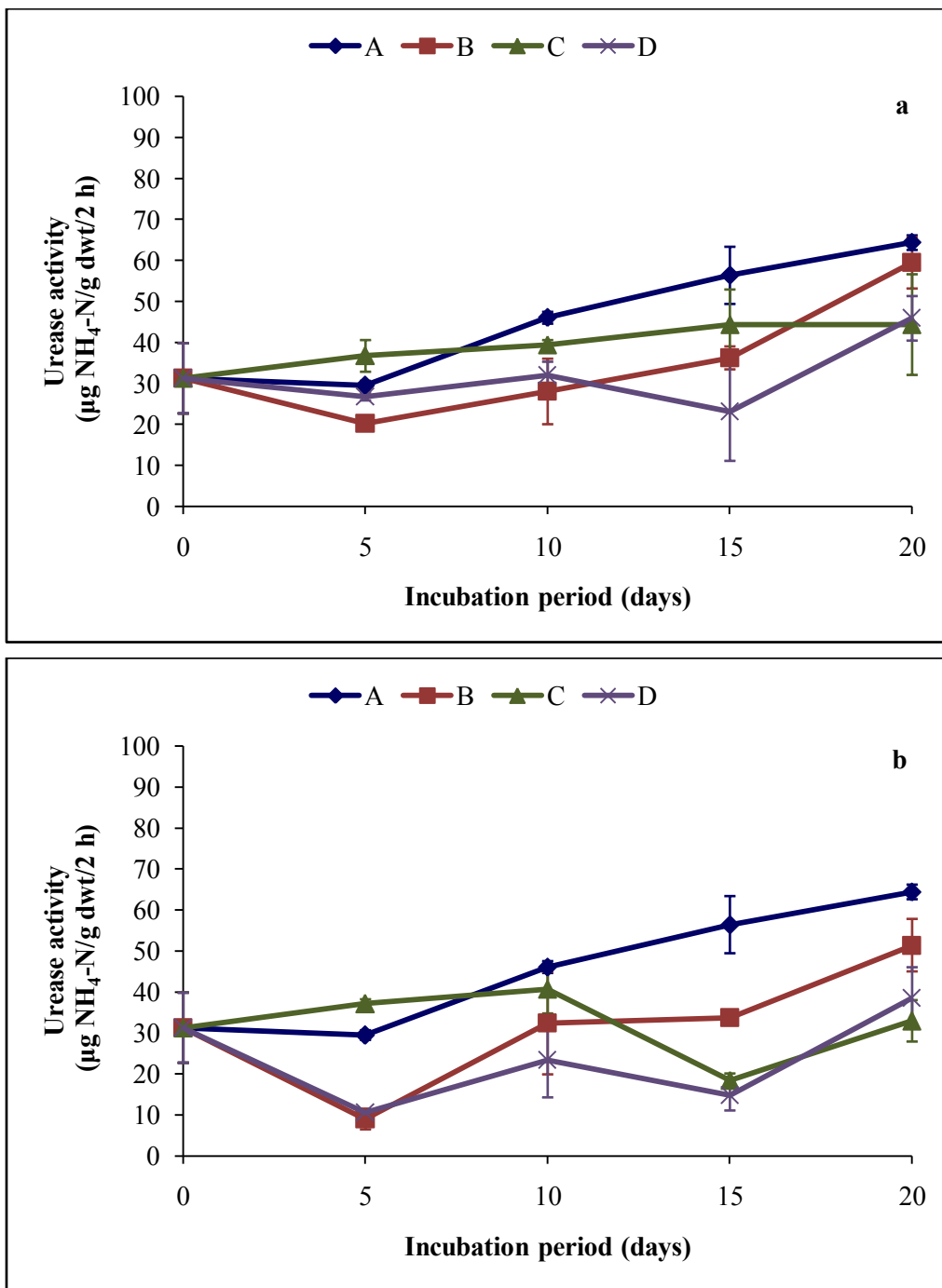
Figure 3.7: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial urease activity in microcosms undergoing bioaugmentation. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + *X. autotrophicus* GJ10; D = soil + 1,2-DCA + HM + dual-bioaugmentation. The results indicate the average of triplicate samples while the error bars show the standard deviation.

3.4   Discussion

Results from this study revealed the capability of the indigenous soil microbial populations to remove 1,2-DCA from co-contaminated soil, with both bioaugmentation and biostimulation approaches playing a fundamental role in enhancing 1,2-DCA degradation, and dual-bioaugmentation having the greatest effect in both As^{3+} and Cd^{2+} contaminated soils. However, the observed degradation profiles obtained varied depending on the heavy metal co-contaminant present in the soil.

The physico-chemical parameters of soil samples have been shown to have a major influence on aeration, water retention, nutrient availability, and consequently on microbial activity and community dynamics (Olaniran *et al*., 2006). In general, a ratio of carbon to nitrogen to phosphorus of 100:10:1 (Norris *et al*., 1994) has been extensively used in the biodegradation processes (Button *et al*., 1992). In addition, bioremediation studies have demonstrated that nitrogen concentrations ranging from 2.0 to 10.0 mg/L are sufficient for near-maximum growth of hydrocarbon-degrading microorganisms (Boufadel *et al*., 1999; Wrenn *et al*., 2006). In this study, the loam soil used in the construction of the microcosms contained relatively low concentrations of soluble organic carbon with fairly high concentrations of nitrogen and phosphorus (mainly in the form of phosphate). Phosphate has a high affinity for most soils which lessens its transport, thus causing it to be unavailable for biological activity (Liebeg and Cutright, 1999).The carbon to nitrogen ratio was considerably low (0.7:1) in the loam soil compared to that recommended for soil hydrocarbon bioremediation which varies greatly and ranges from 100:1 to 10:1 (Atlas and Bartha, 1992; Song and Bartha, 1990). However, it should be noted that these average values depend on the type of microorganisms present in the soil. Thus, in this study, the addition of supplemental substrates such as glucose, fructose and nitrogen fertilizers were shown to enhance 1,2-DCA degradation in the heavy metal co-contaminated soil samples to varying degrees.

In both the As^{3+} and Cd^{2+} co-contaminated soil, biostimulation with fertilizer resulted in the greatest increase in 1,2-DCA degradation after 15 days. It has been demonstrated that biodegradation of large quantities of organic carbon sources by indigenous microorganisms tends to result in a depletion of inorganic nutrients such as nitrogen and phosphorus (Margesin *et al*., 2000), thus limiting the rate of biodegradation. This study demonstrated that under favourable conditions, via the addition of inorganic amendments, biostimulation can enhance the degradation of 1,2-DCA in polluted soils even in the presence of co-contaminants. These findings correlate well with several other research reports on the positive effects of biostimulation with mainly N:P:K fertilizers (Atlas and Bartha, 1992; Margesin *et al*., 2000; Margesin and Schinner, 2001; Olaniran *et al*., 2006; 2009). Siddiqui and Adams (2001) also observed that the best response obtained for diesel oil degradation was also in soil amended with N and P. Biostimulation with glucose also resulted in high 1,2-DCA degradation rates in the presence of As^{3+} and Cd^{2+}, thereby also providing very promising results for effective bioremediation of such co-contaminated soils. Gao and Skeen (1998) reported that glucose, being a simple sugar, is highly soluble in water and

therefore easily metabolized by microorganisms. Similarly, enhanced biodegradation of 1,2-DCA following the addition of glucose to co-contaminated soil has been recently reported (Olaniran *et al*., 2009).

Bioaugmentation, in particular dual-bioaugmentation, was observed to significantly increase the rate of 1,2-DCA biodegradation. Previous studies have demonstrated that, under certain conditions, addition of known degrading organism accelerates the rate and the degree of organic biodegradation (Bento *et al*., 2003; Mueller *et al*., 1992). In particular, the finding in this study correlates well with that of Roane *et al*. (2001) who reported that dual-bioaugmentation, involving inoculation with both metal-detoxifying (*Ralstonia eutropha* JMP134) and organic-degrading (*Pseudomonas* H1) bacteria, facilitates the degradation of 2,4-dichlorophenoxyacetic acid (2,4-D) in the presence of cadmium co-contamination. Based on the results obtained, it was concluded that dual-bioaugmentation with metal-detoxifying and organic-degrading microbial populations is effective for remediation of co-contaminated soil. Implementation of dual-bioaugmentation strategies rely primarily on metal detoxification so that organic-degrading microbial populations are prevented from metal stress and inhibition. This strategy was also found successful at pilot scale with a soil bioreactor, resulting in significant reductions in 2,4-D levels within six weeks. On the other hand, Doelman *et al*. (1994) hypothesized that in soils contaminated with heavy metals, indigenous microorganisms with resistance to these inorganic contaminants may reduce the bacterial bioremediation capability towards chlorinated hydrocarbons. This is in contrast to the results obtained in this study. Inoculation of soil with native microorganisms, provided in a sufficient number to enhance the biodegradation process, has been proposed as a generic bioremediation approach to ameliorate the clean-up of polluted areas (Alisi *et al*., 2009). The bioaugmentation approach assumed in this study was based on strengthening a portion of the native microbial community with an indigenous microorganism resistant to the heavy metals tested. This strain was incorporated in order to indirectly increase 1,2-DCA degradation, by protecting *X. autotrophicus* GJ10 or the other indigenous metal sensitive organic-degrading strains from metal toxicity. In principle, the native strains of co-contaminated matrices, already shaped by selective pressure for heavy metal resistance, are expected to take advantage with respect to sensitive strains in enhancing biodegradation. In this way, they help to overcome an important limitation in bioremediation applications, namely the co-occurrence of toxic heavy metals, which inhibit microbial processes in general (Alisi *et al*., 2009).

Numerous limitations, such as difficulty in delivery of the inoculant to the desired location (Streger *et al*., 2002), rapid decline in introduced microbial numbers and death of the exogenous microorganisms (Goldstein *et al*., 1985), have been reported with the use of bioaugmentation as a bioremediation strategy. However, in this study, the effect of bioaugmentation was more pronounced resulting in a marked increase in 1,2-DCA degradation in both the As^{3+} and Cd^{2+} co-contaminated soils, compared to biostimulation. In addition, a significant increase in the total number of 1,2-DCA degrading

bacterial populations was observed in the bioaugmented microcosms compared to those biostimulated. These increases in total 1,2-DCA degrading populations correspond to a boost in 1,2-DCA degradation in the bioaugmented soil samples. This correlates with the reports of Lendvay *et al*. (2003) who conducted a side-by-side comparison of bioaugmentation and biostimulation; with *Dehalococcoides* and lactate, respectively, in a chloroethene contaminated aquifer. The authors reported that bioaugmentation resulted in a near-stoichiometric dechlorination of both sorbed and dissolved chloroethenes. In addition, the *Dehalococcoides* populations increased by three-to-four orders of magnitude. However, biostimulation with continuous lactate and nutrient injection did result in dechlorination, but only after a prolonged lag period.

The microbial dehydrogenase and urease activity increased in the presence of 1,2-DCA, a readily available carbon source. Dehydrogenase activity plays a central role in the soil environment, primarily in the oxidation of organic matters (Dick *et al*., 1996), therefore it has been considered as an attractive indicator for monitoring various impacts on soil organisms (Maila and Cloete, 2005). In this study, dehydrogenase activity was significantly ($p < 0.05$) inhibited by heavy metals confirming the results from previous studies (Chander and Brookes, 1991; García-Gil *et al*., 2000). The rapid and continuous inhibition in dehydrogenase activity in both the As^{3+} and Cd^{2+} co-contaminated soils throughout the degradation period could be due to the indirect effect of heavy metals, whereby heavy metals alter the microbial community which synthesizes the enzymes (Kandeler *et al*., 2000; Nannipieri, 1994). The decrease in dehydrogenase activity observed in this study may be attributed to the toxic effects of heavy metals on the microorganisms, as evidenced by the decrease in total 1,2-DCA degrading bacterial populations in both heavy metal polluted soils. In addition, it is well documented that the heavy metals react with sulfhydryl groups of enzymes and inhibit and/or inactivate the enzymatic activities. The toxicity of As^{3+} on soil dehydrogenase activity was higher than that of Cd^{2+}, with a corresponding greater inhibitory effect on 1,2-DCA degradation as well as a significant decrease in total 1,2-DCA degrading population. Neither amendment with carbon, nitrogen or phosphorus was able to increase microbial activity to levels obtained prior to co-contamination of the soil. These results suggest that the high soil microbial activity was mainly associated with the presence of the chlorinated hydrocarbon. Shi *et al*. (2005) also stated that the presence of heavy metals may retard enzyme activity in co-contaminated environments, without necessarily preventing aromatic catabolism in soils. Interestingly, the addition of glucose and fructose resulted in a decrease in urease activity below that observed in the untreated co-contaminated soil, whereas the addition of fertilizer resulted in significantly higher increases in urease activity. Urease is a key extracellular enzyme in soil responsible for nitrogen transformation (Xuexia *et al*., 2006). Basically, the enzyme catalyses the hydrolysis of urea to CO_2 and NH_4^+, a significant by-product of the reaction, which can reflect nitrogen availability in soil (Sun *et al*., 2003). Therefore, it is not surprising that addition of fertilizer, which provides a rich source of nitrogen, resulted in a marked

increase in urease activity in both co-contaminated soils. Li *et al*. (2009) recently observed that application of chemical N:P:K fertilizers to As contaminated soil can lead to the release of arsenic from inactive fractions which are bound to mineral matrices to relatively available fractions which may be water soluble or bound to carbonates, iron and manganese oxides. This may explain why rich sources of carbon, nitrogen and phosphorus have a stimulatory effect on microbial activities, leading to the redistribution of arsenic among the fractions (Brouwere *et al*., 2004; Signes-Pastor *et al*., 2007). Harvey *et al*. (2002), Jackson *et al*. (2006) and Rowland *et al*. (2006) also reported that arsenic solubility was positively correlated with organic carbon in soils and sediments, while the leaching of arsenic to soil solution was enhanced by the added carbon sources (Turpeinen *et al*., 1999).

Denaturing gradient gel electrophoresis has proven invaluable for comparative community profiling (Fromin *et al*., 2002; Muyzer *et al*., 1993). Although a number of methodological limitations have been identified, PCR-DGGE is generally accepted to provide a fingerprint of the dominant phylotypes in natural habitats (Jensen *et al*., 1998; Murray *et al*., 1996; Teske *et al*., 1996; Vallaeys *et al*., 1997) and it has been successfully applied to monitor spatial and temporal differences in bacterial communities (Casamayor *et al*., 2002; Giovannoni *et al*., 1990; Muyzer and Smalla, 1998). DGGE produces a unique DNA community fingerprint, where the number, position, and intensity of the bands reflect the number and relative abundance of a particular species in the population, thereby facilitating a comparison of different microbial communities (Casamayor *et al*., 2002; Muyzer and Smalla, 1998). It has been suggested that DGGE in most cases does not reveal diversity unless the community is very simple. Therefore, it should be kept in mind that DGGE profiles only represent the most numerically dominant genospecies, whereas less abundant species are often not represented (Muyzer and deWaal, 1994). The presence of multiple contaminants, including chlorinated organic pollutants and heavy metals, presents extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community (Shi *et al*., 2002), since only microbes that tolerate both heavy metals and toxic levels of hydrocarbons may survive. Therefore, the application of this technique holds great promise in assessing microbial diversity in co-contaminated environments. The relative change in the banding patterns between the As^{3+} and Cd^{2+} treated co-contaminated soil, revealed few differences in the bacterial communities, suggesting the same organisms were able to withstand the toxic effects of these metals as well as utilize 1,2-DCA. Similarly, the changes in DGGE patterns observed in soil contaminated with cis- and trans-dichloroethene also indicated a significant effect of these compounds on indigenous microbial communities and, subsequently, leading to the selection of several microbial populations well-adapted to the contaminants (Olaniran *et al*., 2007). In general, the community structure produced altered PCR-DGGE profiles during the time-course of the degradation process, in which the number of bands was reduced, the intensity of certain bands increased, and new bands appeared. This is indicative of the

selection of microorganisms capable of tolerating the toxic effects of As^{3+} and Cd^{2+} as well as utilizing and/or surviving the toxicity of 1,2-DCA.

These results indicate that the use of dual-bioaugmentation and fertilizer may efficiently speed-up the bioremediation of soil matrices co-contaminated with 1,2-DCA and heavy metals. However, the properties of the contaminated soil should be taken into consideration, as the success of any bioremediation approach is site-specific. The results of this study are encouraging and allow for the identification of some adjustments that could be introduced prior to implementation of such remediation strategies in enhancing the degradation of 1,2-DCA in co-contaminated soils. In particular, the dual-bioaugmentation strategy using strains pre-selected from the co-contaminated soil environment may be used to overcome a critical bottle-neck of the bioremediation technology.

CHAPTER FOUR

EVALUATION OF THE IMPACTS OF DIFFERENT TREATMENT ADDITIVES ON METAL BIOAVAILABILITY AND 1,2-DICHLOROETHANE DEGRADATION IN CO-CONTAMINATED SOIL

4.1   Introduction

The growing awareness and concern regarding the adverse effects induced by elevated levels of arsenic (As) and cadmium (Cd) on soil microorganisms have prompted recent research efforts in evaluating the speciation, mobility and stability of these heavy metals (HM) in soil (Bhattacharyya *et al*., 2008; Kavamura and Esposito, 2010; Száková *et al*., 2009). The total contaminant levels are not necessarily indicative of essentially occurring adverse effects, as the toxic effects are determined not only by their intrinsic toxicity and concentration but also by their physico-chemical forms (Guéguen *et al*., 2004; Sandrin and Maier, 2003). Arsenic and cadmium are potentially hazardous pollutants in the environment and are highly toxic to plants, microorganisms, animals and human beings even at a very low concentration (Flick *et al*., 1971; Vaughan, 2006). Although inorganic arsenic and cadmium in soils are in themselves harmful, immobile heavy metals can be easily transformed into mobile phases as a result of changes in environmental conditions, thereby posing an additional potential environmental hazard (Lee *et al*., 2010; Vig *et al*., 2003). Thus, the toxicity of these heavy metals is related to its oxidation state in the soil environment (Jain and Ali, 2000; Slotnick *et al*., 2006; Vig *et al*., 2003; Zhao and Masaihiko, 2007).

Generally, most analytical measurements deal with the total content of heavy metals or metalloids in an analyzed sample to assess its impact on the environment (Lin *et al*., 2002; Topcuoğlu *et al*., 2002). When considering microorganisms, the chemical form or the oxidation state in which that element is introduced into the environment is crucial. Metals are essentially bioavailable in free ionic and labile forms for microorganisms, whereas particle-bound or ligand-complexed metals are not considered as being directly available for uptake by microorganisms (Campbell, 1995). It has been well documented that the toxicity of these heavy metals are dependent on countless abiotic and biotic factors such as soil pH value, organic matter content, clay minerals and redox conditions (Kavamura and Esposito, 2010; Naidu *et al*., 1997; Vig *et al*., 2003). These complex processes affect heavy metal speciation and distribution, including adsorption onto and desorption from mineral surfaces, incorporation into precipitates, release through the dissolution of minerals, and interactions with microorganisms (Brown Jr *et al*., 1999).

Bioavailability is not deemed a universal attribute; it can be organism and, in some cases, species-specific (Giller *et al*., 1998). Heavy metals that are considered to be bioavailable have the potential to interact with biological vectors. In soil, these vectors may include microorganisms (Langdon *et al*., 2003); thus evaluating uptake of a metal/metalloid (Wolt, 1994), or the impact on growth or activity of the target organism can be used as a sensitive indicator of the effect of pollutants (Giller *et al*., 1998). Microbial parameters appear to be very useful in monitoring soil pollution caused by both chlorinated organic and heavy metal pollutants, since they are more dynamic and often more sensitive than the physical or chemical soil properties (Brookes, 1995). According to Tyler (1974) and Kızılkaya *et al*. (2004), soil

enzymatic activities diminish with increasing concentrations of available heavy metals. Microbial activities (urease and dehydrogenase) and active population densities in such co-contaminated environments appear to provide more sensitive indications of soil pollution by heavy metals than either activity or population measurements alone (Nannipieri *et al*., 1990). It is difficult, however, to select the most sensitive enzymatic response to heavy metals, because the enzyme activity is dependent on the surrounding edaphic microenvironment (Schloter *et al*., 2003).

Due to the complexity of soil-metal interactions and transformations, predicting soil metal bioavailability, mobility and retention is often difficult. To date, there is no generally accepted method of estimating the bioavailability of heavy metals in soil. A single extraction procedure using 1M NH_4NO_3 has been suggested as a simple, cheap and environmentally friendly technique (Meers *et al*., 2007). Moreover, the extracting solution matches the soil solution with respect to pH, concentration and composition. In comparison with divalent exchangeable bases such as Ca, the monovalent cation NH_4 is less competitive for desorption of heavy metals from the soil matrix (Gommy *et al*., 1998). Also, the use of nitrate as a counter ion does not cause additional complexation and mobilization of heavy metals, as is the case for chloride-based extractants (Meers *et al*., 2007). Heavy metal concentrations in soils determined by this extraction procedure give a better indication of bioavailability than total soil contents.

Typical remediation strategies for heavy metal polluted soils have focused on extraction and immobilization of the heavy metals (Montinaro *et al*., 2008). However, given the high risk and cost involved in excavation techniques, the latter process by the action of precipitation, complexation and adsorption are generally preferred as cost a effective technique for treating heavy metal contaminated soils (Lee *et al*., 2004; Montinaro *et al*., 2008). Immobilization procedures are aimed at preventing the migration of heavy metals in the soil environment, either by improving soil physical characteristics, or by limiting the solubility or toxicity of the bioavailable heavy metal (Chen *et al*., 2007; Lombi *et al*., 2004; McGowen *et al*., 2001). The process of immobilizing heavy metals involves mixing contaminated soils with suitable treatment additives which are able to decrease their bioavailability by inducing various sorption processes: formation of stable complexes, promoting ion exchange, adsorption and/or precipitation of the heavy metals (Kumpiene *et al*., 2008). A variety of treatment additives has been investigated to immobilize heavy metals in soils; in particular, application of lime materials, clays, carbonates and phosphates have shown promising results in remediating arsenic and/or cadmium contaminated soils (Brown *et al*., 2005; García-Sanchez *et al*., 2002; Thakur *et al*., 2006; Zhao and Masaihiko, 2007). The most widely used remediation treatment for heavy metal contaminated soils is the application of lime materials. Liming significantly increases the soil pH due to the release of hydroxyl ions by the hydrolysis reaction of calcium carbonate. Consequently, liming can lead to the precipitation of heavy metals as metal-carbonate, resulting in significant decrease in the bioavailable fraction of metals in the contaminated soil (Knox *et al*., 2001).

In view of the above, the aim of this study was therefore to assess the effect of different inorganic treatment additives (calcium carbonate, gypsum and disodium phosphate) on the bioavailability of arsenic and cadmium in soil and the consequent impact on 1,2-DCA degradation in a co-contaminated soil microcosm setting. Moreover, the effects of such treatment additives on selected soil microbial parameters were also investigated.

4.2   Materials and methods

4.2.1   Sample collection and handling

Soil samples were collected and handled as described in Chapter Two; however, only loam soil was used in this section of the study, since the inhibition of 1,2-DCA degradation by the heavy metals was much more pronounced in this soil type.

4.2.2   Experimental design and microcosm set-up

The methodology for experimental design and microcosm set-up is the same as that described in Chapter Two with the following amendments. Microcosms were set-up by artificially co-contaminating loam soil sample with 1,2-DCA and heavy metals. To study the effects of different treatment additives on the bioavailability of arsenic and cadmium, nine treatments were used in total, comprising positive and negative controls, two heavy metals and three treatment additives, with each test conducted in triplicate. Arsenic oxide (As^{3+}) and cadmium chloride (Cd^{2+}) stock solutions were incorporated into the groundwater, prior to the microcosm set-up, to obtain final concentrations of 150 mg/kg and 170 mg/kg, respectively. Each microcosm was amended with either 5 g calcium carbonate ($CaCO_3$) (Ruby *et al*., 1994), 2 g gypsum ($CaSO_4.2H_2O$) (Lombi *et al*., 2004) or a combination of 1.12 g disodium phosphate (Na_2HPO_4) and 0.046g sodium chloride (NaCl) (Ruby *et al*., 1994). Thereafter, the reaction mixtures were manually mixed with a glass rod to allow for even distribution of the treatment additives within the soil. In addition to autoclaving, the biological inhibited controls prepared for each series of reaction mixtures were amended with $HgCl_2$ to achieve a final concentration of 500 mg/kg.

4.2.3   Analytical procedures

The analytical procedures involved in assessing the degradation of 1,2-DCA using gas chromatographic analysis of headspace samples are as described in Chapter Two.

4.2.4   Enumeration of total 1,2-DCA degrading populations

Total 1,2-DCA degrading bacterial populations was determined at different sampling times using a standard spread plate technique (Gerhardt *et al*., 1991), as described in Chapter Three.

4.2.5 Soil analysis

The methodologies used in assessing the physico-chemical properties of the soil sample are described in Chapter Two.

4.2.6 Dehydrogenase and urease activity

The assay procedures used for assessing the microbial dehydrogenase and urease activities in the co-contaminated soil containing the different treatment additives are as described in Chapter Three.

4.2.7 Bioavailability of heavy metals

The bioavailable fraction of heavy metals in the soil microcosms was estimated by extracting 8 g of co-contaminated soil with 1 M NH_4NO_3 (1:2.5 w/v) (Wang *et al*., 2007). The soil suspensions were centrifuged at 4000 rpm for 10 min and filtered through 0.45 μm membrane filters (Whatman). Contents of As and Cd in each filtrate were measured using an inductively coupled plasma-optical emission spectrometer (ICP-OES) 5300 DV and 2100 DV (Perkin-Elmer). Fifteen millilitres of the filtrate were transferred to centrifuge tubes and concentrations of bioavailable heavy metals were estimated by extrapolating from known concentrations of heavy metals. All the extractions were carried out in triplicate, including the analytical blanks which were processed simultaneously with the samples. The specifications of the ICP-OES used for generating calibration curves are indicated in Table 4.1.

Table 4.1: ICP-OES instrument specifications required for generating calibration curves.

Element	Wavelength (nm)	BEC (mg/l)	Detection limit (mg/L)	Signal/Noise (S & T)	Intensity (W)
Arsenic	197.197	2.56	0.0760	-	5.8
Cadmium	226.502	0.11	0.0034	293.1	1000

4.2.8 Statistical analysis

Analysis of the biodegradation results was carried out using student's (paired) *t*-test, 2 tails distribution with significance level of $p < 0.05$.

4.3   Results

4.3.1   Effect of treatment additives on 1,2-DCA biodegradation in co-contaminated soil

The degradation profiles of 1,2-DCA, both in the presence and absence of treatment additives in loam soil co-contaminated with As^{3+} and Cd^{2+}, are illustrated in Figures 4.1a and 4.1b, respectively. 1,2-Dichloroethane was observed to be degraded significantly ($p < 0.05$) by indigenous soil microorganisms with up to 89.78% elimination of 1,2-DCA being achieved in the absence of heavy metals after 20 days. The presence of As^{3+} and Cd^{2+} resulted in a significant ($p < 0.05$) decrease of 9.30% and 5.86% in 1,2-DCA degradation after 20 days, respectively. Addition of treatment additives effectively resulted in an increase in 1,2-DCA degradation with up to 15.84% ($p < 0.05$) and 9.14% in the As^{3+} and Cd^{2+} co-contaminated soil, respectively, within the first 5 days of the incubation period. After 15 days, $CaCO_3$ proved to be the most efficient in the As^{3+} co-contaminated soil, resulting in a significant ($p < 0.05$) increase of 13.52% in 1,2-DCA degradation, compared to Na_2HPO_4 + NaCl and $CaSO_4.2H_2O$ which resulted in a 7.29% and 8.83% increase in 1,2-DCA degradation, respectively (Figure 4.1a). However, in Cd^{2+} co-contaminated soil, similar increases in 1,2-DCA degradation were observed for all treatment additives throughout the degradation period. Calcium carbonate was observed to be more effective in enhancing 1,2-DCA in the As^{3+} co-contaminated soil compared to the Cd^{2+} co-contaminated microcosms, as indicated by an increase of 5.24% in 1,2-DCA degradation after 20 days. The degradation rate constants of 1,2-DCA, as indicated in Table 4.2, ranged variously between 0.081 – 0.167 day^{-1} and 0.091 – 0.152 day^{-1}, in the As^{3+} and Cd^{2+} co-contaminated soil, respectively. The presence of As^{3+} and Cd^{2+} resulted in a decrease of 26.76% and 18.02% in 1,2-DCA degradation rate constant, indicating that the presence of As^{3+} has a greater inhibitory effect on 1,2-DCA degradation. In all microcosms amended with treatment additives, higher degradation rate constants were observed compared to the untreated positive controls (Table 4.2). Moreover, the addition of $CaCO_3$ resulted in an approximately two-fold increase in 1,2-DCA degradation rate constant in both the As^{3+} and Cd^{2+} co-contaminated soil. The effect of all treatment additives were more pronounced in the As^{3+} co-contaminated soil resulting in a 11.19%, 9.25% and 5.63% increase in 1,2-DCA degradation rate constant in the presence of $CaCO_3$, Na_2HPO_4 + NaCl and $CaSO_4.2H_2O$, respectively, compared to the Cd^{2+} co-contaminated soil.
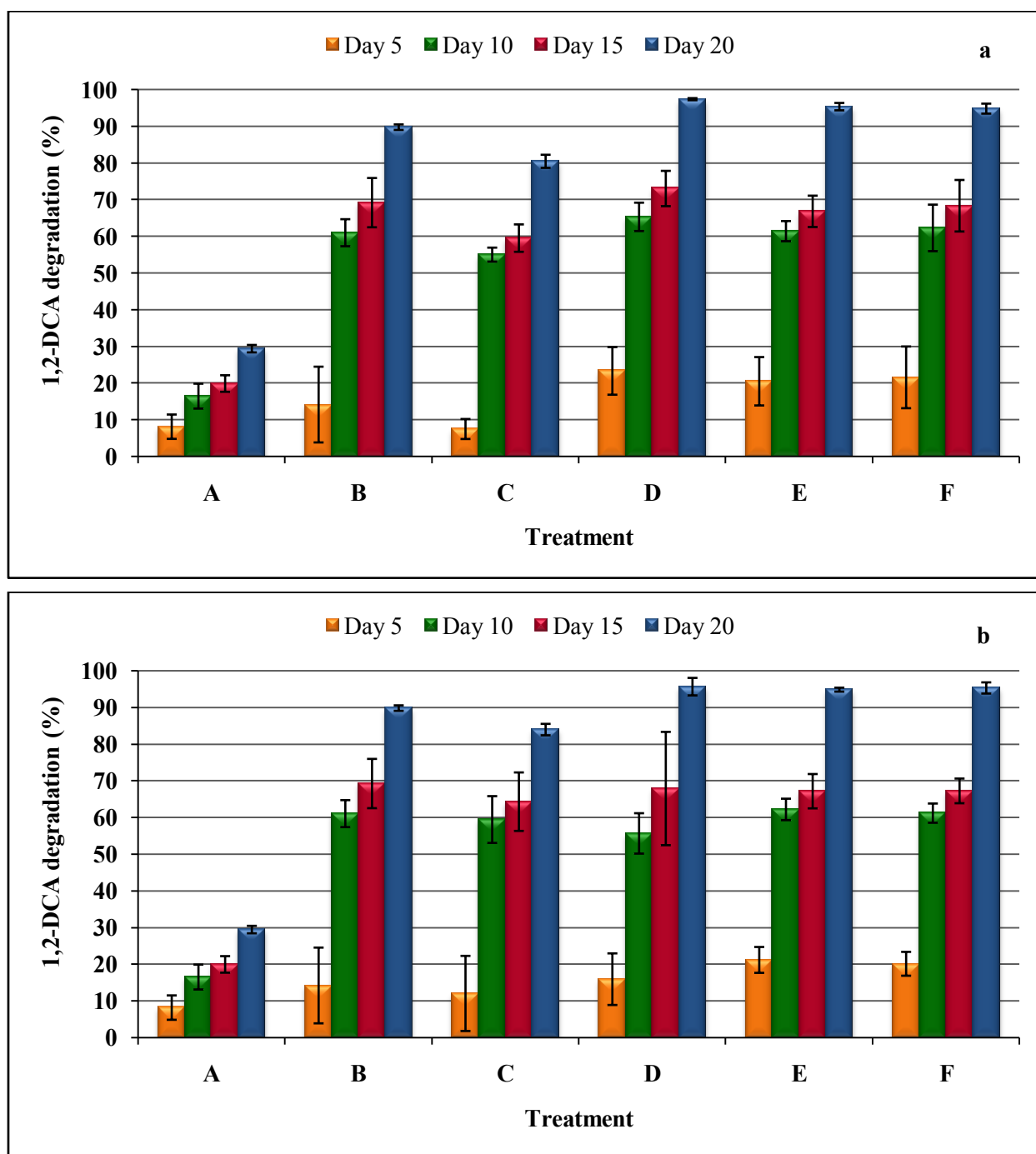
Figure 4.1: Degradation profiles of 1,2-DCA in soil co-contaminated with (a) 150 mg/kg As^{3+} and (b) 170 mg/kg Cd^{2+} in the presence of treatment additives. A = autoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA; C = unautoclaved soil control + 1,2-DCA + HM; D = soil + 1,2-DCA + HM + $CaCO_3$; E = soil + 1,2-DCA + HM + Na_2HPO_4 + NaCl; F = soil + 1,2-DCA + HM + $CaSO_4.2H_2O$. Bars indicate the average of triplicate samples while the error bars show the standard deviation.

Table 4.2: Biodegradation rate constants (day^{-1}) of 1,2-DCA in soil co-contaminated with arsenic (150 mg/kg) and cadmium (170 mg/kg) in the presence of treatment additives.

Treatment	Arsenic	Cadmium
Controls		
Autoclaved soil	0.016 ± 0.001	0.016 ± 0.001
Unautoclaved soil	0.111 ± 0.003	0.111 ± 0.003
Unautoclaved soil + HM	0.081 ± 0.003	0.091 ± 0.003
Treatment additives		
CaCO$_3$	0.167 ± 0.007	0.152 ± 0.004
Na$_2$HPO$_4$ + NaCl	0.141 ± 0.009	0.136 ± 0.006
CaSO$_4$.2H$_2$O	0.138 ± 0.013	0.141 ± 0.014

4.3.2 Total 1,2-DCA bacterial population dynamics in the co-contaminated soil

The total 1,2-DCA degrading bacterial population density in the different microcosm set-ups containing treatment additives is shown in Table 4.3. An alteration in the population of the indigenous microbial community, capable of 1,2-DCA degradation, was observed subsequent to the addition of As^{3+} and Cd^{2+}, resulting in an approximately seventeen-fold and thirteen-fold decrease in the 1,2-DCA bacterial population, respectively after 10 days. The population densities of the total 1,2-DCA degrading bacteria did not seem to follow any regular pattern, although the growth appeared biphasic with an initial decrease in bacterial density in the presence of heavy metals, followed by an increase in treated soils over time, except for the As^{3+} co-contaminated soil amended with Na$_2$HPO$_4$ + NaCl. The bacterial cell density ranged from 3.19 to 34.47 ($\times$ 10^6 cfu/ml) and 2.48 to 11.63 ($\times$ 10^6 cfu/ml) in the treated As^{3+} and Cd^{2+} co-contaminated soil, respectively, after 10 days. The highest 1,2-DCA degrading bacterial cell densities were observed in both Cd and As co-contaminated soil treated with Na$_2$HPO$_4$ + NaCl after 10 days. However, a shift in population densities was observed after 20 days with the peak population observed in the co-contaminated soil treated with CaCO$_3$.

Table 4.3: Total 1,2-DCA degrading bacterial population in heavy metal co-contaminated soil in the presence of different treatment additives.

Treatment	1,2-DCA degrading bacterial population (cfu/ml × 10⁶)	
	Day 10	Day 20
Controls		
Autoclaved soil	0	0
Unautoclaved soil	48.33 ± 7.77	29.07 ± 1.00
Arsenic (As³⁺)		
Unautoclaved soil + As³⁺	2.90 ± 0.34	2.54 ± 0.65
CaCO₃	3.19 ± 0.27	275.67 ± 10.69
Na₂HPO₄ + NaCl	34.47 ± 6.92	33.00 ± 5.66
CaSO₄.2H₂O	9.30 ± 1.11	31.23 ± 2.62
Cadmium (Cd²⁺)		
Unautoclaved soil + Cd²⁺	3.75 ± 0.83	2.85 ± 0.21
CaCO₃	4.43 ± 0.61	46.00 ± 4.58
Na₂HPO₄ + NaCl	11.63 ± 0.80	29.00 ± 1.41
CaSO₄.2H₂O	2.48 ± 0.58	5.37 ± 1.36

Values are averages of triplicate data ± standard deviation.
The initial 1,2-DCA degrading bacterial population was 6.47×10^6 cfu/ml.

4.3.3 Concentrations of soil bioavailable heavy metals

The bioavailable concentrations of As^{3+} and Cd^{2+} in the soil solutions over time are indicated in Tables 4.4 and 4.5, respectively. The concentrations of bioavailable heavy metals varied greatly, with approximately < 2.72% and < 0.62% of the total arsenic and cadmium added to the system being available in NH_4NO_3-extractable fractions. After 5 days, bioavailable Cd^{2+} concentrations were further reduced to undetectable levels in both the $CaCO_3$ and Na_2HPO_4 + NaCl treated soil. In the As^{3+} co-contaminated soil treated with $CaCO_3$ and $CaSO_4.2H_2O$, a 23.91% and 51.028% reduction in the bioavailable fraction was observed after 15 days, respectively. However, treatment with Na_2HPO_4 + NaCl resulted in higher bioavailable fractions compared to the As^{3+} positive control.

Table 4.4:   Concentration of NH_4NO_3-extractable arsenic concentrations (mg/kg) in co-contaminated loam soil amended with treatment additives.

	Day 0	Day 5	Day 10	Day 15	Day 20
Positive control	4.09 ± 0.38	1.71 ± 0.05	1.34 ± 0.05	0.78 ± 0.02	0.77 ± 0.06
$CaCO_3$	4.09 ± 0.38	1.47 ± 0.33	1.23 ± 0.07	0.59 ± 0.04	0.81 ± 0.08
$Na_2HPO_4 +$ NaCl	4.09 ± 0.38	3.36 ± 0.08	2.77 ± 0.23	2.69 ± 0.07	2.14 ± 0.01
$CaSO_4.2H_2O$	4.09 ± 0.38	1.25 ± 0.03	0.97 ± 0.09	0.38 ± 0.02	0.29 ± 0.03

Values are averages of triplicate data ± standard deviation.

Table 4.5:   Concentration of NH_4NO_3-extractable cadmium concentrations (mg/kg) in co-contaminated loam soil amended with treatment additives.

	Day 0	Day 5	Day 10	Day 15	Day 20
Positive control	1.05 ± 0.28	0.25 ± 0.18	0.09 ± 0.05	ND	ND
$CaCO_3$	1.05 ± 0.28	ND	ND	ND	ND
$Na_2HPO_4 +$ NaCl	1.05 ± 0.28	ND	ND	0.02 ± 0.01	ND
$CaSO_4.2H_2O$	1.05 ± 0.28	0.62 ± 0.22	0.01 ± 0.02	ND	ND

Values are averages of triplicate data ± standard deviation; ND-Not detectable.

4.3.4   Combined effects of 1,2-DCA and heavy metals on soil microbial enzyme activities

The activity of soil enzymes was used to estimate the adverse effects of various pollutants on soil quality. Significant variations in urease and dehydrogenase enzyme activities in the co-contaminated soil were observed in this study (Figures 4.2 and 4.3). Dehydrogenase activity was lower in all heavy metal amended samples than those in the control (Figure 4.2). After 20 days, soil dehydrogenase activity was significantly inhibited by 87.26% and 86.33% in the As^{3+} and Cd^{2+} co-contaminated soil, respectively. The combined effect of 1,2-DCA and heavy metals on dehydrogenase activity at the different incubation times was significantly lower than the control sample containing no heavy metals. Overall soil dehydrogenase activities were lower in the heavy metal co-contaminated sample compared to the treated soil, with the exception at day 5, in the Cd^{2+} co-contaminated soil. Moreover, in the As^{3+} co-contaminated soil, an increase in dehydrogenase activity of 67.56%, 76.48% and 45.93% was observed in the presence of $CaCO_3$, Na_2HPO_4 + NaCl and $CaSO_4.2H_2O$ after 20 days, respectively, compared to the untreated co-contaminated soil. The alleviation of the inhibitory effect was more pronounced in As^{3+} co-contaminated soil for both $CaCO_3$ and Na_2HPO_4 + NaCl with up to 7.92% increase in dehydrogenase activity compared to soil co-contaminated with Cd^{2+}. However, the positive effect of $CaSO_4.2H_2O$ in the Cd^{2+} co-contaminated soil resulted in a 6.23% increase in dehydrogenase activity compared to the As^{3+} co-contaminated soil. Soil urease activity was significantly inhibited in the presence of heavy metals, resulting in a 31.37% (Figure 4.3a) and 69.58% (Figure 4.3b) decrease in the presence of As^{3+} and Cd^{2+}, respectively. In the case of treatment additives, a 74.15%, 69.50% and 70.07% increase in urease activity occurred in the Cd^{2+} co-contaminated soil in the presence of $CaCO_3$, Na_2HPO_4 + NaCl and $CaSO_4.2H_2O$ after 15 days, respectively. These values correspond to an increased urease activity of 25.38%, 11.31% and 29.22% compared to As^{3+} co-contaminated soil, over the same period. In addition, a slight decrease in urease activity was observed at day 15 in all additives-treated co-contaminated soil samples.
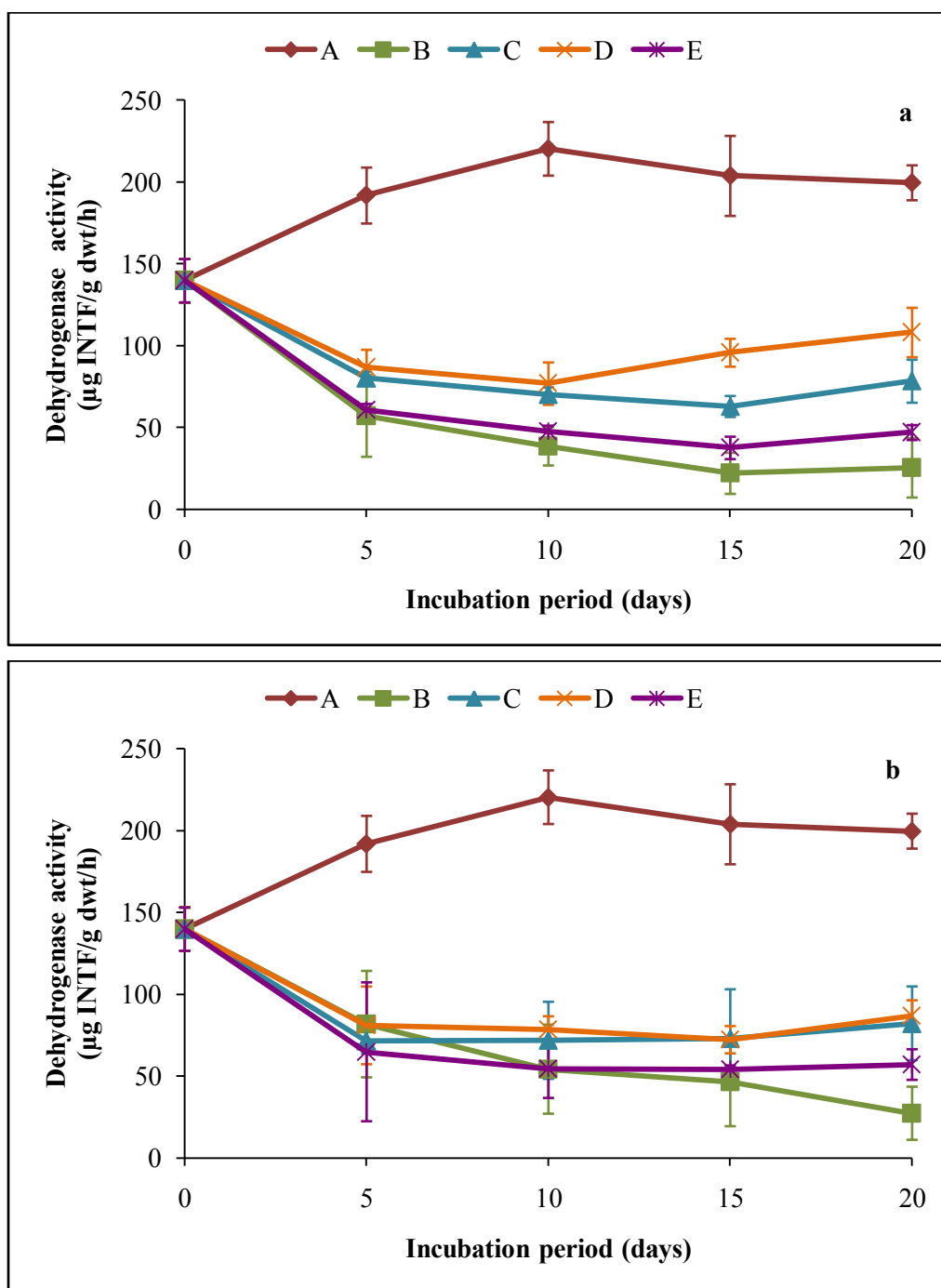
Figure 4.2: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial dehydrogenase activity in microcosms containing treatment additives. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + $CaCO_3$; D = soil + 1,2-DCA + HM + Na_2HPO_4 + NaCl; E = soil + 1,2-DCA + HM + $CaSO_4.2H_2O$. The results are average of triplicate samples while the error bars show the standard deviation.
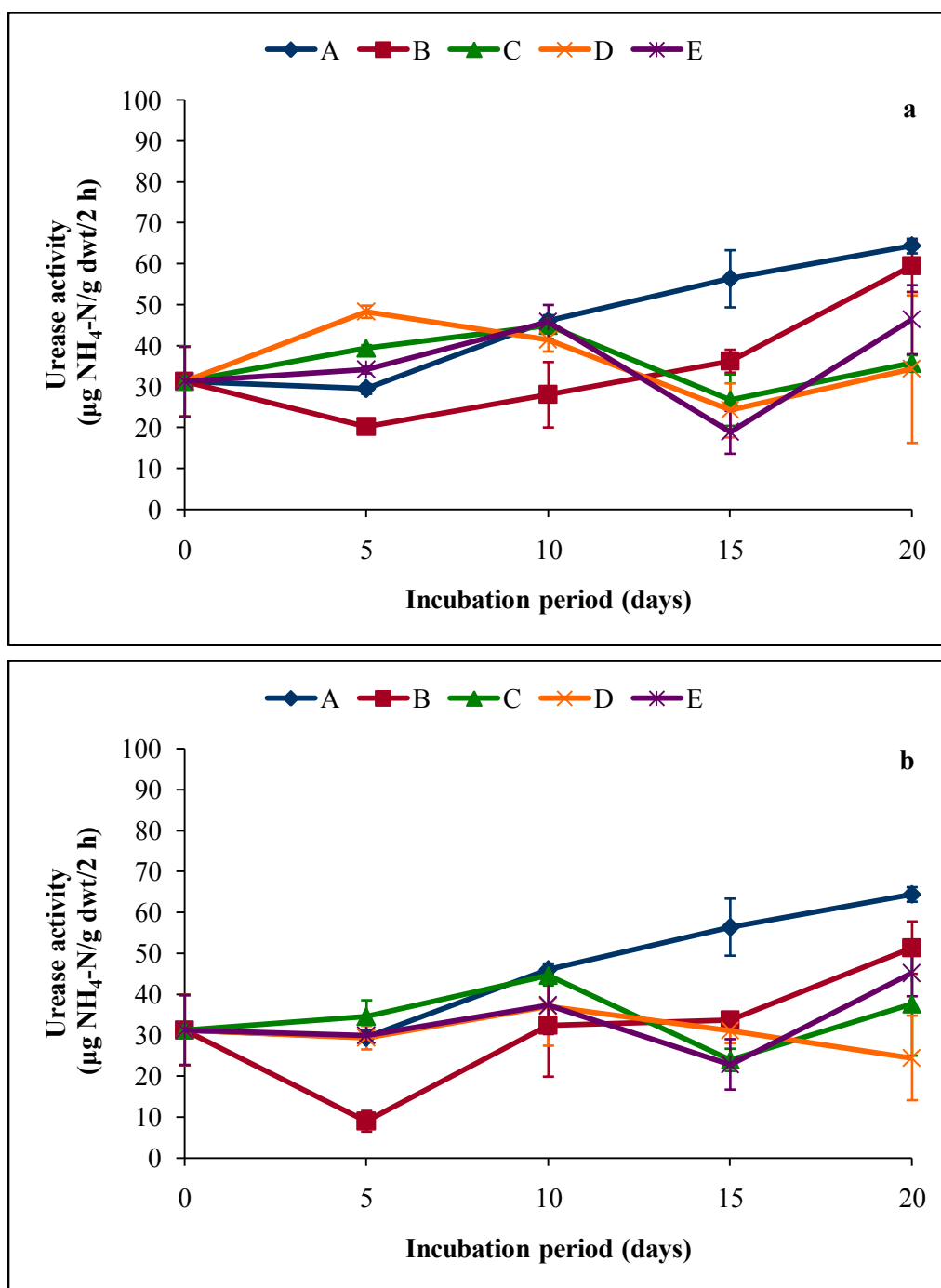
Figure 4.3: Effects of 1,2-DCA and (a) As^{3+}; (b) Cd^{2+} on soil microbial urease activity in microcosms containing treatment additives. A = unautoclaved soil control + 1,2-DCA; B = unautoclaved soil control + 1,2-DCA + HM; C = soil + 1,2-DCA + HM + $CaCO_3$; D = soil + 1,2-DCA + HM + Na_2HPO_4 + NaCl; E = soil + 1,2-DCA + HM + $CaSO_4.2H_2O$. The results are average of triplicate samples while the error bars show the standard deviation.

4.4 Discussion

In the present study, the measured bioavailable concentrations of As^{3+} and Cd^{2+} were significantly lower than the total concentration of the heavy metals added to the system. Metal complexation may be attributed to several physical and chemical properties which affect the sorption of these heavy metals in soil, as they are generally not mobile and strongly adsorbed in soil. This correlates with similar reports, for example, total concentrations of As in the water-extractable fractions of two soil types have been reported to be < 1.2% and < 0.3% of the initial concentration added to the soil (Kavenagh *et al*., 1997). A similar conclusion was drawn by Tye *et al*. (2002) who reported that labile As was between 1.4% and 19% of the total As in soil.

The loam soil used in the construction of the microcosms contained relatively high concentrations of calcium, iron, phosphate, sulphate and soluble organic carbon with a moderate pH value of 6.59. The presence of iron, phosphate, and soluble organic carbon have all been attributed to immobilize free As^{3+} and Cd^{2+} fractions in soils (Hartley *et al*., 2009; Kumpiene *et al*., 2008; Thakur et *al*., 2006; Zhao and Masaihiko 2007). Moreover, the accumulation of both Cd^{2+} and As^{3+} in soils may be distributed among the different soil components, as soil characteristics (pH, adsorption capacity and moisture) affect the mobility, bioavailability and subsequently its toxicity in the complicated soil matrix (deLemos *et al*., 2006; Islam *et al*., 2004; Jiang *et al*., 2005; Song *et al*., 2006; Tang *et al*., 2006). Soil pH generally has the greatest impact on the bioavailability or retention of metals in soil, with a greater retention and lower solubility of metal ions occurring at high soil pH (Marin *et al*., 1993; Martínez and Motto, 2000; Mitchell and Barr, 1995). In general, it has been observed that the adsorption of Cd^{2+} decreases with decreasing pH (Bolan *et al*., 1999; Naidu *et al*., 1994). However, in the case of As^{3+}, the opposite has been observed due to the adsorption effect on iron oxide surfaces (Madejón and Lepp, 2007). A possible reason for arsenic toxicity on indigenous microorganisms is the slightly acidic soil pH, as values in the range of 6.5-7.6 can enhance the risk of As^{3+} mobility (Hartley *et al*., 2009). As shown in this study, the toxic effects of both As^{3+} and Cd^{2+} on indigenous microorganisms involved in the degradation of 1,2-DCA and soil microbial activities were still apparent, albeit at low concentrations.

The application of $CaCO_3$ and $CaSO_4.2H_2O$ to the co-contaminated soil proved to be effective in reducing the bioavailable fractions of As^{3+}, resulting in an increase in 1,2-DCA degradation relative to the untreated control. This result correlates with the findings of several researches suggesting that As immobilization is mainly controlled by the formation of Ca-As precipitates (Dutré and Vandercasteele, 1995; 1998; Dutré *et al*., 1999; Mahuli *et al*., 1997; Vandercasteele *et al*., 2002). These studies have shown that the precipitation of calcium arsenate ($Ca_3(AsO_4)_2$) and calcium hydrogen arsenate ($CaHAsO_3$) controls the immobilization of As in contaminated soils which have been treated with lime among other raw materials. Complexes such as these are precipitated in the presence of Ca under highly oxidizing and moderate pH conditions (Porter *et al*., 2004; Wenzel *et al*., 2001). Hartley *et al*. (2004) observed that lime

reduces As leaching in soil by 8%, and suggested that this phenomenon may be due to the same mechanism of As binding with Ca^{2+} forming As-Ca complexes, thus reducing the mobility and resulting bioavailable fraction.

Treatment with Na_2HPO_4 + NaCl proved to be the most effective in increasing the degradation of 1,2-DCA in the presence of Cd^{2+}, as observed after 5 days. The decrease in the bioavailable concentration of Cd^{2+} in the water soluble fraction (Table 4.4) with a corresponding increase in 1,2-DCA degradation provided indirect evidence for the formation of Cd compounds with low solubility. The possibility of forming $Cd_3(PO_4)_2$ upon the addition of phosphate based compounds to Cd contaminated soils has been proposed (Cotter-Howells and Caporn, 1996; Ma $et\ al.$, 1993). The phosphate-induced decrease of Cd^{2+} in the water soluble fraction infers that the addition of phosphate to the soil resulted in a decrease in the bioavailability of Cd, which in turn progressively intensified the Cd immobilization capacity of the soil (Olaniran $et\ al.$, 2009). Furthermore, it has been observed by Bolan $et\ al.$ (1999) that Cl^- forms a complex with Cd as $CdCl^+$ which reduces the adsorption of Cd by solid phase. Since it is a well-accepted premise that microorganisms derive most of their nutrients from the water soluble fraction, a decrease in bioavailable Cd^{2+} in the water soluble fraction will thus lead to decreased uptake by microorganisms. However, an increase in the bioavailable fraction of Cd^{2+} was observed in soil treated with Na_2HPO_4 + NaCl after 15 days, followed by a decrease by day 20. Investigations have also shown that calcium carbonate and exchangeable Ca^{2+} are important phosphate sorbents in soils (Kuo and Lotse, 1972; Tekchand and Tomar, 1994). These suggest that Cd and phosphate may influence the absorption of each other, either by competing for the Ca^{2+} sorption sites or by influencing the surface charge potential. The high Ca^{2+} ion concentration in the soil may have contributed to the phenomenon of increased bioavailable Cd^{2+} observed in this study. Thakur $et\ al.$ (2006) demonstrated an increase in bioavailable Cd^{2+} with a decreasing Ca:P ratio, indicating that affinity of $CaCO_3$ for surface to Cd^{2+} decreased due to phosphatization. Moreover, phosphate induces variation in soil pH thereby influencing the solubility of Cd^{2+} in soils (Levi-Minzi and Petruzzelli, 1984). However, the effect of phosphate addition on soil pH depends on the buffering capacity of the soil, the extent of phosphate adsorption and the nature of phosphate compounds (Havlin $et\ al.$, 1999).

Lime treatment, which increases soil pH, resulted in a decrease in the bioavailable fraction of Cd^{2+} to undetectable limits after 5 days. Thakur $et\ al.$ (2006) demonstrated that Cd^{2+} is sorbed on $CaCO_3$ by more than one mechanism of action, depending on the concentration of Cd and the presence of phosphate. The high affinity of calcite surface for Cd has been attributed to similarity between the ionic radii of Cd^{2+} and Ca^{2+} (McBride, 1980; Pickering, 1983). At low initial Cd levels, the dominant reaction mechanism is the replacement of Ca^{2+} with Cd^{2+} from accessible surface sites. When all $CaCO_3$ surface sites are covered by chemi-sorbed Cd, nucleation–precipitation of $CdCO_3$ as a surface coating on $CaCO_3$

becomes the dominant mechanism (Thakur *et al.*, 2006). Zhao and Masaihiko (2007) reported that the addition of 2% $CaCO_3$ increased Cd bound to carbonate by 56% as compared to those of the control.

An increase in bioavailable As^{3+} concentrations was observed after treatment with Na_2HPO_4 + NaCl. However, this did not result in decreased degradation of 1,2-DCA. The aerobic conditions maintained in this study may have resulted in the oxidation of As^{3+} to As^{5+} which in turn may have enhanced the sorption and immobilization of As, since the oxidized form As^{5+} adsorbs more strongly to solid phases than As^{3+} (Wang and Mulligan, 2006). Phosphate and arsenate are chemical analogues that demonstrate similar chemical behaviour, competing directly for binding sites within the soil (Davenport and Peryea, 1991; Zhang and Selim, 2008). It has also been demonstrated that phosphate can suppress the adsorption of As^{3+}, while As^{5+} is more strongly affected than As^{3+} (Jain and Loeppert, 2000). In the present study, addition of phosphate as Na_2HPO_4 resulted in a two-fold increase in bioavailable As compared to untreated soil following extraction using NH_4NO_3. It has been reported that As may be mobilized in soils after amendment with phosphorus based chemicals, mainly due to competitive anion exchange (Peryea, 1998; Qafoku *et al.*, 1999). Cao and Ma (2004) reported that addition of phosphate to soils increased soil water-soluble arsenic via replacement of arsenate by phosphate in soil. In some cases, sulphate may also compete with As^{3+} adsorption when the pH is below 7 (Jain and Loeppert, 2000; Meng *et al.*, 2000; Wilkie and Hering, 1996). In addition, 1,2-DCA degrading bacterial populations decreased by 7.80% in As^{3+} co-contaminated soil treated with Na_2HPO_4 + NaCl from day 10 to day 20. A possible explanation is that these oxyanions may have competed with microbial uptake systems (Nies, 1999), in which case the up-take of arsenate may have out-competed that of phosphate.

The untreated co-contaminated soils, with higher bioavailable metal fractions, showed significantly lower enzyme activities and microbial growth, which indicated the inhibitory effect of metals on total 1,2-DCA degrading population and on the microbial urease and dehydrogenase enzymes activities. Upon the addition of treatment amendments, increases in total 1,2-DCA degrading populations were observed on day 10 for all treatments, except for the Cd^{2+} co-contaminated soil treated with $CaSO_4.2H_2O$. Gypsum is known to create air and moisture slots that loosen and break-up the soil structure, thereby creating a greater surface area to which these microorganisms may adhere (Viator *et al.*, 2002). Treatment amended soils resulted in an increase in dehydrogenase enzyme activities. However, urease activity was higher compared to the control containing no treatment amendments within the first 10 days followed by a decrease in activity. An explanation for this trend is that, as the microorganisms degrade organic carbon sources, depletion of inorganic nutrients such as nitrogen may occur (Margesin *et al.*, 2000). Bioavailable heavy metal fractions are generally more toxic than the other forms because they can be easily released into water as ions (Ghosh *et al.*, 2004; Roy *et al.*, 2004). Metal ions react with sulfhydryl (-SH) groups of enzymes, a reaction analogous to the formation of metallic sulfide. This reaction inactivates enzymes since -SH groups serve as integral parts of the catalytically active sites or as

groups involved in maintaining the correct structural relationship of the enzyme protein (Juma and Tabatabai, 1977). Heavy metals can also reduce enzyme activity by interacting with the enzyme-substrate complex, denaturing the enzyme protein, interacting with its active sites (Dick, 1997; Nannipieri, 1994) or by affecting the synthesis of the enzymes within the microbial cells (Vig *et al*., 2003). Moreno *et al*. (2001) reported that the toxicity of Cd on urease and dehydrogenase activities decreased in soil with low pH and high soluble organic carbon, which is apparent in the soil used in this study. The degree of inhibition of urease enzymatic activity with As^{3+} and Cd^{2+} was higher after 5 days than after 20 days of incubation. This indicates a recovery of the extracellular enzyme activities despite heavy metal contamination. The reverse trend was observed with the intracellular dehydrogenase enzymatic activities. In addition, besides being an enzyme inhibitor, heavy metals can have deleterious effects on membrane structure and function by binding to the ligands such as phosphate and the cysteinyl and histidyl groups of proteins (Collins and Stotzky, 1989). The addition of treatment amendments which ultimately lowered the bioavailable fraction of heavy metals increased the activity of soil microorganisms.

In general, amendment of soil with treatment additives increases the residual fraction of heavy metals due probably both to the formation of strong bonds between the metals and the adsorbing surface or metal precipitation in the limed soil (Lombi *et al*., 2002). Among the three amendments, $CaCO_3$ was the most efficient at increasing the degradation of 1,2-DCA in co-contaminated soil. It seems clear that the increased degradation of 1,2-DCA by the indigenous microorganism in the treated soils was related to the decrease of the bioavailable fraction of metals. As shown in this study, bioavailable As^{3+} and Cd^{2+} can reduce the degradation rate of 1,2-DCA, the model organic compound in soil under investigation. Application of treatment additives can be used as a means of reducing bioavailable fractions of these heavy metals, thereby limiting microbial toxicity and ultimately leading to increased degradation of 1,2-DCA in such co-contaminated soil environments. This is important for many industrialized countries as a large proportion of agricultural land is contaminated with both heavy metal and organic compounds (Suhadolc *et al*., 2004). As a consequence, any kind of additive which may lead to increased bioavailability of heavy metals should be avoided, as heavy metals such as As^{3+} and Cd^{2+} interfere with microbial activities. Results from this study suggest that efficacy of treatment additives addition to co-contaminated soil environments for improved organic compound degradation can vary depending on the particular combination of elements present in the soil as well as the range of biological endpoints of concern. In addition, the retention of heavy metals in soils within a given time can be achieved by the addition of sorbing phases and complexing agents. However, the sustainability of maintaining reduced solubility conditions is the key to the long-term success of the treatment (Wang and Mulligan, 2006) in co-contaminated soil.

CHAPTER FIVE

GENERAL DISCUSSION AND CONCLUSION

5.1   The research in perspective

Numerous advanced technologies have emerged in the field of environmental biotechnology to tackle the challenges of cleaning up contaminated sites. Bioremediation is one such emerging technology which takes advantage of the astounding catabolic versatility of microorganisms to degrade and/or detoxify contaminants for alleviation of the noxious effects. *In situ* bioremediation in particular, has shown enormous potential for remediating highly contaminated environments. However, varying degrees of inconsistencies in their application on the field have been reported, raising fundamental questions, viz., (i) how to clarify the biological involvement in the effectiveness of bioremediation and (ii) how to evaluate the environmental impact of bioremediation. In order to address these questions, it is essential to carry out laboratory feasibility tests to determine the effectiveness of bioremediation at a small scale, prior to implementation on a full-scale field application.

Approximately 40% of the hazardous waste sites currently on the National Priorities List sites identified by the U. S. Environmental Protection Agency are co-contaminated with organic and heavy metal pollutants (Cheng, 2003; Fierens *et al*., 2003; Norena-Barroso *et al*., 2004; Sandrin *et al*., 2000; Sandrin and Maier, 2003). Co-contaminated environments are considered difficult to remediate because of the mixed nature of the contaminants and the fact that the two components often must be treated differently (Roane *et al*., 2001). Moreover, all heavy metals are known to be potentially toxic to soil microorganisms at high concentrations and can hinder the biodegradation of organic contaminants (Amor *et al*., 2001; Bååth, 1989; Benka-Coker and Ekundayo, 1998; Riis *et al*., 2002, Roane *et al*., 2001; Sokhn *et al*., 2001; White and Knowles, 2000; 2003). Heavy metals appear to affect organic pollutant biodegradation through interference with the ecophysiology of organic degrading microorganisms, thus imposing a double stress on the microbial populations (Roane *et al*., 2001). Pollutant biodegradation may be inhibited through interaction with enzymes directly involved in biodegradation or those involved in general metabolism, thus rendering the enzyme non-functional (Angel and Chaney, 1989; Sandrin and Maier, 2003). Although broad ranges of heavy metal concentrations have been reported to inhibit biodegradation, the effects of metals on biodegradation processes have not been well-characterized, and different patterns of inhibition have also been reported (Kuo and Genthner, 1996; Roane and Pepper, 1997; Sandrin *et al*., 2000). Also, the effects of metal toxicity on organic pollutant biodegradation in co-contaminated soil environments have not been adequately defined, quantitatively or qualitatively (Sandrin and Maier, 2003). In addition, the presence of multiple contaminants may present extreme challenges to the maintenance of a phylogenetically and functionally diverse microbial community required for the degradation process. The objective of this study, therefore, was to assess the impact of heavy metals on the aerobic biodegradation of 1,2-dichloroethane (1,2-DCA) in soil, on a small scale laboratory test system.

This study incorporated the use of soil microcosms as practical testing systems, to obtain important preliminary information on the biodegradation of 1,2-DCA in heavy metal co-contaminated soil as well as determined the effects of biostimulation, bioaugmentation and treatment additives on the biodegradation process prior to a full-scale field application. Microcosms have previously been used to mimic real world exposures, revealing promising results. Such methods, however, are limited in their direct application to field situations. By using microcosms, soils could be homogenized to evenly distribute both the microbial populations and toxicants, and thereby reduce spatial variability. Prior to implementing *in situ* bioremediation protocols, it is also important to establish the indigenous microbial potential and to assess the limiting factors to be controlled during treatment. This information is crucial, especially in the case of implementing bioremediating strategies such as biostimulation and bioaugmentation. In addition, feasibility tests should reveal that removal of the target compound is due to the effect of biodegradation and that the rate at which this occurs is greater than the natural rate of decontamination (Bento, 2005). In order to establish this, it is important to incorporate and differentiate between non-biological dissipation mechanisms and biodegradation. This involves comparing loss of the compound in sterile treatments with non-sterile treatments. In this study, autoclaved soil amended with mercuric chloride was used to measure abiotic losses of 1,2-DCA. Autoclaving soil samples for sterile controls have been widely used in bioremediation feasibility studies (Carter *et al*., 2007); however, it has been reported to induce changes on the physico-chemical properties of soils. These changes include aggregation of clay particles which leads to a decrease in soil surface area and significant changes in adsorption of organic compounds (Lotrario *et al*., 1995). These factors could account for alterations in solvent extractability or abiotic transformation of 1,2-DCA in the autoclaved soil control.

In this study, 1,2-DCA was readily degraded by indigenous microorganisms in the soil samples tested. It was observed that more degradation occurred in loam soil compared to clay soil over the same period of the degradation process. Thus, it is evident that the soils harboured different microorganisms capable of biodegrading 1,2-DCA at various rates. Moreover, the different physico-chemical properties of the soils such as pH, moisture content and nutrient bioavailability are known to affect the rate of organic biodegradation (Olaniran *et al*., 2009; 2006). In this case, the higher degradation rate of 1,2-DCA in loam soil compared to clay soil could be attributed to the increased levels of inorganic trace elements (e.g., nitrogen, iron and calcium) in loam soil, as increased levels of trace elements have been demonstrated to enhance biodegradation rates in both marine and terrestrial ecosystems considerably (Atlas and Bartha, 1992). The presence of As^{3+}, Cd^{2+}, Hg^{2+} and Pb^{2+} was observed to negatively impact the degradation of 1,2-DCA, with Hg^{2+} having the highest inhibition effect in clay soil and As^{3+} in loam soil. An increase in the half-lives ($t_{1/2}$) of 1,2-DCA was also observed, thus confirming the toxic effects of the heavy metals on indigenous microorganisms involved in 1,2-DCA degradation. Moreover, in clay soil, a dose-dependent

relationship between pseudo-first-order transformation rate coefficient (k_1) and metal ion concentrations in which k_1 decreased with higher initial metal concentrations was observed for all the heavy metals tested except for Hg^{2+}. Of interest were that combinations of metals resulted in non-linear responses, whereby combinations of two heavy metals resulted in a greater negative impact on 1,2-DCA degradation compared to the combinations of three or four heavy metals.

Most reports on the effects of metal toxicity on organic pollutant biodegradation in soil environments have not focused on quantitatively assessing the impact of heavy metals. This is mainly due to related complications which stem from the fact that the physical and chemical state of the metals is affected by environmental conditions (Said and Lewis, 1991). Moreover, the total contaminant levels are not indicative of essentially occurring adverse effects, as the toxic effects are determined not only by their intrinsic toxicity and concentration but also by their physico-chemical forms (Guéguen *et al*., 2004; Sandrin and Maier, 2003). Therefore, when considering inhibition data, it is important to take into account the possible effects of various environmental factors, such as nutrient availability, quality and bioavailability of contaminants, and soil properties. Bioavailable fractions of heavy metals in free ionic and labile forms have the potential to interact with biological vectors such as microorganisms. When measuring bioavailable concentrations of heavy metals, it was noted that this fraction was significantly lower than the total metal concentration of heavy metals added to the system. Several physical and chemical properties affect the adsorption, complexation and distribution of heavy metals among the different soil components. Soil characteristics such as pH, adsorption capacity, moisture content and the presence of certain anions contribute significantly to the mobility, bioavailability and subsequently their toxicity in the complicated soil matrix (deLemos *et al*., 2006; Islam *et al*., 2004; Jiang *et al*., 2005; Song *et al*., 2006; Tang *et al*., 2006). In particular, the presence of iron, phosphate and soluble organic carbon has been attributed to immobilizing free fractions of heavy metals in soils. Generally, soil pH has the greatest impact on the bioavailability or retention of metals in soil, with greater retention and lower solubility of metal ions occurring at high soil pH (Martínez and Motto, 2000; Mitchell and Barr, 1995).

Metals exert their toxic effects on microorganisms by substituting the toxic metal ions for physiologically essential ions within an enzyme, thus rendering the enzyme non-functional (Nies, 1999), and imposition of oxidative stress on microorganisms (Kachur *et al*., 1998). In this regard, the impact of heavy metals on growth or activity of the target organisms can be used as a sensitive indicator of the effects of pollutants (Giller *et al*., 1998). Nannipieri *et al*. (1990) stated that measuring a combination of microbial activities and active population densities provides a more sensitive indication of the effects of heavy metals. However, measuring enzyme activities in soil has a disadvantage as it can be associated with active cells, entirely dead cells, and cell debris which may be complexed with clay minerals and humic colloids (Taylor *et al*., 2002). Activity of many extracellular hydrolases such as urease is probably

a result of enzymes associated with some or all of these components. Ureases are involved in the biogeochemical transformation of nitrogen and are likely to be an essential component of substrate mineralization (Taylor *et al*., 2002). On the other hand, dehydrogenase is present in all microorganisms and provides a more accurate measure of intracellular catalysis. It is considered as an accurate measure of the microbial oxidative activity of the soil and has a direct relationship to total viable microorganisms (Dick, 1997). Measuring both enzyme activities in the co-contaminated soil samples provided a more accurate assessment of the impact of heavy metals on the indigenous soil microorganisms. Interestingly, dehydrogenase enzyme activities were negatively correlated with the presence of heavy metals, confirming the toxicity of heavy metals on indigenous soil microorganisms. Moreover, an accurate and direct correlation between dehydrogenase and total 1,2-DCA degrading populations was observed in the unautoclaved soil control. Since the method for assessing enzyme activity is critical, the buffered method of Kandeler and Gerber (1988) was used in this study. This method measures enzyme activity at the natural pH of the soil, which provides a more realistic indication of the activities likely to occur *in situ*. In the first 5 days of incubation, heavy metals reduced urease activity and thereafter an increase in urease activity was observed. This trend has been attributed to the increased abundance of tolerant microorganisms in co-contaminated environments, due to genetic changes and physiological adaptations involving no alterations in the genotype (Bruins *et al*., 2000; Shen *et al*., 2006). Another interesting observation was the relatively low urease activity in the presence of glucose and fructose, which can be attributed to the depletion/decrease in nitrogen sources. It has been reported that addition of large quantities of organic carbon sources may deplete macronutrients such as nitrogen (Margesin *et al*., 2000). Overall, soil urease activity has been shown to be more sensitive to pollution than that of other soil enzymes (Shen *et al*. 2006). In this study, urease and dehydrogenase activity proved to be sensitive indicators of soil pollution in the co-contaminated environment, with concomitant lower net degradation rates, in the microcosms.

The bioaugmentation strategy employed in this study was effective in enhancing the biodegradation of 1,2-DCA in the co-contaminated soil. In order to overcome some of the problems or limitations which relate to the survival of selected strains, an alternative generic bioremediation approach was adopted, namely dual-bioaugmentation. This technique was based on strengthening a portion of the native microbial community, which in turn inferred multiple resistance to heavy metals, thereby indirectly protecting metal sensitive 1,2-DCA degrading strains from metal toxicity. This is supported by increased proliferation of total 1,2-DCA degrading populations in the dual-bioaugmented microcosms. In particular, the finding in this study correlates well with that of Roane *et al*. (2001) who reported that dual-bioaugmentation, involving inoculation with both metal-detoxifying (*Ralstonia eutropha* JMP134) and organic-degrading (*Pseudomonas* H1) bacteria, facilitates the degradation of 2,4-dichlorophenoxyacetic

acid in the presence of cadmium co-contamination. Alisi *et al*. (2009) reported that bioaugmentation with a microbial formula tailored with selected native strains, in the presence of heavy metals, resulted in an overall reduction of about 75% of the total diesel hydrocarbons in co-contaminated soil. However, monitoring the survival of the heavy metal-resistant strain in the co-contaminated soil will provide a more promising indication of the effectiveness of such an approach.

When considering biostimulation as an option to ameliorate contaminated environments, it is necessary to first determine the physico-chemical parameters of the target compound. In the As^{3+} and Cd^{2+} co-contaminated soil, biostimulation with fertilizer proved most effective in enhancing the degradation of 1,2-DCA. Contradictory opinions about the effect of nutrients on biodegradation of chlorinated organic pollutants have been documented, in particular, conflicting results regarding the impact of nitrogen application. Results showed that nitrogen addition either enhanced microbial activity thus accelerating degradation, or altered enzymatic systems responsible for the degradation (Demoling *et al*., 2007; Ruppel *et al*., 2007). However, in co-contaminated soils, addition of nutrients which are aimed at accelerating the breakdown of the target compound may have a stimulatory effect on microbial activities, leading to redistribution and in most cases leaching of the heavy metal to the soil solution (Browere *et al*., 2004; Signes-Pastor *et al*., 2007). Alternatively, phosphates which are used mainly as a treatment additive may have a two-fold effect by reducing bioavailable concentrations, as well as providing a low source of phosphorus (Liebeg and Cutright, 1999). Moreover, in co-contaminated soil, the presence and fate of nutrients is the key factor determining the overall success of the biostimulation regime. In this regard, amendment with slow-release fertilizers, such as Inipol EAP-22 (Lessard *et al*., 1995) and inorganic fertilizers may be a suitable alternative. Slow-release inorganic fertilizers provide a sustained release of nutrients while being slowly dissolved or degraded by continual or intermittent contact with water (Xu *et al*., 2005).

In general, amendment of soil with treatment additives increased the residual fraction of heavy metals, due probably both to the formation of strong bonds between the metals and the adsorbing surface or metal precipitation in the limed soil (Lombi *et al*., 2002). Among the three amendments, $CaCO_3$ was the most efficient at increasing the degradation of 1,2-DCA in co-contaminated soil. It seems clear that the increased degradation of 1,2-DCA by the indigenous microorganism in the treated soils was related to the decrease in the bioavailable fraction of metals. It was also observed that some treatment additives resulted in increased levels of bioavailable fractions of heavy metals. However, this did not result in an increase in 1,2-DCA degradation. Due to the dynamic nature of metals to interact with various soil components and macronutrients, it is important to consider these factors prior to implementing such strategies to bioremediate co-contaminated soils.

DGGE profiles of PCR-amplified 16S rDNA region revealed close similarities in the banding patterns between the As^{3+} and Cd^{2+} treated co-contaminated soil, suggesting that the same organisms were able to withstand the toxic effects of these metals as well as utilize 1,2-DCA. In the co-contaminated microcosms amended with fertilizer, numerous lighter bands were observed on the gel profile. It could therefore be suggested that the addition of fertilizer led to the enrichment of a small number of genospecies. These observations corroborate with previous studies on community structures in fertile soils which harbour an equitable distribution of a large number of phylotypes which produces a DGGE profile with diffuse staining and few distinct bands (Nakatsu *et al*., 2000).

In co-contaminated soil environments, microbial communities are undoubtedly the key driving force behind the biological treatment of pollutants. As indicated in this study, heavy metals reduced the activity of indigenous microorganisms, thus decreasing the degradation rate of 1,2-DCA. The study emphasizes the toxic effects of heavy metals by quantitatively assessing the possible interference of the metallic species on 1,2-DCA degradation. Application of dual-bioaugmentation strategy and biostimulation with fertilizer was most efficient at enhancing the degradation of 1,2-DCA. Treatment additives, in particular $CaCO_3$ were also effective, and can be used as a means of reducing bioavailable fractions of heavy metals, thereby limiting microbial toxicity and ultimately leading to increased degradation of 1,2-DCA in soil co-contaminated with heavy metals. However, a more thorough understanding of the extent and mechanisms by which metals inhibit 1,2-DCA degradation is needed. These results are encouraging and allow for the identification of some alterations that could be introduced to overcome a critical bottle neck of the bioremediation technology. In this regard, the bioremediation strategies adopted in this study may be used as a rational methodology for remediation of sites co-contaminated with 1,2-DCA and heavy metals, subject to a thorough understanding of the site's ecology and of the local physico-chemical constraints. This is important for many industrialized nations, as a large proportion of agricultural land is co-contaminated with heavy metals and organic pollutants.

5.2   Potential for future development of the study

Firstly, the difficulties inherent in studying the effect of heavy metals on chlorinated organic pollutants may be associated with the methodological choices for devising protocols and the methods used for data treatment (Ren *et al*., 2004). Since application of bioremediation strategies is time consuming, it is essential to design experiments which can cut experimental time and improve the efficiency of experiments. In this respect, experimental uniform design can provide an important contribution to research development in this area (Shen *et al*., 2006). Basically, this design is aimed at distributing the experimental points evenly in the factor space so as to have fewer trials and with each point having full representation. This design has been applied successfully in many other facets of

research (Cheng *et al*., 2002; Liang *et al*., 2001), and has brought about results closer to the facts of combined pollution of organic and heavy metals in real-world exposures (Shen *et al*., 2006). Case studies using the approach employed in this study may be used to large scale treatment of 1,2-DCA co-contaminated soil. Also, it is imperative that feasibility studies incorporate various environmental samples prior to implementing bioremediation strategies *in situ*. Since this study focused primarily on co-contaminated soil microcosms, more research incorporating water microcosm set-up should be conducted. In addition, the effects of co-contaminated soil conditions, such as moisture content and pH, should be investigated as these properties play significant roles on metal bioavailability.

In addition to DGGE, various other culture-independent techniques such as single strand conformation polymorphism (SSCP) and terminal restriction fragment length polymorphism (T-RFLP), along with numerous other techniques, have provided a wealth of information regarding the dominant microbial species as well as detection of specific microorganisms involved in the biodegradation of organic pollutants. Some of these molecular fingerprinting methods were observed to be too sensitive, giving high resolution to provide reliable and robust genotypic characterization at the community level (Torsvik *et al*., 1998) in single pollutant contaminated sites. Therefore, approaches such as these hold great promise for assessing microbial diversity in co-contaminated environments. Combination of different methods that complement each other is a useful strategy for monitoring changes in microbial communities and ecosystems, and should definitely be considered when assessing microbial diversity in co-contaminated environments, such as in the present study. In addition, catabolic gene-specific primers may be used to confirm the direct roles of organisms in 1,2-DCA degradation, thus providing a possible link between phylogeny and function. High-throughput approaches are also making in-roads for characterization of microbial communities in co-contaminated sites. Metagenomics is one such technology which has the potential to provide direct access to the entire pool of environmental genomes, leading to the construction of metagenomic libraries. Such techniques are expected to boost the discovery of new catabolic activities, and provide valuable information for the management and sustainable clean-up of co-contaminated sites.

APPENDIX A

COMPOSITION OF MEDIA AND REAGENTS

Table 1:   Composition of synthetic groundwater per litre of deionized water (pH 7.8).

Reagent	Quantity (mg)
$MgCl_2. 6H_2O$	304.95
KCl	8.95
NH_4NO_3	2.40
$CaCl_2$	110.99
$Ca(OH)_2$	111.15
$NaHCO_3$	714.09

Adjust the pH to 7.8 using NaOH or HCl

Table 2:   Composition of minimal salts medium (MMZ) per litre of deionized water (pH 7).

Reagent	Quantity
KH_2PO_4	1.36 g
$Na_2HPO_4.12H_2O$	5.37 g
$(NH_4)_2SO_4$	0.50 g
$MgSO_4.7H_2O$	0.20 g
Trace element solution	5.00 ml
Bacteriological agar	12.00 g

Table 3:   Composition of trace element solution per litre of deionized water.

Reagent	Quantity (mg)
$CaCl_2$	530
$FeSO_4.7H_2O$	200
$ZnSO_4.7H_2O$	10
H_3BO_3	10
$CoCl_2.6H_2O$	10
$MnSO_4.5H_2O$	4
$Na_2MoO_4.2H_2O$	3
$NiCl_2.6H_2O$	2

Filter sterilized solution using 0.2 μm filter

Table 4:   Chemical composition of fertilizer (Chemicult).

Element	Quantity
N	146 g/kg
P	43 g/kg
K	274 g/kg
Mg	29 g/kg
B	240 g/kg
Fe	1800 mg/kg
Cu	20 mg/kg
Zn	50 mg/kg
Mo	10 mg/kg
Mu	240 mg/kg

Table 5:   Preparation of heavy metal stock solutions per litre of deionized water.

Heavy metal salt	Quantity (mg)
Arsenic oxide (As_2O_3) – 150 mg/kg	198.05
Cadmium chloride ($CdCl_2$) – 170 mg/kg	277.20
Mercuric chloride ($HgCl_2$) – 500 mg/kg	676.80

Table 6:   Percentage moisture in clay and loam soil samples.

	Clay	Loam
Weight prior to drying (g)	150	150
Weight after drying (g)	120.86	134.86
	134.27	124.87
	118.17	126.35
Average	124.43	128.70
Moisture content (%)	**17.05**	**14.20**

- *Urea solution (0.48%) (prepare fresh)*
Urea (Saarchem)	2.4	g
Distilled water (bring up)	500	ml

- *Potassium chloride solution*
Potassium chloride (KCl) (Saarchem)	74.6	g
1 M Hydrochloric acid (32% HCl = 10 M) (Merck)	10	ml
Distilled water (bring up)	1000	ml

- *Sodium hydroxide solution (0.3 M)*
Sodium hydroxide (NaOH) (Saarchem)	12	g
Distilled water (bring up)	1000	ml

- *Sodium salicylate solution*
Sodium salicylate (Na-salicylate) (Fluka)	17	g
Sodium nitroprusside (Merck)	0.12	g
Distilled water (bring up)	100	ml

- *Sodium salicylate/Sodium hydroxide solution (prepare fresh)*
Sodium hydroxide stock solution	100	ml
Sodium salicylate stock solution	100	ml
Distilled water	100	ml

- *Sodium dichloroisocyanide solution (0.1%) (prepare fresh)*
Sodium dichloroisocyanide (Merck)	0.1	g
Distilled water	100	ml

- *Borate buffer (pH 10)*
Disodium tetraborate	56.85	g
Distilled water (warm)	1500	ml
pH adjustment - after cooling (20% sodium hydroxide)	pH 10	
Distilled water (bring up)	2000	ml

- *Ammonium standard solution*

  Solution I (1000 μg NH$_4$-N/ml)
Ammonium chloride (NH$_4$Cl) (Saarchem)	3.82	g
Distilled water (bring up)	1000	ml

  Solution II
Solution I stock solution (varying concentrations)	0.01 - 0.5 ml	
Potassium chloride solution (bring up)	100	ml

- *2-(p-iodophenyl)-3(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT)*
INT (Sigma)	0.03	g
N,N-dimethylformamide (*N,N*-DMF) (Merck, Germany)	100	μl
Distilled water (bring up)	50	ml
Sonicate with gentle heating		

- *0.5 M Disodium ethylenediaminetetraacetate (EDTA)*

EDTA (Saarchem)	186.12	g
Double distilled water (bring up)	1000	ml
pH adjustment (sodium hydroxide pellets ~20 g)	pH 8	

- *50 × Tris-acetate EDTA buffer (TAE)*

Tris base	242	g
Glacial acetic acid (Merck)	57.1	ml
0.5 M EDTA (pH 8)	100	ml
Double distilled water (bring up)	1000	ml
pH adjustment (sodium hydroxide pellets/glacial acetic acid)	pH 8	

- *Phosphate buffered saline (PBS)*

Sodium chloride (KCl) (Saarchem)	8	g
Potassium chloride (KCl) (Saarchem)	0.20	g
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) (Saarchem)	3.58	g
Potassium dihydrogen phosphate (KH_2PO_4) (Saarchem)	0.24	g
Double distilled water (bring up)	1000	ml
pH adjustment (hydrochloric acid)	pH 7.4	

- *Sodium chloride solution (0.85%)*

Sodium chloride (NaCl) (Saarchem)	8.5	g
Distilled water (bring up)	1000	ml

- *Ethidium bromide stain (EtBr)*

Ethidium bromide (Sigma)	50	µl
Double distilled water	500	ml

- *Primer stocks (16S rDNA region) (Inqaba Biotec)*

Double distilled water added to 63F primer	408.73	µl
Double distilled water added to 1387R primer	250.81	µl
Final concentration	100	µM

- *Primer stocks (V3 – V5 region) (Inqaba Biotec)*

Double distilled water added to 341F-GC primer	280.36	µl
Double distilled water added to 907R primer	343.99	µl
Final concentration	100	µM

- *Denaturing solution (0%)*

40% Acrylamide/bisacrylamide (BioRad)	15	ml
50 × TAE buffer (pH 8) (BioRad)	2	ml
Double distilled water	83	ml

- *Denaturing solution (100%)*

40% Acrylamide/bisacrylamide	15	ml
50 × TAE buffer (pH 8)	2	ml
40% (v/v) Deionized formamide (BioRad)	40	ml
7 M Urea (BioRad)	42	g
Double distilled water (bring up)	100	ml

- *Ammonium persulphate (APS)*

APS (10%) (Promega)	0.05	g
Double distilled water	500	µl

APPENDIX B

STANDARD CURVES AND NUMERICAL DATA

Table 1: Gas chromatographic peak area values used for construction of 1,2-DCA standard curve in clay soil.

1,2-DCA (µl)	Sample 1	Sample 2	Sample 3	Average	Standard deviation
5	5 188	8 257	9 096	7 514	2 057
10	17 149	18 711	18 522	18 127	853
15	25 917	16 037	24 774	22 243	5 405
20	31 887	28 945	32 833	31 222	2 028
25	29 043	37 525	35 397	33 988	4 413
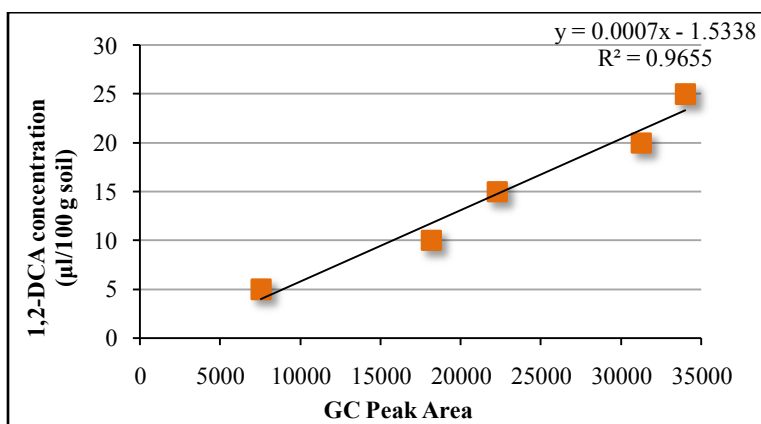
Figure 1: Standard curve for determination of 1,2-DCA concentration in clay soil.

Table 2: Gas chromatographic peak area values used for construction of 1,2-DCA standard curve in loam soil.

1,2-DCA (µl)	Sample 1	Sample 2	Sample 3	Average	Standard deviation
5	4 072	3 910	3 907	3 963	94
10	7 072	8 408	7 534	7 671	679
15	10 097	11 192	10 361	10 550	571
20	14 242	14 202	14 222	14 222	20
25	16 657	17 965	17 474	17 365	661
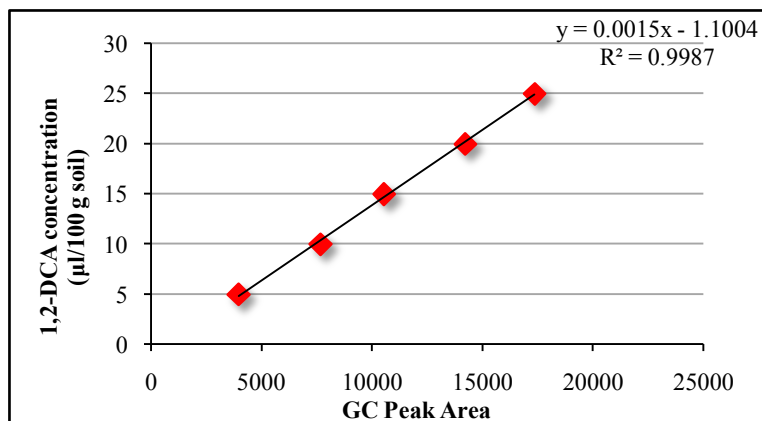
Figure 2: Standard curve for determination of 1,2-DCA concentration in loam soil.

Table 3: Gas chromatographic peak area values used for construction of 1,2-DCA standard curve in loam soil undergoing bioremediation treatments.

1,2-DCA (µl)	Sample 1	Sample 2	Sample 3	Average	Standard deviation
5	4270	4092	3963	4108	154
10	8487	8907	9700	9031	616
15	14041	13273	12637	13317	703
20	16959	18476	19442	18292	1252
25	22993	22730	20910	22211	1134

$y = 0.001x + 0.292$
$R^2 = 0.998$
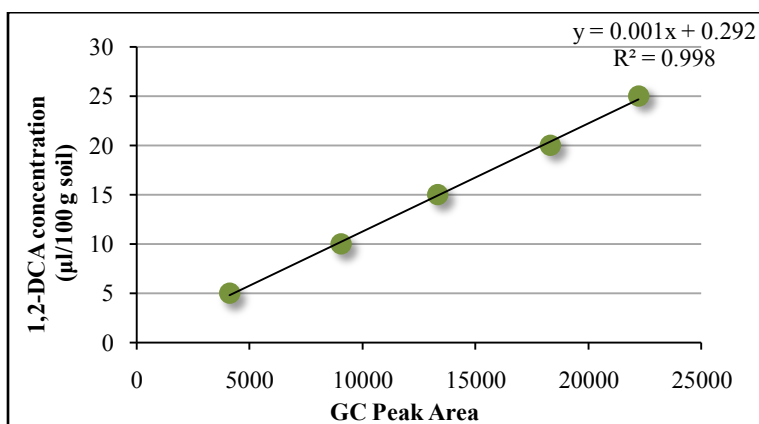
Figure 3: Standard curve for determination of 1,2-DCA concentration in loam soil undergoing bioremediation treatments.

Table 4: Optical density values used for dehydrogenase activity standard curve.

INTF (µg per test)	Sample 1	Sample 2	Sample 3	Average	Standard deviation
10	0.043	0.032	0.036	0.037	0.006
20	0.076	0.084	0.092	0.084	0.008
50	0.199	0.196	0.208	0.201	0.006
70	0.266	0.272	0.296	0.278	0.016
100	0.303	0.387	0.394	0.361	0.051
150	0.563	0.539	0.541	0.548	0.013
200	0.756	0.726	0.722	0.735	0.019
250	0.927	0.898	0.905	0.910	0.015
300	1.067	1.058	1.051	1.059	0.008

$y = 0.003x + 0.016$
$R^2 = 0.999$
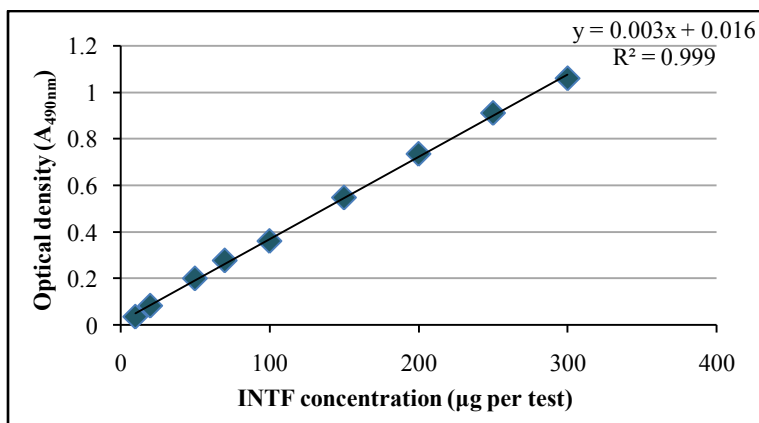
Figure 4: Standard curve for determining INTF concentration in the dehydrogenase activity test.

Table 5:   Optical density values used for urease activity standard curve.

NH$_4$ (µg NH$_4$-N/ml)	Sample 1	Sample 2	Sample 3	Average	Standard deviation
0.1	0.069	0.071	0.079	0.073	0.005
0.2	0.121	0.153	0.114	0.129	0.021
0.3	0.178	0.183	0.196	0.186	0.009
0.4	0.220	0.246	0.262	0.243	0.021
0.5	0.285	0.294	0.286	0.288	0.005
1.0	0.564	0.576	0.593	0.578	0.015
1.5	0.876	0.886	0.907	0.890	0.016
2.5	1.334	1.458	1.401	1.398	0.062
3.5	1.978	1.986	1.972	1.979	0.007
4.5	2.532	2.549	2.539	2.540	0.009
5	2.772	2.848	2.845	2.822	0.043
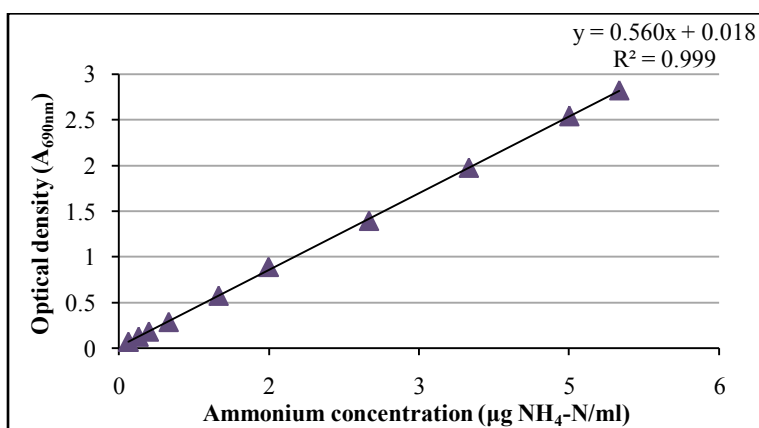
Figure 5:   Standard curve for determining ammonium concentration in the urease activity test.

Table 6: Gas chromatographic peak area values used for clay soil samples co-contaminated with different concentrations of heavy metals.

Microcosm		Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Autoclaved soil control		19 103	16 278	15 704	14 906	14 106	13 583	13 411
		21 244	19 859	17 553	15 856	14 956	14 617	13770
		-	-	-	-	-	-	-
Unautoclaved soil control		19 307	12 230	9 780	6 061	5 475	4 388	3 938
		22 339	15 046	11 803	6 999	6 496	5 054	4 233
		-	-	-	-	-	-	-
$[As^{3+}]$ mg/kg	75	18 283	12 847	11 571	6 813	6 525	4 651	3 770
		21 305	14 918	12 192	5 017	7 292	6 032	4 642
		21 771	15 557	12 635	8 599	8 409	6 576	5 403
	112.5	20 475	14 867	12 873	8 979	8 738	7 027	5 686
		22 805	16 161	13 576	8 869	9 228	7 299	5 379
		22 122	13 185	9 374	6 909	5 763	4 221	2 385
	150	17 531	14 411	12 265	8 612	8 377	6 883	5 714
		21 129	16 602	14 448	10 857	10 454	8 866	7 120
		20 682	13 823	10 878	6 676	6 613	4 929	4 485
$[Cd^{2+}]$ mg/kg	85	18 849	13 820	11 787	8 149	7 794	5 997	4 858
		19 648	12 216	9 716	6 035	5 802	4 399	3 649
		18 314	12 812	11 236	6 450	6 628	5 051	3 897
	127.5	21 111	13 112	11 705	7 749	6 808	5 526	4 662
		21 115	12 653	10 922	8 872	7 084	5 559	4 676
		18 266	10 886	9 656	6 242	5 396	4 148	3 227
	170	20 049	12 538	10 869	8 642	8 406	6 767	6 012
		19 937	11 927	9 476	6 731	6 386	5 080	4 429
		17 494	14 289	11 657	8 159	7 320	5 963	4 980
$[Hg^{2+}]$ mg/kg	840	20 795	15 314	13 940	11 087	10 850	7 502	6 390
		18 905	15 265	13 144	11 269	10 587	7 983	6 277
		18 073	14 324	12 243	8 356	10 015	6 148	4 905
	1260	19 987	12 514	10 638	7 098	8 781	5 465	4 445
		17 999	15 335	14 315	10 680	9 137	6 097	4 846
		18 415	12 432	12 317	8 149	9 897	6 374	5 480
	1680	18 313	15 842	13 499	12 041	11 176	8 524	7 538
		18 331	17 265	15 591	12 254	10 647	6 476	5 069
		19 139	15 226	11 212	8 521	7 983	6 539	5 734
$[Pb^{2+}]$ mg/kg	420	19 074	10 671	9 528	7 146	6 730	4 016	3 426
		18 496	11 497	9 021	5 521	6 079	3 897	3 266
		19 749	11 663	10 870	6 986	6 472	5 044	4 411
	630	19 044	10 317	10 411	7 214	7 069	6 036	5 048
		19 938	13 496	10 678	7 944	7 234	4 955	4 131
		19 299	11 281	8 937	5 193	4 733	3 971	3 134
	840	19 887	12 392	10 077	6 097	7 209	4 717	3 910
		18 533	12 039	9 811	7 943	6 908	4 682	3 836
		18 807	12 736	11 115	8 204	7 583	5 393	4 462

Autoclaved soil control

◆ Sample 1 ■ Sample 2

y = -0.012x + 2.398
R² = 0.911

y = -0.017x + 2.567
R² = 0.961

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

Unautoclaved soil control

◆ Sample 1 ■ Sample 2

y = -0.076x + 2.372
R² = 0.979

y = -0.076x + 2.589
R² = 0.983

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

Arsenic (75 mg/kg)

◆ Sample 1 ■ Sample 2 ▲ Sample 3

y = -0.076x + 2.463
R² = 0.976

y = -0.065x + 2.447
R² = 0.809

y = -0.058x + 2.553
R² = 0.981

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

Arsenic (112.5 mg/kg)

◆ Sample 1 ■ Sample 2 ▲ Sample 3

y = -0.052x + 2.496
R² = 0.981

y = -0.057x + 2.615
R² = 0.965

y = -0.086x + 2.545
R² = 0.990

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

Arsenic (150 mg/kg)

◆ Sample 1 ■ Sample 2 ▲ Sample 3

y = -0.048x + 2.377
R² = 0.982

y = -0.042x + 2.551
R² = 0.983

y = -0.070x + 2.461
R² = 0.970

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

Cadmium (85 mg/kg)

◆ Sample 1 ■ Sample 2 ▲ Sample 3

y = -0.059x + 2.436
R² = 0.985

y = -0.080x + 2.413
R² = 0.982

y = -0.072x + 2.418
R² = 0.967

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

172

Cadmium (127.5 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.065x + 2.477
R² = 0.983

y = -0.063x + 2.467
R² = 0.985

y = -0.086x + 2.394
R² = 0.983

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Cadmium (170 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.047x + 2.339
R² = 0.951

y = -0.065x + 2.335
R² = 0.970

y = -0.057x + 2.397
R² = 0.992

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Mercury (840 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.047x + 2.549
R² = 0.965

y = -0.043x + 2.481
R² = 0.967

y = -0.055x + 2.452
R² = 0.922

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Mercury (1260 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.062x + 2.411
R² = 0.923

y = -0.059x + 2.571
R² = 0.945

y = -0.048x + 2.359
R² = 0.906

Ln 1,2-DC (µl/100g clay soil)
Time (days)

Mercury (1680 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.036x + 2.448
R² = 0.979

y = -0.058x + 2.665
R² = 0.906

y = -0.052x + 2.419
R² = 0.982

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Lead (420 mg/kg)
Sample 1 Sample 2 Sample 3

y = -0.081x + 2.419
R² = 0.953

y = -0.086x + 2.385
R² = 0.963

y = -0.066x + 2.375
R² = 0.973

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

173

Figure 6: Graphical representation of the degradation rate constants of 1,2-DCA in clay soil co-contaminated with different concentrations of heavy metals.
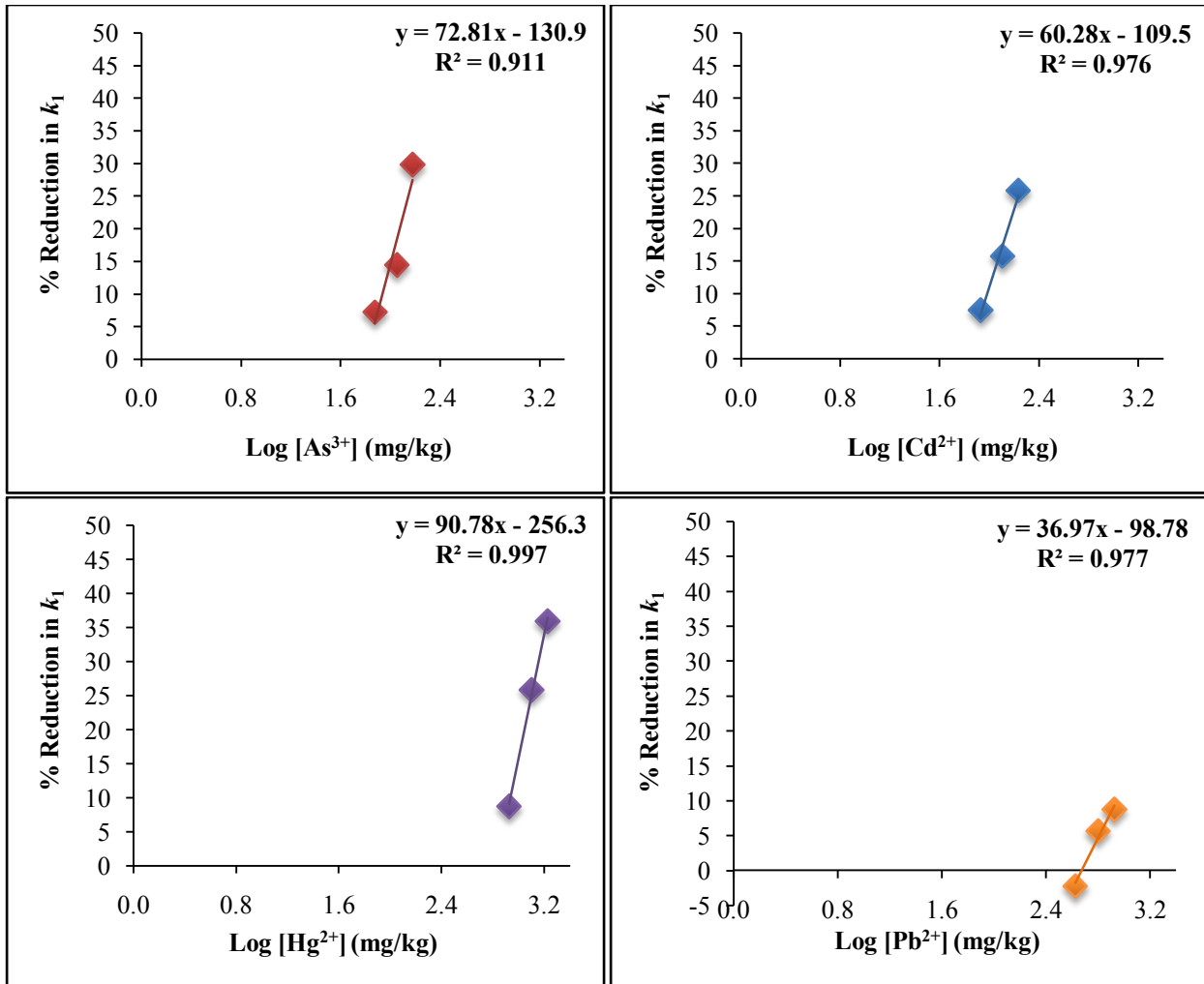
Figure 7: Regression lines representing the effects of the As^{3+}, Cd^{2+}, Hg^{2+}, and Pb^{2+} concentrations in clay soil on 1,2-DCA biodegradation rate coefficients (k_1) relative to k_1 for samples unamended with heavy metals.

Table 7:   Gas chromatographic peak area values used for loam soil samples co-contaminated with different concentrations of heavy metals.

Microcosm		Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Autoclaved soil control		14 683	13 895	12 894	12 213	11 932	10 977	10 519
		12 675	12 111	11 195	10 256	9 782	9 422	8 560
		13 518	13 033	11 709	10 403	9 607	8 878	8 603
Unautoclaved soil control		17 819	9 970	5 943	2 294	1 914	1 694	1 038
		17 195	8 100	5 422	2 773	2 418	1 806	1 242
		16 860	8 404	5 623	2 690	2 375	1 844	1 370
$[As^{3+}]$ mg/kg	**75**	14 854	12 187	7 548	5 782	3 368	2 809	2 325
		15 727	9 906	7 758	5 911	3 577	3 069	2 490
		14 105	10 558	6 719	5 173	3 168	2 678	2 236
	112.5	17 092	11 590	7 788	5 974	3 903	2 962	2 534
		15 523	9 374	5 702	4 606	2 629	2 092	1 707
		13 564	8 562	6 312	4 933	3 112	2 470	2 000
	150	12 014	9 374	6 347	5 024	2 954	2 488	2 004
		14 282	9 669	7 123	5 313	3 225	2 736	2 060
		13 628	8 616	6 739	5 243	3 270	2 500	2 144
$[Cd^{2+}]$ mg/kg	**85**	16 049	8 862	7 495	4 785	3 618	2 946	2 447
		15 989	9 391	6 404	5 025	3 166	2 428	2 079
		14 117	8 793	6 154	5 206	3 260	2 758	2 146
	127.5	15 467	8 220	5 634	4 417	2 813	2 237	1 693
		14 399	8 428	6 118	4 939	3 208	2 518	2 184
		13 603	7 154	4 853	4 119	2 426	1 924	1 715
	170	15 595	9 243	6 271	4 855	3 315	2 708	2 179
		14 668	9 048	6 177	4 886	3 418	2 728	2 164
		13 385	8 304	5 712	4 740	3 196	2 511	1 977
$[Hg^{2+}]$ mg/kg	**840**	14 836	8 987	6 504	5 083	3 498	2 743	2 126
		14 092	8 861	6 268	5 192	3 315	3 048	2 257
		15 239	7 604	5 041	4 223	2 762	2 519	1 702
	1260	14 485	8 128	5 734	4 696	3 094	2 392	1 846
		15 752	9 761	6 886	5 924	4 101	3 103	2 712
		15 526	8 907	5 785	5 124	3 358	2 439	2 124
	1680	14 455	7 852	3 382	4 297	3 065	2 140	1 756
		14 961	8 901	6 496	5 620	3 959	2 502	2 505
		14 844	8 966	6 081	5 196	3 438	2 611	2 194
$[Pb^{2+}]$ mg/kg	**420**	14082	7412	5731	4057	2567	2429	1703
		15 825	9 109	5 284	5 358	3 720	2 880	2 347
		15 865	8 367	6 043	4 726	3 138	2 389	1 986
	630	15 139	7 838	5 568	4 552	2 889	2 195	1 678
		14 864	7 847	5 513	4 498	2 840	2 308	1 705
		14 609	7 470	5 113	3 999	2 657	1 984	858
	840	14 881	8 058	5 744	4 723	2 758	2 243	1 757
		14 058	7 649	5 380	4 459	2 848	2 257	1 779
		14 653	7 024	4 858	3 923	2 794	1 755	1 543

Autoclaved soil control

Sample 1 ■ Sample 2 ▲ Sample 3

$y = -0.011x + 3.035$
$R^2 = 0.99$

$y = -0.015x + 2.921$
$R^2 = 0.945$

$y = -0.017x + 2.967$
$R^2 = 0.980$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Unautoclaved soil control

Sample 1 ■ Sample 2 ▲ Sample 3

$y = -0.129x + 3.214$
$R^2 = 0.969$

$y = -0.109x + 3.041$
$R^2 = 0.982$

$y = -0.104x + 3.007$
$R^2 = 0.976$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Arsenic (75 mg/kg)

Sample 1 ■ Sample 2 ▲ Sample 3

$y = -0.078x + 3.116$
$R^2 = 0.984$

$y = -0.072x + 3.052$
$R^2 = 0.987$

$y = -0.076x + 2.997$
$R^2 = 0.987$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Arsenic (112.5 mg/kg)

Sample1 ■ Sample 2 ▲ Sample 3

$y = -0.075x + 3.158$
$R^2 = 0.993$

$y = -0.091x + 3.026$
$R^2 = 0.989$

$y = -0.077x + 2.909$
$R^2 = 0.993$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Arsenic (150 mg/kg)

Sample 1 ■ Sample 2 ▲ Sample 3

$y = -0.076x + 2.885$
$R^2 = 0.988$

$y = -0.077x + 3.011$
$R^2 = 0.993$

$y = -0.074x + 2.924$
$R^2 = 0.99$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Cadmium (85 mg/kg)

Sample 1 ■ Sample 2 ▲ Sample 3

$y = -0.071x + 2.983$
$R^2 = 0.980$

$y = -0.081x + 3.024$
$R^2 = 0.989$

$y = -0.073x + 2.912$
$R^2 = 0.989$

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

177

Cadmium (127.5 mg/kg)

$y = -0.087x + 2.961$
$R^2 = 0.991$

$y = -0.074x + 2.898$
$R^2 = 0.986$

$y = -0.085x + 2.793$
$R^2 = 0.977$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Cadmium (170 mg/kg)

$y = -0.076x + 2.972$
$R^2 = 0.989$

$y = -0.074x + 2.934$
$R^2 = 0.992$

$y = -0.075x + 2.858$
$R^2 = 0.993$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Mercury (840 mg/kg)

$y = -0.075x + 2.963$
$R^2 = 0.995$

$y = -0.069x + 2.897$
$R^2 = 0.984$

$y = -0.082x + 2.868$
$R^2 = 0.972$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Mercury (1260 mg/kg)

$y = -0.080x + 2.911$
$R^2 = 0.991$

$y = -0.066x + 2.998$
$R^2 = 0.985$

$y = -0.077x + 2.960$
$R^2 = 0.982$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

1,2-DCA (µl/100g loam soil)

Time (days)

Mercury (1680 mg/kg)

$y = -0.079x + 2.749$
$R^2 = 0.911$

$y = -0.070x + 2.951$
$R^2 = 0.970$

$y = -0.074x + 2.943$
$R^2 = 0.988$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Lead (420 mg/kg)

$y = -0.082x + 2.850$
$R^2 = 0.980$

$y = -0.070x + 2.921$
$R^2 = 0.961$

$y = -0.080x + 2.963$
$R^2 = 0.986$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

178

Figure 8: Graphical representation of the degradation rate constants of 1,2-DCA in loam soil co-contaminated with different concentrations of heavy metals.

Table 8: Gas chromatographic peak area values used for clay soil samples co-contaminated with different combinations of heavy metals.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Autoclaved soil control	19 103	16 278	15 704	14 906	14 106	13 583	13 411
	21 244	19 859	17 553	15 856	14 956	14 617	13770
	-	-	-	-	-	-	-
Unautoclaved soil control	19 307	12 230	9 780	6 061	5 475	4 388	3 938
	22 339	15 046	11 803	6 999	6 496	5 054	4 233
	-	-	-	-	-	-	-
As^{3+}+Cd^{2+}	22 905	20 248	12 240	12 415	8 614	8 225	6 143
	22 100	16 634	9 439	9 402	6 799	6 284	4 380
	20 503	18 342	11 487	10 899	8 879	7 816	6 139
As^{3+}+Hg^{2+}	22 582	18 832	12 227	12 471	9 273	8 854	6 754
	15 761	13 368	12 957	11 170	8 597	7 679	6 293
	18 982	16 368	10 756	10 670	8 031	7 432	5 949
As^{3+}+Pb^{2+}	18 362	9 881	8 350	10 486	7 886	7 123	5 770
	21 196	15 213	6 807	8 204	6 341	5 565	4 147
	20 144	14 482	10 684	8 836	6 339	5 292	4 197
Cd^{2+}+Hg^{2+}	21 029	16 081	9 291	8 374	6 323	5 696	4 342
	22 400	15 866	10 380	8 221	5 872	5 153	3 744
	17 001	14 272	9 303	9 758	7 069	6 041	4 668
Cd^{2+}+Pb^{2+}	20 768	15 899	9 699	9 042	6 798	5 838	4 409
	20 707	14 116	8 584	7 388	5 595	4 466	3 352
	20 051	13 427	8 373	6 930	5 186	4 307	3 426
Hg^{2+}+Pb^{2+}	21 727	17 754	11 751	9 450	6 848	6 616	4907
	20 503	13 065	9 702	7 638	5 518	5 086	3 817
	22 148	16 229	10 749	8 915	5 837	5 488	4 198
As^{3+}+Cd^{2+}+Hg^{2+}	21 145	16 683	11 975	9 940	7 994	7 263	5 558
	22 037	16 838	11 972	8 787	7 002	5 700	4 090
	24 911	17 689	11 881	8 946	6 569	5 627	4 270
As^{3+}+Cd^{2+}+Pb^{2+}	24 478	16 890	11 983	9 437	7 209	6 311	4 621
	22 130	17 238	12 431	9 982	7 548	6 660	5 035
	22 473	16 601	11 206	9 270	6 554	5 609	4 307
As^{3+}+Hg^{2+}+Pb^{2+}	20 799	16 611	12 429	10 002	7 570	6 838	5 389
	21 140	17 207	15 249	9 893	7 873	7 134	5 355
	19 479	15 635	16 549	9 275	6 446	5 935	4 820
Cd^{2+}+Hg^{2+}+Pb^{2+}	21 578	13 859	10 621	5 553	4 582	3 650	2 478
	21 345	17 810	14 515	10 015	7 552	6 943	5 371
	21 772	14 279	10 379	7 823	5 746	5 222	4 097
As^{3+}+Cd^{2+}+Hg^{2+}+Pb^{2+}	19 030	14 777	11 474	8 372	5 963	4 758	3 402
	17 582	13 542	9 069	6 183	4 881	3 989	3 088
	17 620	9 452	8 780	5 041	3 449	3 073	2 233

As^{3+} = 112.5 mg/kg; Cd^{2+} = 127.5 mg/kg, Hg^{2+} = 1260 mg/kg, and Pb^{2+} = 630 mg/kg.

Autoclaved soil control
Sample 1 Sample 2

$y = -0.012x + 2.398$
$R^2 = 0.911$

$y = -0.017x + 2.567$
$R^2 = 0.961$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Unautoclaved soil control
Sample 1 Sample 2

$y = -0.076x + 2.372$
$R^2 = 0.979$

$y = -0.076x + 2.589$
$R^2 = 0.983$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

As^{3+} + Cd^{2+}
Sample 1 Sample 2 Sample 3

$y = -0.054x + 2.685$
$R^2 = 0.960$

$y = -0.068x + 2.577$
$R^2 = 0.961$

$y = -0.050x + 2.552$
$R^2 = 0.970$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

As^{3+} + Hg^{2+}
Sample 1 Sample 2 Sample 3

$y = -0.047x + 2.620$
$R^2 = 0.959$

$y = -0.039x + 2.316$
$R^2 = 0.966$

$y = -0.049x + 2.450$
$R^2 = 0.970$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

As^{3+} + Pb^{2+}
Sample 1 Sample 2 Sample 3

$y = -0.039x + 2.141$
$R^2 = 0.791$

$y = -0.069x + 2.410$
$R^2 = 0.895$

$y = -0.071x + 2.531$
$R^2 = 0.996$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

Cd^{2+} + Hg^{2+}
Sample 1 Sample 2 Sample 3

$y = -0.070x + 2.521$
$R^2 = 0.977$

$y = -0.082x + 2.645$
$R^2 = 0.992$

$y = -0.057x + 2.362$
$R^2 = 0.967$

Ln 1,2-DCA (µl/100g clay soil)
Time (days)

181

Cd²⁺ + Pb²⁺

$y = -0.067x + 2.533$
$R^2 = 0.981$

$y = -0.087x + 2.541$
$R^2 = 0.987$

$y = -0.087x + 2.541$
$R^2 = 0.987$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Hg²⁺ + Pb²⁺

$y = -0.065x + 2.621$
$R^2 = 0.983$

$y = -0.076x + 2.472$
$R^2 = 0.989$

$y = -0.076x + 2.622$
$R^2 = 0.987$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

As³⁺ + Cd²⁺ + Hg²⁺

$y = -0.055x + 2.556$
$R^2 = 0.99$

$y = -0.075x + 2.680$
$R^2 = 0.991$

$y = -0.078x + 2.746$
$R^2 = 0.996$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

As³⁺ + Cd²⁺ + Pb²⁺

$y = -0.070x + 2.695$
$R^2 = 0.992$

$y = -0.063x + 2.642$
$R^2 = 0.994$

$y = -0.074x + 2.653$
$R^2 = 0.995$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

As³⁺ + Hg²⁺ + Pb²⁺

$y = -0.058x + 2.571$
$R^2 = 0.994$

$y = -0.060x + 2.652$
$R^2 = 0.980$

$y = -0.067x + 2.625$
$R^2 = 0.945$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Cd²⁺ + Hg²⁺ + Pb²⁺

$y = -0.129x + 2.830$
$R^2 = 0.945$

$y = -0.061x + 2.659$
$R^2 = 0.986$

$y = -0.075x + 2.537$
$R^2 = 0.991$

◆ Sample 1 ■ Sample 2 ▲ Sample 3

Ln 1,2-DCA (µl/100g clay soil)

Time (days)

182

Figure 9: Graphical representation of the degradation rate constants of 1,2-DCA in clay soil co-contaminated with different combinations of heavy metals

Table 9: Gas chromatographic peak area values used for loam soil samples co-contaminated with different combinations of heavy metals.

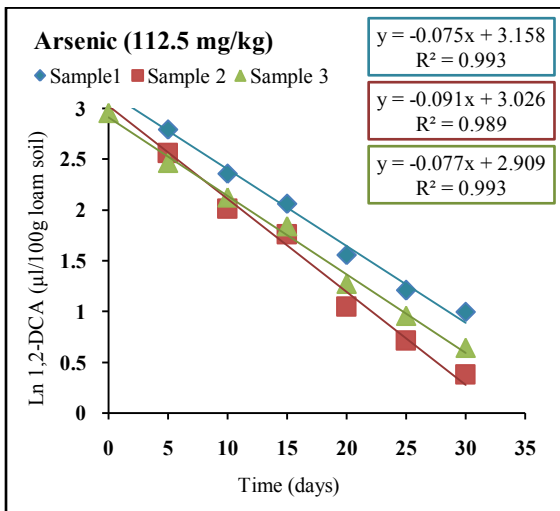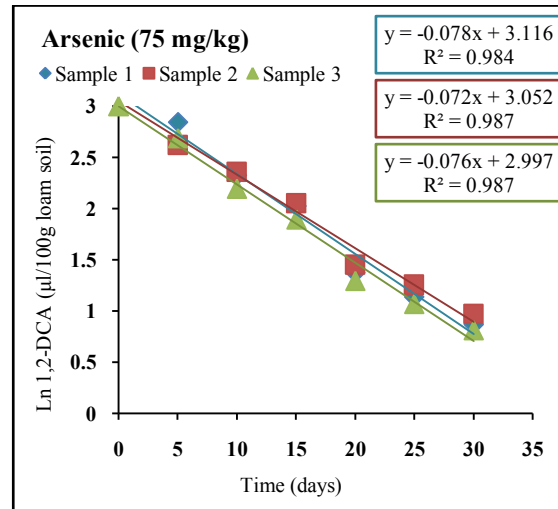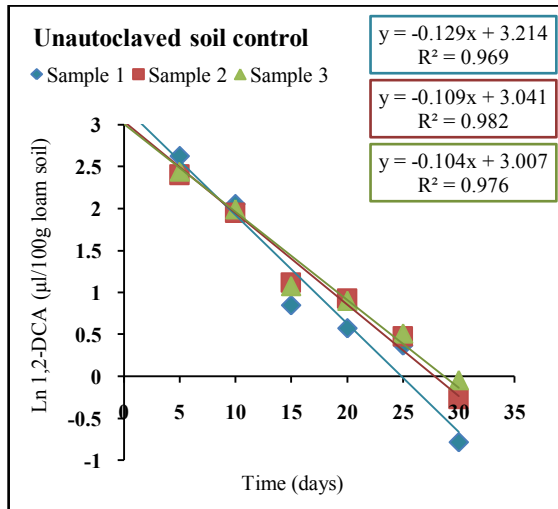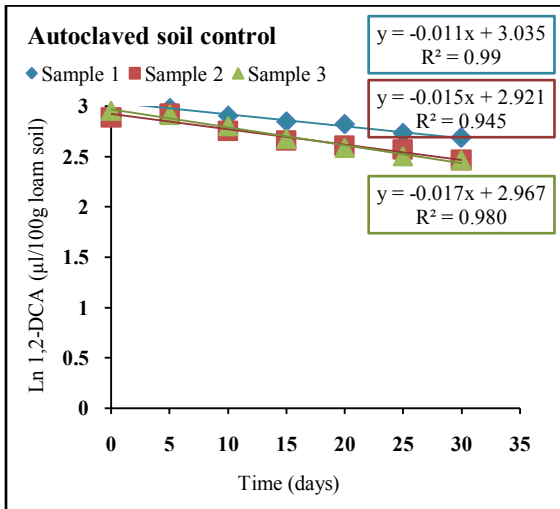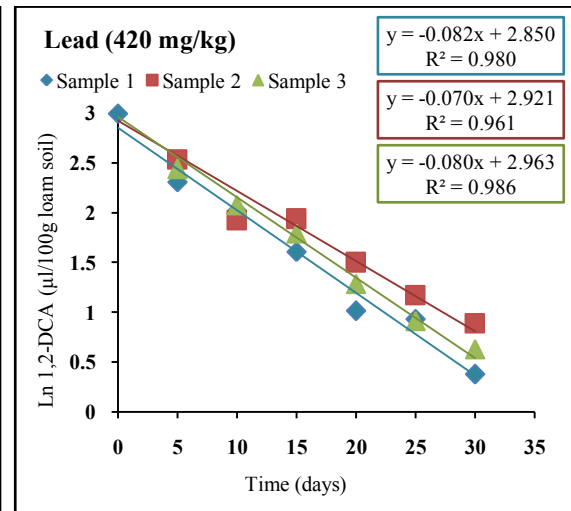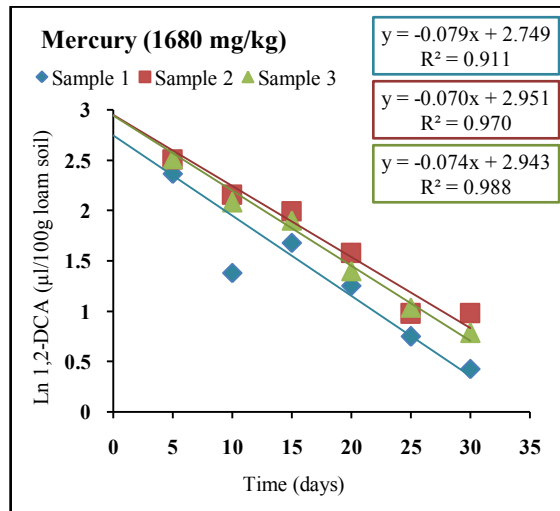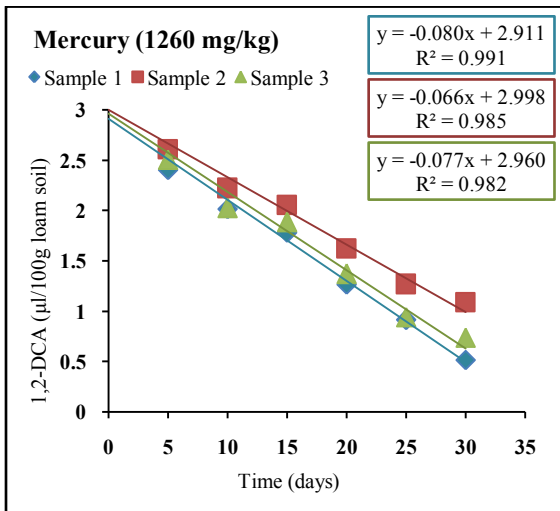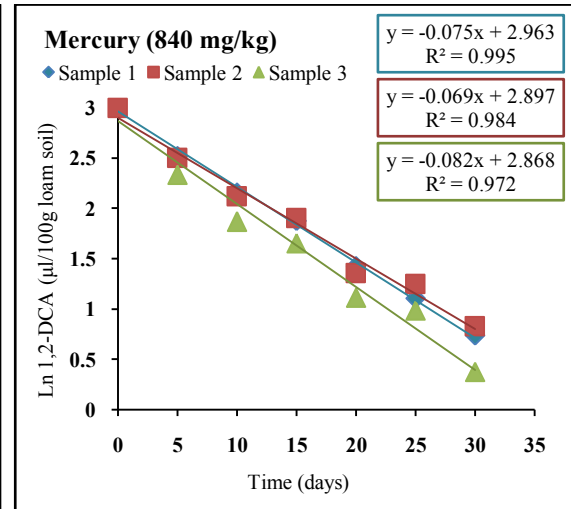| Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20	Day 25	Day 30
Autoclaved soil control	14 683	13 895	12 894	12 213	11 932	10 977	10 519
	12 675	12 111	11 195	10 256	9 782	9 422	8 560
	13 518	13 033	11 709	10 403	9 607	8 878	8 603
Unautoclaved soil control	17 819	9 970	5 943	2 294	1 914	1 694	1 038
	17 195	8 100	5 422	2 773	2 418	1 806	1 242
	16 860	8 404	5 623	2 690	2 375	1 844	1 370
$As^{3+}+Cd^{2+}$	17 434	11 517	6 894	3 755	3 480	2 864	2 369
	17 384	12 457	8 024	4 545	4 378	3 387	3 099
	17 453	11 600	7 238	4 058	3 669	3 108	2 719
$As^{3+}+Hg^{2+}$	17 282	10 142	6 225	3 503	3 231	2 576	2 372
	18 816	15 607	10 689	6 734	6 945	5 540	4 932
	19 295	11 217	10 299	6 348	6 040	5 014	4 646
$As^{3+}+Pb^{2+}$	16 395	14 407	10 214	6 225	6 563	5 379	4 695
	17 640	14 301	10 557	6 381	5 980	5 311	4 660
	17 418	13 622	9 183	5 885	5 780	4 918	4 410
$Cd^{2+}+Hg^{2+}$	17 417	10 534	6 383	3 982	3 630	3 054	2 569
	16 168	11 021	7 251	4 443	4 363	3 215	2 748
	17 403	10 232	6 534	3 877	3 580	2 994	2 517
$Cd^{2+}+Pb^{2+}$	16 075	10 038	5 950	3 760	3 412	2 633	2 352
	16 812	9 458	5 918	3 708	3 120	2 545	2 189
	15 398	9 693	5 782	3 667	3 615	2 490	2 239
$Hg^{2+}+Pb^{2+}$	16 760	9 357	5 192	3 232	3 271	2 208	2 208
	17 916	10 576	6 147	3 738	3 361	2 709	2 645
	17 693	10 577	6 560	4 049	3 666	2 938	2 941
$As^{3+}+Cd^{2+}+Hg^{2+}$	15 968	11 975	8 172	4 345	5 155	4 684	4 488
	17 940	8 095	4 564	2 567	2 014	1 787	1 815
	16 590	11 342	7 764	5 169	4 038	4 174	4 043
$As^{3+}+Cd^{2+}+Pb^{2+}$	16 848	12 457	8 522	5 485	4 199	4 572	4 264
	16 879	12 676	8 378	2 587	3 680	4 814	4 489
	17 802	11 543	5 176	4 839	3 290	3 494	3 342
$As^{3+}+Hg^{2+}+Pb^{2+}$	17 662	10 003	6 255	4 144	3 296	3 171	2 647
	16 502	9 806	5 963	3 836	3 299	2 910	2 550
	15 546	9 202	5 130	3 447	2 878	2 382	2 023
$Cd^{2+}+Hg^{2+}+Pb^{2+}$	18 013	10 357	6 220	3 323	2 884	2 295	2 056
	17 329	9 135	5 398	4 101	3 862	3 234	2 859
	15 054	7 930	4 474	2 956	2 607	2 040	1 883
$As^{3+}+Cd^{2+}+Hg^{2+}+Pb^{2+}$	16 275	9 486	6 141	4 083	3 857	3 098	2 704
	15 995	8 905	5 347	3 825	3 437	2 742	2 520
	14 810	9 108	5 903	3 834	3 491	2 718	2 386

As^{3+} = 112.5 mg/kg; Cd^{2+} = 127.5 mg/kg, Hg^{2+} = 1260 mg/kg, and Pb^{2+} = 630 mg/kg.

Cd²⁺ + Pb²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.079x + 2.957
R² = 0.959

y = -0.075x + 2.944
R² = 0.954

y = -0.076x + 2.918
R² = 0.958

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Hg²⁺ + Pb²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.080x + 2.903
R² = 0.925

y = -0.075x + 2.992
R² = 0.921

y = -0.070x + 2.989
R² = 0.920

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

As³⁺ + Cd²⁺ + Hg²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.048x + 2.937
R² = 0.807

y = -0.094x + 2.852
R² = 0.902

y = -0.055x + 2.973
R² = 0.888

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

As³⁺ + Cd²⁺ + Pb²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.054x + 3.034
R² = 0.886

y = -0.053x + 2.881
R² = 0.543

y = -0.063x + 2.937
R² = 0.842

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

As³⁺ + Hg²⁺ + Pb²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.071x + 2.970
R² = 0.931

y = -0.071x + 2.920
R² = 0.934

y = -0.080x + 2.878
R² = 0.956

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Cd²⁺ + Hg²⁺ + Pb²⁺

Sample 1 ◆ Sample 2 ■ Sample 3 ▲

y = -0.087x + 3.044
R² = 0.950

y = -0.064x + 2.868
R² = 0.896

y = -0.083x + 2.768
R² = 0.939

Ln 1,2-DCA (µl/100g loam soil)

Time (days)

Figure 10: Graphical representation of the degradation rate constants of 1,2-DCA in loam soil co-contaminated with different combinations of heavy metals.

Table 10:   Gas chromatographic peak area values used for arsenic co-contaminated loam soil samples undergoing various bioremediation strategies.

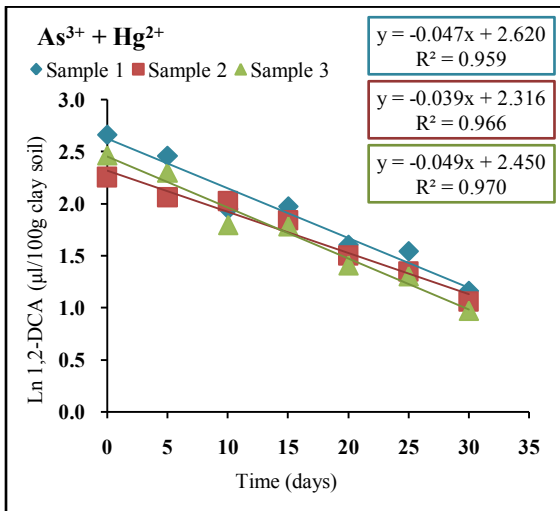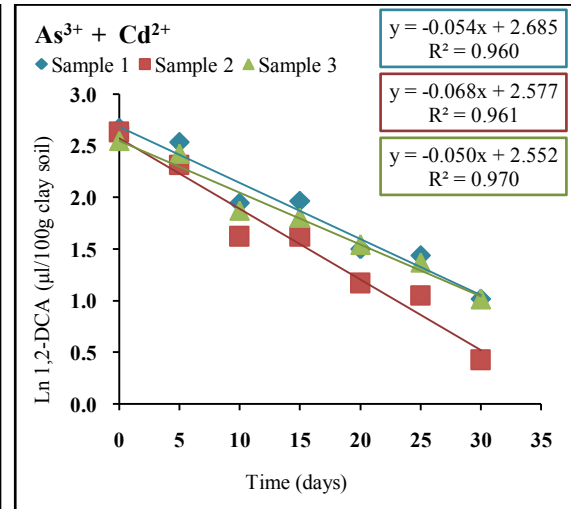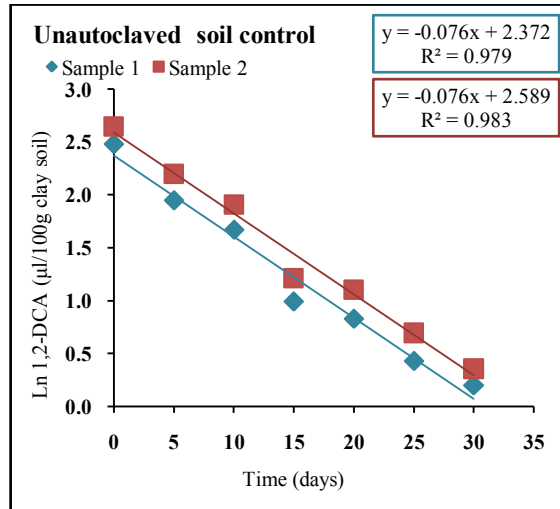| Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Autoclaved soil control	11 603	10 814	9 686	9 526	8 234
	14 495	12 729	11 534	11 255	10 009
	11 743	11 042	10 147	9 295	8 186
Unautoclaved soil control	15 499	14 685	5 633	5 792	1 352
	15 060	13 231	6 336	3 785	1 373
	15 521	11 499	5 442	4 021	1 193
Unautoclaved soil control+As^{3+}	12 835	11 973	5 365	4 977	2 291
	14 635	13 833	6 710	6 326	2 344
	11 765	10 490	5 118	4 155	2 267
Biostimulation					
Glucose	13 581	12 142	5 839	5 374	340
	13 965	13 293	6 804	6 739	455
	13 691	10 099	5 684	4 710	221
Fructose	15 257	13 650	6 432	5 599	406
	15 205	13 647	7 080	6 795	426
	14 997	11 872	5 890	5 171	447
Fertilizer	14 674	12 892	5 734	5 117	343
	14 921	13 659	6 718	6 079	583
	15 938	11 282	5 336	4 337	328
Bioaugmentation					
X. autotrophicus GJ10	13 512	9 307	1 057	451	0
	14 646	9 392	599	215	0
	12 864	8 522	473	868	0
Dual-bioaugmentation	12 380	4 933	366	182	0
	11 091	7 517	849	201	0
	13 213	7 856	434	155	0
Treatment additives					
CaCO$_3$	17 194	13 532	5 615	4 255	178
	18 795	15 284	7 131	5 843	240
	17 886	12 306	5 399	3 826	117
Na$_2$HPO$_4$ + NaCl	16 184	12 980	5 944	4 824	368
	16 181	13 788	6 558	5 981	658
	15 873	11 414	5 563	4 614	362
CaSO$_4$.2H$_2$O	15 369	12 268	5 480	4 399	500
	14 900	12 741	6 450	5 684	707
	16 826	11 562	5 147	4 089	368

Autoclaved soil control

y = -0.015x + 2.480
R² = 0.959

y = -0.016x + 2.670
R² = 0.962

y = -0.017x + 2.505
R² = 0.983

Unautoclaved soil control

y = -0.108x + 2.994
R² = 0.870

y = -0.112x + 2.956
R² = 0.953

y = -0.114x + 2.913
R² = 0.953

Unautoclaved soil control + As³⁺

y = -0.081x + 2.704
R² = 0.926

y = -0.084x + 2.876
R² = 0.898

y = -0.079x + 2.594
R² = 0.958

Glucose + As³⁺

y = -0.139x + 3.040
R² = 0.782

y = -0.131x + 3.088
R² = 0.739

y = -0.146x + 3.010
R² = 0.794

Fructose + As³⁺

y = -0.141x + 3.153
R² = 0.8

y = -0.136x + 3.164
R² = 0.751

y = -0.137x + 3.060
R² = 0.823

Fertilizer + As³⁺

y = -0.144x + 3.105
R² = 0.809

y = -0.129x + 3.103
R² = 0.794

y = -0.148x + 3.092
R² = 0.857

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Figure 11: Graphical representation of the degradation rate constants of 1,2-DCA in arsenic co-contaminated loam soil samples undergoing various bioremediation strategies. (◆) = sample 1; (■) = sample 2; (▲) = sample 3.
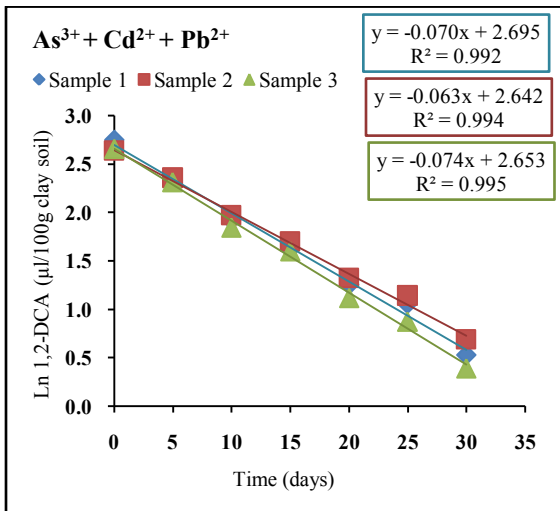
Table 11:   Gas chromatographic peak area values used for cadmium co-contaminated loam soil samples undergoing various bioremediation strategies.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
	11 603	10 814	9 686	9 526	8 234
Autoclaved soil control	14 495	12 729	11 534	11 255	10 009
	11 743	11 042	10 147	9 295	8 186
	15 499	14 685	5 633	5 792	1 352
Unautoclaved soil control	15 060	13 231	6 336	3 785	1 373
	15 521	11 499	5 442	4 021	1 193
	15 124	13 898	5 644	4 967	2 176
Unautoclaved soil control+Cd^{2+}	13 853	13 233	6 463	5 986	1 766
	14 923	11 327	5 114	4 073	2 395
Biostimulation					
	14 691	11 065	5 326	4 841	275
Glucose	13 544	11 094	5 759	5 977	294
	14 219	9 621	4 986	4 262	309
	11 419	10 569	5 944	5 692	429
Fructose	14 398	11 000	5 983	5 839	414
	11 023	9 998	5 869	5 132	405
	13 623	11 645	5 174	3 331	219
Fertilizer	14 393	13 371	6 594	5 200	248
	13 451	10 491	4 861	2 897	225
Bioaugmentation					
	14 358	8 220	264	0	0
X. autotrophicus GJ10	11 468	6 295	339	0	0
	14 659	7 558	319	168	0
	12 643	6 951	296	0	0
Dual-bioaugmentation	12 087	5 429	258	0	0
	12 964	6 244	341	0	0
Treatment additives					
	10 771	9 861	5 265	4 660	357
$CaCO_3$	17 747	14 616	6 798	2 399	0
	11 942	9 243	5 043	4 204	396
	16 137	12 506	5 729	4 801	509
Na_2HPO_4 + NaCl	14 818	12 216	5 929	5 480	586
	15 647	11 810	5 399	4 397	485
	14 742	12 113	5 569	4 598	321
$CaSO_4.2H_2O$	15 903	12 823	6 415	5 583	756
	15 011	11 380	5 243	4 233	264

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Autoclaved soil control

$y = -0.015x + 2.480$
$R^2 = 0.959$

$y = -0.016x + 2.670$
$R^2 = 0.962$

$y = -0.017x + 2.505$
$R^2 = 0.983$

Unautoclaved soil control

$y = -0.108x + 2.994$
$R^2 = 0.870$

$y = -0.112x + 2.956$
$R^2 = 0.953$

$y = -0.114x + 2.913$
$R^2 = 0.953$

Unautoclave soil control + Cd^{2+}

$y = -0.093x + 2.877$
$R^2 = 0.935$

$y = -0.092x + 2.869$
$R^2 = 0.875$

$y = -0.088x + 2.754$
$R^2 = 0.972$

Glucose + Cd^{2+}

$y = -0.146x + 3.054$
$R^2 = 0.813$

$y = -0.138x + 3.016$
$R^2 = 0.748$

$y = -0.142x + 2.957$
$R^2 = 0.841$

Fructose + Cd^{2+}

$y = -0.123x + 2.861$
$R^2 = 0.738$

$y = -0.133x + 3.018$
$R^2 = 0.779$

$y = -0.124x + 2.824$
$R^2 = 0.754$

Fertilizer + Cd^{2+}

$y = -0.156x + 3.045$
$R^2 = 0.864$

$y = -0.150x + 3.167$
$R^2 = 0.782$

$y = -0.155x + 2.983$
$R^2 = 0.889$

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Figure 12: Graphical representation of the degradation rate constants of 1,2-DCA in cadmium co-contaminated loam soil samples undergoing various bioremediation strategies. (◆) = sample 1; (■) = sample 2; (▲) = sample 3.

Table 12:   Optical density values used for dehydrogenase activity in arsenic co-contaminated loam soil undergoing various bioremediation strategies.

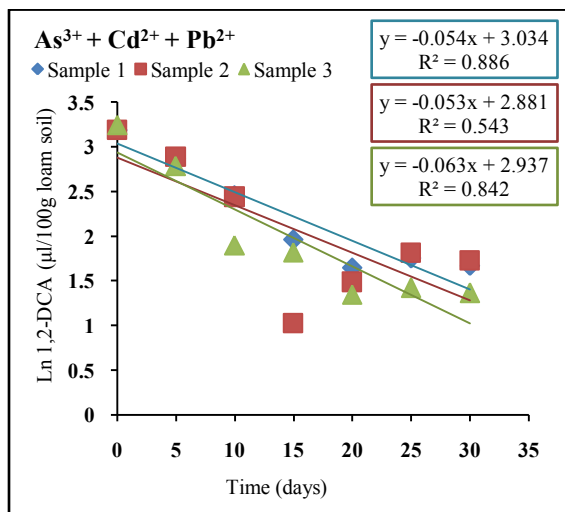| Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Autoclaved soil control	0.023	0.028	0.025	0.031	0.023
	0.018	0.024	0.021	0.038	0.031
	0.029	0.019	0.027	0.026	0.033
Unautoclaved soil control	0.361	0.496	0.571	0.506	0.531
	0.426	0.583	0.628	0.616	0.575
	0.415	0.546	0.659	0.624	0.598
Unautoclaved soil control + As^{3+}	0.361	0.124	0.096	0.082	0.049
	0.426	0.163	0.129	0.069	0.089
	0.415	0.247	0.161	0.123	0.155
Biostimulation					
Glucose	0.361	0.309	0.567	0.506	0.568
	0.426	0.225	0.519	0.564	0.571
	0.415	0.321	0.386	0.447	0.476
Fructose	0.361	0.186	0.209	0.271	0.389
	0.426	0.306	0.189	0.213	0.354
	0.415	0.223	0.204	0.447	0.439
Fertilizer	0.361	0.198	0.249	0.368	0.425
	0.426	0.284	0.197	0.395	0.362
	0.415	0.237	0.368	0.406	0.445
Bioaugmentation					
X. autotrophicus GJ10	0.361	0.376	0.393	0.469	0.525
	0.426	0.434	0.365	0.463	0.489
	0.415	0.426	0.439	0.391	0.463
Dual-bioaugmentation	0.361	0.401	0.396	0.423	0.431
	0.426	0.433	0.31	0.384	0.446
	0.415	0.421	0.343	0.392	0.381
Treatment additives					
CaCO$_3$	0.361	0.252	0.21	0.221	0.273
	0.426	0.245	0.224	0.197	0.236
	0.415	0.223	0.208	0.187	0.213
Na$_2$HPO$_4$ + NaCl	0.361	0.243	0.221	0.289	0.268
	0.426	0.292	0.268	0.32	0.349
	0.415	0.238	0.207	0.262	0.346
CaSO$_4$.2H$_2$O	0.361	0.183	0.142	0.133	0.164
	0.426	0.186	0.153	0.121	0.149
	0.415	0.194	0.163	0.146	0.155

Table 13:   Optical density values used for dehydrogenase activity in cadmium co-contaminated loam soil undergoing various bioremediation strategies.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Autoclaved soil control	0.023	0.028	0.025	0.031	0.023
	0.018	0.024	0.021	0.038	0.031
	0.029	0.019	0.027	0.026	0.033
Unautoclaved soil control	0.361	0.496	0.571	0.506	0.531
	0.426	0.583	0.628	0.616	0.575
	0.415	0.546	0.659	0.624	0.598
Unautoclaved soil control+Cd^{2+}	0.361	0.201	0.221	0.198	0.047
	0.426	0.191	0.083	0.079	0.138
	0.415	0.341	0.208	0.194	0.123
Biostimulation					
Glucose	0.361	0.346	0.298	0.482	0.467
	0.426	0.414	0.446	0.483	0.552
	0.415	0.309	0.404	0.497	0.465
Fructose	0.361	0.179	0.138	0.268	0.352
	0.426	0.143	0.091	0.167	0.306
	0.415	0.287	0.162	0.191	0.305
Fertilizer	0.361	0.229	0.315	0.363	0.411
	0.426	0.321	0.363	0.351	0.392
	0.415	0.364	0.393	0.422	0.463
Bioaugmentation					
X. autotrophicus GJ10	0.361	0.392	0.398	0.404	0.398
	0.426	0.469	0.389	0.433	0.44
	0.415	0.376	0.384	0.423	0.409
Dual-bioaugmentation	0.361	0.382	0.324	0.345	0.352
	0.426	0.397	0.331	0.354	0.396
	0.415	0.432	0.369	0.392	0.426
Treatment additives					
$CaCO_3$	0.361	0.197	0.161	0.191	0.215
	0.426	0.232	0.205	0.179	0.213
	0.415	0.223	0.289	0.316	0.325
Na_2HPO_4 + NaCl	0.361	0.242	0.223	0.252	0.264
	0.426	0.309	0.258	0.221	0.287
	0.415	0.176	0.227	0.207	0.239
$CaSO_4.2H_2O$	0.361	0.103	0.184	0.171	0.154
	0.426	0.324	0.209	0.191	0.212
	0.415	0.169	0.121	0.171	0.183

Table 14: Optical density values used for urease activity in arsenic co-contaminated loam soil undergoing various bioremediation strategies.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Autoclaved soil control	0.125	0.053	0.072	0.134	0.131
	0.105	0.048	0.07	0.147	0.135
	0.116	0.052	0.07	0.128	0.123
Unautoclaved soil control	0.304	0.188	0.289	0.431	0.448
	0.208	0.194	0.299	0.38	0.435
	0.284	0.195	0.287	0.41	0.433
Unautoclaved soil control+As^{3+}	0.304	0.146	0.25	0.323	0.445
	0.208	0.15	0.191	0.31	0.423
	0.284	0.148	0.175	0.298	0.377
Biostimulation					
Glucose	0.304	0.147	0.155	0.24	0.404
	0.208	0.186	0.161	0.259	0.333
	0.284	0.191	0.18	0.259	0.313
Fructose	0.304	0.064	0.117	0.27	0.242
	0.208	0.052	0.153	0.23	0.231
	0.284	0.054	0.139	0.203	0.236
Fertilizer	0.304	1.116	1.498	1.201	1.513
	0.208	1.078	1.216	1.169	1.63
	0.284	1.112	1.284	1.326	1.409
Bioaugmentation					
X. autotrophicus GJ10	0.304	0.231	0.256	0.374	0.359
	0.208	0.242	0.259	0.312	0.283
	0.284	0.209	0.265	0.361	0.386
Dual-bioaugmentation	0.304	0.186	0.228	0.193	0.341
	0.208	0.177	0.206	0.248	0.336
	0.284	0.176	0.238	0.301	0.373
Treatment additives					
CaCO$_3$	0.304	0.246	0.288	0.238	0.294
	0.208	0.234	0.287	0.266	0.317
	0.284	0.241	0.284	0.29	0.292
Na$_2$HPO$_4$ + NaCl	0.304	0.277	0.286	0.246	0.237
	0.208	0.286	0.261	0.234	0.259
	0.284	0.286	0.261	0.278	0.387
CaSO$_4$.2H$_2$O	0.304	0.215	0.27	0.228	0.394
	0.208	0.209	0.295	0.211	0.317
	0.284	0.221	0.307	0.242	0.346

Table 15: Optical density values used for urease activity in cadmium co-contaminated loam soil undergoing various bioremediation strategies.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Autoclaved soil control	0.125	0.053	0.072	0.134	0.131
	0.105	0.048	0.070	0.147	0.135
	0.116	0.052	0.070	0.128	0.123
Unautoclaved soil control	0.304	0.188	0.289	0.431	0.448
	0.208	0.194	0.299	0.380	0.435
	0.284	0.195	0.287	0.410	0.433
Unautoclaved soil control + Cd^{2+}	0.304	0.082	0.168	0.301	0.382
	0.208	0.098	0.286	0.303	0.410
	0.284	0.102	0.224	0.291	0.337
Biostimulation					
Glucose	0.304	0.125	0.166	0.247	0.226
	0.208	0.089	0.104	0.219	0.289
	0.284	0.076	0.113	0.222	0.227
Fructose	0.304	0.061	0.108	0.170	0.308
	0.208	0.06	0.112	0.171	0.265
	0.284	0.085	0.096	0.183	0.307
Fertilizer	0.304	1.367	1.603	1.377	1.306
	0.208	1.359	1.348	1.388	1.478
	0.284	1.354	1.643	1.271	1.218
Bioaugmentation					
X. autotrophicus GJ10	0.304	0.237	0.259	0.223	0.276
	0.208	0.223	0.298	0.227	0.278
	0.284	0.228	0.242	0.224	0.309
Dual-bioaugmentation	0.304	0.105	0.162	0.225	0.275
	0.208	0.096	0.233	0.203	0.336
	0.284	0.104	0.154	0.194	0.333
Treatment additives					
$CaCO_3$	0.304	0.228	0.273	0.258	0.243
	0.208	0.227	0.294	0.268	0.363
	0.284	0.197	0.287	0.228	0.326
Na_2HPO_4 + NaCl	0.304	0.179	0.236	0.270	0.244
	0.208	0.201	0.210	0.294	0.205
	0.284	0.196	0.299	0.293	0.292
$CaSO_4.2H_2O$	0.304	0.193	0.231	0.276	0.339
	0.208	0.189	0.230	0.231	0.330
	0.284	0.202	0.288	0.231	0.370

Table 16:   ICP-OES values used for determining the bioavailable concentrations of arsenic in co-contaminated loam soil.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Unautoclaved soil + As^{3+}	3.788	1.756	1.283	0.788	0.729
	3.958	1.706	1.348	0.788	0.833
	4.513	1.654	1.375	0.757	0.743
Average	4.09	1.71	1.34	0.78	0.77
Standard deviation	0.38	0.05	0.05	0.02	0.06
CaCO$_3$	3.788	1.832	1.306	0.642	0.716
	3.958	1.369	1.236	0.574	0.83
	4.513	1.199	1.161	0.559	0.88
Average	4.09	1.47	1.23	0.59	0.81
Standard deviation	0.38	0.33	0.07	0.04	0.08
Na$_2$HPO$_4$ + NaCl	3.788	3.295	2.53	2.764	2.131
	3.958	3.341	2.987	2.665	2.137
	4.513	3.447	2.799	2.63	2.14
Average	4.09	3.36	2.77	2.69	2.14
Standard deviation	0.38	0.08	0.23	0.07	0.01
CaSO$_4$.2H$_2$O	3.788	1.245	1.048	0.377	0.295
	3.958	1.225	0.99	0.359	0.306
	4.513	1.276	0.879	0.407	0.258
Average	4.09	1.25	0.97	0.38	0.29
Standard deviation	0.38	0.03	0.09	0.02	0.03

Table 17:   ICP-OES values used for determining the bioavailable concentrations of cadmium in co-contaminated loam soil.

Microcosm	Day 0	Day 5	Day 10	Day 15	Day 20
Unautoclaved soil + Cd^{2+}	0.745	0.183	0.039	-0.052	-0.069
	1.302	0.122	0.138	-0.066	-0.058
	1.112	0.456	0.102	-0.067	-0.057
Average	1.05	0.25	0.09	-0.06	-0.06
Standard deviation	0.28	0.18	0.05	0.01	0.01
CaCO$_3$	0.745	-0.061	-0.068	-0.078	-0.081
	1.302	-0.07	-0.07	-0.079	-0.081
	1.112	-0.071	-0.071	-0.079	-0.081
Average	1.05	-0.07	-0.07	-0.08	-0.08
Standard deviation	0.28	0.01	0.00	0.00	0.00
Na$_2$HPO$_4$ + NaCl	0.745	-0.042	-0.047	0.013	-0.012
	1.302	-0.039	-0.044	0.019	-0.012
	1.112	-0.047	-0.04	0.027	-0.014
Average	1.05	-0.04	-0.04	0.02	-0.01
Standard deviation	0.28	0.00	0.00	0.01	0.00
CaSO$_4$.2H$_2$O	0.745	0.372	0.009	-0.079	-0.081
	1.302	0.722	-0.012	-0.079	-0.08
	1.112	0.767	0.03	-0.078	-0.081
Average	1.05	0.62	0.01	-0.08	-0.08
Standard deviation	0.28	0.22	0.02	0.00	0.00

Table 18:   Enumeration of the total 1,2-DCA degrading bacterial population in loam soil co-contaminated with arsenic.

Total 1,2-DCA degrading population (cfu/ml $\times 10^6$)			
Day 0			
	Autoclaved soil control	**Unautoclaved soil control**	
	0	7.60	
	0	6.20	
	0	5.60	
Average	**0**	**6.47**	
SD	**0**	**1.03**	
Day 10			
	Autoclaved soil control	**Unautoclaved soil control**	**Unautoclaved soil + As^{3+}**	**Glucose + As^{3+}**
	0	42.00	2.67	21.80
	0	46.00	2.79	20.90
	0	57.00	2.75	18.20
			3.40	51.00
				33.00
Average	**0**	**48.33**	**2.90**	**28.98**
SD	**0**	**7.77**	**0.34**	**13.55**
	Fructose + As^{3+}	**Fertilizer + As^{3+}**	***X. autotrophicus* GJ10 + As^{3+}**	**Dual bioaugmentation + As^{3+}**
	2.42	8.40	26.70	115.00
	2.63	7.50	28.40	112.00
	2.81	6.20	31.00	108.00
	3.60		38.00	
Average	**2.87**	**7.37**	**31.03**	**111.67**
SD	**0.52**	**1.11**	**4.98**	**3.51**
	CaCO$_3$ + As^{3+}	**Na$_2$HPO$_4$ + NaCl + As^{3+}**	**CaSO$_4$.2H$_2$O + As^{3+}**	
	2.98	28.40	10.50	
	3.50	42.00	8.30	
	3.10	33.00	9.10	
Average	**3.19**	**34.47**	**9.30**	
SD	**0.27**	**6.92**	**1.11**	

SD – standard deviation; cfu/ml – colony forming units per milliliter

Table 19: Enumeration of the total 1,2-DCA degrading bacterial population in loam soil co-contaminated with cadmium.

	Total 1,2-DCA degrading population (cfu/ml × 10⁶)		
	Day 0		
	Autoclaved soil control	**Unautoclaved soil control**	
	0	7.60	
	0	6.20	
	0	5.60	
Average	**0**	**6.47**	
SD	**0**	**1.03**	
	Day 10		
	Autoclaved soil control	**Unautoclaved soil control**	**Unautoclaved soil + Cd^{2+}**	**Glucose + Cd^{2+}**
	0	42.00	2.94	3.81
	0	46.00	4.60	3.58
	0	57.00	3.70	3.63
				2.30
Average	**0**	**48.33**	**3.75**	**3.33**
SD	**0**	**7.77**	**0.83**	**0.69**
	Fructose + Cd^{2+}	**Fertilizer + Cd^{2+}**	***X. autotrophicus* GJ10 + Cd^{2+}**	**Dual-bioaugmentation + Cd^{2+}**
	2.08	4.60	84	480
	1.69	3.90	91	410
	1.97	4.10	71	320
Average	**1.91**	**4.20**	**82.00**	**403.33**
SD	**0.20**	**0.36**	**10.15**	**80.21**
	CaCO$_3$ + Cd^{2+}	**Na$_2$HPO$_4$ + NaCl + Cd^{2+}**	**CaSO$_4$.2H$_2$O + Cd^{2+}**	
	5.10	12.40	1.98	
	4.30	10.80	2.46	
	3.90	11.70	2.18	
			3.30	
Average	**4.43**	**11.63**	**2.48**	
SD	**0.61**	**0.80**	**0.58**	

SD – standard deviation; cfu/ml – colony forming units per milliliter

Table 20: Enumeration of the total 1,2-DCA degrading bacterial population in loam soil treatments co-contaminated with arsenic after 20 days.

	Total 1,2-DCA degrading population (cfu/ml × 10^7)			
	Autoclaved soil control	Unautoclaved soil control	Unautoclaved soil + As^{3+}	Glucose + As^{3+}
	0	3.01	0.23	29.7
	0	2.81	0.21	35.00
	0	2.90	0.23	37.00
			0.35	
Average	0	2.91	0.26	33.90
SD	0	0.10	0.06	3.77
	Fructose + As^{3+}	Fertilizer + As^{3+}	*X. autotrophicus* GJ10 + As^{3+}	Dual bioaugmentation + As^{3+}
	2.85	40.00	275	330.00
	2.82	39.00	308	450.00
	2.76	30.00		510.00
	2.90			
Average	2.83	36.33	291.50	430.00
SD	0.06	5.51	23.34	91.65
	Total 1,2-DCA degrading population (cfu/ml × 10^6)			
	CaCO$_3$ + As^{3+}	Na$_2$HPO$_4$ + NaCl + As^{3+}	CaSO$_4$.2H$_2$O + As^{3+}	
	285.00	37	29.50	
	264.00	29	29.40	
	278.00		31.00	
			35.00	
Average	275.67	33.00	31.23	
SD	10.69	5.66	2.62	

SD – standard deviation; cfu/ml – colony forming units per milliliter

Table 21:   Enumeration of the total 1,2-DCA degrading bacterial population in loam soil treatments co-contaminated with cadmium after 20 days.

	Total 1,2-DCA degrading population (cfu/ml × 10^7)			
	Autoclaved soil control	**Unautoclaved soil control**	**Unautoclaved soil + Cd^{2+}**	**Glucose + Cd^{2+}**
	0	3.01	0.27	29.10
	0	2.81	0.30	23.20
	0	2.90		31.00
				41.00
				50.00
Average	**0**	**2.91**	**0.29**	**34.86**
SD	**0**	**0.10**	**0.02**	**10.62**
	Fructose + Cd^{2+}	**Fertilizer + Cd^{2+}**	***X. autotrophicus* GJ10 + Cd^{2+}**	**Dual bioaugmentation + Cd^{2+}**
	2.63	26.40	370	560
	2.84	28.10	450	350
	2.76	41.00		450
		36.00		
		33.00		
Average	**2.74**	**32.90**	**410**	**453.33**
SD	**0.11**	**5.93**	**56.57**	**105.04**
	Total 1,2-DCA degrading population (cfu/ml × 10^6)			
	CaCO$_3$ + Cd^{2+}	**Na$_2$HPO$_4$ + NaCl + Cd^{2+}**	**CaSO$_4$.2H$_2$O + Cd^{2+}**	
	45.00	30.00	6.80	
	42.00	28.00	4.10	
	51.00		5.20	
Average	**46.00**	**29.00**	**5.37**	
SD	**4.58**	**1.41**	**1.36**	

SD – standard deviation; cfu/ml – colony forming units per milliliter

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